Bone morphogenetic proteins and receptors are over-expressed in bone-marrow cells of multiple myeloma patients and support myeloma cells by inducing ID genes

Grčević, Danka; Kušec, Rajko; Kovačić, Nataša; Lukić, Anita; Lukić, Ivan Krešimir; Ivčević, Sanja; Nemet, Damir; Serventi Seiwerth, Ranka; Kolonić Ostojić, Slobodanka; Croucher, Peter I; ...

Source / Izvornik: **Leukemia Research, 2010, 34, 742 - 751**

Journal article, Accepted version
Rad u časopisu, Završna verzija rukopisa prihvaćena za objavljivanje (postprint)

https://doi.org/10.1016/j.leukres.2009.10.016

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:105:564907

Bone morphogenetic proteins and receptors are over-expressed in bone-marrow cells of multiple myeloma patients and support myeloma cells by inducing ID genes

Danka Grčević[1], Rajko Kušec[2], Nataša Kovačić[3], Anita Lukić[1], Ivan Krešimir Lukić[3], Sanja Ivčević[1], Damir Nemet[4], Ranka Serventi Seiwerth[4], Slobodanka Kolonić Ostojić[5], Peter I Croucher[6], Ana Marušić[3]

[1]Department of Physiology and Immunology, University of Zagreb School of Medicine, Zagreb, Croatia

[2]Department of Hematology, Dubrava University Hospital, University of Zagreb School of Medicine, Zagreb, Croatia

[3]Department of Anatomy, University of Zagreb School of Medicine, Zagreb, Croatia

[4]Department of Hematology, Zagreb University Hospital Centre, University of Zagreb School of Medicine, Zagreb, Croatia

[5]Department of Hematology, Merkur University Hospital, University of Zagreb School of Medicine, Zagreb, Croatia

[6]Academic Unit of Bone Biology, Section of Musculoskeletal Science, University of Sheffield Medical School, Sheffield, UK

Corresponding author: Prof. Danka Grcevic, MD, PhD, Department of Physiology and Immunology, University of Zagreb School of Medicine, Salata 3, 10000 Zagreb, Croatia; Phone: 385 (1) 4566 944; Fax: 385 (1) 4590 222; e-mail: dgrcevic@mef.hr

Abstract

We assessed the expression pattern and clinical relevance of BMPs and related molecules in multiple myeloma (MM). MM bone-marrow samples (n=32) had increased *BMP4*, *BMP6*, *ACVR1* and *ACVR2A*, and decreased *NOG* expression compared with controls (n=15), with *BMP6* having the highest sensitivity/specificity. Within MM bone-marrow, the source of BMPs was mainly $CD138^+$ plasma-cell population, and *BMP6* and *ACVR1* expression correlated with plasma-cell percentage. Using myeloma cell lines NCI H929 and Thiel we showed that BMPs induced *ID1*, *ID2* and *IL6*, and suppressed *CDKN1A* and *BAX* gene expression, and BAX protein expression. Finally, BMPs partially protected myeloma cells from bortezomib- and TRAIL-induced apoptosis. We concluded that BMPs may be involved in MM pathophysiology and serve as myeloma cell biomarkers.

Key words: multiple myeloma, bone morphogenetic proteins, bone marrow, cell lines, ID genes, cell survival

1. Introduction

Multiple myeloma (MM) is at present an incurable B-lymphocyte neoplasia, characterized by slow proliferation of malignant-plasma cells (PC) in the bone-marrow (BM) [1-3]. Multiple anti-apoptotic signaling mechanisms contribute to the accumulation of myeloma cells within the BM and account for their resistance to chemotherapy [4-6]. The most important molecules expressed by myeloma cells which promote their survival and proliferation are anti-apoptotic members of the BCL2 family and a number of growth factors, adhesion molecules and cytokines, especially IL6 [6-9]. Several cytokines have also been reported to inhibit myeloma cell growth in different culture conditions, including bone morphogenetic proteins (BMPs) [10,11]. Despite BMP pro-apoptotic action on several myeloma cell lines and primary myeloma cells in vitro [12-14], other studies on different types of malignancies showed their tumor-promoting effects in vitro and in vivo [15-20]. Importantly, in vitro effects of BMPs need to be further investigated on human MM samples because primary myeloma cells in culture conditions are removed from the interactions with other cells within BM microenvironment which may regulate their response [7-9]. In addition, primary myeloma cells have poor survival and growth in culture [21], so the in vitro observations may not reveal the true biological role of BMPs in tumorigenesis.

BMPs, members of the transforming growth factor-β superfamily, had been originally identified by their bone-inducing activity but were later shown to regulate a broad spectrum of cellular responses including proliferation, differentiation, morphogenesis, chemotaxis and apoptosis in a variety of cell types, including haematopoietic cells [22-24]. Although BMPs affect bone cells and thus may be relevant in MM bone disease [25], we focused on direct BMP action on myeloma cells. BMPs exert their biological effects by signaling through both type I and II

serine/threonine kinase receptors and a family of intracellular signal transducers known as SMADs (small mothers against decapentaplegic) [22,23]. The most common BMP intracellular signaling pathway is through the phosphorylation of SMAD1/5/8 complex, which translocates to the nucleus and either alone or in combination with other non-SMAD transcription factors regulate the transcription of target genes [23]. One of the important direct target of SMAD1/5/8 pathway in many cell systems is the inhibitor of DNA binding (ID) gene family [26-28]. ID genes encode a family of proteins that inhibit basics helix-loop-helix transcription factors to bind to DNA thus interrupting their regulatory role in many developmental and differentiation processes. ID family were initially identified to inhibit cell differentiation, but more recently shown to have a much wider biological role in regulation of cell-cycle progression, migration and invasiveness. Different studies on human tumors proved their oncogenic properties in regulating cell growth and proposed their causal role in malignant transformation, presumably through their deregulated expression caused by the perturbations in upstream signaling pathways [26-30].

Whereas other studies on the effects of BMPs on myeloma cells have been performed only in vitro, this is the first report describing the expression pattern of BMPs, receptors and related molecules in human MM BM samples, as well as their potential clinical relevance as myeloma cell markers. In addition, by using myeloma cell lines we showed that BMPs protect myeloma cells from apoptosis induced by anti-myeloma drug bortezomib [31] or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [32]. Our results collectively indicate that BMPs promote myeloma cell survival by inducing ID family of oncogenes and increasing the ratio between pro-survival and pro-apoptotic molecules.

2. Patients and Methods

2.1. Primary myeloma sample collection

After obtaining approval from the institutional Ethics Committee and informed consent from participants, we studied BM samples from 32 MM patients and 15 control subjects. Patients with MM were included in the study with a minimal diagnostic inclusion criterion of 10% malignant PC in cytologic or histologic BM specimen [33], obtained as part of the routine clinical examination. Patients were assessed clinically for myeloma stage and bone disease [34]. Most patients entered the study at diagnosis (21/32), whereas a few had already been treated and assessed at relapse (8/32) or remission (3/32) (Table 1). Patients were treated with different protocols adjusted for age and clinical status.

Fifteen BM samples, obtained from subjects (nine men and six women) without history of cancer, served as a control group; with median age 54 years (range 30-78). Most of the control BM samples (13/15) were collected from subjects assigned for BM aspiration as healthy donors and two were from patients aspirated for observation (one with leukocytosis and one with megaloblastic anemia). No statistical difference was found for age and sex between patient and control group.

BM specimens were obtained by sterile punction of the iliac crest, followed by mononuclear cell separation using Histopaque (Sigma-Aldrich, St Louis, MO, USA). In selected samples, PC from BM aspirates were purified by immuno-magnetic separation using anti-CD138 antibodies (Caltag, Invitrogen Ltd, Paisley, UK) conjugated to magnetic beads (Dynal, Invitrogen Ltd) with the purity of >90% as confirmed by flow-cytometry.

2.2. Cell culture

The human myeloma cell lines NCI H929 (DSMZ cell line collection, Brunschweig, Germany) and Theil (a gift from Dr. K. Pulford, University of Oxford, UK) were used for in vitro experiments. Cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS) (Gibco, Invitrogen Ltd), 2 mM L-glutamine and 100 U/ml penicillin/streptomycin, in a 5% CO_2 at 37°C. Recombinant human (rh)BMP2, rhBMP4 or rhBMP6 (all from R&D Systems, Abingdon, UK) were used for the cell treatment as indicated in each experiment. In some experiments, the soluble BMP antagonist noggin (NOG) (PeproTech, London, UK) was used to anatagonize BMP effect, whereas bortezomib (a gift from Millenium Pharmaceuticals, Inc., Cambridge, MA, USA) and TRAIL (R&D Systems) were used to induce myeloma cell death and apoptosis. All experiments were repeated three times and the representative data were shown.

2.3. Flow-cytometric analysis

Myeloma cell lines were analyzed by flow-cytometry using a FACCalibur and the Cell-Quest software (BD Biosciences, San Jose, CA, USA). For cell-cycle analyses, cells were resuspended in DNA staining solution (0.01 M Tris, 10 mM NaCl, 700 U/L RNAse, 7.5×10^{-5} M propidium iodide (PI), 0.01% Nonidet P-40) for 40 min in the dark. Viability and apoptosis were evaluated by annexin V/ PI staining (BD Biosciences) according to the manufacturer's instructions. Cells were classified on dot-plots as dead (PI^+), apoptotic (PI^-/annexin V^+) or viable (double negative).

2.4. Quantitative polymerase chain reaction amplification

Total RNA was extracted (TriPure; Roche Mannheim, Germany) from primary MM BM samples or cultured myeloma cells, reversely transcribed to cDNA (MuLV Reverse Transcriptase;

Applied Biosystems, Foster City, CA) and amplified by quantitative (q)PCR using in an ABI Prism 7000 Sequence Detection system (Applied Biosystems). The following commercially available specific TagMan Gene Expression Assays, named by the official gene symbol [35] and the respective assay ID (Applied Biosystems), were used for the analyses: *BMP2* (Hs00154192_m1), *BMP4* (Hs00370078_m1), *BMP6*, (Hs00233470_m1), *BMP7* (Hs00233477_m1), *ACVR1* (Hs00153836_m1), *ACVR2A* (Hs00155658_m1), *ACVR2B* (Hs00609603_m1), *BMPR1A* (Hs01034910_g1), *BMPR1B* (Hs00176144_m1), *BMPR2* (Hs00176148_m1), *SMAD5* (Hs01557454_m1), *SMAD6* (Hs00178579_m1), *BAMBI* (Hs00180818_m1), *NOG* (Hs00271352_s1), *ID1* (Hs00357821_g1), *ID2* (Hs00747379_m1), *IL6* (Hs00174131_m1), *BCL2* (Hs00153350_m1), *TP53* (Hs00153349_m1), *CDKN1A* (Hs00355782_m1), *BAX* (Hs00180269_m1) and *GAPDH* (Hs99999905_m1). Each reaction was performed in triplicate in a 25 μL reaction volume using TaqMan Gene Expression Master Mix (Applied Biosystems), as recommended by the manufacturer. Cycle conditions were: incubation at 50°C for 2 minutes and at 95°C for 10 minutes, then 40 cycles of 15 s at 95°C and 60 s at 60°C. The relative quantities of unknown samples for the each gene were interpolated from the six-point dilution standard curve of the calibrator sample (BM cells or myeloma cell lines) as previously described [36]. To equalize samples according to the amount of input cDNA, the relative quantity of the target gene was normalized to *GAPDH* as the endogenous control.

2.5. Enzime-linked immunosorbent assay

Myeloma cells were solubilized in a lysis buffer for total proteins (1 mM EDTA, 0.5% Triton X-100 in 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH 7.2-7.4) or phosphorylated proteins (1 mM EDTA, 0.5% Triton X-100, 10 mM NaF, 150 mM NaCl, 20 mM

β-glycerophosphate, 1 mM DTT; pH 7.2-7.4) containing protease inhibitors (Protease Inhibitor Cocktail Tablets; Roche). Total protein concentrations were determined in the supernatants of cell lyzates using a commercial kit (BCA protein assay; Pierce Biotechnology, Rockford, IL). The concentration of phospho-TP53 (P53), BAX and BCL2 protein in cellular extracts was determined by ELISA using commercial kits (DuoSet IC; R&D Systems) according to the manufacturer's instructions. OD was determined on a microplate reader (Bio-Rad, Hercules, CA) set to 450 nm excitation wavelength. Protein concentration was interpolated from the seven-point dilution standard curve and expressed per mg total protein.

2.6. Statistics

Gene expression values between MM and control BM samples were expressed as median (range) and compared using non-parametric Mann-Whitey test. Values for RNA relative quantities and protein concentrations in cultured cell lines were expressed as mean±SD and compared using analysis of variance (ANOVA). The receiver operating characteristic (ROC) curve analysis was expressed as area under curve (AUC) with its 95% confidence interval (CI) and used to determine the efficacy of analyzed genes to discriminate between MM and control group. Diagnostic efficacy for gene expression values were assessed through sensitivity and specificity at cut-off point. Gene expression values were correlated to clinical data using rank correlation and Spearman's coefficient of rank correlation rho (ρ) with its 95% CI. Statistical analysis was performed using MedCalc software-package (Mariakerke, Belgium). For all experiments, α-level was set at 0.05.

3. Results

3.1. Expression of BMPs and related molecules in human multiple myeloma bone-marrow samples

Since it has been observed that BMPs modulate growth and survival of myeloma cells in vitro [10-14], we investigated the expression of BMPs, their receptors and intracellular molecules related to BMP signaling in MM BM samples. To detect changes in the whole MM BM microenvironment, we compared the expression of *BMP2*, *BMP4*, *BMP6* and *BMP7* in total mononuclear cell population of 32 MM BM samples (Table 1) with 15 control BM samples by qPCR (Table 2, Fig 1A). The expression of *BMP4* and *BMP6* was significantly higher in MM BM samples compared with control BM samples. *BMP7* expression was below the detection limit of the qPCR assay in most samples.

We also analyzed the expression of several molecules involved in BMP signal transduction, including BMP receptors – *ACVR1* (*ALK2*), *BMPR1A* (*ALK3*), *BMPR1B* (*ALK6*), *BMPR2*, *ACVR2A*, *ACVR2B*, decoy receptor *BAMBI* (bone morphogenetic protein and activin membrane-bound inhibitor), intracellular signaling molecules – activatory *SMAD5* and inhibitory *SMAD6*, BMP antagonist *NOG*, and inhibitors of DNA binding *ID1* and *ID2*. Significant increase in the expression was found for *ACVR1* and *ACVR2A*, both binding BMP6 [22], whereas *NOG* expression was significantly down-regulated in MM BM samples compared with controls (Table 2, Fig 1A).

To assess their clinical importance, we further correlated the expression of different BMPs and corresponding receptors in MM BM samples with the clinical data. Most of the analyzed samples were obtained at diagnosis (21/32) or at relapse (8/32), reflecting active disease at the time of collection (Table 1). Significant positive correlation was found for the PC percentage in MM BM

samples and *BMP6* expression (Fig 1B; Spearman's ρ = 0.56, 95% CI 0.28-0.76, p = 0.001) as well as *ACVR1* expression (Fig 1B; Spearman's ρ = 0.50, 95% CI 0.15-0.73, p = 0.010). Interestingly, the expression of *BMP6* and *ACVR1* also correlated with each other (Spearman's ρ = 0.43, 95% CI 0.07-0.69, p = 0.026).

Finally, we constructed ROC curves from the qPCR data for BMPs and related molecules to assess the ability of gene expression values to discriminate between the MM and control BM samples. The ROC curve analysis revealed the potential usefulness of the gene expression values for *BMP4*, *BMP6*, *ACVR1*, *ACVR2A* and *NOG* as MM molecular markers since they differ significantly between MM and control BM samples (Fig 1C; p (AUC = 0.5) for presented molecules < 0.05). *BMP6* expression showed the greatest area under ROC curve value (AUC = 0.92, 95% CI 0.79-0.98; p (AUC = 0.5) < 0.001) and the best diagnostic efficacy as marker of malignant MM BM cells among tested molecules (cut-off point >1.9, sensitivity 0.89 and specificity 0.86).

BMPs may be produced by several cell types within the mononuclear fraction of BM cells [22]. To identify the source of BMPs in MM BM samples, we analyzed the expression of BMPs and related molecules in purified $CD138^+$ malignant PC population of four MM BM samples obtained during routine clinical assessments (MM1, MM3 and MM4 at diagnosis and MM2 at progression; Table 3) and compared the expression pattern between the whole mononuclear fraction of MM BM aspirates, $CD138^-$ and $CD138^+$ populations (Fig 2). All samples expressed these molecules, albeit with high inter-individual variability (Table 3). The lowest expression was detected in the sample MM1, which was obtained from the patient with the most benign disease stage. Most of the molecules were relatively over-expressed in $CD138^+$ PC population compared with the whole BM aspirate, especially *BMP4*, *BMP6* and *ID1* (Fig 2). This finding indicates that

PC may be the source of BMPs and offers the potential clinical usefulness of BMPs as MM PC biomarkers.

3.2. Protection effect of BMPs on myeloma cells in vitro

Since we found significant over-expression of several BMPs and corresponding receptors in MM BM samples, we tested the effects of BMPs on myeloma cells in vitro. As primary myeloma cells are known for their poor ability to survive and divide in culture [21], we investigated the mechanisms by which BMPs affect myeloma cells using myeloma cell lines. First we tested several myeloma cell lines (data not shown) and than used NCI H929 and Thiel cells to confirm the effect of BMPs on myeloma cell survival. Both MM cell lines expressed BMPs and corresponding receptors but to the very different extent (Fig 3). The greatest difference was found for *BMP7*, *BMPR1A* and *ACVR2B*, which were over-expressed in NCI H929 cells, whereas *BMP4*, *ACVR1* and *ACVR2A* were over-expressed in Thiel cells. Those differences may explain different response of cell lines to BMP treatment, seen after addition of bortezomib and TRAIL (Fig 4).

When those cells were cultures for up to 72 hours with relatively high dose of BMP2, BMP4 or BMP6 (0.5 μg/mL for all), in our experimental setting we could not induce significant changes in the proportion of dead and apoptotic cells determined by annexin V/PI staining and cell-cycle distribution (data not shown). Nevertheless, BMPs were able to partially protect myeloma cells from bortezomib- and TRAIL-induced apoptosis and increase cell survival, especially of bortezomib treated Thiel cells at earlier culture time-points (Fig 4).

3.3. Intracellular effects of BMPs on myeloma cell lines

We then analyzed the gene expression pattern of several groups of intracellular molecules, known to be important for myeloma cell survival, in order to determine the final effect of BMPs on myeloma cells, including tumor suppressor *TP53* (*P53*) that transcriptionally regulate *CDKN1A* (*P21*) and *BAX* [10], *ID* oncogenes [27], anti-apoptotic *BCL2* [4] and growth factor *IL6* [7]. We used BMP2 and BMP6 since they bind to different BMP receptors, whereas BMP2 and BMP4 overlap in receptor molecule usage [22].

Treatment of Thiel cells with BMP2 or BMP6 (0.5 μg/mL for both) for 24 hours produced weak effect on *TP53* (data not shown), but significantly down-regulated *CDKN1A* and *BAX*, indicating pro-survival action (Fig 5A). The expression of *IL6*, an autocrine and paracrine growth factor of myeloma cells [7], was increased in Thiel cells with BMP6 (Fig 5A). Moreover, *ID* genes were induced by BMPs in both cell lines. Interestingly, NCI H929 cells, carrying t(4;14) translocation, had much higher constitutive expression of *ID1*, whereas Thiel plasma-blast cell line had lower *TP53* and higher *BCL2* constitutive expression (data not shown) as well as greater induction of *ID* genes by BMPs (Fig 5A). In addition, we found that BMP treatment up-regulated the expression of extracellular BMP antagonist *NOG* and inhibitory transcription factor *SMAD6* especially in Thiel cell line (Fig 5A). Similar results of gene expression in myeloma cell lines were observed after BMP treatment for 12 h (data not shown). In a dose response experiments, *ID* genes showed dose-dependent induction of expression by BMPs in Thiel cells (Fig 5B). Suppression of BMP induced gene expression of *ID1* with BMP-specific soluble antagonist NOG (2 μg/mL), confirmed that over-expression of *ID* genes was a specific effect of BMPs (Fig 5C). In addition to gene expression, we measured the concentration of phosphorylated TP53 by ELISA to assess the activation of transcription factor TP53 in NCI H929 and Thiel cells with

BMP2 or BMP6 (0.5 μg/mL for both), but did not find significant effect within 12 hours upon treatment (data not shown). We also assessed the concentration of BCL2 and BAX, which has been shown to reciprocally regulate cell survival versus apoptosis [37,38]. Addition of BMP2 or BMP6 did not change BCL2, but significantly decreased BAX concentration within 24 hours upon treatment (Fig 5D). The final effect of BMP treatment was a 45% increase in the ratio of BCL2/BAX in Thiel cell line, indicating pro-survival effect.

Discussion

Other studies on the effects of BMPs on myeloma cells have been performed only in vitro, whereas the role of BMPs in tumorigenesis is still controversial. Therefore we investigated the expression pattern of BMPs, receptors and related molecules in multiple myeloma (MM) bone-marrow samples and demonstrated that BM samples from MM patients over-expressed not only *BMP4* and *BMP6* but also their receptors *ACVR1* and *ACVR2A*, and at the same time had decreased expression of BMP antagonist *NOG*. To the best of our knowledge, this is the first report describing the expression pattern of BMPs, receptors and related molecules in human MM BM samples, as well as its clinical relevance since the ROC curve analysis demonstrated potential usefulness of the gene expression values as MM markers that differ from control BM samples, with *BMP6* having the highest sensitivity and specificity. ROC curve analysis is considered to be more biologically and clinically relevant than simple group comparison (t-test or Mann-Whitney test) [39]. Moreover, once the cut-off value is defined by the ROC curve profile at desirable sensitivity and specificity, additional samples could be classified in respect to this cut-off point. By the analysis of total mononuclear cell population of MM samples we confirmed BMP over-expression in BM microenvironment of MM compared with control samples. In addition, we confirmed that the source of BMP expression was predominantly $CD138^+$ population. A possible mechanism of pro-survival effects of BMPs on myeloma cells was through targeting the ID family of oncogenes, because *ID1* was over-expressed in separated $CD138^+$ myeloma cells compared to whole BM aspirates of MM patients. By using mayloma cell lines, we demonstrated that *ID1* and *ID2* were induced in myeloma cells by BMPs and BMPs were able to partially protected myeloma cell lines from bortezomib- and TRAIL-induced apoptosis.

We believe that our finding of BMP over-expression in primary MM BM samples provides strong evidence that BMPs may have a true biological role in tumorigenesis. BMP6 seems to be specifically important for MM, since the expression of both *BMP6* and its receptors was higher in MM BM samples compared with controls, and *BMP6* and *ACVR1* correlated with the percentage of PC in MM BM samples. Moreover, *BMP6* was relatively over-expressed in purified $CD138^+$ MM PC population compared to $CD138^-$ cells. Several studies on global gene expression profiling observed up-regulation of some BMPs or related molecules in myeloma cells from human samples or myeloma cell lines [40-42]. Therefore we were interested to explore direct effects of BMPs on myeloma cells, aside from the effects on bone cells [25]. The proposed tumor-promoting role of BMPs is in line with the findings of increased BMP activity in other types of hematopoietic malignancies such as acute promyelocytic leukemia [43,44]. Over-expression of BMPs correlates with unfavorable prognosis in melanoma [20], non-small-cell lung carcinoma [18] and diffuse large-B-cell lymphoma [19]. In addition, several BMPs have the ability to promote proliferation in various carcinoma cell lines, including lung [45], breast [15], colon [16] and prostate [17].

However, our findings on myeloma cell lines in vitro differ from previous observations that several BMPs inhibit proliferation and induce apoptosis in myeloma cell lines and separated $CD138^+$ primary cells in vitro [10-14]. The inconsistency of BMP effects on myeloma cells may be at least partially explained by specific culture conditions, since the action of BMPs in vitro depends on the local environment. BMPs signal through several intracellular pathways, including phosphorylation of AHSA1 (P38), STAT3 and SMAD1/5/8 [23], which are not equally activated in different culture conditions. For example, BMP2 stimulate proliferation of lung cancer cells when cultured in medium containing serum compared with serum-free medium, in a way

dependent on the activation of SMAD1/5 and over-expression of BMP target gene ID1 [45]. Moreover, different myeloma cell lines and primary samples are genetically and biologically very heterogeneous [21,45,46] and, probably therefore, response differently to BMPs. We showed that plasma-blast Thiel cells were more sensitive to BMP protective effect after treatment with bortezomib compared with NCI H929 cells and at the same time greatly differ in the expression of BMPs, their receptors and down-stream signaling molecules. In addition, BMPs stimulate the expression of their own antagonists, as we demonstrated for *NOG* and *SMAD6*, which may limit the duration of BMP activity in culture [47].

BMPs, like other growth factors, may affect the expression of a number of genes associated with apoptosis but also with cell-cycle progression, including oncogenes of ID family [22,26,37]. Our study on cell lines demonstrated that BMPs induced *ID1* and *ID2* gene expression, especially in Thiel plasma-blast cells, whereas NCI H929 cells, carrying unfavorable t(4;14) translocation, exhibited higher constitutive expression of *ID1*. In MM BM primary samples, the major source of *ID1* expression was $CD138^+$ population. Over-expression of ID1 was observed in a variety of human malignancies including MM, in which ID1 expression was associated with the t(4;14) translocation that indicates poor prognosis [48]. Since ID1 enhanced proliferation and stimulated the invasiveness of several cancer cell types [29,30], it seems likely that malignant transformation reactivates proliferative machinery, temporarily inhibited in normal differentiated cells, through enhanced expression of ID genes [26].

The unique effect of BMPs on myeloma cell lines, observed in our experimental setting, may be explained by the susceptibility of treated myeloma cells, determined by their intrinsic balance between pro-survival and pro-apoptotic factors. Induction of *ID* genes by BMPs was paralleled by the suppression of pro-apoptotic factors *BAX* and *CDKN1A*. Moreover, BMP treatment increased BCL2/BAX protein ratio, which has been recognized as a key indicator of suppressed

apoptosis [37]. *IL6*, which promotes growth of myeloma cells and counteracts apoptotic signals by regulating BCL2 family [49], was enhanced by BMP treatment in our conditions. It is known that resistance to apoptosis, common in myeloma cells, depends on activated intracellular pathways in the particular circumstances and constitutive expression of molecules acting at cell-cycle checkpoints [6-9,23]. Relatively high basal apoptotic rate and the slow division of myeloma cells in culture may explain why BMPs exhibited the protective role on myeloma cell lines only in conditions of bortezomib- or TRAIL-induced apoptosis.

Conclusions

In the context of their role in myeloma cell survival versus apoptosis, BMPs may initiate several intracellular signaling pathways. Using myeloma cell lines, we demonstrated that BMP treatment up-regulated pro-survival *ID1*, *ID2* and *IL6*, whereas down-regulated pro-apoptotic *CDKN1A* and *BAX* genes as well as suppressed bortezomib- and TRAIL-induced myeloma cell apoptosis. In addition, in primary MM BM samples *BMP6* and *ACVR1* expression significantly correlated with the percentage of PC and purified $CD138^+$ PC expressed BMPs and BMP receptors, suggesting that the biological effect of BMPs in vivo may be to promote survival and accumulation of myeloma cells within the BM by an autocrine action. We also proposed that BMPs may be of clinical relevance in MM, since they were over-expressed in MM BM and discriminated between control and MM samples by ROC curve analysis. Further studies should address the molecular mechanisms of cell sensitivity to BMPs and regulation of BMP expression by clinical treatment of MM. Our findings also indicated that the final effect of cell-cycle regulators, as a novel anti-myeloma therapeutic strategy, may vary depending of the individual biological characteristics of myeloma cells.

Conflict of interest

All the authors declare no conflict of interest.

References:

1. Barlogie B, Shaughnessy J, Tricot G, Jacobson J, Zangari M, Anaissie E, et al. Treatment of multiple myeloma. Blood 2004;103:20-32.
2. Hideshima T, Mitsiades C, Tonon G, Richardson PG, Anderson KC. Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. Nat Rev Cancer 2007;7:585-98.
3. Kyle RA, Rajkumar SV. Multiple myeloma. Blood 2008;111:2962-72.
4. Chen Q, Ray S, Hussein MA, Srkalovic G, Almasan A. Role of Apo2L/TRAIL and Bcl-2-family proteins in apoptosis of multiple myeloma. Leuk Lymphoma 2003;44:1209-14.
5. Yang DH, Park JS, Jin CJ, Kang HK, Nam JH, Rhee JH, et al. The dysfunction and abnormal signaling pathway of dendritic cells loaded by tumor antigen can be overcome by neutralizing VEGF in multiple myeloma. Leuk Res 2009;33:665-70.
6. van de Donk NW, Lokhorst HM, Bloem AC. Growth factors and antiapoptotic signaling pathways in multiple myeloma. Leukemia 2005;19:2177-85.
7. Lauta VM. A review of the cytokine network in multiple myeloma. Cancer 2003;97:2440-52.
8. Oliveira AM, Maria DA, Metzger M, Linardi C, Giorgi RR, Moura F, et al. Thalidomide treatment down-regulates SDF-1alpha and CXCR4 expression in multiple myeloma patients. Leuk Res 2009;33:970-3.
9. Bommert K, Bargou RC, Stühmer T. Signalling and survival pathways in multiple myeloma. Eur J Cancer 2006;42:1574–80.

10. Fukuda N, Saitoh M, Kobayashi N, Miyazono K. Execution of BMP-4-induced apoptosis by p53-dependent ER dysfunction in myeloma and B-cell hybridoma cells. Oncogene. 2006;25:3509–3517.
11. Kawamura C, Kizaki M, Yamato K, Uchida H, Fukuchi Y, Hattori Y, et al. Bone morphogenetic protein-2 induces apoptosis in human myeloma cells with modulation of STAT3. Blood 2000;96:2005-11.
12. Rø TB, Holt RU, Brenne AT, Hjorth-Hansen H, Waage A, Hjertner O, et al. Bone morphogenetic protein-5, -6 and -7 inhibit growth and induce apoptosis in human myeloma cells. Oncogene 2004;23:3024-32.
13. Hjerner O, Hjorth-Hansen H, Børset M, Seidel C, Waage A, Sundan A. Bone morphogenetic protein-4 inhibits proliferation and induces apoptosis of multiple myeloma cells. Blood 2001;97:516-22.
14. Kawamura C, Kizaki M, Ikeda Y. Bone morphogenetic protein (BMP)-2 induces apoptosis in human myeloma cells. Leuk Lymphoma 2002;43:635-9.
15. Clement JH, Raida M, Sänger J, Bicknell R, Liu J, Naumann A, et al. Bone morphogenetic protein 2 (BMP-2) induces in vitro invasion and in vivo hormone independent growth of breast carcinoma cells. Int J Oncol 2005;27:401-7.
16. Deng H, Mikizumi R, Ravikumar TS, Dong H, Yang W, Yang WL. Bone morphogenetic protein-4 is overexpressed in colonic adenocarcinomas and promotes migration and invasion of HCT116 cells. Exp Cell Res 2007;313:1033-44.
17. Ide H, Yoshida T, Matsumoto N, Aoki K, Osada Y, Sugimura T, Terada M. Growth regulation of human prostate cancer cells by bone morphogenetic protein-2. Canc Res 1997;57:5022-7.

18. Langenfeld EM, Bojnowski J, Perone J, Langenfeld J. Expression of bone morphogenetic proteins in human lung carcinomas. Ann Thorac Surg 2005;80:1028-32.
19. Rosenwald A, Wright G, Chan WC, Connors JM, Campo E, Fisher RI et al; Lymphoma/Leukemia Molecular Profiling Project. The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-call lymphoma. New Engl J Med 2002;346:1937-47.
20. Rothhammer T, Poser I, Soncin F, Bataille F, Moser M, Bosserhoff A-K. Bone morphogenic proteins are overexpressed in malignant melanoma and promote cell invasion and migration. Canc Res 2005;65:448-56.
21. Zlei M, Egert S, Wider D, Ihorst G, Wäsch R, Engelhardt M. Characterization of in vitro growth of multiple myeloma cells. Exp Hematol 2007;35:1550-61.
22. Chen D, Zhao M, Mundy GR. Bone morphogenetic proteins. Growth Factors 2004;22:233-41.
23. Herpin A, Cunningham C. Cross-talk between the bone morphogenetic protein pathway and other major signaling pathways results in tightly regulated cell-specific outcomes. FEBS J 2007;274:2977-85.
24. Bhatia M, Bonnet D, Wu D, Murdoch B, Wrana J, Gallacher L, Dick JE. Bone morphogenetic proteins regulate the developmental program of human hematopoietic stem cells. J Exp Med 1999;189:1139-47.
25. Edwards CM, Zhuang J, Mundy GR. The pathogenesis of the bone disease of multiple myeloma. Bone 2008;42:1007-13.
26. Lasorella A, Uo T, Iavarone A. Id proteins at the cross-road of development and cancer. Oncogene 2001;20:8326-33.

27. Norton JD. ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. J Cell Sci 2000;113:3897-905.

28. Ruzinova MB, Benezra R. Id proteins in development, cell cycle and cancer. Trends Cell Biol 2003;13:410-8.

29. Fong S, Itahana Y, Sumida T, Singh J, Coppe JP, Liu Y, et al. Id-1 as a molecular target in therapy for breast cancer cell invasion and metastasis. Proc Natl Acad Sci U S A 2003;100:13543-8.

30. Ouyang XS, Wang X, Ling M-T, Wong HL, Tsao SW, Wong JC. Id-1 stimulates serum independent prostate cancer cell proliferation through inactivation of $p16^{INK4a}$/pRB pathway. Carcinogenesis 2002;23:721-5.

31. Dicato M, Boccadoro M, Cavenagh J, Harousseau JL, Ludwig H, San Miguel J, Sonneveld P. Management of multiple myeloma with bortezomib: experts review the data and debate the issues. Oncology. 2006;70:474-82.

32. Shipman CM, Croucher PI. Osteoprotegerin is a soluble decoy receptor for tumor necrosis factor-related apoptosis-inducing ligand/Apo2 ligand and can function as a paracrine survival factor for human myeloma cells. Canc Res 2003;63:912-6.

33. Smith A, Wisloff F, Samson D; UK Myeloma Forum; Nordic Myeloma Study Group; British Committee for Standards in Haematology. Guidelines on the diagnosis and management of multiple myeloma 2005. Br J Haematol 2006;132:410-51.

34. Durie BG, Salmon SE. A clinical staging system for multiple myeloma. Correlation of measured myeloma cell mass with presenting clinical features, response to treatment and survival. Cancer 1975;36:842-54.

35. Human Genome Organisation. (HUGO) Gene Nomenclature Committee: Guidelines for Human Gene Nomenclature. Available from: http://www.genenames.org/guidelines.html

36. Grcevic D, Lukic IK, Kovacic N, Ivcevic S, Katavic V, Marusic A. Activated T lymphocytes suppress osteoclastogenesis by diverting early monocyte/macrophage progenitor lineage commitment towards dendritic cell differentiation through down-regulation of receptor activator of nuclear factor-kappaB and c-Fos. Clin Exp Immunol 2006;146:146-58.
37. Maddika S, Ande SR, Panigrahi S, Paranjothy T, Weglarczyk K, Zuse A, et al. Cell survival, cell death and cell cycle pathways are interconnected: implications for cancer therapy. Drug Resist Updat 2007;10:13-29.
38. Danial NN, Korsmeyer SJ. Cell death: critical control points. Cell 2004;116:205-19.
39. Bickel DR. Degrees of differential gene expression: detecting biologically significant expression differences and estimating their magnitudes. Bioinformatics 2004;20:682-8.
40. Neri P, Tassone P, Shammas M, Yasui H, Schipani E, Batchu RB, et al. Biological pathways and in vivo antitumor activity induced by Atiprimod in myeloma. Leukemia 2007;21:2519-26.
41. Zhan F, Barlogie B, Arzoumanian V, Huang Y, Williams DR, Hollmig K, et al. Gene-expression signature of benign monoclonal gammopathy evident in multiple myeloma is linked to good prognosis. Blood 2007;109:1692-700.
42. Mattioli M, Agnelli L, Fabris S, Baldini L, Morabito F, Bicciato S, et al. Gene expression profiling of plasma cell dyscrasias reveals molecular patterns associated with distinct IGH translocations in multiple myeloma. Oncogene 2005;24:2461-73.
43. Detmer K, Steele TA, Shoop MA, Dannawi H. Lineage-restricted expression of bone morphogenetic protein genes in human hematopoietic cell lines. Blood Cells Mol Dis 1999;25:310-23.

44. Grcevic D, Marusic M, Grahovac B, Jaksic B, Kusec R. Expression of bone morphogenetic proteins in acute promyelocytic leukemia before and after combined all trans-retinoic acid and cytotoxic treatment. Leuk Res 2003;27:731-8.
45. Langenfeld EM, Kong Y, Langenfeld J. Bone morphogenetic protein 2 stimulation of tumor growth involves the activation of Smad-1/5. Oncogene 2002;25:685-92.
46. Krejčí J, Harničarová A, Streitová D, Hájek R, Pour L, Kozubek S, Bártová E. Epigenetics of multiple myeloma after treatment with cytostatics and gamma radiation. Leuk Res 2009;33:1490-8.
47. Kameda T, Koike C, Saitoh K, Kuroiwa A, Iba H. Developmental patterning in chondrocytic cultures by morphogenic gradients: BMP induces expression of indian hedgehog and noggin. Genes Cells 1999;4:175-84.
48. Hudlebusch HR, Theilgaard-Mönch K, Lodahl M, Johnsen HE, Rasmussen T. Identification of ID-1 as a potential target gene of MMSET in multiple myeloma. Br J Haematol 2005;130:700-8.
49. Schwarze MM, Hawley RG. Prevention of myeloma cell apoptosis by ectopic bcl-2 expression or interleukin 6-mediated up-regulation of bcl-xL. Canc Res 1995;55:2262-5.

Acknowledgement

This work was supported by grants from the Croatian Ministry of Science, Sport and Education (108-1080229-0142, 108-1080229-0140, 108-1080955-3094, 108-1081872-2061). Bortezomib was a gift from Millenium Pharmaceuticals, Inc. We thank Mrs. Katerina Zrinski-Petrović for her technical assistance.

Table 1. Clinical characteristics of patients with multiple myeloma

Clinical feature	No. of patients (n=32)
Sex (male/female)	17/15
Age (years) median (range)	63 (46-86)
Type of sample	
At diagnosis	21
At relapse	8
At remission	3
Outcome (alive/dead)	22/10
Multiple myeloma stage[1]	
I	10 (9 A and 1 B)
II	7 (all A)
III	15 (10 A and 5 B)
Type of myeloma protein	
IgG	21
IgA	4
Bence Jones	7
Bone disease*	
0	10
1	6
2	4
3	12

[1] Multiple myeloma was clinically staged according to Durie–Salmon system and bone disease.

Table 2. Expression of bone morphogenetic proteins and related molecules in multiple myeloma bone-marrow samples compared with bone-marrow from control subjects

Gene[1]	MM samples[2] median (range)	Control samples median (range)	P[3] (Mann-Whitney)
BMPs			
BMP2	0.57 (0-7.03)	0.84 (0.14-9.43)	0.080
BMP4	9.34 (0.20-406.00)	0.85 (0-11.90)	**0.002**
BMP6	43.56 (0.39-519.45)	0.94 (0-5.87)	**<0.001**
BMP receptors			
ACVR1	1.75 (0.42-11.00)	0.97 (0-2.51)	**0.011**
BMPR1A	1.47 (0.12-28.80)	0.62 (0.10-9.16)	0.090
BMPR1B	0.08 (0-13.90)	0.26 (0-2.30)	0.880
BMPR2	0.95 (0.19-3.63)	0.82 (0.11-2.74)	0.149
ACVR2A	0.25 (0.03-6.35)	0.13 (0-0.78)	**0.032**
ACVR2B	0.14 (0.01-1.84)	0.08 (0.01-1.30)	0.263
Related molecules			
ID1	0.23 (0-1.64)	0.12 (0.03-0.89)	0.402
ID2	1.29 (0.21-7.61)	1.44 (0.24-3.62)	0.461
NOG	1.04 (0-37.72)	12.43 (1.25-69.99)	**<0.001**
SMAD5	0.73 (0.11-11.10)	0.49 (0.10-9.80)	0.341
SMAD6	0.22 (0-2.16)	0.29 (0-0.70)	0.747
BAMBI	1.79 (0-83.22)	2.09 (0.91-20.56)	0.379

[1] Gene expression was calculated as RNA relative quantity for target gene and normalized to the expression of *GAPDH*. *BMP*, bone morphogenetic protein; *BMPR*, BMP receptor; *ACVR*, activin receptor; *ID*, inhibitor of DNA binding; *NOG*, BMP antagonist noggin; *SMAD*, small mothers against decapentaplegic; *BAMBI*, bone morphogenetic protein and activin membrane-bound inhibitor. *BMP7* expression was below the detection limit of the qPCR assay in most samples.

[2] Primary samples were obtained after the informed consent from patient with multiple myeloma (MM) and control subjects that were clinically observed for other reasons.

[3] Comparisons with statistically significant difference ($p<0.05$) are bolded.

Table 3. Gene expression analysis of bone morphogenetic proteins and related molecules in purified $CD138^+$ bone-marrow cells of selected multiple myeloma bone-marrow samples

Sample	MM1	MM2	MM3	MM4
Patient characteristics[1]				
Gender/age	F/53	F/57	M/64	F/86
Type of M protein	IgG	BJ	IgA	IgG
Stage	1A	2A	2A	3B
Bone disease	0	0	1	1
Percent of plasma-cells	12	30	30	85
Translocation t(4;14)	–	–	+	–
Serum albumin (g/L)	43	48	45	29
β_2-microglobulin (mg/L)	4.3	6.0	2.5	9.8
Gene expression[2]				
BMP2	0.38	0.69	0.97	1.12
BMP4	0.51	22.02	313.22	42.40
BMP6	5.56	102.89	64.37	52.82
ACVR1	0.88	5.42	2.03	1.68
BMPR1A	0.05	0.83	0.29	1.37
ACVR2A	0.42	1.12	2.98	4.76
ID1	0.12	4.64	2.28	3.06
ID2	1.81	1.04	1.96	1.88

[1] Four primary myeloma samples were obtained from patients during routine clinical assessment (MM1, MM3 and MM4 at diagnosis and MM2 at progression). Multiple myeloma was clinically staged according to Durie–Salmon system and bone disease. F, female; M, male; BJ, Bence Jones.

[2] Gene expression in $CD138^+$ fraction of myeloma bone-marrow aspirates were analyzed by RT-qPCR. Gene expression was calculated as RNA relative quantity for target gene and normalized to the expression of *GAPDH*. *BMP*, bone morphogenetic protein; *BMPR*, BMP receptor; *ACVR*, activin receptor; *ID*, inhibitor of DNA binding.

Figure legends

Figure 1. Gene expression of bone morphogenetic proteins (BMPs) and related molecules in bone-marrow (BM) samples of patients with multiple myeloma (MM) compared with BM obtained from control subjects. A) Expression of *BMP4*, *BMP6*, BMP receptors activin receptor (*ACVR*)*1* and *ACVR2A*, and BMP antagonist noggin (*NOG*) in MM BM samples (n=32) compared with control samples (n=15); lines indicate median value. B) Correlation of *BMP6* and *ACVR1* expression and the percentage of plasma-cells in MM BM samples. ρ, Spearman's coefficient of rank correlation. C) Receiver operating characteristic curve profiles for the expression of *BMP4*, *BMP6*, *ACVR1*, *ACVR2A* and *NOG*. *p*, indicates discriminative ability of gene expression values between MM and control samples.

Figure 1

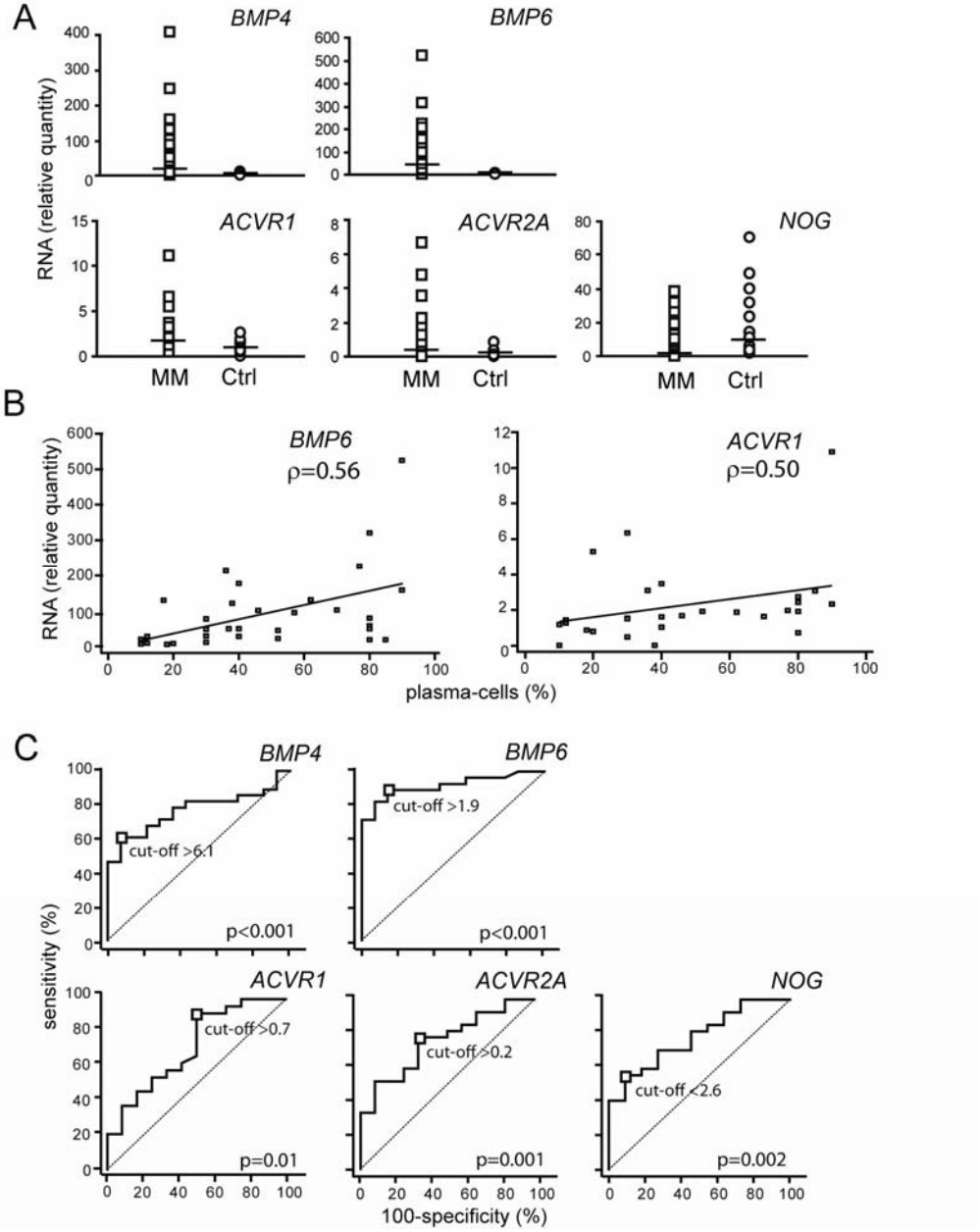

A
BMP4
BMP6
ACVR1
ACVR2A
NOG
RNA (relative quantity)
MM
Ctrl
B
BMP6
ρ=0.56
ACVR1
ρ=0.50
RNA (relative quantity)
plasma-cells (%)
C
BMP4
cut-off >6.1
p<0.001
BMP6
cut-off >1.9
p<0.001
ACVR1
cut-off >0.7
p=0.01
ACVR2A
cut-off >0.2
p=0.001
NOG
cut-off <2.6
p=0.002
sensitivity (%)
100-specificity (%)

Figure 2. Expression of bone morphogenetic proteins (BMPs) and related molecules in purified multiple myeloma (MM) bone-marrow (BM) samples. The whole MM BM aspirates of four patients obtained during routine clinical assessment (MM1, MM3 and MM4 at diagnosis, and MM2 at progression) were separated using magnetic beads conjugated to anti-CD138 antibodies. RNA, extracted from the whole aspirates and separated fractions – $CD138^-$ and $CD138^+$, were analyzed by RT-qPCR. Expression of target genes activin receptor (*ACVR*)*1*, *ACVR2A*, *BMP4*, *BMP6*, and inhibitor of DNA binding *ID1* was presented as RNA relative quantity and normalized to the expression of *GAPDH*.

Figure 2

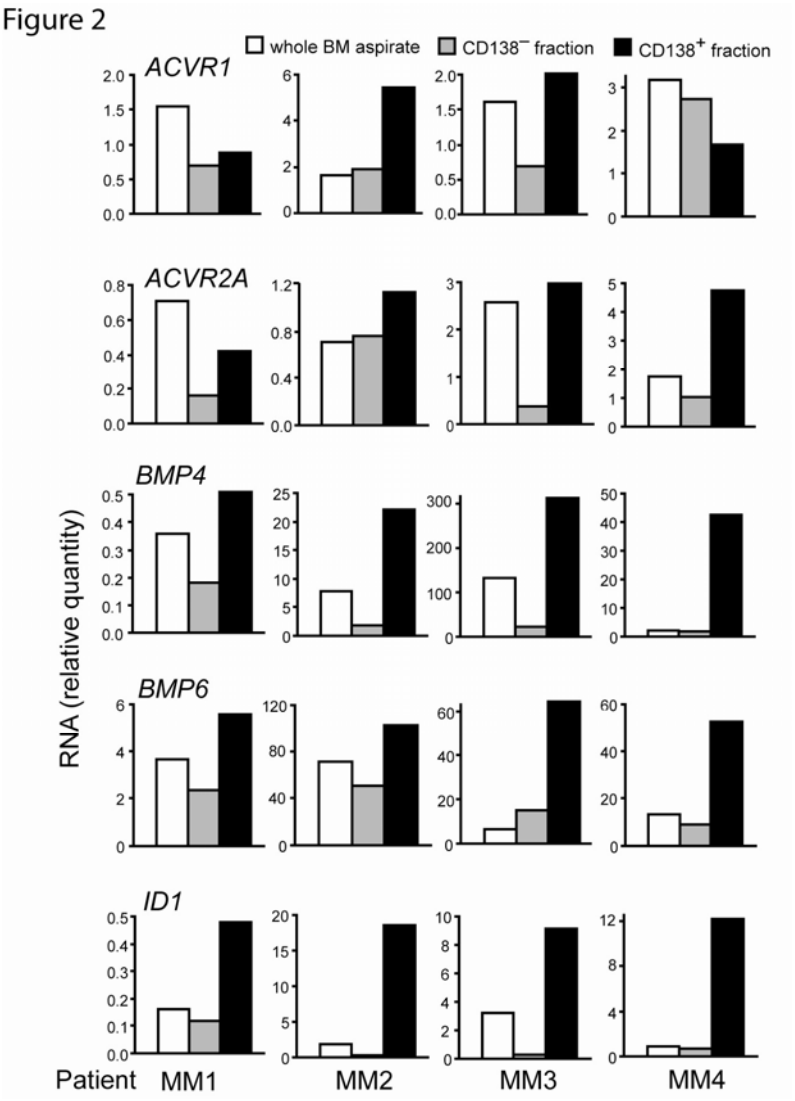

whole BM aspirate
CD138⁻ fraction
CD138⁺ fraction
ACVR1
ACVR2A
BMP4
BMP6
ID1
RNA (relative quantity)
Patient
MM1
MM2
MM3
MM4

Figure 3. Expression of bone morphogenetic proteins (BMPs) and corresponding receptors in myeloma cell lines – NCI H929 and Thiel. RNA was extracted from myeloma cells end analyzed by RT-qPCR. Expression of target genes *BMP2*, *BMP4*, *BMP6*, *BMP7*, activin receptor (*ACVR*)*1*, BMP receptor (*BMPR*)*1A*, *BMPR1B*, *BMPR2*, *ACVR2A*, and *ACVR2B* was presented as RNA relative quantity (mean±SD) and normalized to the expression of *GAPDH*.

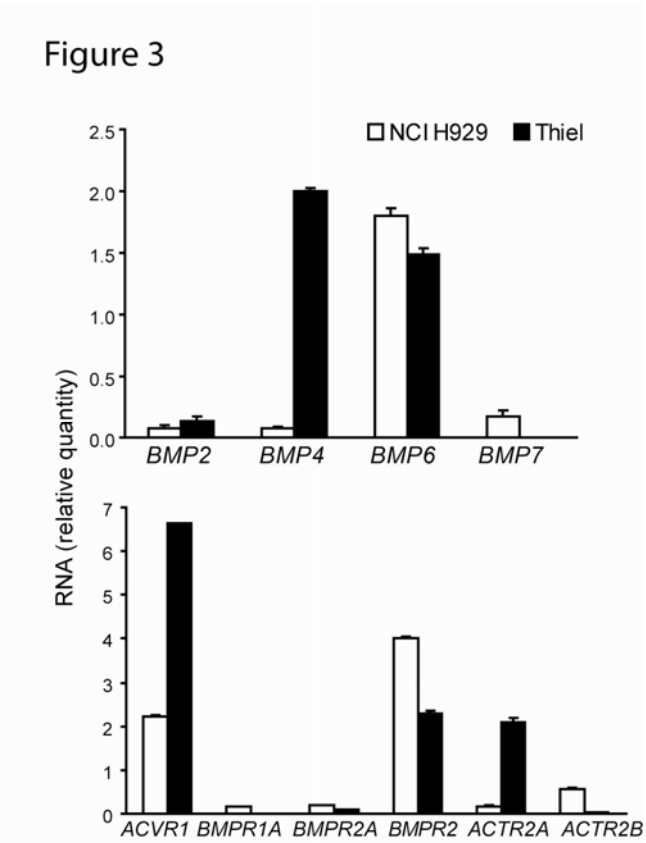


Figure 4. Flow-cytometric analysis of myeloma cell lines treated with bone morphogenetic proteins (BMPs) in combination with bortezomib (Bort) or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Two myeloma cell lines NCI H929 and Thiel were treated with Bort (4 nM or 5.2 nM respectively) or TRAIL (200 ng/mL or 800 ng/mL respectively). In some groups, myeloma cells were also treated with BMP2, BMP4 or BMP6 (0.5 μg/mL for all) and culture for up to 72 hours. A) Representative plots for the determination of the percentage of death/apoptotic cells by annexin V/propidium iodide (PI) staining in Thiel cells harvested 72 hours after the combined treatment with BMP6 and Bort. B) Percentage of annexin V positive cells in two myeloma cell lines, NCI H929 (left panel) and Thiel (right panel) after the combined treatment with BMPs and Bort (upper panel) or TRAIL (lower panel). Cells treated only with BMPs respond similarly as non-treated controls (not shown). Data are the representative of three similar experiments.

Figure 4

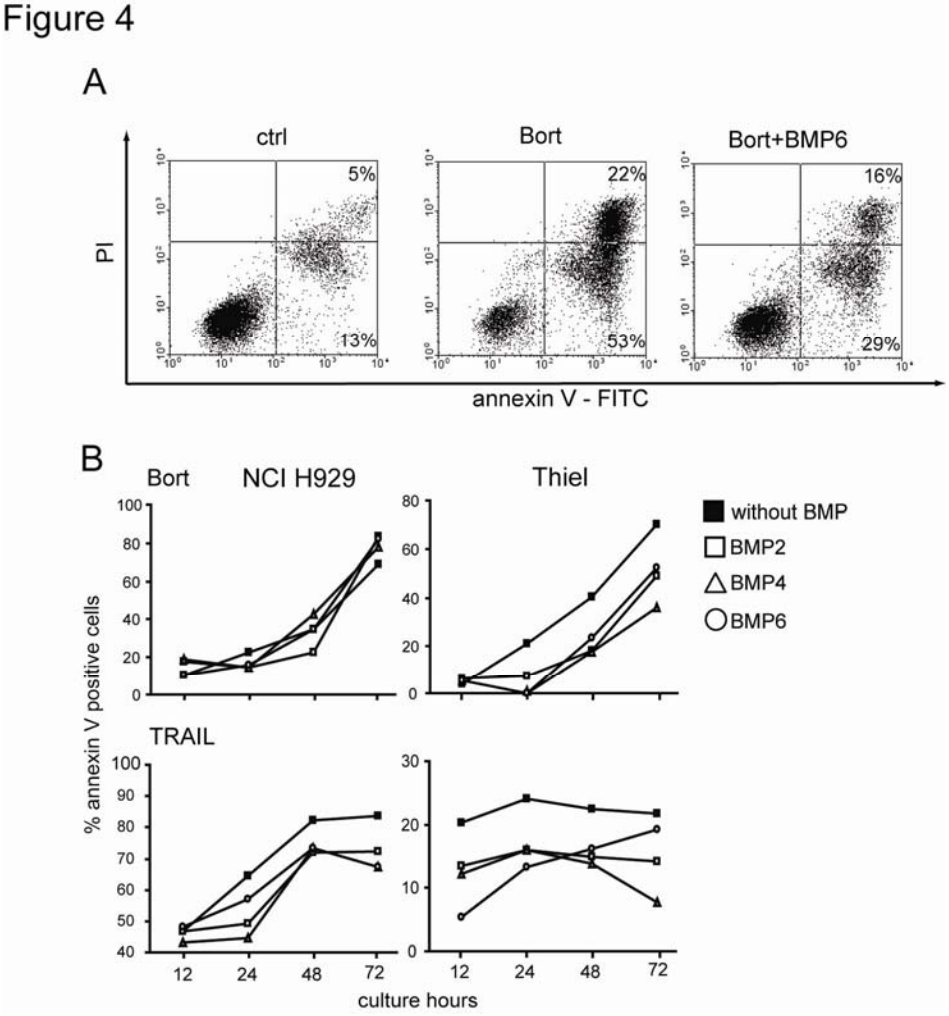
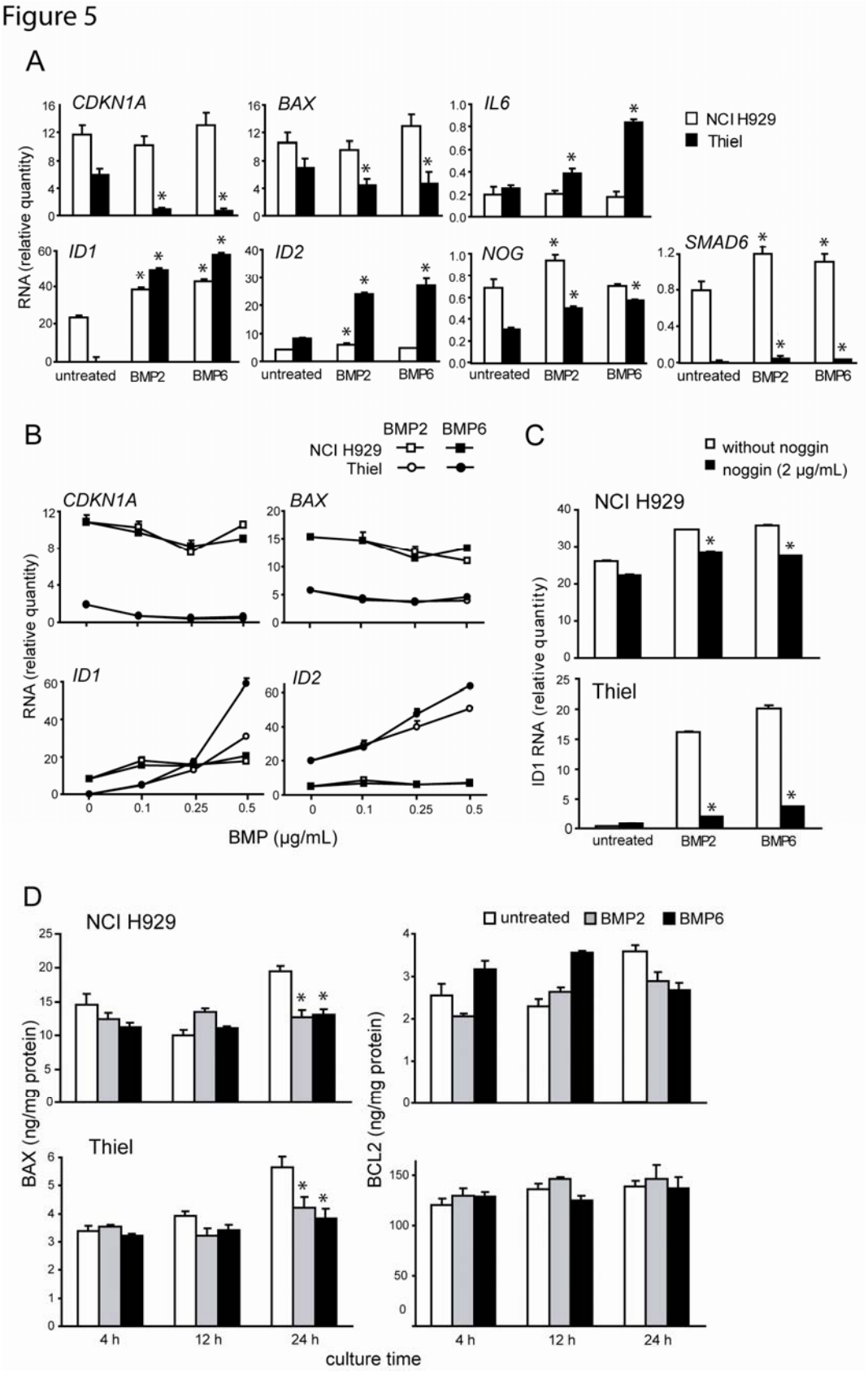

A
ctrl
Bort
Bort+BMP6
5%
13%
22%
53%
16%
29%
PI
annexin V - FITC
B
Bort
NCI H929
Thiel
without BMP
BMP2
BMP4
BMP6
% annexin V positive cells
TRAIL
culture hours
12
24
48
72

Figure 5. Intracellular effects of bone morphogenetic protein (BMP) treatment on myeloma cell lines NCI H929 and Thiel. Expression of target gene normalized to the expression of *GAPDH* was presented as RNA relative quantity (mean±SD). A) Myeloma cells were treated with BMP2 or BMP6 (0.5 μg/mL for both) for 24 hours and analyzed for the expression of pro-apoptotic molecules *CDKN1A* and *BAX*, autocrine myeloma growth factor *IL6*, inhibitors of DNA binding *ID1* and *ID2*, BMP antagonist noggin (*NOG*) and intracellular inhibitor *SMAD6*. Statistically significant differences for the expression of *CDKN1A*, *BAX*, *IL6*, *ID2* and *NOG* in Thiel cells, and for *ID1* and *SMAD6* for both cell lines were found between control and BMP-treated groups ($p<0.05$, ANOVA). B) Several doses of BMP2 and BMP6 were applied for 24 hours to test a dose response effect on the expression of selected molecules in myeloma cell lines. C) Suppression of BMP effect by noggin after 24 hour treatment was assessed by *ID1* gene expression in myeloma cell lines. Statistically significant difference for the *ID1* expression was found between the corresponding groups without and with addition of noggin ($p<0.05$, ANOVA). D) Concentration of BAX and BCL2 protein was determined in myeloma cells after the treatment with BMP2 or BMP6 (0.5 μg/mL for both) for up to 24 hours. Protein concentration was determined in cellular protein extracts by ELISA. Amount of target proteins (mean±SD) were expressed relatively to the total protein cellular content. Statistically significant difference for BAX concentration was found between control and BMP-treated groups for the time-point of 24 hours ($p<0.05$, ANOVA). Data are the representative of three similar experiments.

Figure 5


A
CDKN1A
BAX
IL6
ID1
ID2
NOG
SMAD6
RNA (relative quantity)
NCI H929
Thiel
untreated
BMP2
BMP6
B
BMP2 BMP6
NCI H929
Thiel
CDKN1A
BAX
ID1
ID2
RNA (relative quantity)
BMP (μg/mL)
C
without noggin
noggin (2 μg/mL)
NCI H929
Thiel
ID1 RNA (relative quantity)
untreated
BMP2
BMP6
D
NCI H929
Thiel
untreated
BMP2
BMP6
BAX (ng/mg protein)
BCL2 (ng/mg protein)
4 h
12 h
24 h
culture time