Published in final edited form as:

Br J Haematol. 2010 March ; 148(5): 726–738. doi:10.1111/j.1365-2141.2009.08009.x.

Characterization of Wnt/ β -catenin signalling in osteoclasts in multiple myeloma

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Summary

We recently showed that increasing Wnt/ β -catenin signalling in the bone marrow microenvironment or in multiple myeloma (MM) cells clearly suppresses osteoclastogenesis in SCID-hu mice; however, this regulation of osteoclastogenesis could result directly from activation of Wnt/ β -catenin signalling in osteoclasts or indirectly from effects on osteoblasts. The present studies characterized Wnt/ β -catenin signalling and its potential role in osteoclasts. Systematic analysis of expression of *WNT*, *FZD*, *LRP* and *TCF* gene families demonstrated that numerous Wnt-signalling components were expressed in human osteoclasts from patients with MM. Functional Wnt/ β -catenin signalling was identified by accumulation of total and active β -catenin and increases in Dvl-3 protein in response to Wnt3a or LiCl. Furthermore, Wnt-induced increases in β -catenin and Dvl-3 were attenuated by Wnt antagonists Dkk1 and sFRP1. Finally, Wnt3a-induced *TCF/LEF* transcriptional activity suggests that canonical Wnt signalling is active in osteoclasts. Supernatants from dominant-negative- β -catenin-expressing osteoblast clones significantly stimulated tartrate-resistant acid phosphatase-positive osteoclast formation from primary MM-derived osteoclasts, compared with supernatants from control cells. These results suggested that Wnt/ β -catenin signalling is active in osteoclasts in MM and is involved in osteoclastogenesis in bone marrow, where it acts as a negative regulator of osteoclast formation in an osteoblast-dependent manner in MM.

Keywords

Wnt3a; β -catenin; osteoclast; bone disease; multiple myeloma

Multiple myeloma (MM) is characterized by osteolytic bone lesions that stem from uncoupled bone remodelling, wherein bone resorption is increased because of increases in osteoclast activation and bone formation is decreased due to inhibition of osteoblast differentiation (Roodman, 2004). Although specific molecular events contributing to initiation and progression of bone lesions in MM are still poorly understood, significant advances in understanding osteoblastogenesis and osteoclastogenesis have resulted from the

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Authorship: contribution

YWQ conceptualized and designed the research, designed and performed experiments, analysed and interpreted results, made figures, and wrote the manuscript; YC, NB, BH, and JE performed experiments; BB conceptualized the research, provided clinical samples, and helped write the manuscript; JDS conceptualized the research and helped write the manuscript.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

study of signalling pathway components that regulate osteoblast differentiation and osteoclast activation. Most recently, emerging studies demonstrated that activation of Wnt/ β -catenin signalling is pivotal for healthy bone development and formation (Gong *et al*, 2001; Boyden *et al*, 2002; Kato *et al*, 2002; Little *et al*, 2002; Baron & Rawadi, 2007); therefore, understanding this signalling axis may contribute to advances in the molecular mechanism of MM pathogenesis and MM-triggered bone diseases. Activation of Wnt/ β -catenin signalling prevents mesenchymal stem cells from differentiating into chondrocytes (Day *et al*, 2005; Hill *et al*, 2005) and adipocytes (Ross *et al*, 2000). *In vivo* studies in transgenic mice demonstrated that expression of active β -catenin (Glass *et al*, 2005) or deletion of APC (Holmen *et al*, 2005), a negative regulator of Wnt signalling, led to reduced osteoclastogenesis.

Wnts comprise a family of 19 secreted glycoproteins that have well-characterized roles in stem cell maintenance and development (Nusse, 2005) and, probably, MM pathogenesis (Qiang *et al*, 2003, 2005; Qiang & Rudikoff, 2004). Many effects of Wnts are mediated through β -catenin, which plays a pivotal role in the canonical Wnt signalling pathway (Wodarz & Nusse, 1998). In the absence of stimulation by Wnt ligands, β -catenin is phosphorylated by GSK3 β in a complex with adenomatous polyposis coli protein (APC) and axin, which targets β -catenin for ubiquitination and rapid proteasome degradation. Wnt proteins bind frizzled (Fz) receptors and low-density lipoprotein receptor-related protein (Lrp) 5/6 co-receptors (Tamai *et al*, 2000), triggering phosphorylation of Lrps by serine/threonine kinase CK1. Phosphorylated Lrps bind axin, which inhibits the APC/axin/GSK complex through dishevelled (Dvl) family members (Yanagawa *et al*, 1995; Lee *et al*, 1999). As a result, Dvl, in a complex with FRAT, inhibits GSK3 β phosphorylation of β -catenin and augments dissociation of the destruction complex. Thus, β -catenin protein accumulates in the cytoplasm and translocates to the nucleus, where its association with T cell factors (*TCF1*, *-3*, and *-4*) and lymphoid enhancer-binding factor 1 (*LEF1*) leads to transcriptional activation of target genes that regulate many cellular processes, including cell cycle progression and differentiation (Clevers, 2006).

Recently, we have demonstrated that elevated expression of Dickkopf-1 (Dkk1) by myeloma tumour cells is associated with formation of bone lesions (Tian *et al*, 2003) and deregulated expression of *TNFRSF11B* (previously termed *OPG*) and *TNFSF11* (previously termed *RANKL*) in osteo-blasts (Qiang *et al*, 2008a). Activation of Wnt/ β -catenin signalling by blockage of Dkk1 activation using a neutralising antibody (Yaccoby *et al*, 2007) or by administration of recombinant Wnt3a protein in the bone marrow microenvironment or by injection of Wnt3a-overexpressing myeloma cells into the bone marrow attenuates MM-triggered bone lesions *in vivo* (Qiang *et al*, 2008b), which is associated with reduced osteoclast numbers. Moreover, osteoclasts are of hematopoietic origin and differentiate from monotypic precursors. Several Wnts regulate monocyte expansion and the differentiation of hematopoietic progenitors (Van Den Berg *et al*, 1998; Brandon *et al*, 2000). Thus, Wnt signalling potentially plays a role in regulating osteoclastogenesis. However, the direct effects of Wnt/ β -catenin signalling on osteoclast maturation and activity currently remain unclear. Therefore, we present our systematic analyses of *WNT*, *FZD* and *TCF* gene families and secreted modulators in human osteoclasts isolated from 10 MM patients and in a preosteoclast cell line (Raw264.7), as well as investigations of functional activation of Wnt/ β -catenin signalling and the associated biological effects.

Materials and methods

Cell lines and reagents

The murine macrophage-like cell line Raw264.7, capable of differentiating into osteoclasts (Horwood *et al*, 2001), was purchased from American Type Culture Collection (Manassas,

VA, USA). Cells were cultured in alpha-minimum essential medium (MEM supplemented with 10% fetal bovine serum (FBS) and penicillin (100 U/ml). Recombinant Wnt3a (rWnt3a), secreted frizzled-related protein 1 (sFRP1) and Dkk1 proteins were purchased from R&D Systems (Minneapolis, MN, USA). Anti-Dvl-1, Dvl-2 and Dvl-3 antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA), and an antibody specific for non-phosphorylated β -catenin from Upstate Biotechnology Incorporated (Lake Placid, NY, USA). Anti- α -, β -, or γ -catenin and horseradish peroxidase-conjugated anti-mouse antibodies were purchased from Transduction Laboratories (BD Pharmingen, Lexington, KY, USA).

Generation of primary human osteoclasts from patients with MM

Previously described methods were used to generate mononuclear cells from bone marrow of patients with MM (Yaccoby *et al.*, 2004). Signed informed-consent forms, approved by the Institutional Review Board, are kept on record. Briefly, heparinized bone marrow was aspirated from patients, and mononuclear cells were subsequently isolated by Ficoll-Hypaque density-gradient centrifugation. Plasma cells were removed by immunomagnetic-bead selection using the AutoMACs automated separation system (Miltenyi-Biotec, Auburn, CA, USA) with monoclonal mouse anti-human CD138 antibody. Purity of the mononuclear cell fraction with undetectable plasma cells was confirmed by two-colour flow cytometry using CD138/CD45 staining (Becton Dickinson, San Jose, CA, USA). Mononuclear cells were cultured in MEM with 15% FBS for 24 h. After removing adherent cells, the suspended cells were cultured in alpha-MEM supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 μ g/ml), recombinant human macrophage colony-stimulating factor (rM-CSF; 25 ng/ml), human soluble Rankl (sRankl) (60 ng/ml) and l-glutamine (2 mmol/l) at 37°C with 5% CO₂. The adherent cell population was detached and then subjected to 6-well or 96-well serial assays. The cells were further analysed by flow cytometry with monoclonal antiintegrin V to identify committed preosteoclasts (>90%). To generate mature multinuclear osteoclasts, adherent cells were further cultured for 10–14 d in differentiation medium (MEM supplemented with 10% FBS, 60 ng/ml sRankl and 12.5 ng/ml M-CSF).

Preparation of conditioned media

Wnt3a-conditioned medium (Wnt3a-CM) was prepared from Wnt3a-producing L cells (kindly provided by Shinji Takada, PhD) and control-conditioned medium (Con-CM) was prepared from L cells grown to confluence. Both cell types were cultured in MEM supplemented with 10% FBS. After 10 d, 0.5 μ g/ml G418 was added to maintain selective pressure, as previously described (Qiang *et al.*, 2003). After cells reached confluence, medium was replaced with serum-free MEM, and culture supernatants were collected 72 h later. Supernatants were sterilized by 0.2- μ m filtration and stored at –70°C until use.

Constructs and transfections

To generate dominant-negative (DN)- β -catenin stable clones, a pluripotent mesenchymal precursor cell line (C2C12), was transfected using lipofectamine (Invitrogen, Carlsbad, CA, USA) with empty vector or with pcDNA4 vector containing DN- β -catenin cDNA tagged with X-express (Qiang *et al.*, 2008c). After transfection, stable clones were generated by growing the cells in Dulbecco's modified eagle medium (DMEM) containing 10% FBS in the presence of Zeocin 1 mg/ml (Invitrogen) for 2 weeks. Proteins were isolated, and expression of the target protein was confirmed by immunoblotting using anti-X-express antibody (Invitrogen). Control cells and DN- β -catenin-positive clones were seeded (2.5×10^5 cells/well) in 6-well plates and incubated in medium without Zeocin for 72 h. To measure the concentrations of Opg and Rankl proteins, culture supernatants were harvested

and subjected to enzyme-linked immunosorbent assays according to manufacturer recommendations (R&D Systems).

Osteoclast differentiation assays

Preosteoclasts generated from MM patient samples or Raw264.7 murine cells were cultured in DMEM supplemented with 10% FBS in 6-well plates in the absence or presence of rWnt3a plus sRankl and M-CSF for 10–12 d. The media were replaced with 50% of the fresh growth medium twice weekly. Adherent cells were fixed and stained for osteoclast marker tartrate-resistant acid phosphatase (TRAP), using the leucocyte acid phosphatase kit (Sigma-Aldrich, St Louis, MO, USA), according to instructions. Briefly, cells were gently washed with PBS, fixed with 3.7% formaldehyde and then incubated in TRAP-staining buffer for 1 h at 37°C. After washing with water, cells were counterstained in hematoxylin solution to identify nuclei. TRAP-positive cells with >two nuclei were scored as osteoclasts and were counted from three replicate wells. Pictures of TRAP-stained cells were taken with an Olympus RT Colour microscope with 63× objective lens (Olympus, Tokyo, Japan) and SPOT camera (Diagnostic Instruments, Inc, Sterling Heights, MI, USA). The images were processed with Photoshop CS2.0 (Adobe Inc, San Jose, CA, USA).

Proliferation assay

Viability of preosteoclasts was examined by colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Qiang *et al*, 2004). Briefly, the generated preosteoclasts were seeded (2×10^4 cells/ well) in 96-well plates and incubated with rM-CSF (25 ng/ml), sRankl (60 ng/ml) and rWnt3a (100 ng/ml) or with serial concentrations of LiCl (5–60 μ mol/l) for 48 and 72 h. First, 10 μ l of 5 mg/ml MTT buffer (Sigma, St Louis, MO, USA) was added to each well for 4 h, followed by overnight incubation in 100 μ l 10% sodium dodecyl sulphate. Optical density was read on a Spectra Max340 Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 570 nm.

Immunoblotting analysis

Cells were incubated in MEM containing Wnt3a-CM or con-CM for indicated time points. For inhibition studies, cells were pretreated with purified recombinant Dkk1 or sFRP1 at indicated concentrations for 1 h. Following treatment, cells were lysed in lysate buffer as described (Qiang *et al*, 2002). After cell debris was removed by centrifugation at 9000 *g* for 10 min at 4°C, protein concentrations were determined by bicinchoninic acid assays (Pierce, Rockford, IL, USA). Proteins in whole-cell lysates were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Immunoblotting was performed using indicated antibodies and chemiluminescence (Pierce).

Immunoprecipitation and phosphate treatment

Whole-cell lysates from cells untreated or treated with rWnt3a for 6 h were prepared as described above and precleared by incubation with Protein G-Sepharose. Lysates were incubated with anti-Dvl-3 antibody for 2 h at 4°C. Immune complexes were then adsorbed to protein G-Sepharose beads and washed three times. Phosphatase treatment of immunocomplexes was performed as described (Semenov & Snyder, 1997). Treated complexes were analysed by immunoblotting with anti-Dvl-3 antibody.

Luciferase reporter gene assay

Cells plated at 5×10^4 cells per well in a 12-well plate were transiently cotransfected using Lipofectamine with 1 μ g/ml of either TOPflash or FOPflash along with 50 ng pSV- β -

galactosidase vector to normalize for transfection efficiency. Three independent transfections were performed, each in triplicate. Following transfection, cells were exposed to the media in the presence or absence of 100 ng/ml of rWnt3a for 24 h. Lysates were harvested, and luciferase and β -galactosidase activities in cell extracts were determined using the Bright-Glo luciferase assay system (Promega, Madison, WI, USA) and the β -galactosidase enzyme assay system (Promega) as previously described (Qiang *et al*, 2003). Luciferase activity was monitored with a Veritas microplate Luminometer (Turner Biosystem, Sunnyvale, CA, USA). β -galactosidase activity was measured with a Spectra MAX340 Microplate spectrophotometer (Molecular Devices).

Reverse transcription polymerase chain reaction (RT-PCR) analysis and sequence analysis

First-strand cDNA synthesis was performed as previously described (Qiang *et al*, 2003). All PCR reactions began with a 3-min incubation at 95°C and ended with a 10-min incubation at 72°C. Reactions were carried out for 35 cycles under the following conditions: 94°C for 30 s, 60°C for 45 s, 72°C for 1 min. Primer sequences for human *FZDs*, *WNTs* and *TCFs* were previously described, as were those for mouse *Fzds* and *Tcfs* (Qiang *et al*, 2008c). PCR fragments were subcloned into TOPO-TA cloning vector according to manufacturer instructions (Invitrogen). Sequence analysis was performed as previously described (Qiang *et al*, 2008c). Data were analysed using MacVECTOR software (Accelrys software Inc, San Diego, CA, USA) and compared with Genbank using National Center for Biotechnology Information BLAST (<http://www.ncbi.nlm.nih.gov/blast/>). DNA fragments from RT-PCR-amplified *WNT* mRNA were separated by agarose gel electrophoresis and visualized by ethidium bromide. Images of the DNA bank were captured with GeneFlash System Bio image (SYNGENE, Frederic, MD, USA), supplied with a digital camera and computer, and analysed by National Institutes of Health (NIH) image 6.61 software.

Statistical analysis

The Student's *t*-test was performed, using the Microsoft Excel statistical package, to analyse the statistical significance of differences between experimental groups. *P* values <0.05, as determined by the two-tailed test, were considered significant.

Results

Expression of Wnt signalling components in osteoclasts

RT-PCR was used to systemically analyse the expression of Wnt signalling components in human osteoclasts from bone marrow of 10 patients with MM and from mouse preosteoclast cell line Raw264.7 cells, by using primers specific for human and mouse Wnt receptor *FZD*, Wnt co-receptor *LRP5/6*, *WNT* ligands and *TCF* family, as described in our previous studies (Qiang *et al*, 2003, 2005, 2008a,c). mRNA from human myeloma cells and mouse C2C12 cells served as the positive controls to indicate optimal PCR amplification conditions and specificity of primers for nearly all these Wnt signalling components (data not shown). Multiple *FZD* isoforms, including *FZD1*, *-4*, *-5*, *-7*, *-8* and *-9*, were detectable in Raw264.7 cells, but *FZD2*, *-3*, *-6* and *-10* were absent (Fig 1A). *FZD1* to *-9* were amplified in all 10 patients' osteoclasts, and only *FZD10* was undetectable (Fig 1B); however, *FZD10* was detected in MM cell line OPM-2 (data not shown), used as a positive control as in the previous studies (Qiang *et al*, 2003). We next examined expression of Wnt co-receptors *LRP5* and *LRP6*. RT-PCR analysis using specific primers for mouse *Lrp5* and *-6* revealed relatively high expression levels of both receptors in Raw264.7 cells (Fig 1C). Specific primers for human *LRP5* or *-6* detected mRNA of both co-receptors in primary osteoclasts from all 10 patients; higher levels of *LRP6* mRNA were detected in primary

osteoclasts from patients #3, 4, 6 and 8 (Fig 1D). These results indicate that Wnt coreceptors are expressed in both mouse preosteoclasts and human osteoclasts.

Finally, we examined the presence of Wnt ligands in these cells. mRNA for multiple *Wnt* ligands was expressed in both mouse preosteoclasts (Fig 1E) and human osteoclasts (Table I). While levels of *Wnt2b*, *-4*, *-6* and *-7b* were higher in Raw264.7 cells, *WNT3* and *-WNT5A* were highly expressed in human osteoclasts. Both mouse preosteoclasts and human osteoclasts lacked expression of *Wnt1* and *-3a*, two active ligands important in canonical Wnt signalling.

Wnt3a induced phosphorylation of Dvl protein

After demonstrating the presence of Wnt receptors and coreceptors, we next examined whether the canonical β -catenin pathway was functional in osteoclasts. We first evaluated Dvl proteins, which are important downstream components of Wnt signalling. In primary osteoclasts, Dvl-3 protein level increased (Fig 2A), but Dvl-1 and *-2* proteins were weakly detectable (data not shown). After Wnt3a treatment, Dvl-3 protein level increased with time (Fig 2A), attaining maximum levels by 6 h and remaining for 12 h. Phosphorylated Dvl protein migrated slowly and caused slow migration bands, indicated by a gel mobility shift (Semenov & Snyder, 1997). This change in mobility was dependent on the concentration of Wnt3a, starting at 20 ng/ml of rWnt3a, as demonstrated by treatment with varying concentration of rWnt3a (Fig 2B). Phosphorylation of Dvl-3 protein was further confirmed by phosphatase treatment following immunoprecipitation with Dvl-3 antibody (Fig 2C).

Wnt3a induced accumulation of β -catenin in osteoclasts

Detailed examination of the effects of Wnt3a revealed that β -catenin protein accumulation was time- and dose-dependent (Fig 3). In primary osteoclasts from five patients with MM, β -catenin levels increased at 6 h and continued through to 9 h (Fig 3A). Similar increases in the active form of the protein (Fig 3B) were revealed by using an antibody that specifically recognizes the unphosphorylated form of β -catenin (van Noort *et al*, 2007). In primary osteoclasts, total β -catenin levels increased at 6.5 ng/ml of Wnt3a and were maximal at 25 ng/ml of Wnt3a (Fig 3C). Wnt3a induced stabilisation of β -catenin and increased levels of non-phosphorylated β -catenin in mouse preosteoclasts, with relatively obvious effects at 50 ng/ml of Wnt3a (Fig 3D). Additionally, LiCl, which inhibits GSK3 β phosphorylation of β -catenin (Stambolic *et al*, 1996), similarly stabilized β -catenin in primary osteoclast cells (Fig 3E). LiCl effects on β -catenin protein levels was observed at 10 μ mol/l and with peaked between 20 and 40 μ mol/l. Wnt3a treatment had no effect on α - or γ -catenin protein levels (data not shown). These results suggest that an increase of Wnt3a or an inhibition of GSK3 β specifically induce accumulation of β -catenin protein in the preosteoclast cell line and in primary osteoclast cells from bone marrow of patients with MM.

Dkk1 and sFRP1 inhibited Wnt-induced accumulation of β -catenin

To determine the specificity of Wnt3a on stabilisation of β -catenin in osteoclasts, we took advantage of Wnt signaling antagonist sFRP1 (Uren *et al*, 2000), which prevents Wnt from binding its receptor FZD. Pretreatment of Raw264.7 cells with serial concentrations of sFRP1 inhibited Wnt3a-induced increases in β -catenin in a dose-dependent manner, with a maximum effect at 10 μ g/ml (Fig 4A). To determine whether Lrp5/6 co-receptors were required for Wnt3a signalling in osteoclasts, we used Dkk1, which acts as an antagonist of canonical Wnt signalling by specifically interfering with Wnt binding to Lrp5/6 (Mao *et al*, 2001). As shown in Fig 4B, Dkk1 dose-dependently blocked Wnt3a-stabilized β -catenin, with maximal effects at 100–200 ng/ml. Furthermore, both Dkk1 and sFRP1 blocked unphosphorylated β -catenin induced by Wnt3a treatment in primary osteoclasts from MM patients (Fig 4C). These results indicate that Wnt3a specifically induces stabilisation of β -

catenin protein by binding Wnt receptor and co-receptors in both mouse and human osteoclast cells.

Wnt3a induced TCF transcriptional activity in osteoclasts

Stabilisation of β -catenin in osteoblasts (Qiang *et al*, 2008a,c) or in myeloma cells (Qiang *et al*, 2003, 2005) leads to its formation of a complex with members of *TCF/LEF* families, with subsequent transcriptional activation. RT-PCR analysis of *TCF/LEF* mRNA expressions using the specific primers for human and mouse *Tcf* family members, respectively, revealed expression of *LEF1* and *TCF1*, -3 and -4 in the majority of examined primary osteoclasts from patients with MM (Fig 5A); *Tcf4* was detected in mouse preosteoclasts (Fig 5B). We next transfected the primary osteoclasts with a luciferase reporter construct (TOPflash) containing a β -catenin binding site (Korinek *et al*, 1997). A significant increase in luciferase activity was observed after treating TOPflash-expressing cells with Wnt3a (Fig 5C), indicating that transcriptional activation was a downstream effect of Wnt3a treatment in osteoclasts.

Activation of Wnt/ β -catenin signalling is not sufficient for osteoclast formation, survival and proliferation

Given the characterization of functional Wnt/ β -catenin signalling in osteoclasts, we next investigated biological effects associated with activation of this pathway. First, we determined the effects of Wnt3a on the ability of human preosteoclast cells to differentiate into multinucleate osteoclasts. After 14 d of exposure to 100 ng/ml rWnt3a in differentiation media (Fig 6B), differentiated multinucleate osteoclasts were not morphologically different from those formed in media alone (Fig 6A); neither was a difference in TRAP-positive cells observed in the rWnt3a-treated group (Fig 6D), compared with the cells cultured in differentiation media alone (Fig 6C); Wnt3a alone had no effect on preosteoclast differentiation as determined by counting the number of TRAP-positive cells or by TRAP activity (Fig 6E and data not shown), nor did Wnt3a synergize with Rankl and M-CSF in inducing preosteoclast differentiation (Fig 6E), nor effect on proliferation/survival of preosteoclast cells as determined by MTT assay (Fig 6F). LiCl induced accumulation of β -catenin protein in osteoclasts, and LiCl has been reported to alter human osteoclast formation from peripheral blood mononuclear cells (Modarresi *et al*, 2009). Therefore, we examined the effects of LiCl on osteoclast differentiation from human preosteoclasts isolated from bone marrow of patients with MM. At serial concentrations from 10 to 60 μ mol/l (including concentrations inducing β -catenin stabilisation), LiCl had no effect on osteoclast proliferation or formation, based on the number of TRAP-positive cells (data not shown). Neither Dkk1 nor sFRP1 had an effect on osteoclast differentiation in the presence of Rankl (Fig 6E). Similar results were observed in mouse preosteoclasts (data not shown). These results indicate that inhibiting endogenous Wnt signalling or activating the Wnt/ β -catenin in response to Wnt3a, or inhibition of GSK3 β is not sufficient for osteoclast differentiation and proliferation, nor suppression of this process.

The effects of Wnt3a on osteoclast proliferation were examined by MTT assays of primary preosteoclasts. Wnt3a treatment resulted in cell proliferation comparable to that resulting from positive control agents M-CSF and Rankl (Fig 6F); similar results were observed in mouse preosteoclasts (data not shown). Furthermore, Wnt3a did not prevent the cells from undergoing apoptosis induced by serum starvation or by bortezomib treatment (data not shown). Because bortezomib reportedly induced apoptosis in osteoclasts (Willert *et al*, 2002), this indicates that Wnt3a had no direct effect on osteoclast growth or survival. Taken together, these results suggest that, although Wnt/ β -catenin signalling was activated in osteoclasts, this activation was not sufficient to affect differentiation, proliferation, or survival of these cells.

Supernatants from osteoblasts expressing DN- β -catenin attenuated osteoclast formation

Because activation of Wnt/ β -catenin had no direct effect on osteoclast formation from preosteoclasts of patients with MM, we evaluated the involvement of Wnt-mediated indirect regulation of human osteoclast formation, which might be responsible for Wnt3a-suppressed osteoclastogenesis in our animal model of MM. We examined culture supernatants from osteoblasts (C2C12 cells) stably expressing empty vector (pcDNA3) or one of two separate clones that stably express DN- β -catenin (designated DNBC#4 and #5). These cells produce DN- β -catenin protein and functionally inhibit Wnt/ β -catenin signalling, based on attenuated *TCF/LEF* transcriptional activity (Qiang *et al*, 2008a). Enzyme-linked immunosorbent assays of the culture supernatants revealed, as expected, that Rankl protein levels were significantly higher in supernatants of DNBC#4 or #5 osteoblasts than in those carrying empty vector (Fig 7A); however, Opg protein levels were clearly lower in cells with DNBC#4 or #5 (Fig 7B). When the supernatants were added to cultures of preosteoclasts in differentiation medium, osteoclast differentiation assays demonstrated that culture supernatants from control clones (with high concentrations of Opg) significantly attenuated osteoclast formation (Fig 7C). This inhibition was rescued by using supernatants from DNBC#4 or #5 osteoblasts; these effects were confirmed by microscopy (Fig 7D – G). These results suggest that Wnt- signalling –mediated Opg/Rankl production in osteoblasts regulates osteoclast formation in human primary preosteoclasts in patients with MM.

Discussion

The Wnt signalling pathway has been reported to indirectly mediate osteoclastogenesis via regulation of Rankl and Opg production in normal physiological conditions in a mouse models (Glass *et al*, 2005; Holmen *et al*, 2005). Other groups and ours have reported studies of myeloma bone disease in mouse myeloma models showing that suppression of osteoclastogenesis results from increased Wnt/ β -catenin signalling in the bone marrow microenvironment that follows administration of rWnt3a or LiCl to inhibit GSK3 β activity (Yaccoby *et al*, 2007; Edwards *et al*, 2008; Qiang *et al*, 2008b). However, it is currently unclear whether Wnt/ β -catenin signalling is activated in preosteoclasts in patients with MM and, if so, whether this signalling contributes to regulation of osteoclast formation in the patients. In the present studies, we employed preosteoclast cell lines and primary preosteoclasts from 10 patients with MM to characterize functional Wnt/ β -catenin signalling and investigate the potential direct role of Wnt/ β -catenin signalling in human osteoclast formation.

For the first time, we demonstrated that primary human osteoclasts from MM patients express a wide range of *FZD* receptor mRNAs, including *FZD1*, *-2*, *-3*, *-4*, *-5*, *-6*, *-7*, *-8* and *-9* and co-receptors *LRP5* and *-6*. Presence of these Wnt receptors and co-receptors required for canonical Wnt signalling indicates that osteoclasts in MM are likely to be able to signal through both canonical and non-canonical Wnt signalling pathways. To date, little is known about specific interactions of ligands and *FZD* receptors for signalling activation. In *Drosophila*, *FZD2* loss-of-function mutants reportedly block regulation of armadillo, the *Drosophila* homologue of vertebrate β -catenin (Bhat, 1998; Chen & Struhl, 1999), and overexpression of *FZD2* stabilizes Wingless (Wg), the *Drosophila* homologue of Wnt, in imaginal wing discs (Cadigan *et al*, 1998). These results support the contention that *FZD2* is required for Wnt (Wg) signalling during normal embryo development (Bhat, 1998; Chen & Struhl, 1999). We found that multiple *WNT* mRNAs (*WNT3*, *-5A*, *-7A*, *-11* and *-13*) were expressed in primary human osteoclasts from patients with MM, while other Wnt family members (*WNT1*, *-2B*, *-4*, *-5B*, *-6*, *-7B* and *-10B*) were expressed in mouse preosteoclasts. Expression of Wnt ligands suggests a possible autocrine signalling, but further studies will be necessary to evaluate biological responses associated with endogenous production of these mRNAs.

Functional Wnt/ β -catenin signalling involves downstream effects that include activating Dvl proteins that disrupt the β -catenin degradation complex. Treating osteoclasts with Wnt3a led to phosphorylation of Dvl-3, accompanied by stabilisation of β -catenin. Accumulation of β -catenin protein in osteoclasts was inhibited by sFRP1, a putative antagonist that prevents Wnt from binding to the receptors (Uren *et al*, 2000), as well as by Dkk1 protein, an important antagonist of canonical Wnt signalling that prevents Wnts from binding their co-receptors LRP5/6 (Tamai *et al*, 2000). LiCl, a known inhibitor of GSK3 β in the β -catenin degradation complex, also mimicked Wnt-mediated accumulation of β -catenin. Accumulation of β -catenin leads to its nuclear translocation and binding to members of the *TCF/LEF* families to form transcription-activating complexes (Korinek *et al*, 1997) that regulate *WNT* target gene transcription. Relatively high mRNA expression of *TCF1* and weak expression of *LEF1* and *TCF3* and *-4* were present in most of the tested primary human osteoclasts from MM patients. Luciferase reporter assays demonstrated formation of functional activating complexes of β -catenin and the *TCF/LEF* family in response to Wnt3a. Collectively, these results revealed that human primary osteoclasts have functional canonical Wnt/ β -catenin signalling capable of initiating transcriptional activation upon exposure to Wnt ligand.

Despite functional activation of β -catenin signalling in human osteoclasts, the present studies revealed, unexpectedly, that Wnt3a neither induced formation of TRAP-positive osteoclasts *in vitro* nor affected osteoclast proliferation. These data provide the first evidence that activation of Wnt/ β -catenin signalling is insufficient to directly induce or suppress human osteoclast formation *in vitro* from preosteoclasts of patients with MM. These results are consistent with a previous report showing that activating canonical Wnt signalling by using GSK3 β inhibitor results in attenuated osteoclast formation only when murine preosteoclasts are cocultured with osteoblasts or bone marrow stromal cells (Spencer *et al*, 2006).

It should be noted that, during preparation of this manuscript, recent studies proposed a role for β -catenin in suppression of osteoclast differentiation, based on using LiCl and adenovirus overexpressing β -catenin in human osteoclasts (Modarresi *et al*, 2009): exposure to a high concentration of LiCl (10 mmol/l), a known GSK3 β inhibitor, resulted in decreased numbers of TRAP-positive osteoclasts. This contrasts with our results with LiCl exposure, which showed that preventing β -catenin degradation via GSK3 β inhibition had no effect on osteoclast formation. These apparent discrepancies might be related to the use of different LiCl concentrations. In previous reports, osteoclast formation was suppressed by 10 mmol/l LiCl, which is 1000-fold higher than the concentration available for stabilisation of β -catenin in the present studies (Fig 3E). This raises the possibility that the ability of high concentration LiCl to suppress osteoclast formation might occur via activation of other signalling pathways because GSK3 β is a multiple-tasking kinase (Doble & Woodgett, 2003).

Importantly, our studies provide *in vitro* evidence to support the rationale for increasing Wnt signalling in the bone marrow microenvironment as a potential therapeutic strategy for treating MM and MM-triggered bone disease. We and others demonstrated that an increase of Wnt signalling in the bone marrow microenvironment, by administration of Wnt3a (Qiang *et al*, 2008b), or inhibited GSK3 β (Edwards *et al*, 2008) or anti-Dkk1 activity, by a Dkk1-neutralising antibody (Yaccoby *et al*, 2007), leads to decreased osteoclast numbers and suppressed tumour growth, potentially due to direct inhibition of osteoclastogenesis or to indirect suppression of osteoclastogenesis by the upregulating *TNFRSF11B/TNFSF11* expression in osteoblasts. The results presented here demonstrate that activation of Wnt/ β -catenin signalling in osteoclasts does not directly affected osteoclast formation and proliferation, but acts as a negative regulator for osteoclastogenesis via regulating

TNFRSF11B/TNFSF11 production in osteoblasts. These results are useful to understanding the physiological function of Wnt signalling in regulating osteoclastogenesis and support previous *in vivo* physiological studies of bone development in mice (Glass *et al*, 2005; Holmen *et al*, 2005) and, most importantly, provide *in vitro* evidences for the molecular mechanism by which an increase of Wnt signalling in bone marrow microenvironment suppresses myeloma bone disease in mouse models (Yaccoby *et al*, 2007; Edwards *et al*, 2008; Qiang *et al*, 2008b). Increased Wnt signalling results in increased *TNFRSF11B* mRNA and Opg protein level and decreased *TNFSF11* mRNA expression and Rankl protein in osteoblasts (Qiang *et al*, 2008a) and consequently leads to decreased osteoclast numbers. Increased Wnt signalling would result in suppression of myeloma survival because osteoclasts support myeloma growth in cell to cell contact manner (Yaccoby *et al*, 2004).

This study demonstrates, for the first time, multiple *WNTs*, *FZDs*, *LRP5/6* and *TCF* family members are present in osteoclasts from MM patients, and we characterized functional activation of Wnt/ β -catenin signalling in both osteoclasts from MM patients and a mouse preosteoclast cell line. Furthermore, these data showed that activation of Wnt/ β (using symbol font for β)-catenin signalling functions as a negative factor to suppress osteoclastogenesis in an osteoblast-dependent manner, by regulating osteoblast production of Opg and Rankl. These results provide important *in vitro* evidence to support the mechanism by which increased activity in the Wnt/ β (using symbol font for β)-catenin signalling pathway attenuates osteoclastogenesis in our previous *in vivo* studies (Qiang *et al*, 2008b). Characterization of the Wnt/ β -catenin pathway also provides new insight for understanding the biology of osteoclasts in MM. Our evidence, that Wnt/ β -catenin signalling inhibits osteoclastogenesis via effects on osteoblasts, provides a rational basis for developing therapeutic strategies that aim to increase activity of this pathway (possibly by inhibiting its antagonist Dkk1) in the bone marrow micro-environment to treat bone lesions in MM.

Acknowledgments

The authors would like to thank Drs Stuart Rudikoff and Jeff Rubin, NCI, NIH, for reagents. The authors are grateful for Rohit P. Ojha, University of North Texas Health Science Center, and David R. Williams, Austin Porter III, Rachel Flinchum, and Yan Xiao at the Lambert Laboratory of Myeloma Genetics for technical assistance. The authors also wish to thank the faculty, staff, and patients of the Myeloma Institute for Research and Therapy for their support. This manuscript was edited by the Office of Grants and Scientific Publications, University of Arkansas for Medical Sciences.

Disclosures

This work was supported by a grant from the Multiple Myeloma Research Foundation to YWQ, and by grants CA97513 (BB) and CA113992 (JE) from the National Cancer Institute, NIH.

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Fig 1.

FZD1-10, *LRP5/6*, and *WNT* mRNAs were expressed in osteoclasts. Total RNA was extracted from Raw264.7 cells or primary osteoclasts generated from mononuclear cells from bone marrow of 10 patients with MM. RT-PCR was performed with primers specific to mouse *Fzd1-10* (A), *Lrp5/6* (C), *Wnt*'s (E), and *Gapdh*, as well as primers specific to human *FZD1-10* (B), *LRP5/6* (D), and *GAPDH*.

Fig 2.

Wnt3a modulation of Dvl-3 protein in osteoclasts. Primary osteoclasts were (A) treated with L929 control-CM or Wnt3a-CM for indicated times or (B) cultured with specified serial concentrations of rWnt3a for 6 h. Cell lysates were analysed by immunoblotting with anti-Dvl-3 antibody. (C) Lysates were prepared and immunoprecipitated with anti-Dvl-3 antibody from untreated cells (lane 1) or cells treated with rWnt3a (100 ng/ml, 3 h) (lanes 2–4). Immunoprecipitates were collected and left untreated (lanes 1 and 2), treated with alkaline phosphatase (AP) buffer (lane 3), or AP plus AP buffer (lane 4). Samples were analysed by immunoblotting with anti-Dvl3 antibody. For all blots, anti- β -tubulin antibody was used as loading control.

Fig 3.

Wnt3a induced stabilisation of β -catenin protein in a time- and dose-dependent manner in osteoclasts. (A) Cell lysates from primary osteoclasts generated from five patients with MM were untreated or treated with 100 ng/ml of rWnt3a for indicated times. Lysates were analysed by immunoblotting for total β -catenin protein. (B) Protein lysates from three osteoclast samples were analysed by immunoblotting for nonphosphorylated β -catenin. (C) Primary osteoclasts and (D) Raw264.7 cells were untreated or were cultured with indicated serial concentrations of rWnt3a for 6 h; lysates were analysed by immunoblotting for total β -catenin and nonphosphorylated β -catenin (Non-p- β -catenin). (E) Primary osteoclasts were untreated or treated with indicated serial concentrations of LiCl and analysed by

immunoblotting for total β -catenin. For all blots, anti- β -tubulin antibody was used as a loading control.

Fig 4.

Dkk1 and sFRP1 inhibition of Wnt3a-induced β -catenin stabilisation. Raw264.7 cells were pretreated with indicated concentrations of (A) recombinant sFRP1 or (B) Dkk1 and then treated with rWnt3a (100 ng/ml, 6 h); lysates were analysed by immunoblotting for β -catenin. (C) Primary osteoclasts generated from three patients with MM were cultured alone (lane 1) or were treated with rWnt3a alone (lane 2), rWnt3a plus sFRP1 (10 μ g; lane 3), or Dkk1 (200 ng/ml; lane 4). Cell lysates were analysed by immunoblotting for nonphosphorylated β -catenin (Non-p β -catenin). For all blots, anti- β -tubulin antibody was used as a loading control.

Fig 5.

Wnt3a induced *TCF/LEF* transcriptional activity in human osteoclasts. Total RNA was extracted (A) from primary osteoclasts generated from bone marrow of 10 patients with MM or (B) from Raw264.7 cells. RT-PCR analysis was performed with primers specific to (A) human or (B) mouse *Tcf1*, *-3*, and *-4* and *Lef1* to quantitate the mRNAs. A β -actin primer specific for both mouse (*Actb*) and human (*ACTB*) was used as the control for both A and B. (C) Primary osteoclasts were transiently transfected with expression constructs, wild-type (TOPflash) or mutant (FOPflash) *TCF/LEF* luciferase reporter constructs, and pSV- β -galactosidase vector (internal control for transfection efficiency). Cells were untreated (control) or treated with rWnt3a (100 ng/ml), and luciferase activity was measured; results

are representative of three independent experiments and are shown as the mean $\pm$ SD ($n = 3$). ** $P < 0.01$, based on comparison of treated groups *versus* control using the Student t -test.

Fig 6.

Effects of Wnt3a on formation and proliferation of osteoclasts. Mononuclear premature osteoclasts generated from bone marrow of patients with MM were cultured in whole growth medium in the absence (A and C) or presence (B and D) of 100 ng/ml rWnt3a for 2 weeks; medium was changed twice weekly. Cells were fixed and stained for TRAP (C and D). The osteoclast cells (A and B) and stained samples (C and D) were photographed as described in Materials and methods. (E) Human preosteoclasts from MM patients were cultured for 14 d in growth medium alone or with rWnt3a (100 ng/ml), Rankl (60 ng/ml) (positive control), Rankl plus rWnt3a (100 ng/ml), Dkk1 (100 ng/ml), or sFRP1 (500 ng/ml); half of the growth medium was replaced twice weekly. Cells were analysed by acid

phosphatase assays to determine TRAP activity. (F) Cells were cultured in growth medium as a control (Con) or in the presence of rWnt3 (100 ng/ml) or Wnt3a-CM, using M-CSF (25 ng/ml) and Rankl (60 ng/ml) as positive controls for 72 h. Cell proliferation was determined by MTT assays. Data represent the mean $\pm$ SD ($n = 3$) of a representative experiment. ** $P < 0.01$, based on comparison of treated groups *versus* control using the Student t -test.

Fig 7.

Supernatants from osteoblasts expressing DN- β -catenin inhibited osteoclast formation. Osteoblasts (C2C12 cells) stably expressing empty vector (pcDNA3) or two separate clones stably expressing DN- β -catenin (DNBC4 and DNBC#5) were cultured in growth medium for 72 h, and supernatants were collected. Protein levels of (A) Rankl and (B) Opg were measured by enzyme-linked immunosorbent assays of supernatants. (C) Mononuclear preosteoclasts from bone marrow of patients with MM were cultured for 1 week in 6-well plates in whole growth medium alone (Cont) or with addition of supernatant from C2C12 cells expressing empty vector (SpcDNA), DNBC#4, or DNBC#5; medium was changed twice weekly. Multinucleated TRAP-positive cells were counted and photographed under

the microscope. Osteoclasts were cultured for 1 week in (D) whole growth medium alone or with addition of supernatants of C2C12 cells expressing (E) empty vector, (F) DNBC#4, or (G) DNBC#5. Each experimental group was cultured in triplicate; results are presented as the mean $\pm$ SD ($n = 3$) of a representative experiment. ** $P < 0.01$, *** $P < 0.001$, based on comparison of treated groups *versus* control using the Student *t*-test.

Table I

RT-PCR analysis of Wnt expression in primary osteoclasts*.

Osteoclast sample	WNT3	WNT5A	WNT7A	WNT11	WNT13	GAPDH
1	++	+	-	-	-	+++
2	++	-	+	+	-	+++
3	++	+++	+++	-	+	+++
4	+++	++	-	-	+++	+++
5	++	+	-	-	-	+++
6	+++	+++	+++	+++	+	+++
7	++	+++	+	-	-	+++
8	++++	++++	+	++	+	+++
9	++	+++	+	+	+	+++
10	+	-	+	-	-	+++

* Data were scored as five groups (from - to +++) according to staining intensity of DNA bands as described in Materials and methods.