Published in final edited form as:

Biochem Biophys Res Commun. 2012 January 6; 417(1): 223–230. doi:10.1016/j.bbrc.2011.11.089.

Noncanonical Wnt signaling promotes osteoclast differentiation and is facilitated by the human immunodeficiency virus protease inhibitor ritonavir

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Abstract

Wnt proteins that signal via the canonical Wnt/ β -catenin pathway directly regulate osteoblast differentiation. In contrast, most studies of Wnt-related effects on osteoclasts involve indirect changes. While investigating bone mineral density loss in the setting of human immunodeficiency virus (HIV) infection and its treatment with the protease inhibitor ritonavir (RTV), we observed that RTV decreased nuclear localization of β -catenin, critical to canonical Wnt signaling, in primary human and murine osteoclast precursors. This occurred in parallel with upregulation of Wnt5a and Wnt5b transcripts. These Wnts typically stimulate noncanonical Wnt signaling, and this can antagonize the canonical Wnt pathway in many cell types, dependent upon Wnt receptor usage. We now document RTV-mediated upregulation of Wnt5a/b protein in osteoclast precursors. Recombinant Wnt5b and retrovirus-mediated expression of Wnt5a enhanced osteoclast differentiation from human and murine monocytic precursors, processes facilitated by RTV. In contrast, canonical Wnt signaling mediated by Wnt3a suppressed osteoclastogenesis. Both RTV and Wnt5b inhibited canonical, β -catenin/T cell factor-based Wnt reporter activation in osteoclast precursors. RTV- and Wnt5-induced osteoclast differentiation were dependent upon the receptor-like tyrosine kinase Ryk, suggesting that Ryk may act as a Wnt5a/b receptor in this context. This is the first demonstration of a direct role for Wnt signaling pathways and Ryk in regulation of osteoclast differentiation, and its modulation by a clinically important drug, ritonavir. These studies also reveal a potential role for noncanonical Wnt5a/b signaling in acceleration of bone mineral density loss in HIV-infected individuals, and illuminate a potential means of influencing such processes in disease states that involve enhanced osteoclast activity.

Keywords

β -catenin; human immunodeficiency virus; osteoclast; pathogenesis; protease inhibitor; Wnt signaling

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1. Introduction

Signaling by Wnt proteins that stimulate the canonical β -catenin pathway, including Wnt3a and Wnt10b, is crucial for bone formation [1]. Binding of these secreted proteins to their primary receptors on osteoblasts, composed of Frizzled proteins and low density lipoprotein receptor-related protein (LRP) 5 and 6, results in inhibition of glycogen synthetase-kinase 3 β (GSK3 β)-mediated phosphorylation of β -catenin. This leads to stabilization of β -catenin, its nuclear translocation, and interaction with T cell factor (TCF) transcription factors to control target gene expression [2]. Noncanonical Wnt signaling, which is independent of β -catenin, may also play a role in bone formation through promotion of osteoblast differentiation [3]. In contrast, most studies of Wnt-specific effects on bone resorbing osteoclasts (OC) have been limited to indirect influences: changes in soluble stromal or osteoblast-derived factors that modulate osteoblast-OC coupling [2,4,5]. Yet β -catenin also localizes to nuclei of isolated OC in vitro [4] and in vivo [6], consistent with endogenous Wnt/ β -catenin signaling.

Indeed, Wnt proteins affecting noncanonical pathways may have a direct role in OC differentiation. In one preliminary study, noncanonical signaling via Wnt5a enhanced receptor activator of nuclear factor κ B ligand (RANKL)-induced OC formation from primary murine precursors [7]. Daily injection of a soluble GST-receptor tyrosine kinase-like orphan receptor 2 (Ror2) fusion protein, acting as a Wnt5a binding decoy, into mice with a rheumatoid arthritis-like condition prevented bone mineral density (BMD) loss [7].

We became involved in Wnt-dependent bone metabolism through our focus on mechanisms by which the human immunodeficiency virus (HIV) protease inhibitor (PI) ritonavir (RTV) accelerates human immunodeficiency virus (HIV)-mediated loss of BMD clinically [8] and in ex vivo models [9,10]. RTV is the key component of all PI-boosted antiretroviral therapies (ART), one of the two recommended regimens for treatment of HIV disease. We used oligonucleotide microarrays to examine transcripts from adherent human peripheral blood mononuclear cell (PBMC) OC precursors cultured with the OC differentiating cytokines RANKL and M-CSF and RTV as well as antiretrovirals not linked clinically to BMD loss. We documented increases in Wnt5a and Wnt5b transcripts in the presence of RTV, but no change with other antiretrovirals [10]. As both Wnt5a and -5b can inhibit β -catenin/TCF signaling in a variety of cell systems [11-14], we postulated that alterations in β -catenin in OC precursors directly impact OC differentiation, and would be influenced by RTV.

We now explore the hypothesis that noncanonical Wnt signaling can increase OC differentiation, and that RTV accelerates this process via Wnt5a/b. We also sought to identify receptor(s) mediating these noncanonical Wnt effects, as the nature of the intracellular signals generated by a given Wnt ligand is highly context-related, and is restricted by receptor type and availability [13,15-18]. Our studies should help to define the role of Wnt signaling in HIV/ART mediated BMD loss. It may also illuminate potential means for suppressing such processes in disease states associated with accelerated OC activity.

2. Materials and Methods

2.1. Reagents

Human recombinant RANKL and M-CSF and murine RANKL were purchased from Peprotech. The HIV PIs ritonavir, indinavir, atazanavir, lopinavir and amprenavir, the non-nucleoside reverse transcriptase inhibitor (RTI) efavirenz, the nucleoside RTIs AZT and d4T, and the nucleotide RTI tenofovir were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program and prepared in Me₂SO. Rabbit polyclonal

anti-Ryk (Abgent, San Diego, CA) and goat polyclonal anti-murine Wnt5a (Sigma) antibodies were purchased. Purified recombinant (r) Wnt5b and Wnt3a proteins and murine and human IFN- γ were obtained from R&D Systems (Minneapolis, MN). GeneSilencer, a proprietary siRNA transfection reagent, was obtained from Genlantis (San Diego, CA), and OptiMEM media from Invitrogen.

2.2. Cell lines

Murine osteoclast precursor RAW 264.7 monocytic cells and murine osteoblast line 7F2 were obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM plus 10% FCS. $0.5-1 \times 10^6$ RAW cells per condition were differentiated into OC, using methods described below, in the presence of RANKL (50ng/ml) and physiologic levels of IFN- γ (1ng/ml, equivalent to 10U/ml).

2.3. Isolation, propagation, and differentiation of human osteoclast precursors

PBMCs were derived from heparinized venous blood by density gradient centrifugation using Ficoll-Paque. They were cultured in RPMI 1640 plus 10% heat-inactivated FBS in polystyrene flasks for 2-4 hrs at 37 C. Non-adherent cells were depleted by rinsing the flasks with DMEM; adherent cells were collected using a cell scraper. 1×10^5 adherent cells per experimental condition were incubated in 8 well glass slide Lab-Tek chambers (Nalge Nunc, Naperville, IL) with 0.2ml RPMI 1640 plus 10% FBS. 50ng/ml human RANKL, 100ng/ml M-CSF, and human IFN- γ (1ng/ml) were employed in differentiation experiments with these cells.

Osteoclastogenesis was quantitatively assessed using tartrate-resistant acid phosphatase (TRAP) activity as an index, as previously described [9]. The assay was performed according to the manufacturer's instructions (Sigma), with p-nitrophenol phosphate in acid phosphatase buffer as substrate, and enzyme activity measured colorimetrically at OD 405 nm. The validity of this assay as a measure of precursor cell differentiation into functional OC had been established in our lab by parallel determinations of membrane expression of TRAP and bone resorption function utilizing Osteologic discs and dentine slices [9].

2.4. Reverse transcriptase polymerase chain reaction (RT-PCR)

Single-stranded cDNA was reverse transcribed from 2 μ g of random hexamer primed RNA using the TaqMan Reverse Transcriptase Reaction Kit (Applied Biosystems, Foster City, CA) and PCR performed as described by our lab [10]. The following murine-based primer pairs were used:

Ryk: forward, 5'-CGCTCTGTCCTTTAACCTGC
reverse, 5'-CCAGTTCAATCCTTTTCATGC
Ror2: forward, 5'-ATCCAAGACCTGGACACAACAGA
reverse, 5'-GAACCCCACTGGCAGTGATG

2.5. Wnt5a gene transfer

Retroviral vectors based on LNCX, bearing hemagglutinin (HA)-tagged Wnt5a or the HA tag alone, were previously described [19]. Briefly, GP293 packaging cells, 5×10^6 (Clontech, Palo Alto, CA), were seeded and transfected with 10 μ g LNCX DNA and pVSVG, and retroviral-containing supernatants collected 48 hours later. Retrovirus (0.1ml of stock cell supernatant) plus 8 μ g/ml polybrene were added to pelleted RAW 264.7 cells (1×10^6) for 2 hours at 37°C, followed by washing three times with PBS and resuspension in DMEM plus 10% FBS with geneticin (100-200 μ g/ml). After a 48 hour culture the cells were washed and utilized in OC induction protocols.

2.6. Gene knockdown

A small interfering RNA/DNA chimera with validated target specificity for Ryk was prepared by Novus Biologicals (Littleton, CO) and used to suppress Ryk expression. 2nM of the chimeric RNAi was transfected into cells using 100 μ L GeneSilencer and OptiMEM media. After 4 hours of incubation, media and additional reagents were added. Alteration in Ryk transcripts at 24 hours was verified by immunoblotting. A control RNAi chimera (Novus) lacking target specificity was employed in a parallel manner.

2.7. Immunoblotting

0.5-1 $\times 10^6$ cells per condition were treated with lysis buffer (20mM dTT, 6% SDS, 0.25M Tris, 10% glycerol, pH 6.8). 50 μ g of protein lysate, quantitated by bicinchoninic acid (BCA) assay (Pierce), were separated by SDS/PAGE. Proteins were transferred to PVDF membranes using an LKB transfer apparatus and probed with 1:500 dilutions of an anti-Wnt5a goat polyclonal antibody, which detects both Wnt5a and -5b (unpublished observation), or an anti-Ryk rabbit polyclonal antibody. The secondary antibody was either a horseradish peroxidase (HRP)-rabbit anti-goat or goat anti-rabbit IgG. Bands were detected by a chemiluminescence kit (Amersham, GE Healthcare). Controls involved immunoblotting for β -actin.

2.7. β -catenin/TCF reporter assay

2 $\times 10^6$ RAW264.7 cells were infected with WntRgreenL1, a lentivirus vector that expresses green fluorescent protein (GFP) in a β -catenin/TCF-responsive manner. WntRgreenL1 was constructed by inserting 7 copies of a TCF binding site into the HIV-based vector pTRH1-mCV (System Biosciences, Mountain View, CA). 48 hours after viral infection, the cells were washed, plated as 1 $\times 10^5$ cells/well, and cultured with RANKL (50ng/ml), RANKL + RTV (5 μ M), RANKL + tenofovir (25 μ M), or RANKL + rWnt5b (100ng/ml) overnight. Cells were then fixed in 2% p-formaldehyde and percent fluorescent cells assessed by flow cytometry on gated viable cells, counting 10,000 cells per condition.

3. Results

3.1. RTV synergizes with RANKL in upregulating Wnt5 protein

We exposed RAW cells to RANKL, RTV (5 μ M), RANKL + RTV, or RANKL + tenofovir (25 μ M) for 18 hours and measured total cellular Wnt5 by western blotting. We cannot distinguish between Wnt5a and Wnt5b as available monoclonal reagents react with both forms. However, both Wnt5a [11] and Wnt5b [12] inhibit canonical Wnt/ β -catenin signaling in similar cell types, and they have an additive effect in this inhibition [20]. The ratio of Wnt5: β -actin expression increased above control levels (Buffer) in the presence of RANKL or RTV alone (Fig. 1, $p < 0.01$), with RTV-mediated changes greater than those effected by RANKL ($p < 0.05$). The addition of RTV to RANKL treated cultures led to a further increase in Wnt5 levels (Fig. 1, $p < 0.03$).

We had previously shown that activation of the canonical Wnt pathway via LiCl suppressed OC formation, an effect which was partially blocked by RTV [10]. We now show that LiCl has no impact on RANKL/RTV-mediated enhancement of Wnt5 expression (Fig. 1), suggesting that RTV could override the effects of LiCl-enhanced canonical signaling via persistent upregulation of Wnt5.

Tenofovir, which has also been associated with BMD loss in the setting of HIV, but possibly acts via renally-mediated phosphate loss and effects on the osteoblast rather than the OC [21], had no impact on Wnt5 when added alone, and led to a decrease in Wnt5 protein in RANKL treated cultures (Fig. 1, $p < 0.03$). Another HIV PI, indinavir, and the non-

nucleoside reverse transcriptase inhibitor efavirenz, which have not been linked clinically to BMD loss, also had no effect on Wnt5a expression at concentrations to 25 μ M (data not shown).

3.2. Wnt proteins associated with noncanonical vs. canonical signaling pathways differentially affect OC differentiation

We compared the direct effects of Wnt5 vs. Wnt3a on OC differentiation. rWnt5b alone, at doses from 25 to 100ng/ml, had no impact on differentiation of murine RAW 264.7 precursors (Fig. 2A). However, there was a marked synergy between RANKL and doses of rWnt5b >50ng/ml in promotion of TRAP activity (Fig. 2A; $p<0.01$). At 100ng/ml, Wnt5b increased TRAP activity over that seen with RANKL alone by 2.3-fold (Fig. 2A). In contrast, while rWnt3a also had little effect on differentiation of these OC precursors in the absence of RANKL (Fig. 2B) it decreased, in a dose-dependent manner, OC formation driven by RANKL (Fig. 2B).

Distinct effects of these Wnt proteins were also seen using primary human adherent mononuclear cells. There was an increase in TRAP activity when either RTV ($p<0.005$) or rWnt5b (100ng/ml; $p<0.002$) was added along with RANKL, while rWnt3a (100ng/ml) suppressed OC formation in the presence of RANKL ($p<0.0002$) (Fig. 2C). Effects of RTV together with Wnt5b were additive in augmentation of TRAP activity (Fig. 2C; $p<0.0007$). In contrast, other common antiretroviral drugs, including tenofovir (1-25 μ M), efavirenz (2 μ M), the PIs indinavir (10 μ M), atazanavir (2 μ M), lopinavir (5 μ M), and amprenavir (5 μ M), the nucleoside RTIs AZT (2 μ M) and d4T (2 μ M), and the integrase inhibitor raltegravir (2 μ M) had no effect on RANKL/M-CSF-mediated OC differentiation, either in the presence or absence of rWnt5b (data not shown).

The increased RANKL-driven OC differentiation observed by treatment with rWnt5b was replicated utilizing a retrovirus vector to overexpress Wnt5a in RAW cells. This demonstrates the functional redundancy of Wnt5a and Wnt 5b. In agreement with the high dose rWnt5b data reported above, overexpression of Wnt5a in the absence of RANKL led to increased OC formation (Fig. 3; $p<0.002$). Addition of RTV to RANKL-treated, Wnt5a-overexpressing cells enhanced OC activity over RANKL plus Wnt5a alone ($p<0.002$). Enhancement of β -catenin signaling with LiCl resulted in marked suppression of TRAP activity in RANKL-treated cultures ($p<0.001$). Consistent with the ability of noncanonical Wnt signaling to antagonize signaling generated through the canonical Wnt/ β -catenin pathway in several cell systems [12,13], this suppression was partially reversed by overexpression of Wnt5a (Fig. 3; $p<0.004$).

3.3. Expression of Ror2 and Ryk, receptors linked to noncanonical Wnt signaling in osteoblasts, on OC precursors and mature OC

RNA from murine 7F2 osteoblasts gave a strong signal for Ror2 which was not seen in the murine OC precursors at baseline nor following RANKL-induced differentiation. (Fig. 4A). RTV had no impact on this pattern (Fig. 4A). In contrast, Ryk was expressed in both osteoblasts and OC precursors and was unaltered by RANKL-mediated differentiation or addition of RTV (Fig 4A).

3.4. Promotion of OC differentiation by ritonavir requires Ryk expression

Use of a DNA/RNA chimeric RNAi against Ryk reduced detection of Ryk protein in RAW cells exposed to RANKL plus RTV by >90% (Fig. 4B). In parallel, it blocked the ability of rWnt5b to enhance TRAP activity in RANKL-stimulated RAW cells, and the ability of RTV to enhance TRAP (Fig. 4C). These results suggest that RTV induces Wnt5a/5b expression which in turn acts via Ryk to mediate noncanonical Wnt signaling.

3.5. Ritonavir inhibits canonical Wnt reporter activation

We had previously shown that RTV suppressed the cytoplasmic to nuclear translocation of β -catenin in human OC precursors and murine RAW cells [10]. Data now presented in Fig. 3 suggest that Wnt5a/b can antagonize β -catenin signaling, mitigating the ability of LiCl to inhibit OC formation. We therefore sought to test directly whether RTV affects canonical Wnt signaling in OC precursors. To do this we used RAW cells infected with WntRgreenL1, a lentiviral reporter virus that expresses GFP from a β -catenin/TCF-responsive promoter (Fig. 4D). When these cells were exposed to RTV there was a mean 51% suppression in percent fluorescent cells, as compared to control cells incubated only with RANKL (Fig. 4D). rWnt5b had a similar effect on GFP, suppressing it by 50%. In contrast, tenofovir + RANKL treated cells showed a mean 94.5% increase in fluorescence over cells treated with RANKL alone (Fig. 4D). This facilitation of canonical β -catenin/TCF-based signaling may be reflected in tenofovir-associated suppression of RANKL-associated increases in Wnt5b (Fig. 1).

Discussion

In our continuing investigation of the mechanisms by which certain antiretroviral agents accelerate loss of BMD in the setting of HIV infection, we initially focused on RANKL production in CD4+ T cells infected with HIV or exposed to HIV soluble envelope protein gp120, and the ability of the HIV protease inhibitor RTV to enhance osteoclastogenesis mediated by RANKL. We now document that RTV upregulates Wnt5 protein expression, and that Wnt5a and 5b directly enhance OC formation. The RTV effect on OC differentiation was dependent on the putative Wnt receptor Ryk and occurred in the context of suppression of β -catenin signaling. In contrast, canonical Wnt/ β -catenin signaling, mediated by rWnt3a, suppressed OC formation. We believe this is the first demonstration of a direct role for Wnt signaling pathways and Ryk in regulation of OC differentiation, and its modulation by a clinically important drug.

The mechanism by which RTV induces Wnt5 expression is the focus of our continued study. Our working hypothesis is based on the fact that Wnt5a is upregulated by proinflammatory cytokines induced by HIV [22,23]. We had shown that RTV facilitates signaling pathways related to these cytokines in ex vivo models [10] and in vivo [24], based upon its ability to block immunoproteasome-mediated degradation of the cytokine nuclear signaling adapter protein TRAF6 [9,10].

In contrast to Wnt3a effects and the β -catenin pathway, the molecular determinants subserving activation of β -catenin-independent Wnt pathways are poorly defined, although there is consensus that such signaling can antagonize the Wnt- β -catenin pathway in vertebrates [17]. Our observations in OC are consistent with this. We also recognize that the clinical effect of any drug must be assessed in the context of interacting cellular systems, noting the prominent coupling between OC and osteoblasts in bone biology. However, in support of our OC differentiation model, PIs as a class are linked to low BMD in the setting of HIV [25], and RTV alone had a strong clinical link to bone mineral density loss in at least one recent clinical trial [8] RTV-treated individuals also show elevated levels of OC differentiation from peripheral precursors [24]. In addition, RTV has no effect on Wnt-related transcripts in osteoblasts [26]. Further investigation of a direct role for Wnt and its inhibitors in OC biology, as suggested by our studies, may open additional avenues for modulation of OC function in HIV/ART-associated osteopenia as well as other disease states linked to accelerated loss of BMD.

Acknowledgments

This work was supported by National Institutes of Health Grants DK65511 and AI65200 (to J.L.) and CA123238 (to A.B.), Qatar National Research Fund Grant NPRP 08-632-3-132 (to A.B.), and the Angelo Donghia Fund (to J.L.). We are grateful to Kate McCarty for assistance with the siRNA experiments, Jordan Doyle for help with construction of β -catenin/TCF reporters, and Mariya A. Babayeva for help with flow cytometry. We thank the National Institutes of Health AIDS Research and Reference Reagent Program for providing the antiretroviral drugs and recombinant HIV proteins.

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Highlights

First demonstration of direct role for noncanonical Wnt in osteoclast differentiation

Demonstration of Ryk as a Wnt5a/b receptor in inhibition of canonical Wnt signaling

Modulation of noncanonical Wnt signaling by a clinically important drug, ritonavir

Establishes a mechanism for an important clinical problem: HIV-associated bone loss

Fig. 1.

Ritonavir augments Wnt5 protein expression in osteoclast precursors. 1×10^6 RAW 264.7 murine monocytic cells per ml were cultured in the presence of buffer, RANKL (50ng/ml), the HIV protease inhibitor RTV (5 μ M), the HIV nucleotide reverse transcriptase inhibitor tenofovir (25 μ M), or LiCl (10mM) for 24 hours. Cell lysates were prepared and used in an immunoblot protocol for Wnt5 and β -actin, followed by densitometry scanning. %change in Wnt5:actin ratios were compared to buffer. RTV, RANKL + RTV, and RANKL + RTV + LiCl all increased these ratios above that seen with buffer alone ($p < 0.01$). RTV + RANKL increased Wnt5 expression over that seen with RTV or RANKL alone, while RANKL + tenofovir led to a decrease in Wnt5 compared to RANKL ($p < 0.03$). Each experimental data point represents 3 separate experiments.

Fig. 2.

Recombinant Wnt5b enhances OC differentiation in murine and human precursor cells, while rWnt3a suppresses OC differentiation. 1×10^6 RAW 264.7 murine OC precursors were exposed to buffer or RANKL (50ng/ml) in the presence of varying concentrations of rWnt5b (A) or rWnt3a (B). TRAP activity, used as a measure of OC differentiation, was assessed on day 7. A significant increase in TRAP activity was seen for RANKL-treated cultures at all concentrations of Wnt5b ≥ 100 ng/ml ($p < 0.01$). In contrast, significant suppression of TRAP activity was seen for all concentrations of Wnt3a ≥ 25 ng/ml ($p < 0.01$). These experiments were performed three times. The TRAP OD obtained in the absence of exogenous Wnt is equivalent to that seen for the anticipated number of mature, TRAP+ multi-nucleated giant cell OC obtained after a 7 day differentiation of 10^6 murine precursors in 50ng/ml of RANKL.

(C) Primary human peripheral blood mononuclear adherent cells (1×10^5 /well) were cultured in the presence of M-CSF (100ng/ml) and either RANKL (50ng/ml) alone, RANKL + rWnt5b (100ng/ml), RANKL + rWnt3a (100ng/ml), or RANKL + RTV (5 μ M). TRAP activity was assessed on day 7. Each bar represents 3 separate experiments. The TRAP OD obtained in the presence of RANKL + M-CSF only is equivalent to that seen for the

anticipated number of mature, TRAP+ multi-nucleated giant cell OC obtained after a 7 day differentiation of 10^5 human precursors in 50ng/ml of RANKL (10).

Fig. 3.

Overexpression of Wnt5a enhances OC differentiation in murine precursors. 0.5×10^6 RAW 264.7 cells, either without manipulation (Control) or following infection with retrovirus bearing a Neo resistance gene alone (LNC-X) or genes encoding Neo resistance plus Wnt5a (LNC-Wnt5a) were exposed to buffer, RANKL (50ng/ml), LiCl (10mM), or RTV (5 μ M). TRAP activity was measured on day 7. Each bar represents 4 separate experiments.

A 1- 7F2 murine osteoblast

5- RAW cells + RANKL + RTV 24hrs

2- H₂O

6- RAW cells 72hrs

3- RAW cells 24hrs

7- RAW cells + RANKL 72hrs

4- RAW cells + RANKL 24hrs

8- RAW cells + RANKL + RTV 72hrs

1 2 3 4 5 6 7 8

1- Human Kidney K293

5- RAW cells + RANKL + RTV 24hrs

2- 7F2 Murine Osetoblast

6- RAW cells 72hrs

3- RAW cells 24hrs

7- RAW cells + RANKL 72hrs

4- RAW cells + RANKL 24hrs

8- RAW cells + RANKL + RTV 72hrs

B

C

Fig. 4.

A. Expression of receptors Ror2 and Ryk, implicated in noncanonical Wnt signaling, in murine OC precursors and osteoblasts. RT-PCR for Ror2 (upper panel) and Ryk (lower panel) was performed with the 7F2 murine osteoblast cell line and the RAW 264.7 monocytic OC precursor line at baseline and following a 24 or 72 hour exposure to RANKL (50ng/ml) in the presence or absence of RTV (5μM). These gels are representative of 2 separate experiments.

B. Ryk knockdown blocks the ability of Wnt5b and RTV to enhance RANKL-driven osteoclastogenesis. Use of a chimeric DNA/RNA construct against Ryk markedly suppressed Ryk expression in RAW 264.7 cells. These western blots are representative of 4 separate experiments

C. Enhancement of TRAP activity by RTV was abrogated following inhibition of Ryk expression. Each bar represents 4 separate experiments.

D. Wnt5b and RTV suppress canonical Wnt reporter activation. Top: Schematic of WntRgreenL1, a lentivirus vector containing 7 copies of a TCF binding site cloned into an HIV-based vector (pTRH1-mCV). Bottom: 0.5×10^6 RAW264.7 cells were infected with WntRgreenL1, permitting green fluorescent protein to be expressed in a β -catenin/TCF-responsive manner. 48 hours after infection, the cells were washed, plated at 1×10^5 cells/well, and cultured overnight with buffer, RANKL (50ng/ml), RANKL + RTV (5μM), RANKL + tenofovir (25μM), or RANKL + Wnt5b (100ng/ml). Cells were then fixed in 2% p-formaldehyde and % fluorescent cells assessed by flow cytometry. Background fluorescence (vector lacking the TCF-responsive element) was subtracted from all measures prior to calculating the percent change in fluorescent cells relative to those treated with RANKL alone. Each bar represents 4 separate experiments.