Control of Bone Formation by AP-1 Transcription Factor and Rho GTPases: Implications for Bone Regeneration and Tissue Repair

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Summary: Understanding the basic molecular pathways that control the proliferation of osteoprogenitor cells and their subsequent differentiation into mature osteoblasts capable of synthesizing bone, forms the fundamental basis of all strategies aimed at growing new bone in a reparative or regenerative setting. We have focussed on two intracellular pathways that we have shown previously using *in vivo* and *in vitro* approaches to be critical for regulating osteoblast growth and bone formation. First, the c-Fos/AP-1 transcription factor causes osteoblast transformation and osteosarcoma formation when overexpressed in transgenic mice, and we suggest here that one possible underlying mechanism is the ability of c-Fos to deregulate osteoblast growth and proliferation by targeting specific components of the cell cycle machinery, such as cyclin A. Second, we show that the activity of the Rho family GTPase, RhoA and its downstream target Rho kinase (ROCK) are crucial for regulating bone formation *in vitro*. Specifically, we show that Rho activation inhibits calvarial osteogenic cell differentiation and bone formation *in vitro*, while treatment of osteogenic cultures with ROCK inhibitors gives an anabolic response, markedly stimulating bone formation. The implications for bone remodelling and for bone tissue repair and regeneration are discussed.

Key words: osteoblast, osteoclast, differentiation, remodelling, signalling pathways

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INTRODUCTION

The repair and regeneration of bone tissue is extremely complex because bone itself is one of the most complex tissues of the body. Its development and function throughout life are dependent on multiple cell types that must communicate and function with extremely tight controls at the structural, hormonal, local and molecular levels. Indeed, the cellular and molecular concepts underlying tissue repair and tissue regeneration follow on from concepts that have been in place in stem cell biology and biomaterials for several decades. Nevertheless, it is clear that, in order to understand and carry out protocols aimed at regenerating or repairing specific tissues such as bone, it is essential to have a detailed understanding of how bone develops in the embryo, in addition to how bone remodels throughout life. Indeed, any disruption of this process forms the basis of metabolic bone diseases and bone disorders, the same diseases that tissue repair and regeneration protocols aim to alleviate. This paper will review briefly the basic features of bone cell biology and bone remodelling, and focus on two signalling pathways that control the growth and differentiation of osteoblast differentiation and bone formation, with particular implications for bone growth and repair.

BONE CELL DIFFERENTIATION AND BONE REMODELLING

The vertebrate skeleton is continuously remodelled throughout life in order to fulfil certain essential requirements such as calcium homeostasis, for the precise replacement of old or damaged bone to occur, and for the maintenance of bone mass and mechanical integrity. For this to occur efficiently, there must be a tight coupling and balance of bone degradation and bone formation. Bone remodelling occurs within discrete anatomical units (basic multicellular unit – BMU) where bone resorption by osteoclasts is tightly coupled to bone formation by osteoblasts both in space and time, a process which occurs throughout life at a rate of approximately 10% per year (Manolagas, 2000). Disruption of this balance leads to an uncoupling of remodelling and ultimately skeletal abnormalities characterised either by excessive bone loss (e.g. osteoporosis) or, alternatively, by increases in bone mass (e.g. osteopetrosis). At the cellular level, this process is controlled by two major cell types, bone-resorbing osteoclasts, and bone-forming osteoblasts.

Osteoclasts are large, multinucleated cells whose function is to resorb or degrade bone. They are derived from haematopoietic stem cells, specifically from cells of the monocyte/macrophage lineage (Fig 1). Osteoclastogenesis is dependent on osteoblasts and stromal cells which produce the two cytokines that are essential for osteoclast differentiation: Receptor Activator of NF-κB Ligand (RANKL) and Macrophage Colony-Stimulating Factor (MCSF) (Boyle et al, 2003). RANKL and MCSF bind to their respective receptors on marrow-derived osteoclast precursors and peripheral blood monocytes: RANKL binds to RANK, a member of the TNF receptor family, and MCSF binds to *c-fms*, a receptor tyrosine kinase, and stimulation of both receptors leads to the activation of several intracellular signalling cascades involving TNF-receptor-associated factors (TRAFs), MAPKs, *c-src*, and Akt/PKB, leading to activation of the transcription factors NF-κB, *c-fos* and NFATc1 which are essential for osteoclastogenesis (see Teitelbaum and Ross, 2003 for review).

The physiological effects of RANKL are negatively regulated by the expression of a soluble decoy receptor termed osteoprotegerin (OPG) which is also synthesised by osteoblasts/stromal cells and which binds to and sequesters RANKL preventing it from binding to RANK (Fig 1). Once formed, osteoclasts undergo a well-defined series of events which include attachment to bone, cytoskeletal rearrangements, polarization and matrix degradation. Thus, the relative ratio of RANKL to OPG is the most important determinant of osteoclast formation *in vivo* and many osteotropic factors which induce osteoclast formation do so via osteoblasts (Fig 1). Thus, besides being responsible for bone formation (see below), osteoblasts are also responsible for the production of all the factors that are essential for osteoclastogenesis and therefore critically control the whole process. This highlights an important feature of osteoblasts that goes beyond their ability to form bone.

Osteoblasts are cuboidal, post-mitotic cells that are situated on active bone-forming surfaces. Their basic

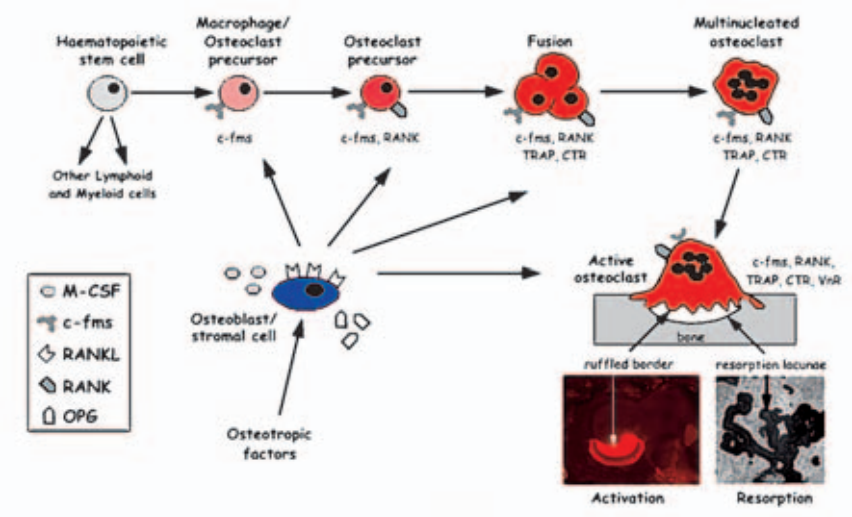

Fig 1 The osteoclast lineage and regulation of differentation and activation. Osteoclasts are derived from haematopoietic stem cells and monocyte/macrophage precursors. The MCSF/*c-fms* and RANKL/RANK signalling pathways provide essential signals for proliferation, differentiation, fusion and survival of osteoclasts. RANKL is also inhibited by the soluble decoy receptor, OPG. Osteoclasts can be identified by the expression of markers like TRAP, CTR, the αvβ3 integrin, vitronectin receptor (VnR). Osteotropic factors regulate osteoclast formation and/or activity indirectly by influencing the expression ratio of RANKL:OPG on osteoblastic/stromal cells. The micrographs show F-actin staining of the ruffled border in active osteoclasts, and resorption lacunae formed on a dentine substrate. Reproduced with permission from Cambridge University Press (see McGowan et al, 2005).

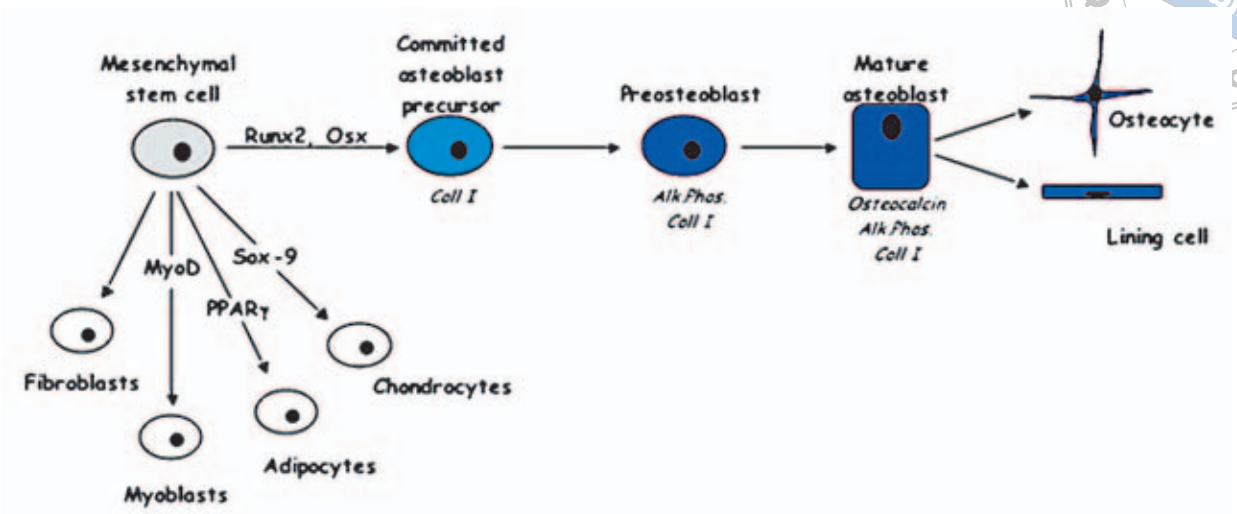

Fig 2 The osteoblast lineage. Osteoblasts are derived from mesenchymal stem cells which have the potential to differentiate into other mesenchymal cells such as muscle, fat, cartilage and other connective tissue cells. The commitment to each lineage is dependent upon lineage-specific transcription factors, such as MyoD for muscle, PPARγ for adipocytes, Sox-9 for chondrocytes, and Runx2/cbfa1 and Osterix (Osx) for osteoblasts. The expression of genes such as type I collagen (coll I), alkaline phosphatase (Alk.Phos.) and osteocalcin are commonly used as markers for the osteoblast lineage (see Aubin, 1998 for details). Reproduced with permission from Cambridge University Press (see McGowan et al, 2005).

function is to secrete the organic matrix of bone that becomes mineralized and undergoes calcification. Osteoblasts are derived from pluripotent mesenchymal stem cells and their differentiation is a linear, multi-step process, requiring the sequential activation and suppression of specific genes. They are related at the precursor cell level to other mesenchymal cell derivatives, such as muscle cells, adipocytes, chondrocytes and other connective tissue fibroblasts (Fig 2; see also Karsenty and Wagner, 2002). The identification of osteoblasts is aided by marker genes which are expressed at different stages of differentiation. Recent gene knockout studies in mice have identified two transcription factors, Runx2/cbfa-1 and osterix, that are essential for the commitment of cells to the osteoblast lineage and for differentiation to mature, bone-forming osteoblasts. Besides Runx2/cbfa-1 and osterix, the expression of several marker genes are commonly used to characterise cells of the osteoblast lineage, namely type I collagen, alkaline phosphatase, and osteocalcin, the latter being specific to osteoblasts (see also Ducy et al, 2000; Karsenty and Wagner, 2000 for further details).

The study of the mechanisms controlling bone formation has been aided greatly by the establishment of efficient *in vitro* models of osteoblast differentiation and bone formation. Primary cultures of osteoblasts derived either from perinatal rodent calvariae or from adult bone marrow stroma, contain osteoprogenitor cells that can undergo the full differentiation cascade from committed precursor cells to fully functional osteoblasts that are capable of forming three-dimensional, mineralised bone nodules (Aubin, 1998). Such systems have been essential for determining the effects of specific growth factors and transcription factors on osteogenesis by gain- and loss-of-function studies, and are ideal for screening compounds that are thought to regulate bone formation.

Thus, it is clear that understanding the molecular mechanisms controlling osteoblast growth, proliferation and differentiation, in addition to how osteoblasts control osteoclast activity in a remodelling sequence, is essential knowledge which will underpin the strategies aimed at growing bone either from stem cells, or from committed precursors.

REGULATORS OF OSTEOBLAST PROLIFERATION AND DIFFERENTIATION

Any strategy which aims to grow bone either from uncommitted mesenchymal stem cells, or from committed osteoprogenitors, must take into account the molecules and signal transduction pathways that control the number and activity of the cells which form bone, i.e. osteoblasts. In this presentation, two intracellular signalling pathways will be described, which have been shown using *in vivo* and *in vitro* approaches, to control osteoblast proliferation and differentiation.

The c-Fos/AP-1 Transcription Factor and Osteoblasts

The proto-oncogene c-*fos* is a component of the AP-1 family of transcription factors that includes members the Fos family (c-Fos, Fra-1, Fra-2, FosB), Jun family (c-Jun, JunB, JunD), and ATF and CREB families (Shaulian

and Karin, 2002). The AP-1 complex converts extracellular signals to changes at the nuclear level, and this is achieved by formation of specific heterodimeric complexes, binding to AP-1 binding sites in the promoters of AP-1 target genes, and regulation of a number of cellular functions, such as proliferation, differentiation, apoptosis and oncogenic transformation.

With respect to osteoblasts, c-Fos is an important protein, as we have shown that overexpression of c-Fos in transgenic mice causes malignant transformation of osteoblasts leading to osteosarcoma formation (Grigoriadis et al, 1993; see also Wagner, 2002 for review) (Fig 3a). While there is intense current research on aspects of oncogenic transformation using these mice, this model system is also extremely useful for studying the growth control of osteoblasts, in particular, examining the mechanisms through which high c-Fos levels stimulate osteoblast proliferation. To address this, we have developed an inducible system *in vitro* in MC3T3-E1 osteoblasts, in which an exogenous c-*fos* transgene is expressed from a tetracycline-regulatable promoter, and we have investigated specifically whether c-Fos controls osteoblast proliferation and the cell cycle machinery (Sunters et al, 2004). First, we demonstrated that induction of c-Fos resulted in enhanced cell proliferation, and this was confirmed by flow cytometric analysis which showed that cells overexpressing c-Fos exhibited accelerated S-phase entry (Fig 3b). To investigate the mechanisms of this increased cell proliferation, we examined in detail the expression profile of all cell cycle-associated proteins, namely cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (CKIs). The results demonstrated that while most cell cycle-related proteins remained unchanged, osteoblasts overexpressing c-Fos showed higher levels of cyclin A and to a lesser extent, cyclin E, following cell cycle re-entry (Fig 3c). Significantly, not only were the levels higher, but their kinetics of expression were also altered, suggesting deregulated expression of these cyclins. As cyclins A and E bind specifically to CDK2, we also confirmed that the observed increases in protein levels were functional, since kinase assays showed an enhanced ability of CDK2 to phosphorylate the retinoblastoma protein (pRb), which is essential for cell cycle progression (Fig 3d). These data have suggested that in osteoblasts, the enhanced proliferation observed following induction of c-Fos, is mediated by altered cell cycle regulation, primarily increases in cyclin A and cyclin A/CDK2 activity. Thus, cyclin A has emerged as an additional c-Fos/AP-1 target gene, and preliminary cyclin A promoter-reporter experiments have confirmed that c-Fos enhances cyclin A promoter activity (data not shown).

Finally, if deregulated cyclin A is important in mediating the proliferative effects of c-Fos overexpression, then it is predicted that osteoblasts derived from the transgenic osteosarcomas will have deregulated cyclin A. Indeed, analysis of osteoblastic osteosarcoma cell lines derived from the transgenic tumours (Grigoriadis et al, 1993), showed that these cells had constitutively high levels of cyclin A and higher CDK2 kinase activity (Fig 3e).

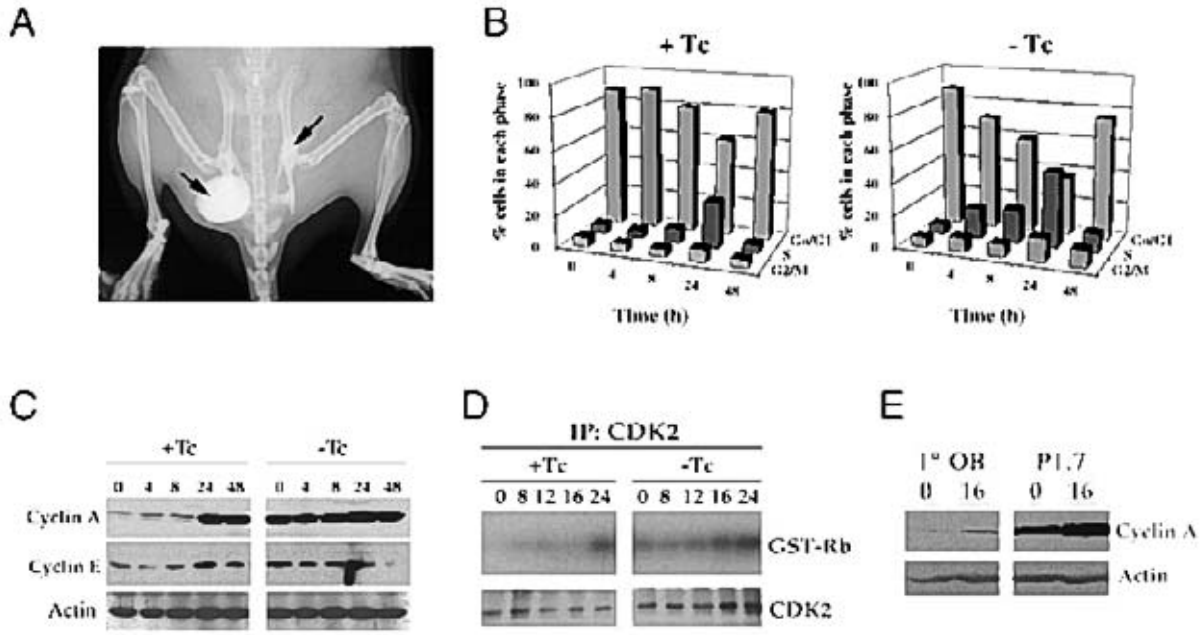

Fig 3 (a) Induction of osteosarcomas (arrows) in c-Fos overexpressing transgenic mice (see also Grigoriadis et al, 1993). **(b)** Accelerated S-phase entry in osteoblasts following induction of a c-Fos transgene after withdrawal of tetracycline (Tc). Increased cyclin A, cyclin E **(c)** and increased CDK2 kinase activity **(d)** in osteoblasts following c-Fos induction (-Tc). **(e)** P1.7 osteosarcoma cells derived from c-Fos transgenic mice express constitutively high levels of cyclin A (see also Sunters et al, 2004).

Thus, an interesting story is emerging, whereby specific cell cycle proteins, such as cyclin A (as well as cyclin D1, a known AP-1 target gene; data not shown), critically regulate osteoblast proliferation. Moreover, the fact that overexpression of the other Fos-related genes, Fra-1 and DFosB, result in increased bone formation in transgenic animals with no apparent effects on proliferation (Jochum et al, 2000; Sabatakos et al, 2000), point to specificity amongst the Fos family proteins to regulate osteoblast proliferation. The future challenge is to define the role that Fos proteins and their specific cell cycle targets have on the growth control of osteoblasts, first during normal development and bone remodelling, in addition to during pathological disorders, such as metabolic bone disease and skeletal malignancies. This is currently being done using siRNA and specific cell cycle gene knockout strategies in osteoblasts.

Rho Family GTPases and Osteoblasts

Rho proteins are members of the Ras superfamily of small GTP-binding proteins (small GTPases) (Etienne-Manneville and Hall, 2002). These proteins function as molecular switches, transducing changes in the extracellular environment into intracellular signals via specific effector molecules, resulting in changes in cell behaviour and gene expression. The Rho subfamily contains many proteins, of which Rho, Rac and Cdc42 are the best characterised. The main function of these proteins is to regulate the actin cytoskeleton, although Rho GTPases have also been shown to be involved in a variety of other cellular functions like cell cycle control, cell differentiation, transformation and apoptosis (Bishop and Hall, 2000). In general, Rho appears to be involved in the formation of stress fibres and focal adhesions, Cdc42 induces the formation of filopodia and Rac is involved in the formation of lamellipodia and membrane ruffles (Nobes and Hall, 1995).

We have become interested in Rho GTPases through studies involving the unique bacterial protein toxin, *Pasteurella multocida* toxin (PMT), a large 146kDa intracellularly-acting protein that has been shown previously to be a potent mitogen. Bacterial toxins such as PMT have proved to be extremely useful tools for analysing signal transduction pathways in different cell types, as they have very specific molecular targets (Lax et al, 2004). PMT is an interesting toxin and an ideal tool for the study of signalling cascades in bone cells, as this toxin has profound effects on bone physiology *in vivo*. PMT is the causal agent of atrophic rhinitis in pigs, which is characterised by abnormal bone remodelling resulting in the progressive loss of the nasal turbinate bones (Lax and Chanter, 1990). PMT activates the Rho GTPase in fibroblasts, leading to cytoskeletal rearrangements, although the molecular target is thought to be the related G-protein coupled receptors, Gq/11 and G12/13. The toxin also stimulates signalling cascades linked to phospholipase C, leading to increased formation of diacylglycerol and inositol phosphates, increased protein kinase C activity and calcium mobilisation, in addition to indirect activation of MAPK pathways (for review see Lax et al, 2004 and references therein).

The bone remodelling phenotype observed in atrophic rhinitis suggests that PMT targets specific bone cell populations, and that this may occur via modulation of Rho GTPase signalling. Below, we summarise our recent evidence using PMT as a molecular tool, that the Rho GTPase pathway is an important regulator of osteoblast differentiation and bone formation, and that this signalling pathway is a potential target for therapeutic and tissue regeneration strategies.

Using a well-characterised osteoblast differentiation and bone formation system *in vitro*, whereby primary mouse or rat osteoblast precursors are stimulated to undergo differentiation into functional osteoblasts and lay down bone matrix in three-dimensional nodules, we demonstrated that PMT was a potent inhibitor of osteoblast differentiation and bone nodule formation (Fig 4a). This inhibitory effect was also confirmed at the molecular level by the downregulation of osteoblast marker gene expression such as Runx2, osteocalcin and alkaline phosphatase (Harmey et al, 2004). That this effect was related to the activation of Rho GTPase by PMT was demonstrated by inhibitor studies, which showed that the inhibition of osteoblast differentiation was restored by treatment with the Rho inhibitor, C3 transferase, or by inhibition of Rho Kinase (ROCK), one of the major downstream target gene effectors of Rho (Fig 4b, c). These data suggested that activation of the Rho pathway was inhibitory to osteoblast differentiation and bone formation.

However, one of the more revealing aspects of these results, was that in the absence of toxin, inhibition of ROCK stimulated bone nodule formation (Fig 4c), prompting us to investigate this further. Indeed, treatment of primary osteogenic cells with ROCK inhibitors markedly stimulated both the expression of alkaline phosphatase activity, and more importantly, stimulated the number of bone nodules in a dose-dependent manner (Fig 4d, e). This provided further functional evidence of the role of the Rho pathway in osteoblasts.

Taken together, these findings have demonstrated that the Rho-ROCK GTPase is a critical regulator of osteogenesis, whereby activation of this pathway downregulates, whereas inhibition of this pathway is stimulatory to bone formation. This is a novel pathway for osteogenesis, in particular, the emergence of ROCK inhibitors as potential anabolic agents. Future experiments targeting constitutively active or dominant-negative Rho or ROCK to osteoblasts *in vivo* are currently underway.

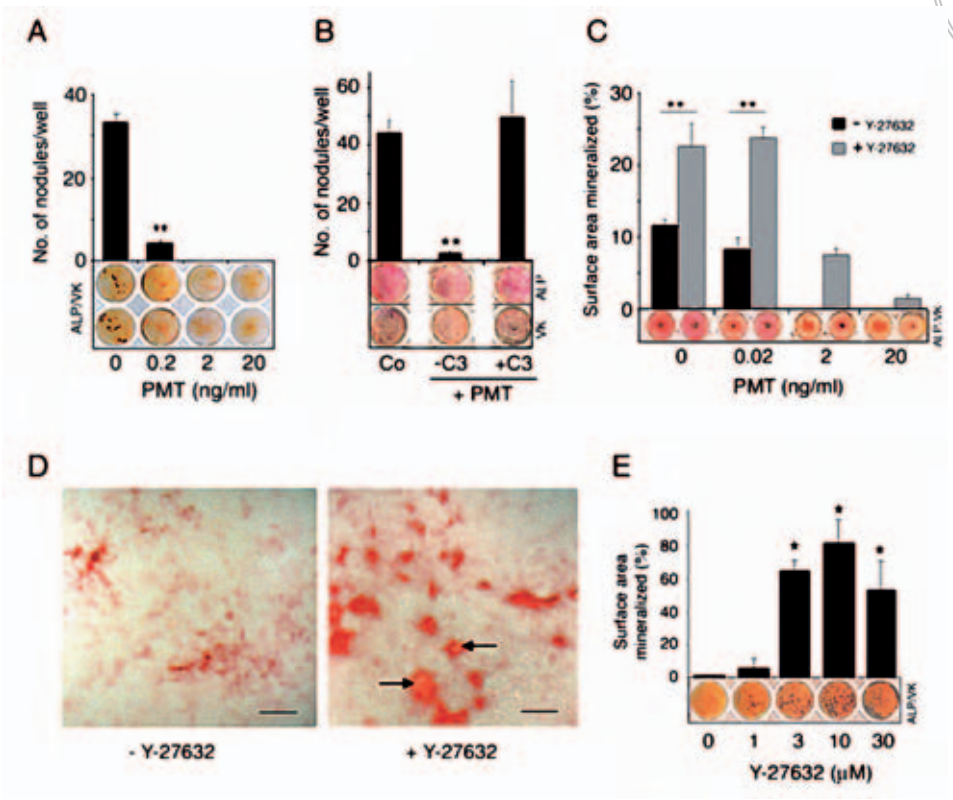

Fig 4 PMT inhibits osteoblast differentiation and bone nodule formation **(a)**. This is rescued by the Rho inhibitor, C3 exoenzyme **(b)** and by the ROCK inhibitor, Y-27632 **(c)**. The ROCK inhibitor Y-27632 stimulates nodule formation in primary calvarial osteoblasts **(d, e)**. Reproduced with permission from J Bone Mineral Res (see Harmey et al, 2004).

ROLE OF OSTEOCLASTS AND BONE REMODELLING

Finally, as mentioned above, the close interaction between osteoblasts and osteoclasts in a basic multicellular unit during a bone remodelling sequence, suggests that any potential strategy to increase bone *in vivo* must take into account the role of osteoclasts. Thus, in terms of the effects of c-Fos/AP-1 and Rho GTPases on osteoblast properties, the role of these important regulators on osteoclasts must also be considered. Indeed, in terms of AP-1, we have shown that c-Fos is essential for osteoclast differentiation, as c-Fos knockout mice lack osteoclasts, have defective tooth eruption and develop osteopetrosis (Grigoriadis et al, 1994). We and others have also suggested that osteoclasts are required or c-Fos-induced osteosarcoma formation in transgenic mice, highlighting the importance of osteoclasts in the accelerated osteoblast proliferation and progression of bone tumours (Wang et al, 1995; David et al, 2005). We have also shown recently that PMT and Rho GTPases also affect osteoclast differentiation (data not shown), and it is already well-established that Rho GTPases are essential for podosome assembly, actin ring formation and osteoclastic bone resorption (Chelliah et al, 2000). Thus, it is clear that in the course of investigating the roles of c-Fos/AP-1 or Rho GTPases as potential regulators of osteoblast proliferation, bone formation and bone anabolism, it is essential to consider simultaneously the contribution these regulators have on osteoclast biology.

CONCLUSIONS

Specific signalling pathways that regulate multiple aspects of cell differentiation and cell physiology are being uncovered rapidly by many laboratories. We have highlighted only two such pathways that we have shown to be important for regulating osteoblast proliferation and differentiation. Elucidating further the molecular mechanisms explaining how the AP-1 transcription factor or the small GTPase Rho modulate osteoblast proliferation and differentiation, respectively, will undoubtedly provide critical insights for understanding their roles in diseases of the skeleton, and ultimately, for the development of novel therapeutic strategies. Regarding specific strategies related to bone formation in a therapeutic or regenerative setting, whether inves-

tigating the commitment of stem cells to the osteogenic lineage, cellular interactions with specific scaffolds and biomaterials, or recombinant proteins and therapeutic compounds which aim to target the osteoblast lineage, it is ultimately the fundamental properties of cell growth and differentiation that will shed light on osteoblast proliferation and apoptosis, matrix deposition and mineralization, which will culminate in a structure that is functionally and mechanically competent.

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