

Opposing SMAD-6 and SMAD-7 Expression Profiles Correlate with the Sensitivity of Multiple Myeloma Cells to Bone Morphogenetic Protein (BMP)-2

Axel Seher¹, Charlotte Lagler¹, Thomas Dieter Müller² and Joachim Nickel^{3,4*}

¹Department of Oral and Maxillofacial Plastic Surgery, University Hospital Würzburg, Würzburg, Germany

²Department of Molecular Plant Physiology and Biophysics, Julius-von-Sachs-Institute, University of Würzburg, Würzburg, Germany

³Chair Tissue Engineering and Regenerative Medicine, University Hospital Würzburg, Würzburg, Germany

⁴Fraunhofer ISC, Translational Center Regenerative Therapies (TLC-RT), Würzburg, Germany

*Corresponding author: Joachim Nickel, Chair Tissue Engineering and Regenerative Medicine, University Hospital Würzburg, Würzburg, Germany, E-mail: joachim.nickel@uni-wuerzburg.de

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Abstract

Objective: Multiple myeloma (MM) is a hematologic disease characterized by hyper-proliferation of antibody secreting B-cells. One of the most severe accompanying symptoms in MM is the destruction of bone tissue. Thus, the use of bone morphogenetic proteins (BMPs) for MM treatment seems to be a good option since these proteins are capable to induce formation of bone tissue *in vivo* and additionally can suppress cell proliferation of malignant B-cells. But, the different BMP family members vary in their biological activities also in terms of donor specific differences of addressed cells. In this study we analyzed signal transduction of two different TGF β family members, BMP2 and GDF5, in different MM cell lines.

Methods: Ten MM cell lines were stimulated either with BMP2 or GDF5 and cell proliferation was analyzed by WST-1 assays. Receptor expression levels were determined by qRT-PCR for relevant BMP receptors. SMAD-1/5/8 phosphorylation was analyzed by Western blot and correlated to the expression levels of inhibitory (i)-SMADs, SMAD-6 and SMAD-7 proteins, respectively.

Results: Only three out of ten investigated cell lines were BMP2 responsive, one of which was additionally sensitive to GDF5. Depending on the expression of the required receptors the different cell lines could be divided into three subgroups. The first group expressed all receptor chains which are crucial for proper signal transduction and was ligand sensitive, the second also expressed the required receptors, but appeared ligand-resistant. The third subgroup instead missed at least one or more essential receptor rendering these cells also resistant to ligand exposure. Western blot analyses addressing phosphorylated SMAD-1/5/8 proteins revealed that the second group showed no or at least extremely reduced levels of SMAD-1/5/8 phosphorylation levels upon ligand exposure. Furthermore, Western blot analyses also showed that non-responsive cells expressed the inhibitory SMAD-7 protein at high levels prior to ligand stimulation. In contrast, the BMP2 responsive cells did not express SMAD-7 but instead expressed SMAD-6 at high levels prior to ligand stimulation.

Conclusion: Aside of the expression of essential receptors, further factors are decisive for proper BMP signal transduction in MM cells such as the individual expression levels of SMAD-6 and SMAD-7 in unstimulated cells. The conspicuous opposing basal expression levels of either SMAD-6 or SMAD-7 might be used as prediction whether a particular MM cell line appears ligand-responsive or ligand-resistant.

Keywords: Multiple myeloma; BMP2; GDF5; SMAD-6; SMAD-7; Receptor expression; B-cell; Bone

Introduction

Multiple myeloma (MM) is a hematologic disease characterized by hyper-proliferation of B-cells and an extreme production and secretion of monoclonal antibodies and/or antibody fragments [1]. The incidence of MM is approximately 4-6 new cases per 100,000 people per year. MM represents 10% of all hematological and 1% of all cancer types [2]. A serious and, above all, painful symptom during the course of the disease is the destruction of bone by osteolysis due to strong deposition of antibody fragments in bone tissue [3]. Interestingly, over

the last 10 years, proteins have been identified which play an important role in the pathology of the MM but are also relevant for bone homeostasis - the bone morphogenetic proteins (BMPs) [4]. The BMPs belong to the TGF β family and are capable to induce ectopic bone formation in mesenchymal tissues and are used as gold standard for the treatment of non-union critical-size bone defects [5]. In the context of MM it could be shown that BMP2, -4, -5, -6, -7 and BMP9 also share further biological functions. They act anti-proliferative and/or apoptotic on primary cells and also on MM cell lines [6-10]. For this reason, we postulated a potential use of molecular designed BMP (mdBMPs) variants in MM, which on the one hand facilitate the reduction and/or elimination of malignant B cells and, on the other hand, simultaneously promote bone regeneration [11]. BMPs are

native antagonists of activin A, another TGF β family member [11,12]. In MM activin A expression levels are increased which significantly contributes to the observed bone loss since overexpression of activin A on the one hand inhibits osteoblastogenesis and on the other hand simultaneously activates osteoclast activity [13]. The successful use of activin A antagonists has been demonstrated in both, mouse models [13,14] as well as in clinical studies [15]. Thus, BMPs might act similarly effective as activin A antagonists since they also ameliorate the overall clinical manifestation [11].

The decisive criterion for a successful use of particular BMPs in the clinic relies on their individual, patient-specific efficacy. Thus, it is important to know whether BMPs can exert their function in every MM patient but also to what extent. It has been shown in both, cell lines and primary cells, that the biological activities of BMPs vary strongly between the particular cells. A particular BMP ligand can reduce the cell number in different cells/cell lines from 10% to 90% (anti-proliferation and/or apoptosis) but also insensitivities exist in that cells isolated from individual donors or particular cell lines do not respond to this BMP ligand at all [6-10].

The individual biological activity of the BMPs certainly depends on expression of the correlating receptors relevant for signal transduction but also to the expression of cytoplasmic inhibitors, such as the so-called iSMADs. BMP mediated signaling is initiated by binding of the dimeric ligand to two type I- and two type II receptor serine/threonine kinase receptors. For signal transduction the intrinsic kinase domain of the type-II-receptor phosphorylates multiple serine and threonine residues in the cytoplasmic GS-region of the type I receptor. The activated type I receptor subsequently can phosphorylate the so-called rSMADs (for BMP2,-4 and GDF5: SMAD-1/5/8) which after complex formation with the co-SMAD (SMAD-4) translocate into the nucleus, where these complexes bind to specific SMAD-response elements thus regulating the expression of specific genes [16]. BMP signal transduction is regulated at various cellular levels but usually there are two causes for a BMP resistance; first, an essential receptor at least of one receptor type, either an essential type I- or type II receptor (or from both subtypes) is missing. Ro et al., for example, showed that the MM cell lines INA6 and RPMI-8226 lack the receptor BMPR-IA most likely causing the observed BMP2 and BMP4 resistance. A second cause might rely on the expression of specific inhibitory SMAD proteins (iSMADs). The iSMADs SMAD-6 and -7 interfere with the canonical SMAD signaling cascade in two different ways. Either they can prevent complex formation of the phosphorylated rSMADs (SMAD-1/5/8) with the co-SMAD (SMAD-4) or they can prevent the phosphorylation of the rSMADs by the type I receptor [16]. While SMAD-7 is considered to be a general signaling inhibitor initiated by various TGF β superfamily members, SMAD-6 specifically blocks signal transduction induced by BMPs [17-19]. Furthermore, it has already been demonstrated that in context of MM the presence of either SMAD-6 or SMAD-7 fundamentally influences the responsiveness to particular BMPs [17,18].

In this study, ten different MM cell lines were exposed to two different ligands, BMP2 and GDF5. Since the majority of the investigated cell lines was found to be ligand resistant the expression levels of the relevant type I-(BMPR-IA, BMPR-IB) and type II receptors BMPR-II, ActR-II and -IIB) were analyzed by qRT-PCR. Since ligand-resistance did not rely in every case on the lack of essential type I- or type II receptors the expression levels of the two iSMADs, SMAD-6 and SMAD-7 were also investigated.

Material and Methods

Ligand expression

Recombinant human BMP2 and human GDF5 were expressed in *E.coli*, refolded and purified as described previously [19,20].

Cell culture

The human MM cell lines MM.1S, RPMI-8226, AMO1, U266, L363, JFN3, OPM2, KMS12-BM, KMS11 and INA6 cells were cultured in RPMI 1640 (PAA, Pasching, Germany) supplemented with 10% (v/v) of heat-inactivated fetal calf serum (PAA, Pasching, Germany). Recombinant human IL6 (ImmunoTools, Friesoythe, Germany) was added to a final concentration of 2 ng/ml when culturing INA6 cells. All assays were carried out in the described culture media. The cell lines MM.1S, RPMI-8226 and U266 were obtained from the American Type Culture Collection (ATCC). The cell lines KMS12-BM, AMO1, OPM2, L363, JFN3 and KMS11 were obtained from the DSMZ (Leibniz-Institut, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). The cell line INA6 was a friendly gift from Dr. Martin Gramatzki, Erlangen.

WST-1 assay

Cell numbers of the cell lines KMS12-BM and L363 were determined using a cell counter (CASY[®], OMNI Life Science, Bremen, Germany). 2×10^4 cells per sample were seeded at 100 μ l/well into 96-well plates and stimulated with 250 nM of either BMP2 or GDF5 for 96 h. The control cells remained unstimulated. To measure reduction equivalents (e.g. NADH) WST-1 (10 μ l/well) (Roche, Germany) was added, and the cells were further incubated for exactly 3 h at 37°C. WST-1 was measured at 450 nm using a micro plate reader (TECAN RAINBOW[®], Germany). All assays were performed in duplicate, in three individual experiments.

Real time qRT-PCR

The following primers were used:

- (1) *bmpr1a_1_SG* Qiagen, Venlo, The Netherlands
- (2) *bmpr1b_1_SG* Qiagen, Venlo, The Netherlands
- (3) *bmpr2* (forward) AGGGGAATCCGTACCAGAGT (reverse) CATCCTGGTCCCAACAGTCT
- (4) *actr2* (forward) GTTGCCATTTGAGGAGGAAA and (reverse) CCAGCTGATAACCTGGCTTC
- (5) *actr2b* (forward) CTGACTTTGGCTTGGCTGT and (reverse) AGGGCAGCATGTACTCATCC and
- (6) *hprt* (hypoxanthine-guanine phosphoribosyltransferase) (forward) GACCAGTCAACAGGGGACAT, (reverse) ACACCTCGTGGGGTCCTTTT (primer 3-6 are purchased from Thermo Fisher, Germany).

Total RNA was prepared using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. For cDNA synthesis, 1 μ g of total RNA was reverse-transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Germany). qRT-PCR was performed using 20 ng of the cDNA synthesis mix per reaction and the QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany). Three independent PCR analyses were

performed in duplicate for each gene. Relative expression levels were calculated from a comparison with the house keeping gene *HPRT* and the following equation: $\text{rel. expression (\%)} = [2^{(\text{CtS} - \text{CtR})}] * 100$, where CtS

is the Ct value for *HPRT* gene expression and CtR is the Ct value for the individual receptor gene expression.

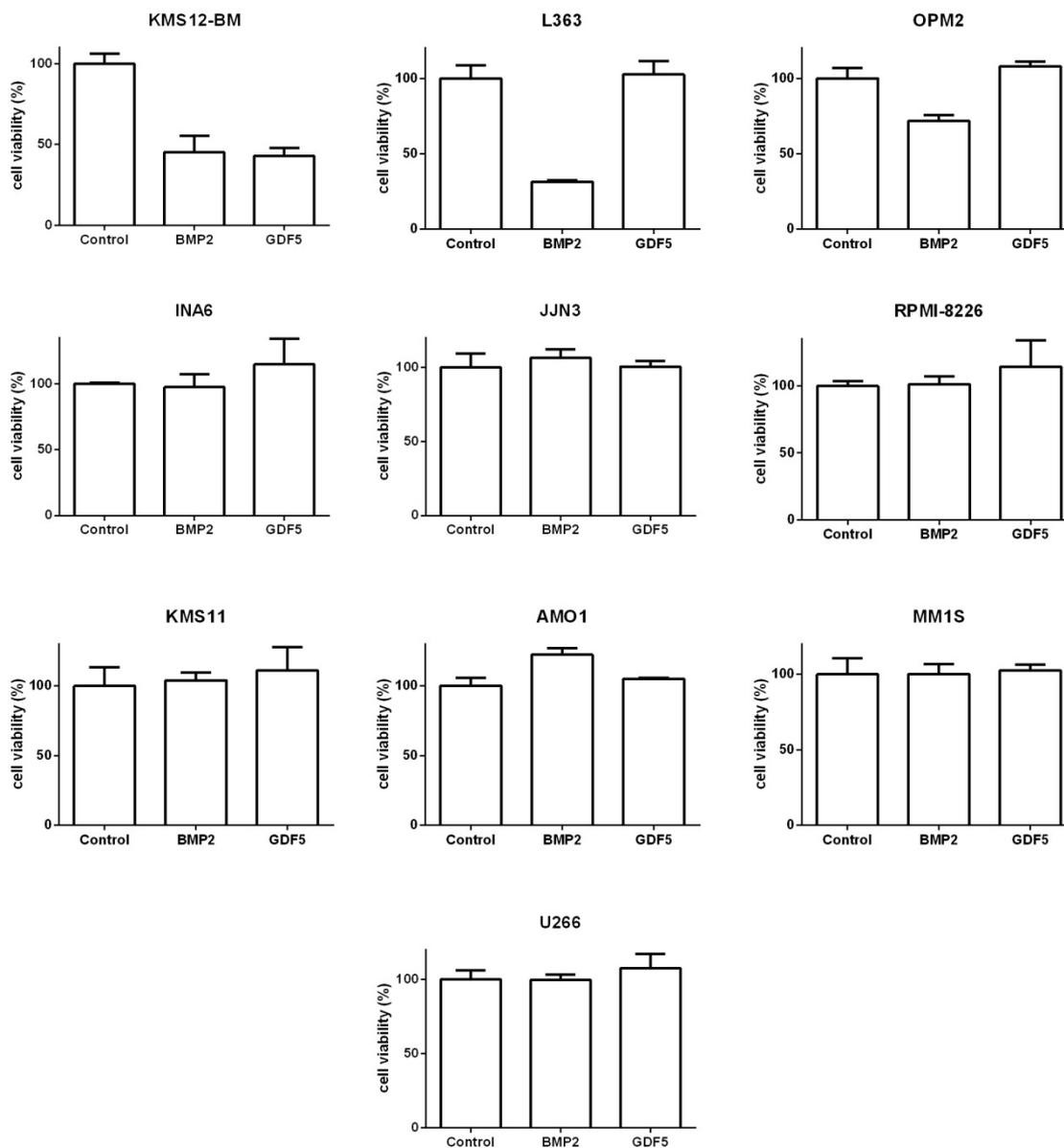


Figure 1: Influence of BMP2 and GDF5 on cell proliferation. To analyze the biological response to BMP2 and GDF5 ten MM cell lines (KMS12-BM, L363, OPM2, INA6, JLN3, RPMI-8226, KMS11, AMO1, MM1S and U266) were stimulated with 250 nM BMP2 or GDF5. Cells were analyzed *via* WST-1 assays.

Western blot

For Western blot analysis of whole cell lysates, the cells were collected into ice-cold PBS, centrifugated, sonicated and subsequently lysed by boiling (5 min at 96°C) in 4 × Laemmli sample buffer (8% SDS, 0.1 M DTT, 40% glycerol, and 0.2 M Tris, pH 8.0). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes.

After blocking non-specific binding sites by incubating the membranes in TBS buffer containing 0.1% Tween 20 and 5% dry milk, immunoblotting was performed. Primary antibodies that were specific for SMAD-1 (#9743S), pSMAD-1/5/8 (#9511S) (both Cell Signaling Technology, Danvers, USA), SMAD-6 (H-150) (#sc-13048) (Santa Cruz Biotechnology, Heidelberg, Germany) or SMAD-7 (#420-400) (Invitrogen™, Carlsbad, CA, USA) and HRP-conjugated secondary

antibodies (Dako, Hamburg, Germany) were used. Detection was performed by enhanced chemiluminescence using ECL Plus reagents (Amersham-Pharmacia, Freiburg, Germany). Exposed films were processed with AGFA Curix60 machine. Experiments were repeated three times. As loading control an antibody raised against alpha-tubulin (tubulin alpha (DM1A), Neo Markers, Fremont, CA (now Invitrogen, #1983313)) was used.

Results

Not all investigated BMP2 sensitive MM cells are also sensitive to GDF5

In a first experiment we tested the influence of BMP2 and GDF5 on cell proliferation of ten different MM cell lines (KMS12-BM, L363, OPM2, INA6, JN3, RPMI-8226, KMS11, AMO1, MM1.S and U266) using WST-1 assays. In three cell lines - KMS12-BM, L363 and OPM2 - a significant anti-proliferative effect could be detected upon BMP2 stimulation. For the first time we could show that also GDF5 can act anti-proliferative but this effect was only seen in case of KMS12-BM cells (Figure 1).

BMP2 insensitivity cannot be explained in every case by missing receptors

One reason for the resistance of MM cells to BMP2 or GDF5 exposure might rely on missing type I- or type II receptors. We thus analyzed the presence of all relevant receptors, i.e. BMPR-IA, BMPR-IB, ActR-II, ActR-IIB and BMPR-II on transcriptional level by real-time qRT-PCR (Figure 2). Based on receptor expression the cells can be classified into three different subgroups. The first subgroup comprises the three BMP2 responsive cell lines KMS12-BM, L363 and OPM2, respectively (Figure 2A). These cell lines express at least one essential receptor of either receptor type. Noteworthy, the cell line KMS12-BM, the only cell line being sensitive for GDF5, lacks expression of BMPR-IB, the preferred receptor for GDF5 binding. Thus, despite of an approximately 15-fold weaker binding affinity being reported for the GDF5-BMPR-IA interaction [21], it can be assumed, that signal transduction here - like it is reported for ATDC-5 cells - is also mediated *via* BMPR-IA [22].

The second subgroup (INA6, JN3 and RPMI-8226 cells) showed no or only extremely low expression levels for the essential type I receptors BMPR-IA or BMPR-IB (Figure 2B) which is in agreement to already published data [10]. Ro et al. showed that INA6 and RPMI-8226 are resistant to BMP2 and BMP4 and the authors already correlated their findings to the missing type I receptors BMPR-IA and BMPR-IB, respectively [10]. However the third subgroup represented by the cell lines KMS11, AMO1, MM1.S and U266 is of special interest (Figure 2C) since these cells express all essential receptors but are nevertheless resistant to both, BMP2 and GDF5 ligands.

All BMP2-resistant cell lines showed no or at least strongly reduced levels of SMAD-1/5/8 phosphorylation

For more detailed analysis signal transduction of BMP2 induced SMAD-1/5/8 phosphorylation was investigated (Figure 3). The three BMP2 sensitive cell lines KMS12-BM, L363 and OPM2 showed robust SMAD-1/5/8 phosphorylation despite of low BMPR-IA expression levels particularly observed in i.e. OPM2 cells. As expected, for INA6, JN3, and RPMI-8226 cells, no pSMAD-1/5/8 signal could be detected.

However, the subgroup of cells which, based on the expression profile of essential BMP receptors should be ligand-sensitive, unexpectedly also showed no (i.e., AMO1 and MM1.S cells) or only weak (i.e., KMS11 cells) SMAD-1/5/8 phosphorylation if compared to a positive control (C). Astonishingly, U266 cells showed a SMAD phosphorylation being comparable to that of the control (C).

MM cell lines either express SMAD-7 or SMAD-6

As mentioned before, expression of the inhibitory SMAD-6 and -7 proteins influences canonical SMAD signaling either by preventing the formation of rSMAD/co-SMAD complexes or by inhibiting type I receptor mediated rSMAD phosphorylation. *Via* Western blot we analyzed the native expression of these inhibitory SMADs in the investigated MM cell lines (Figure 4). Surprisingly, SMAD-6 and SMAD-7 showed an opposing expression profile. With the exception of INA6 cells all unstimulated BMP2 resistant cell lines showed high SMAD-7 expression levels whereas all unstimulated BMP2-sensitive cell instead express SMAD-6 (Figure 4).

Discussion

Multiple myeloma is a malignant neoplastic disease of bone marrow, characterized by plasmocytosis with increased production of inoperable and mostly fragmented immunoglobulins [1]. MM is the second most common cancer of the blood-forming system with ten percent of all malignant hematological disorders and represents one percent of all cancer cases [2]. The disease is considered to be non-curable, but can be treated by partial remission of the disease by age- and condition-dependent primary therapies [23-25].

Therefore, further innovations are needed in MM treatment. We have postulated that molecular designed BMPs could potentially be suitable for clinical use [11]. In addition to the anti-proliferative/apoptotic effect on neoplastic B cells, the native osteogenic property of BMPs could help to treat one of the outstanding and extremely painful symptoms of the MM - the osteolysis of bone tissue and the associated destruction of the skeleton [3].

But, one premise to use mdBMPs for the treatment of MM is the same as it is for other drugs; it must be active and effective. This, however, is not given in every case since different efficacies in the inhibition of MM cell proliferation was observed which is linked to patient specific sensitivities to individual BMPs [6-12]. Since for therapy we intend to use different BMP2 muteins in the future, we have investigated BMP2 induced signal transduction in ten different MM cell lines. In addition, GDF5 was involved in our investigations since this ligand induces cellular responses only in a limited set of cells and/or tissues.

Surprisingly, BMP2-induced cellular responses (inhibition of cell proliferation) were observed in just three out of ten cell lines (KMS12-BM, L363, and OPM2). In case of GDF5 the number of responsive cell lines was further diminished in that only one cell line appeared GDF5 responsive. Astonishingly only in three cases (i.e. in INA6, JN3 and RPMI-8226 cells) the observed resistance to the applied factors can be explained by missing receptors. Despite a seemingly functional signal transduction machinery phosphorylation of the downstream signal mediators did not occur (i.e., in AMO1 and MM1.S cells) or was only induced to minor extents (i.e., in KMS11 and in U266 cells). A comparison of the native expression levels of the inhibitory SMAD proteins SMAD-6 and -7 revealed that, with the exception of INA6

cells, all BMP2-resistant cells solely expressed SMAD-7 whereas all BMP2-sensitive cells solely expressed SMAD-6.

These new findings highlight the complexity of cellular signaling provided by a multitude of factors which utilize overlapping sets of cellular receptors. The simplified scheme that explains resistance by missing receptors is certainly relevant but not true in all cases. A causal relationship between ligand-resistance and missing signaling receptors as shown by Ro et al. seems valid only in some minor cases [10]. A new finding, however, is the responsiveness of the cell line KMS12-BM also

to GDF5. This at first sight is surprising since the receptor preferentially bound by GDF5, BMPR-IB, is absent in these cells. Thus, signal transduction seems here also to be mediated *via* the type I receptor BMPR-IA whose function for GDF5 mediated signal transduction was also reported in other cell lines [21]. But, GDF5 is not active in the other two BMP2-sensitive cell lines (i.e., L363 and OPM2). This most likely relies on (a) missing GDF-specific co-receptor(s) currently being discussed in the literature [22].

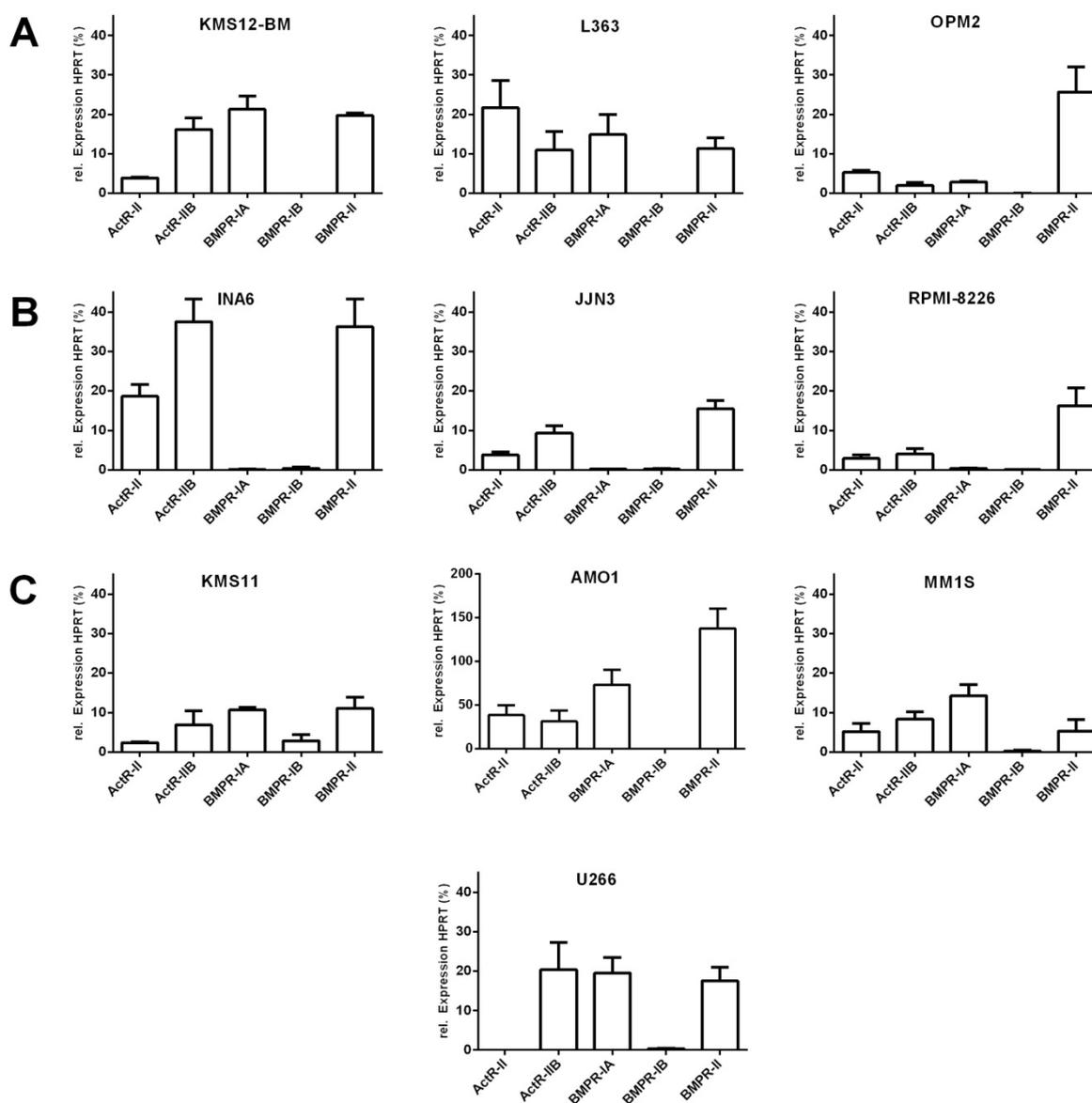


Figure 2: Expression levels of BMP receptors in MM cell lines. The indicated MM cell lines were analyzed for expression of ActR-II, ActR-IIB, BMPR-IA, BMPR-IB and BMPR-II by real-time qRT-PCR. (A) Cell lines are BMP2-responsive and express at least one essential receptor of each type. (B) Cells lack expression of essential receptors of at least one type. (C) Cell lines express at least one essential receptor of each type but are BMP2-resistant.

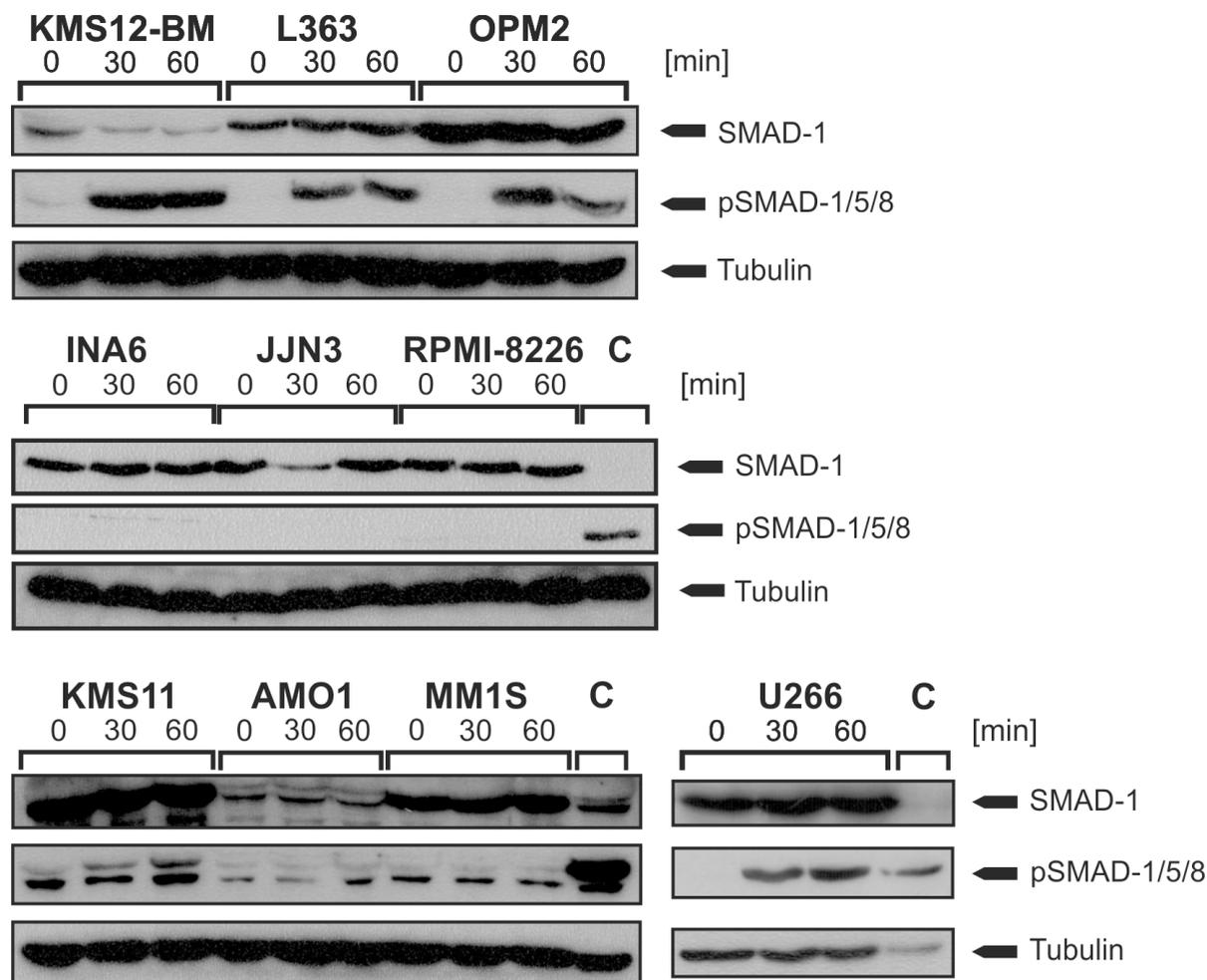


Figure 3: Western Blot analysis of BMP2 induced SMAD-1/5/8 phosphorylation in different MM cell lines. Cell lines were incubated with 250 nM BMP2 for 0, 30 or 60 min, respectively. As positive control (C) a whole cell lysate of BMP2 stimulated (60 min) KMS12-BM cells were used. Analysis of Tubulin expression serves as loading control.

However, the fact that a matching receptor expression profile is not solely decisive for ligand-sensitivity responsiveness has been shown by the work of Huse et al. The analysis of different lymphoma cells revealed, that also BMP-resistant cells express all necessary BMP receptors [17]. Thus, another aspect has to be taken into account addressing the activation of the downstream mediators of BMP signaling, SMAD-1/5/8. Huse et al. showed that the degree of ligand-resistance in cells differs amongst particular BMPs but is in general reflected by strongly reduced SMAD-1/5/8 phosphorylation levels [17]. The consequence of reduced signaling *via* either canonical SMAD pathway for apoptosis in the context of MM cells was shown by Holien et al. They showed that BMP4 or BMP6 induced apoptosis is SMAD dependent *via* downregulation of the oncogene myc, a master regulator of cell growth and protein synthesis. So an over expression of the iSMADs SMAD-6 and SMAD-7 results in the inhibition of apoptosis by preventing the downregulation of myc expression [26].

In our study, these results basically could be confirmed, but the investigated cell lines showed inhibition of SMAD phosphorylation to different extents. This might indicate that these effects are caused by different inhibitory mechanisms. As already mentioned the iSMADs

(i.e. SMAD-6 and -7) can interfere with the canonical signaling cascade induced by BMPs in two different ways. Either they prevent complex formation of pSMAD-1/5/8 with the co-SMAD SMAD-4 or they prevent the phosphorylation of the rSMADs *via* the type I receptor [16]. While no phosphorylation of the rSMADs was detected in the cell lines AMO1 and MM1.S, a weak phosphorylation was found in the cell line KMS11 but without a translation into any measurable biological activity. It is possible that rSMAD phosphorylation in AMO1 and MM1.S cells is already blocked by the iSMADs at the receptor level whereas in KMS11 cells the iSMADs might impede rSMAD/co-SMAD complex formation. The latter mechanism most likely also renders U266 cells ligand-resistant since in these cells only slightly reduced rSMAD phosphorylation levels were observed but no anti-proliferative effects could be observed. Huse et al. did not discuss in their manuscript any plausible explanation for the BMP-resistance but they could show that ectopic expression of either SMAD-6 or SMAD-7 results in BMP-resistance of B-cells derived from lymphomas [17]. While SMAD-7 seems to inhibit signaling of all TGF β family members, SMAD-6 seems to act more specifically on ligands which transduce signals *via* the SMAD-1/5/8 [17,18] pathway. However, in

both reports a correlation between the native basic expression levels of the inhibitory SMAD-6 and SMAD-7 proteins to ligand-sensitivity was not drawn. Neither gene expression profiling of normal or malignant B-cell lines nor that of cells comparing healthy and malignant donors allowed such a direct correlation [17]. Thus, the data presented here

clearly indicate, that such a correlation might exist: BMP-resistant cells strongly express SMAD-7 whereas SMAD-6 expressing cells are in principle BMP-sensitive as long as the essential receptors are expressed at the cell surface.

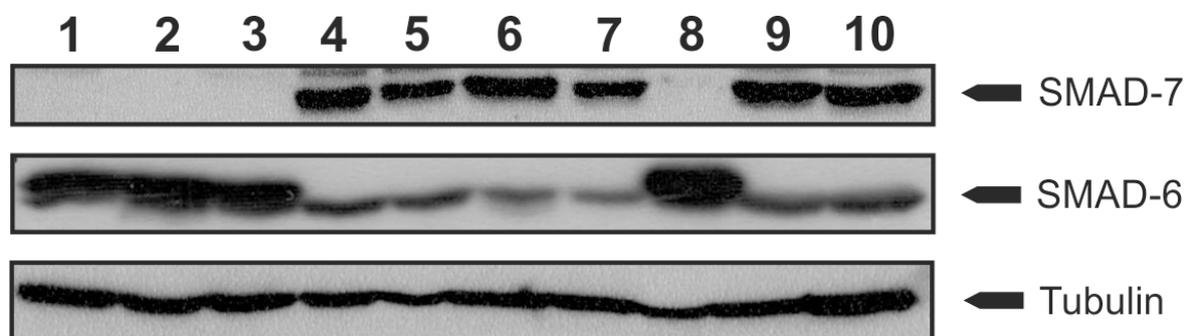


Figure 4. Western Blot analysis of SMAD-6 and SMAD-7 expression in different MM cell lines. (1 L363, 2 KMS12-BM, 3 OPM2, 4 KMS11, 5 AMO1, 6 MM1.S, 7 U266, 8 INA6, 9 JN3 and 10 RPMI-8226) Tubulin served as a loading control. Noteworthy: The faint bands with slightly faster electrophoretic mobilities as those representing SMAD-6 are unspecific.

This also applies in particular to the INA6 cells. Lacking BMPR-IA and BMPR-IB these cells appear activin A but not BMP2 sensitive [11]. But how can this result be explained, especially since the SMAD-6 expression contradicts the previously published results that the ectopic expression of SMAD-6 specifically inhibits the group of BMPs [17,18]? The answer to this might be provided by Ishisaki et al. who showed that the expression of SMAD-6 and SMAD-7 is basically inducible by particular TGF β family members, i.e. BMP2 and activin A, respectively. Although basal expression of the two iSMADs in the murine cell line HS-72 was not detectable at the RNA level, BMP2 and activin A induced SMAD-7 expression occurred after 1 h. Interestingly, SMAD-7 expression decreased strongly after 3 h and 6 h whereas SMAD-6 expression was upregulated. Therefore, as observed in our work, the two iSMADs showed completely opposing expression profiles, but here only upon ligand stimulation.

It seems obvious that only a permanent expression of iSMADs results in a sustained inhibition of signaling by TGF β family members. However, both the transient expression profile in HS-72 cells and the opposing basal expression levels of SMAD-6 and SMAD-7 observed in our ten investigated cell lines suggest, that SMAD-6 and SMAD-7 expression might be causally related. The extent to which SMAD-6 and SMAD-7 possibly regulate each other and whether the expression of the iSMADs is regulated by e.g. degradation remains the subject of further investigations.

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