

## **Pim-2 is a critical target for treatment of osteoclastogenesis enhanced in myeloma**

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**Running head:** The role of Pim-2 in myeloma bone destruction

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Multiple myeloma (MM) has a unique propensity to develop and expand almost exclusively in the bone marrow, and generates bone destruction. MM bone disease is characterized by increased osteoclastic activity and suppressed osteoblastic differentiation causing devastating bone destruction with rapid loss of bone (Raje and Roodman 2011, Roodman 2006). In pursuing novel factors responsible for MM tumor expansion in the bone lesions, Pim-2 has been found to be constitutively over-expressed as an anti-apoptotic mediator in MM cells and further upregulated through an interaction with osteoclasts and/or bone marrow stromal cells with defective osteoblastic differentiation (Asano, *et al* 2011, Johrer, *et al* 2012, Lu, *et al* 2013). We subsequently reported that Pim-2 is also induced in bone marrow stromal cells in MM and acts as a negative regulator for osteoblastogenesis, and that Pim inhibition is able to restore bone formation in addition to the suppression of the tumor burden in MM animal models (Hiasa, *et al* 2015). Thus, Pim-2 appears to be an important therapeutic target in MM; clinical studies with Pim inhibitors have been launched. However, the effects of Pim inhibition on osteoclastogenesis enhanced in MM has not been studied. Therefore, we explored the expression of Pim-2 in osteoclastic lineage cells, its role in osteoclastogenesis, and the impact of Pim inhibition on MM-induced bone resorption.

Pim-2 was expressed in cathepsin K-positive mature osteoclasts on the surface of bone in addition to MM cells in mouse MM models with intra-tibial inoculation of mouse 5TGM1 MM cells (Fig 1A, upper). Pim-2 was also found to be clearly expressed exclusively in cathepsin K-positive osteoclasts, but not in other bone marrow cells in the tibia from normal control mice (Fig 1A, lower). Receptor activator of NF- $\kappa$ B ligand (RANKL), a critical mediator of osteoclastogenesis, is overproduced to extensively enhance bone resorption in MM (Giuliani, *et al* 2002, Pearse, *et al* 2001). Addition of RANKL time-dependently induced the expression of Pim-2 in parallel with c-fos, NFATc1, critical transcription factors for osteoclastogenesis, and cathepsin K, a functional marker of mature osteoclasts, in RAW264.7 preosteoclastic cells (Fig 1B, left). Inhibition of NF- $\kappa$ B pathway by addition of SN50 or IMG2001, inhibitory peptides for the nuclear translocation of p65 or p50, respectively, suppressed the Pim-2 upregulation by RANKL (Fig 1B, right), suggesting Pim-2 upregulation via activation of the canonical NF- $\kappa$ B pathway by RANKL.

We next looked at the effects of Pim inhibition on RANKL-induced osteoclastogenesis. Treatment with Pim inhibitor SMI-16a or Pim-2 siRNA suppressed

the RANKL-induced expression of c-fos, NFATc1 and cathepsin K in RAW264.7 cells (Figs 1C and 1D, respectively), and abolished their formation of TRAP-positive multinucleated cells (Fig 1C). SMI-16a also markedly suppressed RANKL-stimulated resorption by RAW264.7 cells as shown by a reduction of resorption areas on hydroxyapatite-coated dishes (Fig 1E). These results demonstrate the critical role of Pim-2 in RANKL-induced osteoclast formation and activation.

To dissect the mechanisms of suppression of RANKL-induced osteoclastogenesis by the Pim inhibitor SMI-16a, we examined the effects of SMI-16a on the transcriptional activity of NF- $\kappa$ B in NF- $\kappa$ B reporter vector-transfected RAW264.7 cells upon treatment with RANKL. RANKL induced the luciferase reporter activity; however, SMI-16a did not affect the RANKL-induced transcriptional activity of NF- $\kappa$ B (Fig 2A). Together with the observation of Pim-2 upregulation downstream of the canonical NF- $\kappa$ B pathway (Fig 1B, right), these results demonstrate that NF- $\kappa$ B activation by RANKL triggers Pim-2 upregulation, and that Pim-2 upregulation does not affect NF- $\kappa$ B activation by RANKL. NFATc1 is known to be auto-amplified by RANKL-triggered intracellular  $Ca^{2+}$  ( $[Ca^{2+}]_i$ ) oscillation to act as a critical transcription factor for osteoclastogenesis (Takayanagi 2007).  $[Ca^{2+}]_i$  oscillation was induced after treatment with RANKL; however, addition of SMI-16a abrogated the RANKL-induced  $[Ca^{2+}]_i$  oscillation (Fig 2B), suggesting a critical role of Pim-2 in  $[Ca^{2+}]_i$  oscillation and NFATc1 upregulation by RANKL.

We next examined whether Pim inhibition is able to reduce osteoclastogenesis enhanced by MM cells. MM cells potently induced osteoclast formation in cocultures with bone marrow cells. However, treatment with SMI-16a almost completely abolished the osteoclast formation (Fig 2C). We further confirmed the effects of the Pim inhibition on osteoclastogenesis in MM animal models with intra-tibial injection of murine 5TGM1 MM cells. The mice were treated with SMI-16a intraperitoneally at 20 mg/kg every other day after confirming MM cell growth. Cathepsin K-positive osteoclast were increased in number on the surface of bone in the tibiae; however, treatment with SMI-16a reduced cathepsin K-positive osteoclast numbers nearly to those in control mice (Fig 2D and Supplementary Fig 1). These results suggest the therapeutic efficacy of Pim inhibition for osteoclastic bone destruction in MM.

Consistent with our previous observation in MM cells (Asano, *et al* 2011), in the present study, we observed a predominant role of NF- $\kappa$ B activation in Pim-2 induction,

but not the involvement of Pim-2 in NF- $\kappa$ B activation in osteoclastic lineage cells. However, there was one report demonstrating that Pim-2 activates NF- $\kappa$ B-dependent gene expression by inducing phosphorylation of Cot in lymphoid lineage cells (Hammerman, *et al* 2004). Although we need to further elucidate the cross-talk between Pim-2 upregulation and NF- $\kappa$ B activation, from the present study, the hierarchical RANKL-NF- $\kappa$ B-Pim-2 pathway appears to play a predominant role in osteoclastogenesis in MM. Furthermore, we found Pim-2 as a critical downstream mediator of RANKL, triggering  $[Ca^{2+}]_i$  oscillation and thereby sustaining the up-regulation of NFATc1 and osteoclastogenesis. These observations may serve as rationale for Pim-2 as an important therapeutic target for osteoclastogenesis markedly enhanced in MM.

We previously reported that Pim-2 expression is also up-regulated as a negative regulator for osteoblastogenesis in bone marrow stromal cells and preosteoblastic cells in the presence of MM cells as well as cytokines known as inhibitors of osteoblastogenesis in MM, and that Pim-2 knockdown as well as the Pim inhibitor SMI-16a successfully restore osteoblastogenesis suppressed by inhibitory factors, as well as by MM cells (Hiasa, *et al* 2015). The present study further added another important role of Pim-2 in bone metabolism pathologically skewed in MM. These observations collectively corroborate that Pim-2 is a pivotal therapeutic target for MM bone disease and tumor progression.

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**Authorship:** JT and MA designed study and wrote the manuscript; JT performed all of the experiments; MH, AO, TH, SN and HT performed the *in vivo* experiments; MH, RA and HT helped histological section and analysis; All authors were involved in the analyses and the interpretation of the data; All authors approved the submission of manuscript.

## Competing interests

The authors have no competing interests.

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## Figure Legends

**Figure 1.** Pim-2 expression in osteoclasts and its role in RANK ligand-induced osteoclastogenesis. **(A)** Double immunofluorescence staining of Pim-2 (red) and cathepsin K (green) in the tibia from a mouse inoculated with 5TGM1 MM cells (upper) and a normal control mouse (lower). Bone surfaces are demarcated by dashed lines (B; bone, M; bone marrow). The scale bar indicates 50  $\mu$ m. **(B)** RAW264.7 cells were cultured in the absence or presence of RANK ligand at 25 ng/ml. After the indicated time periods, the protein levels of Pim-2 and osteoclast differentiation markers, c-fos, NFATc1 and cathepsin K, were analyzed by Western blotting (left). Pim-2 protein levels were determined after culturing for 2 days with or without addition of NF- $\kappa$ B inhibitors, SN50 or IMG2001 at 10 pg/ml (right). **(C)** RAW264.7 cells were cultured in the absence or presence of RANK ligand at 25 ng/ml. SMI-16a (SMI) was added at the indicated concentrations. The expression of c-fos, NFATc1 and cathepsin K, were analyzed at day 2 by Western blotting (left). The cells were fixed at day 3, and stained with TRAP (middle). TRAP-positive multinucleated cells (MNCs) containing 3 or more nuclei were counted (right). Data are expressed as means  $\pm$  SD (n=4). \*, p < 0.05. **(D)** RAW264.7 cells were transfected with either scramble (CTL) or Pim-2-specific siRNA, and cultured in the presence of RANK ligand at 25 ng/ml. The expression of c-fos, NFATc1 and cathepsin K were analyzed at day 2 by Western blotting (left). The cells were fixed and stained with TRAP at day 3. MNCs with 3 or more nuclei were counted (right). Data are expressed as means  $\pm$  SD (n=4). \*, p < 0.05.  $\beta$ -actin was used as a protein loading control. **(E)** RAW264.7 cells were cultured on osteo-assay plates for 5 days with RANK ligand at 25 ng/ml in the presence or absence of SMI-16a (SMI) at 10  $\mu$ M. Resorption areas were measured by Image J after visualization with von Kossa staining (left). Results were expressed as ratios of the summation of resorption areas in the whole well areas (right). Data are expressed as means  $\pm$  SD (n=4). \*, p < 0.05.

**Figure 2. (A)** NF- $\kappa$ B promoter activity. NF- $\kappa$ B reporter vector-transfected RAW264.7 cells were cultured in the absence or presence of SMI-16a (SMI) at 20  $\mu$ M. After addition of RANK ligand at 25 ng/ml, cell lysates were collected and luciferase activity was measured. Results are expressed from 3 separate experiments as means  $\pm$  SD of ratios from the control samples. “n.s.” indicates not significant (p>0.05). **(B)** RAW264.7 cells were cultured in the absence or presence of SMI-16a (SMI) at 20  $\mu$ M.

After culturing for 24 hours with RANK ligand at 25 ng/ml, time courses of intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) levels in a single cell were detected as described in Materials and Methods in Supplementary Information, and compared to those maximally increased by ionomycin. The results were expressed as per cent of the  $[\text{Ca}^{2+}]_i$  levels by ionomycin. Each color corresponds to different cells in the same fields. **(C)** Primary mouse bone marrow cells were pretreated with RANK ligand at 25 ng/ml for 24 hours. After washing the cells to remove exogenous RANK ligand, they were co-cultured with various MM cell lines as indicated. The cells were cultured in quadruplicate in the absence or presence of SMI-16a (SMI) at 20  $\mu\text{M}$ . After culturing for 5 days, the cells were fixed and stained with TRAP. MNCs with 3 or more nuclei were counted. Data are expressed as means  $\pm$  SD (n=3). \*,  $p < 0.05$ . **(D)** Mice with or without intra-tibial inoculation of murine 5TGM1 MM cells were treated with an intraperitoneal injection of SMI-16a at 20 mg/kg or saline every other day from day 5 to day 20 after confirming MM cell growth. The tibiae were taken out on day 21, and cathepsin K (CTK) immunoreactivity were analyzed on 5- $\mu\text{m}$  thick paraffin-embedded serial sections. The numbers of CTK-positive osteoclasts were counted in 4 different areas (500  $\text{mm}^2$ ) of three independent sections (lower graph). Data are expressed as means  $\pm$  SD. \*,  $p < 0.05$ .



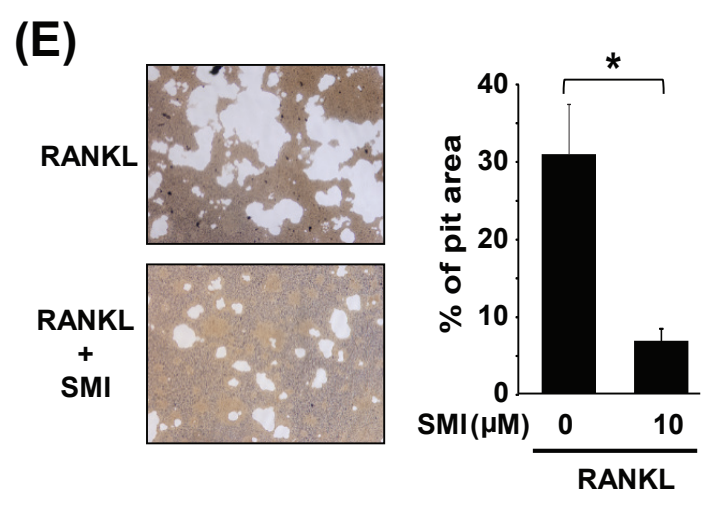
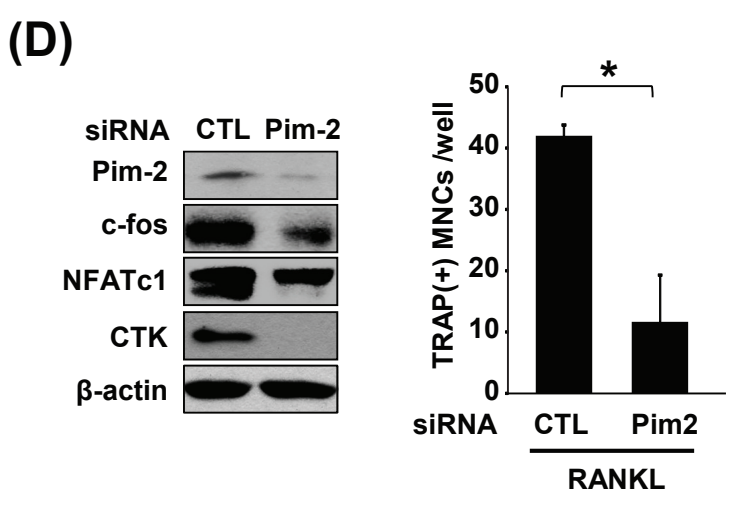
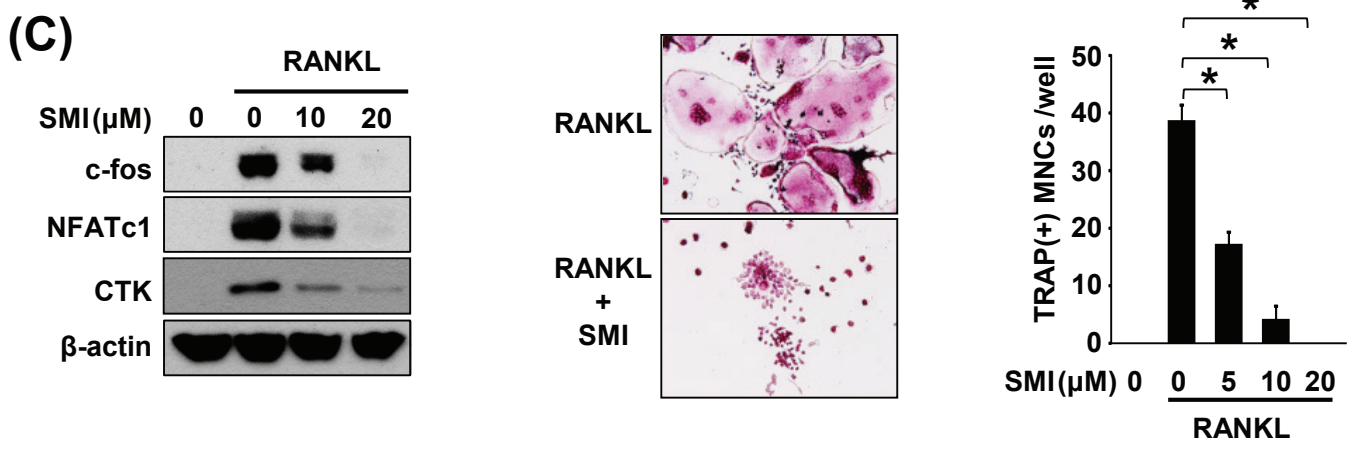
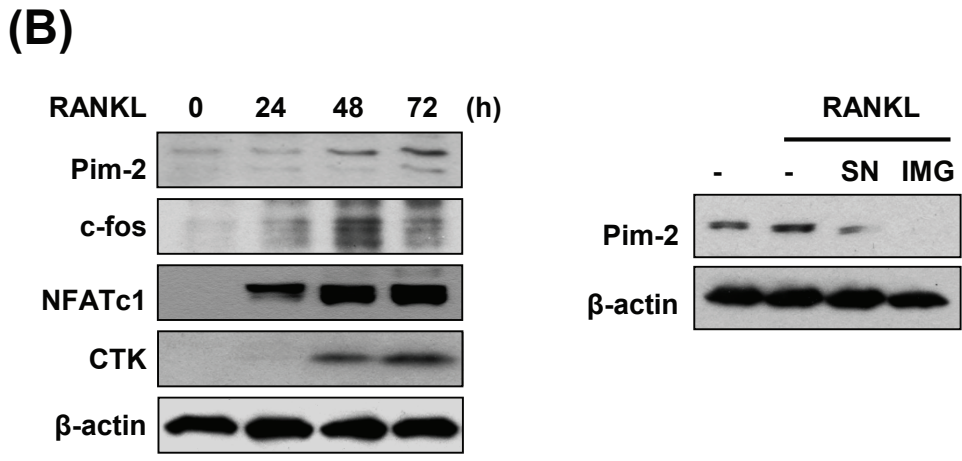
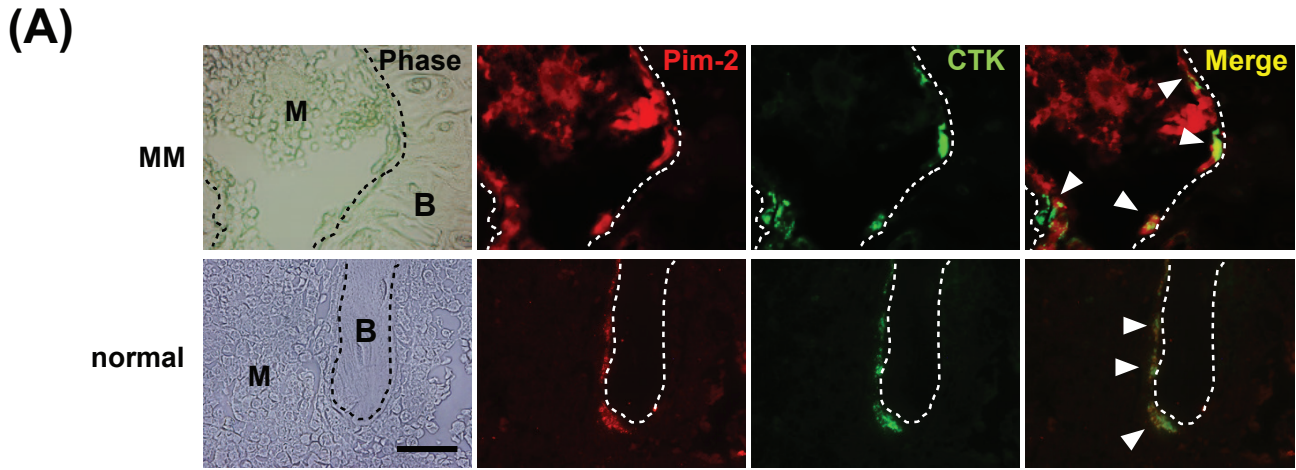


Figure 2

