

Title: The serine/threonine kinase Pim-2 is a novel anti-apoptotic mediator in myeloma cells

Running head: Pim-2 in myeloma cells

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## **Abstract**

Bone marrow stromal cells (BMSCs) and osteoclasts (OCs) confer multiple myeloma (MM) cell survival through elaborating factors. We demonstrate herein that IL-6 and TNF family cytokines, TNF $\alpha$ , BAFF and APRIL, but not IGF-1 cooperatively enhanced the expression of the serine/threonine kinase Pim-2 in MM cells. BMSCs and OCs up-regulate Pim-2 expression in MM cells largely via the IL-6/STAT3 and NF- $\kappa$ B pathway, respectively. Pim-2 siRNA reduces MM cell viability in cocultures with BMSCs or OCs. Thus, up-regulation of Pim-2 appears to be a novel anti-apoptotic mechanism for MM cell survival. Interestingly, the mTOR inhibitor rapamycin further suppresses the MM cell viability in combination with the Pim-2 silencing. The Pim inhibitor (Z)-5-(4-propoxybenzylidene) thiazolidine-2, 4-dione and the PI3K inhibitor LY294002 cooperatively enhance MM cell death. The Pim inhibitor suppressed 4E-BP1 phosphorylation along with the reduction of Mcl-1 and c-Myc. Pim-2 may therefore become a new target for MM treatment.

Keywords: Pim-2, myeloma, bone marrow stromal cell, osteoclast, IL-6

## **Introduction**

Multiple myeloma (MM) cells enhance osteoclast (OC) formation and suppress osteoblast (OB) differentiation of bone marrow stromal cells. Such effects of MM cells not only create destructive bone lesions but also provide a cellular microenvironment to protect MM cells from various apoptotic insults.<sup>1-5</sup> IL-6 and TNF family cytokines, TNF $\alpha$ , BAFF and APRIL, are among predominant anti-apoptotic factors for MM cells elaborated by the bone marrow microenvironment surrounding MM.<sup>1, 6-8</sup> IL-6 is mainly produced by bone marrow stromal cells (BMSCs),<sup>1, 6</sup> whereas OCs are a major producer of BAFF and APRIL in the MM bone marrow microenvironment.<sup>9, 10</sup> The serine/threonine kinase Pim-2 has been demonstrated to be transcriptionally up-regulated to promote survival of hematopoietic cells in response to ambient growth factors and cytokines.<sup>11</sup> We demonstrate herein that Pim-2 is up-regulated in MM cells by BMSCs and OCs, and acts as an important pro-survival mediator.

## **Materials and methods**

### **Reagents**

The following reagents were purchased from the indicated manufactures: rh IL-6, BAFF and SDF-1 $\alpha$  from PEPROTECH EC (London, UK); rh TNF $\alpha$ , APRIL, IGF-1, VEGF, neutralizing mouse monoclonal antibodies against IL-6 and against IL-6 receptor from R&D Systems (Minneapolis, MN); cucurbitacin I, parthenolide, rapamycin, LY294002 and the Pim inhibitor (Z)-5-(4-propoxybenzylidene)thiazolidine-2,4-dione from

Calbiochem (Darmstadt, Germany); Mouse monoclonal antibody against human Pim-2 and rabbit polyclonal antibodies against STAT-3 and Mcl-1 from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit polyclonal antibodies against phosphorylated NF $\kappa$ B p65, NF- $\kappa$ B p65, 4E-BP1, phosphorylated 4E-BP1 (Ser 65), phosphorylated 4E-BP1 (Thr 37/46) and phosphorylated STAT-3, mouse monoclonal antibody against phosphorylated Bad, rabbit monoclonal antibodies against human Pim-2 and c-Myc, horseradish peroxidase (HRP)-conjugated horse anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG from Cell Signaling Technology (Beverly, MA); mouse monoclonal anti-Bad from BD Transduction Laboratories (Franklin-Lakes, NJ); rabbit anti- $\beta$ -actin from Sigma (St. Louis, MO).

### **Cells and cultures**

Human non-hematopoietic cell lines, MG63, MCF7 and Colo205, human myeloid cell lines, KU812F, HL-60 and U937, human lymphoid cell lines, CEM and IM-9, and human MM cell lines, RPMI8226 and U266, were obtained from American Type Culture Collection (ATCC) (Rockville, MD). The MM cell line INA6 and MM.1S was kindly provided by Dr. Renate Burger (University of Kiel, Kiel, Germany) and Dr. Steven Rosen (Northwestern University, Chicago, IL), respectively. TSPC-1 and OPC MM cell lines were established in our laboratory.<sup>12</sup> Primary MM cells and BMSCs were isolated from fresh bone marrow aspirates from patients with MM and cultured as previously described.<sup>11</sup> BMSCs were a homogeneous population of spindle-shaped cells expressing CD44, CD73, CD90 and CD105, but neither CD45 nor factor VIII. OCs

were generated from peripheral blood mononuclear cells (PBMCs) as previously reported.<sup>11</sup> Cells were cultured in  $\alpha$ MEM supplemented with 10% Fetal Bovine Serum (FBS), 2 mM of L-Glutamine (Sigma), 100 U/ml of penicillin G and 100  $\mu$ g/ml of streptomycin (Sigma). All procedures involving human specimens were performed with written informed consent according to the Declaration of Helsinki and using a protocol approved by the Institutional Review Board for human protection.

### **Western blot analysis**

Cells were collected and lysed in lysis buffer (Cell Signaling) supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail solution (Sigma). Cell lysates were subjected to Western blot analysis as described previously.<sup>13</sup>

### **Immunohistochemistry**

Bone marrow clot sections from MM patients were fixed in neutral-buffered formalin and embedded in paraffin. Paraffin-embedded tissue sections were deparaffinized and hydrated. For antigen retrieval the sections were microwaved in 10mM sodium citrate buffer (pH 6.6). After a 10-minute blocking process, the sections were incubated with rabbit monoclonal anti-human Pim-2 antibody (Cell Signaling) or control rabbit IgG (Vector Laboratories, Burlingame, CA) overnight at 4°C. Immunoreactivity was detected by biotinylated secondary antibodies and horseradish peroxidase-conjugated streptavidin (DAKO LSAB+ System, HRP; DAKO, Carpinteria, CA) followed by diaminobenzidine substrate (DAKO).

### **Quantitative real-time PCR**

RNA isolation and quantitative real-time PCR were performed as described previously.<sup>13</sup> Primers used were as follows: Human *Pim-2* sense 5'-AGGGATTGAGGATCAGGGGT-3' and antisense 5'-CACAGGTTCTGGGAGGAAGG-3'. Human *GAPDH*, used as a housekeeping gene for quantity normalization, sense 5'-AATCCCATCACCATCTTCCA-3', antisense 5'-TGGACTCCACGACGTACTCA-3'.

### **Transfection**

MM cells were transfected with 6-carboxyfluorescein (6-FAM)-labeled Pim-2 short interfering RNA (siRNA) (sense, 5'-GUGCCAAACUCAUUGAUUUTT-3' and antisense, 5'-AAAUCA AUGAGUUUGGCACTT-3') and scrambled siRNA (sense, 5'-AUCCGCGCGAUAGUACGUATT-3' and antisense, 5'-UACGUACUAUCGCGCGGAUTT-3') (B-Bridge, Mountain View, CA) using a Human Nucleofector Kit (Amaxa Biosystems, Cologne, Germany). The transfected cells were sorted by a flow cytometer (EPICS ELITE; Beckman Coulter, Brea, CA).

### **Cell viability assays**

Viable cell numbers were counted by trypan blue dye exclusion assay as we previously described.<sup>11</sup> Cell viability was also determined by Cell Counting Kit-8 assay (DOJINDO, Kumamoto, Japan) according to the manufacture's instructions. The

absorbance of each well was measured at 450 nm with a microtiter plate reader (Model 450 micro plate reader; Bio-Rad Laboratories, Hercules, CA). Apoptosis was evaluated by staining cells with an annexin V-FITC and propidium iodide (PI) labeling kit (MEBCYTO Apoptosis Kit; MBL, Nagano, Japan) according to instructions.

### **Statistical analysis**

Data are expressed as means +/- standard deviation unless otherwise specified.

Statistical significance was determined by a one-way analysis of variance (ANOVA) with Scheffe's post hoc tests. The minimal level of significance was P=0.05.

## **Results and Discussion**

### **BMSCs and OCs enhance Pim-2 expression in MM cells**

Pim-2 protein was constitutively expressed at higher levels in MM cell lines and some other hematopoietic cell lines than in non-hematopoietic cell lines and normal peripheral blood mononuclear cells (Figure 1a and Supplementary Figure 1). Pim-2 protein expression was also observed in MM cells in bone marrow specimens of patients with MM (Figure 1b). Interestingly, *Pim-2* mRNA levels were up-regulated substantially (about 2- to 9-fold) in all MM cell lines and MM cells from all patients with MM when cocultured with BMSCs (Figure 2a upper). Consistently, Pim-2 protein levels were also enhanced by coculturing with BMSCs (Figure 2a lower). Although PBMC-derived OCs also up-regulated Pim-2 expression in most of these MM cells



(Figure 2b), BMSCs more potently enhanced Pim-2 expression in MM cells than OCs. These results demonstrate that Pim-2 is over-expressed in MM cells and that BMSCs and OCs stimulate Pim-2 expression in MM cells.

### **IL-6 and TNF family cytokines cooperatively enhance Pim-2 expression in MM cells**

To determine the factors responsible for Pim-2 up-regulation in MM cells, we examined Pim-2 expression in MM cells under stimulation with anti-apoptotic cytokines over-produced in the MM bone marrow microenvironment. IL-6 and TNF family cytokines, TNF $\alpha$ , BAFF and APRIL, up-regulated Pim-2 expression in MM cells (Figure 3a). Interestingly, IL-6 and these TNF family cytokines cooperatively enhanced Pim-2 expression in MM cells (Figure 3a). However, another important anti-apoptotic cytokine, IGF-I, did not increase but rather suppressed Pim-2 expression (Supplementary Figure 2). SDF-1 and VEGF showed no significant effect. These results suggest that IL-6 and these TNF family cytokines are among predominant factors responsible for Pim-2 up-regulation in MM cells in the bone marrow microenvironment in MM.

IL-6 induced STAT3 phosphorylation followed by Pim-2 up-regulation in MM cells (Supplementary Figure 3). Addition of cucurbitacin I, a STAT3 inhibitor, suppressed the Pim-2 up-regulation by IL-6 (Figure 3b). TNF $\alpha$  induced Pim-2 up-regulation following phosphorylation of NF $\kappa$ B in MM cells (Supplementary Figure 3). The Pim-2 up-regulation by TNF $\alpha$  was suppressed by an IKK $\alpha$ / $\beta$  inhibitor,

parthenolide, (Figure 3c). Thus, STAT3 and NF- $\kappa$ B activation mediates Pim-2 up-regulation in MM cells. These results are consistent with the notion that IL-6 and these TNF family cytokines act together to up-regulate Pim-2 expression in MM cells in the bone marrow microenvironment. However, basal expression levels of Pim-2 were associated with neither the levels of phosphorylation of STAT3 nor NF- $\kappa$ B activation in MM cell lines without external stimuli (Supplementary Figure 1). Other signaling pathways may also be involved in endogenous up-regulation of basal Pim-2 levels in MM cells.

#### **Inhibition of IL-6/STAT3 and NF- $\kappa$ B pathways abolishes Pim-2 up-regulation in MM cells by BMSCs and OCs, respectively**

Because BMSCs produce a large amount of IL-6 and regarded as a major source of IL-6 in the bone marrow microenvironment in MM,<sup>1,6</sup> and because IL-6 potently up-regulates Pim-2 expression in MM cells, we next explored the role of IL-6 in the Pim-2 up-regulation in MM cells by BMSCs. The up-regulation of *Pim-2* mRNA expression in INA-6 and RPMI8226 cells by BMSCs was almost completely suppressed by antibodies against IL-6 and IL-6 receptor in combination (Figure 3d). The inhibition of STAT3 by cucurbitacin I also abolished the *Pim-2* up-regulation in MM cells by BMSCs (Figure 3e left). The inhibition by LY294002 of a PI3K/Akt pathway, a major downstream signaling pathway of IGF-1, did not affect *Pim-2* expression in MM cells up-regulated by BMSCs (Figure 3e right), which is consistent with the observation with no induction of *Pim-2* expression in MM cells by IGF-1 (Supplementary Figure 2).

Thus, IL-6 appears to be a predominant BMSC-derived factor responsible for Pim-2 up-regulation in MM cells. The IKK $\alpha/\beta$  inhibitor parthenolide abolished *Pim-2* up-regulation in MM cells cocultured with OCs (Figure 3f), suggesting a major role of the NF $\kappa$ B pathway in Pim-2 up-regulation in MM cells by OCs.

### **Pim-2 inhibition and rapamycin cooperatively suppress MM cell survival**

To clarify the roles of Pim-2 in MM cell survival, we next examined the effect of Pim-2 silencing in MM cells. Pim-2 silencing by Pim-2 siRNA reduced Pim-2 expression in RPMI8226 cells to about 62% and 29% in the absence and presence of IL-6, respectively, compared with that by control siRNA (Supplementary Figure 4a). IL-6, BMSCs or OCs are able to enhance the survival of the IL-6-dependent INA-6 cell line, which otherwise undergoes apoptosis. The viability of INA-6 cells was partially but significantly reduced by Pim-2 silencing in the presence of IL-6, BMSCs or OCs (Figure 4a). Because Pim-2 up-regulation is largely independent of the PI3K/Akt pathway (Figure 3e), and because the PI3K/Akt pathway is an important survival pathway in MM cells<sup>14, 15</sup>, we examined the effects of inhibition of both Pim-2 and PI3K/Akt pathways on MM cell viability in the presence of BMSCs. Rapamycin is an inhibitor of mammalian target of rapamycin (mTOR), a downstream signaling molecule of the PI3K/Akt pathway. Pim-2 silencing or addition of rapamycin alone partially reduced the viability of INA-6 cells supported by BMSCs (Figure 4b left), whereas the two treatments in combination further reduced INA-6 cell viability. The combinatory treatment also potently suppressed the survival of RPMI8226 cells (Figure 4b right)

which can grow in the absence of BMSCs. These results suggest a cooperative role of Pim-2 and mTOR pathways in MM cell survival.

### **The Pim inhibitor (Z)-5-(4-propoxybenzylidene)thiazolidine-2,4-dione suppresses MM cell viability**

Because Pim-2 knockdown was partial in MM cells by Pim-2 siRNA due to low transfection efficiency in MM cells, we extended the experiments with the Pim inhibitor (Z)-5-(4-propoxybenzylidene)thiazolidine-2,4-dione, which preferentially suppress Pim-2 rather than Pim-1<sup>16,17</sup>. The Pim inhibitor dose-dependently suppressed the viability of MM cell lines including INA6, RPMI8226, MM.1S, TSPC-1 and OPC (Figure 4c). Addition of the Pim inhibitor increased the %distribution of annexinV-positive cells in INA-6 cells in the absence or presence of BMSCs (Figure 4d in the revised manuscript), suggesting anti-apoptotic activity mediated by the Pim pathway. The Pim inhibitor in combination with the PI3K inhibitor LY294002 cooperatively reduced numbers of viable INA-6 cells both in the absence or presence of BMSCs (Figure 4e).

We further examined the effects of the Pim inhibitor on phosphorylation of 4E-BP1 and expression of growth and survival mediators, because the Pim pathway has been demonstrated to be responsible for the phosphorylation of 4E-BP1 to trigger protein translation for cell growth and survival.<sup>11,18</sup> Treatment with the Pim inhibitor markedly suppressed the phosphorylation of 4E-BP1 along with the reduction of Mcl-1 and c-Myc protein levels in INA6 and RPMI8226 cells (Figure 4f). However,

phosphorylation of Bad and the levels of other apoptosis-related factors including Bcl-xL, Bcl-2 and Bim showed no appreciable change in RPMI8226 cells upon the treatment with the Pim inhibitor (Supplementary Figure 4b). Thus, the induction of apoptosis by the Pim inhibitor is suggested to be associated at least in part with the reduction of phosphorylated 4E-BP1, Mcl-1 and c-Myc.

### **Conclusion**

The present observations demonstrate that Pim-2 is a novel pro-survival mediator for MM cells, and suggest that the MM bone marrow microenvironment up-regulates Pim-2 expression in MM cells through activation of the JAK2/STAT3 pathway for IL-6 and the NF- $\kappa$ B pathway for TNF family cytokines to promote MM cell survival. Therefore, Pim-2 over-expressed in MM cells in the MM bone marrow microenvironment appears to be an important therapeutic target. IGF-1 is another critical microenvironment-derived survival factor for MM cells,<sup>14, 15</sup> and inhibition of the IGF-1/PI3K/Akt pathway by Akt or mTOR inhibitors has drawn considerable attention as a new therapeutic modality against MM.<sup>19, 20</sup> Because Pim-2 up-regulation is largely independent of the PI3K/Akt pathway (Figure 3e), and because inhibition of Pim-2 and PI3K/Akt pathways cooperatively reduce MM cell survival (Figure 4b and 4e), Pim-2 should be targeted to improve anti-MM efficacy together with PI3K/Akt pathway inhibitors. A therapeutic impact of Pim-2 inhibition on MM survival would be further warranted when potent specific inhibitors for Pim-2 become available.

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(<http://www.nature.com/leu>)

## References

1. Raab MS, Podar K, Breitkreutz I, Richardson PG, Anderson KC. Multiple myeloma. *Lancet* 2009 Jul 25; **374**(9686): 324-339.
2. Roodman GD. Targeting the bone microenvironment in multiple myeloma. *J Bone Miner Metab* May; **28**(3): 244-250.
3. Matsumoto T, Abe M. Bone destruction in multiple myeloma. *Ann N Y Acad Sci* 2006 Apr; **1068**: 319-326.
4. Abe M, Hiura K, Wilde J, Shioyasono A, Moriyama K, Hashimoto T, *et al.* Osteoclasts enhance myeloma cell growth and survival via cell-cell contact: a vicious cycle between bone destruction and myeloma expansion. *Blood* 2004 Oct 15; **104**(8): 2484-2491.
5. Yaccoby S, Wezeman MJ, Henderson A, Cottler-Fox M, Yi Q, Barlogie B, *et al.* Cancer and the microenvironment: myeloma-osteoclast interactions as a model. *Cancer Res* 2004 Mar 15; **64**(6): 2016-2023.
6. Podar K, Chauhan D, Anderson KC. Bone marrow microenvironment and the identification of new targets for myeloma therapy. *Leukemia* 2009 Jan; **23**(1): 10-24.

7. Novak AJ, Darce JR, Arendt BK, Harder B, Henderson K, Kindsvogel W, *et al.* Expression of BCMA, TACI, and BAFF-R in multiple myeloma: a mechanism for growth and survival. *Blood* 2004 Jan 15; **103**(2): 689-694.
8. Moreaux J, Legouffe E, Jourdan E, Quittet P, Reme T, Lugagne C, *et al.* BAFF and APRIL protect myeloma cells from apoptosis induced by interleukin 6 deprivation and dexamethasone. *Blood* 2004 Apr 15; **103**(8): 3148-3157.
9. Moreaux J, Cremer FW, Reme T, Raab M, Mahtouk K, Kaukel P, *et al.* The level of TACI gene expression in myeloma cells is associated with a signature of microenvironment dependence versus a plasmablastic signature. *Blood* 2005 Aug 1; **106**(3): 1021-1030.
10. Abe M, Kido S, Hiasa M, Nakano A, Oda A, Amou H, *et al.* BAFF and APRIL as osteoclast-derived survival factors for myeloma cells: a rationale for TACI-Fc treatment in patients with multiple myeloma. *Leukemia* 2006 Jul; **20**(7): 1313-1315.
11. Hammerman PS, Fox CJ, Birnbaum MJ, Thompson CB. Pim and Akt



oncogenes are independent regulators of hematopoietic cell growth and survival. *Blood* 2005 Jun 1; **105**(11): 4477-4483.

12. Abe M, Hiura K, Wilde J, Moriyama K, Hashimoto T, Ozaki S, *et al.* Role for macrophage inflammatory protein (MIP)-1alpha and MIP-1beta in the development of osteolytic lesions in multiple myeloma. *Blood* 2002 Sep 15; **100**(6): 2195-2202.
13. Hiasa M, Abe M, Nakano A, Oda A, Amou H, Kido S, *et al.* GM-CSF and IL-4 induce dendritic cell differentiation and disrupt osteoclastogenesis through M-CSF receptor shedding by up-regulation of TNF-alpha converting enzyme (TACE). *Blood* 2009 Nov 12; **114**(20): 4517-4526.
14. Menu E, van Valckenborgh E, van Camp B, Vanderkerken K. The role of the insulin-like growth factor 1 receptor axis in multiple myeloma. *Arch Physiol Biochem* 2009 May; **115**(2): 49-57.
15. Sprynski AC, Hose D, Caillot L, Reme T, Shaughnessy JD, Jr., Barlogie B, *et al.* The role of IGF-1 as a major growth factor for myeloma cell lines and the prognostic relevance of the expression of its receptor. *Blood* 2009 May 7; **113**(19): 4614-4626.

16. Xia Z, Knaak C, Ma J, Beharry ZM, McInnes C, Wang W, *et al.*  
Synthesis and evaluation of novel inhibitors of Pim-1 and Pim-2  
protein kinases. *J Med Chem* 2009 Jan 8; **52**(1): 74-86.
17. Beharry Z, Zemsanova M, Mahajan S, Zhang F, Ma J, Xia Z, *et al.* Novel  
benzylidene-thiazolidine-2,4-diones inhibit Pim protein kinase  
activity and induce cell cycle arrest in leukemia and prostate cancer  
cells. *Mol Cancer Ther* 2009 Jun; **8**(6): 1473-1483.
18. Fox CJ, Hammerman PS, Cinalli RM, Master SR, Chodosh LA,  
Thompson CB. The serine/threonine kinase Pim-2 is a  
transcriptionally regulated apoptotic inhibitor. *Genes Dev* 2003 Aug 1;  
**17**(15): 1841-1854.
19. Hideshima T, Catley L, Yasui H, Ishitsuka K, Raje N, Mitsiades C, *et al.*  
Perifosine, an oral bioactive novel alkylphospholipid, inhibits Akt  
and induces in vitro and in vivo cytotoxicity in human multiple  
myeloma cells. *Blood* 2006 May 15; **107**(10): 4053-4062.
20. McMillin DW, Ooi M, Delmore J, Negri J, Hayden P, Mitsiades N, *et al.*  
Antimyeloma activity of the orally bioavailable dual

phosphatidylinositol 3-kinase/mammalian target of rapamycin

inhibitor NVP-BEZ235. *Cancer Res* 2009 Jul 15; **69**(14): 5835-5842.

## Figure legends

**Figure 1.** Pim-2 is constitutively expressed in MM cells. **(a)** Pim-2 protein expression in MM cell lines. Pim-2 protein expression was examined by Western blot analysis in various malignant cell lines as well as normal peripheral blood mononuclear cells (PBMC). Cell lysates were harvested from human non-hematopoietic cell lines, MG63, MCF7 and Colo205, human myeloid cell lines, KU812F, HL-60 and U937, human lymphoid cell lines, CEM and IM-9, and human MM cell lines, RPMI8226, U266 and INA-6, and PBMC.  $\beta$ -actin was blotted as a loading control. **(b)** Pim-2 expression in primary MM cells. Bone marrow clot sections obtained from 6 patients with MM were stained with control IgG (upper) or anti-Pim-2 antibody (lower). Pim-2 protein expression was observed in MM cells in all bone marrow specimens. Cells were visualized under an Olympus BX50 microscope equipped with UMPlanFI 40X/0.75 objective lens (Olympus, Tokyo, Japan) to achieve an original magnification of 400X. Images were recorded with an Olympus SC35 CCD camera and Viewfinder Lite Software (Pixera, Los Gatos, CA).

**Figure 2.** Pim-2 up-regulation in MM cells by BMSCs and OCs. After co-culturing in quadruplicate with BMSCs ( $1 \times 10^5$ /mL) **(a)** or PBMC-derived OCs ( $1 \times 10^4$ /mL) **(b)** for 2 days, MM cells were harvested. *Pim-2* mRNA expression in primary MM cells and MM cell lines was analyzed by real-time RT-PCR (upper). Human *GAPDH* was used as a housekeeping gene for quantity normalization. The results with BMSCs and OCs are

expressed as a fold increase from the baseline ratios of *Pim-2* mRNA /*GAPDH* mRNA. *Pim-2* protein levels were also examined by Western blot analysis (lower).  $\beta$ -actin was blotted as a loading control.

**Figure 3.** Regulation of *Pim-2* expression in MM cells. **(a)** INA-6 cells were cultured with rh IL-6 (10 ng/ml), TNF $\alpha$  (10 ng/ml), BAFF (100 ng/ml) and APRIL (100 ng/ml) alone or in combination as indicated. **(b)** The effects of STAT-3 inhibition on *Pim-2* up-regulation in MM cells by IL-6. INA-6 cells were cultured in the absence or presence of rhIL-6 at 10 ng/mL. The JAK/STAT3 inhibitor cucurbitacin I was added at 10  $\mu$ M as indicated. Total RNA and cell lysates were collected from the MM cells after culturing for 4 and 24 hours, respectively. *Pim-2* mRNA expression in MM cells was analyzed by real-time RT-PCR (left). The results are expressed as ratios of *Pim-2* mRNA /*GAPDH* mRNA. \*, p<0.05. *Pim-2* protein levels were also examined by Western blot analysis (right).  $\beta$ -actin was blotted as a loading control. **(c)** The effects of NF- $\kappa$ B inhibition on *Pim-2* up-regulation in MM cells by TNF $\alpha$ . RPMI8226 cells were cultured in the absence or presence of rhTNF $\alpha$  at 10 ng/mL. The IKK $\alpha$ / $\beta$  inhibitor parthenolide was added at 10  $\mu$ M as indicated. Total RNA and cell lysates were collected from the MM cells after culturing for 4 and 24 hours, respectively. *Pim-2* mRNA expression in MM cells was analyzed by real-time RT-PCR(left). \*, p<0.05. *Pim-2* protein levels were also examined by Western blot analysis (right). **(d)** The effects of IL-6 blockade on *Pim-2* up-regulation in MM cells by BMSCs. *Pim-2* mRNA expression in INA-6 and RPMI8226 cells was analyzed by real-time RT-PCR after

co-culturing with BMSCs for 2 days in the absence or presence of neutralizing anti-IL-6 and anti-IL-6 receptor antibodies in combination. (e) The effects of STAT-3 inhibition on Pim-2 up-regulation in MM cells by BMSCs. INA-6 cells were cultured alone or with BMSCs for 4 hours in the absence or presence of cucurbitacin I at different concentrations as indicated as well as the PI3K/Akt inhibitor LY294002 at 10  $\mu$ M. (f) The effects of NF- $\kappa$ B inhibition on Pim-2 up-regulation in MM cells by OCs. TSPC-1 MM cells were cultured alone or with PBMC-derived OCs ( $1 \times 10^4$ /mL). Parthenolide was added at 10  $\mu$ M as indicated. Total RNA was extracted from the MM cells after culturing for 4 hours. *Pim-2* mRNA expression in MM cells was analyzed by real-time RT-PCR. The results are expressed as ratios of *Pim-2* mRNA /*GAPDH* mRNA. \*,  $p < 0.05$ .

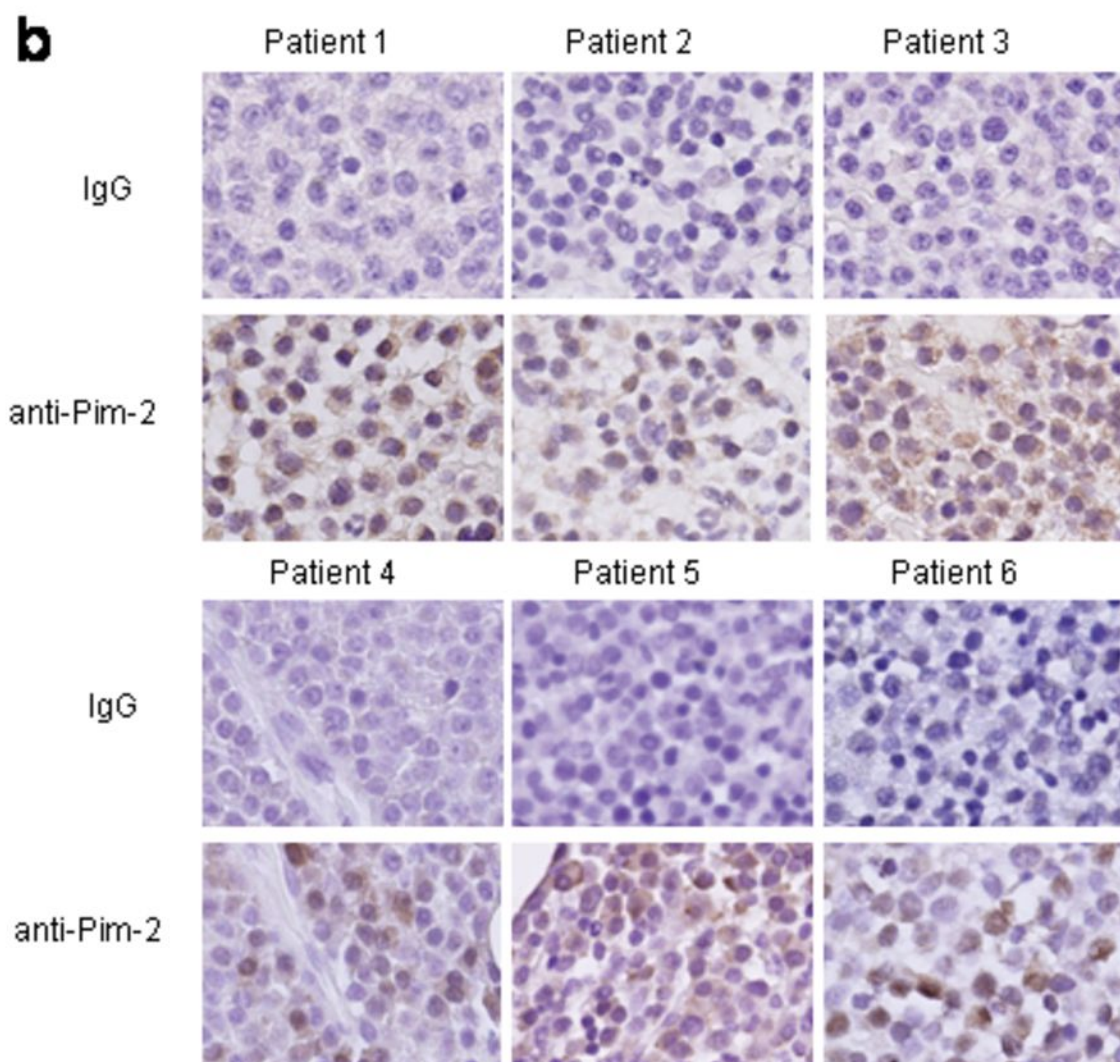
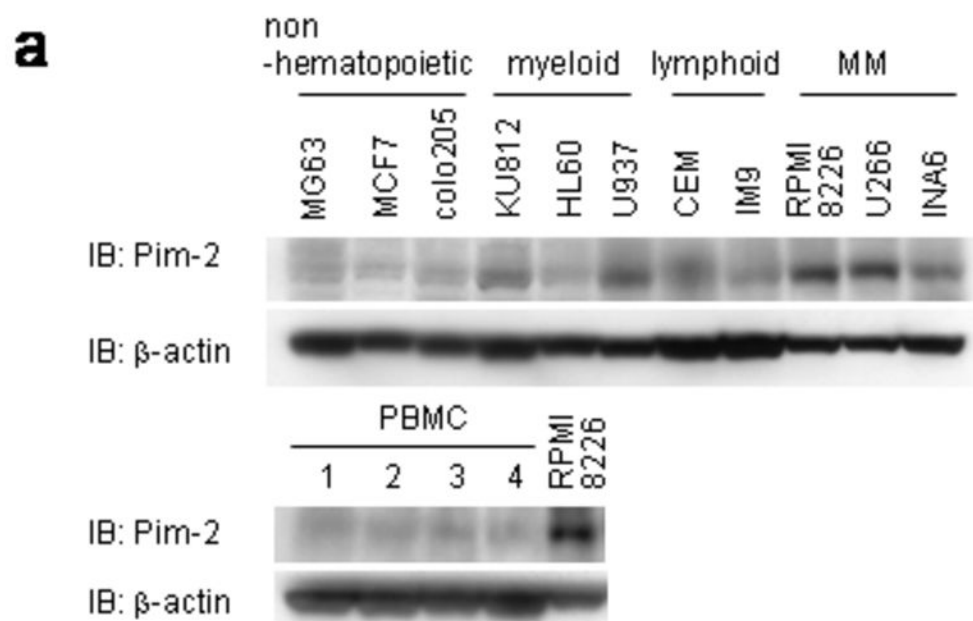
**Figure 4.** Pim-2 inhibition suppresses MM cell survival. (a) The suppression of MM cell viability by Pim-2 knockdown. INA-6 cells were transfected with either 6-FAM-labeled scrambled (control) or Pim-2 siRNA. The transfected cells were sorted by a flow cytometer, and cultured in quadruplicate in the absence or presence of rhIL-6 at 10 ng/mL, or cocultured with BMSCs or OCs. INA-6 cells were harvested after culturing for 2 days and their viability was analyzed. The results are expressed as % change from the baseline. \*,  $p < 0.05$ . (b) Cooperative effects of Pim-2 siRNA and rapamycin on MM cell survival. INA-6 (left) and RPMI8226 (right) cells were transfected with either control or Pim-2 siRNA. INA-6 cells were cocultured with BMSCs. RPMI8226 cells were cultured alone in serum-depleted media. Rapamycin was

added at 10 nM as indicated. After culturing for 2 days, MM cells were harvested and their viability was analyzed. Representative results from 3 independent experiments are shown. The results are expressed as % change from the baseline. (c) Suppression of MM cell viability by the Pim inhibitor (Z)-5-(4-propoxybenzylidene)thiazolidine-2,4-dione. INA6, RPMI8226, MM.1S, TSPC-1 and OPC MM cell lines were cultured in triplicate at  $3 \times 10^5$ /mL in the absence or presence of (Z)-5-(4-propoxybenzylidene)thiazolidine-2,4-dione as the indicated concentrations. After culturing for 48 hours, the viability of MM cells was analyzed. The results were expressed as %change of viable cell numbers in the presence of the Pim inhibitor as indicated concentrations from those in control. (d) Induction of apoptosis by the Pim inhibition. INA-6 cells were cultured alone or with BMSCs. (Z)-5-(4-propoxybenzylidene) thiazolidine-2,4-dione was added as indicated. After culturing for 24 hours, induction of apoptosis was analyzed by AnnexinV and PI dual staining. (e) Suppression of MM cell viability by the Pim inhibition in combination with LY294002. INA-6 cells were cultured alone or cocultured with BMSCs. (Z)-5-(4-propoxybenzylidene) thiazolidine-2,4-dione, LY294002, or both in combination were added as indicated. After culturing for 24 hours, the viability of MM cells was analyzed. The results were expressed as %change from viable cell numbers in INA6 cells cultured alone. (f) The reduction of phosphorylated 4E-BP1, Mcl-1 and c-Myc by the Pim inhibition. INA6 and RPMI8226 cells were cultured in 0.1% FBS in the absence or presence of (Z)-5-(4-propoxybenzylidene) thiazolidine-2,4-dione at 50  $\mu$ M. Cell lysates were collected after culturing for 6 hours. Protein levels of

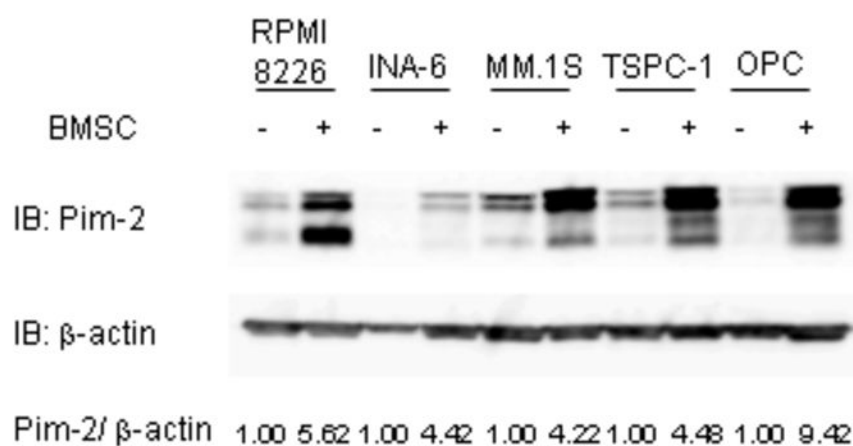
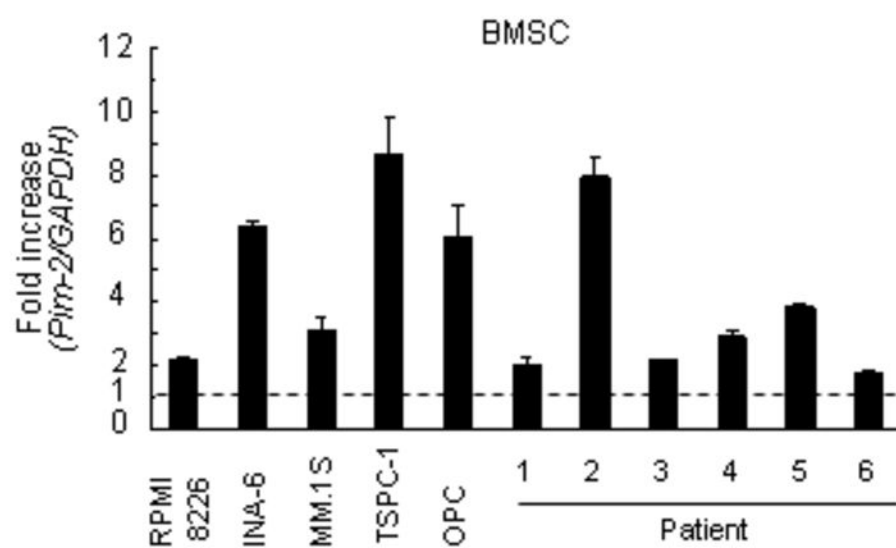
phosphorylated 4E-BP1, Mcl-1 and c-Myc were analyzed by Western blot analysis.

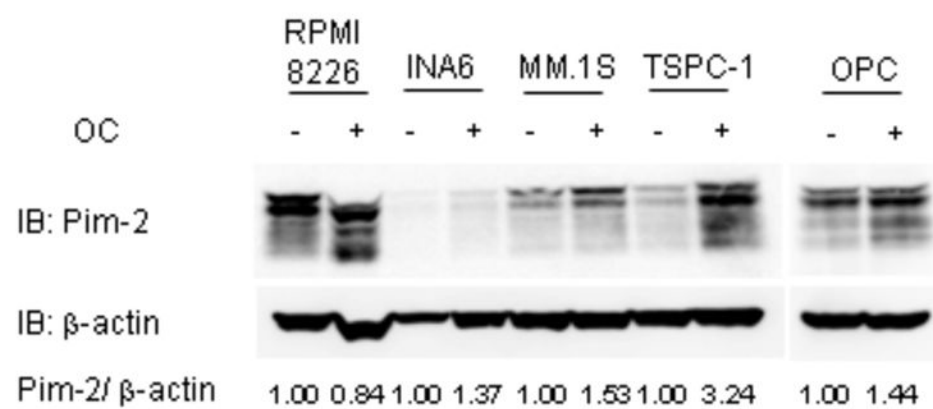
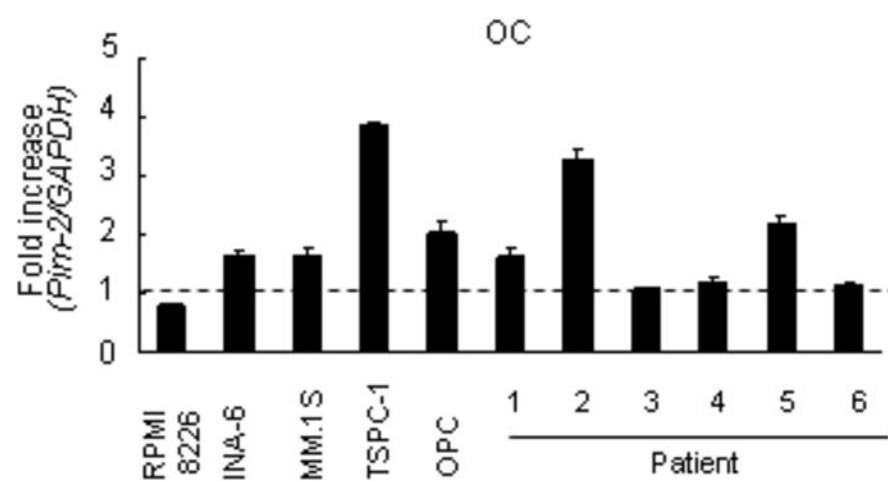
$\beta$ -actin was blotted as a loading control.

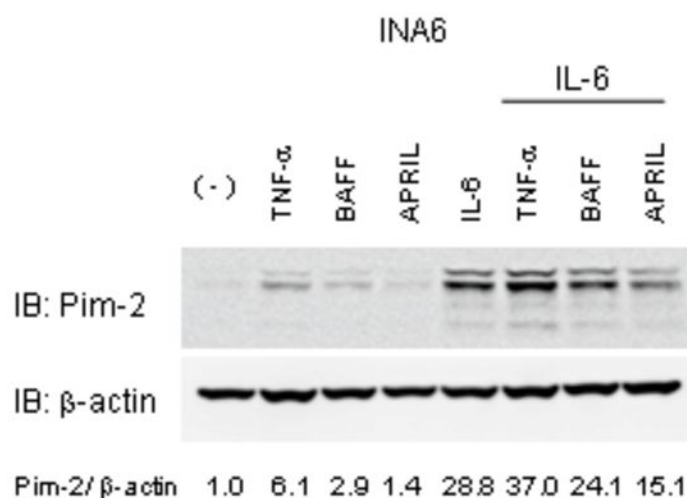
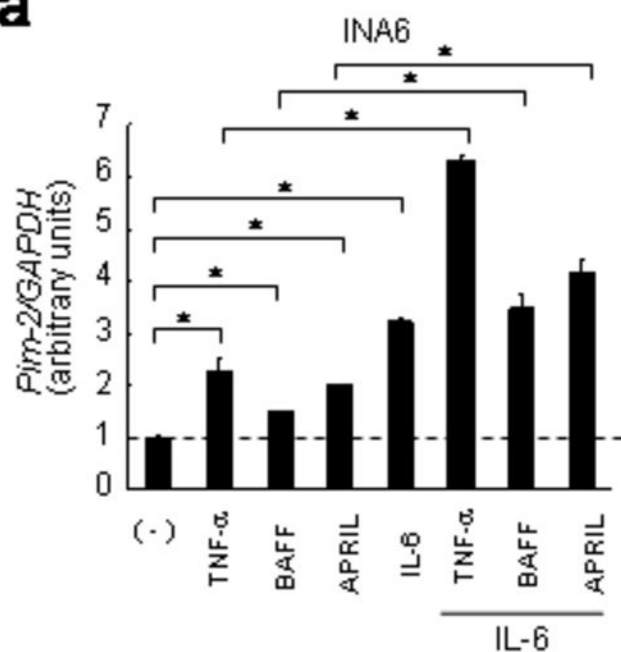
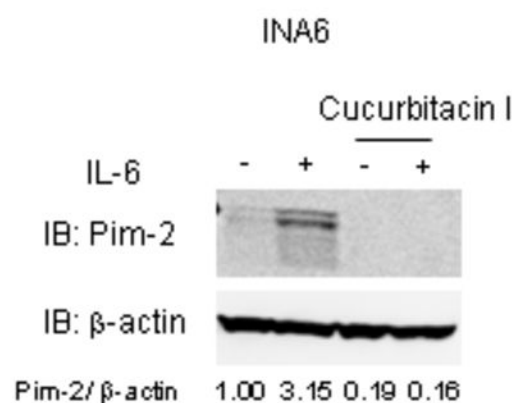
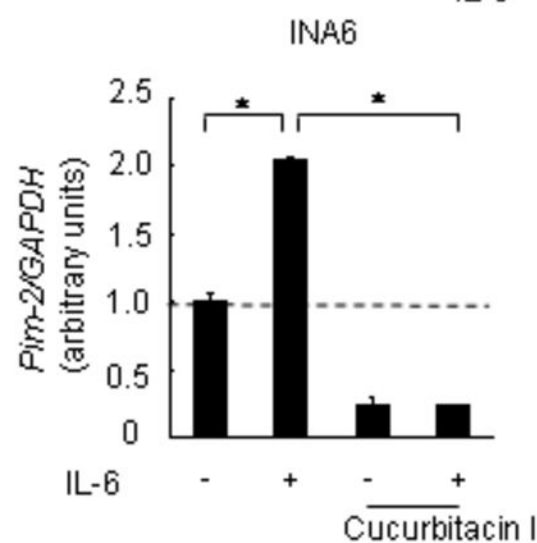
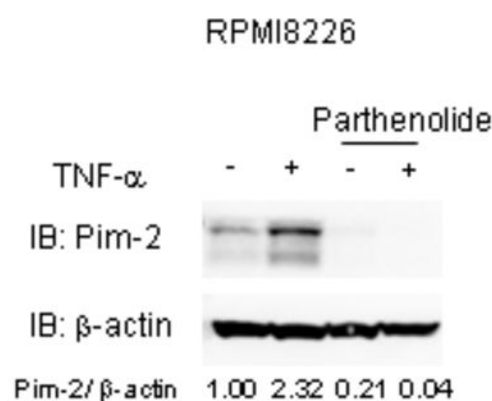
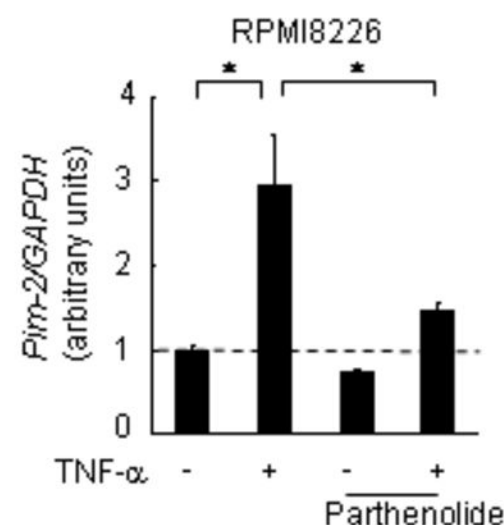


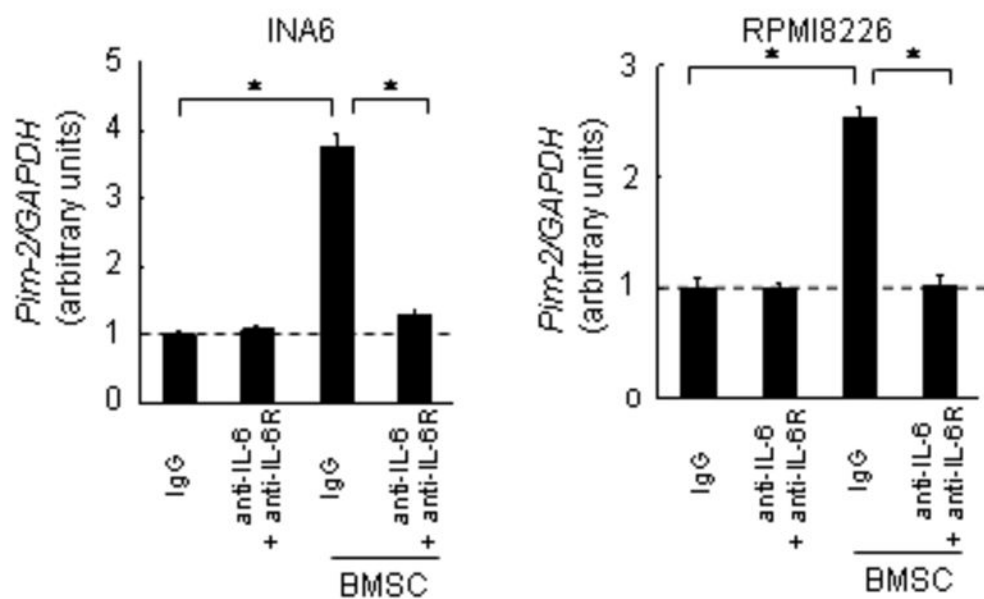
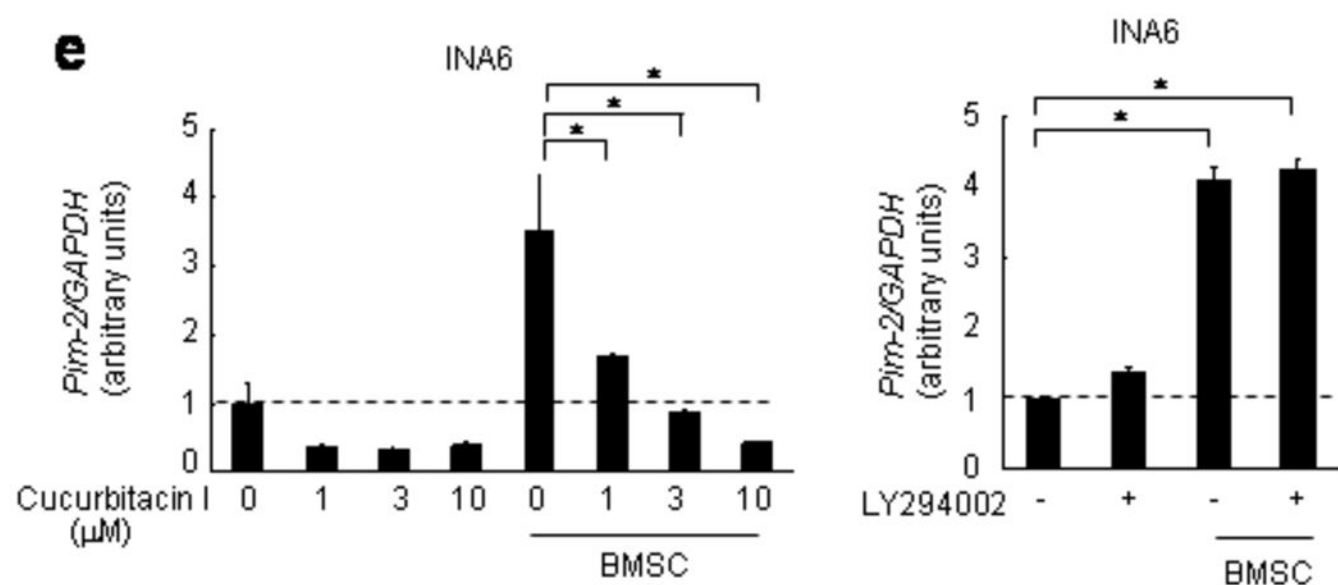
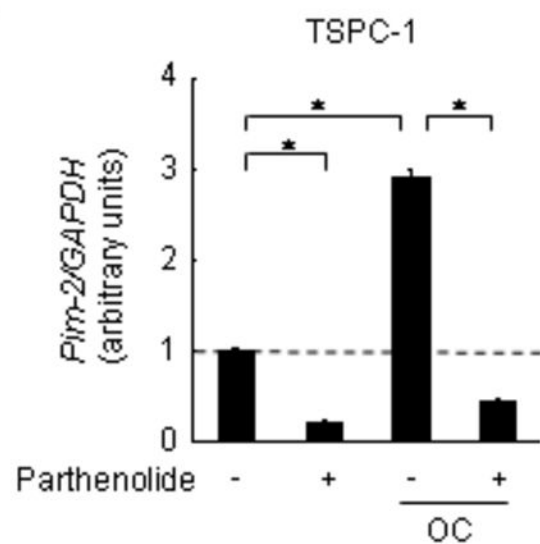


**Figure 1**

**a****Figure 2**

**b****Figure 2**

**a****b****c****Figure 3**

**a****b****c****Figure 3**

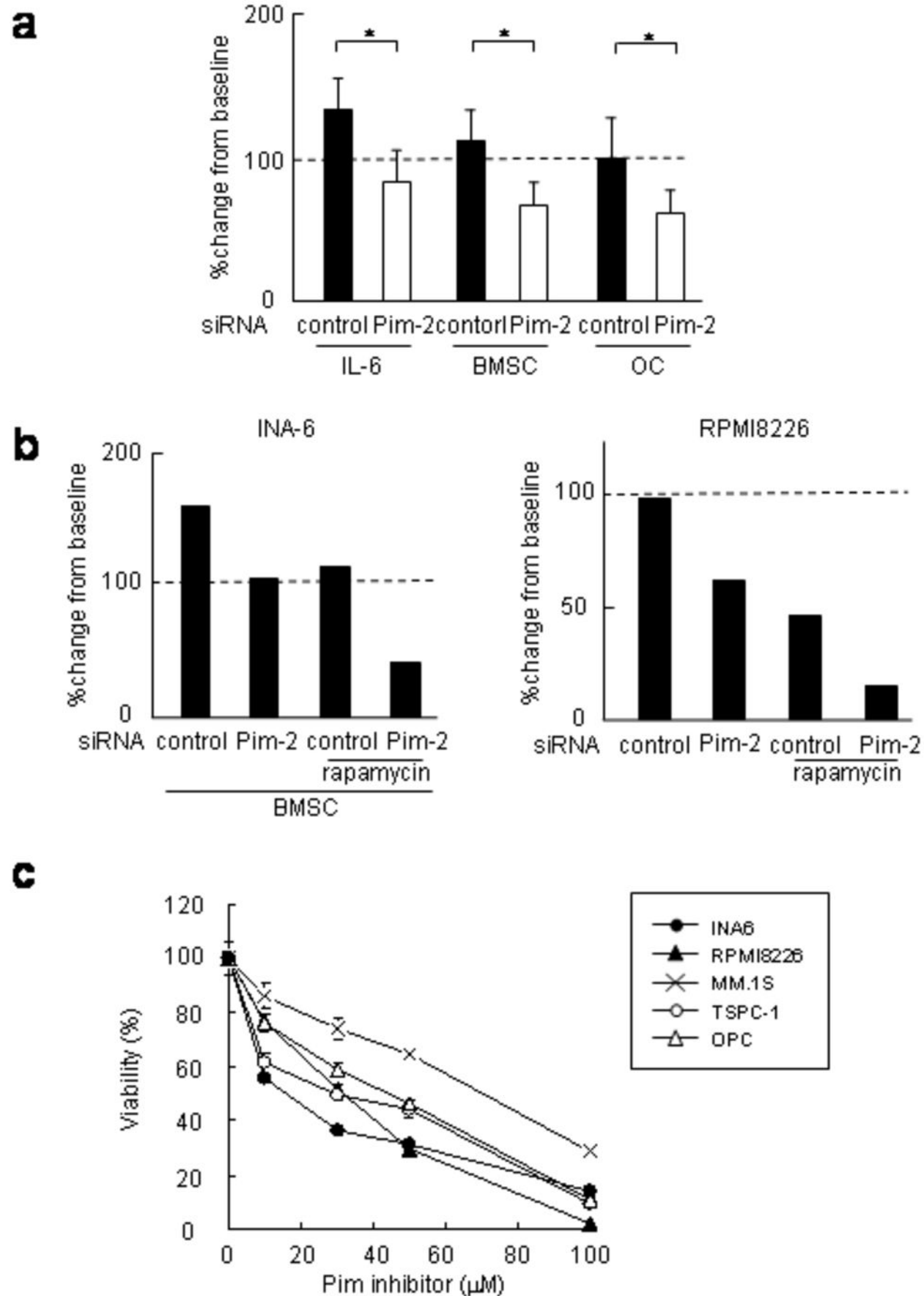
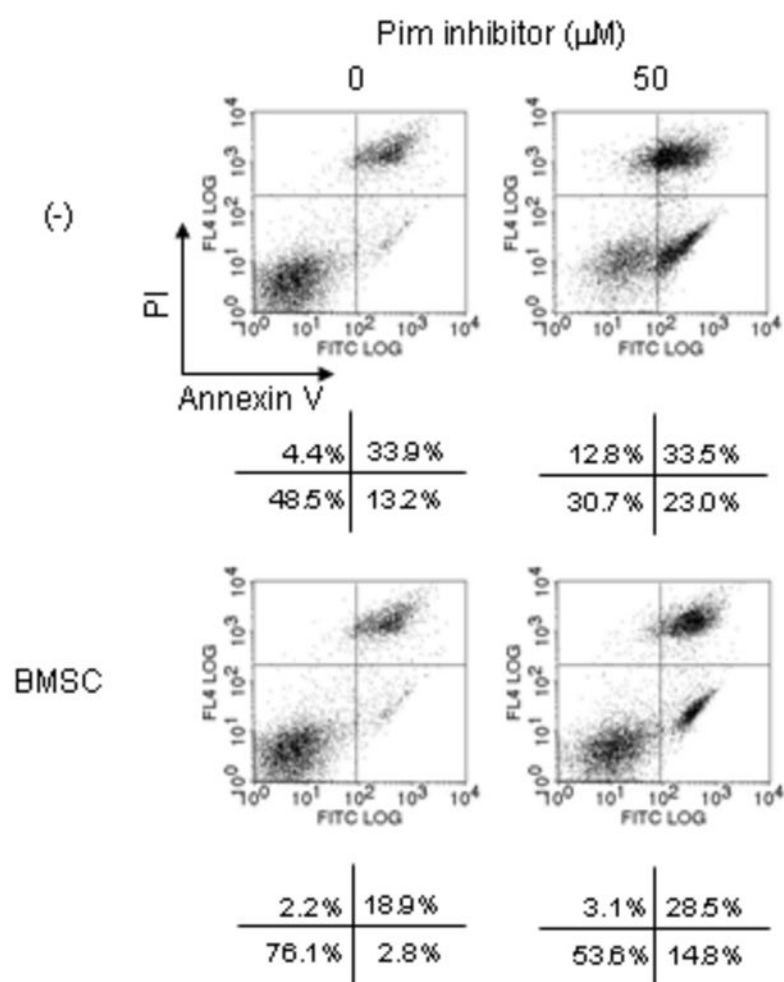
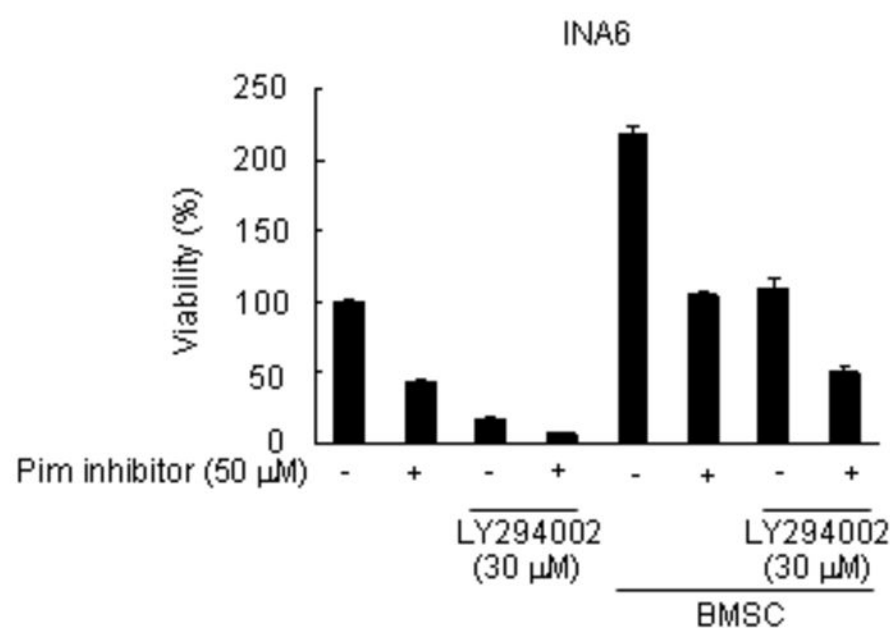
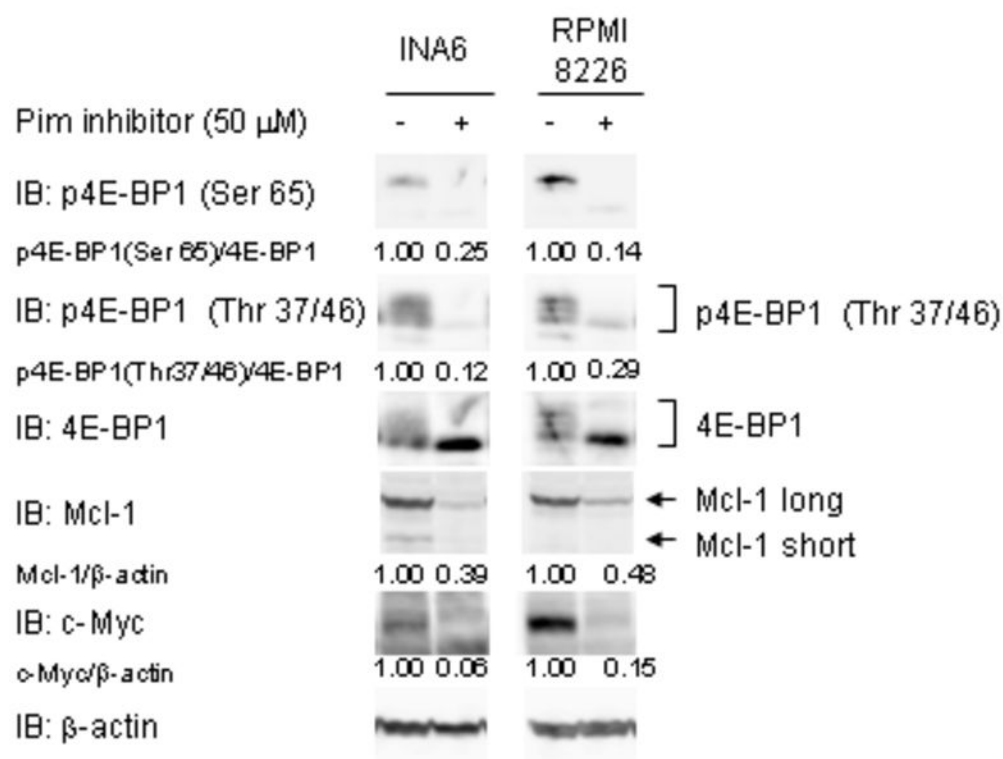


Figure 4

**d****e****Figure 4**

**f****Figure 4**