The role of bone morphogenetic proteins in myeloma cell survival

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ABSTRACT

Multiple myeloma is characterized by slowly growing clones of malignant plasma cells in the bone marrow. The malignant state is frequently accompanied by osteolytic bone disease due to a disturbed balance between osteoblasts and osteoclasts. Bone morphogenetic proteins (BMPs) are present in the bone marrow and are important for several aspects of myeloma pathogenesis including growth and survival of tumor cells, bone homeostasis, and anemia. Among cancer cells, myeloma cells are particularly sensitive to growth inhibition and apoptosis induced by BMPs and therefore represent good models to study BMP receptor usage and signaling. Our review highlights and discusses the current knowledge on BMP signaling in myeloma.

ABBREVIATIONS

BMP, bone morphogenetic protein; TGF, transforming growth factor; GDF, growth and differentiation factor; SMAD, (small) mothers against dpp (decapentaplegic) homolog; ALK, activin receptor-like kinase; BMSC, bone marrow stromal cells

1. INTRODUCTION

1.1 General introduction

The BMP family of ligands has been shown to play a role in a multitude of processes throughout the body, in particular in cellular lineage commitment, morphogenesis and patterning, differentiation, proliferation, cellular maintenance and survival.[1-3] Also in multiple myeloma, BMP signaling has been proposed to influence important processes such as growth control, bone homeostasis, iron metabolism and angiogenesis. However, BMP signaling is highly dependent on cell type and context. Multiple myeloma is characterized by the presence of slowly growing, malignant plasma cells in the bone marrow.[4] Dependent on receptor expression, many BMPs potently induces growth arrest or apoptosis in myeloma cells, making them unique among cancer cells. Myeloma cells are therefore particularly interesting tools to study BMP receptor use and signaling. Moreover, a hallmark of myeloma is severe osteoporotic or osteolytic bone disease and the BMPs are known as potent mediators of bone formation. Thus, BMPs have the potential not only to suppress survival of myeloma cells but also to restore bone in these patients. This review focuses on the roles of BMPs, their related ligands and receptors in regulation of myeloma cell growth.

1.2 An introduction to bone morphogenetic proteins

Bone morphogenetic proteins (BMPs) constitute the largest subgroup of the transforming growth factor (TGF)-β family of ligands that also include growth and differentiation factors (GDFs), activins and nodal. The bone inducing activity of BMP was discovered in the 1960s and the first proteins of the family were characterized in the late 1980s.[5, 6] Further studies have revealed multiple functions for BMPs, such

as involvement in embryogenesis, hematopoiesis and neurogenesis (reviewed by Bragdon *et al.*),[2] as well as both tumor promoting and growth inhibiting effects in various cancers.[3] The signal is usually transduced through the (small) mothers against dpp (decapentaplegic) homolog (SMAD) pathway that is unique for the TGF-β family.[7] The pleiotropic effects of BMP suggest the need for tight control of its activities. This is achieved by multiple regulatory mechanisms, including regulation of ligand activity, availability of ligand, particularly regulated by highly specific antagonists, expression of receptors, decoy receptors, co-receptors and the inhibitory (I)-SMADs, SMAD6 and SMAD7.[2]

1.3 BMP signal transduction

BMPs and other TGF-β family members signal by binding as active homo-, or sometimes, heterodimers to their respective serine/threonine kinase receptors. They either bind to a preformed receptor complex, or alternatively, a receptor complex is formed by ligand binding to high-affinity receptors followed by recruitment of receptors with lower affinity. For most BMPs this means initial binding to a type 2 receptor, followed by binding to a type 1 receptor. Upon ligand binding, the constitutively active type 2 receptor phosphorylates the type 1 receptor in the juxtamembrane glycine/serine rich domain (Figure 1). This activating step enables binding and phosphorylation of receptor activated (R)-SMADs. Which one of the R-SMADs that is activated depends on the type 1 receptor and the composition of the receptor complex. In general, the R-SMADs are divided into two groups: (1) BMP-activated R-SMADs; SMAD1, SMAD5 and SMAD8 that are activated by ALK1, ALK2, ALK3 and ALK6. (2) TGF-β/Activin-activated R-SMADs; SMAD2 and SMAD3 that are activated by ALK4, ALK5 and ALK7. Activated R-SMADs bind the Co-SMAD, which

in humans is SMAD4. Trimers consisting of SMAD4 and two R-SMADs translocate to the nucleus to repress or activate expression of SMAD-responsive genes.[2]

The ligand-receptor interaction of the BMP/TGF-β family of ligands is highly promiscuous, as evident by the presence of only seven type 1 receptors and five type 2 receptors for over 30 different ligands.[3] Typically, one receptor can bind different ligands, whereas one ligand can activate different sets of receptors. Historically, one has thought that four type 1 receptors of the activin receptor-like kinases (ALK) are involved in BMP signaling. Thus, BMPs are believed to signal through ALK1, ALK2, ALK3, and ALK6, as well as through three type 2 receptors, namely BRII, ActRIIa, and ActRIIb (summarized in table 1). Now it has become clear that there is another level of complexity in that heteromeric receptor complexes exist that enables ligands to signal to both R-SMAD branches. Thus, TGF-β can activate heteromeric receptor complexes consisting of both ALK5 and ALK1, thereby activating both SMAD2/3 and SMAD1/5/8.[8, 9] Also, BMP-9 has been shown to induce phosphorylation of SMAD2 by signaling through ActRIIa in pulmonary endothelial cells.[10] More recently, it was shown that BMP-2 also could signal through heteromeric complexes consisting of TGFBR2/ALK5/ALK3, leading to SMAD2 phosphorylation, and BMPR2/ALK5/ALK3 or BMPRII/ALK7/ALK6 leading to Smad3 phosphorylation.[11] BMPs also share all type 2 receptors with activin A, which should be kept in mind when developing targeted therapies to these TGF-β family members.[3, 12] Additionally, TGF-β family type 3 receptors and coreceptors exist that contribute to regulation and fine-tuning of formation of the ligand-receptor complex.[13] The affinity of a single ligand to a single receptor will thus be of less importance than the affinity of a given ligand to the complete receptor-signaling complex, including coreceptors. Receptor expression will

vary between cell types, the state of the cells and the microenvironment in which they reside.

1.4 Non-SMAD signaling pathways

Besides signaling through the canonical SMAD pathway, receptors of the TGF-beta superfamily may activate mitogen-associated protein kinases (MAPKs) p38, Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), the PI3 kinase-AKT-mTOR pathway and nuclear factor kappa beta (NFkB).[2] The focus of this review, however, is on the SMAD signaling pathway.

1.5 TGF-β/BMP induced growth arrest

TGF-β/BMPs potently induce cell cycle arrest in the G1 phase, but the mechanism varies between cell types. Here, a simplified version of the TGF-β/BMP induced cytostatic response is presented. MYC promotes cell proliferation and growth and TGF-β induced downregulation of MYC is a key event in this cytostatic process that was discovered over 20 years ago.[14] Cell cycle progression from G1 to S phase is dependent on the activity of cyclin-dependent kinases (CDKs). *CDKN2B* and *CDKN1A*, encoding the CDK inhibitors p15 and p21, are suppressed by MYC. MYC inhibits MYC-interacting zinc finger 1 (MIZ1) mediated activation of *CDKN2B* and *CDKN1A*. Thus, downregulation of MYC is necessary for their induction.[15, 16] CDC25A is involved in cell cycle progression by dephosphorylating and activating CDKs. Downregulation of CDC25A also seems to be important for the TGF-β/BMP cytostatic response. The inhibitor of DNA binding (ID) proteins are also involved in the cytostatic response although it is not entirely clear how. The ID proteins (ID1, ID2 and ID3) are transcriptionally downregulated by TGF-β activated SMADs, whereas

BMP upregulates their transcription.[17] This might explain the fact that BMP exerts a much weaker growth-inhibitory effect on epithelial cells than TGF-β, in spite of higher induction of *CDKN1A*.[18]

1.6 TGF-β/BMP induced apoptosis

The mechanism for TGF-β/BMP induced apoptosis varies between cell types. The TRAF6-TAK1-JNK/p38 pathway is essential in TGF-β induced apoptosis in prostate cancer cells.[19] This pathway also depends on SMAD7 as a scaffolding protein, enabling activation of p38.[20] R-SMAD-dependent pathways are also involved in apoptosis and one important regulator of apoptosis, the tumor suppressor gene *TP53*, is regulated by both p38 and SMAD. Proposed mechanisms for TGF-β/BMP induced apoptosis in the B cell lineage include accumulation of proapoptotic Bim in B-lymphocytes and upregulation of Bik concomitant with downregulation of Bcl-xL in Burkitt's lymphoma cells and B-lymphocytes.[21, 22]

1.7 Regulation of BMP signaling

BMP signaling must be tightly controlled and aberrant signaling has been implicated in cancer and other diseases. Regulation includes the access of receptor to active ligands which is regulated by expression, activation by cleavage and the presence of antagonists. Other levels of regulation includes expression, activity and trafficking of receptors, the presence of coreceptors or pseudoreceptors, regulation of R-SMAD activity by I-SMADs, phosphatases and proteosomal degradation of receptors or SMADs.[2]

BMPs form homodimers or heterodimers before secretion and have to be activated by cleavage (reviewed in Umulis *et al*.).[23] In the extracellular space, availability of ligands is regulated by over 15 secreted cystine knot containing antagonists that bind specifically to BMPs or other members of the TGF-β superfamily and block BMP-signaling. The antagonists can be divided into three subgroups based on the size of the cystine knot: the DAN (differential screening-selected gene aberrative in neuroblastoma) family (eight-membered ring), twisted gastrulation (nine-membered ring), and chordin, noggin and follistatin (ten-membered ring).[24] The cysteine knot antagonists noggin, gremlin, follistatin, and sclerostin are all produced by osteoblasts and thus may be present in bone marrow (reviewed by Rosen)[25]. Noggin and gremlin caused endocytosis of BMPs, as opposed to chordin, that inhibited BMPs by sequestration.[26] The impact of extracellular BMP antagonists in cancer may be positive or negative, and the cellular context as well as the local concentration of BMP and extracellular antagonists is important for the outcome.

The type 3 receptors endoglin/CD105 and betaglycan/TGF-β receptor III (TBRIII) are transmembrane glycoproteins that have come forth as important regulators of TGF-β signaling in cancer.[13] They bind TGF-β family ligands and may act by presenting them to their type 1 and type 2 receptors. Both endoglin and betaglycan also exist in soluble forms that may prevent binding of ligand to receptor. Several other coreceptors for BMP exist that either inhibit or promote receptor assembly. One is the pseudoreceptor BMP and activin membrane-bound inhibitor (BAMBI) that prevents the formation of activated receptor complexes.[27] Other coreceptors that enhance BMP signaling are the repulsive guidance molecule (RGM) family, including DRAGON and hemojuvelin.[13]

There are also other molecules that modulate BMP-signaling, either by having antagonistic functions, by acting as coreceptors, or by influencing the downstream SMAD-signaling. Heparan sulphate (HS), *in vivo* mostly found as part of HS proteoglycans (HSPG) such as glypicans and syndecans, serves as a coreceptor for BMP, facilitating the formation of signaling complexes. HS also may serve to regulate the transport of BMP *in vivo*, limiting the range of BMP signaling. BMP antagonists may also bind HS, adding further complexity (reviewed in Umulis *et al*.).[23] Moreover, we found that CpG-oligodeoxynucleotide with a phosphorothioate backbone (PTO-CpG-ODN) antagonized BMP-induced activation of SMADs in mesenchymal stem cells as well as myeloma cell lines.[28] Other BMP binding molecules in the extracellular space include fibrillin and type IV collagen.[23]

BMP signaling involves internalization of ligand-receptor-complexes by endocytosis, either by clathrin-mediated endocytosis or by endocytosis of detergent resistant membrane fractions. BMP is thought to activate canonical SMAD signaling through clathrin-mediated endocytosis and SMAD-independent pathways through detergent resistant membrane fractions such as caveolae.[2]

BMP signaling is also regulated at many different intracellular levels. For instance, BMP receptors as well as R-SMADs are subject to post-translational modifications such as phosphorylation and ubiquitylation, regulating their stability and availability. Negative feedback loops involving I-SMADs (SMAD6 and SMAD7) and SMURFs (E3 ubiquitin ligases that target receptors and SMADs for proteosomal degradation) regulate the strength and duration of the signal.[27] I-SMADs bind activated receptors

and may compete with R-SMAD for binding, thus inhibiting R-SMAD phosphorylation. Other mechanisms by which I-SMADs regulate SMAD signaling include prevention of R-SMAD/SMAD4 heteromerization by binding to SMAD4, recruitment of SMURFs to receptors leading to receptor degradation, and direct inhibition of transcriptional responses.[27] SMAD7 inhibits both TGF-β and BMP signaling, whereas SMAD6 mainly inhibits BMP signaling, and more efficiently signaling by ALK3/6 than ALK1/2.[29] BMP signaling is also regulated by phosphatases that dephosphorylate C-terminal phosphorylated R-SMADs.[27] Other intracellular regulatory mechanisms also exist, but will not be discussed here.

2. BMPS IN MULTIPLE MYELOMA

2.1 Expression of components of the BMP signaling pathway

Myeloma cells in general express all known BMP receptors except ALK1 (ACVRL1), which is predominantly expressed by endothelial cells.[30-34] ALK6 (BMPR1B) is variably expressed.[31, 35] The SMADs -1, -4 and -5 are also expressed, but not SMAD8.[33] Expression of mRNA encoding the ligands BMP-6 and -4 has been found to be increased in plasma cells from multiple myeloma patients compared to healthy individuals.[31, 33] Normal bone marrow stromal cells (BMSC) that have been passaged in cell culture have been shown to express BMP mRNA and protein.[36, 37] In supernatants of human myeloma cell lines and in BM plasma, low levels of BMP-6 were found using ELISA.[33] Another study found more BMP-2 in sera from multiple myeloma patients than in normal sera, but still at low levels (~ 60 pg/mL).[38] We found increased levels of BMP-9 in myeloma sera compared to

normal sera.[35] However, the total BMP-activity in the bone marrow niche of multiple myeloma patients, as measured by *in situ* SMAD1/5/8-phosphorylation, is not known.

2.2 Expression of related receptors and ligands

Due to extensive sharing of receptors and possible heterogeneity of assembled ligand-receptor signaling complexes, expression of related TGF-β superfamily receptors and ligands is highly relevant. ALK5 (TGFBR1) is expressed in myeloma cells and is capable of being activated in response to TGF-β.[39] Both myeloma cell lines and primary cells were shown to express TGF-β mRNA and active protein,[40, 41] however it was also reported that myeloma cells do not produce active TGF-β.[39] Epigenetic silencing of TGFBR2 correlated with poor outcome in myeloma patients.[42] Using quantitative RT-PCR, mRNA expression of the TGF-β family type 1 receptors ALK4 and ALK7 was found in all eight myeloma cell lines tested (O E Olsen *et. al.,* NTNU). These are receptors for activins, nodal, myostatin, GDF-1,-3,-9,-10 and -11.[3]

Expression of the TGF-β type 3 receptor, betaglycan (TGFBRIII) is decreased in myeloma cells compared to normal cells.[43] Restoration of expression inhibited myeloma cell growth and proliferation independently of its ligand presentation role. Expression of the other type 3 receptor, endoglin, is induced by TGF-β and hypoxia,[44, 45] and increased levels of soluble endoglin in myeloma patients were associated with advanced disease and tumor growth.[46, 47] Membrane-bound endoglin is found on some myeloma cell lines, but lack on others.[35] Expression of selected receptors and their putative ligands in myeloma cells is summarized in Table 1.

2.3 Myeloma cell growth suppression by BMP

Only a few cytokines have been shown to inhibit myeloma cell growth, including BMP-2, -4, -5, -6, -7, and -9.[30, 33, 35, 48-50] BMP induces growth arrest and/or apoptosis in myeloma cell lines as well as primary cells in a dose- and time-dependent manner. Generally speaking, TGF-β and BMP share several events in the cytostatic response, including downregulation of c-MYC and upregulation of cyclin-dependent kinase (CDK) inhibitors. In contrast to TGF-β, BMPs upregulated ID expression in epithelial cells, whereas in B cells both TGF-β and BMP upregulated ID proteins concomitantly with induction of growth arrest.[37, 51-54] We also found *ID1-3* to be the most upregulated genes by BMP in MM cells.[50] However; the exact role of ID proteins in this setting remains elusive.

Downregulation of phosphorylated signal transducer and activator of transcription 3 (STAT3) and concomitant downregulation of the anti-apoptotic Bcl-xL, have been proposed to be involved in the mechanism for BMP-induced apoptosis.[48] Additionally, BMP-induced apoptosis has been linked to endoplasmic reticulum (ER)-stress and *TP53* status in myeloma cell lines.[55] We discovered that BMP induced apoptosis by downregulation of c-MYC, and further that many myeloma cells seemed to be addicted to c-MYC for survival.[50, 56, 57] The BMP-induced c-MYC downregulation was dependent on SMAD1/5/8 activity. Interestingly, patients with myeloma cells harboring translocations that placed *MYC* in the proximity of immunoglobulin enhancers were resistant to BMP, although SMAD1/5/8 was activated.[50]

The presence of BMPs in the bone marrow as described above raises the question: How do myeloma cells evade the action of BMPs, and may the presence of BMPs be one of the reasons why myeloma cells grow so slowly in the bone marrow? This is a complex matter since the activities of BMP depend on many other factors such as the presence of extracellular antagonists and coreceptors. What has been shown, is that a high expression of BMP-6 mRNA in primary myeloma cells predicted superior overall survival.[33] Furthermore, expression of the gene encoding the pseudoreceptor BAMBI correlated with poor prognosis.[58] These findings indicate that increased BMP activity might be advantageous for patient prognosis. Knowledge of the total activity of R-SMADs downstream of the complete pool of BMPs, and taking the presence of antagonistic activity into account might be a better measure of how BMPs impact myeloma cell growth. Thus, measuring SMAD1/5/8 phosphorylation in cancer cells *in situ* using bone marrow biopsies could shed more light on these questions. This has not yet been done in multiple myeloma, but in a xenograft mouse model of breast cancer, both TGF-β and BMP pathways were activated in bone metastatic lesions.[59] Moreover, BMP signaling has also been implicated in osteolytic lesions in prostate cancer.[60]

2.4 Effects of other TGF-β family members on myeloma cell growth

Mutations in the TGF-β signaling pathway are not common in hematological cancers.[61] Abundant TGF-β in the areas of destructive bone lesions in bone marrow may contribute to suppression of bone formation in myeloma patients.[45] TGF-β has been shown to induce production and secretion of IL-6 and vascular endothelial growth factor (VEGF) by BMSC and myeloma cells, contributing to increased proliferation and survival of the malignant cells.[40, 62] TGF-β does not

induce growth arrest in myeloma cells *in vitro*.[40] This could be explained by disturbed localization or trafficking of TGF-β receptors.[63] Another explanation may be that the cyclin dependent kinases (CDK) CDK2 and CDK4 can phosphorylate SMAD2/3, thereby inhibiting transactivation of genes.[64] In primary bone marrow myeloma cells SMAD2 was phosphorylated by CDK2 and the transcriptional regulation by TGF-β was disrupted.[39] The SMAD2/3 binding protein Pin1 regulates TGF-β signaling by inducing degradation of SMAD proteins.[65] Pin1 is overexpressed in most myeloma cell lines and may thus play a role in the defective TGF-β response in these cells.[45] Although TGF-β does not directly affect myeloma cell growth, inhibition of TGF-β in multiple myeloma still may be a potent therapeutic approach. By inhibiting TGF-β the number of osteoblasts may increase, which in turn may lead to enhanced growth suppression of myeloma cells.[45]

Like TGF-β, activin A activates SMAD2/3 proteins. Activin A primarily signals through ALK4 or ALK7 in complex with type 2 receptors. In murine myeloma cells, activin A was found to induce apoptosis.[66] In our panel of human myeloma cell lines, however, activin A did not significantly affect cell proliferation or survival (O E Olsen *et. al.,* NTNU).

The GDF-15 ligand is a distant member of the TGF-β superfamily. Myeloma BMSCs have increased expression GDF-15 compared with normal BMSCs, and GDF-15 could replace BMSC in supporting long-term growth of a stromal cell-dependent myeloma cell line.[67] Moreover, GDF-15 induced the expansion of tumor-initiating myeloma cells and supported the self-renewal potential of myeloma cells.[68] To date it is not known through which receptors GDF-15 signals.

2.5 Modulation of BMP-signaling by TGF-beta family members

Sharing of receptors and components of downstream signaling pathways influences the outcome of BMP-signaling in myeloma cells. In a cell culture dish the effects of a single ligand can be evaluated in a controlled manner. *In vivo*, however; a complex mixture of available ligands and antagonists exists and the repertoire of receptors on neighboring cells also influences the final outcome. Possible mechanisms for modulation of BMP-signaling in myeloma cells by other TGF-β family members may involve competition for common receptors or downstream pathway components such as SMAD4 or influence on regulatory feedback mechanisms, such as inhibitory SMADs.

2.6 BMP and bone disease in multiple myeloma

Myeloma cells in the bone marrow usually proliferate slowly and have the capability to influence bone cells, causing both osteolytic lesions and systemic osteoporosis. Thus, BMPs were initially discovered by their ability to induce bone formation. Numerous *in vitro* and *in vivo* studies have shown that BMPs have osteogenic effects. Moreover, overactive signaling by BMP receptors may cause severe diseases with increased bone formation, such as fibrodysplasia ossificans progressiva (FOP).[69]

Factors in bone marrow that are known to be elevated in multiple myeloma, such as hepatocyte growth factor (HGF) and dickkopf-1 (DKK-1), may inhibit the osteoinductive effects of BMPs.[70, 71] HGF was found to inhibit BMP-induced osteoblast differentiation of human mesenchymal stem cells.[72] This effect was partly explained by the fact that HGF abrogated nuclear translocation of BMP-

activated R-SMADs. In contrast, HGF was found to induce the expression of BMP-2 in human osteoblast-like cells, in part by activating Runx2.[73] Autocrine Wnt signaling is necessary to promote BMP-2 induced differentiation in pre-osteoblasts.[74] Expression of the Wnt inhibitor DKK-1 correlated with lytic bone disease in MM and DKK-1 antagonized BMP induced osteoblast differentiation.[74] Sclerostin also antagonized BMP and/or Wnt activity, although the mechanism is not clear.[75] Recombinant sclerostin was proposed to antagonize the growth inhibitory effect of BMP-6 on a myeloma cell line *in vitro*, but the specificity was not entirely clear from this assay.[33] Sclerostin is expressed by osteocytes but can also be expressed by myeloma cells and high serum levels in myeloma patients correlated with advanced disease stage.[76, 77] Another Wnt inhibitor, sFRP-2, is secreted by myeloma cells and suppresses bone formation.[78]

Myeloma cells may secrete interleukin (IL)-3, and IL-3 from the bone marrow plasma of myeloma patients inhibited both basal and BMP-2 induced osteoblast formation.[79] IL-3's bone remodeling effects were shown to depend on increased activin A secretion from $CD14^+$ bone marrow monocytes.[80] Activin A levels were increased in bone marrow plasma of myeloma patients with osteolytic disease and has been proposed to play an important role in myeloma bone disease, at least in part by inhibiting phosphorylation of SMAD1/5/8 during osteoblastogenesis.[81, 82] Lenalidomide treatment has been shown to cause activin A secretion by myeloma BMSC, providing a rationale for a combination treatment of lenalidomide with activin A inhibition.[83] TGF-β is abundant in the bone marrow of myeloma patients, and represses bone formation in osteolytic lesions. At first, TGF-β induces expansion of osteoblast progenitors and promotes bone mineralization. What happens next is that

TGF-β inhibits osteoblast differentiation and matrix mineralization, making TGF-β a possible target for therapy of myeloma bone disease.[45]

2.7 BMP and hepcidin-related anemia in multiple myeloma

In MM, the most common cause of anemia is the anemia of chronic inflammation characterized by hypoferremia, normal to increased ferritin levels, and reduced saturation of transferrin. Hepcidin has been described as the principal iron-regulating hormone and is encoded by the gene *HAMP*.[84] BMP-2, -4, -6, and -9 all stimulated *HAMP* promoter activity *in vitro* whereas TGF-beta did not.[38] Thus, the conclusion drawn from this work was that BMP-2 was the main mediator of hepcidin induction in sera from myeloma patients. Targeting BMP-2 or other BMPs with the intention to treat anemia of multiple myeloma may not be a good approach, considering that there is substantial redundancy in the system regulating hepcidin. To counteract hepcidin induction other factors may have to be targeted simultaneously. Also, interfering with the positive effects induced by BMPs on inhibition of myeloma cell growth and promotion of bone formation could give unwanted side-effects. Therefore, a better way of treating anemia in multiple myeloma would be to target hepcidin itself.

3. THERAPEUTIC TARGETING OF THE BMP SIGNALING PATHWAY

3.1 Recombinant human BMP

BMP-2 and BMP-7 have been used in preclinical and clinical studies of different scenarios of bone regeneration including non-union, bone defects, open tibial fractures and spinal fusion (reviewed in Nauth *et al*.).[85] The only reports found on use of BMPs in myeloma patients were two case-reports with use of rhBMP-2. The

first report describes a patient that experienced induction of heterotopic bone formation after spinal fusion surgery.[86] The other report describes a patient initially not diagnosed with myeloma, where the disease exacerbated after lateral lumbar interbody fusion supplemented with BMP-2.[87] Despite good effects on bone formation, there are concerns that BMP treatment may have adverse effects such as seroma formation, neurologic deficits and heterotopic induction of bone.[88] Moreover, the potential roles of use of recombinant BMPs in neoplasia are not fully understood.

3.2 Ligand traps

The activin ligand trap Sotatercept (ACE-011; Celgene/Acceleron Pharma) is a fusion product of the extracellular domain of activin receptor type IIA (ActRIIA) and an IgG-Fc fragment. This fusion protein may bind and inhibit the activity of several members of the TGF-β family of ligands, including BMPs. Activin A levels are increased in bone marrow plasma of MM patients with osteolytic disease and activin A was shown to inhibit osteoblastogenesis.[81] Sotatercept has been employed in mouse models of MM and shown reduced osteolytic bone disease and metastases as well as inhibition of tumor growth.[81, 82] Sotatercept has also been tested in a phase I/II clinical study of MM patients with osteolytic lesions, and preliminary data showed increased bone formation, improvement in skeletal metastases, decreased bone pain as well as antitumor activity.[89] Activin receptor signaling has also been implicated in erythropoiesis and Sotatercept may be effective in anemia of multiple myeloma and other cancers.[90, 91] Recently, treatment with different doses of Sotatercept combined with melphalan, prednisolone, and thalidomide was evaluated in 24 myeloma patients and found to be safe and generally well-tolerated.[92] The

immunomodulatory drug lenalidomide was shown to induce activin A secretion from BMSC and an ongoing phase I trial will combine Sotatercept with lenalidomide and dexamethasone.[83]

An ALK1-based ligand trap named Dalantercept (ACE-041; Acceleron Pharma) has also been developed and was tested in a phase I study with advanced solid tumors and some patients with relapsed or refractory multiple myeloma.[93] ALK-1 is predominantly expressed by endothelial cells and is only known to bind the two ligands BMP-9 and -10.[94] Thus, the proposed beneficial effects of this treatment would be inhibition of angiogenesis. As we have shown that BMP-9 is an inducer of growth arrest and apoptosis in myeloma cells, a possible side-effect could be increased growth of myeloma cells.

A third ligand trap, a soluble BMPR1A/ALK3 fusion protein, inhibited signaling by BMP2 and BMP4 concomitantly with a surprising increase in bone mass.[95] This finding highlights the complexity of BMP signaling in bone. We previously showed that soluble ALK3 inhibited BMP-4-induced growth arrest in myeloma cells *in vitro*.[30] Possible effects of this treatment on myeloma tumor growth *in vivo* remains to be investigated. In view of what is known regarding hepcidin regulation, it would also be interesting to know if any of the ligand traps influence serum hepcidin levels in myeloma patients.

3.3 Receptor-targeting antibodies

Drugs developed to prevent ligand binding to receptor using variants of soluble receptors may neutralize all ligands that bind to the receptor and thus block ligand

signaling through other receptors. This may be particularly problematic in the TGF-β family where there is a high degree of promiscuity between ligands and receptors. Signaling is clearly context dependent. Thus, *in vivo* studies are necessary to understand how ligand-receptor inhibition actually functions in the body. More specific targeting the ligand of interest could be safer, but the many different functions of each ligand make also this approach challenging. Moreover, development of such antibodies is not easy as the BMP receptors are highly conserved among different species. Examples of antibodies targeting BMP receptors to influence biological processes are anti-ALK1.[96]

3.4 Synthetic inhibitors of BMP signaling

The discovery of the small molecule inhibitor dorsomorphin, that prevents activation of R-SMADs, but not MAPKs upon ligand binding, has been important in understanding how BMPs signal.[97] Dorsomorphin inhibits activation of BMP type I receptors, but has minor effects on activation of TGF-β or activin type I receptors. The exact mechanism for the inhibitor is not clear; however it is likely that the kinase domain of the type I receptor is inhibited via competition for ATP-binding. The fact that MAPK-activation is unaffected by dorsomorphin, suggests other sites are important for MAPK-activation than for SMAD-activation. However, high concentrations of dorsomorphin affected SMAD-, as well as p38 MAPK- and AKT-signaling.[98] Homologues of dorsomorphin have been developed which have a higher degree of specificity for the type I BMP-receptors.[99] Recently, an inhibitor that more specifically targets ALK2 was developed, that possibly could help distinguishing ALK2 signaling from signaling through other type 1 BMP receptors.[100]

4. CONCLUDING REMARKS

By their involvement in a number of processes in development and cancer, and adding the complexity of their regulation and modes of signaling, BMPs are difficult to control in a specific manner. We have here reviewed the roles of BMPs in the hematological malignancy multiple myeloma with regards to inhibition of cancer cell growth, osteolytic bone disease and hepcidin-related anemia. It would be of interest to investigate the *in situ* situation in the bone marrow of myeloma patients with regards to SMAD1/5/8 activation. *In vitro* studies suggest that it could be of advantage to try to increase bone marrow BMP activity in multiple myeloma. Relevant *in vivo* studies on this are however needed to prove this concept. It should also be noted that many attempts to treat cancer by manipulating TGF-β/BMP-signaling are focused on inhibition of signaling, whereas in myeloma an opposite effect could be of greater benefit. Thus, in multiple myeloma there is a chance that by increasing BMP activity one could kill two birds with one stone; namely eradicate myeloma cells and counteract bone disease.

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FIGURE LEGEND

Figure 1. BMP signal transduction

Ligand binding enables the constitutively active type 2 receptor to activate the type 1 receptor. R-Smads are phosphorylated after binding to the activated type 1 receptor. Two activated R-SMADs and one Co-SMAD form a heteromeric complex that translocates to the nucleus where it can regulate transcription of specific target genes. The canonical pathway is regulated at several steps including: Regulation of the access to ligand by BMP binding to extracellular BMP antagonists (like noggin, gremlin, follistatin etc.), membrane-bound or soluble type 3 receptors that either facilitate or inhibit formation of a ligand/receptor signaling complex, and by inhibitory SMADs-6 and -7 that regulate signaling by different mechanisms. In myeloma cells, activation of R-SMADs leads to downregulation of target genes such as the oncogene c-MYC, concomitantly with induction of growth arrest and/or apoptosis.

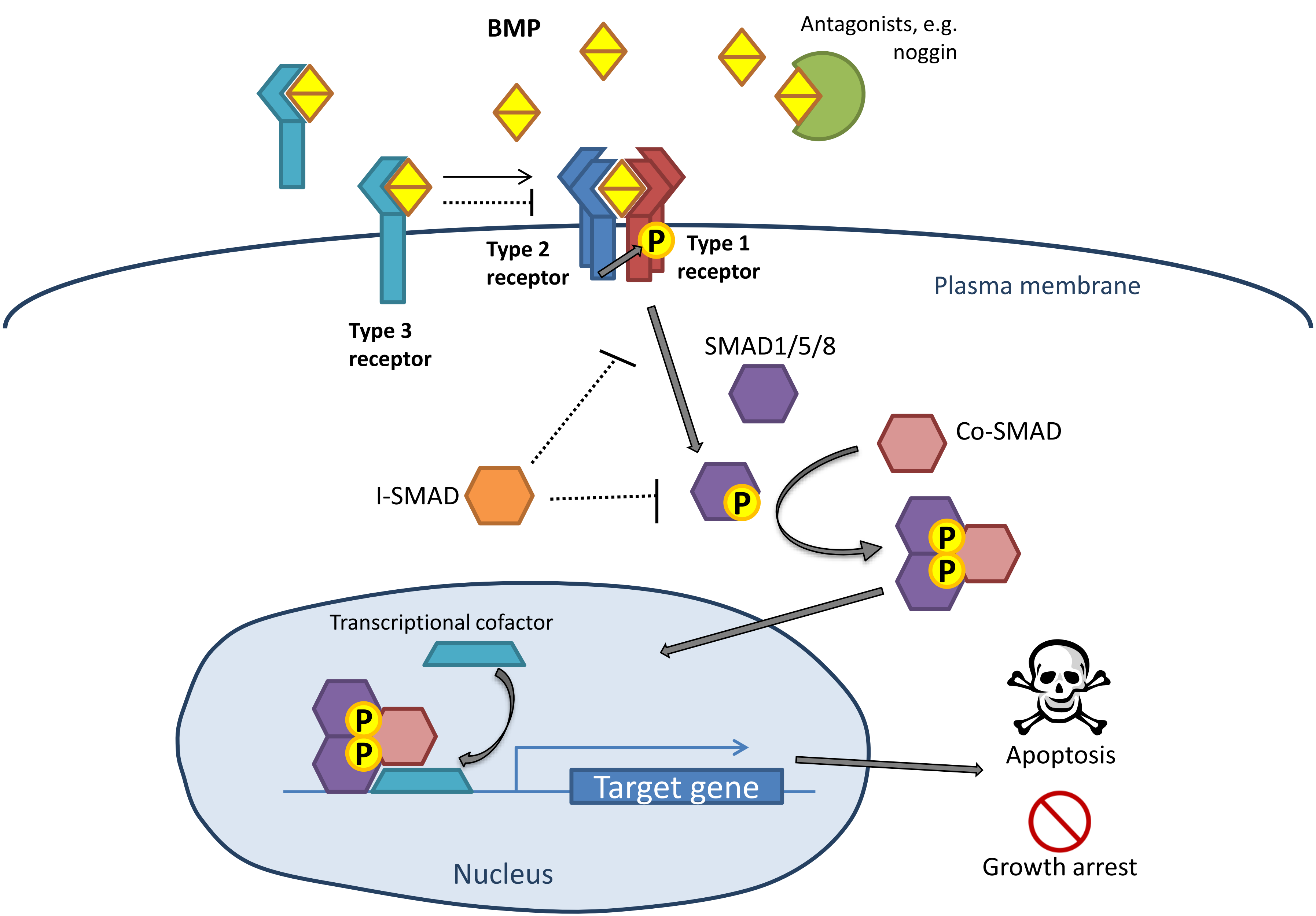
BMP
Antagonists, e.g. noggin
Type 2 receptor
Type 1 receptor
Type 3 receptor
Plasma membrane
SMAD1/5/8
Co-SMAD
I-SMAD
P
Transcriptional cofactor
Target gene
Nucleus
Apoptosis
Growth arrest

Table 1. BMP-, activin-, and TGF-β-receptors and their putative ligands in multiple myeloma

	Receptor	Alternate names	Myeloma cell expression	Putative ligands
Type 1	ALK1	ACVRL1	No	BMP9, BMP10, TGFβ
	ALK2	ACVR1	Yes	BMP6, BMP7, BMP9, BMP2
	ALK3	BMPR1A	Yes	BMP2, BMP4, BMP5, BMP6, BMP7, BMP10, BMP12-14
	ALK4	ACVR1B	Yes	Activin A
	ALK5	TGFBR1	Yes	TGFβ
	ALK6	BMPR1B	Yes/No	BMP2, BMP4, BMP6, BMP7, BMP10, BMP12-15
	ALK7	ACVR1C	Yes	Activin A
Type 2	ACTRII	ACVR2A	Yes	BMP2, BMP4, BMP6, BMP7, BMP9, BMP10, BMP-12, BMP14
	ACTRIIB	ACVR2B	Yes	BMP2, BMP6, BMP7, BMP9, BMP10, BMP14
	BMPRII	BMPR2	Yes	BMP2, BMP4, BMP6, BMP7, BMP9, BMP10, BMP12-15
	TGFBR2		Yes/No	TGFβ
Type 3	TGFBR3	Betaglycan	No	TGFβ
	Endoglin	CD105	Yes/No	BMP9, TGFβ