Wnt signalling in osteoblasts regulates expression of the receptor activator of NFκB ligand and inhibits osteoclastogenesis in vitro

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Summary

Reports implicating Wnt signalling in the regulation of bone mass have prompted widespread interest in the use of Wnt mimetics for the treatment of skeletal disorders. To date much of this work has focused on their anabolic effects acting on cells of the osteoblast lineage. In this study we provide evidence that Wnts also regulate osteoclast formation and bone resorption, through a mechanism involving transcriptional repression of the gene encoding the osteoclastogenic cytokine receptor activator of NFκB ligand (*RANKL* or *TNFSF11*) expressed by osteoblasts. In co-cultures of mouse mononuclear spleen cells and osteoblasts, inhibition of GSK3β with LiCl or exposure to Wnt3a inhibited the formation of tartrate-resistant acid phosphatase-positive multinucleated cells compared with controls. However, these treatments had no consistent effect on the differentiation, survival or activity of osteoclasts generated in the absence of supporting stromal cells. Activation of Wnt signalling downregulated *RANKL* mRNA and protein expression, and overexpression of full-length β-catenin, but not transcriptionally inactive β-catenin ΔC(695-781), inhibited *RANKL* promoter activity. Since previous studies have demonstrated an absence of resorptive phenotype in mice lacking LRP5, we determined expression of a second Wnt co-receptor *LRP6* in human osteoblasts, CD14$^+$ osteoclast progenitors and mature osteoclasts. *LRP5* expression was undetectable in CD14-enriched cells and mature human osteoclasts, although *LRP6* was expressed at high levels by these cells. Our evidence of Wnt-dependent regulation of osteoclastogenesis adds to the growing complexity of Wnt signalling mechanisms that are now known to influence skeletal function and highlights the requirement to develop novel therapeutics that differentially target anabolic and catabolic Wnt effects in bone.

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Introduction

Inappropriate changes in bone formation and bone resorption compromise normal skeletal function, and aberrant bone mass homeostasis is a common feature of many degenerative skeletal disorders. Effective prevention or treatment of these changes depends on a greater understanding of the cellular mechanisms that control the formation, proliferation, differentiation and apoptosis of bone cells, and their function under normal and pathological conditions (Rodan and Martin, 2000).

Recent studies have implicated an important role for Wnt signalling in the regulation of skeletal function and have prompted widespread interest in the use of Wnt mimetics as novel therapies aimed at manipulating bone cells to normalise bone mass and correct developmental defects. Wnt proteins belong to a large, highly conserved family of secreted glycoproteins which help guide developmental and post-developmental physiology by regulating a diverse range of cellular processes including proliferation, fate determination and differentiation. During embryogenesis, Wnt signalling tightly regulates skeletal development to control limb patterning (Zakany and Duboule, 1993), joint formation (Hartmann and Tabin, 2000; Hartmann and Tabin, 2001) and chondrogenesis (Rudnicki and Brown, 1997). Expression of functional Wnt signalling molecules have been identified in mesenchymal stem cells and have been demonstrated to help regulate proliferation and guide cell fate specification (Gregory et al., 2003; Shea et al., 2003; De Boer et al., 2004; Etheridge et al., 2004), whereas genetic mutations linked to osteoporosis-pseudoglioma syndrome (OPPG) and phenotypic analysis of mice lacking the Wnt co-receptor Lipoprotein receptor-related protein-5 (LRP5) have demonstrated a fundamental role for canonical Wnt signalling in the accrual of bone mass (Boyden et al., 2002; Kato et al., 2002; Patel and Karsenty, 2002; Gregory et al., 2003; Van Wesenbeeck et al., 2003). *LRP5*-knockout mice have a low bone-mass phenotype and decreased osteoblast mitotic index, consistent with impaired pre-osteoblast proliferation and mature osteoblast activity (Kato et al., 2002). Conversely, activating mutations in *LRP5* are associated with high bone mass and increased osteoblast proliferation (Boyden et al., 2002; Little et al., 2002; Babij et al., 2003; Van Wesenbeeck et al., 2003). Many of these phenotypic changes are thought to be mediated by alterations

in canonical Wnt signalling that affect gene expression and cell function. In canonical signalling, gene transcription is repressed in the absence of Wnt ligands (off state) and the intracellular effector β-catenin is phosphorylated by glycogen synthase-3-kinase β (GSK3β) in association with adenomatous polyposis coli (APC) and axin, forming a multi-protein destruction complex through which the protein is ubiquitylated and constitutively degraded by the proteasome (Ikeda et al., 1998; Kishida et al., 1998; Willert and Nusse, 1998; van Noort et al., 2002). In the presence of Wnt signals (on state), Wnt binds to Frizzled (FZD) and Wnt co-receptors, LRP5 or -6, under the regulation of secreted Frizzled-related peptides (SFRPs) and Dickkopf proteins (DKK), which act as soluble decoy receptors and LRP5 antagonists respectively, inhibiting signalling (Moon et al., 1997; Tamai et al., 2000; Mao et al., 2001). Activated FZD stimulates the intracellular protein Dishevelled (Dvl) and inhibits GSK3β, stabilising cytoplasmic β-catenin. Stabilised β-catenin translocates to the nucleus and stimulates Wnt target-gene expression through the transactivation of T-cell factor (TCF)/lymphoid enhancer factor (LEF) family of transcription factors relieving constitutive transcriptional repression mediated by Groucho/Transducin-like Enhancer of Split (TLE) proteins and C-terminal binding protein (CTBP) (Tamai et al., 2000; Zhurinsky et al., 2000; Brantjes et al., 2001). Alkaline phosphatase has been identified as a Wnt target gene whose activity is positively regulated in preosteoblastic cells in response to BMP2 via a Wnt autocrine loop and canonical pathway (Rawadi et al., 2003), although mechanisms of Wnt-mediated transcription repression also exist in osteoblasts. In a recent study, LEF1 and β-catenin have been demonstrated to inhibit Runx2/Cbfa1-dependent transcriptional activation of the osteocalcin gene promoter (Kahler and Westendorf, 2003).

In this study we performed a detailed analysis of the expression of Wnt signalling components in osteoblasts and determined the effects of mimicking signalling on osteoblastic TCF/LEF-dependent gene transcription. Our data demonstrate a novel role for Wnt signalling in the regulation of osteoclastogenesis, and provide evidence for an indirect osteoblast-mediated mechanism of inhibition, involving transcriptional repression of the receptor activator of NFκB ligand gene (*RANKL* or *TNFSF11*). To account for the lack of resorptive phenotype in *LRP5*-knockout mice we also determined expression of a second Wnt co-receptor, *LRP6* by osteoblasts, osteoclast progenitors and mature osteoclasts. Taken together, these data provide evidence for Wnt-mediated regulation of osteoclastogenesis which acts to functionally antagonise the anabolic effects of Wnts in bone and as such, it is likely that the future therapeutic value of using Wnt mimetics for the treatment of bone disorders will have to be weighted against their net anabolic and catabolic effects in bone.

Results

Expression of Wnt signalling components by osteoblasts

RT-PCR was performed to identify expression of Wnt signalling components in human osteoblastic cells. Using cDNAs derived from a range of clonal osteoblast-like cells (MG-63, SaOS-2 and TE85) and primary human osteoblasts we identified expression of multiple Wnt ligands (*WNT2*, *WNT2B2*, *WNT3A*, *WNT4*, *WNT5A*, *WNT10* and *WNT11*, Fig. 1A), Frizzled receptors (*FZD2*, *FZD3*, *FZD4*, *FZD5*, *FZD6*, Fig. 1B) and secreted Frizzled-related peptides (*SFRP1, SFRP2*, *SFRP3*, *SFRP4* and *SFRP5*, Fig. 1C). In addition to genes encoding receptor/ligand components, we also identified expression of genes encoding signalling molecules required for intracellular signal transduction including β-catenin (*CTNNB1*), APC and GSK3β (*GSK3B*), and components of the core Wnt-signalling transcriptional apparatus including TCF/LEF transcription factors TCF1, LEF1, TCF3 and TCF4 (*TCF7*, *LEF1*, *TCF7L1* and *TCF7L2*) and the transcriptional co-repressors Groucho/TLE (*TLE1*) and C-terminal binding protein (*CTBP1*, Fig. 1E,F). Osteoblasts therefore express the necessary cellular machinery to send, receive and transduce extracellular Wnt signals into a specific intracellular transcriptional response. Despite some variation in expression of these molecules between individual cell lines, *WNT2B2*, *WNT4*, *WNT5A*, *FZD2*, *FZD4*, *FZD6*, *SFRP2*, *CTNNB1*, *APC*, *GSK3B*, *TCF3* and *TLE1* showed consistent expression by all osteoblastic cells tested. No products were obtained using water as a control or samples generated in the absence of reverse transcriptase (data not shown). Cloning and sequencing were performed to confirm the identity of amplified PCR products. All cloned cDNAs were found to be 100% homologous to published GenBank sequences.

Mimicking Wnt signalling induces β-catenin nuclear translocation and stimulates TCF gene transcription in osteoblasts

To determine whether Wnt signalling molecules identified by RT-PCR were functional in osteoblasts, MC3T3-E1 cells were exposed to conditioned medium from Wnt3a-overexpressing cells and changes in β-catenin localisation determined by fluorescent immunocytochemistry (Fig. 2). In the presence of control conditioned medium (1-50%), β-catenin immunoreactivity was identified in the cytoplasm of cells and at peripheral sites of cell-cell contact. By contrast, following exposure to Wnt3a-conditioned medium (1-50%) for 24 hours β-catenin was predominantly localised in the nucleus of cells, determined by colocalisation with the nuclear stain DAPI (Fig. 3A). Wnt3a-induced nuclear translocation was dose-dependent and increased from 0% of cells with nuclear β-catenin in the absence of Wnt to approximately 80% in the presence of 50% Wnt3a-conditioned medium (Fig. 2B). Furthermore, nuclear translocation of β-catenin induced by exposure to Wnt-conditioned medium (25%) was significantly reduced by the LRP antagonist Dickkopf-1 (0.01-0.1 μg/ml), as determined by automated image analysis, reducing nuclear β-catenin levels by more than 50% at all concentrations tested (Fig. 2C).

To examine the intercellular mechanism of Wnt-induced β-catenin translocation, LiCl was used to inhibit the activity of GSK3β, mimicking the inhibitory effects of Dvl following activation of FZD by Wnt. LiCl is a widely available and commonly used Wnt mimetic with demonstrated effectiveness in a number of in vitro and in vivo systems (Berridge et al., 1989; Stambolic et al., 1996; Hedgepeth et al., 1997). SaOS-2 osteoblastic cells exposed to LiCl (20 mM, 24 hours) exhibited markedly elevated levels of nuclear β-catenin compared with controls (NaCl, 20 mM), which displayed prominent peripheral β-catenin localisation (Fig. 3A). Similarity between the cellular distribution of β-catenin in Wnt3a and LiCl-treated

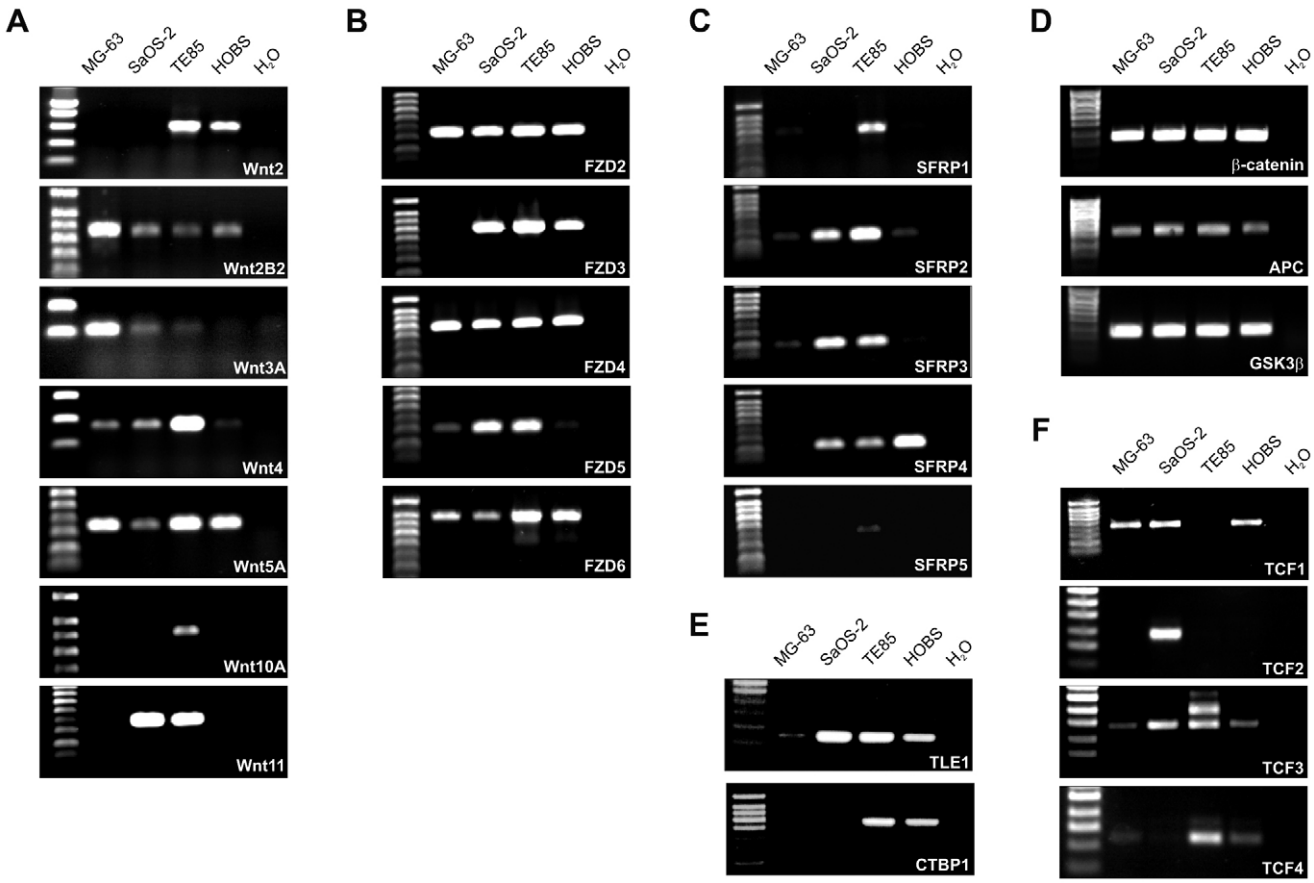

Fig. 1. RT-PCR analysis of Wnt signalling components in osteoblastic cells. (A) Secreted Wnt ligands, (B) Frizzled receptors (FZD), (C) secreted frizzled related peptides (SFRPs), (D) intracellular signalling components, (E) transcriptional machinery and (F) TCF/LEF transcription factors.

osteoblastic cells strongly implicated a central role for the inhibition of GSK3β and canonical Wnt signalling pathway in Wnt-mediated effects in osteoblasts.

Specific reporter gene assays were performed to determine whether mimicking canonical Wnt signalling stimulated gene expression in osteoblastic cells by β-catenin-mediated transactivation of TCF/LEF transcription factors. In SaOS-2 cells, nuclear translocation of β-catenin accompanied a significant increase in gene expression from a TCF/LEF specific luciferase reporter plasmid (TOPFLASH, Fig. 3B). By contrast, no transcriptional activation was observed in control cells incubated with vehicle alone or cells transfected with a reporter gene containing mutant TCF binding sites (FOPFLASH). In time-course experiments we determined that LiCl-induced TCF/LEF transcription was stimulated from 5 hours post-treatment, correlating temporally with the observed changes in β-catenin accumulation in the nucleus of osteoblastic cells (Fig. 3C).

The involvement of β-catenin in Wnt-induced TCF/LEF-dependent transcription was investigated by co-transfecting osteoblastic cells with full-length and truncated β-catenin-EGFP fusion constructs and TOPFLASH TCF/LEF reporter plasmid using empty EGFP vector and mutant FOPFLASH reporter plasmid as controls (Fig. 4). Overexpression of full-length β-catenin-EGFP potently stimulated TOPFLASH reporter gene transcription in osteoblastic cells compared with cells transfected with FOPFLASH vector or empty vector alone. Deletion of the C-terminus of β-catenin (ΔC695-781) was sufficient to reduce TCF/LEF transcriptional responses to those observed with control vector (FOPFLASH), demonstrating the importance of the C-terminal transactivation domain of β-catenin in canonical Wnt-induced changes in TCF gene transcription in osteoblastic cells. By fluorescent microscopy, full-length β-catenin EGFP and β-catenin ΔC695-781 EGFP specifically localised to the nucleus of osteoblasts consistent with observed stimulatory and inhibitory transcriptional responses.

Wnt signalling regulates expression of receptor activator of NFκB ligand (RANKL) in osteoblasts

To identify potential target genes regulated by Wnt signalling in osteoblasts we searched candidate promoter sequences for TCF/LEF consensus binding sites (ctttgww) using MATInspector online search software (http://www.genomatix.de). Using this approach we identified five TCF/LEF binding sites in the 5′ proximal region of the human *RANKL* promoter (GenBank accession number, AF333234) located at positions –1594 to –1600, –1891 to –1897, –2430 to –2436, –3358 to –3364 and –3794 to –3800, relative to the translational start codon, implicating *RANKL* as a potential transcriptional target for Wnt signalling in bone (Fig. 5A). To test this hypothesis we treated osteoblastic cells with LiCl and performed

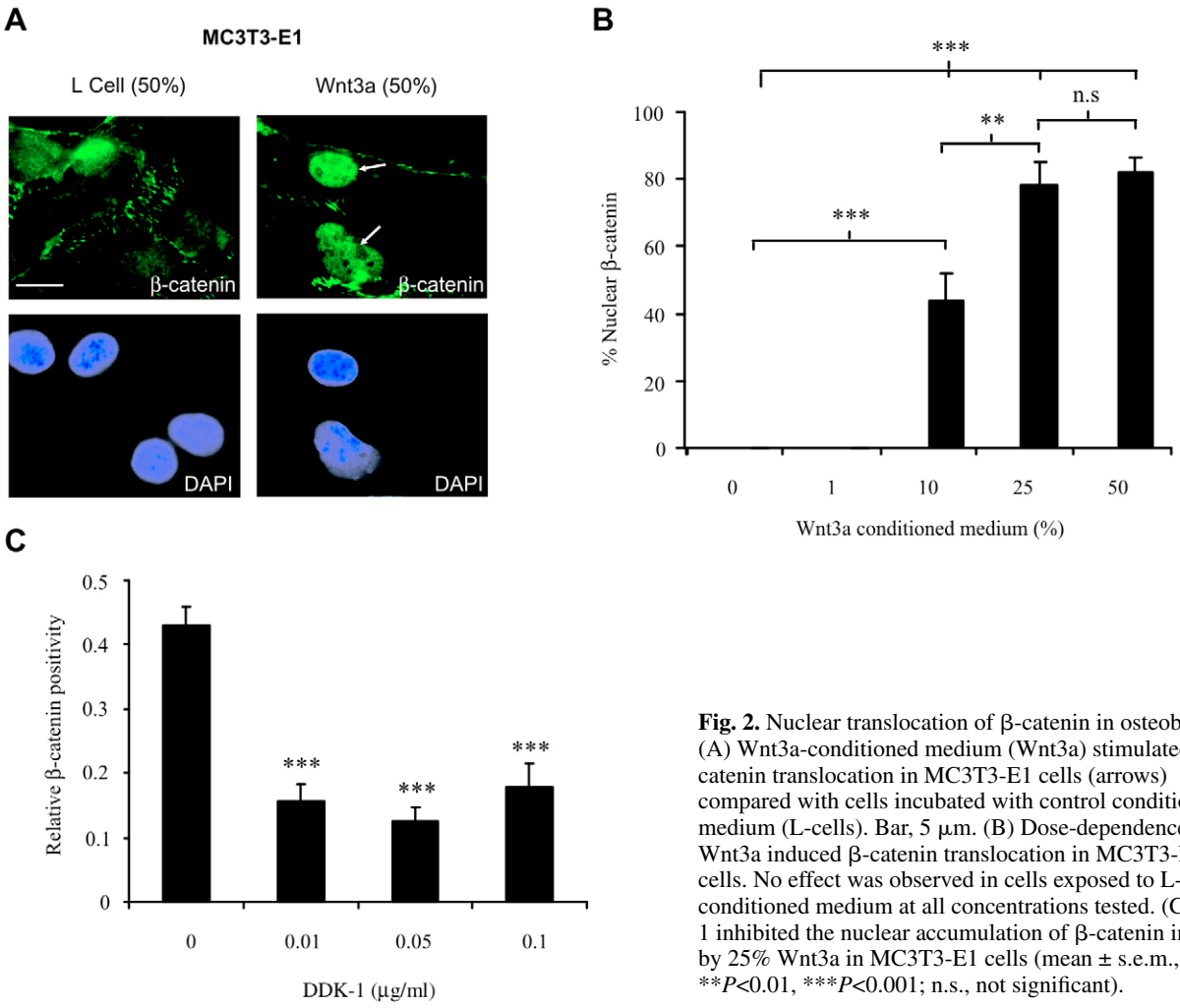

Fig. 2. Nuclear translocation of β-catenin in osteoblasts. (A) Wnt3a-conditioned medium (Wnt3a) stimulated β-catenin translocation in MC3T3-E1 cells (arrows) compared with cells incubated with control conditioned medium (L-cells). Bar, 5 μm. (B) Dose-dependence of Wnt3a induced β-catenin translocation in MC3T3-E1 cells. No effect was observed in cells exposed to L-cell conditioned medium at all concentrations tested. (C) Dkk-1 inhibited the nuclear accumulation of β-catenin induced by 25% Wnt3a in MC3T3-E1 cells (mean ± s.e.m., n=12; $^{**}P$<0.01, $^{***}P$<0.001; n.s., not significant).

Northern and western blot analyses to determine the effects of mimicking Wnt signalling on expression of RANKL in MG-63 and MC3T3-E1 osteoblastic cells (Fig. 5B). Exposure of osteoblastic cells to LiCl (20 mM) for 24 hours completely inhibited *RANKL* mRNA and protein expression compared with cells incubated with NaCl (20 mM). GSK3β is known to participate in a number of other signalling pathways including those mediated by phosphatidyl lipids, in addition to its role in Wnt signal transduction. To address whether effects on RANKL expression were specifically mediated by canonical Wnt signalling, osteoblastic cells were incubated with recombinant Wnt proteins and their effects determined on RANKL mRNA expression by RT-PCR. Treatment of MC3T3-E1 cells with Wnt3a (100 ng/ml) or LiCl (20 mM) for 24 hours markedly reduced RANKL mRNA expression compared with cells incubated with NaCl (20 mM) or vehicle alone. By contrast, RANKL expression was unaffected by treatment with the non-canonical Wnt ligand, Wnt5a (500 ng/ml; Fig. 5C). Significantly, Wnt5a at this dose was also unable to antagonise the inhibitory effects of Wnt3a on RANKL mRNA expression, which has been described for TCF-gene transcription in reporter gene assays in heterologous cells (Topol et al., 2003). To assess the role of β-catenin in mediating Wnt3a effects on RANKL expression, ST2 stromal cells were transfected with full-length β-catenin or β-catenin ΔC695-781 and a luciferase reporter gene construct containing a 7 kb fragment of the mouse *RANKL* promoter (–6880 to +115 relative to the transcriptional start site). Overexpression of full-length β-catenin significantly reduced endogenous *RANKL* promoter activity in these cells. By contrast, activity was unaffected by overexpression of β-catenin lacking the C-terminal domain which is required for TCF/LEF-dependent gene transcription in osteoblasts (β-catenin ΔC695-781) (Fig. 5D).

Mimicking Wnt signalling inhibits osteoclast formation in vitro

Since RANKL is required to support the formation of osteoclasts in vitro and in vivo we hypothesised that Wnt signalling may inhibit osteoclast formation through regulation of RANKL expression by osteoblasts. To test this hypothesis, co-culture assays of mouse primary osteoblasts and mononuclear spleen cells were performed to determine the effects of mimicking Wnt signalling on osteoclast formation in vitro. Treatment of co-cultures with LiCl (5-20 mM) over 12 days, caused a significant (P<0.001) dose-dependent decrease in the formation of TRAP-positive multinucleated cells compared with cultures treated with NaCl (Fig. 6A). These effects were associated with decreased cell fusion, as determined by light microscopy. In LiCl-treated cultures, mononuclear cells remained predominant after 6 days in

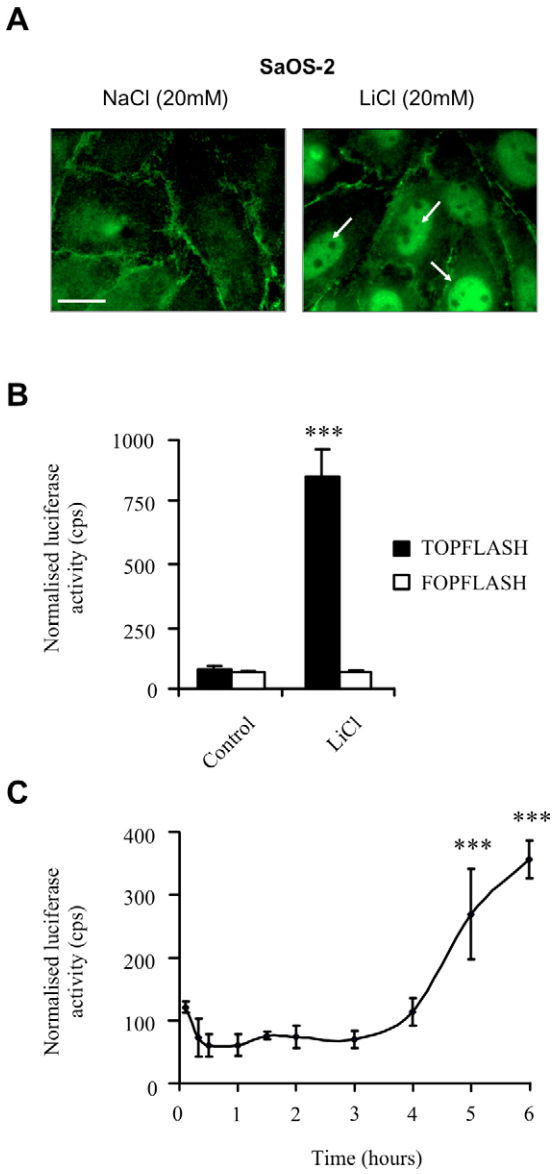

Fig. 3. Mimicking Wnt signalling in osteoblasts stimulates β-catenin nuclear translocation and TCF/LEF-dependent transcriptional activation. (A) Inhibition of GSK3β with LiCl (20 mM) stimulated β-catenin nuclear translocation in SaOS-2 cells (arrows) compared with NaCl controls. Bar, 5 μm. (B) Inhibition of GSK3β with LiCl (20 mM) stimulated TOPFLASH TCF/LEF gene transcription in SaOS-2 cells compared with cells incubated with vehicle alone or those transfected with control reporter containing mutant TCF/LEF binding sites (FOPFLASH). Mean values are shown ± s.d., n=4. (C) Time course of LiCl-stimulated gene transcription in MG-63 cells. LiCl stimulated TCF/LEF reporter gene transcription from 5 hours post treatment (mean ± s.e.m., n=8; ***$P \leqslant 0.001$).

culture. By contrast, treatment with NaCl resulted in the formation of large cells typical of cell fusion and osteoclastogenesis after 6 days (Fig. 6B). To address the potential issue of the lack of specificity of LiCl for the Wnt signalling pathway, parallel experiments were performed to determine the effects of Wnt3a conditioned medium on osteoclast formation in vitro. Treatment of cultures with Wnt3a (1-25%) over 12 days significantly (P<0.001) inhibited the formation of TRAP-positive multinucleated osteoclasts at levels comparable with LiCl treatment. By contrast, normal osteoclast numbers were obtained following culture in the presence of L-cell conditioned medium (Fig. 6C).

Expression of LRP5 and LRP6 by bone cells

It has been suggested that Wnt/LRP5 signalling has no effect on osteoclast formation and bone resorption in vivo and in vitro (Gong et al., 2001; Boyden et al., 2002; Patel and Karsenty, 2002; Babij et al., 2003). To determine whether LRP6 signalling could be responsible for mediating the effects of Wnts on osteoclastogenesis we used an RT-PCR approach to determine the expression of *LRP5* and *LRP6* by bone cells and mouse spleen, which contains a rich source of osteoclast progenitors (Fig. 7). We identified expression of both *LRP5* and *6* by a range of osteoblastic cells (MG-63, SaOS-2 and TE85), primary human osteoblasts and mouse spleen, suggesting Wnt signals could be transduced by either co-receptor in these cells. By contrast, only expression of *LRP6* was identified using cDNA from mature human osteoclasts generated from long-term bone marrow cultures. Since mouse spleen contains multiple haematopoietic and non-haematopoietic cell types, RT-PCR was also performed using cDNA generated from CD14-enriched and CD14-depleted cells isolated by magnetic activated cell sorting from the mononuclear fraction of human peripheral blood. Using this approach we found that CD14-depleted cells expressed both LRP5 and 6. However, CD14-enriched cells only expressed *LRP6* mRNA and not *LRP5*.

Effects of Wnt3a on osteoclast formation in the absence of stromal cells/osteoblasts

To test the hypothesis that LRP6 mediates the direct effects of Wnt signalling on osteoclast differentiation in the absence of supporting stromal cells/osteoblasts, effects of recombinant Wnt3a were determined on the formation of TRAP-positive multinucleated cells generated from peripheral blood monocytes (PBMCs) cultured in the presence of exogenous M-CSF and RANKL (Fig. 8). PBMCs were incubated in the presence of recombinant Wnt3a (10-100 ng/ml) over 14 days in culture. At the end of the culture period cells were stained for TRAP and the number TRAP-positive cells with more than two nuclei counted. Wnt3a had no consistent inhibitory or stimulatory effect on the formation of osteoclasts or their resorptive activity compared with cells incubated with vehicle alone.

β-catenin immunolocalises to nuclei of mature osteoclasts

The identification of *LRP6* expression by mature osteoclasts raises the question of whether Wnt signalling mechanisms operate in these cells. To test this hypothesis using disaggregated rat osteoclasts we performed β-catenin immunolocalisation experiments to determine whether endogenous Wnt signalling operates in these cells. In the absence of exogenous Wnt signals we identified prominent β-catenin immunoreactivity in the cell nuclei, which colocalised with DAPI (Fig. 9). These data are consistent with active Wnt signalling in mature osteoclasts and provide evidence for autocrine Wnt signalling mechanisms that regulate their activity.

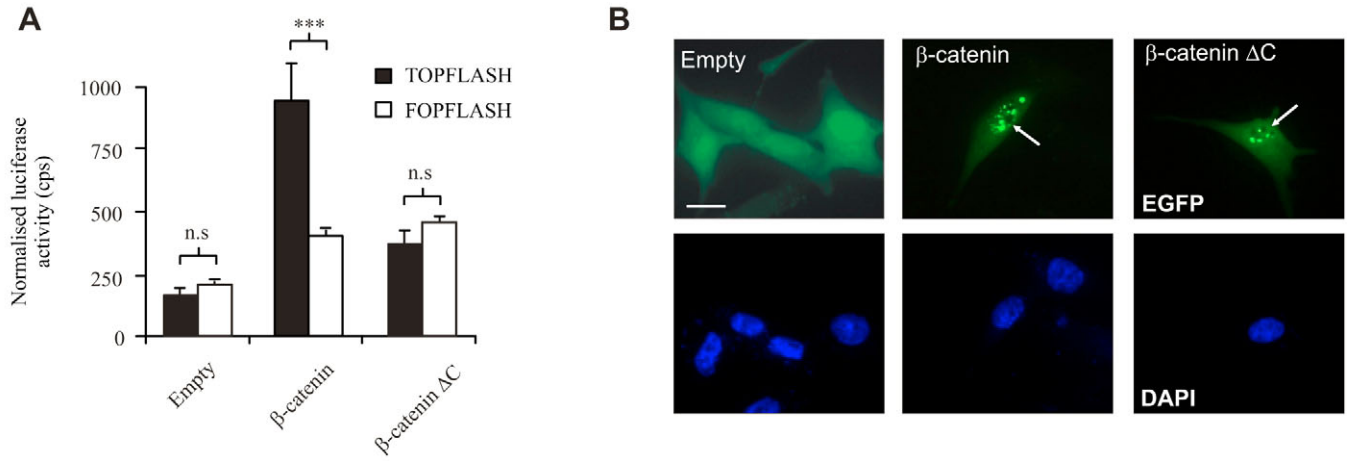

Fig. 4. β-catenin transactivates TCF/LEF gene transcription in osteoblasts. (A) Overexpression of β-catenin EGFP fusion protein significantly increased TCF/LEF-dependent transcription of a luciferase reporter gene (TOPFLASH) in MG-63 cells compared with cells transfected with control reporter containing mutant TCF/LEF binding sites (FOPFLASH). By contrast, overexpression of a β-catenin C-terminal mutant lacking the transactivation domain (β-catenin ΔC695-781) failed to activate transcription (values are mean ± s.d., n=4; ***P≤0.001; n.s., not significant). (B) Both full-length β-catenin and β-catenin ΔC localised to the nucleus of osteoblasts (arrows). EGFP, Green; DAPI, blue. Bar, 10 μm.

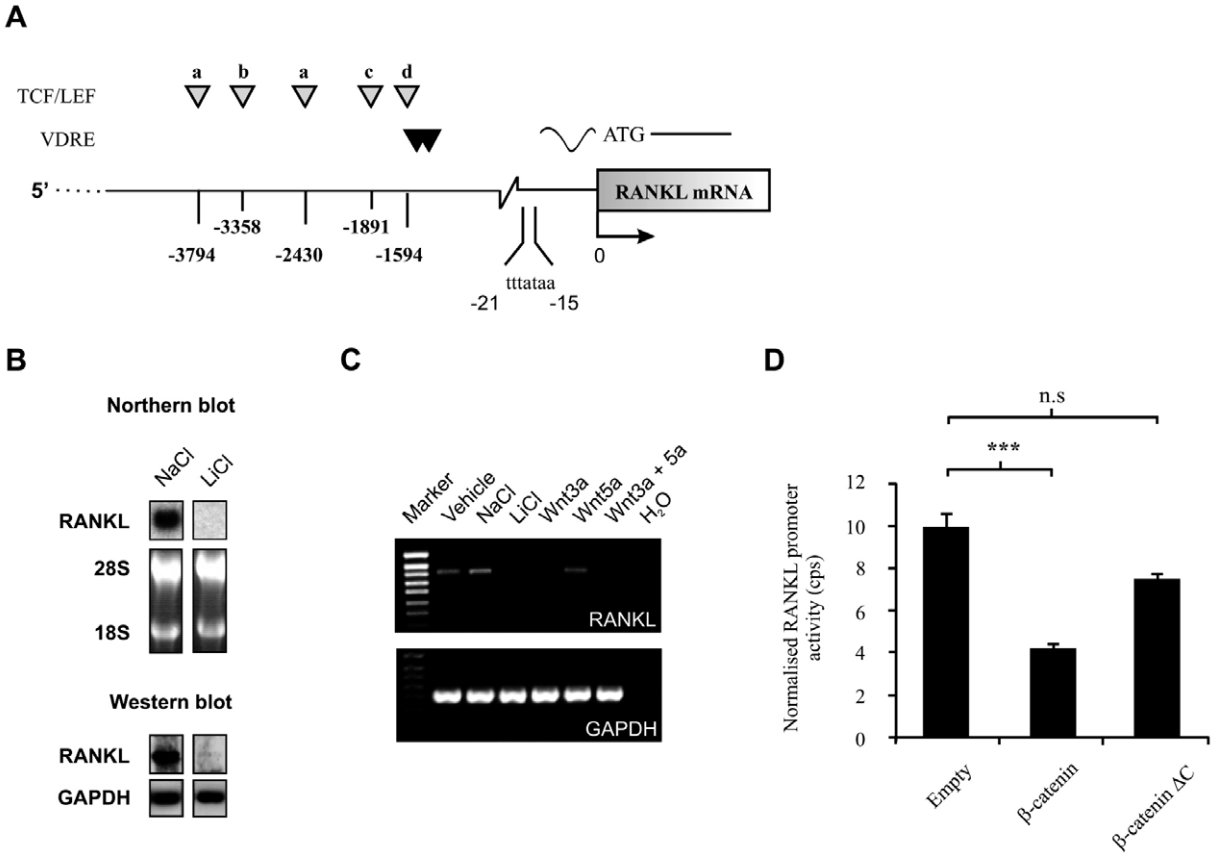

Fig. 5. (A) Schematic representation of the human *RANKL* promoter illustrating putative TCF/LEF transcription factor binding sites. MattInspector software was used to examine the human RANKL promoter (GenBank accession number AF333234) for the presence of the consensus TCF/LEF binding sequence ctttgww. Relative positions and nucleotide sequences of putative binding sites are shown (a-d: a, ctttgaa; b, ttcaaag; c, ctttgat; d, ctttgta). Positions indicated are relative to the translational start codon of the *RANKL* mRNA. Functional vitamin D_3 response elements (VDRE) are shown for comparison (Kitazawa et al., 2003). (B) Mimicking Wnt signalling in osteoblasts inhibited expression of receptor activator of NFκB ligand (RANKL). Inhibition of GSK3β with LiCl (20 mM) potently inhibited *RANKL* mRNA expression by MC3T3-E1 cells and protein expression by MG-63 osteoblastic cells compared with the NaCl control. (C) RT-PCR analysis illustrating the inhibitory effects of recombinant Wnt on *RANKL* mRNA expression by MC3T3-E1 cells. Exposure to Wnt3a (100 ng/ml) inhibited expression at levels comparable with LiCl (20 mM). Expression was unaffected by treatment with recombinant Wnt5a (500 ng/ml). (D) Overexpression of full-length β-catenin inhibited basal activity of the *RANKL* promoter in ST2 stromal cells. Inhibitory effects were dependent on the C-terminal transactivation domain of β-catenin deleted in β-catenin ΔC695-781. Values are mean ± s.e.m., n=8; ***P≤0.001; n.s., not significant.

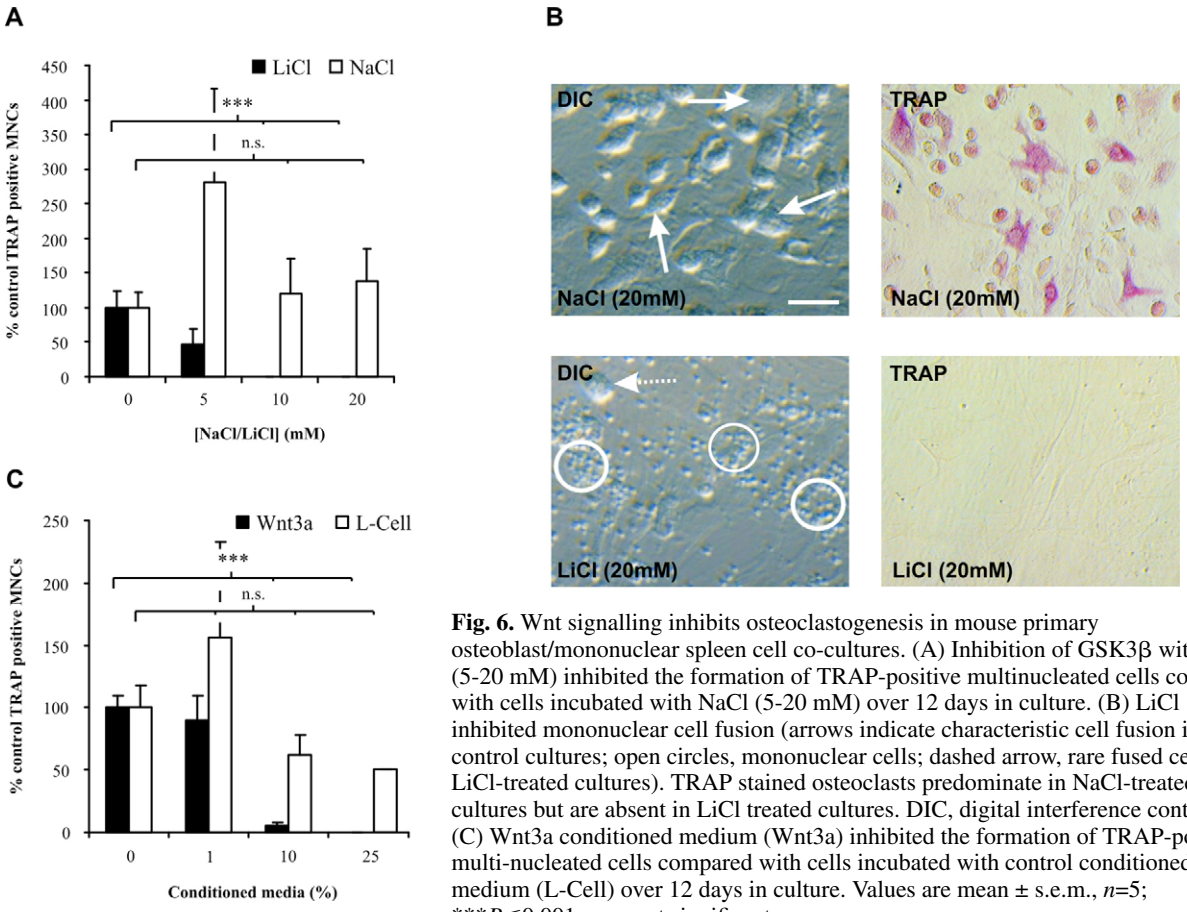

Fig. 6. Wnt signalling inhibits osteoclastogenesis in mouse primary osteoblast/mononuclear spleen cell co-cultures. (A) Inhibition of GSK3β with LiCl (5-20 mM) inhibited the formation of TRAP-positive multinucleated cells compared with cells incubated with NaCl (5-20 mM) over 12 days in culture. (B) LiCl inhibited mononuclear cell fusion (arrows indicate characteristic cell fusion in control cultures; open circles, mononuclear cells; dashed arrow, rare fused cell in LiCl-treated cultures). TRAP stained osteoclasts predominate in NaCl-treated cultures but are absent in LiCl treated cultures. DIC, digital interference contrast. (C) Wnt3a conditioned medium (Wnt3a) inhibited the formation of TRAP-positive multi-nucleated cells compared with cells incubated with control conditioned medium (L-Cell) over 12 days in culture. Values are mean ± s.e.m., n=5; ***$P \leq 0.001$; n.s., not significant.

Effects of Wnt3a on osteoclast survival and bone resorption

To determine whether canonical Wnt signalling affected survival or the resorptive activity of mature osteoclasts, osteoclasts were generated from peripheral blood mononuclear cells by culturing them for 12 days with M-CSF and RANKL, at which point mature osteoclasts predominate. Osteoclasts were then incubated with recombinant Wnt3a (50 ng/ml) or vehicle alone for the final 2 days of culture, during which time the pH of the medium was reduced to initiate resorption (Fig. 10). Wnt3a had no reproducible stimulatory or inhibitory effect on the survival of osteoclasts and failed to significantly affect bone resorption.

Discussion

The data presented in this study support the emerging view that Wnt signalling plays an important role in bone physiology. Using RT-PCR we have demonstrated that osteoblasts express multiple Wnt ligands, FZD receptors and SFRPs, consistent with their ability to send, receive and inhibit Wnt signals. Although expression of Wnt signalling molecules varied between cells, we identified consistent expression of *WNT2b2*, *WNT4*, *WNT5a*, *FZD2*, *FZD4*, *FZD6* and *SFRP2* by all osteoblastic cells tested. To date most experimental studies on the responses of bone cells to Wnt have been performed on multipotent cell lines (Bradbury et al., 1994; Gong et al., 2001; Rawadi et al., 2003). Given the paucity of data describing the expression of Wnt signalling molecules by osteoblasts, this Wnt/FZD/SFRP profile provides a useful insight into the identity of Wnt molecules and their potential availability for signalling in the bone microenvironment. Considering the growing number of reports that implicate Wnt signalling in the regulation of multiple cell types and cell lineages in bone, these data may help clarify some studies in which the origin of paracrine Wnt-signalling molecules mediating specific effects remain unclear.

Assuming limited functional redundancy, the expression of multiple FZD receptors by osteoblasts suggests that downstream transcriptional responses are likely to depend on highly specific, Wnt/FZD interactions. As the identity of individual Wnt ligands responsible for regulating target genes is not clear, we chose to use LiCl to mimic Wnt signalling and determine transcriptional effects of global signalling in osteoblasts, thereby circumventing the need to test multiple Wnts for ligand-specific activity. Lithium has been demonstrated to affect development in a wide range of organisms, inducing axis duplication and altered cell-fate specification, phenotypes that are consistent with overstimulation of canonical Wnt signalling (Kao et al., 1986; Van Lookeren Campagne et al., 1988). Lithium exerts these effects through inhibition of GSK3β, inducing nuclear translocation of β-catenin and stimulating gene transcription, leading to its widespread use as mimetic of canonical Wnt signalling (Klein and Melton, 1996; Stambolic et al., 1996;

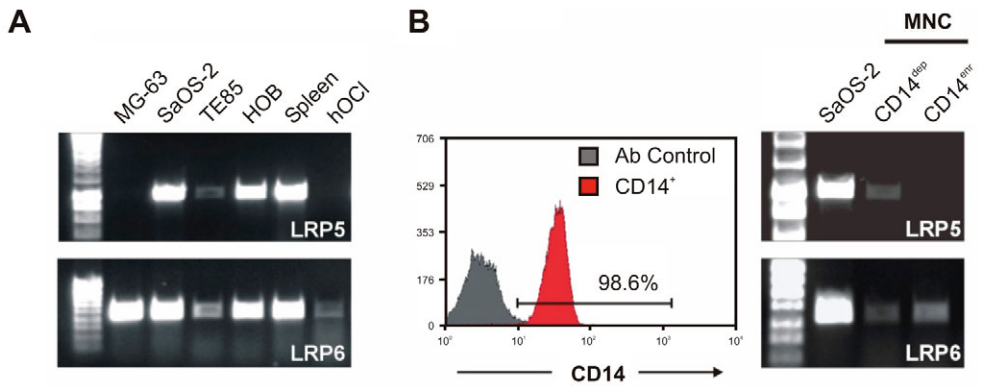

Fig. 7. Expression of LRP5 and LRP6 Wnt co-receptors by osteoblasts, osteoclast progenitors and mature osteoclasts. (A) RT-PCR demonstrating expression of *LRP5* and *LRP6* using cDNA generated from human osteoblastic cells (MG-63, SaOS-2 and TE85), primary human osteoblasts (HOB), mouse spleen and mature osteoclasts derived from human long-term bone marrow cultures. (B) Histogram illustrating the purity of $CD14^+$ cells determined by flow cytometry following isolation from the mononuclear (MNC) fraction of human peripheral blood by magnetic activated cell sorting. RT-PCR demonstrating expression of *LRP5* and *LRP6* by $CD14^-$ (depleted) and $CD14^+$ (enriched) cells is shown on the right. The PCR product obtained using SaOS-2 cDNA is shown for comparison.

Hedgepeth et al., 1997). However, lithium also affects other signalling pathways and inhibits inositol monophosphatase (IMPase) (Berridge et al., 1989), decreasing inositol trisphosphate (IP_3) synthesis and negatively regulating phospholipase C signalling. Complete inhibition of IMPase with an inhibitor 1000 times more potent than lithium however, had no effect on the morphogenesis of *Xenopus* embryos (Klein and Melton, 1996), and *Dictyostelium discoideum* mutants lacking the ability to generate IP_3 developed normally (Drayer et al., 1994). These data suggest that any inhibition of IMPase by lithium does not interfere with Wnt-mediated developmental processes. In our hands, LiCl induced β-catenin nuclear translocation at levels comparable to Wnt3a and stimulated TCF/LEF transcription in osteoblasts at levels comparable to overexpression of full-length β-catenin known to activate TCF/LEF transcription in heterologous expression systems (Sadot et al., 1998). We performed a search of promoters containing consensus TCF/LEF binding motifs (ctttgww) to identify candidate genes regulated by Wnt signalling in osteoblasts. Using MatInspector software, five consensus TCF/LEF binding sites were identified in the 5′ proximal region of the human *RANKL* promoter, implicating *RANKL* as a potential target of Wnt signalling in osteoblastic cells. Since RANKL plays a fundamental role in osteoclastogenesis by interacting with the RANK receptor on osteoclast progenitors, driving osteoclast cell lineage commitment, monocyte cell fusion and osteoclast maturation through regulation of NFκB-mediated gene expression (Suda et al., 1999), this raised the intriguing possibility that catabolic Wnt signalling mechanisms exist alongside anabolic Wnt pathways to regulate osteoclast formation in bone. Supporting its putative role as a Wnt signalling target, we demonstrated that LiCl or Wnt3a treatment suppressed *RANKL* expression and overexpression of transcriptionally active full-length β-catenin inhibited *RANKL* promoter activity in osteoblastic cells. By contrast, overexpression of β-catenin ΔC695-781, which lacks an essential transactivation domain and transcriptional activity in osteoblasts, failed to inhibit basal activity of the promoter. Although activation of Wnt signalling is commonly associated with stimulation of gene transcription, suppression of Runx2/Cbfa-1 induced activation of the osteocalcin gene promoter by β-catenin/LEF-1 has recently been described in osteoblasts, providing a precedent for Wnt-mediated suppression of bone cell promoters (Kahler and Westendorf, 2003). The precise mechanism responsible for this suppression is unclear, one possibility is that β-catenin translocates to the nucleus and sequesters transcription factors necessary for basal activation of the promoter. Consistent with Wnt-mediated suppression of RANKL expression by osteoblasts, both LiCl and Wnt3a inhibited osteoclast formation in co-culture assays of murine osteoblasts and mononuclear spleen cells in vitro; effects similar to those reported for the effects of LiCl on 1, 25 $(OH)_2$ vitamin D_3-stimulated osteoclast formation in human long-term marrow cultures (Pepersack et al., 1994a).

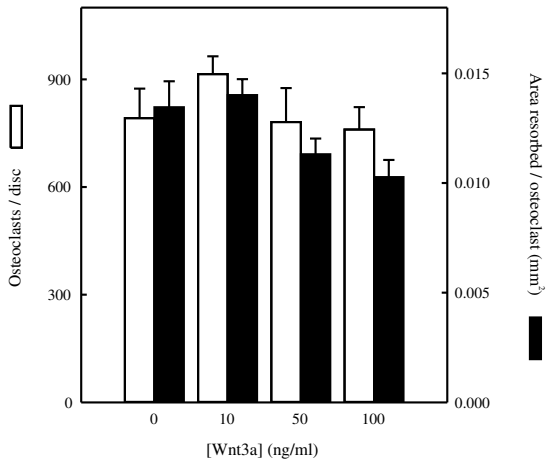

Fig. 8. Effects of Wnt signalling on human osteoclast formation and activity. Wnt3a (10-100 ng/ml) had no effect on the formation of TRAP-positive multinucleated osteoclasts generated from peripheral blood mononuclear cells cultured in the presence of RANKL and M-CSF (open bars) and had no effect on their resorptive activity (closed bars). Data are mean ± s.e.m., n=5.

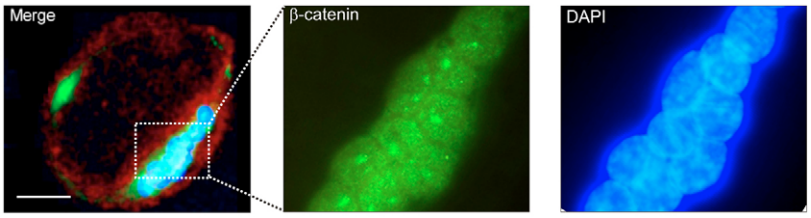

Fig. 9. Evidence for endogenous Wnt signalling in mature osteoclasts. Fluorescent immunolocalisation of β-catenin in mature disaggregated rat osteoclasts. β-catenin predominately localised to nuclei with some peripheral staining (green). Multinucleation is disclosed by DAPI (blue) and peripheral actin ring formation, typical of active bone resorbing osteoclasts is stained with Rhodamine-phalloidin (red). Bar, 10 μm.

Wnt proteins have been demonstrated to play an important role in the proliferation and cell fate specification of haematopoietic stem cells from which osteoclasts are derived (Van Den Berg et al., 1998; Yamane et al., 2001; Willert et al., 2003). Specifically, Wnt3a has been reported to inhibit the differentiation of a number of haematopoietic lineages including osteoclast-like TRAP-positive multinucleated cells in co-cultures via an indirect mechanism, which was dependent on interactions with stromal cells and could be compensated by the addition of exogenous RANKL in the absence of supporting stroma (Yamane et al., 2001). Our demonstration of Wnt-mediated regulation of RANKL provides evidence for one mechanism through which Wnt3a could mediate these effects. Reciprocal changes in the expression of the RANKL decoy protein osteoprotogerin (OPG) may also contribute to Wnt-mediated regulation of osteoclastogenesis in bone. Wnt3a has been demonstrated to upregulate OPG mRNA expression by micro-array analysis in embryonic carcinoma cells (Willert et al., 2002) and elevated OPG mRNA levels are observed following mechanical loading of gain of function LRP5 mice (LRP5 G171V), engineered to contain the same mutation found in human patients with high-bone-mass disease Johnson et al., 2004). It is tempting to speculate that an increased OPG/RANKL ratio could result in enhanced osteoclastogenesis if translated to a functional change in protein levels and that high bone mass associated with this mutation may be partially attributed to changes in Wnt signalling that affect catabolic regulatory pathways in bone (Johnson et al., 2004).

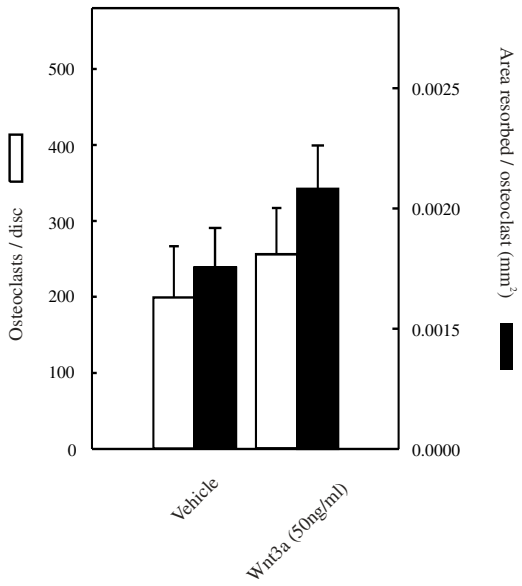

Fig. 10. Effects of recombinant Wnt3a on the survival and activity of mature osteoclasts. Osteoclasts were generated from peripheral blood mononuclear cells cultured in the presence of RANKL and M-CSF for 14 days, and treated with recombinant Wnt3a (50 ng/ml) or vehicle alone for the final 48 hours of culture. The number of TRAP-positive osteoclasts and the area of bone resorption were quantified. Wnt3a had no reproducible effect on osteoclast survival or their resorptive activity. Data are mean ± s.e.m., n=5.

Wnt signalling has recently emerged as candidate pathway in the pathogenesis of multiple myeloma, a plasma cell neoplasia associated with increased osteoclast number and bone resorption, resulting in the formation of osteolytic lesions. DKK-1 is aberrantly expressed by plasma cells in patients with multiple myeloma and directly correlates with the presence of characteristic resorption foci (Tian et al., 2003; Derksen et al., 2004). Interpretation of these data based on our experiments may account for increased osteoclast number in multiple myeloma, if excessive antagonism of osteoblastic Wnt signalling by DKK-1 causes an increase in expression of RANKL and promotes osteoclastogenesis.

The lack of a reproducible effect of Wnt3a on the generation of osteoclasts from peripheral blood monocytes cultured in the presence of exogenous M-CSF and RANKL and absence of stromal cells/osteoblasts provides strong evidence for a mechanism of inhibition for Wnt3a involving the downregulation of RANKL expression by osteoclast supporting stroma/osteoblasts. Interestingly, reciprocal downregulation of OPG expression has been recently reported in response to Wnt3a in vitro and transgenic manipulation of canonical Wnt signalling in vivo, implicating a global shift in the OPG/RANKL ratio to favour inhibition of osteoclastogenesis when Wnt signalling is activated in bone. In this study we were only able to determine the effects of a single commercially available canonical Wnt ligand (Wnt3a) for direct effects on osteoclast progenitors, although to date 19 human Wnt proteins have been identified.

It remains possible that some or all of the effects of manipulating Wnt signalling in co-cultures of mononuclear cells and osteoblasts are mediated independently of effects on osteoblastic RANKL expression, by a direct action on osteoclast progenitors. By RT-PCR we have demonstrated that a second Wnt co-receptor, *LRP6* is expressed at high levels by these cells, which provides evidence for their ability to receive and transduce Wnt signals in the absence of LRP5. However, we were unable to demonstrate any reproducible effect of recombinant Wnt3a on osteoclast differentiation or bone resorption in PBMC-derived osteoclast cultures generated with exogenous RANKL and M-CSF in the absence of stromal cells. These data suggest that Wnt3a specifically regulates osteoclast differentiation via an indirect mechanism, involving the downregulation of RANKL expression by osteoblasts and

raises a question over the function of LRP6 expressed by osteoclast progenitors. It is possible that other Wnts have direct effects on these cells, mediated by LRP6, which regulate as yet unidentified functions in progenitor cells. Similarly, we were unable to demonstrate any effect of Wnt3a on osteoclast survival or resorptive activity but cannot exclude a role for other Wnts in the regulation of mature osteoclast biology. In preliminary experiments we also identified expression of *LRP6* by mature human osteoclasts and have demonstrated that β-catenin frequently localises to nuclei of disaggregated rat osteoclasts, consistent with endogenous Wnt signalling and transcription of Wnt target genes in mature osteoclasts. Expression of β-catenin and APC have previously been described in mature osteoclasts in vivo (Monaghan et al., 2001) and LiCl has been reported to inhibit bone resorption in cultured foetal rat long bones (Pepersack et al., 1994b). The precise role of Wnt signalling and the mechanisms by which these effects are mediated are currently unclear but warrants further investigation, one possibility is that nuclear β-catenin influences osteoclast activity by regulating the DNA binding activity of the transcription factor NFκB in a manner similar to that recently described in breast cancer cells (Deng et al., 2004). Consequently, specific manipulation of Wnt signalling in osteoclast progenitors or mature cells may be an attractive therapeutic avenue for the treatment of bone disorders such as osteoporosis.

It is also possible that some of the observed effects of Wnts on osteoclastogenesis are mediated by reduced RANKL expression which results from decreased osteoblast differentiation. RANKL expression decreases with osteoblast differentiation (Deyama et al., 2000; Atkins et al., 2003) and some researchers (De Boer et al., 2004), including ourselves (unpublished observations) have demonstrated that Wnt signalling impairs the osteogenic differentiation of human mesenchymal stem cells in vitro. However, we feel that this is unlikely to solely account for the Wnt-induced down regulation of pro-osteoclastogenic cytokines. If this were the case one would expect to see increased osteoclast numbers in association with the low bone mass phenotype of LRP5 knockout mice, which display a phenotype of delayed osteoblast proliferation and mineralisation. LRP5 knockout mice have normal osteoclast numbers and indicators of bone resorption including urine deoxypyridinoline:creatinine ratio compared with age-matched wild-type littermates (Kato et al., 2002).

Although the body of evidence from transgenic and knockout studies support an exclusive role for LRP5/Wnt signalling in the regulation of bone mass through the control of osteoblast activity, in vivo evidence also suggests that catabolic Wnt-signalling pathways may contribute to the regulation of osteoclast formation and activity in bone. Previous studies have demonstrated that targeted knockout of the Wnt co-receptor *LRP5* has no effect on osteoclastogenesis in vitro, which is consistent with the absence of osteoclastic LRP5 expression in vivo (Kato et al., 2002). Our data demonstrating negative regulation of RANKL expression by Wnt signalling in osteoblasts however, supports an indirect osteoblast-mediated effect on osteoclast formation, which may not have been observed in ex vivo cultures performed using osteoclast progenitors derived from *LRP5*$^{-/-}$ mice, if osteoclasts were generated by the addition of exogenous RANKL (Kato et al., 2002). Following our hypothesis, an increase in RANKL expression caused by inhibition of Wnt signalling (by *LRP5* knockout) could cause increased osteoclast numbers and contribute to the low-bone-mass phenotype of *LRP5*$^{-/-}$ mice. Alternatively the lack of resorptive phenotype could result from compensatory mechanisms which counteract Wnt-induced changes to maintain 'normal' osteoclast numbers and bone resorption. Our identification of *LRP6* expression by osteoclast progenitors, mature osteoclasts and osteoblasts, raises the possibility that catabolic effects of Wnt signalling, including those involving Wnt-mediated changes in *RANKL* expression, are transduced specifically by LRP6 acting in opposition to anabolic effects mediated by LRP5. Measurements of osteoclast number, bone resorption markers and adult bone mass in mice with homozygous deletion of *LRP6* have not been possible because of embryonic lethality, although *LRP6*$^{+/-}$ heterozygotes have lower total and trabecular BMD compared with age-matched controls (Kharode et al., 2003). Delayed ossification at birth and reduced bone mass have also been described for ringelschwanz mutant mice, caused by a point mutation in *LRP6* and disrupted canonical Wnt signalling, in addition to a number of skeletal abnormalities mirroring multiple Wnt-phenotypes. An elegant genetic study of compound mutations in *LRP5* and *6* using mice heterozygous for *LRP6* and either heterozygous or homozygous for a mutation in *LRP5* (*LRP6*$^{+/-}$; *LRP5*$^{+/-}$ or *LRP6*$^{+/-}$; *LRP5*$^{-/-}$) has revealed that BMD decreases as mice are progressively more deficient in each co-receptor and demonstrated LRP6 haploinsufficiency further exacerbates the low-bone-mass phenotype of *LRP5*$^{-/-}$ mice (Holmen et al., 2004). Although overall LRP5 and -6 appear to act independently of one and another to affect bone mass, by acting on osteoblasts at different skeletal sites (trabecular versus cortical) the potential exists that LRP5 and -6 operate to transduce Wnt signals and regulate bone mass by acting on different cellular targets (osteoblasts and osteoclasts). As such, it will be interesting to determine whether some or all of these phenotypes described for *LRP6* mutant mice are transduced through Wnt-mediated changes in osteoclast number or activity. Osteoblast-specific knockout of APC, a protein involved in constitutive β-catenin degradation in the absence of Wnt signals, has been demonstrated to cause a dramatic increase in bone acquisition and accelerated osteoblast differentiation, in addition to dramatically reducing the number of osteoclasts. By contrast, osteoblast-specific deletion of β-catenin results in severe osteopenia and marked increases in osteoclast number (Holmen et al., 2005). Both osteoclast phenotypes were associated with changes in the expression of RANKL by osteoblasts. Combined with our study, these data strongly implicate RANKL as a direct target of Wnt signalling in osteoblasts. Glass and co-workers (Glass et al., 2005) performed a similar study in which they showed that stabilisation of β-catenin in differentiated osteoblasts resulted in high bone mass whereas its deletion caused osteopenia. In this instance phenotypes were found to be specifically associated with changes in the expression of the RANKL decoy protein, OPG by osteoblasts. Taken together these data implicate reciprocal changes in RANKL and OPG expression in mediating the effects of Wnt signalling on osteoclast differentiation in vivo.

Further support for a role for Wnt signalling in bone resorption have come from demonstrations that the Wnt antagonist SFRP-1 inhibit osteoclastogenesis in co-cultures of osteoblasts and spleen cells and mononuclear cell cultures induced by exogenous RANKL and M-CSF. These effects are thought to be mediated by a mechanism involving SFRP-1 binding to RANKL preventing its interaction with RANK in a manner similar to OPG, independent of effects on osteoblastic Wnt signalling. However, it remains a possibility that some or all of the anti-osteoclastogenic effects of SFRP-1 in co-cultures are mediated by osteoblasts or a direct action on osteoclast progenitors, possibly by allowing Wnt signalling to proceed unopposed in these cells (Hausler et al., 2004). Paradoxically, mice homozygous for SFRP-1 deletions have high bone mass and increased osteoclast formation ex vivo but normal bone resorption markers and a phenotype more consistent with enhanced osteoblast function in vivo. However, it remains possible that negative regulators of osteoclast formation compensate or act to limit enhanced osteoclastogenesis caused by loss of SFRP-1 in vivo (Bodine et al., 2004).

Although strong evidence exists to support a functional role for Wnt signalling in the regulation of osteoblast proliferation, differentiation and function (Gong et al., 2001; Boyden et al., 2002; Kato et al., 2002; Little et al., 2002; Patel and Karsenty, 2002; Babij et al., 2003; Van Wesenbeeck et al., 2003), until now little has been reported on the effects of Wnt signalling on osteoclastic cells and their progenitors. In this study we provided evidence to support an indirect role for osteoblastic Wnt signalling in the regulation of osteoclast formation. These data suggest that net changes in bone mass induced by Wnt signalling may result from changes in both bone formation and bone resorption through regulation of osteoblast and osteoclast formation and activity.

Considering the growing number of cells responsive to Wnt in the bone microenvironment it is becoming increasingly important to determine precisely which ligands and receptors are responsible for mediating different effects of signalling, in order to realise their true therapeutic potential. Differential effects of Wnt signalling in bone could be mediated at several levels by cell-specific Wnt ligands, differential expression of Frizzled receptors, cross-talk with co-regulatory signalling pathways or by changes in expression of lineage-specific Wnt-target genes.

Materials and Methods

Cell culture

Human primary osteoblasts (HOBs) and human clonal MG-63 and TE85 osteoblastic cells were cultured in Dulbecco's modified Eagle's medium (DMEM), 10% foetal bovine serum (FBS). Mouse primary osteoblasts (MOBs) and mononuclear spleen cells (MNCs), mouse clonal MC3T3-E1 preosteoblastic cells, human SaOS-2 osteoblastic cells were cultured in α-minimum essential medium (α-MEM), 10% FBS. Mouse stromal ST2 cells were cultured in RPMI medium, 10% FBS. All media contained 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Cells were cultured in a humidified atmosphere at 37°C in 5%CO_2:95% air.

HOBs were isolated from explants of trabecular bone derived from femoral heads removed during hip replacement surgery following informed consent and MOBs were isolated by sequential collagenase digestion as previously described (Beresford et al., 1984; Bellows et al., 1986; Peet et al., 1999). MNCs were prepared from 12-16-week-old male B6Cba mice. Briefly, spleens were rapidly dissected and triturated repeatedly in Hank's buffered saline solution (HBSS) and passed through a 40 μm sieve. Mononuclear cells were fractionated by Ficoll density gradient centrifugation using Ficoll Paque (Amersham) according to the manufacturer's instructions.

Generation of Wnt3a-conditioned medium

Mouse Wnt3a overexpressing cells (L-Wnt3a) and control non-transfected L-cells were obtained from the American Type Culture Collection (ATCC, Manassas, USA; http://www.atcc.org) and cultured in DMEM with glutamax, 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. L-Wnt3a culture medium was supplemented with 400 μg/ml geneticin to maintain selective pressure. Conditioned medium from L-Wnt3a and control L-cells was collected according to the manufacturer's instructions. Briefly, cells were passaged 1:10 in 8 ml medium without geneticin and left to grow for 10 days. Medium was collected from each cell line and replaced with 8 ml fresh medium for a further 3 days. This second batch of medium was then collected and the cells discarded. First and second batches were combined, sterile filtered (0.2 μm) and stored at –20°C until required.

Co-culture assays

Osteoclasts were generated from MNCs (2×10^6 cells/ml) co-cultured with MOBs (5×10^4 cells/cm^2) in α-MEM, 10% heat-inactivated FBS supplemented with 10 nM 1,25 $(OH)_2$ Vitamin D_3 (Sigma) and 100 nM dexamethasone. Cells were maintained in culture for 12 days, replenishing 50% of the medium every 3 days. Cells were incubated with LiCl (5-20 mM), NaCl (5-20 mM), Wnt3a-conditioned medium (1-25%) or control conditioned medium (1-25%) throughout the entire culture period, after which cells were stained for the osteoclast marker, tartrate-resistant acid phosphatase (TRAP) and counterstained with DAPI to identify nuclei. Briefly, cells were washed gently in phosphate-buffered saline (PBS) and sequentially fixed in 3.7% formaldehyde for 10 minutes and 50% ethanol:acetone (v/v) for 1 minute at room temperature. TRAP staining was performed at 37°C for 10 minutes by incubating cells in TRAP buffer [70 mM sodium acetate, 30 mM acetic acid, 0.1 mg/ml Napthol AS-MX phosphate disodium salt, 0.1% Triton X-100 (v/v) pH 5.0] containing 0.3 mg/ml Fast Red Violet LB stain. Numbers of TRAP-positive osteoclasts were counted from five replicate wells and TRAP-positive cells with greater than two nuclei were scored as osteoclasts.

Generation of osteoclasts from human peripheral blood monocytes

Mononuclear cells were isolated from peripheral blood from normal human volunteers. Briefly, 20 ml whole blood samples were diluted with 15 ml PBS, gently layered over 15 ml Ficoll Paque Plus (Amersham Pharmacia Biotech, Sweden) and centrifuged at 750 ***g*** for 30 minutes. The mononuclear cell layer was then removed from the Ficoll-plasma interface, washed in 10 ml PBS, centrifuged at 750 ***g*** for 10 minutes and resuspended in minimum essential medium (MEM) with 15% heat-inactivated foetal calf serum (FCS). The mononuclear cells (200,000 cells in 100 μl) were sedimented onto 16 mm^2 slices of dentine in 96-well plates for 4 hours (Arnett and Dempster, 1986). The dentine slices were then rinsed by dipping gently in PBS, transferred to fresh 96-well plates containing 200 μl MEM, 15% FCS, 5 ng/ml M-CSF and 5 mEq/l OH^- (as NaOH), and cultured for 4 days. After this initial 4 day culture period, dentine slices were transferred to 25 cm^2 flasks containing 1 ml per slice of the same culture medium, plus 1 ng/ml RANKL, and cultured for a further 10 days in a 5% O_2:5% CO_2:90% N_2 atmosphere. The hypoxic environment stimulates osteoclast formation strongly (Arnett et al., 2003); addition of alkali to the culture medium at this stage results in an operating pH of ~7.40 at equilibrium with 5% CO_2, further promoting osteoclast differentiation while largely maintaining osteoclasts in an inactive state, so that little excavation of resorption pits occurs (Hoebertz and Arnett, 2003). For the final 48 hours, cells were incubated in medium containing either 5 MEq/l OH^- or 10 MEq/l H^+ (as HCl). To determine the effects of Wnt on osteoclast differentiation and resorption, cell medium was supplemented with 0, 10, 50 or 100 ng/ml recombinant mouse Wnt3a throughout the entire culture period. To determine effects of Wnt on the survival and resorptive activity of mature osteoclasts, cells were incubated with 50 ng/ml recombinant Wnt3a or with vehicle alone for the final 48 hours of culture only. Culture medium pH and PCO_2 were monitored by blood gas analyser (ABL 705, Radiometer, Crawley, UK). Cultures were fixed in 2% glutaraldehyde and stained to demonstrate TRAP for 40 minutes. Osteoclasts were defined as TRAP-positive cells with two or more nuclei. The total number of TRAP-positive cells on each dentine slice was counted 'blind' using transmitted light microscopy. The plan surface area of resorption pits on dentine slices was measured 'blind' using reflected light microscopy and dot-counting morphometry (Hoebertz and Arnett, 2003).

Isolation of disaggregated rat osteoclasts

Neonatal rat osteoclasts were isolated from the long bones of 4-day-old male Wistar rats. Briefly, long bones (femora, tibiae and ulnae) were rapidly dissected from six rats, separated from the surrounding muscle tissue and temporarily stored in HBSS. Bones were finely minced using a scalpel blade and the mixture vortexed for 1 minute. Bone chips were allowed to sediment for 30 seconds, the supernatant was carefully removed and the cells were pelleted by centrifugation at 1500 ***g*** for 5 minutes. Cells were resuspended in α-MEM containing 10% FBS and seeded onto glass coverslips by sedimentation for 30 minutes at 37°C. Media were carefully removed, coverslips washed gently in PBS and fresh media added. Cells were maintained for up to 2 days at 37°C in a humidified atmosphere in 95% air:5% CO_2.

Reverse-transcriptase PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and contaminating genomic DNA removed by DNAse digestion using DNA-free™ (Ambion). Purity and integrity of RNA was determined by agarose gel electrophoresis and spectrophotometry. All RNA had an A260/A280 absorbance ratio of ⩾1.8. cDNA was synthesised from 1-5 μg of total RNA using SuperScript II (Invitrogen). PCR amplification of Wnt genes was performed using sequence-specific primers (Genosys) (see Table 1 in supplementary material) in a total reaction volume of 50 μl containing 1× PCR buffer, 0.2 μM dNTPs, 2.5 mM $MgCl_2$, 0.2 μM of forward and reverse primers, 0.5 U Platinum Taq polymerase (Invitrogen) and 5 μl of 1:10 diluted cDNA. Annealing temperatures were optimised for individual primer pairs. PCR was performed for a total of 35 cycles of 94°C for 10 seconds, x°C for 20 seconds (where x is the annealing temperature), and 72°C for 1 minute following an initial denaturation step of 3 minutes at 94°C. A final extension at 72°C was performed at the end of cycling for 10 minutes. RANKL PCR was performed for a total of 40 cycles of 94°C for 10 seconds, 60°C for 20 seconds and 72°C for 1 minute, using the following primers; 5′-taatacgactcactata-gggatcgctctgttcctgtactttcgag-3′ and 5′-taatacgactcactatagggtggacacctgaatgctaattt-cttc-3′. To control for genomic DNA contamination, parallel cDNA synthesis reactions were carried out in the absence of reverse transcriptase (no-RT controls). Purity and integrity of cDNAs were confirmed by PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). No products were amplified using no-RT control samples. PCR products were purified from agarose gels using Qiaquick PCR purification kit (Qiagen), and cloned into pCR2.1 (Invitrogen) or PCRScript (Stratagene) plasmids. Inserts were sequenced using an ABI 377 automated DNA sequencer (Perkin Elmer). Sequences of the amplicons were used to query the EMBL and Genbank DNA databases using basic local alignment search tool (http://www.ncbi.nlm.nih.gov/BLAST/).

Treatment of osteoblastic cells with Wnt3a, LiCl and Dickkopf-1 (Dkk-1)

For immunocytochemisty, SaOS-2 and MC3T3-E1 cells were seeded overnight onto glass coverslips at an initial density of 5×10^4 cells/cm^2 and incubated for 24 hours in media containing LiCl or NaCl (20 mM), Wnt3a-conditioned medium or L-cell-conditioned medium (1-50%) or 25% Wnt3a-conditioned medium in the absence and presence of recombinant Dkk-1 (0.01-0.1 μg/ml). For protein and RNA extraction, osteoblastic cells were grown to approximately 80% confluence and incubated with either LiCl (20 mM) or NaCl (20 mM), or recombinant Wnt3a (100 ng/ml) and/or Wnt5a (500 ng/ml) for 24 hours as indicated.

Northern blot analysis

10 μg total RNA was fractionated on a 0.8% denaturing gel and transferred overnight by capillary blotting to Hybond N^+ nylon membrane. RANKL cDNA probe was prepared using the PCR product as a template by random prime labelling using [α-^{32}P]dCTP (Rediprime II, Amersham Biosciences). Hybridisations were performed at 68°C for 1 hour and excess probe removed by washing twice for 15 minutes in 2× sodium citrate buffer (SSC), 0.1% SDS at room temperature and twice in 0.1× SSC, 0.1% SDS at 60°C. Autoradiographs were generated using BioMAX film and two intensifying screens following overnight exposure at –80°C.

Western blot analysis

Whole cell lysates were prepared by incubating cells on ice for 10 minutes with vigorous cell scraping in lysis buffer containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate and 1 mg/ml leupeptin in 20 mM Tris-HCl pH 7.5. Cell lysates were centrifuged at 12,000 ***g*** at 4°C for 10 minutes to remove cellular debris and protein concentration was determined by BCA protein assay (Pierce). 10 μg protein were fractionated by denaturing polyacrylamide gel electrophoresis and transferred to PVDF membrane (Amersham Biosciences). Membranes were blocked in 4% dried milk/Tris-buffered saline Tween-20 (TBS-T) for 1 hour at room temperature, rinsed twice, washed for 15 minutes in TBS-T and incubated with mouse monoclonal anti-RANKL antibody (2 μg/ml, Imgenex) for 1 hour at room temperature. Following further washes in TBS-T, membranes were incubated with horseradish peroxides (HRP)-conjugated anti-mouse secondary antibody (Santa Cruz, 1:1000 dilution) for 45 minutes at room temperature. HRP activity was disclosed by enhanced chemiluminescence (Super Signal West Femto, Pierce) and autoradiographs generated by exposing membranes to Hyper film (Kodak) at room temperature for up to 5 minutes.

Fluorescent immunolocalisation of β-catenin

SaOS-2 and MC3T3-E1 cells were seeded overnight onto glass coverslips at an initial density of 5×10^4 cells/cm^2 and incubated for 24 hours. Following treatment, medium was removed, cells were fixed in 4% paraformaldehyde for 5 minutes and incubated in 10% goat serum/0.1% Triton X-100 (v/v) for 30 minutes to block non-specific binding and facilitate access to intracellular epitopes. Excess serum was removed and the cells incubated overnight at 4°C with β-catenin antibody (c2206, 1:1000 dilution, Sigma), or normal rabbit IgGs (1.0 μg/ml, Sigma) as a negative control followed by FITC-conjugated goat anti-rabbit secondary antibody (Sigma, 1:100) for 45 minutes. Coverslips were mounted in Vectorshield containing DAPI to identify nuclei (Vector Laboratories Ltd) and cells viewed under UV illumination using a Leica DMLA microscope (Leica). Images were captured using a DC500 CCD camera and number of DAPI-positive and β-catenin-positive nuclei quantified by automated image analysis (QWin, Leica). With the exception of blocking, cells were washed three times for 5 minutes in phosphate buffered saline (PBS) following each incubation. All washes and incubations were performed at room temperature unless otherwise stated.

Plasmid constructs and transfections

TCF/LEF-Firefly luciferase reporter plasmid (TOPFLASH) and control reporter containing mutant TCF binding sites (FOPFLASH) were purchased from Upstate Biotechnology. pcDNA3.1(–), $pEGFPN_2$ and phRL-CMV were obtained from Invitrogen, Clontech and Promega respectively. P7 RANKL luciferase reporter construct containing the mouse *RANKL* promoter (–6880 to +115 relative to the transcriptional start site) was a kind gift from C. A. O'Brien, University of Arkansas for Medical Sciences, Little Rock, AR. Full-length β-catenin pEGFP and β-catenin ΔC695-781 pEGFP were generated by directional cloning into *Sal*I and *Bam*HI restriction sites of $pEGFPN_2$ and full length and β-catenin ΔC685-781 pcDNA3.1-constructed by directional cloning into *Xho*I and *Bam*HI sites. cDNAs were generated by RT-PCR using MG-63 cDNA as template and oligonucleotide primers containing 5′ restriction sites designed to flank the coding region of cDNA to be amplified. Primers used to generate β-catenin constructs were: FL β-catenin $pEGFPN_2$, 5′-ACAGGTCGACGTGGACAATGGCTACTCAAGCTGATTTG-3′ and 5′-TAAGGATCCGTAACAGGTCAGTATCAAACCAGGCCAGC-3′; β-catenin ΔC695-781 $pEGFPN_2$, 5′-ACAGTCGACGTGGACAATGGCTACT-CAAGCTGATTTG-3′ and 5′-TACGGATCCGATCAGCAGTCTCATTCCAAGC-CATTG-3′; Full-length β-catenin pcDNA3.1(–), 5′-ACAGGTCGACGTGGA-CAATGGCTACTCAAGCTGATTTG-3′ and 5′-TAAGGATCCTTACAGGTC-AGTATCAAACCAGGCCAGC-3′; β-catenin ΔC695-781 pcDNA3.1(–), 5′-ATATCTAGAATGGTGAACATGCAGTTGTAAACTTGATTAAC-3′ and 5′-TACGGATCCGATCAGCAGTCTCATTCCAAGCCATTG-3′. ΔC constructs encode β-catenin in which the C-terminal 86 amino-acids containing the transactivation domain of the protein have been removed by the introduction of an artificial stop codon. MG-63 and MC3T3-E1 cells were transiently transfected in 96-well plates using Lipofectamine plus reagent (Invitrogen) according to the manufacturer's instructions. Typical transfection efficiencies ranged from 40-60% as assessed by fluorescent imaging of cells transfected with EGFP vector alone.

TCF reporter gene assays

MG-63 cells were transfected with 0.005 μg TOPFLASH or 0.005 μg FOPFLASH plasmid and 0.025 μg empty $pEGFPN_2$ vector (Clontech), full-length β-catenin pEGFP (FL β-catenin) or β-catenin ΔC (695-781) pEGFP (β-catenin ΔC), using a constitutively active phRL-CMV Renilla luciferase vector (0.005 μg, Promega) as an internal control for transfection efficiency. Firefly and Renilla luciferase activity were sequentially determined 24 hours after transfection using the Dual Glo luciferase assay system according to the manufacturer's instructions (Promega). To determine TCF responses to LiCl, cells were transfected with TOPFLASH or FOPFLASH reporter gene plasmids (0.025 μg) and (0.005 μg) phRL-CMV Renilla luciferase. 24 hours after transfection cells were treated with LiCl (20 mM) or vehicle for the duration indicated and Firefly and Renilla luciferase activities determined as above.

RANKL reporter gene assay

MC3T3-E1 cells were transfected with 0.01 μg p7 mRANKL luc, 0.01 μg phRL-CMV and 0.04 μg empty pcDNA3.1(–) vector, full-length β-catenin pcDNA3.1(–) or ΔC β-catenin pcDNA3.1(–). 24 hours after transfection Firefly and Renilla luciferase activities were determined as above.

Magnetic-activated cell sorting and flow cytometry

Human peripheral blood was collected following informed consent from healthy human volunteers and mononuclear cells extracted by Ficoll density centrifugation as described above. $CD14^+$ cells were isolated from mononuclear cells by two rounds of magnetic activated cell sorting using anti-CD14-labelled micro-beads and a MACs MS^+ column (Miltenyi Biotech), according to the manufacturer's instructions, generating CD14-enriched ($CD14^+$) and CD14-depleted ($CD14^-$) fractions. Purity of $CD14^+$ and $CD14^-$ cells were quantified by flow cytometry using a FITC-conjugated CD14 antibody (Caltag Laboratories) and Cyan flow cytometer (DakoCytomation), using unlabelled cells as a control. Following cytometry, remaining cells were pelleted by centrifugation at 1500 ***g*** for 5 minutes and cDNA synthesised from DNAse-treated total RNA. RT-PCR was performed as described above using the following sequence specific primers to LRP5 (CT-GCTGGGGGACTTCATCTACTGGAC and GGGAGGAGTGGAACACCAGGAT-GTC) and LRP6 (AGACTGGGGAGAAGTGCCAAAGATGAAC and GACTGGA-GACATCAAACACAAATGGGAAC).

Statistical analyses

Statistical significance was determined by ANOVA and Dunnett's post-hoc analysis.

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