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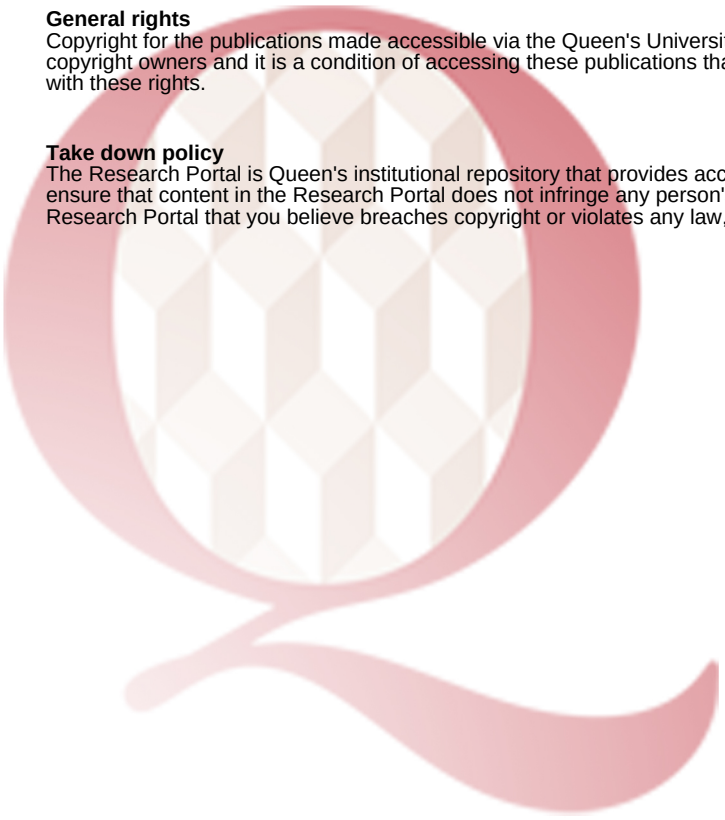
Brazil, D. P., Church, R. H., Surae, S., Godson, C., & Martin, F. (2015). BMP signalling: agony and antagony in the family. Trends in Cell Biology, 25(5), 249-264. DOI: 10.1016/j.tcb.2014.12.004

Published in:
Trends in Cell Biology

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal



BMP signalling: Agony and Antagony in the family

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Keywords

Bone morphogenetic proteins, antagonist, miRNA, Gremlin, disease

Abstract

Bone morphogenetic proteins (BMPs) are secreted extracellular matrix-associated proteins that regulate a wide range of development processes, including limb and kidney formation. A critical element of BMP regulation is the presence of secreted antagonists that bind and inhibit BMP binding to their cognate Ser/Thr kinase receptors at the plasma membrane. Antagonists such as Noggin, Chordin, Gremlin (Grem1) and twisted gastrulation-1 (Twsg1) have been shown to inhibit BMP action in a range of different cell-types and developmental stage-specific contexts. Here, we review new developments in the field of BMP and BMP antagonist biology during mammalian development, and suggest strategies for targeting these proteins in human disease.

Introduction

The first bone morphogenetic protein (BMP) was discovered by Dr. Marshall Urist, an orthopaedic surgeon in UCLA, in the 1960s. These proteins were shown to trigger the formation of bone and cartilage from mesenchymal stem cells in culture [1]. Since then, more than 22 members of the BMP family have been identified, along with a smaller set of plasma membrane receptors that activate a well-defined canonical signalling pathway involving the Smad1/5/8 proteins. Today, it is clear that BMP signalling extends beyond bone and cartilage formation, and is involved in such diverse biological processes as stem cell and organ formation, muscle development, iron metabolism, vascular biology and cancer. In addition, it is increasingly appreciated that a counterbalance of BMP and TGFβ signalling exists in many physiological processes and disease states. In 2010, we published a review in this journal summarising, to the best of our ability, the “state of play” regarding BMP signalling. It is an

indication of the pace of progress in the BMP field that a new review updating readers on developments is warranted a mere four years later. The emerging data describing BMP-TGFβ counter-regulatory signalling will also be discussed herein.

BMP signalling

BMPs are secreted members of the transforming growth factor-beta (TGFβ) family of signalling molecules. Both secreted BMPs and their antagonists are thought to associate with the extracellular matrix, restricting their diffusion and action to neighbouring cells [2]. Glycosylation of these proteins likely affects their interaction with the ECM and their function [3]. A range of BMP ligands bind to type I receptors (BMPRI or activin-like kinase (ALK)-2, ALK3 or ALK6). This complex then binds to a type II receptor (BMPRII), which phosphorylates the type I receptor in the GS glycine-serine repeat domain [4, 5]. The activated type I receptor phosphorylates a set of Smad proteins called receptor-Smads (R-Smad1/5/8), which bind to a nuclear Smad called Smad4. This complex accumulates in the nucleus, where it is recruited to transcriptional complexes to mediate BMP-dependent gene transcription (Fig. 1). Smad-response elements are present in BMP gene targets such as inhibitor of differentiation (Id 1-3) genes, SnoN, and inhibitory Smad6 [6-8], which mediate many of the downstream effects of BMP signalling.

A similar pathway is utilized by TGFβ ligands, which engage a distinct set of membrane receptors, and involve Smad2/3 as the R-Smads that regulate TGFβ-mediated gene expression. Each level of the BMP pathway is tightly regulated, emphasising the critical nature of maintaining tight control of BMP signalling in cells and tissues. BMP ligands are synthesised and secreted as larger propeptides that are then cleaved by extracellular pro-protein convertases such as Furin [9, 10]. Mature BMPs form dimers which interact with BMPRI/II receptors forming a hexameric complex (Fig. 1).

New data has identified additional membrane proteins that may regulate BMP signalling. Endoglin (CD105), a type I membrane glycoprotein, is a novel co-receptor for TGFβ1/BMP signalling [11]. Endoglin regulates BMP-9 and BMP-10 signalling via interaction with the ALK1/type I receptor, and TGFβ1 signalling via ALK5/type II TGFβ receptor binding [12]. Members of the repulsive guidance molecule (RGM) family of receptors have also been shown to be required for BMP, but not TGFβ signalling [13]. Receptors such as RGMa and DRAGON (RGMb) are required for BMP-2 and BMP-12 mediated gene expression, whereas Hemojuvelin (RGMc) is involved in regulating BMP-dependent iron homeostasis via hepcidin expression in liver [14]. Another co-receptor called Cripto interacts with the ALK4 type I receptor for Nodal, a member of the TGFβ family [15].

Both BMP (Smad1/5/8) and TGFβ (Smad2/3) signalling requires Smad complexes to transduce their signals to the nucleus. Anchor proteins such as Endofin recruit and present Smad1 proteins to the BMP receptors for phosphorylation, and also mediate receptor dephosphorylation via its protein phosphatase binding motif [16]. SARA (Smad anchor for receptor activation) regulates TGFβ1-mediated Smad2/3 phosphorylation in a similar manner [17]. Additional proteins such as ERBIN and C18ORF1 compete with SARA for binding to Smad2/3 to influence TGFβ1 signalling [18, 19]. Both Endofin and SARA bind to PI3K in the endosomes, and are regulated by EGFR signalling [20, 21]. Binding of SARA to RNF11 as part of the ESCORT-0 complex also regulates lysosomal degradation of EGFR [21, 22].

In contrast to rapid substrate phosphorylation observed with receptor tyrosine kinases engaged by growth factors such as insulin and epidermal growth factor, the kinetics of BMP-mediated Smad1/5/8 phosphorylation are much slower [23]. One reason for this may be the competition between Smad1/5 and inhibitory Smad6 for binding to the type I receptor [24, 25]. The methyltransferase PRMT1 methylates Smad6 on Arginine, leading to Smad6

dissociation from the type I receptor, thereby facilitating Smad1/5/8 phosphorylation and BMP signalling (Fig. 1, [23]). Similar repression of BMP signalling is facilitated by FK-binding protein 12 (FKBP12), which binds to BMP type I receptors and inhibits their activation (Fig. 1 [26]). Both biochemical and crystal structure data analysing the interaction of ALK2 receptor with FKBP12 has provided critical insights into the protein complex, suggesting reasons for why the R206H ALK2 mutation decreases FKBP12 binding, and leads to overactive BMP signalling and heterotopic ossification [27, 28]. Interestingly, FK506, a drug that binds to FKBP12 was shown to relieve this inhibition and reverse dysfunctional BMP-2 signalling in models of pulmonary artery hypertension [26]. A new protein in the BMP pathway called protein associated with Smad1 (PAWS1) also binds to Smad1 and is phosphorylated by ALK3/BMPR1A [29]. PAWS1 is required for Smad4-independent BMP-2 activation of ASNS and NEDD4 genes in PC3 prostate cancer cells [29].

Recent findings are providing evidence for crosstalk between BMP and other pathways such as TGFβ, Wnt, and Hedgehog. The type III TGFβ receptor (TGFβR3, also known as betaglycan [30] is required for BMP-2 signalling in epicardial cells [31, 32]. Endoglin, another co-receptor for BMP/TGFβ proteins has been shown to regulate crosstalk of TGFβ1 and fibronectin/αvβ1 integrin signalling in endothelial cells [33]. BMP pathways can engage Smad2 and Smad3 in embryonic cells and in invasive ovarian, prostate and breast cancer cells [34], while TGFβ1 can activate Smad1/5/8 phosphorylation in a range of epithelial cells, regulating breast cancer cell migration [35, 36]. Furthermore, TGFβ→ALK5→Smad3 signalling potently inhibits BMP-induced gene transcription and cell invasion via the formation of a Smad3 and pSmad1/5 complex that binds to BMP-response elements, ultimately repressing BMP target gene transcription [37]. This finding suggests that Smad3 is not only critical for TGFβ-induced inhibition of BMP signalling, but also contributes to limit the transcriptional output in response to TGFβ [37].

Crosstalk between BMP and Wnt/β-catenin signalling has been identified in several cell types. Indeed, activation of Wnt3a or overexpression of β-catenin/TCF4 activated BMP-2 expression in osteoblasts [38]. Also, BMP-2 induced osteoblast differentiation via the rapid generation of reactive oxygen species (ROS), linking BMP-2 to NADPH oxidase-4 (Nox4)-generated ROS and osteoblast differentiation [39]. In addition, Dishevelled/Par1b can facilitate TGFβ1 signalling during *Xenopus* mesoderm development and in mammalian HEK293 cells [40]. Others demonstrated that BMP-2 mediated chemotaxis of mesenchymal C2C12 mouse myoblast cells occurs via PI3Kinase signalling, with BMPRII binding to the p55γ/p110α class 1a of the PI3Kinase family [41]. BMP-2 mediated generation of PIP3 triggered recruitment of the LL5β protein, and was required for actin reorganisation and chemotaxis in these cells [41].

Negative regulation of BMP signalling

BMP signalling is regulated on multiple levels in cells, including intracellularly by inhibitory Smads (Smad 6, 7), miRNAs, methylation and extracellularly by pseudoreceptors such as BMP and Activin Membrane Bound Inhibitor (BAMBI) and BMP antagonists including Grem1 (Fig. 1, [7, 8]). For example, expression of BAMBI in endothelial cells reduces non-canonical TGFβ1-mediated Smad1/5 and ERK1/2 phosphorylation, resulting in the inhibition of angiogenesis [42]. Below, we discuss emerging mechanisms controlling BMP signalling.

BMP Antagonists: new insights from crystal structures

BMP signal transduction is closely regulated by a set of structurally diverse extra-cellular secreted protein antagonists, which bind BMPs with high and specific affinity and disrupt ternary receptor complex formation. These antagonists range in size from 170-250 amino acids for the DAN/Cerberus family (including Gremlin1, PRDC and Coco) to larger multi-

domain proteins such as Chordin (948 aa) and Follistatin (344 aa). BMP antagonists are secreted in a pro-form and the leucine/valine rich signal sequence (20aa) is cleaved by proprotein convertases, revealing the N-terminus BMP-interacting domain [43].

BMP-antagonist crystallography has provided new insights into the activity and nature of their molecular interactions [44-47]. Human BMP antagonists do not share significant sequence similarity overall (Fig 2); however, identity increases towards the C-terminus, also termed the cystine knot domain (or Von Willebrand type C domain). The cystine knot is a defining feature of BMP antagonists, and is formed by 6 cysteine residues: two pairs of intramolecular disulphide bonds that form a ring, and a third cysteine pair which bonds through the ring completing the knot. TGFβ family members have seven conserved cysteine residues, whereas BMP antagonists have 6 cysteine residues. Other conserved structural features of the TGFβ family members are that of the wrist and knuckle epitopes [48]. The knuckle epitope is formed by four anti-parallel β-sheets and the wrist is formed by a four-turn alpha-helix at the region of dimerization. Two BMP monomers form an antiparallel dimer, covalently linked through a disulfide bond. Ternary co-crystal 3D structures of BMP-BMP-receptor complexes show that type I receptors interact with the wrist motif and type II receptors interact with the knuckle region [49-51]. The BMP antagonists Noggin and Chordin have 4 additional amino acids, generating ten-membered rings. The disulphide bridges in the cysteine rings ensure a strict structural conformation of the antagonists by ensuring correct folding of the peptide, backbone stability and exposure of key hydrophobic residues [43, 48].

Two co-crystal structures of BMP-BMP antagonist vividly demonstrate the similarities and differences in antagonist binding. The first co-crystal, BMP-7 in complex with Noggin, reveals a butterfly structure (Fig. 3a). The structure also reveals that the Noggin dimer forms a two-fold axis of symmetry with a head-to-head conformation rather than the overlapping

antiparallel conformation of its BMP ligand [44]. The Noggin clip extends and interacts with both wrist and knuckle residues, thus obstructing the BMP ligand to type I and type II receptor binding [44]. The second co-crystal, BMP-2 in complex with von Willebrand type C (VWC1) domain of Crossveinless-2 (CV2), shows considerable similarity in the prevention of BMP receptor binding, with CV2 antagonist interactions occurring at both wrist and knuckle epitopes of BMP-2 (Fig. 3b). Sequence similarity in the clip regions of Noggin and CV2, however, is not significantly shared [47]. A third structure, Follistatin in complex with Activin, highlights further antagonistic diversity by blockade of type I and type II receptor binding sites by a peripheral clamp mechanism and not with clip domains as observed with Noggin and CV2 [52, 53].

The VWC1 domain of CV2 is responsible for binding BMPs and is not only found in Chordin family members, but has also been identified in a diverse range of other extracellular proteins [47]. This X-ray resolved co-complex structure reveals the interaction of the VWC1 domain, but does not fully explain the intricacies of its binding. It still remains unclear as to how the linear peptide of the clip segment contributes strongly to the overall binding energy, yet is assumed to be highly flexible when unbound. A second structural ensemble of VWC1 unbound to other proteins resolved by NMR revealed that the clip segment and a 30-residue subdomain termed SD1 of the VWC domain is preformed in its unbound state (Fig. 3c). The highly flexible nature of the clip segment exhibited strong affinity to BMP-2. The NMR structure showed that the N-terminal segment of the clip was flexible and disordered, whereas subdomain 1 exhibited a small and rigid three-stranded β sheet core. This rigidity contributed to the pre-defined orientation of the clip in a paperclip or hook-like architecture that brought the clip in close proximity to its final BMP binding site; therefore, likely lowering the overall binding energy cost and increasing affinity to the complex [54, 55].

Further, a recently detailed set of data demonstrates that the DAN family of protein antagonists form highly stable non-covalent dimers [56]. The antagonists, Protein Related to Dan or Cerberus (PRDC, also known as Gremlin2) and DAN, form non-covalent homodimers that do not require the unpaired cysteine residue of the cystine knot [56]. PRDC and DAN dimers are highly stable, as they did not dissociate after treatment with DTT, heating to 100 ^{o}C, or incubation with 4M urea [56]. The crystal structure of PRDC/Gremlin2 has also been resolved, and it shows that PRDC forms a non-covalent head-to-tail growth factor-like dimer with an extensive hydrogen bond network between monomers (Fig. 3d, [46]). Mutagenesis of PRDC identified residues belonging to the DAN domain on the convex surface, rather than the N-terminus that are critical for BMP binding affinity. An N-terminal latch mechanism for BMP binding was therefore proposed due to the observed flexibility and potential for conformational sampling of the N-terminal domain that exposes the DAN domain residues upon interaction with a BMP ligand [46].

The diversity of structures already seen within the family of BMP antagonists provides mechanistic and functional information that contributes to our understanding of the finely tuned specificities and affinities for BMP antagonists to BMP ligands and, in turn, to BMP signal transduction. The structures of many more cysteine knot domain containing proteins, BMP antagonists and BMP co-complexes, remain to be resolved, and this information will aid in the understanding of BMP antagonist-mediated regulation of BMP signalling in physiological and disease conditions.

Interactions between BMP antagonists

A complex choreography of interactions between BMP antagonists has recently been demonstrated. Noggin and Grem1 interact to maintain a BMP signalling-free zone in the

mouse embryo, which is required for Sonic hedgehog (Shh)-mediated induction of the sclerotome or early vertebrae [57]. Moreover, limb development requires the regulation of Grem1 and Fgf10 expression by HoxA and HoxD genes, further supporting a link between Grem1 signalling and Shh signalling [58]. Noggin and Grem1, but not Chordin, were shown to be important for BMP-4 mediated clathrin-dependent endocytosis in mouse endothelial cells [59]. Using fluorescently labelled BMP-2, BMP-2 was found to be internalised in HeLa cells via a clathrin-dependent pathway, with Noggin and Grem1 increasing BMP-2 uptake. In contrast, Chordin decreased BMP-2 uptake, suggesting BMP ligand and receptor interactions on the cell surface involve cooperative binding of BMP antagonists such as Noggin and Grem1, as well as other proteins such as the Endoglin CD105 co-receptor [60]. Another example of antagonist cooperation was recently demonstrated for the BMP modulators BMP endothelial cell precursor derived regulator (BMPER) and twisted gastrulation (Twsg1). BMPER is the human ortholog of crossveinless-2 found in Drosophila, and was shown to activate BMP-4 at low concentrations, but inhibit BMP-4 signalling at higher concentrations, in an endocytic trap-and-sink mechanism in mouse endothelial cells [59]. BMPER has also been implicated in endothelial cell biology and angiogenesis, where the BMP antagonist Twsg1, but not Noggin or Chordin, was found to increase HUVEC sprouting in vitro and endothelial cell growth in a Matrigel plug assay in vivo [61, 62]. Interestingly, these Twsg1-dependent effects were inhibited by the addition of recombinant BMPER, suggesting a delicate equilibrium exists whereby Twsg1 and BMPER interact to control each other's pro-angiogenic activity in endothelial cells [61].

MicroRNA regulation in BMP signalling

There has been a dramatic increase in the identification of miRNAs that regulate BMP signalling (Table 1). Among these is miR-21, which has been detected in skin epidermis, specifically keratinocytes, and is highly expressed in hair follicle tumours [63]. miR-21 is a downstream target of BMP-4 in mouse keratinocytes, and treatment of these cells with BMP-4 dramatically reduced miR-21 levels, an effect that was reversed by overexpression of the BMP antagonist Noggin [63]. Furthermore, miR-21 regulates two groups of BMP-4 target genes in keratinocytes that are involved in tumour suppression and cell differentiation. In addition, BMP-4 downregulates the miR302~367 cluster in a Smad1/5 dependent manner in human primary pulmonary artery smooth muscle cells (PASMCs) [64]. BMPRII was found to be the target of miR302, and therefore inhibition of miR-302 by BMP-4 increases BMP-4 signalling by stabilizing the BMPRII transcript [64]. Also, miR-656 represses the expression of BMPR1A in U87 glioma cells and inhibits glioma tumorigenesis [65]. Similarly, BMP-2 mediated glioma growth was inhibited by lentiviral miR-656 expression in mice suggesting a tumour suppressor role for miR-656 [65]. MiR-130a also targets BMP type I receptors, in this case ALK2 in liver cells [66]. The levels of miR-130a are increased by iron deficiency, which leads to a decrease in BMP-6/Smad1/5 signalling. As a result, levels of hepcidin, the main iron regulatory hormone in the body, are reduced, leading to increased iron availability in the circulation [66]. miR-22 has been identified as a master regulator of BMP-7/6 in the kidney [67], where BMP-7/6 have been proposed to act as anti-fibrotic BMPs in chronic diseases of the kidney, lung and other tissues (e.g. [68]). miR-22 deletion reduces the severity of kidney injury induced by unilateral ureteral obstruction (UUO), with higher levels of both BMP-7 and BMP-6 evident in miR-22-/- kidneys post-UUO [67]. A concomitant increase in BMPRIb levels and pSmad1/5/8 phosphorylation was also observed in miR-22-/- kidneys, with miR-22 binding sites identified in the 3' untranslated region of BMP-7, 6 and BMPRIb [67]. Interestingly, miR-22 is itself a transcriptional target of BMP-7/6 signalling, with

several BMP response elements identified in the miR-22 promoter. This study identifies miR-22 as a key regulator of kidney fibrosis, and suggests that an auto-feedback loop likely exists between BMP-7/6 and miR-22 in the normal kidney and regulates kidney physiology (Table 1).

As well as inhibiting the expression of BMPs and their membrane receptors, some miRs have been shown to target BMP antagonists. Noggin expression is repressed by miR-200c/141 in dental epithelial-like cells through transcriptional upregulation of miR-200c by Pitx2, which binds to promoter elements in the miR200c/141 cluster to control the development of mouse incisors [69]. Similar to miR-22, expression of miR-200c is regulated by BMP signalling, creating a negative feedback loop during tooth development [69]. Noggin3 expression is also controlled by miR-92a during cartilage and skeletal formation in Zebrafish [70]. Degradation of Noggin3 mRNA by miR-92a allows sustained BMP activity, which facilitates the survival and differentiation of chondrocytes [70]. Therefore, miR-92a and Noggin3 act in opposition to regulate BMP signalling during cartilage formation. In addition, miR-27b directly targets the 3' UTR of Grem1, and regulates Grem1-mediated gene expression changes in lung fibroblast cells, adding to the efforts to identify the as-yet-undefined role of miR-27b in fibrosis in vivo (Table 1, [71]).

BMP antagonist signalling: focus on Gremlin1

Grem1 has been well characterised as a secreted antagonist that regulates BMP action during development, controlling limb and kidney formation [73, 74]. New data have identified that Grem1 may have its own intrinsic signalling capability, independent of BMP antagonism (Fig. 5). In kidney studies, treatment of mouse mesangial cells with high glucose or conditioned medium containing Grem1 increased the expression of TGFβ1, CTGF and collagen type IV proteins associated with diabetes-induced damage to the glomerulus [121].

Increased ERK1/2 phosphorylation was also observed in cells treated with Grem1, likely contributing to the enhanced mesangial cell proliferation observed under these conditions [121]. Exposure of human tubular epithelial cells (HK-2) to recombinant Grem1 caused phenotypic changes resembling epithelial-mesenchymal transition (EMT), with decreased E-cadherin and increased myofibroblast markers such as vimentin and alpha smooth muscle actin (α-SMA) [122]. Grem1 had a similar profibrotic effect on renal fibroblasts, and silencing of Grem1 using siRNA prevented TGFβ1-induced EMT in HK-2 cells [122]. Grem1 has also been implicated in aristolochic acid-induced EMT and fibrosis [123].

Several reports have identified novel non-BMP binding partners for Grem1. Grem1 can bind to Slit proteins to negatively regulate monocyte chemotaxis [124], and Grem1 can bind to fibrillin microfibrils in mesothelioma cells ([89]). A novel function for Grem1 is as a proangiogenic regulator where Grem1 can bind to VEGFR2 in a similar manner to that of VEGF in endothelial cells and can increase angiogenesis in vitro and in vivo [125]. This effect involves Grem1 binding to heparin and heparin sulphate proteoglycans on the surface of endothelial cells [126]. In addition, the engagement of $\alpha_v\beta3$ integrins and the formation of $\alpha_v\beta3$/VEGFR2 complexes are involved in Grem1-mediated angiogenesis [127]. The identification of Grem1 as a novel proangiogenic factor has implications in highly vascularised tumours and also in the field of endothelial cell biology. Recently the effect of Grem1 on human umbilical cord haematopoietic progenitors was explored, showing that the balance between Grem1 and BMP-2 and BMP-4 are involved in atherosclerotic plaques [128, 129] . The phosphorylation of ERK1/2 is a downstream effect of Grem1 activation (e.g. [89, 121]. Consistently, embryonic fibroblasts isolated from *grem1*-/- mice display reduced ERK phosphorylation compared to wild-type cells [130]. The BMP antagonist Gremlin2 (also called PRDC) has recently been shown to activate JNK signalling in embryonic stem cells during their differentiation into atrial cardiomyocytes [131].

BMP and BMP antagonist signalling in development and disease

The critical role of BMPs and their secreted antagonists in development and disease has been highlighted by the identification of dramatic phenotypes in mice lacking either BMPs or BMP antagonists (e.g. [72-76]). In the adult, it is increasingly appreciated that subversion of the equilibrium between the activities of BMP agonists and antagonists may underlie several pathologies including cancer, skeletal disorders and fibrosis of kidney, lung, liver, eye and heart. In addition, a counterbalance between BMP and TGFβ signalling exists in many tissues and disease contexts, whereby BMP signalling can act to "dampen" TGFβ signalling and vice versa (Fig. 4). In addition, BMP antagonists can act to amplify TGFβ signalling via inhibition of BMP signalling. Some recent examples of this are discussed below.

Cancer

BMPs and their antagonists play a critical role in stem and progenitor cell biology regulating the balance between differentiation and expansion respectively. In basal cell carcinoma, cancer-associated fibroblasts secrete the BMP antagonists follistatin and Grem1 [77]. These antagonists act in a paracrine fashion to facilitate self-renewal and continued proliferation of cancer cells, overwhelming BMP control of proliferation. In human basal cell carcinoma Grem1 expression was detectable in the tumour stroma but not in adjacent normal skin [77]. Recently, Grem1 was identified at the cancer invasion front, suggesting a role for this BMP antagonist in colorecetal cancer metastasis [78, 79]. Grem1 has also been identified as a prognostic marker of pancreatic neuroendocrine tumours, and correlates with increased angiogenesis and increased patient survival [80].

In melanoma, autocrine inhibition of cell proliferation by BMP-7 was attenuated by the BMP antagonist Noggin which promotes tumour progression [81]. The BMP antagonist Coco has also been demonstrated to play an important role in promoting proliferation of breast cancer

cells which have extravasated to the lung. Initially, local production of BMPs limits the proliferative capacity of these cells, which is overcome by the antagonistic activities of Coco. Importantly, the Coco expression signature has been shown to predict metastatic relapse to the lung in humans [82]. In contrast to this oncogenic role, inhibition of BMP signalling has been shown to suppress tumour growth and lung metastases in a murine model of breast cancer [83].

Within a tumour microenvironment, progression versus stasis may be dependent on cancer stem cell (CSC) mediated-self renewal or differentiation. BMP-2 regulates CSC-induced differentiation, suggestive of a net tumour suppressive role. Increased BMP-2 expression, but conversely, decreased BMP-2 activity was detected in CSCs isolated from glioblastomas [84]. This apparent paradox was explained by the enhanced secretion of Grem1 from CSCs, leading to inhibition of BMP-2 and increased p21 signalling [84, 85]. TGFβ1, in contrast, acts to maintain cancer stem cells in their undifferentiated state, and antibodies such as 1D11 which target the TGFβ1 receptor have been shown to have efficacy in certain cancer subtypes (Fig. 4, [86, 87]).

The CSC example above provides a useful example of the opposing actions of BMPs versus TGFβ1 to maintain homeostasis in different cells and tissues, which is an important theme emerging from the field. The crosstalk in BMP and TGFβ1 signalling has been discussed above, and other examples of BMP versus TGFβ1 signalling in tissue fibrosis and EMT and regulation by KCP-1 will be discussed below. A further example of BMP versus TGFβ balance involves the formation of muscle mass, where BMP-mediated signalling increases muscle mass, whereas myostatin, a member of the TGFβ/activin family negatively regulates this process (summarised in Fig. 4, [88]).

Grem1 is highly expressed in mesothelioma tumour samples and primary mesothelioma cells. The high expression of Grem1 along with Slug, a transcriptional regular of E-cadherin, is connected with resistance to paclitaxel-induced cell death. Interestingly, silencing Grem1 with siRNA inhibits cell proliferation and induces a reduction in cancer cell survival upon treatment with paclitaxel [89]. It was suggested that upregulation of fibrillin-2 provides a mechanism for Grem1 localisation to the extracellular matrix of the tumour (Fig. 5, [89]). Grem1 has been shown to bind to A549 lung cancer and HeLa cells in a BMP and VEGFR2 independent manner [90]. Additionally, stably transfected A549 cells expressing Grem1 increased tumour growth in vivo compared to mock transfected A549 cells, further suggesting that Grem1 may potentiate tumour growth (Fig. 5, [90]).

Diabetes and Diabetic Retinopathy

The dual BMP/Wnt antagonist Sostdc1 (also known as USAG-1) plays a role in pancreatic islet function. Levels of Sostdc1 were upregulated in islets from non-immune-mediated lean diabetic mice, and a subset of *sostdc1*-/- mice displayed enhanced insulin secretion and improved glucose tolerance after high-fat diet feeding compared to wild-type controls [91]. Interestingly, *sostdc1*-/- islets displayed significant reductions in Grem1 and CTGF expression, suggesting a complex interplay between the BMP modulators may exist in islets [91].

Both diabetic nephropathy (DN) and retinopathy (DR) are microvascular complications of diabetes that develop in a significant number of diabetic patients. The underlying mechanisms involved in DR overlap with DN (see below). For example, exposure of retinal pericytes to high glucose increased Grem1 expression [92]. A potential role of Grem1 in proliferative vitreoretinopathy was also identified [93]. Transition of lens epithelia to mesenchymal cells and subsequent matrix accumulation is a feature of glaucoma [94]. Grem1

expression is increased in the glaucomatous trabecular meshwork cells and tissues and elevates intraocular pressure (IOP) [95]. In this context, Grem1 potentiates the effects of TGFβ matrix accumulation by attenuating BMP-4 signalling [95]. Furthermore, treatment of human trabecular meshwork cells with recombinant Grem1 induced ECM cross-linking lysyl oxidase (LOX) genes [96]. Grem1-mediated LOX gene induction involved both canonical (Smad) and non-canonical (JNK and p38 MAPK) signalling [96]. These data provide important insights into the potential contribution of Grem1 to increased intraocular pressure and glaucoma.

Kidney disease

Human Greml1 was first described in the context of experimental models of diabetic nephropathy (DN), a chronic complication of diabetes associated with glomerulosclerosis and tubulointerstitial fibrosis [97, 98]. Further investigation revealed that i) increased expression of Grem1 correlated with DN disease severity [99], ii) a Grem1 gene variant was associated with DN in patients and iii) *grem1*+/- mice were protected from early stage sequelae of DN [100]. siRNA-mediated targetting of Grem1 in the kidney also resulted in protection from DN in a murine model, linked to increased BMP-7 activity [101] Consistently, tubular epithelial overexpression of Grem1 exacerbated injury in response to folic acid-induced nephropathy [102]. In podocytes, Grem1 aggravates injury to cells grown in high glucose, and triggers a downregulation of nephrin and synaptopodin, key proteins of the glomerular basement membrane [103]. siRNA targetting of Grem1 rescued podocytes from high glucose-induced injury, supporting the hypothesis that Grem1 is a primary driver of renal cell damage during diabetes. This study suggests that this effect may be due to Grem1 inhibition of BMP signalling, leading to increased TGFβ1-mediated Smad2/3 phosphorylation [103].

Mice lacking Grem1 die shortly after birth due to the absence of kidneys, arising from a failure of ureteric bud outgrowth and GDNF/Wnt11 signalling during embryogenesis [73]. The allelic reduction of BMP-4 reverses this phenotype, and *grem1−/−;BMP-4+/−* mice develop normal kidneys as a result of a corrected "volume" of BMP signalling [104]. Similarly, the complete inactivation of BMP-7 restored ureteric bud outgrowth in *grem1-/-* mice, but did not restore normal kidney formation due to the loss of nephrogenic progenitor cells [105]. BMP-6 null mice manifest increased tubulointerstitial damage and renal fibrosis in response to unilateral ureteric obstruction compared to wild-type mice [106], identifying BMP-6 as another major regulator of renal fibrosis in the kidney [107].

Further evidence for the importance of BMP agonist antagonist interactions in the mature kidney was provided by investigations of USAG-1 and Twsg-1. USAG-1 is the most abundant BMP antagonist expressed in the kidney and negatively regulates renoprotection by BMP-7 in numerous experimental models of glomerular and tubular injury [108]. Using a model of Alport syndrome (a hereditary form of nephritis), the deletion of USAG-1 attenuated renal injury likely due to enhanced BMP-7 suppression of MMP-12 expression [109]. Interestingly, the ability of the lipid lowering agent simvastatin to ameliorate renal fibrosis has been linked to the repression of USAG-1 expression, thus enhancing anti-fibrotic BMP-7 signalling [110]. This USAG-1/BMP-7 axis has also been implicated in supernumerary incisor formation, with enhanced BMP-7 signalling in *usag1*-/- mice thought to drive this process [111]. Podocyte injury and loss is considered an important factor in initiating glomerular injury and proteinuria in DN and other renal conditions. Twisted Gastrulation (Twsg1) has been shown to be the dominant BMP antagonist secreted by podocytes, and acts in synergy with chordin or chordin-like molecules to modulate BMP activity [112]. Twsg1 antagonises BMP-7-induced podocyte differentiation, and is expressed in damaged glomeruli of a mouse model of podocyte injury and proteinuria. Consistently,

twsg1-/-mice were relatively resistant to podocyte injury suggesting that future pharmacological strategies targetting Twsg1 may be a useful avenue for the treatment of renal disease [112].

Disorders of the liver

Gremlin, along with follistatin, was identified as a marker of liver fibrosis using gene array screens of hepatic stellate cells induced to undergo transdifferentiation into myofibroblasts [113]. Upregulation of Grem1 was also identified in chronic hepatitis, liver cirrhosis and liver cancer as a result of hepatitis C, with Grem1 expression correlating with the stage of liver cancer in the patients [114]. Using a CCl_4 mouse model of liver fibrosis, it was shown that treatment with BMP-7 could attenuate the severity of damage and improve liver function [115]. Levels of Grem1 were increased in the fibrotic liver, and treatment with BMP-7 further increased Grem1 expression, which is difficult to rectify given the current dogma regarding the pro-fibrotic role of Grem1 and the anti-fibrotic role of BMP-7. Furthermore, adenoviral delivery of BMP-7 suppressed CCl_4 induced liver fibrosis in mice [116]. Many of these effects are likely related to changes in TGFβ1 expression, which is thought to be the major cytokine driving liver fibrosis and regulating liver carcinogenesis [117].

Miscellaneous

BMPs and their antagonists such as BMP-4, BMP-7, Grem1 and Twsg1, are involved in lymphopoiesis, where they are expressed in specific compartments in the bone marrow and thymus [118]. Surprisingly, the conditional knockout mice lacking BMP-7 or Twsg1 in haematopoietic cells had no effect on B and T cell number [118]. However, Twsg1-deficient B cells demonstrated hyperresponsiveness after B-cell receptor stimulation [119]. Conditional knockout of Grem1 in the ovaries of female mice altered early folliculogenesis, but did not affect overall fertility compared to wild-type mice [120].

All of the data above point to a critical role for BMP and BMP antagonist signalling in serious human diseases such as cancer, diabetic kidney disease and liver fibrosis. It is clear that a delicate balance between BMP and TGFβ signalling exists in many cells, and perturbations in this balance as a result of changes in BMP antagonists such as Grem1 can contribute to the development of human disease. The following section will highlight recent efforts to develop new treatments for diseases where an imbalance of BMP/TGFβ signalling is implicated.

Therapeutic potential of BMP and BMP antagonists in human disease

Targeting BMPs in human disease

Pharmacological targeting of BMP action has long been a focus point for many. Given their key role in bone formation, the delivery of recombinant human BMPs has been developed to accelerate impaired fracture healing in the long bones and spinal cord (reviewed in [132, 133]). Recombinant human BMP-2 (available as InFuse® from Medtronic), and rhBMP-7 (available as OP-1 from Olympus) are sometimes used as adjunct therapies for the treatment of non-union fractures [134]. However, the therapeutic benefit of these rhBMPs is hampered by the high costs of treatment, a shortage of robust data from double blind clinical trials, and a range of adverse effects in patients [132, 135].

BMP-7 signalling has been a key target for reversing fibrosis or scar formation in the kidney, heart, lung and other organs. A wealth of *in vitro* and *in vivo* evidence suggests that BMP-7 possesses anti-fibrotic activity, due to its ability to reverse TGFβ1-mediated fibrosis in many tissues. For example, in the mouse heart, subcutaneous delivery of rhBMP-7 reduced cardiac fibrosis as a result of pressure overload, and also decreased vascular calcification due to excess vitamin D levels [136, 137]. Intracolonically delivered adeno-associated virus-mediated delivery of rhBMP-7 (AAV-BMP-7) reduced the severity of acute ulcerative colitis

in rats [138]. Oral administration of AAV-rhBMP-7 suppressed CCl_4-hepatic fibrosis in mice [116]. Delivery of AAV-rhBMP-7 also reduced the infarct size in a stroke model of middle cerebral artery occlusion in mice [139]. A gene therapy approach using gold nanoparticles containing the BMP-7 gene inhibited fibrosis in a rabbit model of corneal damage [140].

In the kidney, administration of rhBMP-7 has been shown to attenuate the severity of renal fibrosis induced by a range of insults including ischaemic injury [141], nephrotoxic serum nephritis [142] and diabetic nephropathy (DN) [143]. Despites its potential benefits, rhBMP-7 displayed a lack of efficacy in treating lung, skin or kidney fibrosis [144, 145]; however, several groups are still developing therapeutic agents based on BMP-7 and/or activation of the ALK3 BMPRIA receptor.

A peptide mimetic of BMP-7 called THR123 was recently developed. THR123 is a 16-amino acid cyclic peptide corresponding to the finger 2 region of BMP-7 and was designed based on the predicted BMP-ALK3 binding regions using TGF-β2 and BMP-7 crystal structures [146]. THR123 binds to the ALK3 receptor *in vitro*, and administration of THR123 reverses kidney fibrosis in a range of mouse models including nephrotoxic serum nephritis, diabetic nephropathy and the *col4a3* knockout mouse model of Alport syndrome [146]. However, some questions have been raised regarding the ability of THR123 to activate the ALK3 receptor, and whether a hydrophyllic peptide containing a C-terminal sequence that would favour digestion in the GI tract would reach therapeutic doses after oral administration [147]. Other small molecule activators of BMP signalling have been identified through a library screen of bioactive compounds using a BMP responsive luciferase assay in human cervical cancer cells [148]. Two lead compounds, both members of the flavonoid chalcone family, were identified and shown to have both canonical (Smad1/5/8 phosphorylation) and non-canonical (ERK phosphorylation) activity [148]. *In vivo*, these chalcone molecules induced

ventralisation of Zebrafish embryos, a hallmark of BMP activation during development [148]. Screening the Spectrum collection of drug compounds, natural products and bioactive molecules (2320 compounds in total) using BMP-responsive luciferase activity identified tilorone as a strong inducer of BMP activity. Importantly, tilorone decreased the degree of fibrosis in a mouse model of silica-induced lung fibrosis [149]. Increased pSmad1 phosphorylation was detected in the lungs of these mice, with concomitant reductions in TGFβ1 signalling [149]. These data, along with previous results using THR123 indicate that inducers of BMP-7 signalling may have therapeutic benefit for the treatment of fibrosis in the lung and kidney. Other strategies aimed at boosting BMP signalling in disease have focussed on the kielin/chordin-like protein-1 (KCP-1). KCP-1 (also called Crim2) binds to BMP-7 and enhances its engagement with the BMPRI receptor [150]. *Kcp1*-/- mice developed severe renal fibrosis in response to unilateral ureteric obstruction (UUO) and folic acid-induced nephropathy [150]. Conversely, KCP-1 binds to TGFβ1 and inhibits it interaction with its receptor [151]. Indeed, transgenic mice overexpressing KCP-1 in the proximal tubules displayed attenuated fibrosis in the kidney, and revealed that pSmad1 levels (BMP target) were increased, while pSmad3 (TGFβ1 target) was reduced (Fig. 4, [152]).

TGFβ1 is the primary pro-fibrotic cytokine that mediates tissue fibrosis, and strategies aimed at inhibiting TGFβ1 signalling (such as through BMP-7 and its analogues) have been pursued by many. Recently the administration of lipoxin A4 (LXA4), an anti-inflammatory lipid mediators that inhibits injury in the kidney and other tissues (e.g. [153-155]), have proven effective in reducing renal fibrosis in response to unilateral ureteric obstruction (UUO) in mice. The mechanism of LXA4 was a reduction in TGFβ1-mediated signalling and a corresponding decrease in extracellular matrix-associated gene expression in kidney epithelial cells [153]. The anti-fibrotic effect of LXA4 involves the induction of let7c miRNA, which targets several elements of the TGFβ1 signalling pathway [156]. MiRNA-

200b was also identified as a repressor of TGFβ1-induced epithelial-mesenchymal transition (EMT) via targeting of the E-box binding transcription factors ZEB1 and ZEB2 [157].

Targetting BMP Antagonists in human disease

While the therapeutic benefit of boosting BMP signalling is evident in fibrosis of the kidney and lung, other diseases, as a result of excessive BMP signalling, may benefit from BMP inhibition. An inhibitor of BMP signalling called Dorsomorphin was identified in a screen for molecules that disrupt dorsoventral patterning in Zebrafish embryos [158]. Dorsomorphin blocked pSmad1/5/8 phosphorylation via inhibition of ALK2, ALK3 and ALK6 receptor signalling [158]. Dorsomorphin also provided evidence for an essential physiological role for hepatic BMP signalling and iron metabolism [158]. Dorsomorphin and its derivatives (e.g. LDN-193189) reduced the severity of fibrodysplasia ossificans progressive (FOP) in mouse models, by inhibiting of BMP signalling [158, 159]. Moreover, Dorsomorphin induced the myocardial differentiation of mouse embryonic stem cells via inhibition of BMP signalling [160]. The ability of Dorsomorphin to disrupt dorsoventral patterning in zebrafish, due to "off-target" anti-angiogenic effects on the VEGF type 2 receptor (Flk1/KDR) [161]. Further structure activity studies identified a potent and selective inhibitor of ALK2 called DMH1 that disrupted zebrafish dorsoventral patterning but not vascular development [161]. DMH1 induced the formation of beating cardiomyocytes from mouse embryonic stem cells, highlighting a novel role for BMP inhibition during cardiomyogenesis [162]. In addition, a novel class of BMPRI ALK2 inhibitors, based on the structure of Dorsomorphin have been identified and the lead compound, K02288 inhibits BMP-4-mediated Smad1/5/8 phosphorylation at nanomolar concentrations in C2C12 cells. In addition, K02288 induced dorsalization of Zebrafish embryos, similar to that seen with Dorsomorphin [158, 163].

Targeting Grem1 in human disease

Given the wealth of data implicating increased Grem1 in diseases of the kidney, lung, liver and in cancer, an obvious strategy is to design therapeutic inhibitors of Grem1 to treat these conditions. Data supporting this hypothesis was provided by reports showing that *grem1*+/- mice developed less severe early symptoms of DN compared to wild-type [100]. In addition, siRNA-mediated targeting of Grem1 reduced the severity of kidney injury [101]. Furthermore Grem1 may be a potential target for lung disease, in particular idiopathic pulmonary fibrosis (IPF) and pulmonary artery hypertension (PAH). Grem1 is expressed in macrophages and the alveolar epithelial lining of the normal lung [164], and in the interstitium of lungs with IPF [164]. Transient overexpression of Grem1 in rat lungs using adenovirus resulted in alveolar epithelial cell activation and thickening, along with an increase in inflammatory cell infiltration [165]. Collagen deposition and accumulation of α-SMA myofibroblasts were observed in fibroblastic foci. Interestingly, the BMP-4 precursor protein co-immunoprecipitated with Grem1, suggesting that Grem1 binding to BMP-4 causing the reduction in Smad1/5/8 phosphorylation [165]. In parallel with Grem1 activation, FGF-10, an epithelium protectant, was elevated in fibrotic lung epithelial cells, whereas FGF-7 and 9 were decreased, suggesting that a Grem-BMP-FGF-10 loop may exist in the fibrotic lung [165].

It has previously been shown that mutations in the BMPRII are implicated in heritable PAH [166]. Levels of Grem1 are also increased in lung biopsies from PAH patients, likely as a result of hypoxia-induced upregulation in pulmonary endothelial cells [167, 168]. Similar to DN in the kidney, *grem1* haploinsufficiency protects against hypoxia-induced increases in vascular resistance in mice [167]. A novel strategy to target Grem1 using a therapeutic monoclonal antibody was recently developed and tested in a mouse model of PAH. Mice treated with the Grem1 targeting antibody showed a reduction in pulmonary vascular remodelling and right ventricular pathology [169]. In addition, a Grem1 antibody reduced

cancer cell migration and invasiveness, independent of BMP and VEGFR2 binding [90]. These data are an important proof-of-principle demonstrating that therapeutic targeting of Grem1 may provide new avenues to improve the treatment of cancer, as well as fibrotic conditions of the lung and kidney and other organs (summarised in Fig. 5).

Concluding remarks

This review has attempted to summarise the numerous, recent findings regarding BMP signalling. Despite a number of important advances in deciphering the signalling modalities of BMPs and their antagonists, many challenges remain. More experiments are needed to 195 antagonists during developmental processes, physiology and disease. A clear pattern of crosstalk and competing effects between BMPs and TGFβ is emerging in different tissues. The identification of cross-interactions between BMP antagonists such as Noggin and Grem1 presents additional complexities in elucidating BMP signalling [170]. There is a strong possibility that tissue and disease context may determine the specific interactions of BMPs and their antagonists, as well as with TGFβ. Identifying these interactions will increase the opportunities for pharmacological intervention to modify BMP/BMP antagonist signalling, similar to the Grem1 targeting approach developed in pulmonary artery hypertension. We eagerly anticipate future developments in this field, and emerging BMP-targeting therapies that will improve disease treatment and patient outcomes.

Figure Legends

Figure 1. Complex regulation of BMP signalling. BMPs are processed by proprotein peptidases to generate mature dimers which then bind to two copies of the type I and type II BMP receptors, generating a heterohexameric complex. Binding of BMP homodimers to their cognate receptors leads to phosphorylation of the type I receptor by the type II receptor in the

GS domain. Activated BMP receptors then phosphorylate Smad1/5/8 proteins which dimerise with Smad4 and accumulate in the nucleus, where they mediate changes in BMP-regulated gene expression. Regulation of this pathway occurs extracellularly via the binding of extracellular antagonists such as Grem1 and Noggin (1), or in the plasma membrane via the action of pseudoreceptors such as BAMBI (2). In addition, inhibitory constraints on receptor-mediated Smad1/5/8 phosphorylation occur via FKBP12 binding and inhibitory Smad6 binding, which is relieved by the action of a PRMT1 methyltransferase (3). Additional regulation of BMP signalling occurs via cytosolic phosphatases and ubiquitin ligases such as Smurf (4), and via miRNA (5) and methylation (6) mediated control of BMP-mediated gene expression.

Figure 2. Sequence homology of BMP antagonists. (a) Multiple sequence alignment of the cysteine knot regions of BMP antagonists. Red boxes indicate highly conserved cysteine residues. (b) Phenogram of BMP antagonists based on sequence similarity.

Figure 3. Structures of BMPs and BMP antagonists. Cartoon representation of protein structure of (a) BMP-7 in complex with Noggin (PDB entry 1M4U), (b) BMP-2 in complex with VWC1 domain of Crossveinless-2 (PDB entry 3BK3), (c) PRDC dimer (PDB entry 4JPH) and (d) NMR resolved unbound structure of VWC1 of CV2 (PDB entry 2MBK) superimposed to X-ray resolved bound structure of VWC1 of CV2 in complex with BMP-2 (PDB entry 3BK3). All protein structure representations generated using PyMol (DeLano 2002).

Figure 4. BMP and TGFβ signalling play counteregulatory roles in some cases of physiology and disease. Some examples of the counteracting regulation of cellular responses by BMP-7 and TGFβ are shown. BMP-7 signalling acts to inhibit fibrosis in kidney and lung, whereas TGFβ is well established as a primary fibrotic driver in many tissues. BMP-7

signalling is potentiated by the binding of Kielin/Chordin-like protein-1 (KCP-1), which facilitates BMP-7 binding to its cognate receptors. In contrast, KCP-1 binds to TGFβ and prevents it binding to its receptors, thus inhibiting its signalling. BMP-7 and TGFβ signalling are also counter balanced in cancer stem cell differentiation and the regulation of muscle mass (see text for details).

Figure 5. Grem1 signalling occurs via diverse mechanisms in cells. (a) Grem1 dimers bind to BMP dimers and prevent engagement of BMP receptors, preventing BMP signalling and gene expression (see text for details). (b) Grem1 binds to VEGFR2 in endothelial cells and promotes angiogenesis. Heparin sulphate proteoglycans (HSPGs) and αvβ3 integrins are required for this response [125, 126]. (c) Grem1 has been shown, via an unidentified mechanism, to activate cancer cell invasion and proliferation. This effect occurs independently of BMP VEGFR2 signalling [90]. (e) Grem1 can bind to Slit1 and 2 and facilitates their binding to the Robo receptor, leading to inhibition of monocyte chemotaxis [124]. (f) Grem1 associates with fibrillin microfibrils and triggers Slug expression, leading to EMT and mesothelioma cell survival [89]. (g) Grem1 can bind to and sequester BMP-4 precursor protein, preventing mature BMP-4 secretion [171].

Acknowledgements

We apologise to colleagues whose work was not cited in this review due to space limitations. Work in the Brazil laboratory is supported by Diabetes UK, Northern Ireland Kidney Research Fund and DEL Northern Ireland. Rachel Church is supported by a BBSRC CASE PhD studentship. Satnam Surae is funded by an Irish Research Council for Science Engineering and Technology PhD programme in Bioinformatics and Systems Biology.

Catherine Godson is supported by Science Foundation Ireland, The NIDDK Diabetes Complications Consortium and Roche Pharmaceuticals, Basel.

Table 1. Summary of miRNAs regulating BMP signalling.

miRNA	Target	Biological Function/Consequence	Reference
Osteoblast and Bone			
miR-140-5p	BMP-2	Enriched miRNA in undifferentiated hMSCs which directly represses BMP-2 expression and subsequent BMP-2 mediated osteogenesis, thereby negatively regulating osteogenic lineage commitment	Hwang S, 2014 [172]
miR-542-3p	BMP-7	Inhibits BMP-7-mediated osteogenesis, suppressing osteoblast differentiation and	Kureel J, 2014 [173]

		promoting apoptosis	
miR-208	Ets1	Regulates BMP-2 stimulated preosteoblast differentiation in a mouse cell line	Itoh T, 2010 [174]
miR-30 family	Smad1 Runx2	Negatively regulate BMP-2 mediated osteogenic differentiation in vitro	Wu T, 2012 [175]
miR-155	SOCS1	Induced by TNF-α. Targets SOCS1. Plays a role in modulating TNF-α inhibition of BMP induced osteoblast differentiation of MC3T3-E1 cells	Wu T, 2012 [176]
Cancer			
miR-885-3p	BMPR1A	Inhibits Smad1/5/8 phosphorylation and Id1 expression, supresses angiogenesis in vitro and in vivo, impairs HT-29 colon cancer cell xengraft growth in vivo	Xiao F, 2014 [177]
miR-656	BMPR1A	Downreguated in glioma cell lines and tissues. Overexpression of miR-656 suppresses glioma cell proliferation, neurosphere formation, migration and invasion, as well as tumour growth in vivo	Guo M, 2014 [65]
miR-365	SHC1 BAX	Induces gemcitabine resistance in pancreatic cells, Downregulation of apoptosis-promoting genes and upregulation of invasion-promoting genes in pancreatic cancer cells.	Hamada S, 2014 [178]
miR-192	RB1	Downregulated in breast cancer. BMP-6 treatment of MDA-MB-231 cells results in upregulation of miR-192. BMP-6 caused inhibition of cell proliferation in vitro and decreased tumour growth in vivo.	Hu F, 2013 [179]
miR-17-92a	TGFβR2 Smad2 BMP genes	Upregulated in cancer stroma, may contribute to cancer progression	Nishida N, 2012 [180]
Muscle			
miR-675-3p, 5p	Smad1 Smad 5 Cdc6	Promotes muscle differentiation and regeneration	Dey BK, 2014 [181]
miR-26a	Smad1 Smad4	Required for skeletal muscle differentiation and regeneration in vivo	Dey BK, 2012 [182]
Miscellaneous			
miR-30b	BMP-7	Inhibits BMP-7, is involved in EMT induced by methylglyoxal in peritoneal mesothelial cells in rat model	Liu H, 2014 [183]
miR-135a	BMPR1A BMPR1B	Overexpression of miR-135a inhibits transcription of BMPR1A and BMPR1B. May play a role in regulating tooth formation via regulation of BMP signalling	Kim EJ, 2014 [184]
miR-26a	Smad1	Overexpression of miR-26a inhibits pulmonary surfactant synthesis in type II epithelial cells from pulmonary alveolus	Zhang XQ, 2014 [185]

miR-26a	Smad1	Regulates angiogenesis in vitro and in vivo. Inhibits BMP/Smad signalling pathway. Targeting miR-26a, triggered angiogenesis and decreased myocardial infarct size in a mouse model	Icli B, 2013 [186]
miR-21	BMPRII RhoB	Hypoxia and BMPRII signalling upregulate miR-21 *in vitro* in human pulmonary artery endothelial cells. miR-21 expression is increased in pulmonary hypertension	Parikh VN, 2012 [187]
miR-21	BMP-dependent tumour suppressor genes	miR-21 expressed in epidermis and skin follicle epithelium. Downstream target of BMP-4 in mouse keratinocytes e.g. ID1-3, Msx-2	Ahmed MI, 2011 [63]
miR-302-367	TOB2 DAZAP2 SLAIN1	Maintaining pluripotency and self-renewal of human embryonic stem cells by targeting BMP inhibitors. Modulation of TGF-β, BMP signalling during neural induction	Lipchina I, 2011 [188]
miR-24	Trb3	miR-24 targets Trb3, decreasing Smad expression and BMP signalling PDGF inhibits BMP mediated changes in pulmonary smooth muscle cells and also induces expression of miR-24	Chan MC, 2010 [189]
miR-22	BMP-6 BMP-7 BMPR1B	Inhibits BMP-7 and -6 but also induced by BMP-7 and -6 via a negative feedback loop. BMP-7 and -6 expression are increased in kidneys of miR-22 null mice. Targeted deletion of miR-22 attenuated renal fibrosis in UUO model	Long J, 2013 [67]
miR-27b	Grem1	Regulates Grem1-mediated fibrotic gene expression changes in vitro	Graham JR, 2014 [71]
miR-92a	Noggin3	Targets Noggin3. Maintains BMP signalling during pharyngeal cartilage formation	Ning G, 2013 [70]
miR-302-367	BMPRII	BMP signalling downregulates miR 302-367 expression. Overexpression of miR-302 downregulates BMP signalling	Kang H, 2012 [64]

Table 2. Targetting BMP signalling in human disease.

Disease	Target	Novel treatment	Rationale	Outcome	Reference
Kidney	Alk-3	THR123	A peptide mimetic of BMP-7. Evidence for BMP-7 being anti-fibrotic	Reversed renal fibrosis in a range of mouse models including DN.	Sugimoto H, 2012 [146]

	Grem1	Grem1 siRNA	Grem1 contributes to pathogenesis of DN	Attenuated DN characteristics and recovered BMP-7 signalling	Zhang Q, 2010 [101]
Lung	BMPR2	FK506 (tacrolimus)	Dysfunctional BMPR2 signalling is implicated in pathogenesis of PAH	Reversed dysfunctional BMPR2 signalling in vitro Reversed severe PAH in vivo	Spiekerkoetter E, 2013 [26]
	Grem1	Grem1 antibody	Grem1 contributes pathogenesis of PAH	Reduced pulmonary vascular remodelling and right ventricular pathology in mouse model of PAH	Ciuclan L, 2013 [169]
	BMP	Tilorone	Increased Grem1 expression and decreased BMP signalling in idiopathic pulmonary fibrosis	Reduced degree of fibrosis in mouse model of silica-induced lung fibrosis	Lepparanta 0, 2013 [149]
Liver	ALK3 ALK2	LDN-193189 DMH2 VU0465350 (Antagonists of BMP receptors) VU0469381 (Antagonists of BMP receptors)	Inhibiting BMP signalling promotes liver regeneration	Inhibited Smad1/5/8 phosphorylation and in vitro and in vivo. Enhanced liver regeneration after partial hepatectomy. No effect on liver regeneration	Tsugawa D, 2014 [190]
	Hepcidin BMP-6	Neutralizing BMP-6 antibody	Hepcidin and hemojuvilin gene mutations implicated in juvenile hemochromatosis.	Inhibited hepatic hepcidin expression Increased serum iron and transferrin saturation in vivo	Andriopoulos Jr B, 2009 [191]
Skeletal	TGF-β	1D11 (Neutralizing antibody)	Altered TGF-β signalling contributes to pathogenesis of osteogenesis imperfect	Restored bone phenotype in *Crtap-/-* and Col1a2*tm1.1Mcbr* models of osteogenesis imperfecta and corrected lung abnormalities in *Crtap-/-* mice.	Grafe I, 2014 [192]
	ALK2	LDN-193189 (Inhibitor or BMP type I receptor kinases)	ACVR1 gene mutation that results in constitutive activation of ALK2	LDN-193189 inhibited Smad1/5/8 and reduced ectopic ossification in vivo	Yu, P 2008 [193]

			in patients with fibrodysplasia ossificans progressive (FOP)		
	rhGDF-5/β-TCP	rhGDF-5/β-TCP	rhGDF-5 has been shown to have osteoinductive properties and a rhGDF-5/β-TCP device has shown to promote periodontal regeneration in vivo	2- to 3-fold higher amount of new bone and new cementum formation with rhGDF-5/β-TCP compared to OFD alone Potential therapy for periodontal regeneration	Windisch P, 2012 [194]
Cancer	Grem1	Grem1 antibody	Grem1	Reduced cancer cell migration and invasiveness in a BMP and VEGFR2 independent manner	Kim M, 2012 [90]
Anaemia	Activin/TGF-β	RAP-011 (Soluble, activin receptor type IIA ligand trap)	RAP-011 is a novel erythroid stimulating agent that inhibits downstream signalling of activin or TGF-β members	Increased haemoglobin concentration, did not deplete splenic iron stores in hepcidin antimicrobial peptide overexpressing mice. Potential therapeutic for human anaemia	Langdon JM, 2014 [195]

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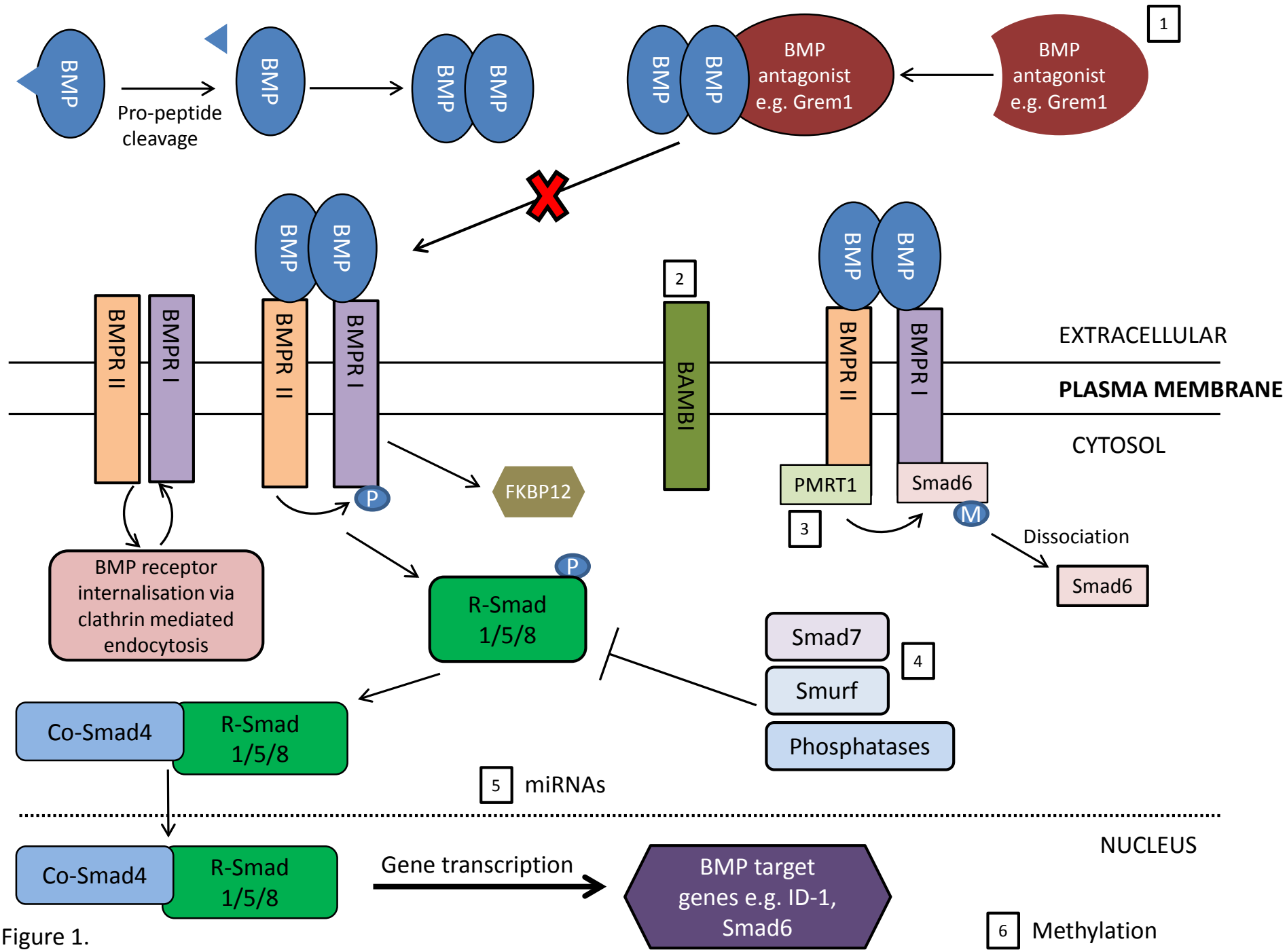
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Dissociation
Smad6
BMP receptor internalisation via clathrin mediated endocytosis
P
R-Smad 1/5/8
Smad7
Smurf
Phosphatases
4
Co-Smad4
R-Smad 1/5/8
5
miRNAs
NUCLEUS
Co-Smad4
R-Smad 1/5/8
Gene transcription
BMP target genes e.g. ID-1, Smad6
6
Methylation

Figure 1.

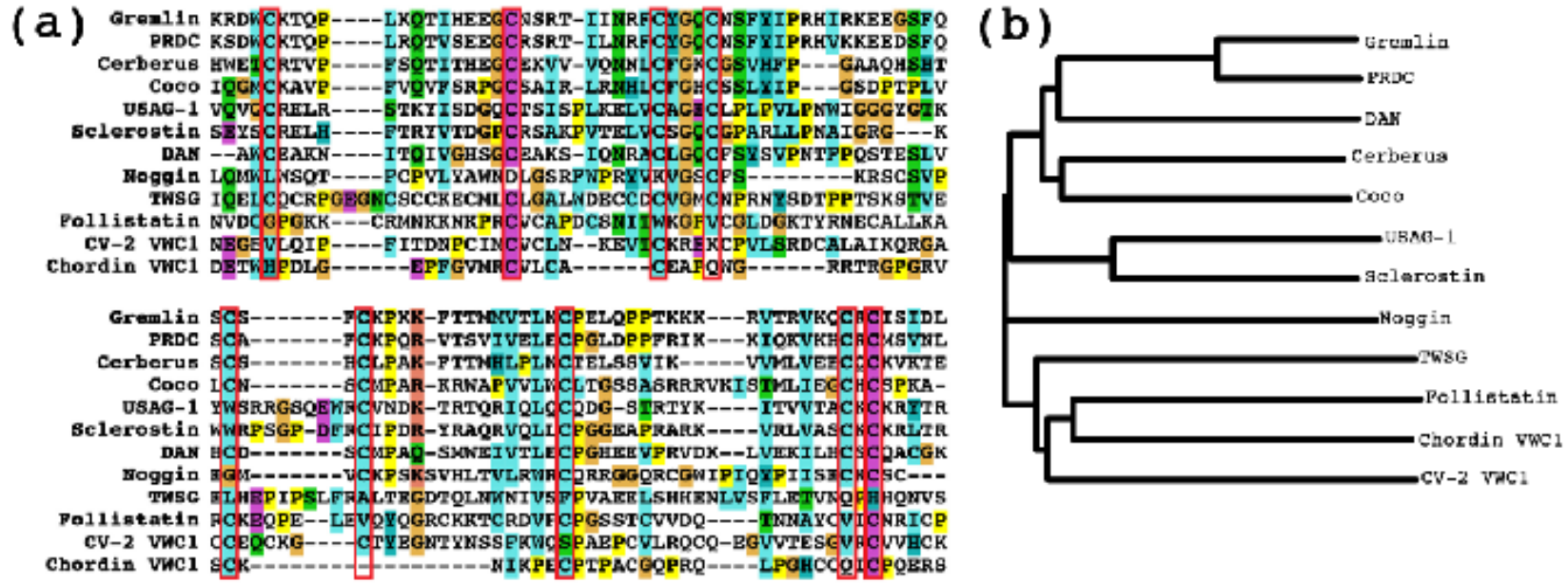

Figure 2.

Brazil et al., 2014

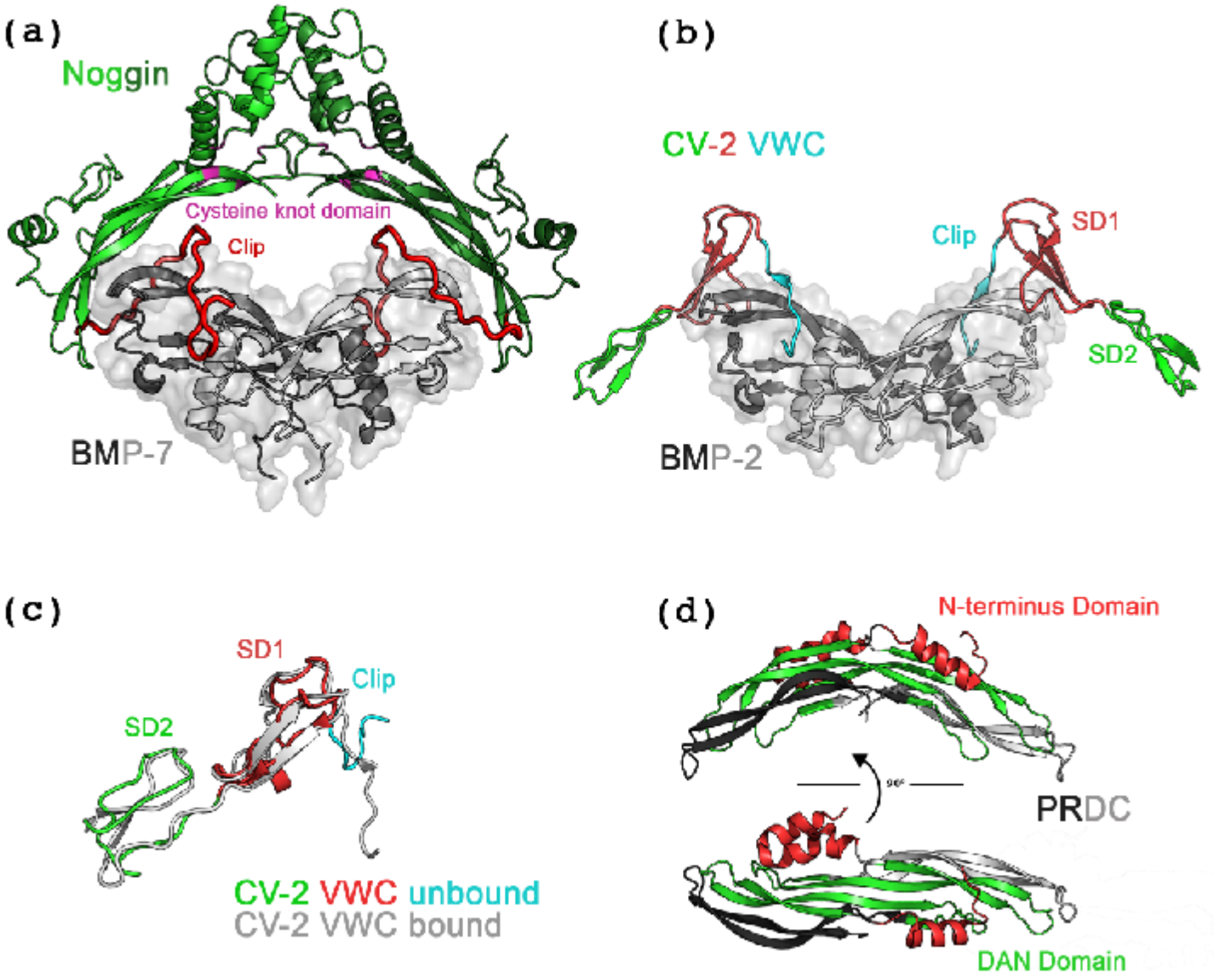

Figure 3.

Brazil et al., 2014

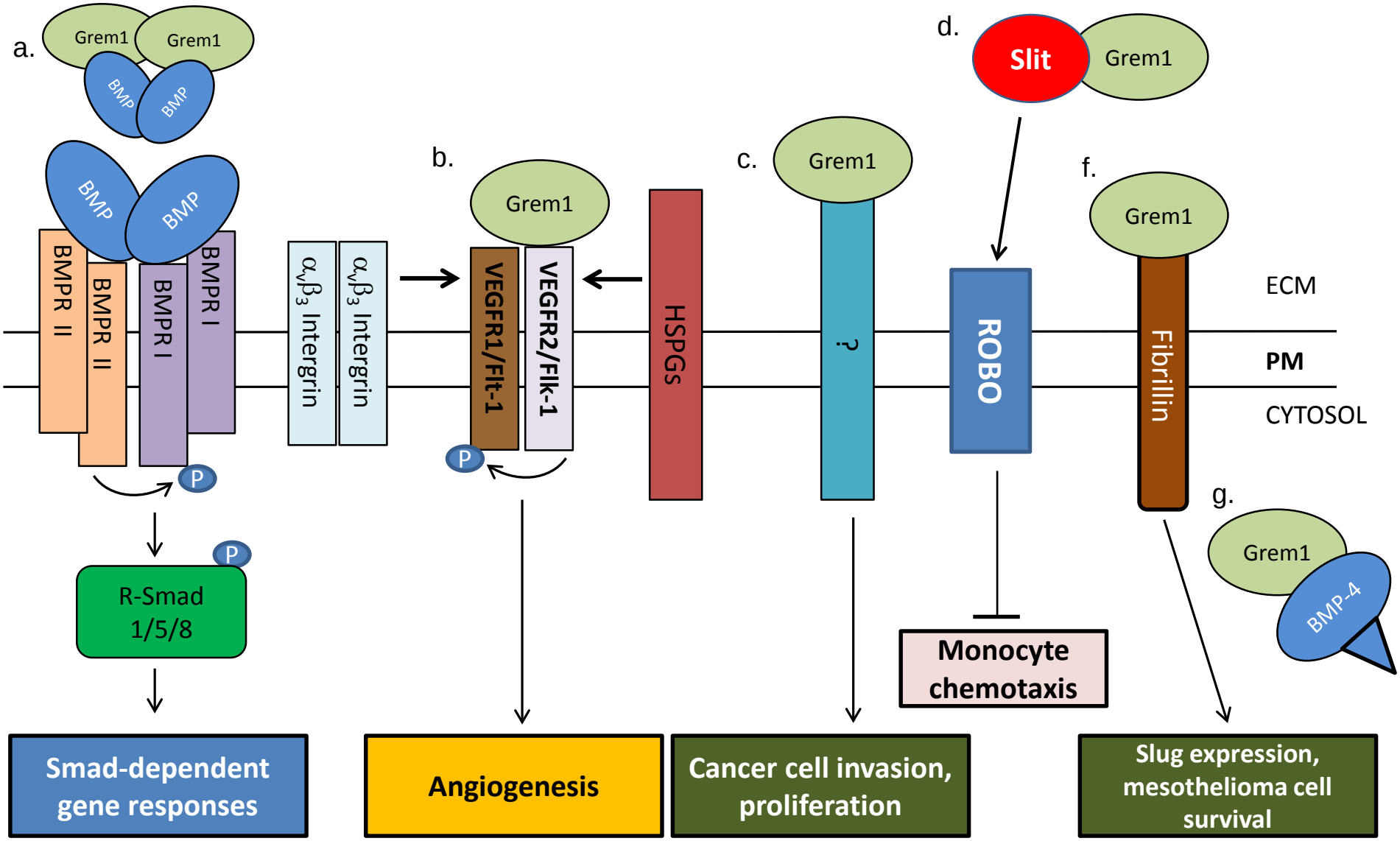

Figure 4.

Brazil et al., 2014

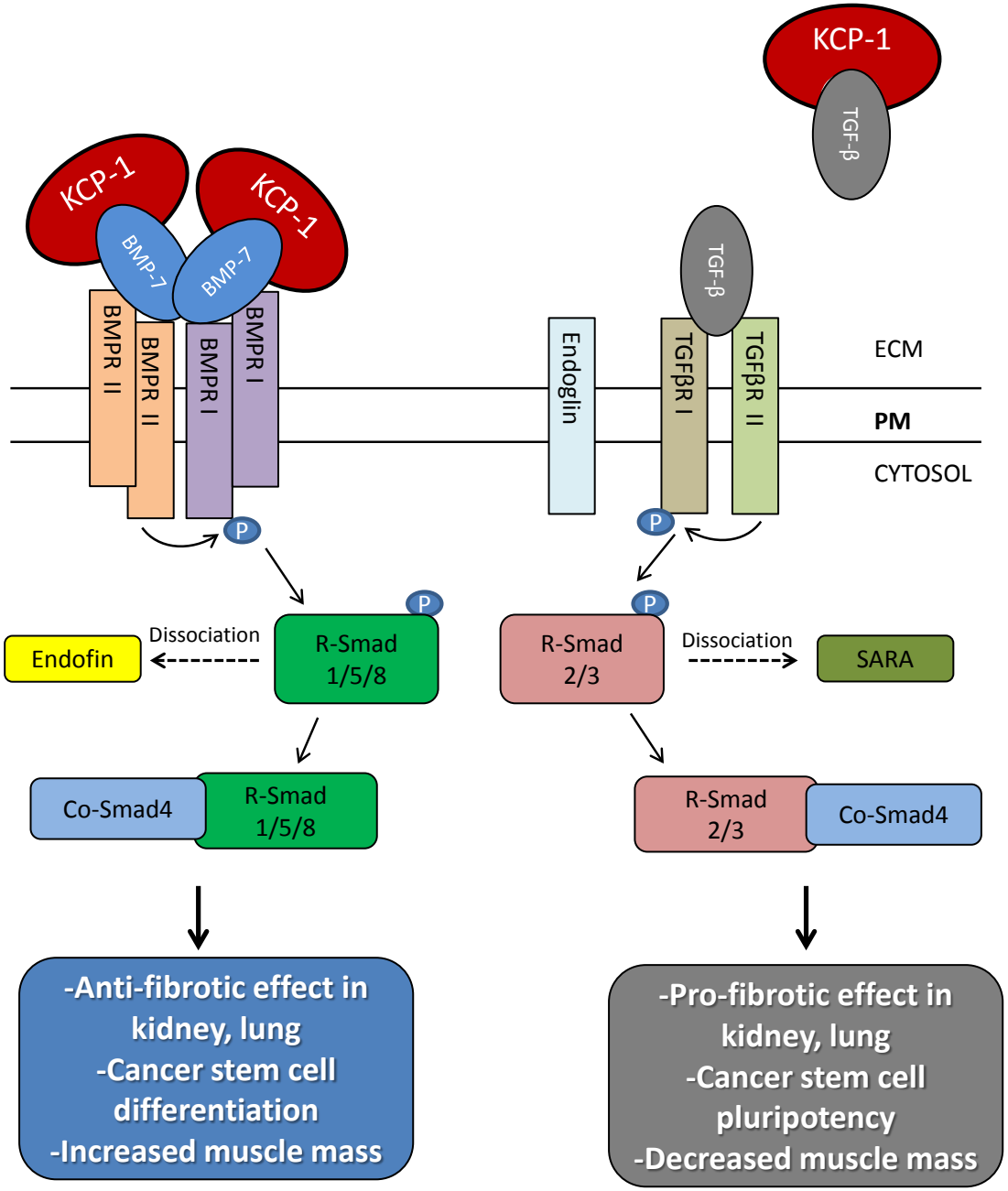

Figure 5.

Brazil et al., 2014