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Review

Perspective on post-menopausal osteoporosis: establishing an interdisciplinary understanding of the sequence of events from the molecular level to whole bone fractures

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Current drug treatments for post-menopausal osteoporosis cannot eliminate bone fractures, possibly because the mechanisms responsible for bone loss are not fully understood. Although research within various disciplines has significantly advanced the state of knowledge, fundamental findings are not widely understood between different disciplines. For that reason, this paper presents noteworthy experimental findings from discrete disciplines focusing on post-menopausal osteoporosis. These studies have established that, in addition to bone loss, significant changes in bone micro-architecture, tissue composition and micro-damage occur. Cellular processes and molecular signalling pathways governing pathological bone resorption have been identified to a certain extent. Ongoing studies endeavour to determine how such changes are initiated at the onset of oestrogen deficiency. It emerges that, because of the discrete nature of previous research studies, the sequence of events that lead to bone fracture is not fully understood. In this paper, two sequences of multi-scale changes are proposed and the experimental challenges that need to be overcome to fully define this sequence are outlined. Future studies must comprehensively characterize the time sequence of molecular-, cellular- and tissue-level changes to attain a coherent understanding of the events that ultimately lead to bone fracture and inform the future development of treatments for post-menopausal osteoporosis.

Keywords: bone; post-menopausal osteoporosis; tissue composition; biomechanics; cell biology

1. INTRODUCTION

Osteoporosis is a disease which causes bone loss and fractures and leads to severe pain, deformity and in certain cases secondary complications that result in death (Johnell *et al.* 2004). The disease is classified clinically to be either primary or secondary osteoporosis. Primary osteoporosis refers to both bone loss occurring in post-menopausal women (type I) and bone loss owing to the normal ageing process (type II). Secondary osteoporosis refers to bone loss that ensues as a secondary effect of other diseases or drug treatment. Post-menopausal osteoporosis (type I) is the most common form of the disease and is believed to initiate when oestrogen production is deficient following the menopause (Riggs *et al.* 2002). During normal physiology, bone can renew itself to maintain bone strength and mineral homeostasis by means of coordinated cellular activity whereby osteoclasts continuously resorb aged or damaged bone and osteoblasts reform new bone tissue in its place (Parfitt 1984). However, during oestrogen deficiency, osteoclasts remove excess bone without adequate formation by osteoblasts. Bone loss ensues when the basic trabecular bone units (trabeculae) become thin and resorb completely or fracture (Eriksen *et al.* 1990), and ultimately bone fractures occur under minimal trauma in the bones of the hip, wrist and spine.

Approximately 40 per cent of women over 50 will suffer a fracture related to post-menopausal osteoporosis during their lifetime (Melton *et al.* 2005). Current diagnosis of the disease involves the use of dual energy X-ray absorptiometry (DEXA) to quantify bone mass as a surrogate to predict the likelihood of

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fracture. However, these techniques are inefficient as changes in bone mass alone do not fully explain susceptibility to bone fracture (Dempster 2000; Garnero & Delmas 2004; Wainwright *et al.* 2005). It is now clear that other features of bone quality including tissue properties, composition, micro-architecture (Parfitt 1987; Compston *et al.* 1989; Lane *et al.* 1998) and micro-damage (Burr *et al.* 1997; Dai *et al.* 2004) also influence susceptibility to fracture in combination with the changes in bone mass, albeit that ongoing research seeks to determine the precise contribution.

Hormone therapy and anti-resorptive therapy (e.g. bisphosphonates, selective tissue oestrogenic activity regulators, calcitonins) are popular treatments for bone loss, and, while they have different modes of operation, the common primary aim is to reduce fracture incidence by inhibiting osteoclast resorption and maintaining bone mass (Kloosterboer & Ederveen 2002). However, even with continuous use, these drugs reduce the propensity to fracture by only approximately 50 per cent (Randell *et al.* 2002), suggesting that the quality of the remaining tissue may be compromised (Flora *et al.* 1981; Hirano *et al.* 2000). More recently developed drugs, including parathyroid hormone (PTH), alendronate, ciclosporin A and strontium ranelate, have an anabolic effect on bone and thereby promote new bone formation (Liberman *et al.* 1995; Neer *et al.* 2001; Reginster *et al.* 2003; Yeo *et al.* 2006). Nonetheless, these drugs still reduce fracture risk by only approximately 40 per cent (Liberman *et al.* 1995; Neer *et al.* 2001; Reginster *et al.* 2003). Furthermore, even with drug treatment, the quality of life of a sufferer of osteoporosis cannot be fully restored as 50 per cent experience disability and 75 per cent never regain the same level of health or independence as they had before suffering an osteoporotic fracture (Compston & Rosen 2002).

While abundant research has been undertaken to identify the exact cause of post-menopausal osteoporosis, the disease is not yet fully understood and the associated bone fractures cannot be eliminated. The high prevalence, inaccuracy in diagnostic techniques and insufficiencies of treatment have made post-menopausal osteoporosis the subject of intense scientific investigation for research disciplines such as biomechanical engineering, materials science and cell and molecular biology. For the most part, these studies have been discrete with little interdisciplinary interaction at the interface of these disciplines. As a result, fundamental findings are not widely understood between different disciplines and, even with the wealth of data produced, there is still no coherent understanding of the cause of bone loss, or the sequence of events that ultimately lead to bone fractures. The objective of this review is to identify significant experimental findings from discrete research fields with a view to promoting an interdisciplinary knowledge base regarding the complex changes that occur at the onset of oestrogen deficiency at the molecular, cellular, tissue and organ levels. Furthermore, this review seeks to correlate the findings where possible, and define important topics for future research.

2. BONE STRENGTH DURING OSTEOPOROSIS

The fundamental concern with the disease of osteoporosis is that the mechanical properties of the bone are compromised to such a degree that bone fractures occur under normal loading conditions, which are not associated with fracture in healthy bones. Consequently, much research has sought to determine how bone loss alters bone mechanical strength. Different measures of mechanical strength include yield strength (load required to initiate failure), ultimate strength (maximum load experienced), elastic modulus (tendency to deform) and toughness (energy absorbed before fracture). Experimental testing of whole bones or bone samples from post-menopausal subjects and animal models has been performed under various types of loading, such as compression, tension, bending or shear. At the whole bone level, the ultimate stress and maximum elastic modulus are reduced in the human osteoporotic vertebrae (Hasegawa *et al.* 1993), and the maximum compressive strength of vertebral bodies and the bending strength of femora are decreased in the ovariectomized animal model of osteoporosis (Kasugai *et al.* 1998; Yoshitake *et al.* 1999). Mechanical tests of volumes of trabecular bone (approx. 5 mm^3) have shown a reduction in the continuum (apparent) compressive strength and elastic modulus of osteoporotic bone compared with normal bone (Li & Aspden 1997; Sugita *et al.* 1999; Yoshitake *et al.* 1999; Ciarelli *et al.* 2000). The cause of these changes in mechanical behaviour is much debated; the overall mechanical strength of bone is determined by two features, bone mass and bone quality (NIH 2000; Judex *et al.* 2003). Bone mass is a quantitative measure of the total bone present in a specific volume of bone selected for clinical analysis as is discussed further below. Bone quality encompasses a number of features of the bone tissue including (i) morphology, (ii) micro-architecture, (iii) composition, and (iv) the degree of micro-damage. Each of these features contributes to different degrees to create the material properties that allow bone to bear the loading conditions experienced during life (figure 1).

2.1. Bone mass during osteoporosis

The reduction in bone mass, occurring during oestrogen deficiency, has long been regarded as the critical event leading to bone fracture. Bone mineral density (BMD) is a quantitative measure of bone mass and represents the total mineral in a selected volume of bone in the hip or in the spine. This has become the gold standard for the clinical diagnosis of osteoporosis (Brunader & Shelton 2002; Kanis *et al.* 2002). The World Health Organization defines that a person is diagnosed with osteoporosis if their BMD falls below 2.5 standard deviations (T-score) of the BMD of a young adult (Kanis *et al.* 2003). A fracture is commonly classified to be osteoporotic if it arises in a patient over 50 and the BMD of the fracture site is sufficiently low (Kanis *et al.* 2001). DEXA is the preferred technology for quantifying BMD (Cummings *et al.* 2002; Leib *et al.* 2004), but other diagnostic tools, including quantitative

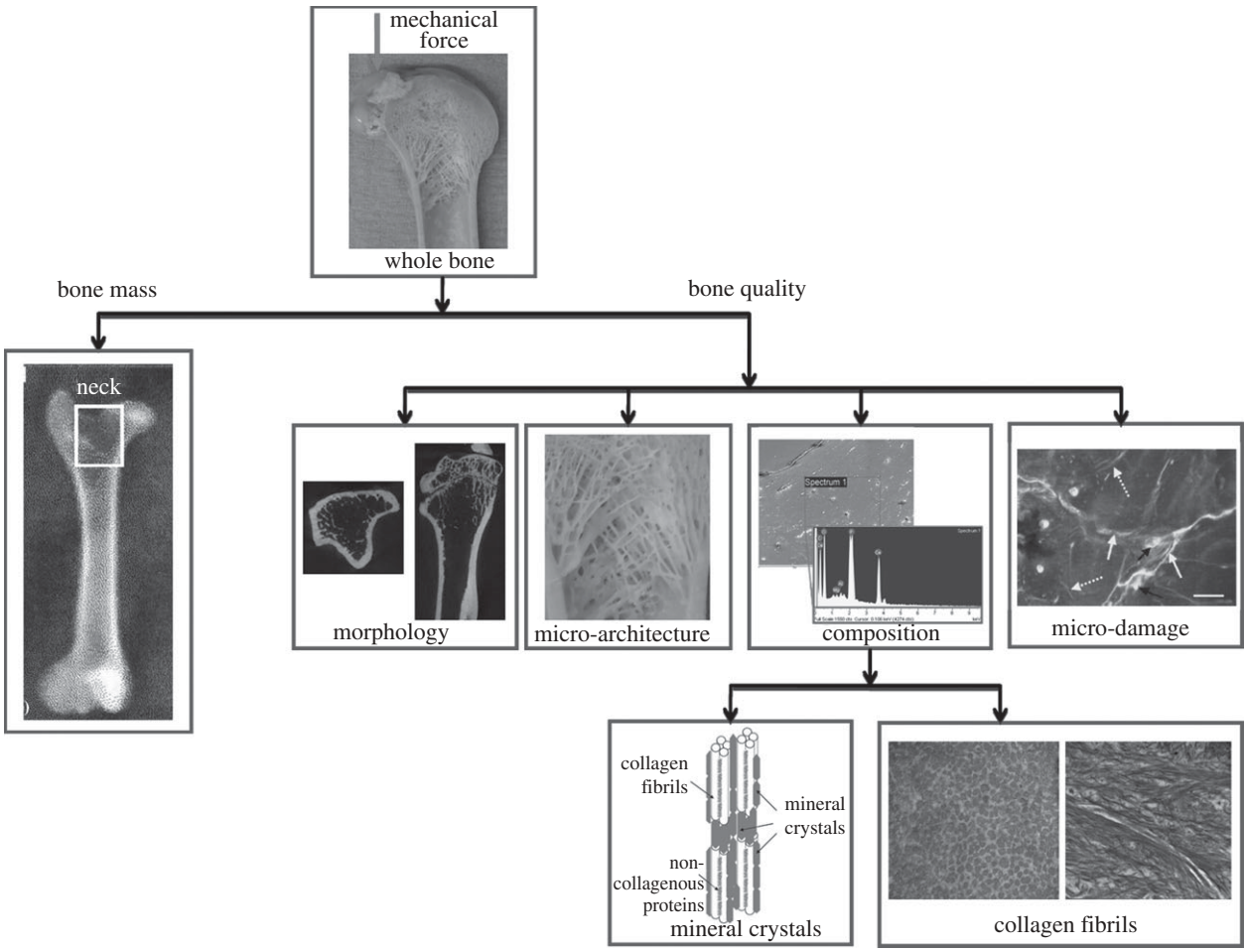

Figure 1. Contribution of bone mass and bone quality in load bearing in normal bone; bone strength is determined by bone mass, morphology, micro-architecture, tissue composition and micro-damage (micro-damage image courtesy of Professor F. J. O'Brien, Royal College of Surgeons in Ireland).

computed tomography (QCT), absorptiometry, quantitative roentgen micro-densitometry and quantitative ultrasound (QUS), may provide more accurate measures of BMD (Richardson *et al.* 1985; van Berkum *et al.* 1989). Nonetheless, BMD is a poor predictor of fracture risk as changes in bone mass alone do not fully explain susceptibility to bone fracture; for example, only 10–53% of bone fractures that occur in female post-menopausal patients over the age of 65 can be attributed to a BMD level low enough (i.e. T-score<-2.5) for clinical diagnosis of osteoporosis (Stone *et al.* 2003; Garnero & Delmas 2004; Wainwright *et al.* 2005). Hence, current research methods now seek to understand whether changes in bone quality might account for the fractures that are not detected by BMD changes alone (Dempster 2000; Stauber *et al.* 2006).

2.2. Bone micro-architecture and morphology during osteoporosis

Micro-architecture is a term which refers to the microscopic morphology and organization of both trabecular and cortical bone. It is characterized using bone histomorphometry and micro-CT scanning (μCT) to obtain two-dimensional/three-dimensional measurements of the architecture at various resolutions. The estimation of the three-dimensional properties of the architecture from two-dimensional histological sections is known as stereology. Stereology is typically used to characterize bone micro-architecture by quantifying cortical porosity, cortical thickness, trabecular number, trabecular thickness and trabecular connectivity. Connectivity is a stereological measurement used to quantify the topology of various structures by quantifying the number of redundant connections between locations (Russ & Dehoff 2000). Using such approaches, it has been demonstrated that, at the onset of post-menopausal osteoporosis, trabecular thinning, deeper resorption cavities, micro-fracture and loss of trabecular connectivity occur (Parfitt 1987; Compston *et al.* 1989; Lane *et al.* 1998). The micro-architecture is statistically significantly more anisotropic than normal, with fewer trabeculae transversing to the primary load axis (Ciarelli *et al.* 2000). Until recently, it was believed that bone loss occurred throughout the trabecular architecture to the same extent, and that this loss was irreversible. However, with the recent generation of high-resolution *in vivo* micro-CT scanners, new findings have challenged this premise considerably; Waarsing *et al.* (2004, 2006) found that, following the initial trabecular thinning in OVX rats, the few remaining trabeculae subsequently slowly increase in thickness. Interestingly, this was not merely due to the disappearance of thin trabeculae,

but rather certain trabeculae were preserved and in fact increased in thickness or merged with each other (Waarsing *et al.* 2006). Computational finite element models have been developed that incorporate the trabecular micro-architecture using data from high-resolution micro-CT scanning (Faulkner *et al.* 1991; Ulrich *et al.* 1997; Homminga *et al.* 2001; Testi *et al.* 2001). By applying these models, it has been shown that fracture risk prediction is improved by approximately 13 per cent as compared with predictions based on bone mass indicators (BMD) alone (Testi *et al.* 2001). This improvement is a result of the inclusion of changes in the trabecular micro-architecture such as resorption cavities, which act as stress concentrations (McNamara *et al.* 2006*a*), and thinning and loss of trabecular connectivity.

There is also evidence that the micro-architecture of cortical bone is altered in hip fracture patients by means of an increase in porosity in the bone tissue of the femoral neck compared with controls (Bell *et al.* 1999). Localized thinning of the cortical bone of the femoral neck also occurs in hip fracture patients (Crabtree *et al.* 2001).

While much research has been dedicated to assessing bone micro-architecture, the whole bone morphology (geometry) has received much less attention. It has been reported that the cross-sectional geometry of femora is altered during osteoporosis to compensate for bone loss (Cordey *et al.* 1992; Jiang *et al.* 2008). A separate study reported that a profound loss of cortical bone dramatically alters the shape and dimensions of the medullary canal of the femur, whereas little or no changes occur in the metaphysis or external dimensions of the femur (El-Zaim *et al.* 2007).

As a result of these studies, osteoporotic fractures are now believed to occur when *both* the bone mass and the micro-architecture of the bone are degraded to such an extent that bone strength is reduced and they occur under normal physiological loads. It is unknown whether these changes occur as a direct result of oestrogen deficiency or whether alternative mechanisms are responsible. By studying the other aspects of bone quality such as tissue composition, it may be possible to address this question.

2.3. Bone tissue properties and composition during osteoporosis

While bone loss and architecture have been extensively characterized, it is less clear how the remaining bone tissue is altered. Bone is a porous composite material, consisting of a mineral phase (mainly calcium and phosphorus in the form of hydroxyapatite crystals) and an organic phase (collagen, non-collagenous proteins and cells). The mechanical properties of this tissue are determined by the quantity and mechanical integrity of each phase, the structural organization of the different phases and the physical interaction between them. The organic phase of bone comprises approximately 35 per cent of bone mass and provides post-yield behaviour and strength, whereas the mineral phase allows the tissue to resist deformation under applied loading, which is known as the stiffness of the tissue. Recent studies have now established that, while overall bone mass and BMD are reduced during oestrogen deficiency, the yield strength and elastic modulus of the remaining tissue increased by 40–90% of control values (McNamara *et al.* 2005, 2006*b*). These tissue-level changes suggest that post-menopausal osteoporosis may alter the primary constituents of bone tissue, namely collagen, mineral and non-collagenous proteins (NCPs) and previous studies have focused on identifying such changes.

2.3.1. Collagen. Collagen is synthesized by osteoblast cells during the initial phase of formation when unmineralized bone (osteoid) is formed. The collagen molecules are assembled extracellularly and immature ketomine and aldimine cross-links are formed, which contribute to the formation of mature pyridinium or pyrrole cross-links. Together, these cross-links result in fibril formation and these fibrils act as a scaffold for bone minerals and provide strength (Eyre *et al.* 1988; Viguet-Carrin *et al.* 2006). The collagen in bone is primarily type I collagen, but types III, IV and VI are also present (Miller 1969; Miller 1984). Various changes in the compositional properties of the collagen have been reported at the onset of post-menopausal osteoporosis; a recent study suggests that type I collagen synthesis increases during osteoporosis, whereas earlier studies reported a reduction in the amount of type VI collagen and type III collagen (Bailey *et al.* 1993). Increased hydroxylation of lysine residues occurs (Batge *et al.* 1992; Kowitz *et al.* 1997) and this modulates the nature of the collagen cross-links that are formed extracellularly. Indeed, decreases in the quantity of ketoimine, pyridinium (Mansell & Bailey 2003), aldimine (Bailey *et al.* 1993; Oxlund *et al.* 1996) and pyrrole cross-links have been reported for osteoporotic bone (Knott *et al.* 1995). Interestingly, these changes were inversely correlated to bone volume and were more prevalent in the femoral neck, a site which is highly susceptible to fracture (Bailey *et al.* 1993). The details of these experimental studies are summarized in table 1. It has been proposed that such changes increase fracture susceptibility by altering the strength of the collagen network or the normal mineralization of the matrix (Bailey *et al.* 1993; Bailey & Knott 1999). Increased collagen metabolism (Mansell & Bailey 2003) or molecular-level defects (polymorphisms) in the type I procollagen genes (COLIA1, COLIA2) (Spotila *et al.* 1991; Grant *et al.* 1996; Harris *et al.* 2000; Efstathiadou *et al.* 2001) may be responsible for changes in collagen content and organization. Further studies are required to fully understand these changes and determine whether altered collagen plays a role in bone loss and fracture. Given that collagen acts as a scaffold for bone minerals, alterations in the collagen framework are likely to be accompanied by altered tissue mineralization.

2.3.2. Non-collagenous proteins. The NCPs in bone, in particular osteopontin, osteocalcin, alkaline phosphatase and bone sialoprotein, are essential for matrix organization by acting as mineral crystal nucleation sites on the organic matrix (Hunter & Goldberg 1993; Tye *et al.* 2003; Qiu *et al.* 2004), binding of mineral crystals to the collagen matrix (Tye *et al.* 2005) and

facilitating cell attachment (Fantner *et al.* 2005). Therefore, these proteins indirectly regulate the mechanical properties of the collagen–mineral interface. In addition, recent studies now suggest that NCPs influence bone matrix material properties in a direct fashion by forming tough bonds with molecular self-healing abilities (Fantner *et al.* 2007; Thurner *et al.* 2009). Interestingly, *in vitro* studies have shown that these bonds may have both energy dissipation (Fantner *et al.* 2005) and energy storage capabilities (Fantner *et al.* 2005; Zappone *et al.* 2008). These findings are relatively new and further studies are required to determine how such bonds could facilitate energy storage. However, if it can be confirmed that these bonds play a mechanical role *in vivo*, then this will dramatically alter the current understanding of bone fracture mechanics.

There is limited evidence that NCPs are altered during post-menopausal osteoporosis. While the overall levels of NCPs have been reported to be considerably reduced (Grynpas *et al.* 1994), other studies report that the levels of circulating alkaline phosphatase, osteocalcin and bone sialoprotein are increased following oestrogen deficiency (Jilka *et al.* 1998; Fassbender *et al.* 2000; Zhu *et al.* 2008). Furthermore, the number of osteopontin-expressing osteocytes increases in bones that tend to resorb after OVX (Ikeda *et al.* 1996). The details of these experimental studies are summarized in table 1. Future studies are required to determine whether such changes relate to the overall reduction in bone, or whether tissue-level changes in NCP content also occur. Furthermore, the link between mechanical properties, in particular fracture susceptibility, and NCPs must be delineated.

2.3.3. Mineral. Bone mineralization occurs when mineral crystals are deposited by osteoblasts on the organic matrix, bind in the presence of NCPs and over time the size of the mineral particles increases slowly (secondary mineralization). It is widely accepted that bone strength and stiffness are correlated to the degree of tissue mineralization (Currey 1984). As such, micro-structural changes in tissue properties (McNamara *et al.* 2006*b*) suggest that a corresponding change in tissue mineralization should occur. However, conflicting data exist from previous studies; some studies report a decrease in tissue mineral content (Gadeleta *et al.* 2000), and others reveal an increase in the mineral content (Dickenson *et al.* 1981; Boyde *et al.* 1998; Zioupos & Aspden 2000; McNamara *et al.* 2006*b*). These findings have been shown to differ for trabecular and cortical bone; while iliac cortical tissue has a lower mineral : matrix ratio than control, this was not seen for trabecular tissue (McCreadie *et al.* 2006). There have also been varying reports that the mineral crystal size is increased (Paschalis *et al.* 1997; Gadeleta *et al.* 2000), decreased or not altered (Baud *et al.* 1988). Variations in experimental methods, choice of animal model or anatomical location (table 1) might explain this large variability. Indeed, a recent study observed that mineral distribution is more heterogeneous in bone tissue from female fracture patients, with both higher and lower mineralization values than non-fracture control subjects (Ciarelli *et al.* 2003). Further studies are required to delineate the exact nature of such changes, the mechanisms by which such changes initiate and whether such changes are limited to specific anatomical locations or occur in human osteoporotic bone.

2.4. Osteoporosis and bone micro-damage

Bone micro-damage has long been considered to be a key determinant of bone strength (Burr *et al.* 1998) and it is also believed that, during normal bone remodelling, osteoclasts maintain bone strength by resorbing mature bone thereby preventing damage accumulation (Frost 1960*b*; Burr *et al.* 1985). There is some evidence that the extent of micro-damage is increased during oestrogen deficiency (Dai *et al.* 2004). This damage may have accumulated as a result of the increased tissue mineralization (McNamara *et al.* 2006*b*). Such changes might reduce the fracture toughness of bone tissue and thereby render the tissue more brittle and susceptible to damage, as has been well explored (Mashiba *et al.* 2001; Burr 2003; Schaffler 2003). Alternatively, bone tissue may be more susceptible to damage following bone loss when the remaining tissue bears higher loads than normal. It is not known whether such damage is sufficient to deteriorate the mechanical behaviour of the tissue to such an extent as to predispose it to failure. Nonetheless, increased remodelling may be attributable to an accelerated removal of damaged tissue (Burr *et al.* 1997) and as such this increased damage may indeed play a significant role in bone loss and subsequently fracture. Future studies are required to definitively prove that micro-damage repair is increased during post-menopausal osteoporosis. Such understanding might be gained through a greater understanding of the physiological activity of bone cells throughout the disease state.

3. BONE PHYSIOLOGY DURING OSTEOPOROSIS

The normal bone remodelling process is regulated by bone cells, osteoblasts and osteoclasts, which operate simultaneously in a unit known as the basic multi-cellular unit (BMU). The process initiates when bone-lining cells degrade unmineralized osteoid and increase the expression of growth factors to recruit osteoclast progenitors to differentiate from the haematopoietic progenitors in the bone marrow. These osteoclasts differentiate into mature multi-nucleated cells that attach to bone surfaces, via binding of the integrin $\alpha_v\beta_3$ and vitronectin, and secrete acids and enzymes to digest bone matrix. Following resorption, additional growth factors are released which recruit osteoblast precursors to differentiate and produce new bone matrix (Parfitt 2005) in the place of resorbed tissue. Osteoblasts also produce growth factors that regulate osteoclast activity. Osteoblasts are derived from osteoprogenitors that reside in the bone marrow and periosteal lining of bone surfaces. These progenitors are induced to differentiate into mature osteoblasts in the presence of various growth factors. Certain osteoblasts become encased in mineralized bone matrix and

Table 1. Changes in bone tissue constituents during oestrogen deficiency (−− indicates a reduction in the amount of constituent; ++ increase in the amount of constituent; +/− the variable changes in tissue constituent).

tissue constituent	origin	function/role	study design	key findings	reference
collagen					
type I collagen	collagen molecule produced by osteoblasts	provides mechanical strength and acts as a scaffold for mineralization	enzyme-linked immunosorbent assay (ELISA) of bone tissue from normal and osteoporotic human bone	++	Mansell & Bailey (2003)
type III collagen	collagen molecule produced by osteoblasts	provides mechanical strength and acts as a scaffold for mineralization	SDS-PAGE electrophoresis of femoral bone tissue from normal and osteoporotic human bone	−−	Bailey *et al.* (1993)
type VI collagen	collagen molecule produced by osteoblasts	regulates collagen fibre diameter and orientation of the fibres in the tissue	SDS-PAGE electrophoresis and western blotting of femoral bone tissue from normal and osteoporotic human bone	−−	Bailey *et al.* (1993)
hydroxylation of lysine residues	intracellular (osteoblast) post-translational modification of collagen molecules	governs the nature of cross-links formed and regulates collagen fibre diameter	amino acid analysis (AAA) of femoral bone tissue from normal and osteoporotic human and avian bone	++	Batge *et al.* (1992), Knott *et al.* (1995), Kowitz *et al.* (1997), Mansell & Bailey (2003)
ketoimine collagen cross-links	immature collagen cross-link formed extracellularly	provides mechanical strength between collagen fibrils	AAA of femoral bone tissue from normal and osteoporotic human bone	−−	Mansell & Bailey (2003)
aldimine collagen cross-links (DHLNL, HLNL)	immature collagen cross-link formed extracellularly	provides mechanical strength between collagen fibrils	AAA of femoral and vertebral bone tissue from normal and osteoporotic human bone	−−	Bailey *et al.* (1993), Oxlund *et al.* (1996)
pyridinium collagen cross-links	mature collagen cross-link derived from the immature ketoimine cross-links	provides mechanical strength between collagen fibrils	AAA of femoral bone tissue from normal and osteoporotic human bone	−−	Mansell & Bailey (2003)
pyrrole collagen cross-link	mature collagen cross-link derived from the immature aldimine cross-links	provides mechanical strength between collagen fibrils	biochemical analysis of bone tissue from normal and osteoporotic hens by reaction with *p*-dimethylaminobenzaldehyde	−−	Knott *et al.* (1995)
non-collagenous proteins					
alkaline phosphatase	non-collagenous protein produced by osteoblasts	act as sites for mineral crystal nucleation	biochemical analysis (autoanalyser with *p*-nitrophenyl phosphate) of bone tissue from normal and osteoporotic human bone enzyme immune assay (EIA) of bone tissue from normal and ovariectomized rats	++	Mansell & Bailey (2003) Zhu *et al.* (2008)

(*Continued.*)

Table 1. (*Continued.*)

tissue constituent	origin	function/role	study design	key findings	reference
osteopontin	non-collagenous protein produced by osteoblasts	act as sites for mineral crystal nucleation, provide mechanical bonding between collagen fibrils	*in situ* hybridization of bone tissue of normal and ovariectomized rats	++	Ikeda *et al.* (1996)
osteocalcin	non-collagenous protein produced by osteoblasts	act as sites for mineral crystal nucleation, provide mechanical bonding between collagen fibrils	radioimmunoassay of serum obtained from normal and ovariectomized mice	++	Jilka *et al.* (1998)
bone sialoprotein	non-collagenous protein produced by osteoblasts	act as sites for mineral crystal nucleation and regulate crystal growth, provide mechanical bonding between collagen fibrils	radioimmunoassay of serum obtained from human osteoporotic patients	++	Fassbender *et al.* (2000)
mineral					
tissue mineral content	calcium phosphate produced by osteoblasts and binds to collagen in the presence of non-collagenous proteins	regulates the mechanical stiffness of bone tissue	fourier transform infrared micro-spectroscopy (FTIRM) of femoral bone tissue from normal and osteoporotic human bone	−−	Boyde *et al.* (1998)
			quantitative backscattered electron imaging (qBEI) of bone tissue from ovariectomized monkeys	−−	Gadeleta *et al.* (2000)
			micro-CT scanning calibrated for mineral content assessment of trabecular bone tissue from normal and ovariectomized rats	++	McNamara *et al.* (2006*b*)
size of mineral crystals	calcium phosphate crystals mature and grow over time	regulates the mechanical stiffness of bone tissue	FTIRM of iliac and vertebral bone tissue from normal and osteoporotic human and monkey bone	++	Paschalis *et al.* (1997), Gadeleta *et al.* (2000)
mineral distribution	distribution of mineral crystal deposition within the bone matrix	regulates the mechanical stiffness of bone tissue	backscattered electron microscopy of bone tissue from normal and osteoporotic human bone	++	Ciarelli *et al.* (2003)

differentiate to become osteocytes, which are essential for secondary mineralization (Frost 1960*a*; Inoue *et al.* 2006; Gluhak-Heinrich *et al.* 2007) and can transduce mechanical signals into biochemical signals to osteoclasts and osteoblasts to alter bone mass (Cowin *et al.* 1991; Lanyon 1993). During normal physiology osteoclasts, osteoblasts and osteocytes work in concert to maintain bone mass.

3.1. Osteoclasts and haematopoietic progenitors

Osteoclasts possess oestrogen receptors (ERα, ERβ), which are normally activated to permit protein synthesis when they bind to oestrogen (Braidman *et al.* 2001). Oestrogen has an inhibitory effect on osteoclasts as the proteins produced following oestrogen binding inhibit bone resorption by decreasing the formation of mature osteoclasts (Oursler *et al.* 1991*b*) and increasing osteoclast apoptosis (Hughes *et al.* 1996). When oestrogen levels are deficient, the number of haematopoietic progenitors increases (Jilka *et al.* 1992, 1995), thereby providing a larger pool from which osteoclast progenitors can be recruited and ultimately an increase in the numbers of osteoclasts forming (Jilka *et al.* 1992, 1995; Brockstedt *et al.* 1993; Rosen 2000*b*). In addition, osteoclast apoptosis

Table 2. Bone physiology during oestrogen deficiency (−− indicates a reduction in cell activity; ++ is an increase in cell activity; +/− the variable changes in cell activity).

cell activity	function/role	study design	key findings	reference
haematopoietic progenitors and osteoclasts				
haematopoietic progenitors (circulating monocytes)	source of progenitor cells in the bone marrow that can differentiate into osteoclast progenitors and osteoclasts	*ex vivo* cultures of bone marrow cells from normal and ovariectomized mouse tissue	++	Jilka *et al.* (1992, 1995)
osteoclast formation	differentiation of haematopoietic progenitors into osteoclasts	*ex vivo* cultures of bone marrow cells from normal and ovariectomized mouse tissue	++	Jilka *et al.* (1992)
mature osteoclasts	active bone resorbing cells that release hydrogen and cytokines to digest the bone matrix	histomorphometry analysis of bone tissue from tibia of normal and ovariectomized mice and rats	++	Turner *et al.* (1988), Jilka *et al.* (1992)
osteoclast surface	determines the amount of surface through which osteoclasts digest bone matrix	histomorphometry analysis of bone tissue from tibia of normal and ovariectomized rats	++	Turner *et al.* (1988)
osteoclast apoptosis	inhibits the bone resorption process	oestrogen deficiency induced *in vitro* using the oestrogen antagonist ICI 164 384. Apoptosis characterized by morphological changes in nuclei	−−	Kameda *et al.* (1997)
osteoprogenitors, osteoblasts and osteocytes				
pool of mesenchymal progenitors	provides a source of cells that can differentiate into osteoblasts	*in vitro* cell culture of marrow cells isolated from sham-operated or ovariectomized mice	+/−	Jilka *et al.* (1998)
osteoblastogenesis	process of differentiation of osteoprogenitors into osteoblasts	*in vitro* cell culture of marrow cells isolated from sham-operated or ovariectomized mice	++	Jilka *et al.* (1998), Rosen (2000*b*)
organization of osteocyte cell process network	acts to transmit biochemical and electrical stimuli between osteocyte cells, may also act as strain or fluid flow sensors	histological imaging of bone samples from human osteoporotic subjects	−−	Knothe Tate *et al.* (2004)
osteocyte apoptosis	initiates an inflammatory response to remove apoptotic cells	TUNEL staining of bone tissue sections from normal and ovariectomized mice	++	Tomkinson *et al.* (1997), Kousteni *et al.* (2001)
osteocyte density	may regulate the sensory capacity of the tissue	histomorphometry of normal and osteoporotic human bone	−−	Mullender *et al.* (1996)
mechanosensitivity	initiates a biochemical response to mechanical stimuli	*in vitro* cell culture of primary human osteoblasts under fluid flow	−−	Sterck *et al.* (1998), Jessop *et al.* (2004)

is inhibited and these events together lead to prolonged resorption, deeper resorption cavities and trabecular perforation (Bell *et al.* 1996). The perforated trabeculae are removed by further remodelling and this loss of bone mass increases the fragility of bones, resulting in a greater propensity for fracture. These findings and details of the experimental studies are summarized in table 2.

Although osteoclasts are the primary target for conventional drug treatments, inhibiting osteoclast activity alone does not completely prevent osteoporotic fractures from occurring. Interestingly, a recent study

found that oestrogen inhibited osteoclast formation *in vitro* when osteoclast precursors were cocultured with osteoblastic cells, but that the hormone did not directly affect osteoclast precursors when they were cultured alone (Michael *et al.* 2005). This study strongly suggests that oestrogen's regulation of osteoclast activity is governed by osteoblasts (Michael *et al.* 2005) and hence the role of osteoblasts during post-menopausal osteoporosis merits further research.

3.2. Osteoblasts, osteocytes and osteoprogenitors

Osteoblasts and osteocytes also possess receptors for oestrogen (Braidman *et al.* 2001), hence their function may be affected when oestrogen production is deficient during post-menopausal osteoporosis. Evidence suggests that oestrogen allows the normal response of osteoblasts and osteocytes to loading (Lanyon 1996) and osteoblastic cells deprived of oestrogen display deficient osteogenic responses to mechanical stimuli *in vitro* (Sterck *et al.* 1998; Jessop *et al.* 2004). Oestrogen deficiency induces osteocyte apoptosis (Tomkinson *et al.* 1997; Kousteni *et al.* 2001), which might result in hypermineralization of the surrounding tissue (Frost 1960*a*; Kingsmill & Boyde 1998; Boyde 2003). In addition, the organization of the osteocyte network is altered (Knothe Tate *et al.* 2004) and osteocyte density is increased in post-menopausal bone, which might indicate that osteoblasts produce less bone per cell during osteoporosis (Mullender *et al.* 1996). Together, these changes might alter the mechanoresponsiveness of the tissue (Tatsumi *et al.* 2007).

Changes in osteoblastic activity during oestrogen deficiency might initiate when the cells are in the immature osteoprogenitor state. Indeed, it has been reported that the number of mesenchymal progenitors capable of committing to the osteoblast lineage (osteoblastogenesis) increases in the murine bone marrow in an ovariectomized animal model of osteoporosis (Jilka *et al.* 1998; Rosen 2000*b*). In contrast to these findings, no difference has been found in the proliferation of osteoprogenitors from normal in human osteoporotic bone (Stenderup *et al.* 2001). *In vitro* cell culture experiments confirm that mesenchymal stem cells from osteoporotic patients maintain their ability to differentiate along the osteogenic lineage (Justesen *et al.* 2002). Further studies are required to fully understand the differences in these experimental findings (table 2) and to comprehensively characterize the physiology of osteoprogenitors, osteoblasts and osteocytes during post-menopausal osteoporosis. Given the tightly coupled nature of bone cell activity, such studies may provide further insight into osteoclast activity and bone loss.

4. MOLECULAR SIGNALLING AND GENE EXPRESSION DURING OSTEOPOROSIS

A number of cytokines and growth factors govern the activity of bone cells during normal bone remodelling. In particular, cytokines and growth factors, namely the receptor activator of nuclear factor kappa B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) and osteoprotegrin (OPG) secreted by osteoblasts and bone-lining cells, are the primary regulators of the process. Normal osteoclast activity is governed by RANKL and M-CSF, which bind to the paracrine receptor RANK on osteoclast precursors and stimulate proliferation and differentiation into mature osteoclasts (Sarma & Flanagan 1996; Lerner 2006). OPG acts to inhibit osteoclast differentiation at the end of the resorption process by binding to RANKL and blocking RANK (Lerner 2006). Many studies have sought to understand post-menopausal bone loss by studying the expression of these factors. RANKL expression is upregulated in osteoporotic bone (Ikeda *et al.* 2001; Eghbali-Fatourechi *et al.* 2003) and MCS-F expression is increased in primary cells harvested from the animal models of post-menopausal osteoporosis (Srivastava *et al.* 1998). Decreased serum levels of OPG have been reported in osteoporotic patients (Yano *et al.* 1999), albeit that the overexpression of OPG in an animal model leads to a severe osteoporotic phenotype (Bucay *et al.* 1998; Mizuno *et al.* 1998).

Other growth factors and cytokines also regulate osteoclast activity: tumour necrosis factor (TNF-α), the interleukin cytokines (interleukin-1 (IL-1), IL-6, IL-7), transforming growth factor (TGF-β), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) (Lam *et al.* 2000; Lerner 2006; Teitelbaum 2006). Therefore, an alternative view is that bone loss initiates through the altered expression of such factors, instead of changes in RANK, M-CSF or OPG expression. Of these, TNF-α production is increased in post-menopausal osteoporosis (Zheng *et al.* 1997; Cenci *et al.* 2000; Lam *et al.* 2000), TGF-β1 and TGF-β3 exhibit a marked decrease in expression during oestrogen deficiency (Orlic *et al.* 2007), IL-1 and IL-6 expression is upregulated in human osteoporotic bone (Ralston 1994; Zheng *et al.* 1997), while IL-7 and the IL-7 receptor are increased in ovariectomized animal models (Weitzmann *et al.* 2002; Orlic *et al.* 2007; Sato *et al.* 2007). Therefore, osteoclast recruitment may occur as a secondary response to these changes. The origin of these factors, observed changes and implication of such changes are summarized in table 3.

There is also evidence that the expression of molecules that modulate osteoblast activity may be altered during post-menopausal osteoporosis. During normal bone resorption, growth factors are released from the extracellular matrix, including insulin growth factor-I (IGF-I) and TGF-β, which recruit and activate osteoblasts to begin collagen synthesis. *In vitro* studies have shown that the proliferation of osteoblasts from osteoporotic donors is altered as a result of impaired IGF-I receptor signalling (Perrini *et al.* 2008) and also that IGF-I expression is decreased during oestrogen deficiency (Lerner 2006). However, it is still unclear whether the expression is indeed altered during post-menopausal osteoporosis (Rosen 2000*a*). Deposition of TGF-β is also reduced during oestrogen deficiency *in vitro* and *in vivo* (Oursler *et al.* 1991*a*; Finkelman *et al.* 1992). These findings are summarized in table 3.

Table 3. Differentially expressed molecules and genes during oestrogen deficiency (−− indicates a reduction in expression; ++ is an increase in expression; +/− the variable changes in expression).

molecule	origin	function in bone	expression during oestrogen deficiency	reference
molecules modulating osteoclast function				
RANKL	cytokine produced by osteoblasts and bone lining cells	binds to RANK on the osteoclasts to stimulate osteoclast proliferation and differentiation	++	Ikeda *et al.* (2001), Eghbali-Fatourechi *et al.* (2003)
M-CSF	cytokine produced by osteoblasts and bone lining cells	stimulates osteoclast proliferation and differentiation, prevents apoptosis	++	Srivastava *et al.* (1998)
OPG	cytokine produced by T cells and osteoblasts	binds to RANKL on osteoblasts to inhibit the differentiation of osteoclast precursors into osteoclasts	−−	Yano *et al.* (1999)
TNF-α	cytokine produced by T cells, macrophages and fibroblasts	stimulates differentiation of osteoclast progenitors and increases the activity of mature osteoclasts	++	Zheng *et al.* (1997), Cenci *et al.* (2000), Lam *et al.* (2000)
TGF-β1	protein produced by osteoblasts and released from extracellular matrix during resorption	stimulates the differentiation of monocytes into osteoclast precursors and enables osteoclastogenesis	−−	Ikeda *et al.* (1993) Oursler *et al.* (1991*a*), Finkelman *et al.* (1992)
TGF-β3	protein produced by osteoblasts and released from extracellular matrix during resorption	stimulates the differentiation of monocytes into osteoclast precursors and enables osteoclastogenesis	−−	Orlic *et al.* (2007)
IL-1	cytokine produced by monocytes in the bone marrow	stimulates the differentiation of osteoclast precursors and bone resorption	++	Ralston (1994), Zheng *et al.* (1997)
IL-6	cytokine produced by T cells in the bone marrow	promotes osteoclastogenesis	++	Ralston (1994), Zheng *et al.* (1997)
IL-7	growth factor secreted by marrow stromal cells and osteoblasts	stimulates osteoclastogenesis by upregulating T-cell-derived cytokines, including RANKL	++	Weitzmann *et al.* (2002), Orlic *et al.* (2007), Sato *et al.* (2007)
molecules modulating osteoblast function				
IGF-1	hormone produced by osteoblasts and released from extracellular matrix during resorption	recruits and activates osteoblasts to begin collagen synthesis	−−	Lerner (2006)
TGF-β1	protein produced by osteoblasts and released from extracellular matrix during resorption	promotes osteoprogenitor proliferation and osteogenesis, inhibits mineralization	−−	Oursler *et al.* (1991*a*), Finkelman *et al.* (1992)
genes modulating osteoclast function				
TREM-2	gene encoding cell surface receptors expressed on osteoclast precursors	regulates the differentiation of osteoclast precursors into mature multi-nucleated osteoclasts	++	Hopwood *et al.* (2009)
CCL2	gene expressed by mature osteoclasts that encodes the chemokine (C–C motif) ligand 2	regulates the recruitment and fusion of osteoclast precursors	++	Hopwood *et al.* (2009)
RANK	gene encoding the protein RANK expressed by osteoclasts	regulates osteoclastogenesis by binding to RANKL on osteoblasts	++	Hopwood *et al.* (2009)

(*Continued.*)

Table 3. (*Continued.*)

molecule	origin	function in bone	expression during oestrogen deficiency	reference
IL-6	gene encoding the cytokine IL-6 secreted by osteoblasts	promotes osteoclastogenesis	++	Hopwood *et al.* (2009)
CD14	gene encoding the surface protein CD14 expressed by monocytes in the marrow	cell surface protein that may regulate bone mass	++	Hopwood *et al.* (2009)
CTSB	gene encoding the production of cathepsin B produced by osteoblastic cells	activates enzymes involved in the osteoclast bone resorption process	++	Hopwood *et al.* (2009)
genes modulating osteoblast function				
TGFB1	gene encoding the production of TGF-β1	recruits and activates osteoblasts to begin collagen synthesis	−−	Langdahl *et al.* (1997), Yamada *et al.* (1998), Hopwood *et al.* (2009)
TGFB3	gene encoding the production of TGF-β3	promotes osteoprogenitor proliferation, osteogenesis, inhibits mineralization	−−	Orlic *et al.* (2007)
SPP1	gene encoding the production of the protein osteopontin	faciliates the attachment of osteoclasts to mineralized matrix	++	Hopwood *et al.* (2009)
CTGF	gene encoding the connective tissue growth factor	promotes proliferation of osteoblast progenitors	++	Hopwood *et al.* (2009)
CTSB	gene encoding the production of cathepsin B produced by osteoblastic cells	may regulate the osteoblast mineralization process	++	Hopwood *et al.* (2009)
RUNX2(Cbfa1)	transcription factor produced by mesenchymal cells and osteoblasts	inhibits mesenchymal cell proliferation but promotes differentiation towards the osteoblast lineage and matrix production	alleles identified which are correlated to BMD	Geoffroy *et al.* (2002), Vaughan *et al.* (2002)

A vast number of genes regulate the normal bone turnover process (Garnero *et al.* 1994; Lerner 2006) and various genetic mutations have been identified in subjects with lower bone mass (Baldock & Eisman 2004) or who have experienced an osteoporotic fracture (Tsangari *et al.* 2004; Hopwood *et al.* 2009). In particular, polymorphisms in the ER promoter have been identified that are correlated to bone loss during osteoporosis (Weel *et al.* 1999). Putative candidate genes for the development of osteoporosis are RUNX2 (also known as Cbfa1) (Vaughan *et al.* 2002); Chemokine receptor 3 (CCR3), histidine decarboxylase (HDC) and glutocorticoid receptor (GCR) (Liu *et al.* 2005); matrix metallopeptidase 8 (MMP8), procollagen types I and VI, PTH receptor 1 and WD repeat domain 5 (Orlic *et al.* 2007), all of which are involved in skeletal development or have regulatory effects on bone cells. While these studies provide much insight, each of the studies focused on a particular subset of genes and could not provide a complete picture of alterations that occur.

It must be noted that only one study (Hopwood *et al.* 2009) has comprehensively characterized differential gene expression in post-menopausal osteoporotic human bone. This study confirmed alterations in several genes that regulate osteoclastogenesis and bone resorption, in particular TREM-2, CCL2, RANK and IL-6 identified in previous studies (Tsangari *et al.* 2004). Furthermore, this study confirmed that genes responsible for osteoblast maturation are differentially expressed in human osteoporotic tissue, in particular CTSB, TGFB1 which had previously been identified (Langdahl *et al.* 1997; Yamada *et al.* 1998), but also SPP1 and CTGF (Hopwood *et al.* 2009). As these genes are known to regulate mineralization and NCP production *in vitro*, this study provides further evidence that tissue-level changes in bone composition occur. The details of the studies which identified these changes are summarized in table 3. It is crucial that these findings are widely understood across multiple disciplines and further investigated.

Several molecular signalling candidates and genes that regulate osteoclast behaviour have been identified as putative candidates for the development of post-menopausal osteoporosis through *in vitro* and *in vivo* studies. More recent studies indicate that molecules that govern osteoblast activity are also altered. Future studies are required to delineate the sequence with which these molecules are expressed, specifically

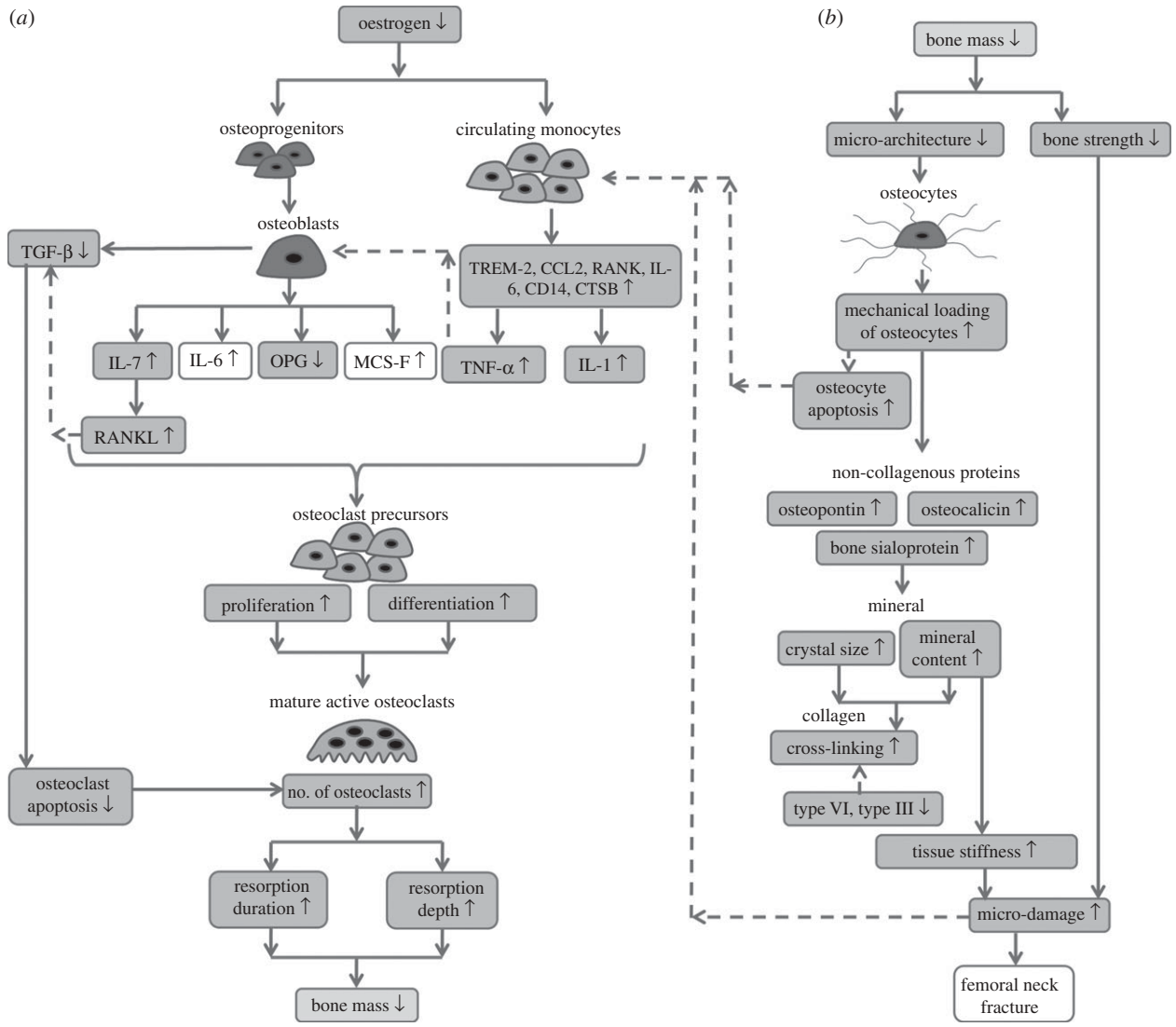

Figure 2. (*a*) Primary sequence of events; molecules governing osteoclast activity are upregulated leading to increased osteoclastogenesis, while molecules inhibiting osteoclasts are downregulated. These events reduce apoptosis and result in excess bone resorption. (*b*) Secondary responses to bone loss; mechanical loading on the remaining tissue is increased and tissue mineralization is altered to compensate for bone loss. Increased mineralization may dissociate collagen or non-collagenous protein bonds and lead to damage accumulation and ultimately bone fracture. Feedback mechanisms are indicated by dashed lines. Unfilled rectangle, *in vitro*; filled rectangle, osteoporosis/OVX.

whether expression is altered as a primary or secondary effect of oestrogen deficiency.

5. UNDERSTANDING THE SEQUENCE OF EVENTS IN THE BONE LOSS CASCADE

A vast amount of research has been undertaken to understand the pathogenesis and biomechanical consequences of post-menopausal osteoporosis and has provided strong evidence of complex changes in molecular and cellular biology, tissue composition, tissue integrity and architecture. However, significant findings from distinct disciplines are not widely reported or understood in an interdisciplinary fashion. As a result, the sequence with which such changes occur in the bone loss cascade is unknown.

It is intriguing to speculate on this sequence based on the existing knowledge. It has long been presumed that osteoclasts are primarily affected by oestrogen deficiency and there is sufficient evidence to support a subsequent sequence of events within this context. Firstly, molecules that promote osteoclast activity (MCS-F, RANKL, TNF-α, interleukins) are upregulated during oestrogen deficiency, whereas those that inhibit osteoclasts (OPG, TGF-β) are downregulated (figure 2*a*). Accordingly, osteoclastogenesis increases and osteoclast apoptosis decreases leading to deeper resorption for a prolonged duration (figure 2*a*). Together, these changes accelerate bone resorption and thereby reduce bone mass. The complex changes in osteoblast behaviour, tissue composition and micro-damage may occur as a secondary response to bone loss (figure 2*b*) because the remaining tissue and mechanosensitive osteocytes will be subject to elevated loading. This might initiate an adaptive response whereby osteoblasts and osteocytes alter local tissue composition (NCPs and mineral) and mechanical properties of this remaining tissue to

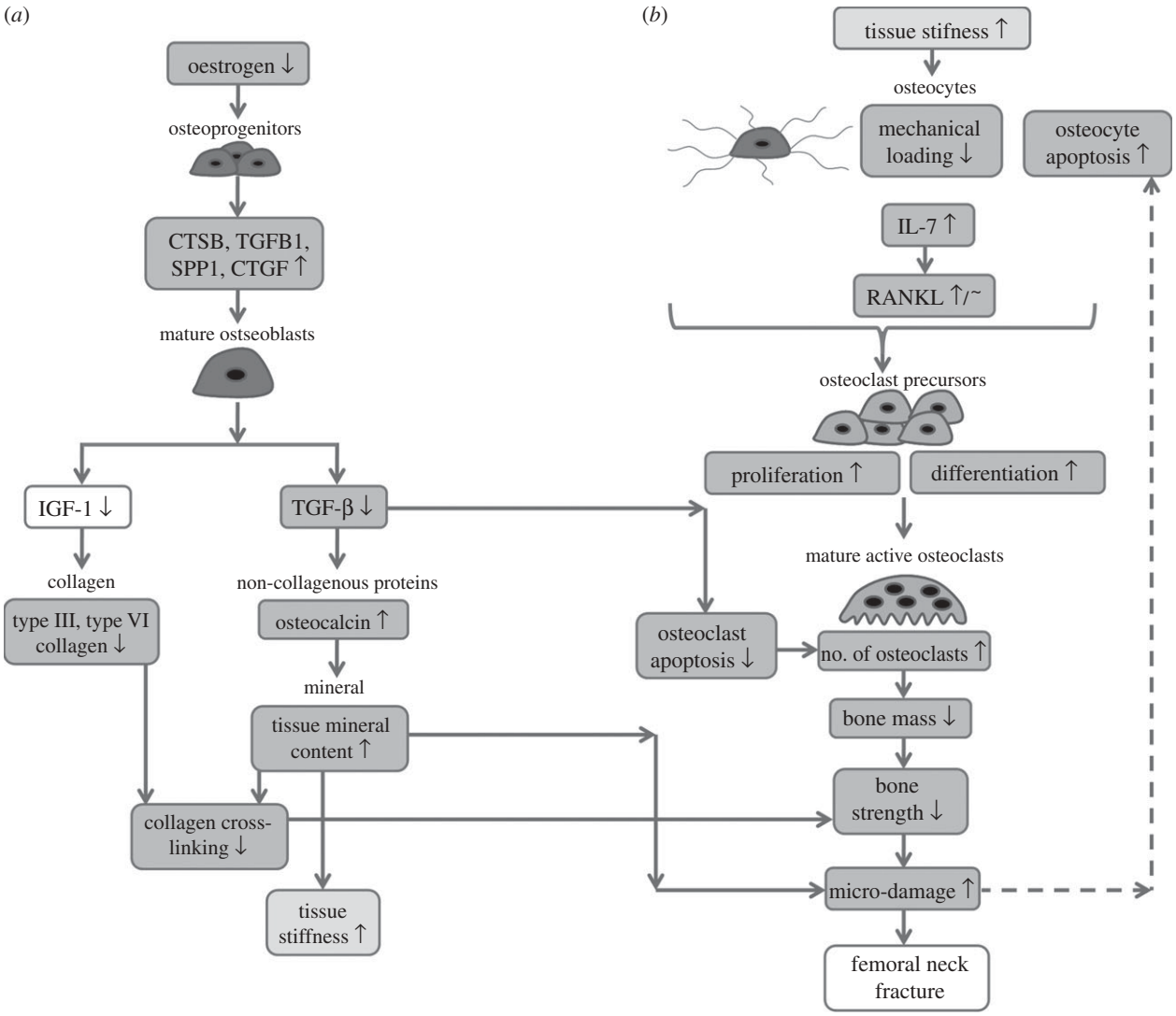

Figure 3. (*a*) Primary sequence of events; genetic and molecular regulators of osteoblasts alter their activity to increase the production of proteins that govern osteoblastogenesis, tissue mineralization and collagen synthesis, which subsequently alters tissue stiffness. (*b*) Secondary responses; as a consequence of increased stiffness the mechanical loading to osteocytes reduces and, in response, osteoclastic resorption occurs to remove excess bone tissue. The changes in collagen cross-linking and damage accumulation ensue as a result of increased mineral and these events culminate and increase the fracture susceptibility of the tissue. Feedback mechanisms are indicated by dashed lines. Unfilled rectangle, *in vitro*; filled rectangle, osteoporosis/OVX.

compensate for bone loss (figure 2*b*). Concurrently, larger or more numerous mineral crystals might dissociate collagen cross-links or prevent the formation of mature cross-links (Otsubo *et al.* 1992). Damage may accumulate as a result of the increasingly brittle nature of the tissue and/or the increased loading. These events combined will increase the fracture susceptibility of the tissue. Although substantial evidence exists to support such a sequence of events, it has not yet been established whether changes in tissue composition are indeed a secondary response to bone loss. Furthermore, it remains that suppressing osteoclast resorption does not prevent osteoporotic fractures, which suggests that osteoclastic activity may not be primarily responsible for the bone loss cascade.

An alternative premise proposed here is that post-menopausal bone loss is not a straightforward case of accelerated osteoclast bone resorption, but rather that the bone loss cascade initiates through oestrogen's effects on osteoblasts. Osteoblasts maintain bone strength by producing, organizing and regulating the tissue matrix, but also possess oestrogen receptors suggesting that these functions may be altered when oestrogen levels are deficient. An alternative sequence is therefore proposed; firstly, differential expression of genes known to regulate osteoblast activity may occur (CTSB, TGFB1, SPP1 and CTGF) followed by the decreased production of IGF-I and TGF-β (figure 3*a*). As these proteins are the regulators of collagen matrix and NCP production (table 3) it would be expected that the changes in both tissue constituents occur (figure 3*a*). It is known that the NCP osteocalcin promotes tissue mineralization (Roy *et al.* 2001) so increased secondary mineralization may occur as a result of the increased levels of this protein (figure 3*a*). This would thereby increase the elastic modulus of the tissue (McNamara *et al.* 2006*b*). Within this context, bone resorption is proposed to be

a secondary response to altered tissue stiffness (figure 3*b*); firstly, the deformation of the tissue matrix would be reduced under normal loading and this may in turn initiate an osteoclastic resorptive response to unloading, similar to stress shielding. Increased crystal size may dissociate collagen cross-links and lead to increased micro-damage accumulation, and together these events would ultimately result in bone fractures. Experimental evidence to support such a sequence is limited, but recent computational studies applied mechanoregulation models and predicted that (i) osteocytes residing in stiffer tissue are subject to reduced strain levels and initiate resorption leading to trabecular perforation (Mulvihill *et al.* 2008) and (ii) if bone tissue stiffness increases, the structure adapts to achieve a lower mass and increased anisotropy similar to osteoporotic bone (van der Linden *et al.* 2004). Future studies must provide the experimental evidence to delineate whether such a sequence occurs during post-menopausal osteoporosis.

6. KEY CHALLENGES TO UNDERSTANDING THE DISEASE OF OSTEOPOROSIS

While two putative sequences have been considered here, further studies are required to definitively describe the sequence of events in the bone loss cascade. A fundamental challenge to understanding the sequence of events lies in the fact that bone adaptation is tightly coupled and any alteration in one of the features of bone quality has resultant effects on each of the others. Therefore, it is imperative to determine whether the observed changes in gene expression, molecular signalling, cell physiology and tissue composition occur immediately at the onset of oestrogen deficiency, and whether they may be causative, or occur later and are thereby secondary responses to bone loss.

To answer such questions, and advance current understanding of the disease, the key challenges are to perform comprehensive multi-disciplinary experiments using both animal models and human subjects. In particular, these studies will require

(i) Comprehensive monitoring of gene and molecular expression immediately post-ovariectomy or in the early stages of oestrogen deficiency (<1 week) to determine the sequence with which regulatory molecules are altered. Such studies would define the initial event postulated in figures 2 and 3 and thereby distinguish whether osteoclast or osteoblast activity is primarily altered in post-menopausal osteoporosis.

(ii) A combination of *in vivo* and *in vitro* studies to monitor osteoblastic activity, e.g. molecular expression, matrix production and mineralization, in the early stages of oestrogen deficiency (>1 week) and at multiple time points as oestrogen deficiency continues. These studies would provide the essential knowledge to understand how bone tissue composition is altered and whether this is a primary (figure 2) or a secondary (figure 3) effect of oestrogen deficiency. *In vitro* experiments are confounded by the difficulties associated with extrapolating *in vitro* results to understand *in vivo* physiology and pathology. Therefore, a greater understanding will be gained by combining *in vivo* histology and *in vitro* culture models to focus on the effects of oestrogen deficiency on osteoblastic cells.

(iii) Quantification of complex changes in tissue composition and micro-architecture in the early-stage oestrogen deficiency and at multiple later time points. Such studies would delineate the exact nature of tissue-level changes and when these arise thereby identifying whether these are altered as a primary or a secondary response to oestrogen deficiency.

(iv) Mechanical characterization at the tissue level and whole bone level at multiple time points from the early-stage oestrogen deficiency. Correlation between mechanical properties, bone mass and tissue composition will delineate how these properties are interrelated and contribute to fracture risk.

If these quantities were better understood, then it might be possible to understand the origins of post-menopausal osteoporosis and inform the development of effective treatments to inhibit the disease. For example, if osteoblastic activity is found to alter tissue composition prior to bone loss, then such findings could pave the way for developing a drug treatment that targets osteoblastic activity rather than osteoclast resorption. Alternatively, if tissue composition is altered to compensate for bone loss, then treatments may be developed to capitalize on this phenomenon. Given the inadequacies of the current treatments, advancements are undoubtedly required and further interdisciplinary research at the interface of biomechanical engineering, materials science and cell and molecular biology is required to derive the necessary information to assist in such developments.

7. CONCLUSION

It remains the case that bone fracture during post-menopausal osteoporosis has not yet been eliminated. Future research studies should include multi-disciplinary analyses at multiple time points to comprehensively characterize the sequence of changes in molecular signalling, cell physiology, tissue composition, micro-architecture, damage and bone mass. Such research will be fundamental to deriving a coherent understanding of the disease and defining the sequence of events that ultimately lead to bone fractures.

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