

Osteoblast physiology in normal and pathological conditions

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Abstract Osteoblasts are mononucleated cells that are derived from mesenchymal stem cells and that are responsible for the synthesis and mineralization of bone during initial bone formation and later bone remodelling. Osteoblasts also have a role in the regulation of osteoclast activity through the receptor activator of nuclear factor κ -B ligand and osteoprotegerin. Abnormalities in osteoblast differentiation and activity occur in some common human diseases such as osteoporosis and osteoarthritis. Recent studies also suggest that osteoblast functions are compromised at sites of focal bone erosion in rheumatoid arthritis.

Keywords Osteoblasts · Wnt/ β -catenin pathway · RANK/RANKL/OPG · Osteoporosis · Osteoarthritis · Rheumatoid arthritis

Introduction

Osteoblasts are mesenchymal origin cells responsible for creating and maintaining skeletal architecture; these cells produce extracellular matrix proteins and regulators of matrix mineralization during initial bone formation and later bone remodelling. In addition to bone formation, osteoblasts regulate osteoclast differentiation and resorption

activity by the secretion of cytokines or by direct cell contact. In many human diseases, bone formation and bone remodelling are deregulated and osteoblasts have an important role in the pathogenesis of these disorders. In this review, we summarize the current knowledge of osteoblast physiology in normal and pathological bone.

Osteoblast physiology

Osteoblast differentiation and maturation

Bone formation is a prolonged, strictly regulated process that takes place during embryonic development, growth, remodelling and fracture repair (Aubin 2001). Bone formation is characterized by a sequence of events starting with the commitment of osteoprogenitor cells and their differentiation into pre-osteoblasts and then into mature osteoblasts whose function is to synthesize the bone matrix that becomes progressively mineralized.

Osteoblasts derive from pluripotent mesenchymal stem cells (Caplan 1991; Owen 1988; Pittenger et al. 1999), which prior to osteoblast commitment can also differentiate into other mesenchymal cells lineages such as fibroblasts, chondrocytes, myoblasts and bone marrow stromal cells including adipocytes, depending on the activated signalling transcription pathways (Friedenstein et al. 1987; Yamaguchi et al. 2000). Thus, the transcription factors of the MyoD family are necessary for the differentiation and maturation of muscle cell lineage (Weintraub 1993), whereas the peroxisome proliferator-activated receptor γ 2 (PPAR γ 2) is essential in determining the differentiation of adipocyte lineage cells (Tontonoz et al. 1994). Several specific transcription factors are responsible for the commitment of pluripotent mesenchymal cells into the osteoblast cell

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lineage. One of the most important of these is represented by *Cbfa1* (core-binding factor $\alpha 1$), a transcription factor belonging to the *runt*-domain gene family, which plays a critical role in osteoblast differentiation, although it is not sufficient alone to support the achievement of the mature osteoblast phenotype (Banerjee et al. 1997; Ducy et al. 1997; Komori et al. 1997; Komori and Ozawa 1999; Lee et al. 1999; Otto et al. 1997). *Cbfa1* is highly expressed in osteoblast lineage cells and regulates the expression of various osteoblast-specific genes (Banerjee et al. 1997; Ducy et al. 1997; Ji et al. 1998; Harada et al. 1999; Tsuji et al. 1998); *Cbfa1*-deficient mice are completely lacking in bone formation (Hoshi et al. 1999), because of the maturational arrest of their osteoblasts, whereas the over-expression of *Cbfa1* induces non-osteogenic cells to express osteoblast-related genes (Yamaguchi et al. 2000).

Another *runt*-related gene that plays an important role in the commitment of multipotent mesenchymal cells to the osteoblastic lineage and for osteoblast differentiation at an early stage is *Runx-2*. *Runx-2* is involved in the production of bone matrix proteins (Komori et al. 1997; Otto et al. 1997), as it is able to up-regulate the expression of major bone matrix protein genes, such as type I collagen, osteopontin, bone sialoprotein and osteocalcin (Ducy et al. 1997; Miyoshi et al. 1991; Ogawa et al. 1993) leading to an increase of immature osteoblasts from pluripotent stem cells; the immature osteoblasts form immature bone (Komori 2010). *Runx-2* expression is down-regulated in the late stage of osteoblast maturation, when phenotypically mature osteoblasts form mature bone (Komori 2010). *Runx-2*-deficient mice are completely lacking in bone formation, because of an absence of osteoblasts (Komori et al. 1997; Otto et al. 1997). *Osterix* (*Osx*) is also an essential transcription factor for osteoblast differentiation at an early stage (Ogawa et al. 1993), whereas it inhibits osteoblast differentiation at a late stage (Komori 2003).

Other transcription factors might participate in the regulation of the proliferation and maturation of osteoblasts, including the zinc-finger proteins, runt-domain proteins and proto-oncogenes such as *c-myc*, *c-jun*, and *c-fos* (Aubin and Liu 1996).

Osteoblast commitment, differentiation and growth are controlled by several local and systemic factors that can also act in a paracrine and/or autocrine way and that can regulate the activity of specific transcription factor (Aubin and Liu 1996). They include bone morphogenetic proteins (BMPs; Centrella et al. 1994), hedgehog proteins, cell growth factors (Canalis et al. 1993) such as fibroblast growth factor (FGF) and insulin-like growth factor (IGF), hormones (Cheng et al. 1994), cytokine modulators (Goldring and Goldring 1990), canonical Wingless (Wnt)/ β -catenin (Ambrosetti et al. 2008; Hu et al. 2005; Mukherjee and Rotwein 2009) and mechanical physical

forces (Baumbach et al. 1984; Buckley et al. 1990). These factors can exhibit different and often opposite effects in modulating cell metabolism depending on the maturation stage and cell phenotype (Canalis et al. 1988; Globus et al. 1988; MacDonald et al. 1993).

BMP-2, BMP4 and BMP-7 have been shown to be able to induce immature cells to differentiate into osteoblasts (Ahrens et al. 1993; Asahina et al. 1996; Wang et al. 1993). BMP-7 induces the expression of *Cbfa1* mRNA (Ducy et al. 1997), indicating that *Cbfa1* represent a nuclear target of BMPs signalling during osteoblast differentiation, even if other transcription factors might also be involved in BMPs signalling. Conversely, many factors can affect *Cbfa1* expression, such as transforming growth factor-beta (TGF- β), which can up-regulate *Cbfa1* (Lee et al. 1999).

The progressive development of the osteoblast phenotype from a proliferating immature cell to a mature osteoblastic cell synthesizing specific bone proteins is characterized by a definite sequential expression of tissue-specific genes that identifies three distinct periods of osteoblast phenotype development: proliferation, maturation and extra-cellular matrix synthesis, and matrix mineralization.

Several studies support the hypothesis that proliferation is strictly dependent upon the synthesis of bone-specific extracellular matrix, whose maturation contributes to up-regulate the proliferation stage. During the active proliferation phase, osteoblast-committed progenitor cells (pre-osteoblasts) express genes that support proliferation and several genes encoding for extracellular matrix proteins, such as type I collagen and fibronectin. The precursors that undergo proliferation and differentiate into pre-osteoblasts are elliptical cells that are unable to deposit bone matrix but are still capable to proliferate. In this phase, BMP-2 and BMP-5 play a significant role in increasing alkaline phosphatase activity, osteocalcin synthesis (Yamaguchi et al. 1991) and parathyroid hormone (PTH) responsiveness (Kodama et al. 1982; Takuwa et al. 1991).

Immediately after growth arrest, a developmental sequence involving the selective expression of specific genes that characterize the differentiated osteoblast phenotype (alkaline phosphatase, osteocalcin) occurs (Collart et al. 1991; Stein et al. 1992). The accumulation of matrix proteins contributes, in part, to the cessation of cell proliferation.

The active bone-matrix-secreting osteoblasts are cuboidal cells, with a large Golgi apparatus and an abundant rough endoplasmic reticulum, and are provided with regions of plasma membrane specialized in the trafficking and secretion of vesicles that facilitate the deposition of bone matrix (Anderson 2003); these cells communicate with each other through tight junctions. During the post-proliferative phase, which is characterized by the high

synthesis of alkaline phosphatase, the extra-cellular matrix progresses into the mineralization phase in which osteoblasts synthesize several proteins that are associated with the mineralized matrix *in vivo* (Franzen and Heinegard 1985; Hauschka et al. 1989; Whitson et al. 1984), including sialoprotein (Nagat et al. 1991), osteopontin and osteocalcin (Gerstenfeld et al. 1987; Owen et al. 1990). Osteopontin is expressed during the stage of active proliferation (25% of maximal level; Lian and Stein 1995), decreases immediately after the post-proliferative stage and increases again at the onset of mineralization, achieving the greatest level of expression during mineralization. Osteopontin might be involved in the control of the relationship between the cells and extra-cellular matrix, as its amino acid sequence containing arg-gly-asp can mediate cell attachment (Oldberg et al. 1986). Unlike osteopontin, osteocalcin (bone Gla protein) is expressed by osteoblasts only in the post-proliferative phase. Osteocalcin is maximally expressed during mineralization *in vivo* (Hauschka et al. 1989) and *in vitro* (Owen et al. 1990). Several studies suggest that osteocalcin is involved in the regulation of mineral deposition and that it acts as a bone matrix signal that promotes osteoblast differentiation and activation (Chenu et al. 1994; DeFranco et al. 1991; Lian et al. 1984; Liggett et al. 1994), confirming that osteocalcin is a marker of mature osteoblasts (Lian et al. 1989, 1991). Osteocalcin synthesis is regulated by various hormones, 1,25 OH Vitamin D, and growth factors (e.g. TGF- β).

The onset and progression of matrix mineralization processes might be responsible for the down-regulation of genes expressed by mature osteoblasts during the same processes of extra-cellular matrix maturation and organization.

At the end of the synthesis and mineralization of the extra-cellular matrix, cellular levels of alkaline phosphatase mRNA decline (Lian and Stein 1995) and 50%–70% of mature osteoblasts undergo apoptosis, whereas the remainder can differentiate into lining cells or osteocytes or transdifferentiate into cells that deposit chondroid bone (Tamara 2006; Lynch et al. 1994). Lining cells remain on the bone surface, regulate the influx and efflux of mineral ions and retain the ability to re-differentiate into secreting osteoblasts upon exposure to various stimuli (hormones, mechanical forces; Clark 2008). Osteocytes are metabolically quiescent osteoblasts embedded in bone matrix; they communicate with other bone cells through cell processes and function as strain and stress sensors (Lozupone et al. 1996).

Osteoblasts and bone formation

Osteoblasts play a crucial role in the process of bone formation, in the induction and regulation of extra-cellular matrix mineralization and in the control of bone remodelling. During bone formation, mature osteoblasts

synthesize and secrete type I collagen (which represents the greatest part of the organic extra-cellular bone matrix) and various non-collagen proteins such as osteocalcin, osteopontin and bone sialoprotein (which exert various essential functions, including the regulation of bone turnover, the control of bone mineral deposition and the regulation of bone cell activity).

Osteocalcin is a vitamin-K-dependent osteoblast-specific protein, which is characterized by 3-gammaparboxyglutamic acid residues (Gla) and whose synthesis is enhanced by 1,25 OH Vitamin D3 and reflects metabolic cellular activity (Cantatore et al. 2005). Of the *de novo* synthesized osteocalcin, 60%–90% is incorporated into the bone matrix where it binds to hydroxyapatite during matrix mineralization. The remainder is released into the circulation where it can be measured as a sensitive marker of bone formation. Osteopontin (OPN) is a phosphorylated acidic glycoprotein that is present in large amounts in immature bone. OPN is synthesized by osteoblasts but is expressed by other cellular types, such as chondrocytes; it is involved in various physiological and pathological events. The expression of OPN has been reported to be regulated by mechanical stress, both *in vitro* and *in vivo*, and the ability of this protein to influence bone homeostasis through the inhibition of mineral deposition is well known (Fujihara et al. 2006; Ishijima et al. 2007).

Bone sialoprotein is a glycosylated, phosphorylated and sulfated protein that promotes hydroxyapatite crystal nucleation and osteoblast differentiation (Gordon et al. 2007). This has been confirmed by the observation that bone-sialoprotein-knockout mice present hypo-mineralized bone, a reduction in the size of their long bones and aberrant levels of osteoblast markers (Malaval et al. 2008). Similar to OPN, bone sialoprotein expression is increased in osteoblasts subjected to mechanical stimulation (Carvalho et al. 2002) but the role of this protein in bone mineralization is different.

Osteoblasts also synthesize IGF-I, interleukin-1 (IL-1) and IL-6, which control bone cells in an autocrine and/or paracrine manner. IGF-I secreted from osteoblasts in the bone tissue has been demonstrated to be a potent chemotactic factor that might play a major role in the recruitment of osteoblasts during bone formation (Nakasaki et al. 2008). Moreover, IGF-I induces cell migration of both MC3T3-E1 (an osteoblast-like cell line) and mouse osteoblasts and, in the same cells, positively regulates wound healing, including the initial polarization stage (Nakasaki et al. 2008).

Various *in vitro* studies of human and murine osteoblastic cell lines suggest that IL-1 can affect proliferation, collagen and osteocalcin synthesis and alkaline phosphatase production (Kim et al. 2002; Evans et al. 1990). Furthermore, human recombinant IL-1 induces IL-6 production in MTC3T3-E1 cells treated with 1,25 (OH)₂ Vitamin D3 (Lacey et al. 1993). The cellular effects of IL-6 are unclear,

because of the contradictory results of *in vitro* studies, and often appear to be opposing, depending on the experimental model employed. IL-6, in combination with its soluble receptor, has been shown to decrease or enhance osteoblast differentiation (Li et al. 2008; Erices et al. 2002); in addition, IL-6 seems to regulate (Bellido et al. 1998) osteoblast apoptosis (Silvestris et al. 2004). The regulation of IL-6 receptor (IL-6R) expression in osteoblasts is also unclear: *in vivo* studies suggest that bone marrow osteoblasts express the IL-6R (Wognum et al. 1993), whereas some *in vitro* studies suggest that this receptor is weakly expressed or absent from the stromal/osteoblastic cell line (Bellido et al. 1996).

Osteoblasts express receptors for various hormones including PTH (Dempster et al. 1993), $1,25(\text{OH})_2\text{D}_3$ (Lian et al. 1999), oestrogens (Boyce et al. 1999) and glucocorticoids (Ishida and Heersche 1998), which are involved in the regulation of osteoblast differentiation and activity. Vitamin D is able to modulate the metabolic activity of osteoblasts through the activation of a series of Vitamin-D-responsive genes that reflect a more mature osteoblast phenotype.

Control of bone remodelling by osteoblasts

Bone is constantly undergoing remodelling, a complex process in which osteoblasts play an essential role. Bone remodelling is strictly regulated by several local and systemic stimuli, including bone micro-damage, the reduction or increase of mechanical loading, blood calcium levels, hormones, cytokines and growth factors. The process of bone remodelling occurs in small “packets” of cells called basic multicellular units (BMUs), characterized by the coordinated action of osteoclasts and osteoblasts; at any one time, about 20% of the cancellous bone surface is undergoing remodelling (Hill 1998). The lifespan of a single BMU is about 6–9 months during which several generations of osteoclasts (average life of about 2 weeks) and osteoblasts (average life of about 3 months) are formed. A bone remodelling cycle consists of four distinct and sequential phases: activation, resorption, reversal and formation.

During the activation phase, osteoclastic precursors are recruited from circulating and bone-marrow mononuclear monocyte-macrophages (Roodman 1999), which differentiate into multinucleated cells and active resorbing osteoclasts that begin the resorption process. Osteoclast action is strictly related to their interaction with bone matrix proteins, including osteopontin and bone sialoprotein (Ross et al. 1993), which have been secreted by osteoblasts during the previous cycle of bone formation.

When resorption has been completed, the reversal phase starts: the osteoclasts die through apoptosis and osteoblast precursors locally proliferate, differentiate into mature

osteoblasts and migrate into the resorption lacuna made by osteoclasts. In the following formative phase, osteoblasts synthesize new un-mineralized bone matrix that fills the resorption lacuna and becomes mineralized in the resting phase.

The concept that the activation and regulation of bone resorption requires an interaction between osteoblasts and osteoclasts was proposed many years ago (Grano et al. 1990; Teti et al. 1991) on the basis of the *in vitro* demonstration that, in order to obtain mature osteoclasts, the presence of osteoblasts was necessary. However, the molecular mechanism underpinning this relationship was understood only some years later, with the identification of the RANK (receptor activator of nuclear factor κB)/RANKL (RANK ligand)/OPG (osteoprotegerin) system (Anderson et al. 1997; Simonet et al. 1997; Yasuda et al. 1998).

RANKL, also called OPG-ligand or osteoclast differentiation factor (ODF), is a tumour necrosis factor (TNF) superfamily member expressed by osteoblasts, both in a membrane-bound form and as a secreted protein (Burgess et al. 1999). RANKL is an essential factor for the recruitment, differentiation, activation and survival of osteoclastic cells through binding to its specific receptor RANK, which is present on the surface of osteoclast precursors and mature osteoclasts.

RANK is a homotrimeric trans-membrane protein member of the TNF-receptor superfamily and is expressed by mature osteoclasts, dendritic cells and some cancer cells, including breast and prostate cancers.

The other essential regulating component of the RANK/RANKL system is OPG. OPG is a soluble receptor of RANK and is synthesized by osteoblasts, stromal cells, vascular smooth muscle cells, B lymphocytes and articular chondrocytes. Studies of animal mouse models have demonstrated that the major biological effect of OPG is to inhibit osteoclast differentiation and activity, whereas its role in other tissues remains unknown (Lacey et al. 1998). OPG-deficient mice exhibit an osteoporotic phenotype and present an increased number of osteoclasts (Bucay et al. 1998), whereas the over-expression of OPG reduces osteoclast formation and leads to osteopetrosis (Simonet et al. 1997). Through the modulation of RANKL and OPG, osteoblasts can control osteoclast differentiation and activity and consequently bone remodelling: RANKL binds to RANK on the surface of osteoclast precursors leading to the activation of Nuclear Factor κB and the transcription of genes involved in osteoclastogenesis. OPG, by interacting with RANKL, prevents RANKL/RANK binding and subsequently inhibits osteoclastogenesis, osteoclast activity and bone resorption (Khosla 2001). Thus RANKL/OPG ratio is a major determinant of bone mass (Hofbauer and Schoppert 2004) and better reflects environmental signals.

In vitro and in vivo studies have suggested that the gene for OPG expression is regulated by the Wnt/ β -catenin signalling pathway (Glass et al. 2005). Wnt proteins (Wnts) are secreted glycoproteins with a post-translational modification represented by the addition of lipid (palmitate). There are 19 known Wnts and four related different signalling pathways: the Wnt/ β -catenin pathway, the Wnt/ Ca^{2+} pathway (Kuhl et al. 2000), the Wnt/planar cell polarity pathway (Mlodzik 2002) and the Wnt/protein kinase A pathway involving CREB (cAMP response element-binding protein-1; Chen et al. 2005). All Wnt pathways regulate various physiological and pathological processes, including cell proliferation, migration, polarity and differentiation, through the activation of diverse transcription factors. The Wnt/ Ca^{2+} , Wnt/planar cell polarity and Wnt/protein kinase A pathways, known as non-canonical pathways, are less well understood but appear to activate the transcription genes in a β -catenin-independent manner.

The Wnt/ β -catenin pathway is known as a canonical pathway; it promotes osteoblast commitment, proliferation and differentiation and enhances osteoblast and osteocyte survival (Bonewald and Johnson 2008). The Wnt/ β -catenin pathway is activated by Wnt binding with a co-receptor complex involving low-density lipoprotein-related protein (LRP5 or LRP6) and one of the frizzled family member (Fz). The complex Wnt/Lrp/Fz leads to the release of non-phosphorylated β -catenin into the cytoplasm from where it translocates into the nucleus to modulate gene transcription. The involvement of the canonical Wnt pathway in bone cells has been revealed in various studies showing that loss-of-function mutations in LRP5 (Gong et al. 2001) decrease bone mass, whereas gain-of-function mutations increase bone mass in both humans and mice (Boyden et al. 2002). Wnt signalling is tightly regulated by secreted antagonists, such as the secreted frizzled-related protein family (sFRP) and Wnt inhibitory factor (WIF-1; Aberle et al. 1997), which antagonize the interaction of Wnt with its receptor Fz.

On the other hand, LRP5/6 activity is antagonized by sclerostin (produced by osteocytes) and by members of the Dickkopf (Dkk) family (Westendorf et al. 2004). The LRP5 mutations associated with high bone mass prevent sclerostin from binding LRP5, thus confirming an in vivo role for sclerostin in depressing bone formation (Krishnan et al. 2006).

The evidence that OPG expression is enhanced in osteoblasts derived from mice with loss-of-function mutations in *LRP5*, whereas it is reduced in osteoblasts from gain-of-function mutations suggests that the Wnt signalling pathway regulates osteoclasts by increasing the OPG/RANKL ratio (Kubota et al. 2009).

Bone remodelling is also regulated by a range of hormones such as PTH, Vitamin D, oestrogen, calcitonin, serotonin and leptin, which primarily act on the osteoblasts

modifying RANKL and OPG expression but minimally affect RANK expression. Parathyroidectomized weanling rats fed a calcium-free diet and infused with PTH exhibit increased RANKL mRNA expression and decreased OPG mRNA expression (Lee and Lorenzo 1999). In vitro (Rogers and Eastell 2005) and in vivo (Buxton et al. 2004) human studies have confirmed these data. Similar to PTH, $1,25(\text{OH})_2\text{D}_3$ increases RANKL mRNA expression and decreases OPG mRNA expression but these changes in gene expression can vary depending on the maturation stage of the osteoblastic cells: in primary calvarial osteoblast cultures treated with $1,25 \text{ OH Vitamin D}_3$, RANKL mRNA expression is increased during all differentiation stages, except in mature cells, whereas OPG mRNA expression is increased at the onset of mineralization (Thomas et al. 2001). Oestrogens, acting directly on osteoblasts, have a dual effect; they increase bone formation and reduce bone resorption by enhancing osteoblast proliferation and function (Ernst et al. 1989; Majeska et al. 1994) and further reduce osteoclast activity by increasing OPG production in osteoblasts (Hofbauer et al. 1999).

A murine in vitro study suggests that calcitonin, a known inhibitor of bone resorption, can act directly on osteoblasts by increasing proliferation, enhancing OPG mRNA expression and inhibiting RANKL mRNA expression (Tian et al. 2007). Insulin has also been demonstrated to be involved in the regulation of bone remodelling. A murine in vivo study has shown a negative regulatory effect of insulin on bone resorption and formation, which leads to decreased bone turnover (Huang et al. 2010). However no evidence is available that insulin can act directly on osteoblasts, modifying OPG and/or RANKL expression. The involvement of circulating serotonin in bone cell function and bone remodelling has recently been proposed. An in vitro study has revealed that serotonin increases OPG and decreases receptor activator RANKL secretion in osteoblasts, suggesting a role in the osteoblast-induced inhibition of osteoclast differentiation (Gustafsson et al. 2006).

Several investigators have also reported the involvement of leptin in the control of bone remodelling. This cytokine-like hormone is secreted by adipocytes and controls food intake and energy expenditure. Leptin has also been reported to be expressed by osteoblasts (Reseland et al. 2001). The evidence that **ob/ob** mice, defective for leptin, present a high bone mass in spite of their hypogonadism and hypercorticozonaemia was the first to reveal that leptin is a potent inhibitor of bone formation in vivo, possibly acting via a central relay (Eleftheriou 2008). Subsequently, the finding that leptin receptors are present in hypothalamus rat (Elmqvist et al. 1998) and the observation that the destruction of these receptors results in increased cancellous bone mass have confirmed that leptin can control bone formation via the central nervous

system (Takeda et al. 2002). Following these observations, a number of central nervous mediators able to modulate bone remodelling have been identified in animal studies, among these the neuropeptide Y system (Baldock et al. 2002), supporting the idea that the central nervous system is involved in the control of bone remodelling.

In addition to RANKL, another factor produced by osteoblasts and required for osteoclast formation is macrophage-colony-stimulating factor-1 (M-CSF; Tsurukai et al. 2000). M-CSF is secreted by osteoblasts and promotes osteoclast precursor proliferation and RANK expression in osteoclast precursors.

More recently, Zhao and colleagues (2006) have proposed a new cell communication system involved in the coupling of bone formation and bone resorption. By using a combination of *in vitro* and *in vivo* studies, they demonstrate the expression of ephrin B2 and its receptor ephrin B4 (EphB4) in osteoclasts and osteoblasts, respectively, and have revealed that ephrinB2-EphB4 bidirectional signalling links the suppression of osteoclast differentiation to the stimulation of bone formation.

Ephrin ligands and Ephrin receptors (Ephs) are membrane-bound proteins; both receptors and ligands are able to transduce a signalling cascade upon interaction. Ephrin-ligand-activated signalling is known as “reverse signalling” and Ephs-activated signalling as “forward signalling.” Ephrins are divided into two classes: EphrinAs, which are attached to the extra-cellular membrane with a glycosylphosphatidylinositol anchor, and EphrinBs, which are transmembrane proteins containing a short cytoplasmic domain. The receptors are also divided into two classes based upon their interaction with the ligands for EphrinAs or EphrinBs. EphrinB2 associated with osteoclast precursors triggers the reverse signalling that suppresses osteoclast differentiation, whereas EphB4-mediated forward signalling in osteoblasts enhances differentiation.

Osteoblasts in pathological conditions

Osteoblasts and osteoporosis

Osteoporosis is a disorder characterized by reduced bone mineral density and an alteration of bone micro-architecture that results in an increased risk of fracture (Raisz 2005). Loss of bone mineral density is attributable to a pathological imbalance between bone resorption and bone formation during the remodelling process. Whereas the postmenopausal osteoporosis is mainly attributable to the increased bone resorbing activity of osteoclasts caused by oestrogen deficiency, senile osteoporosis is attributed to inadequate osteoblastic function (Beil et al. 2008). Various systemic and local factors, both in physiological than in pathological

conditions, can influence the strictly coupled activity of osteoblasts and osteoclasts, determining an imbalance in bone remodelling in favour of resorptive activity (Horwitz and Lorenzo 2002). However, in the pathogenesis of osteoporosis, a constitutive alteration of osteoblast behaviour might play a significant role.

A large number of experimental studies indicate that, in osteoporotic conditions, osteoblasts are characterized by lower proliferation and defective function compared with normal osteoblasts. The evidence that osteoblasts derived from patients with osteoporosis present, under basal conditions, an increased tyrosine phosphorylation of the IGF-I receptor and the blunted stimulation of receptor phosphorylation by the IGF-I receptor suggests that the impaired cell proliferation and decreased bone formation in osteoporosis can be correlated with the abnormalities of the IGF-I signalling system (Perrini et al. 2008).

Mesenchymal stem cells derived from osteoporotic postmenopausal women differ from cells obtained from healthy donors, as the ability of these cells to differentiate into the osteogenic lineage has been shown to be defective (Rodriguez et al. 2008). The finding that osteoblasts and adipocytes derive from common precursors and the evidence that increased adipose tissue volume in bone marrow in patients with osteoporosis is associated with decreased bone tissue volume (Justesen et al. 2001) suggest the involvement of adipogenic process in bone loss. Other studies have shown that, in osteoporosis, the decreased production of osteogenic cells is counterbalanced by increased adipocyte differentiation (Rodriguez et al. 1999; Verma et al. 2002). A human *ex-vivo* study has revealed the lower production of osteocalcin in osteoporotic osteoblasts compared with normal osteoblasts and a lower response to 1,25 (OH)₂ Vitamin D3 in terms of osteocalcin production in osteoporotic osteoblasts compared with normal osteoblasts. These data confirm the occurrence of a different metabolic phenotype in osteoporotic osteoblasts and indicate the presence of reduced, but not totally absent, anabolic function (Maruotti et al. 2009).

Human osteoblastic cells isolated from donors with osteoporosis also show a different production pattern of cytokines involved in the regulation of bone metabolism, including IL-6 (Torricelli et al. 2002) and TGF- β (Neidlinger-Wilke et al. 1995).

As previously described, the OPG/RANK/RANKL system represents the main regulatory factors of bone remodelling. Animal models and *in vitro* studies have demonstrated that the OPG/RANK/RANKL system is involved in the pathogenesis of osteoporosis. OPG-deficient mice present an osteoporotic phenotype associated with the high incidence of fractures (Bucay et al. 1998; Mizuno et al. 1998). Moreover, a human *in vitro* study has suggested that the up-regulation of RANKL on bone

marrow cells is an important determinant of increased bone resorption induced by oestrogen deficiency (Eghbali-Fatourehchi et al. 2003) and a human monoclonal antibody against RANKL has proved to be effective in reducing resorbing processes in postmenopausal women in a randomized double-blind placebo-controlled trial (Bekker et al. 2004). Other clinical trials have confirmed the therapeutic potential of RANKL neutralizing antibody in the treatment of postmenopausal osteoporosis and also suggest its effectiveness in other diseases involving bone loss.

However, various studies analysing OPG and RANKL levels have given contrasting results (Grigorie et al. 2003; Khosla et al. 2002; Oh et al. 2005). This is probably because (1) the amount of circulating OPG and RANKL originates from nonskeletal sources, (2) the majority of RANKL is cell-bound and thus not detectable in the circulation or (3) the commercial assay detects all forms of OPG (monomeric, dimeric and conjugate) but only dimeric OPG has been described as the active form (Rogers and Eastell 2005).

Chronic glucocorticoid therapy is well known as one of the major causes of osteoporosis. High doses of glucocorticoids and long exposure periods to corticosteroids inhibit osteoblast proliferation and activity and enhance osteoblast and osteocyte apoptosis; they also increase bone resorption enhancing the expression of RANKL and decreasing OPG production (Canalis 2003).

An *in vitro* study has shown that DKK-1 mRNA is over-expressed in cultured human osteoblasts treated with dexamethasone (Ohnaka et al. 2004). This suggested that glucocorticoids induce the impairment of bone formation through the suppression of Wnt signalling. On the other hand, glucocorticoids decrease the expression of BMP-2 and enhance the expression of its antagonist, follistatin (Leclerc et al. 2004; Luppen et al. 2003). A subsequent study has demonstrated that bisphosphonates and PTH, which are currently used in clinical practice for the treatment of post-menopausal and glucocorticoid-induced osteoporosis, are able to reverse the effects of dexamethasone on BMP and Wnt signalling (Hayashi et al. 2009).

Nicotine and alcohol consumption are well established as being responsible for decreased bone density and increased fracture risk (Benson and Shulman 2005; Giuliani et al. 1999; Lalor et al. 1986; Kapoor and Jones 2005). Indeed, nicotine acts directly on osteoblasts inhibiting their proliferation and differentiation (Nakayama et al. 2009). Recently, however, a possible positive effect of nicotine has been demonstrated, for the first time, on bone metabolism; at a low concentration, corresponding to those acquired by a moderate smoker, nicotine appears to be able to increase osteoblast proliferation and to improve bone metabolism (Rothem et al. 2009). Alcohol has also been reported to reduce osteoblast proliferation and bone metabolism but a

wealth of evidence suggests that a moderate consumption (1 drink per day) is associated with a decreased risk of osteoporotic hip fractures (Berg et al. 2008). Further studies are needed to understand the precise effects of moderate nicotine and alcohol consumption on osteoblast metabolism.

Osteoblasts and osteoarthritis

Osteoarthritis (OA) is a chronic degenerative joint disease characterized by loss and degradation of cartilage, inflammation of the synovium and peri-articular bone alteration consisting of the formation of osteophytes and subchondral bone sclerosis (Davis et al. 1988; Valdes and Spector 2010). Radin and Rose (1986) were the first to suggest the involvement of the subchondral bone in the progression and initiation of cartilage degradation. Successive studies have confirmed this hypothesis and demonstrated the abnormal behaviour and metabolism of OA osteoblasts (Corrado et al. 2005; Dequeker et al. 1993; El Miedany et al. 2000; Hilal et al. 1998; Lajeunesse and Rebol 2003).

Some investigators have examined the molecular basis of bone OA changes by comparing microarray gene expression profiling of bone obtained from individuals with no evidence of joint disease and from individuals with degenerative hip OA (Hopwood et al. 2007). Several genes that influence osteoblast function, bone remodelling and mineralization exhibit a different expression in OA. Many of these genes are components of the Wnt and TGF- β /BMP signalling pathway. Moreover, a subset of genes are differentially expressed between females and males; this might in part explain the sex disparity in OA.

La Jeunesse's group has reported elevated alkaline phosphatase activity and increased osteocalcin levels in primary human OA subchondral osteoblasts (Hilal et al. 1998) and this data has been confirmed by the results of several clinical *ex/in vivo* and *in vitro* studies (Cantatore et al. 2004; Hilal et al. 2001; Mansell et al. 1997). Differences in the metabolic response to 1,25(OH) Vitamin D3 stimulation, consisting of a significant increase of osteocalcin after Vitamin D3 treatment, have been found in osteoarthritic osteoblasts, proportional to the degree of joint damage (Cantatore et al. 2004; Corrado et al. 2005; Gevers and Dequeker 1987), suggesting that the abnormal behaviour of OA osteoblasts includes an altered response to systemic or local factors (Cantatore et al. 2004).

Other investigators have distinguished two different groups of OA osteoblasts: low OA osteoblasts, associated with low levels of prostaglandin E₂ (PGE₂) and IL-6, similar to normal cells, and high OA osteoblasts associated with high levels of PGE₂ and IL-6 (Massicotte et al. 2002). Recent data have suggested a close relationship between the OPG/RNK/RANKL system and the subchondral bone

changes observed in OA. Studies performed on osteoblasts derived from patients with OA have demonstrated an abnormal expression of OPG and RANKL and consequently OPG/RANKL ratio (Kwan Tat et al. 2008a, 2008b). Low OA osteoblasts show a marked decrease in OPG and increased level of RANKL, whereas high OA osteoblasts exhibit a marked increase of OPG and a reduction of RANKL-t (Tat et al. 2006). Moreover, low and high OA subchondral osteoblasts express membranous and RANKL isoforms differently and are modulated differently by osteotropic factors (Tat et al. 2008). This might explain the different metabolic states of human subchondral bone osteoblast subpopulations: low OA osteoblasts promote bone resorption, whereas high OA osteoblasts favour bone formation.

Recently, human osteoblasts derived from subchondral OA bone have been shown, for the first time, to express ephrin B2 and its receptor EphB4. EphB4 receptor is expressed in OA osteoblasts and its levels are increased in low OA cells but no differences have been observed between normal and high OA cells. Moreover, EphB4 activation by the specific ligand ephrin B2 inhibits the expression of IL-1 β , IL-6 and RANKL, but not of OPG (Kwan Tat et al. 2008a, 2008b). These data suggest that the activation of EphB4 by ephrin B2 affects the abnormal metabolism in OA subchondral bone by inhibiting resorption factors and their activities.

Dequeker's group (1993) has demonstrated an elevated production of IGF-I, IGF-II and TGF- β in bone explants from the iliac crest of OA patients. The same results have subsequently been obtained in vitro (Massicotte et al. 2006).

The altered osteoblast metabolism might also explain the presence of an abnormal mineralization of subchondral bone in OA. Type I collagen levels are elevated in OA bone tissue (Mansell and Bailey 1998) and should lead to excessive mineralization. This might be the reason for the subchondral bone sclerosis that characterizes OA, even if, in the early stage of disease, this tissue is hypomineralized. A rapid and aggressive OA has recently been demonstrated to develop in the Brittle IV (Brtl) mouse model of osteogenesis imperfecta, which is characterized by a defect in Type I collagen (Blair-Levy et al. 2008). These data confirm the idea that the alterations in subchondral bone tissue microarchitecture play a key role in the progressive destruction of joint cartilage observed in OA. Human OA osteoblasts present increased collagen type I deposition, but with an altered ratio of α_1 and α_2 chains, in particular with an increase of the α_1 chain. This abnormal production of type I collagen leads to abnormal mineralization and can be correlated with the high levels of TGF- β detected in OA osteoblasts (Couchourel et al. 2009). TGF- β is a potent inducer of osteophytes and acts

directly or via the inhibition of BMP-2-induced mineralization.

A human in vitro study has demonstrated the abnormal production of leptin in OA osteoblasts: leptin expression is increased five-fold in OA osteoblasts compared with normal osteoblasts (Mutabaruka et al. 2010). This increased production of leptin might be responsible, at least in part, for the elevated levels of bone markers observed in OA osteoblasts (osteocalcin, alkaline phosphatase) and confirms the key role of leptin in OA pathophysiology, as previously demonstrated by the Dumond group (2003).

Osteoblasts and rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease characterized by a chronic inflammatory process within the synovial membrane leading to the formation of an invasive pannus responsible for the progressive destruction of bone and joint cartilage. This disease is characterized by an increase in bone resorption that is associated with impaired bone formation resulting in imbalanced bone remodelling and reduced bone mass.

Three forms of bone loss, involving various regions of skeleton, are observed in RA: focal bone loss affecting the bone at the joint margins resulting in erosive changes; periarticular osteopenia adjacent to inflamed joints; and generalized bone loss involving the axial and appendicular skeleton. As is well known, osteoclasts are the principal cell type responsible for bone loss in RA (Keffer et al. 1991; Pettit et al. 2001; Shealy et al. 2002), whereas other cells, including synovial fibroblasts and macrophages, might directly contribute to the pathogenesis of bone erosion (Maruotti et al. 2007; Pap et al. 2000). Nevertheless, osteoblast differentiation and functions have recently been suggested to be abnormal at sites of focal bone erosion in RA.

A murine study, involving the use of dynamic bone histomorphometry, has shown that the rate of bone formation is similar in arthritic and non-arthritic bone, suggesting that, in RA, increased osteoclast resorbing activity is not counterbalanced by osteoblast bone formation. Furthermore, within arthritic bone, mineralization of the newly formed bone in areas adjacent to inflammation sites is reduced compared with bone surfaces adjacent to normal bone marrow suggesting that inflammatory tissue impairs osteoblast activity (Walsh et al. 2009).

Several factors produced by cells involved in the RA inflammatory process, such as members of the Wnt signalling pathway and the proinflammatory cytokine TNF- α , seem to inhibit osteoblast maturation and functions at sites of focal bone erosion. The Wnt signalling antagonist DKK-1 is increased in the animal RA model of mice overexpressing TNF- α and prophylactic treatment with an

antibody to DKK-1 results in protective effects against focal bone erosion (Diarra et al. 2007), probably through the suppression of the negative regulation of osteoblastogenesis and osteoblast activity.

TNF- α is a pro-inflammatory cytokine involved in the pathogenesis of RA and actually represents a therapeutic target in RA. This cytokine inhibits, in a dose-dependent manner, the differentiation of osteoblasts from fetal calvaria precursor cells, which spontaneously differentiate into the osteoblast phenotype over 21 days; the addition of TNF α after 7–8 days only partially inhibits differentiation and, after day 14, has no effect, suggesting that it acts during an early stage of osteoblast phenotype selection (Gilbert et al. 2000).

In vitro studies have demonstrated that TNF- α induces a reduction in alkaline phosphatase activity, in osteocalcin expression and in collagen type I synthesis (Bertolini et al. 1986; Centrella et al. 1988; Li and Stashenko 1992). Moreover osteoblasts treated with TNF- α are unable to regulate matrix mineralization (Panagakos et al. 1996).

TNF- α might also inhibit osteoblast function in the RA microenvironment through the modulation of Wnt signalling, thereby enhancing DKK-1 expression in synovial fibroblast cells (Diarra et al. 2007). Additionally, osteoblast-like cells, exposed to sera of RA patients treated with Infliximab, a TNF- α blocking agent, show a reduced synthesis of IL-6, a cytokine directly involved in arthritis-related bone loss (Musacchio et al. 2009).

Other factors that are present in the arthritic bone microenvironment and that seem to be involved in impaired bone formation include hypoxia and reduced pH. In vitro studies have demonstrated that these factors are able to down-regulate alkaline phosphatase synthesis in osteoblasts and to prevent mineralization (Utting et al. 2006; Brandao-Burch et al. 2005). In addition, hypoxia has been found to inhibit Wnt signalling in osteoblast-like cells, both directly, by sequestering β -catenin and therefore inhibiting transcriptional activity (Almeida et al. 2007), and indirectly, by the up-regulation of DKK-1 (Colla et al. 2007).

Concluding remarks

Osteoblasts are mesenchymal cells involved in bone formation processes and in the mineralization of extracellular bone matrix. They play a fundamental role in the modulation of bone remodelling and in the regulation of the metabolic activity of other bone cells. Inadequate osteoblastic function is crucially involved in the pathogenesis of a number of common human bone diseases. An understanding of the molecular mechanisms that underlie osteoblast function, under both physiological and pathological conditions, could lead to the development of new

therapeutic strategies in diseases characterized by a reduced bone mass.

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