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Review Article

Secreted BMP antagonists and their role in cancer and bone metastases

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ABSTRACT

Bone morphogenetic proteins (BMPs) are multifunctional secreted cytokines that act in a highly context-dependent manner. BMP action extends beyond the induction of cartilage and bone formation, to encompass pivotal roles in controlling tissue and organ homeostasis during development and adulthood. BMPs signal via plasma membrane type I and type II serine/threonine kinase receptors and intracellular SMAD transcriptional effectors. Exquisite temporospatial control of BMP/SMAD signalling and crosstalk with other cellular cues is achieved by a series of positive and negative regulators at each step in the BMP/SMAD pathway. The interaction of BMP ligand with its receptors is carefully controlled by a diverse set of secreted antagonists that bind BMPs and block their interaction with their cognate BMP receptors. Perturbations in this BMP/BMP antagonist balance are implicated in a range of developmental disorders and diseases, including cancer. Here, we provide an overview of the structure and function of secreted BMP antagonists, and summarize recent novel insights into their role in cancer progression and bone metastasis. Gremlin1 (GREM1) is a highly studied BMP antagonist, and we will focus on this molecule in particular and its role in cancer. The therapeutic potential of pharmacological inhibitors for secreted BMP antagonists for cancer and other human diseases will also be discussed.

1. Introduction

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β (TGF-β) family of cysteine knot-containing secreted cytokines. BMPs are present in all multi-cellular organisms where they perform pivotal roles in cell communication, and appeared earlier than TGF-β during evolution [1]. BMP proteins were first isolated from demineralized bone matrix as a factor with an ability to induce bone, hence their name [2]. Studies thereafter revealed that their actions extend far beyond a role in regulating musculo-skelelal system, including controlling cardiovascular, hematopoietic, immune and nervous system. BMPs are pleiotropic cytokines that mediate a range of effects depending on cellular context [3,4]. Dysregulation of BMP signalling is implicated in a broad range of human diseases, including cancer [5]. The activity of BMPs is kept in check at extracellular level by secreted BMP interacting proteins that block or modulate BMP function [6]. BMP antagonists are expressed in a highly controlled temporospatial manner, and BMP antagonist structure, function and role in cancer progression and bone metastasis will be the focus of this review.

BMPs exert their cellular effect via heteromeric complexes of cell surface BMP type I and type II serine/threonine kinase receptors [7]. BMPs can utilise three type II receptors (BMP-specific BMPRII as well as ActRIIA and ActRIIB (that are also receptors for activins), and four BMP type I receptors, also termed activin receptor-like kinase (ALK)1, ALK2, ALK3 (BMPRIA) and ALK6 (BMPRIB) [8] (Figs. 1, 2). Each BMP ligand partners with a specific set of heteromeric type I/type II receptor complexes. The BMPRII kinase is constitutively active, and upon BMP-

Abbreviations: BAMBI, BMP and activin membrane-bound inhibitor; BCC, basal cell carcinoma; BMP, bone morphogenetic protein; BMPER, BMP binding endothelial regulator; BMPR, BMP receptor; CAF, cancer associated fibroblast; CHRD, Chordin; CRC, colorectal cancer; CS, cancer stem cell; CMS, consensus molecular subtypes; CV, Crossveinless; DAN, Differential screening selected gene aberrative in neuroblastoma; ERK, extracellular signal-regulated kinase; FST, Follistatin; GREM1, Gremlin1; GWAS, genome wide association study; HMPS, hereditary mixed polyposis syndrome; miRNA, micro RNA; MSC, mesenchymal stem cell; NOG, Noggin; RCC, renal cell carcinoma; SDF-1α, stromal-derived factor-1α; SMAD, Sma and Mad related protein; SNP, single nucleotide polymorphism; TWSG, Twisted Gastrulation; UTR, untranslated region; VEGFR, vascular endothelial growth factor receptor

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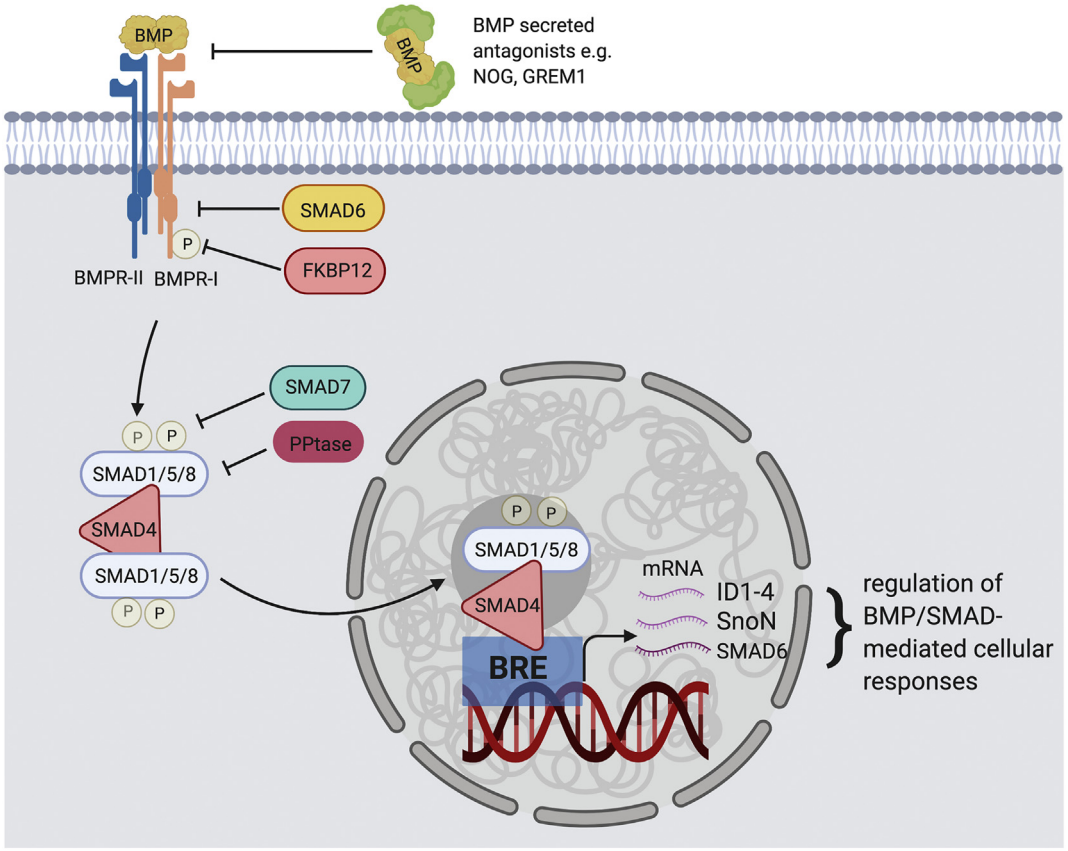

Fig. 1. Schematic diagram summarising BMP signalling pathways. BMP dimers bind to their cognate heterotetrameric receptors (BMPR-II/BMPR-I) at the plasma membrane. This leads to phosphorylation of BMPR-I by BMPR-II, which then leads to phosphorylation of R-SMAD1/5/8 on Ser463 and Ser465 C-terminal residues. Phospho-SMAD1/5/8 then forms a complex with SMAD4. The complex of phospho-SMAD1/5/8 and SMAD4 then translocates to the nucleus where it can bind to BMP-response elements (BREs) on a range of promoters for genes such as *ID1*, *SnoN* and *SMAD6*. Transcriptional changes induced by this binding then leads to BMP-mediated cellular responses. Regulation of this pathway occurs at multiple levels, including secreted BMP antagonist (e.g. GREM1, NOG) binding to BMP ligands in the extracellular matrix, SMAD6 and FKBP12 inhibiton of receptor activation and SMAD7 and protein phosphatase inhibition of SMAD1/5/8 phosphorylation. More in-depth detail on the regulation of BMP signalling is provided in [6]. Image generated using BioRender.com.

induced heteromeric complex formation, the BMPRI becomes phosphorylated by BMPRII and initiates intracellular signalling by phosphorylating SMAD1, SMAD5 and/or SMAD8 (Fig. 1). These activated receptor-regulated SMADs (R-SMADs) then form complexes with SMAD4, and R-SMAD/SMAD4 complexes translocate to the nucleus where they act as transcription factors at BMP/SMAD-response elements (BREs) to regulate gene transcription. By recruiting co-activators or co-repressors, SMADs can participate in upregulation or inhibition of transcriptional responses. Each step in the canonical BMP/SMAD pathway is subject to positive and negative regulation. For example, a type I decoy receptor termed BAMBI (BMP and activin membrane-bound inhibitor) was identified that resembles BMPRI but lacks the intracellular kinase domain [9]. BAMBI can form a BMP-induced heteromeric complex with BMPRII and prevent effective BMP signalling. In contrast, multiple auxiliary BMP cell surface receptors have been identified, which have either small intracellular domains lacking intrinsic enzymatic motifs, or are attached to the cell surface with a GPI anchor that can promote the binding of BMP to signalling receptors [10]. Many such receptors (e.g. endoglin) can be shed from the plasma membrane by proteases, and these secreted extracellular regions can sequester BMP ligand and prevent its binding to BMP receptors and their subsequent activation [11,12]. In a similar fashion, numerous BMP ligand binding proteins have been identified and can be divided in multiple subgroups (Fig. 2). Similar to BMP ligands, some of the BMP antagonists (e.g. noggin and the DAN family members) contain cysteine-knot motifs [13], and both BMPs and their antagonists may have evolved from a common ancestral gene [14].

In this review we will discuss the structure-function relationships of BMP antagonists, as well as their role in cancer progression and bone metastasis. In particular, we will focus on GREMLIN1 (GREM1) as one of the best-studied secreted BMP antagonists. Indeed, studies on GREM1 may serve as a paradigm to elucidate novel roles for other secreted BMP antagonists. The role of BMP antagonists in developmental processes and fibrosis is beyond the scope of this review. We refer readers to other excellent recent reviews on this topic [13,15–17]. We end our review with some future perspectives in which we will discuss emerging approaches for the pharmacological targeting of secreted BMP antagonists as a novel therapeutic strategy for cancer.

2. Secreted antagonists and their mechanism of action: insights from structure-function studies

Perhaps the most unique aspect of BMP signalling among growth factors is the plethora of secreted, secreted antagonists that can modulate BMP signalling by binding directly to the growth factor ligands. The structural diversity of these antagonists is also surprising, ranging from large, multidomain Chordin (CHRD) and crossveinless-2 (CV-2, also known as BMPER) through modular follistatin (FST) and FST-like (FSTL) protein to dimeric Noggin (NOG) and DAN family proteins, including GREM1 (Figs. 2, 3 [18]). This diversity suggests BMP antagonism has risen multiple times independently during the course of evolution, with different structural frameworks purposed for this task.

CHRD and CV-2 contain four von Willebrand factor type C (vWC) domains, which are the sites of interaction with BMPs, preventing

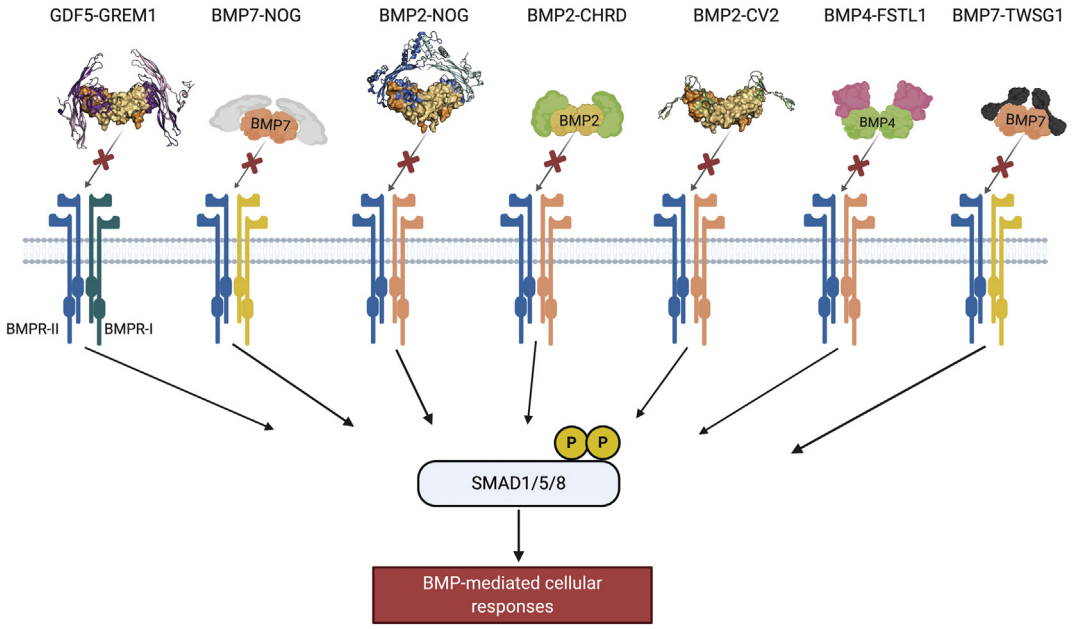

Fig. 2. Mechanism of secreted BMP antagonists-mediated inhibition of BMP signalling. Examples of BMP ligands and their secreted antagonists are shown either as their crystal structures (GDF5-GREM1; BMP2-NOG; BMP2-CV2) or representative models of dimeric BMP ligands bound to 2 secreted antagonist molecules (BMP7-NOG; BMP2-CHRD; BMP4-FSTL1; BMP7-TWSG1). The different BMP membrane receptors are shown as heterotetramers of BMP type II receptors (ActRII/ActRIIB/BMPRII, blue) or type I receptors (ALK2 (yellow), ALK3 or 6 (orange), ALK6 (green)). Binding of secreted BMP antagonists to their BMP ligands prevents activation of the BMP receptor serine kinases, preventing SMAD1/5/8 phosphorylation on Ser463/Ser465 C-terminal residues and preventing BMP-mediated transcriptional responses in the cell. Image created using BioRender.com. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

receptor engagement, but also mediate interaction between these to agonists [19,20]. Interestingly, CV-2 has also been reported to promote BMP signalling in zebrafish embryos via binding and inhibition of CHRD [19]. FST and FSTL are modular proteins best known for their role in activin and myostatin (GDF8) inhibition, but are also known to bind BMPs with high affinity [21]. While no structural information is available, it is predicted that FST and FSTL bind to BMPs in a similar manner to that of activin-like ligands. The DAN family contains seven proteins in humanwith GREM1 and GREM2 (also called as PRDC) being the best characterised BMP antagonists in this family. DAN proteins share a cystine-knot core domain and typically form very stable non-covalently bound dimers [173]. NOG has also a cystine knot fold, but it dimerises in a head-to-head manner with a helical dimerization domain, stabilised by an intramolecular disulfide bond in the loops of the cystine knot domain [22]. Despite their lack of sequence similarity, the BMP antagonists that have been structurally characterised share remarkable mechanistic similarities in their modes of BMP inhibition. As described earlier, BMPs require two different receptors for their signalling. The type II receptor, with its small disulfide linked extracellular domain, binds to the convex “knuckle epitope” of the ligand. The type I receptor, which for many BMPs is the higher affinity receptor, interacts with the cleft formed at the interface of the two protomers in the dimeric BMP ligand (Fig. 3A [1,23]). This cleft contains a small pocket to which type I receptors insert an aromatic side chain, and this pocket is also used by the antagonists GREM2, CV-2 and Noggin (Fig. 3B-D). In all of these cases, a hydrophobic residue (or two in GREM2) bind to this BMP pocket. But unlike the type I receptor where the interacting residue is part of a globular domain, all three inhibitors engage with the BMP with an extended linear binding segment, which interacts and blocks both the type I and type II receptor binding sites on BMPs. In NOG, the N-terminal part of the protein that wraps around the “fingers” of the target BMP (e.g. BMP7) and sterically block both receptor binding sites [14] (Fig. 3B). GREM1 and − 2 also utilise an N-terminal segment that is not part of the cysteine-knot domain to engage with the BMP ligand, but this segment is shorter and structured in GREM1 that is not bound to a BMP ligand [24–26]. The interaction of the vWC domain of CV2 (that is predicted to be the same with CHRD) is also mediated by a non-globular extension of the domain, interacting with the type I binding site pocket via an isoleucine residue (Fig. 3C [27]). Another shared feature between BMP antagonists is that they all interact with the type II receptor binding site on BMPs, with the edge of the β-sheet resting on the BMP while the linear epitope wraps around the BMP molecule (Fig. 3B-D). GREM1/2 interaction with BMPs is unique in that they have been observed both in crystal structures [28] and in biophysical analyses to form concatenated complexes of alternating GREM1/2 and BMP ligands [24,26] (Fig. 3E). The physiological significance of this oligomerisation is still unclear, but it is remarkably similar to that seen with BMP2 and its non-canonical receptors repulsive guidance molecules (RGMs) and Neogenin [29].

Another feature shared between many of the antagonists as well their BMP ligands is their interaction with cell surface heparan sulfates (HS, [30]). Many BMPs are known to interact with HS, which is regulating their function by restricting their distribution in tissues, as well as their bioactivity [31]. The HS binding sites in BMP2–2 and − 4 have been mapped to the extended N-terminal part of the mature domain, whereas BMP5/6/7 appear to have HS binding site in the C-terminal part of the cystine knot domain [32]. In some cases, proteolytic removal of the HS binding region can release the BMP ligand from the extracellular matrix and activate signalling [33]. The BMP antagonists CV-2, CHRD, NOG, GREM1/2 and FST are also well-characterised HS binding proteins [20,34–36], The HS interactions are likely to limit the tissue distribution and range of extracellular inhibitory effects of these BMP antagonists. This is also consistent with the fact that these (and other) antagonists are often induced by the BMP ligands themselves, as part of a feedback inhibition mechanism [37,38]. Therefore, restriction in the localisation of BMP antagonists is an essential part of their biological function. It is also worth noting that complexes of BMP antagonists and their BMP targets often have higher HS affinity than either of the individual proteins alone [35], potentially affecting clearance of these complexes from tissues. Whether HS actively facilitates the BMP

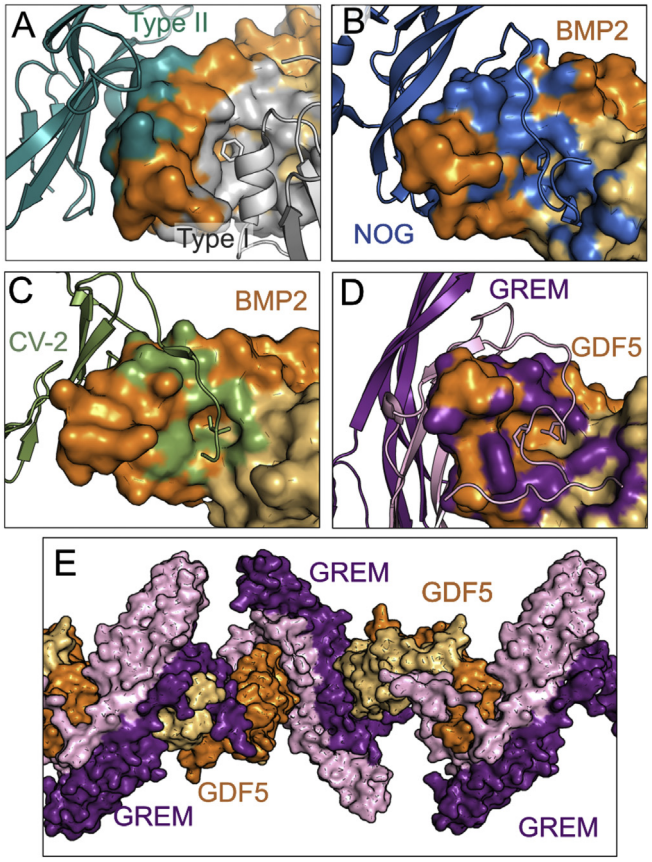

Fig. 3. Mechanism of BMP inhibition by blocking receptor binding sites. (A) Complex of BMP2 (two shades of orange for the protomers in the dimeric ligand) with extracellular domains of type I (light gray) and type II (teal) receptors with the surface of BMP2 coloured in the same colour as the receptor domain at sites of direct interaction (PDB: 2h64). (B) Close-up view of NOG (blue) interacting with BMP2, with interface colours on the surface of BMP2 in blue and showing Pro35 that interacts with type I receptor pocket as sticks (PDB: 1m4u). (C) Close-up view of CV-2 (green) binding to the BMP2 with Ile2 inserted into type I receptor pocket shown as sticks (PDB:3bk3) (D) Closed-up view of GREM2 (purple) binding to GDF5, with Ile49 and Leu53 binding to type I pocket shown as sticks (PDB: 5hk5). (E) A daisy chain of alternating GDF5 and GREM2 dimers as found in the crystal lattice of the complex and demonstrated in solution (PDB: 5hk5). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

neutralising activites of BMP antagonists through co-localisation is unclear.

3. Secreted BMP antagonists: role in cancer

There are numerous reports describing distinct, and at times, opposing roles for secreted BMP antagonists in cancer (summarized in Table 1). DAN is the founding member of the DAN family of secreted BMP antagonists that was identified in neuroblastoma cells [15]. DAN has been reported to restrain neural crest and melanoma metastasis by slowing cell migration [39] and suppressing osteosarcoma growth [40]. While mainly involved in dorsoventral patterning during embryogenesis, CHRD is protective against tumour migration and invasion, as it regulates epithelial to mesenchymal transition (EMT) by maintaining epithelial cell characteristics [41]. Reduced expression of CHRD has been reported in the epithelium of ovarian cancer [41,42], hepatocellular carcinoma [43], melanoma [44] and colorectal cancer [45,46]. In contrast, BMP pathway suppression by CHRD (as well as NOG and GREM1) in sporadic colon cancer appears to simulate the transition from adenoma to carcinoma [47]. FST was first classified as an activin antagonist with key roles in regulating pituitary FSH secretion. FST expression has been implicated in breast [48], skin [49,50], adrenal [51], pituitary [52–54], lung [55], colorectal [56,57] and gastric cancers. Mainly through the antagonism of activin rather than BMP signalling. Twisted Gastrulation (TWSG) both promotes and inhibits BMP signalling by binding to both BMP and BMP antagonist CHRD and CHRD-like Crossveinless-1 and 2 (CV1, CV2). The same duality of TWSG regulation is observed with regard to tumour suppression. Loss of TWSG alleles have been recorded in familial colorectal cancer [58], and reduced expression reported in gastric cancer [59]. In contrast, TWSG expression was significantly upregulated in hepatocellular carcinoma [60] and papillary thyroid cancer [61], and was found to promote cell proliferation and motility. CV2 (also call BMPER) is a pro-angiogenic secreted BMP antagonist that inhibits BMP2, BMP4 and BMP6. In lung, colon and cervical cancers, BMPER is highly expressed and is associated with tumour angiogenesis and malignancy [62]. Coco, a soluble BMP antagonist sometimes called Cerberus-like 2, is essential for left-right and anterior-posterior axis establishment. Breast cancer relapse due to metastasis in the lung may involve Coco-mediated reactivation of dormant breast cancer cell by blocking BMP signalling in the lung [63]. NOG helps maintain homeostasis of intestinal crypt stem cell proliferation and differentiation by suppression of BMP signalling and Wnt activation [64]. NOG has been reported to protect against pancreatic cancer [65] and oesophageal squamous cell carcinoma [66]. In contrast, other reports found that NOG expression is detrimental function in renal, thyroid papillary carcinoma [67]. In melanoma the melanocytic cells were found to secrete NOG that mediated an evasion to BMP7-induced growth inhibition [68]. Noggin also plays a key role in metastasis of prostate cancer to bone (see Section 5 below).

4. Focus on GREM1 and cancer

The secreted BMP antagonist GREM1 in human cancer has been subject of numerous studies. The original report identifying GREM1 (or Drm) by Topol and colleagues suggested it's activity was to inhibit the growth of normal, but not cancer cells [69,70]. The function of GREM1 as a secreted BMP antagonist and regulator of chondrogenesis and limb development was subsequently identified [71]. This key physiological role for GREM1 in bone development was underscored by the phenotype of embryonic and newborn mice lacking both copies of the GREM1 allele [72,73]. These mice present with severely deformed forelimbs and an absence of digits, and also die at P2 due to renal agenesis [72,73]. GREM1 has been identified as a marker of stem cell progenitors of connective tissue in bone (osteochondroreticular stem cells) and intestine (intestinal reticular stem cells) [74]. It is assumed that the canonical function of GREM1 during development, and as a marker of bone stem cells is predominantly as a BMP antagonist. However, it is likely that other, non-canonical signalling modalities for GREM1 exist. Other groups also suggested tumour suppressor function for GREM1, and demonstrated that overexpression of GREM1 inhibited the tumorigenesis of Daoy neuroectodermal and Saos-2 osteoblastic cell lines [40,75]. One potential mechanism for this effect was increased levels of the cyclin-dependent kinase inhibitor-1 p21Cip1 and reduced ERK signalling observed when GREM1 was overexpressed [75]. There is little or no mechanistic data relating to how GREM1 may be mediating these anti-cancer effects in cells, or indeed whether these anti-cancer effects are related to BMP inhibition. Importantly, the majority of published data support a pro-oncogenic role of GREM1 in human cancer (see below).

One of the earliest reports on a cancer-promoting role for GREM1 was published in 2006. Namkoong and colleagues showed that GREM1 was overexpressed in cervical cancer, as well as carcinomas of the lung, breast and sarcoma [76]. An intracellular interaction between GREM1 and 14–3-3η (eta) (YWHAH protein) was suggested as a potential mechanism for GREM1-mediated oncogenic signalling [76]. Overexpression of GREM1 has been reported in malignant mesothelioma

Table 1
Summary of the reported roles for secreted BMP antagonists in cancer. The name and abbreviation of the soluble BMP antagonist is shown, together with the described role in cancer, and whether this effect may have a pro- or anti-oncogenic effect in different cancers. Supporting literature for these observations are listed in the Reference column.

BMP Antagonist	Abbreviation	Primary Tumour Growth	Migration & invasion	Tumour Angiogenesis	Immune invasion	Pro-oncogenic	Anti-oncogenic	Reference
Crosveinless-2/Bone Morphogenic Protein Endothelial Regulator	CV-2/BMPER	✓	✓	✓		Colon Cervix Lung		[61,160]
Cerberus-Like 2	Coco		✓			Breast		[63]
Chordin	CHRD		✓			Colon	Colon Ovary Liver Skin	[41–47]
Chordin-Like 1	Chrdl1		✓				Breast	[161]
Differential Screening-Selected Gene Aberrative in Neuroblastoma	DAN	✓	✓				Bone Skin	[39,40,162]
Follistatin	FST	✓	✓	✓	✓	Adrenal Cortex Breast Colon Lung Pituitary Skin Stomach Thymus		[48–57,163]
Follistatin-Like 1	FSTL1	✓	✓		✓	Brain Lung Melanoma		[164,165]
Gremlin 1	GREM1	✓	✓			Lung Breast Mesothelial		[108,166–168]
Noggin	NOG	✓	✓		✓	Breast Kidney Prostate Skin	Pancreas Oesophagus	[67,68,140,169–171]
Sclerostin	SOST		✓				Prostate	[172]
Twisted Gastrulation	TWSG	✓	✓			Liver Thyroid	Colon Stomach	[58–61]

[77], pancreatic neuroendocrine tumours [65], gastric cancer and hepatocellular carcinoma associated with hepatitis-C infection [78]. In liver, Guimei et al. suggested that high levels of GREM1 inhibit BMP7 signalling and facilitate the proliferation of cancer stem cells [78]. Yan and colleagues demonstrated a key role for GREM1 in maintaining the undifferentiated state of glioma cancer stem cells (CSCs, [79]). High levels of GREM1 inhibit BMP-induced CSC differentiation into astrocytes, maintaining a proliferative phenotype in glioblastomas [79]. GREM1 gene silencing suppressed migration and promoted apoptosis of glioblastoma cells [80]. A similar role for GREM1 in maintaining CSC-like properties was identified in cervical cancer cells [81]. GREM1 appears to play an important role in mesenchymal stem cells (MSCs) and lung cancer. The phenotype of MSCs is modulated by the tumour microenvironment in lung cancer, with GREM1 levels upregulated in lung biopsies from patients [82]. Secretion of GREM1 from MSCs was reported to promote EMT in human oesophageal carcinoma, and silencing of GREM1 expression reversed the malignancy of oesophageal tumours in mice [83]. This secretion of GREM1 was also identified in mouse intestine, where stromal fibroblasts that were positive for *GREM1* RNA secreted GREM1 protein that was taken up by crypt-based epithelial cells that stained negative for GREM1 RNA [84].

How is GREM1 mediating these pro-cancer signalling effects in this diverse array of human tumours? Similar to the reports of GREM1 as a protective factor in cancer, detailed insights into the molecular mechanisms of GREM1-mediated oncogenesis are not available. There are sporadic reports that GREM1 was associated with AKT/mTOR signalling in malignant mesothelioma of the lung [85], p21/CKDN1A accumulation in Caco2 colon cancer cells [86] and ERK activation in breast cancer cells [28]. Some reports identified a potential role for GREM1 in amplifying TGFβ1 signalling to drive EMT in colorectal cancer [47] and human oesophageal squamous cell carcinoma [83]. TGF-β1-induced Grem1-mediated EMT in breast cancer cells was shown to be inhibited by the ω-3-fatty acid docosohexaenoic acid (DHA) which is abundant in fish oils [28]. High levels of GREM1 have been implicated in tumour angiogenesis in colon cancer and pancreatic neuroendocrine tumours [65,87,88]. The $\alpha_v\beta_3$ integrin receptor was also purported to regulate GREM1-mediated angiogenesis in human umbilical cord endothelial cells (HUVECs) [89]. Further research is required to reveal additional and as yet undiscovered mechanisms of GREM1 signalling in the tumour environment.

4.1. Genetic alterations and dysregulated GREM1 expression in cancer

There are numerous reports in the literature detailing dysregulated GREM1 expression as a contributing factor in human cancer. However, we do not yet have a full understanding of the precise molecular mechanisms that underelie these GREM1 effects. A significant number of papers have described a key role for GREM1 in colorectal cancer (CRC). A genome wide association study (GWAS) in 2011 by Tomlinson and colleagues showed that several common susceptibility variants for heritable CRC localised to chromosomal locus 15q13.3, close to the location of the GREM1 gene [90]. This same group then identified that hereditary mixed polyposis syndrome (HMPS), a rare autosomal dominant inherited form of colorectal cancer identified in Ashkenazi Jewish families, was caused by a 40-kb chromosomal duplication at chromosome 15q13.3 [91]. This chromosomal duplication led to > 2000-fold increase in *GREM1* mRNA levels in colonic crypts compared to normal tissue. The Tomlinson/Leedham group shed

further light on this condition by showing that ectopic expression of GREM1 in colonic *epi*thelial cells in mice recapitulated the abnormal intestinal morphology seen in HMPS patients, due to disruption of homeostatic morphogen gradients in the intestine [92]. Importantly, several groups have demonstrated that increased intestinal epithelial *GREM1* mRNA is a feature of the more common sporadic, traditional serrated adenomas seen in patients [92,93]. Consistently, GREM1 was identified as a major component of the tumour invasion front in CRC, regulating the migration of cancer cells into the proximal stromal tissue toward adjacent blood vessels [94].

In the absence of chromosomal duplication at 15q13.3, what other (epi)genetic mechanisms could lead to upregulated GREM1 expression in colorectal cancer? A number of single nucleotide polymorphisms (SNPs) have been identified that may contribute to dysregulated *GREM1* expression. Lewis et al. identified SNPs that are located in an enhancer region that was associated with increased *GREM1* expression and higher risk of CRC [95]. These authors demonstrated that the presence of these SNPs enhanced the recruitment and activity of CDX2 and TCF7L2 transcription factors, leading to enhanced *GREM1* expression [95]. Others have demonstrated that low frequency polymorphisms in the 3′ untranslated region (UTR) of *GREM1* alter binding of miR-185-3p and are associated with increase CRC risk in a Chinese patient cohort [96]. *GREM1* was identified as a target of hsa-miR-1/miR-203 in oesophageal cancer cells [97], and these authors suggest that down-regulation of these miRs may lead to upregulation of *GREM1* and other genes that facilitate cancer cell growth [97]. Others identified miR-137 as a negative regulator of *GREM1* in cervical cancer, with reduced miR-137 and increased *GREM1* mRNA present in cervical cancer tissues and cells [98].

In contrast to the wealth of data identifying upregulation of GREM1 expression in human cancers (see above), the *GREM1* gene has also been reported to be frequently methylated and inactivated in several human cancers including lung, breast and bladder cancers [99,100]. Li et al. demonstrated that hypermethylation of *GREM1* gene is a biomarker for early detection of breast cancer [101]. In renal clear cell carcinoma (RCC), hypermethylation of *GREM1* promoter CpG islands in 3 distinct regions was associated with ccRCC progression [102]. Vlodrop and colleagues suggested that methylation of the 3' UTR of *GREM1* was associated with increased tumour size, higher tumour grade and stage, as well as worse prognosis [102]. In contrast, a study reported that in cancer associated fibroblasts (CAFs) in gastric cancer, trimethylation of histone H3 lysine27 (H3K27me3) was detected, and was more extensive than DNA methylation. Several genes displaying loss of H3K27me3, including *GREM1*, are associated with upregulation of genes with tumour-promoting effects [103]. A summary of how genetic and epigenetic changes may alter GREM1 expression is shown in Fig. 4.

The evidence that epigenetic marking of *GREM1* correlates with a range of human cancers suggests that silencing of *GREM1* expression may contribute to cancer progression [104]. Some have suggested that hypermethylation of *GREM1* may lead to lower *GREM1* expression in human cancers including renal cell carcinoma (RCC, [105]). Methylation and other epigenetic-mediated reductions in *GREM1* expression as a contributory element to cancer progression would contrast with the wealth of data identifying high *GREM1* expression as a marker of a number of human cancers (see below). The specific effect of CpG methylation or H3K27me3 loss on *GREM1* mRNA expression remains to be determined. The levels of BMP ligands present in each context also need to be carefully considered, as tumours with low levels of BMPs may liberate GREM1 to activate other non-canonical signalling pathways. In contrast, tumours with high levels of BMP expression may limit the signalling of GREM1 to inhibition of BMP action, which may also contribute to the regulation of cancer cells. The ability of BMPs to induce the expression of GREM1 and other soluble BMP antagonists should also be factored into any model of how GREM1 is contributing to tumour formation and cancer (summarized in Fig. 5).

4.2. Prognostic value of GREM1 expression in human cancer

The prognostic value of high *GREM1* mRNA levels in CRC and other cancers has been assessed by multiple groups. The majority of reports suggest that high levels of *GREM1* mRNA correlates with poor patient prognosis e.g. in CRC [84,92,87] and breast cancer [106,107]. Increased *GREM1* mRNA has been identified in consensus molecular subtype-4 (CMS4), a mesenchymal, stromal, metastatic subtype of CRC [84], as well as the metastatic recurrence stage, supporting the association with poor patient prognosis [87]. In contrast, elevated *GREM1* expression in traditional serrated adenomas has been suggested to be prognostic for better clinical outcomes and improved patient survival in CRC [108,109]. Increased *GREM1* levels were reported to associate with elevated levels of angiogenesis and favourable prognosis in pancreatic neuroendocrine tumours [65]. Despite these reports, the overwhelming bulk of published data suggests that high levels of GREM1 in tumour tissue contribute to lower patient survival in CRC and other cancers. More recently, Neckmann and colleagues demonstrated that high levels of *GREM1* associated with a more metastatic tumour subtype and shorter survival times in ER-negative breast cancer [107]. Similar data was published by Sun et al., who showed that high levels of *GREM1* mRNA was associated with poorer survival in gastric cancer in a Chinese patient cohort [110]. In contrast to data from Chen et al. [65], Yu and colleagues identified that *GREM1* levels were induced by sonic hedgehog (SHH) signalling in human pancreatic cancer cells, an effect mediated by the Gli1 transcription factor [111]. These authors also identified that pancreatic cancer patients with higher levels of *GREM1* expression had poorer survival outcomes [111]. Overexpression of GREM1 in idiopathic pulmonary fibrosis (IPF) correlates with reduced recruitment of lymphocytes and chemokine expression (e.g. CXCL10) in the bronchoalveolar lavage fluid from alveolar epithelial cell GREM1 transgenic mice [112]. These GREM1-mediated changes in immune cell function may contribute to the increased risk of lung cancer in IPF patients.

4.3. How does increased GREM1 expression contribute to cancer progression?

What is the mechanism by which increased expression of GREM1 promotes tumour formation and growth? As we alluded to earlier in this reviewer, the diversity of GREM1 signalling reported in the literature suggests that non-canonical, BMP-independent signalling activities of GREM1 likely exist. Secretion of GREM1 from cancer-associated stromal cells was first identified by Sneddon et at [113]. These authors demonstrated that the growth of basal cell carcinoma (BCC) was promoted by GREM1 secretion from stromal cells, a pattern that was also identified in other human cancers such as oesophageal, breast and pancreas [113]. The authors suggest that secretion of GREM1 from the stromal cell niche can act to block BMP-mediated inhibition of tumour cell expansion. CAFs were also shown to be the source of GREM1 in breast cancer, and in particular at the invasion fronts [106]. These authors showed that GREM1 derived from these CAFs inhibited BMP signalling and promoted a stem-like, mesenchymal, invasive phenotype in breast cancer cells, and that high expression correlated with poor prognosis in breast cancer [106]. GREM1 expression was potently induced by TGF-β produced by cancer cells and inflammatory cytokines. GREM1 produced by CAFs was critical for the CAF activation phenotype and may well contribute to the desmoplastic phenotype that promotes cancer cell invasion and metastasis. Similar data were obtained by Kim and colleagues, who showed that GREM1 levels acted as a marker for activated myofibroblasts in scar tissue and the cancer stroma [114]. In glioma, amplified GREM1 expression in cancer stem cells (CSCs) acts to inhibit BMP2-mediated CSC differentiation, maintaining CSC pluripotency within the tumour hierarchy [79]. Importantly, a recent report identified that GREM1 protein could be actively taken up by intestinal epithelial cells, supporting an earlier report that

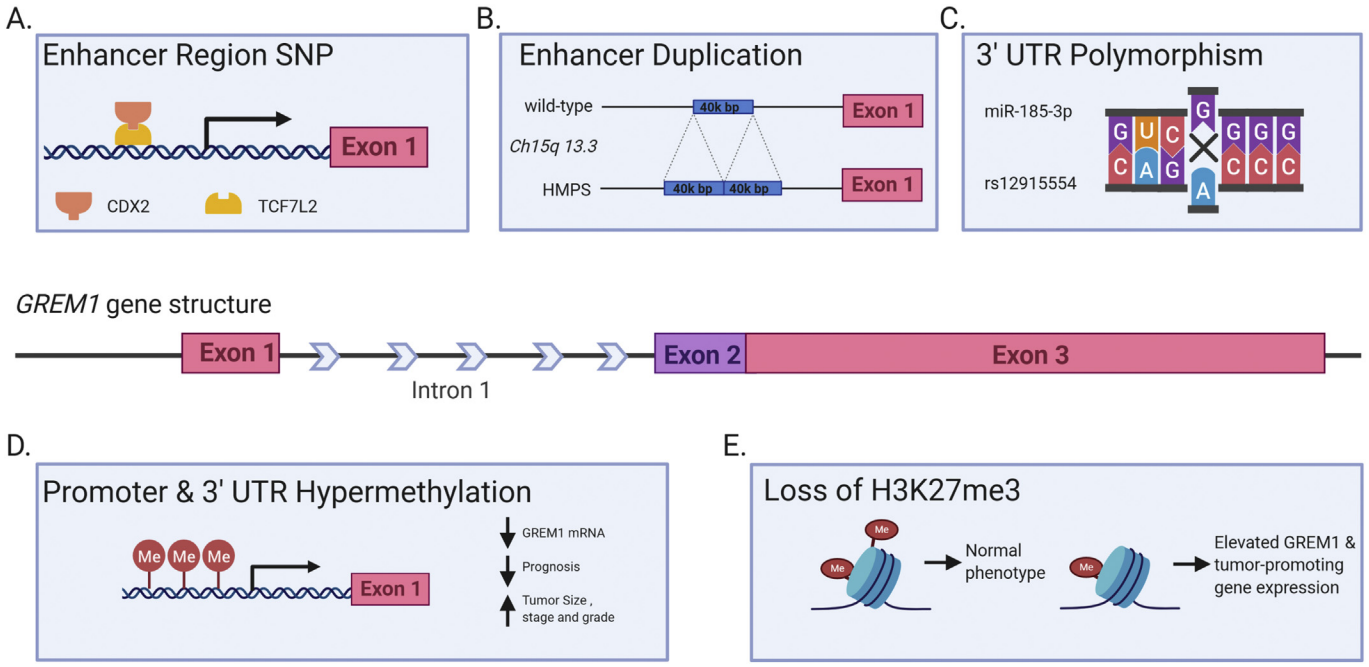

Fig. 4. Schematic of the *GREM1* locus and summary of epigenetic changes and modifications reported. A schematic of the *GREM1* gene locus is shown in the centre of the figure. A. Single nucleotide polymorphisms (SNPs) upstream from the *GREM1* promoter in the enhancer region increases the recruitment of transcription factors CDX2 and TCF7L2. The interaction of CDX2 opens up the chromatin and recruits TCF7L2 complexed with β-catenin to increase *GREM1* gene transcription (91). B. Duplication of a chromosomal region at 15q13.3 (containing an enhancer region for *GREM1*) is an heritable variant within Ashkenazi Jew populations known as hereditary mixed polyposis syndrome (HMPS). As a result GREM1 mRNA expression is greatly increased within intestinal crypts and contributes to colorectal cancer (87). C. GREM1 expression is regulated by miR-185-3p interactions. A SNP (rs12915554) within the 3' UTR of *GREM1* was identified within a cohort of Chinese colorectal cancer patients which results in the substitution of a Cytosine base for Adenine, significantly impacting miR-185-3p binding affinity, reportedly leading to upregulation of GREM1 (92). D. Hypermethylation of the promoter and 3' UTR region of the *GREM1* locus alters GREM1 mRNA expression. Loss of GREM1 due to hypermethylation has been reported as a potential prognostic tool in breast (97) and renal cancer (98). Methylation of the *GREM1* 3'UTR is associated with an increase in the size, grade and stage of tumours that negatively correlate with GREM1 mRNA levels (98). E. GREM1 expression is elevated in fibroblasts isolated from gastric cancers due to loss of trimethylation in histone H3 lysine27. Apart from increased *GREM1*, other genes related to tumour promotion are also enhanced by histone modifications (99). Image created using BioRender.com.

intracellular GREM1 could act to bind to and inhibit BMP4 action [84,115].

There are a number of reports suggesting that non-canonical GREM1 signalling (i.e. not directly related to antagonism of BMPs) may contribute to GREM1 signalling in cancer. Direct interaction between GREM1 and DAN with SLIT proteins has been demonstrated to inhibit SDF-1 induced monocyte chemotaxis [116]. Others have identified a negative crosstalk loop where GREM1 binding to SLIT2 inhibits activation of the ROBO receptor in nephron progenitor cells [117]. Several reports have suggested that GREM1 acts as a ligand for the vascular endothelial growth factor receptor (VEGFR)2, driving angiogenesis during vascular development and tumour neovascularisation [88,118]. Others have identified a role for GREM1 as an antagonist of the VEGFR2 in endothelial cells [119] and in the lung [120]. A more recent report casts doubt on the ability of GREM1 to activate VEGFR2 signalling, suggesting that other mechanisms may underpin the pathogenic role of GREM1 in human cancer [121]. A tantalising hypothesis is that a specific, cognate receptor exists for GREM1 at the plasma membrane that mediates many of the oncogenic signalling effects of GREM1 in cells. Research efforts to test this provocative hypothesis are underway and will shed new light on how GREM1 mediates many of its cancer-associated effects independent of its canonical BMP targets.

5. Secreted BMP antagonists and bone metastasis

Bone is the third most common site of metastasis in cancer, after lung and liver (reviewed in [123]). The majority of bone metastases emanate from prostate and breast cancer, likely due to the large number of patients and long disease course for these patients [122]. Other cancers such as thyroid, lung and bladder can also metastasise to bone, which are characterised by increased fracture risk and extreme pain in patients [123]. Bone metastasis is typically an indicator of poor patient prognosis, with lung cancer patients who develop bone metastases having a 6–7 month median survival [124]. There are a number of proposed mechanisms by which primary tumours metastasise to bone [122]. One of these mechanisms involves support for invasive, metastatic cancer cells by the bone microenvironment ("seed and soil" hypothesis, [125]). This hypothesis suggests that growth factors that regulate physiological bone formation and resorption can facilitate the development of bone metastases in a range of cancers.

BMPs and their secreted antagonists have been identified as important suppressors and promoters of cancer [126]. A role for BMP signalling has also been identified in bone metastasis. BMP6 expression is increased in prostate cancer, inhibiting the proliferation of PC3 cells [123]. However, BMP6 also increases the expression of the NOG [123], which may antagonize the anti-proliferative effects of BMPs BMP2 and BMP6 were shown to increase the in vitro invasive ability of prostate cancer cells, supporting the idea of the microenvironment as a key component of cancer metastasis to bone [127]. Altered levels of BMPs have been identified in breast cancer, and appear to modulate oestrogen receptor signalling (reviewed in [128]). BMP-mediated tumour invasion to bone may occur via regulation of matrix metalloproteinases (MMPs), immune cell signalling or inflammatory cytokines [128]. BMP7 has been suggested to act as an inhibitor of bone metastasis from primary tumours in prostate [129] and breast cancer [130]. Buijs and colleagues also identified that BMP2/7 heterodimers inhibited breast cancer stem cell metastasis to bone [131]. Interestingly, the BMP2/7 heterodimers were more potent than BMP homodimers in this action;

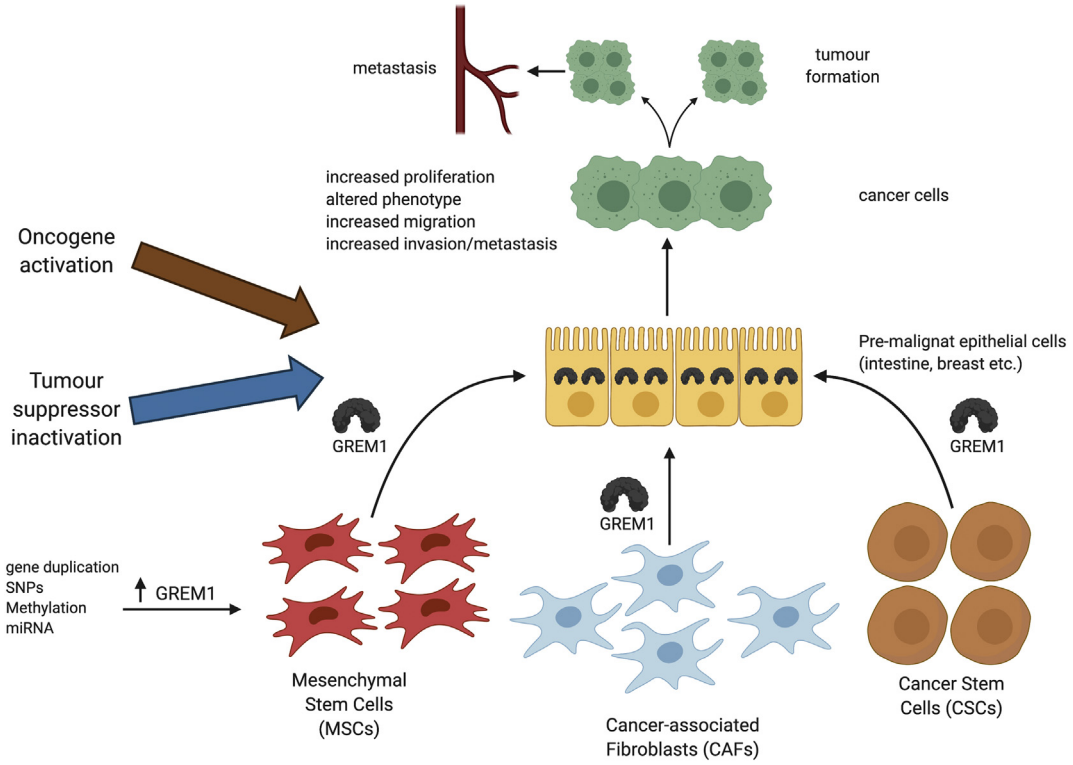

Fig. 5. Schematic diagram summarising potential mechanisms of GREM1 signalling in human cancer. Dysregulated GREM1 expression may occur via gene duplication, SNPs, epigenetic methylation events or altered miRNA levels, leading to increased GREM1 expression in mesenchymal stem cells (MSCs), cancer-associated fibroblasts (CAFs), cancer stem cells (CSCs) etc. Secreted Grem1 protein (black) can be taken up by pre-malignant epithelial cells in the intestine, breast etc. This uptake (or overexpression) of GREM1, together with well-described oncogene (e.g. K-Ras, MYC) and tumour suppressor (e.g. p53, APC) mutations can lead to altered cell phenotypes including increased proliferation and enhanced migration of epithelial cells leading to tumour formation and metastasis in many cases. Image generated using BioRender.com.

however, the activity of heterodimers is not inhibited as efficiently by NOG compared to homodimers [132]. Kobayashi et al. demonstrated that BMP7 secretion from bone stromal cells triggered senescence in prostate cancer stem cells (CSCs) via induction of the p21 cell cycle inhibitor [133]. In contrast, several reports have identified a metastasis-promoting role for BMP7, BMP2 and BMP4 in breast cancer models [134–137] .

BMP3 and the BMP antagonist NOG are expressed in PC3 prostate cancer cells, but at low levels in SaOS2 osteosarcoma cells [138]. Conditioned medium from PC3 prostate cancer cells stimulated a marked induction of BMP3 and NOG expression in SaOS2 cells (88.3-fold induction of NOG [138]). Knockdown of NOG expression in PC3 cells reduced the ability of conditioned medium from these cells to increase SaOS2 cell proliferation [138] and also limited the growth of PC3 cells in a bone xenograft model in mice [139]. This increased expression of NOG is thought to antagonize bone formation, preventing repair of bone and contribute to osteolytic bone metastasis [139,140]. Low levels of NOG expression have been reported to be associated with osteolytic cell lines, and osteosclerotic metastasis in prostate and breast cancer [141]. Overexpression of antagonists of BMP signalling has been shown to reduce the metastasis of prostate cancer to bone, as well as other tumour cell growth [142,143]. Analogues of a BMP type I receptor kinase inhibitor Dorsomorphin were also shown to reduce breast cancer metastases in mouse models, potentially by altering immune cell responses to tumours [144]. A natural small molecule compound called ZL170 was shown to inhibit BMP receptor signalling in triple-negative cancer models in vitro and in vivo [145]. Virtual drug screening has identified potential NOG inhibitors that could potentially be developed as novel drugs to reduce cancer metastasis to bone [146]. These efforts to develop pharmacological tools to modulate BMP signalling in cancer and bone metastasis will hopefully bear fruit in the coming years and improve clinical outcomes for patients with bone metastases.

6. Conclusions and future perspectives

Secreted BMP proteins have been shown modulate cell surface BMP-BMP receptor binding. The vast majority of these BMP interacting proteins are antagonists, including GREM1, NOG, CHRD and FST as prominent examples [147]. However, some secreted BMP antagonists were found (under specific conditions) to potentiate BMP signalling, e.g. CV2 [148] and KCP/Kielin [149]. Moreover, the extracellular domains of BMP receptors that are shed by proteases from the plasma membrane can sequester BMPs and prevent binding to their receptors [10]. The secreted BMP antagonists appears to be a still-growing family, as proteins with novel structures that act as BMP antagonists continue to be discovered. For example, multiple extracellular matrix proteins and members of CCN family of matricellular proteins are emerging as key modulators of BMP activity via direct interaction with BMP ligands (e.g. BMP2) [150] via their vWC domain, but this has not been possible to demonstrate directly [151].

Here, we have focussed on the role of secreted BMP antagonists and their role in cancer and bone metastasis. In some ways, the action of BMP antagonists resembles that of BMPs, despite their countervailing antagonist/agonist actions. BMPs and their antagonists have been shown to effect cancer cell function, but also cells from tumour microenvironment (TME). Effects on CAFs and endothelial cells have been reported and were reviewed here. However, it will be interesting to explore the function of BMPs and their antagonist on immune cells.

Both BMPs and their antagonists can have tumour promoting and tumour suppressive activities depending on cancer cell-type and tumour stage. For example, DAN was initially identified as a putative tumour suppressor in neuroblastoma [152]. In contrast, GREM1

promotes cancer cell stemness, and a mesenchymal phenotype, activates CAFs and promotes angiogenesis. Thus, targeting certain BMP antagonists in carefully selected patients/tumours by neutralising antibodies and/or activation of BMP signalling with BMP mimetics may have therapeutic benefit for cancer patients (e.g. [153]).

To date, however, clinical advancement of such pharmacological agents has been limited. Of note, some antagonists were shown to sequester BMPs inside the cells and prevent secretion [115,154], thereby eliciting an inhibitory effect on BMP signalling. This intracellular pool of BMP antagonists will likely not be inhibited by the action of a neutralising antibody [116,154]. The development of cell-permeable small molecule inhibitors that inhibit BMP antagonist/BMP interaction has the potential to overcome this. The multifunctional action of BMP antagonists may also pose a problem for clinical translation by systemic administration of agents targeting these proteins. In the case of bone metastasis, one might be able to target anti-BMP antagonist antibodies to bone by coupling to bisphosphonates to promote BMP signalling, thereby inhibiting the vicious cycle of TGF-β mediated bone destruction [155].

Another emerging area to pursue will be to develop reliable assays to measure serum levels of secreted BMP antagonists as diagnostic/prognostic markers for cancer patients. Development of such assays has been limited by the availability of well-characterised, high affinity, specific antibodies reactive to soluble BMP antagonists such as GREM1. Encouragingly, recent reports have made progress toward this objective, and initial assays for GREM1 and DAND5 have been reported and shown to correlate with disease phenotype in pulmonary artery hypertension, breast cancer and type 2 diabetes [156–158].

Another future area of interest will be to investigate if secreted BMP antagonists have targets other than BMPs. A number of alternative targets have been proposed including VEGFR, Slit proteins and Notch, but conflicting and incomplete data have created an unclear picture of this important area. The development of small molecule inhibitors will allow the addition of secreted BMP antagonists in the absence or presence of these inhibitors in different cell types. This will then allow the interrogation of the activity of different signalling pathways triggered by secreted BMP antagonists using transcriptional reporter activity, expression of key signalling intermediates or proteomic/genomic and/or metabolomic profiling, to reveal novel and exciting results. In this regard, a recent paper has demonstrated that mechanical loading promotes a GREM1-NFκB signalling pathway in chondrocytes and may contribute to cartilage degeneration during osteoarthritis [159]. Of note, a series of small molecule benzoxazole have been identified as potent direct activators of SMAD1/5/8 phosphorylation and BMP signalling in kidney cells by bypassing BMP receptor activation at the plasma membrane [153].

As the wealth of papers cited in this review demonstrates, the complex interplay between BMPs and their soluble antagonists still contains many unknowns that need to be discovered. We look forward to new advances and discoveries by researchers in the field that will further clarify the signalling modalities of BMP antagonists in cancer, bone metastasis and other cellular contexts relevant to human disease.

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Declaration of competing interest

None of authors have competing interests.

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