Research Article

BMP antagonists in tissue development and disease

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Abstract

Bone morphogenic proteins (BMPs) are important growth regulators in embryogenesis and postnatal homeostasis. Their tight regulation is crucial for successful embryonic development as well as tissue homeostasis in the adult organism. BMP inhibition by natural extracellular biologic antagonists represents the most intensively studied mechanistic concept of BMP growth factor regulation. It was shown to be critical for numerous developmental programs, including germ layer specification and spatiotemporal gradients required for the establishment of the dorsal–ventral axis and organ formation. The importance of BMP antagonists for extracellular matrix homeostasis is illustrated by the numerous human connective tissue disorders caused by their mutational inactivation. Here, we will focus on the known functional interactions targeting BMP antagonists to the ECM and discuss how these interactions influence BMP antagonist activity. Moreover, we will provide an overview about the current concepts and investigated molecular mechanisms modulating BMP inhibitor function in the context of development and disease.

Introduction

Bone morphogenetic proteins (BMPs) are pluripotent growth factors of the transforming growth factor- β (TGF-β) superfamily. They play essential roles in several crucial biological processes ranging from dorsal-ventral axis patterning during early embryogenesis to postnatal homeostasis in various organs and tissues. Thereby BMPs drive a multitude of cellular processes such as differentiation, proliferation, and apoptosis [1,2].

To initiate a cellular response, BMPs interact with type I and type II BMP receptors, which form an active heterotetrameric receptor complex upon phosphorylation of type I receptors by

constitutively active type II receptors [3]. For canonical BMP signalling, BMP ligands bind to a preformed receptor complex of BMP type I (ALK2, ALK3, ALK6) and type II receptors (BMPRII, ActRIIA, ActRIIB), resulting in type I receptor mediated phosphorylation of SMAD proteins SMAD1, SMAD5, and SMAD8 and clathrin-dependent internalisation into endosomes. These receptor-regulated SMADs form a complex and bind to the co-mediator SMAD4. The established complex translocates into the nucleus and acts as transcription factor with co-repressors or -activators to regulate BMP target gene expression. In contrast, non-canonical BMP signalling is SMAD-independent [4,5]. In this signalling route, the ligand initially binds and dimerises its high affinity BMP type I receptor followed by subsequent recruitment of type II receptors, resulting in the formation of the BMP-induced signalling complex (BISC) [5,6]. Characteristic for BISC is caveolae-dependent internalisation and activation of non-SMAD pathways via p38 mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinases (ERK), and phosphoinositide 3-kinase (PI3K). These activated kinases translocate into the nucleus where they activate activating transcription factor 2 (ATF2), c-Jun or c-Fos to regulate the expression of BMP target genes [5,7].

Major differences between BMP and TGF-β signalling are that TGF-β binds exclusively to TGF-β type I (ALK5) and type II (TGFBR2) receptors [8]. After TGF-β binding to its receptors, mainly receptor-regulated SMAD2 and SMAD3 are phosphorylated which then bind to SMAD4 and translocate into the nucleus. Since SMAD proteins differ in the positioning of DNA-binding hairpins, different SMAD complexes recognise specific DNA sequences via SMAD-binding elements and different DNA-binding partners [5]. Similar to BMPs, TGF-β also triggers SMAD-independent pathways such as MAPK activation, JNK/p38, or PI3K/Akt [9,10].

Various control mechanisms have evolved to regulate the level, positioning, and timing of BMP signals in the extracellular space [11]. BMP signalling is dependent on tissue specific BMP ligand and BMP receptor expression and presence. Furthermore, local BMP bioavailability is regulated by diffusible BMP antagonists, which presents the most established mechanism of extracellular regulation of BMPs, preventing BMPs from accessing their signalling receptors [12]. If the regulation by antagonists is disrupted, developmental processes of dorsoventral patterning are impaired. For instance, knockout of the BMP antagonist chordin in mice causes an extensive array of malformations that include most features of DiGeorge syndrome in humans such as cleft palate, indicating a role for chordin in pharyngeal development [13]. Chordin/noggin double knockout mice display defects in the development of the forebrain, eye, and facial structures, as well as a disrupted mesoderm development and left to right patterning, highlighting their importance for proper establishment of the body axes [14].

Crucial to this concept is the diffusion of BMP growth factors and their specific antagonists from distant cells through the extracellular space, to opposite directions. The exact mechanism by which this diffusion is facilitated is still part of current research. However, BMP growth factors and their antagonists are also known to fulfil various functions in extracellular matrix (ECM) rich postnatal tissues making the concept that they reach their place of action through pure gradient diffusion, incomplete. Alternatively, it is thought that in late embryonic stages and postnatal life BMPs and their inhibitors are targeted to and sequestered by the ECM-rich cellular microenvironment that controls and integrates BMP signalling in a context-specific manner [15] (Fig. 1).

BMP antagonist families

The most prominent BMP inhibitors are represented by noggin, follistatin and follistatin like proteins, the CAN family (cerberus and DAN; differential screening selected gene aberrative in neuroblastoma), and the chordin family of BMP antagonists [16]. All chordin family members contain two to five cysteine rich von Willebrand factor type C (vWC) homology domains which are responsible for BMP binding. Unique to chordin is the presence of chordin specific domains (CHRD) which are of unknown function but are also found in bacterial proteins [17]. In chordin, the N- and C-terminal BMP-binding vWC domains are mostly spaced by CHRD domains so that a BMP growth factor dimer can be positioned between them suggesting that chordin can bind BMPs co-operatively thereby covering two BMP receptor interaction sites simultaneously. This may serve to stabilise the complex through multiple recognition sites and increase steric interference between BMPs and their receptors [18].

A phenogram of BMP antagonists based on sequence similarity is shown in Fig. 2A (adapted and modified from [19]). CAN family antagonists are small (<20 kDa), single domain proteins, characterised by a core 'DAN' domain that contains a cystine knot motif. The knot structure is observed in a number of proteins including BMP growth factors and consists of a conserved eight-residue ring formed by a pair of disulphide bonds that link two anti-parallel β-strands followed by an additional disulphide bond that reaches through the ring [20]. Members of the DAN family include DAN, gremlin (also referred to as gremlin-1), PRDC/gremlin-2 (protein related to DAN and cerberus), cerberus, sclerostin (SOST), coco, and USAG-1. A schematic

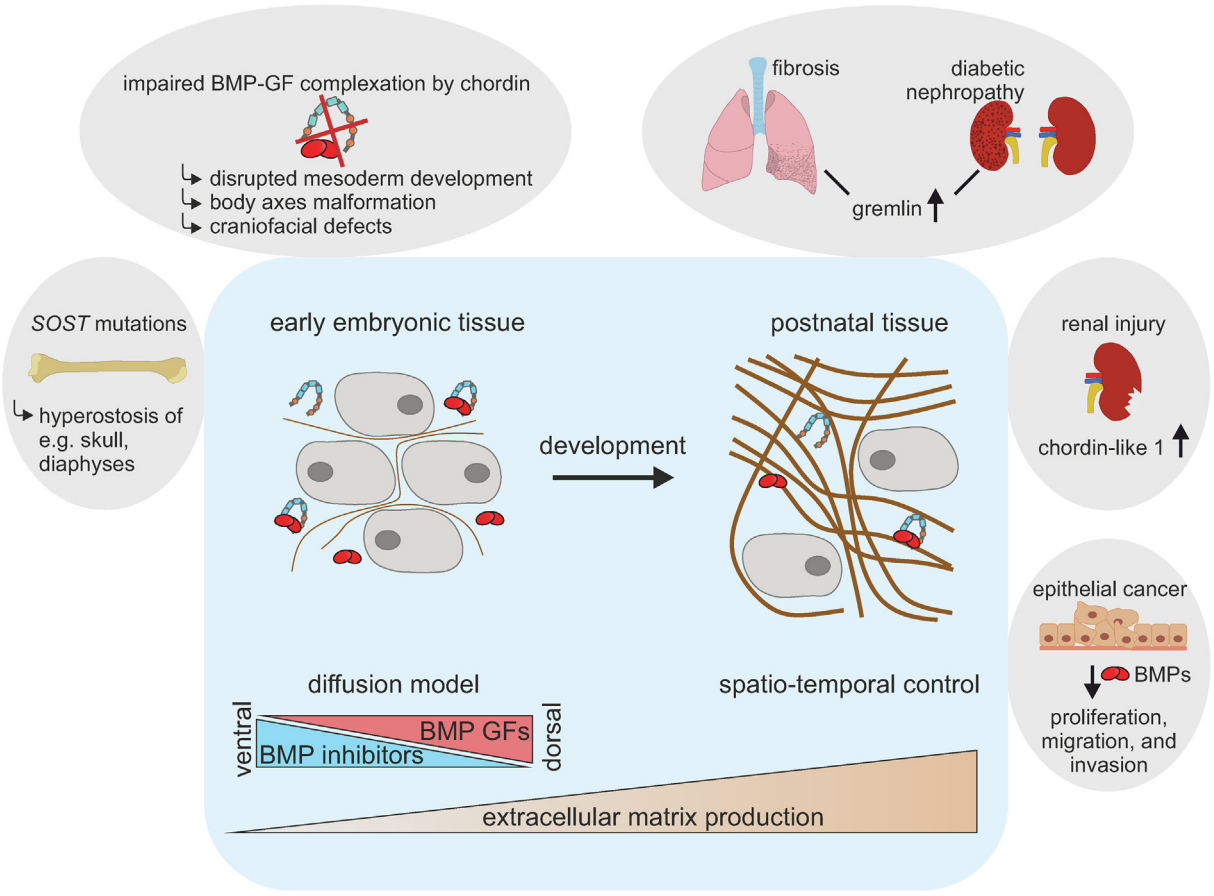

Fig. 1. Concepts to control BMP signalling: BMP growth factor and antagonist diffusion versus ECM-dependent spatio-temporal control. In early embryonic tissue, ECM production is minor, so that diffusion of BMP growth factors (GFs) and inhibitors in opposite directions is a plausible concept. By ventral expression of BMP inhibitors and dorsal expression of BMP GFs and their diffusion in opposing directions a gradient is formed, which determines the cell fate in a concentration-dependent manner. In postnatal tissues, the presence of the ECM is more pronounced. Pure gradient diffusion as explanation for BMP GFs and inhibitors to reach their place of action is therefore an insufficient model. Instead, spatio-temporal control might take place to target, locally concentrate and release BMP GFs and antagonists when needed. Several diseases have been implicated in this context. *SOST* mutations can lead to hyperostosis of e.g. the skull and diaphyseal cortices of the long bones. Impaired complexation of BMP GFs by chordin can result in disrupted mesoderm development, body axes malformation and craniofacial defects. In postnatal tissue, increased levels of gremlin have been associated with pulmonary fibrosis and diabetic nephropathy. Upon renal injury, chordin-like 1 was found to be upregulated. In epithelial cell cancers, BMPs are involved in proliferation, migration, and invasion.

representation of the CAN family and the position of the core DAN domain is shown in Fig. 2C. Little is known about the mechanism of BMP ligand inhibition by CAN family members. X-ray crystallography of gremlin-2 revealed a growth factor like appearance with a dimerization mechanism and that the complexation of BMP growth factors is largely mediated by hydrophobic interactions [21]. Important for BMP inhibition by gremlin-2 is a large hydrophobic interface on the convex surface which is built by the central part of the DAN domain [21,22]. It was suggested that the flexible N-terminus first shields the protein core and dissociates to enable binding [21]. However, this mechanism cannot be transferred to all DAN domain containing proteins since their structures and dimerization ability differs. For instance, gremlin-2 forms head-to tail dimers in contrast to SOST which acts as monomer [21]. Chordin is the prototypic member of a family of proteins, characterised by multiple copies of cysteine-rich von Willebrand factor C (vWC) repeats including chordin-like-1, chordin-like-2, crossveinless-2 (Cv2)/BMPER (BMP binding endothelial regulator), brorin, and brorin-like [16]. The domain structure of members of the chordin family is illustrated in Fig. 2B.

However, regulation of BMP signalling is highly complex and BMP antagonists like Cv2/BMPER, or twisted gastrulation (Tsg) are capable of both inhibiting and enhancing BMP signalling [23].

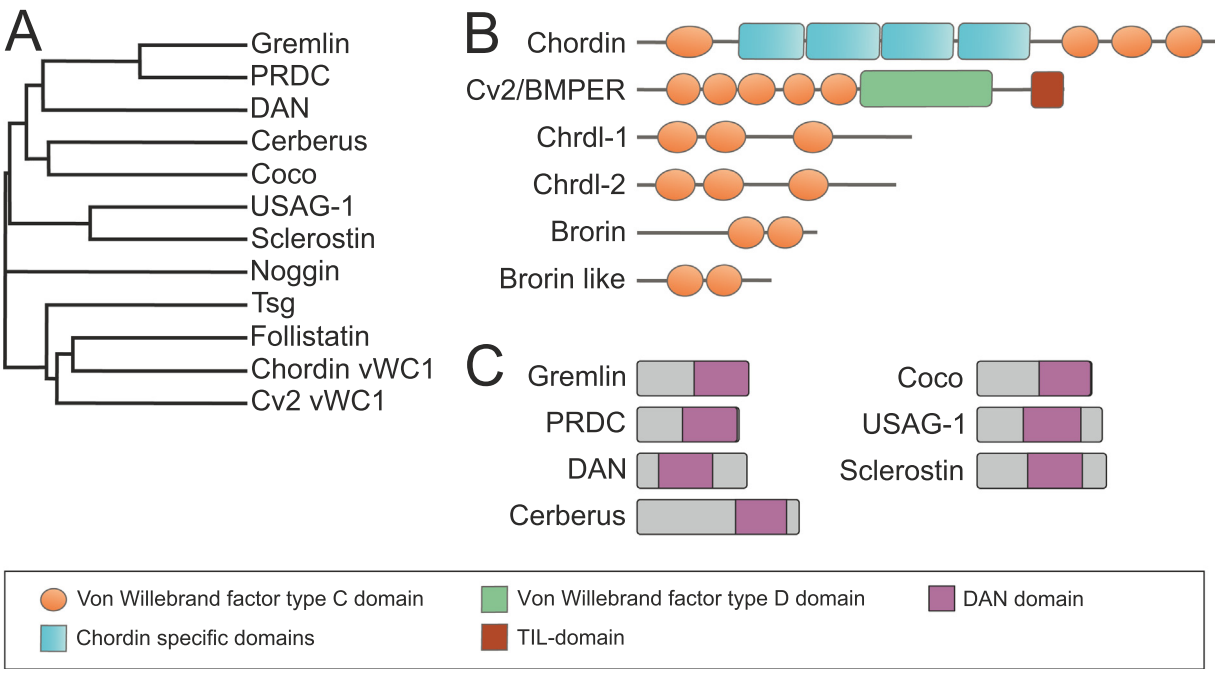

Fig. 2. BMP antagonists and their implications in diseases. **A.** Phenogram of BMP antagonists based on sequence similarity adapted from Brazil *et al* 2015. **B.** Domain structure of chordin family members. Von Willebrand factor type C (vWC) domains are represented as orange ovals. Chordin specific domains are illustrated as blue rectangles, the von Willebrand factor type D domain is depicted as a green rectangle and the brown rectangle represents the TIL-domain of Cv2/BMPER. **C:** Domain structure of CAN family members. The purple box illustrates the relative size and position of the DAN domain in each protein. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Moreover, interactions between different antagonists can lead to synergistic or inhibitory effects on their activity towards BMPs [24].

Role of BMP antagonists in tissue development

Deficiency of BMP antagonists leads to a multitude of defects in tissue development. An overview is given in Table 1.

Chordin/Sog

BMP antagonists play important roles in the development of various organisms from *Drosophila* to humans. A well-characterised example is the BMP-binding protein short gastrulation (Sog), the *Drosophila* homolog of the vertebrate protein chordin [25], which is crucial for early dorsal tissue patterning. Sog is secreted by cells in the ventral-lateral region and forms a gradient through diffusion [26]. In this developmental stage, decapentaplegic (Dpp), and screw (Scw), *Drosophila* homologues of the vertebrate BMPs, are expressed dorsally and ubiquitously, respectively. They form a heterodimer circulating towards the ventral region and back to dorsal if bound to Sog. The heterodimer is released and recycled after BMP capture by Sog and cleavage of Sog through the metalloprotease tolloid (Tld) [27]. For this mechanism, tight binding between Sog and Dpp-Scw must be assumed [11,25], but requires the formation of a collagen IV matrix, which binds BMPs and Sog thereby promoting their spatial concentration and interaction [28]. The dissociation of Sog-BMP-collagen is mediated by Tsg, which is found in *Drosophila* and vertebrates [29]. Tsg was shown to displace Sog-BMP from collagen IV leading to the assembly of a ternary Sog-BMP-Tsg complex [28]. Further, it could be demonstrated that Tld interacts via its N-terminal non-catalytic CUB domains with collagen IV, which enhances Tld activity towards Sog, and facilitates Tsg-dependent stimulation of cleavage [30]. Therefore, Tld binding to collagen IV represents an elegant way to fine-tune Tld activity to a particular ECM-dependent developmental context.

In zebrafish and *Xenopus* embryos, two opposing signalling centres establish a graded BMP signal to mediate dorsoventral patterning. For this, the Spemann's organiser in the dorsal tissue secretes BMP-binding proteins, including cerberus, chordin, follistatin, and noggin, whereas Bmp4 and 7 and the Tld homolog xolloid-related metalloprotease (Xlr/Tll) are produced in the ventral tissue. Tsg also plays an important role in these organisms by increasing the rate of chordin/Sog cleavage by Tld/Xlr [31–33].

Table 1 BMP antagonists and their respective loss of function features, including references.

BMP antagonist	loss of function phenotypes	references
Gremlin	pulmonary fibrosis, diabetic nephropathy	PMID: 16816361 [42],15957132 [43]
DAN	neuroblastoma	PMID: 8084583
Cerberus	defects in head formation	PMID: 12952900
Coco	defects in left/right body axis formation	PMID: 15466485
USAG-1	extra teeth, fused molars, altered cusp patterns	PMID: 16179481
Tsg	defects in neural arch development in cervical vertebrae, forebrain defects	PMID: 15013800
Follistatin	skeletal and cutaneous abnormalities e.g. decreased mass of the diaphragm, shiny taut skin	PMID: 7885475
Chordin	ventralised gastrulation, malformations in mice with most features of human DiGeorge and Velo-Cardio-Facial syndromes: pharyngeal malformations, lack of thymus and parathyroid glands, lack of heart colonisation by neural crest	PMID: 12810603 [13]

Crossveinless 2/BMPER

Crossveinless 2 (Cv2), also termed BMPER as the human ortholog [34], regulates BMP signalling in early axis formation in *Xenopus* [35], axis formation, haematopoiesis and vascular development in zebrafish [25,36], neural crest formation in chick [37], and skeletogenesis in mice [38]. Cv2 acts in close proximity to the cell surface [39], likely inhibiting signalling by increasing endocytosis and degradation of BMPs by targeting Cv2-BMP to the cell surface via the von Willebrand factor type D (vWD) domain of Cv2 [40]. In addition, a more direct effect is possible since the N-terminal cysteine-rich domain of Cv2 was shown to compete with type I and type II BMP receptors for overlapping binding sites on BMPs in zebrafish [41]. However, Cv2 can also promote BMP signalling. Two mechanisms are possible to explain the opposing functions of Cv2. In the first model, BMP bound to Cv2 is in equilibrium with a ternary complex formed with the type I BMP receptor. The close association between Cv2 and the type I BMP receptor is thought to allow the exchange of BMPs. At low Cv2 concentrations, BMPs are provided for the receptor, which switches to BMP sequestration at high Cv2 concentrations [39]. In the second model, the ability of Cv2 to promote BMP signalling is independent of its capability to bind BMPs, possibly by interactions with molecules like chordin/Sog or Tsg. Mammalian Cv2 can bind Tsg and chordin independently or in complexes with BMPs [35]. How this binding affects Cv2 function is not yet understood and does not exclude the first model, but it shows the close interplay between different BMP modulators in a context-dependent manner. As an alternative, the endocytic trap-and-sink mechanism was hypothesised, which leads to the efficient degradation of BMPER and Bmp4 by the lysosome. BMPER-mediated internalization of Bmp4 reduces the duration and magnitude of Bmp4-dependent SMAD signalling [40]. Thereby BMPER is able to decrease local extracellular BMP concentrations over a long range which leads to an increase of the diffusion gradient [11].

Mutations in BMP antagonists lead to congenital disorders primarily affecting the skeletal system

The various diseases caused by mutations or dysregulated levels of BMP antagonists illustrate the importance of BMP antagonists for pre- and postnatal development and life. For instance, increased levels of the BMP antagonist gremlin are associated with pulmonary fibrosis [42] and diabetic nephropathy [43], and mutations in gremlin-2 lead to isolated tooth agenesis (STHAG9: OMIM#617275), microdontia, short tooth roots, taurodontism, sparse and slow-growing hair, and dry and itchy skin [44].

Chordin-like 1 was found to be upregulated upon renal injury [45] and mutations can independently cause megalocornea 1 (MGC1: OMIM#309300) characterised by intellectual disability, facial dysmorphism, hypotonicity and seizures [46,47].

Craniodiaphyseal dysplasia (CDD: OMIM#122860) [48], autosomal dominant sclerosteosis 1 (SOST1: OMIM#269500) [49], and Van Buchem disease (VBCH: OMIM#239100) [50] are caused by mutations in sclerostin (*SOST*). *SOST* mutations lead to hyperostosis of the skull, mandible, clavicles, ribs, and diaphyseal cortices of the long bones. The most striking clinical features of patients with *SOST* mutations are the enlargement of the jaw and the thickness of the skull, which may lead to facial nerve palsy, hearing loss, and optic atrophy [51,52]. Sost null mice have a high bone mass phenotype characterised by significant increases in bone mineral density, bone volume, bone formation, and bone strength, further demonstrating SOST as negative regulator of bone formation [53]. Also, mutations in noggin primarily affect the skeletal system leading to congenital bone dysplasias such as brachydactyly type B2 (BDB2: OMIM#611377) [54], multiple synostoses syndrome 1 (SYNS1: OMIM#186500) [55], or the tarsal-carpal coalition syndrome (TCC: OMIM#186570) [56], among others (OMIM#602991) [57].

Clinical features of patients affected by noggin mutations include proximal symphalangism, multiple joint fusions, usually commencing in the hands, conductive deafness, and characteristic facial features, including a broad, tubular-shaped nose and a thin upper vermilion. Other features include brachydactyly, hypoplastic or absent middle phalanges, radial head dislocation, and pectus carinatum [58].

BMPER mutations cause skeletal disorders such as diaphanospondylodysostosis (DSD: OMIM#608022) [59], with phenotypic similarities to the BMPER-null mouse [60], and the milder ischiospinal dysostosis [61]. Skeletal characteristics of the phenotype include a small chest, abnormal vertebral segmentation, and posterior rib gaps containing incompletely differentiated mesenchymal tissue. Consistent craniofacial features include ocular hypertelorism, epicanthal folds, a depressed nasal bridge with a short nose, and low-set ears.

A summary of skeletal dysplasias caused my mutations in BMP antagonists is given in Table 2.

Role of BMP antagonists in cancer progression

Furthermore, the BMP pathway and BMP antagonists have been implicated in various stages of carcinogenesis in multiple cancers. Exemplarily, BMPs are involved in proliferation, migration, and invasion of epithelial cancer cells [62], but also sensitize tumor-initiating cells to mechanical cues from the extracellular tumor microenevirnment [63]. The role and influence of BMP antagonists has recently been reviewed in detail by Ouahoud and colleagues [64]. In human carcinomas elevated levels of certain antagonists such as gremilin-1 [65,66], and chordin-like-1 [67] have been found which may block the general anti-proliferative functions of BMP ligands, while elevated levels of BMP signalling, possibly through dysregulation of BMP antagonist expression, can promote certain tumours [68]. For instance it was shown that chordin overexpression in melanoma cells was sufficient to inhibit BMP4-induced cell migration and invasion [68], as well as BMP-induced expression of matrix metalloproteinases (MMPs) which lowers the metastatic potential [69]. Moreover, it was found that chordin was downregulated in ovarian tumours compared to normal tissue and in the epithelial lining covering the surface of the ovaries. Re-expression of chordin in ovarian cancer cell lines decreased migration and invasion [70].

SOST expression was found to be dysregulated in a number of cancers that metastasise to the bone [71]. This raises the possibility of targeting SOST for the treatment of cancer patients with bone metastasis [71]. In this context it was proposed that the bone microenvironment is a major contributor to metastasis. Analysis of co-culture models showed that elevated Wnt signalling derived from Sost deficient osteoblasts promoted prostate cancer cell invasion, while the application of recombinant SOST had an inhibitory effect [72]. Also, factors secreted by prostate cancer cells were shown to downregulate SOST in osteoblasts which may promote bone metastasis [73]. These findings illustrate the need for a better understanding of how the ECM of the tumour microenvironment modulates the functional behaviour of BMP antagonists during cancer progression.

Extracellular mechanisms modulating BMP antagonist function

Mechanisms modulating chordin function

Chordin is a specific antagonist of BMP-2, -4, -7, and anti-dorsalising morphogenic protein [74]. As mentioned above, chordin plays a key role in early embryogenesis, but it is thought to also have important functions in the adult organism like adult tissues, such as the brain [75]. Chordin exhibits a compact horseshoe-shaped structure comprising four von Willebrand factor type C (vWC) homology domains and four chordin specific (CHRD) domains

Table 2 Skeletal dysplasias caused by mutations in BMP antagonists. AD: autosomal dominant, AR: autosomal recessive, XLR: X-linked recessive.

genetic disorder	affected gene	OMIM#	inheritance	reference
STHAG9	GREM2	617275	AD	[34]
MGC1	CHRDL1	309300	XLR	[36]
CDD	SOST	122860	AD	[38]
SOST1	SOST	269500	AR	[39]
VBCH	SOST	239100	AR	[40]
BDB2	NOG	611377	AD	[44]
SYNS1	NOG	186500	AD	[45]
TCC	NOG	186570	AD	[46]
SYM1A	NOG	185800	AD	[48]
STAPESANKYLOSIS	NOG	184460	AD	[47]
DSD	BMPER	608022	AR	[59]

positioned between the first and second vWC domains [76] (Fig. 2B). BMP binding and biological activities are mediated by the vWC domains, especially vWC1 and -3 [77]. One chordin molecule is thought to interact with one BMP dimer by interactions of the terminally protruding BMP-binding domains with the growth factor [76]. The CHRD domains provide spacing to support the cooperative binding event in which both N- and C-terminal vWC domains interact with the BMP dimer [76,77].

By masking the binding sites on the BMP molecule, the receptor interaction and thus BMP signalling is inhibited by chordin. This inhibitory complex is strengthened by Tsg binding with high affinity to the C-terminal region of chordin [78]. Being complexed and inactive, BMP growth factors are thought to diffuse through the extracellular space [79] until chordin binds to cell surface anchored components such as BMPER [40], integrins [80], and collagen IV [81]. Hence, BMP can be targeted to a specific cellular microenvironment for subsequent release by tolloid proteases or taken up by endocytosis.

Tolloid proteinases abolish BMP inhibition by cleavage of chordin at two specific sites, downstream of the first and third vWC domain (see Fig. 3), leaving the BMP-binding vWC domains intact [82]. Interestingly, following partial, single cleavage of chordin by tolloids the resulting fragments appear to retain their BMP-inhibitory capacity [76]. Thereby, cleavage of either terminal vWC domain had little effect on the affinity of chordin for BMP-4 and BMP-7 but C-terminal cleavage increase the efficacy of chordin as a BMP-4 inhibitor [76]. One regulation mechanism of chordin cleavage by tolloids is alternative splicing which allows the generation of tolloid proteinases with differing biological activities and specificities [78]. Structural analyses showed that tolloids adopt a compact conformation and can dimerise, which together can restrict substrate access. This substrate exclusion mechanism provides substrate specificity and prevents unwanted ECM degradation [83–85].

Tsg plays an agonistic as well as antagonistic role in the regulation of BMP signal transduction. Tsg induces a conformational change in chordin, leading to an increased cleavage by tolloid proteinases, representing the BMP agonistic effect of Tsg (Fig. 3) [31,78]. In addition, Tsg not only increases the inhibitory capacity of full-length chordin, but also competes with the residual chordin fragments for BMP binding and increases their rate of degradation in vivo [31,32,86]. Interestingly, Tsg is also able to selectively inhibit BMP-7 signalling directly while no effect on BMP-4 signalling was observed [78]. Moreover, it could be demonstrated that Tsg interacts with partially cleaved chordin to increase BMP inhibition [78]. These findings suggest a type-specific regulation of BMPs by Tsg that allows fine-tuned regulation of BMP signalling in conjunction with other regulators [18].

The activity of chordin may also be influenced directly by additional ECM factors. As already mentioned, the *Drosophila* homolog Sog, interacts with collagen IV to form shuttling complexes to maintain long range BMP signalling [81]. This interaction is facilitated via vWC4, which when deleted may result in only short distance diffusion of growth factors. Alternatively, following cleavage of vWC4, the remaining C-terminal domains such as vWC2 may be more accessible to other binding partners. For instance, it was proposed that chordin forms ternary complexes with BMPs and Cv2. In chordin-Cv2-BMP complexes, both BMP and chordin directly interact with Cv2, where Cv2 induces a conformational change in chordin which favours the Cv2-BMP interaction [87].

Mechanisms impacting BMPER function

BMPER is composed of five vWC domains, one vWD domain and a trypsin inhibitor like domain (TIL) domain (Fig. 2). The conserved, acid-catalysed cleavage motif of BMPER, GDPH, is found within the vWD domain where a disulphide bridge tethers the cleavage products. BMPER is targeted to the plasma membrane via binding of its C-terminal region to cell surface heparan sulphate proteoglycans (HSPGs), therefore acting as a short-range modulator of BMP signalling [39]. However, after auto-catalytic cleavage of full length BMPER, most BMPER remains intact due to the internal disulphide bond but some is released. The N-terminal cleavage fragment, is soluble, diffuses away from the cell surface and is a better inhibitor of BMP4 [23]. As aforementioned, BMPER can both inhibit and enhance BMP signalling in a context and concentration-dependent manner. Crystallographic data showed that BMPER directly binds BMPs via its first vWC domain, which blocks the BMP type I and II receptor binding sites [41]. It is thought that the activity of BMPER as a BMP agonist is mediated by displacing the BMP growth factor from the chordin-Tsg-BMP inhibitory complex [87]. Thereby BMPER and chordin directly interact via the BMPER vWC-1 and −4 domains and vWC2 of chordin [25,35,87]. It could be also demonstrated that BMPER binds to Tsg through the N-terminal BMP-binding region and cooperatively inhibits BMP-4 signalling, exhibiting most likely a synergistic function in antagonising BMP activity [23].

Interactions of BMP antagonists with heparin/ heparan sulphate

Interestingly, the majority, but not all, of the BMP antagonists also bind heparin and heparan sulphate (HS) and get thereby targeted to the tissue –specific architecture of cellular microenvironments [88]. Out of the CAN family, gremlin-1, gremlin-2, and

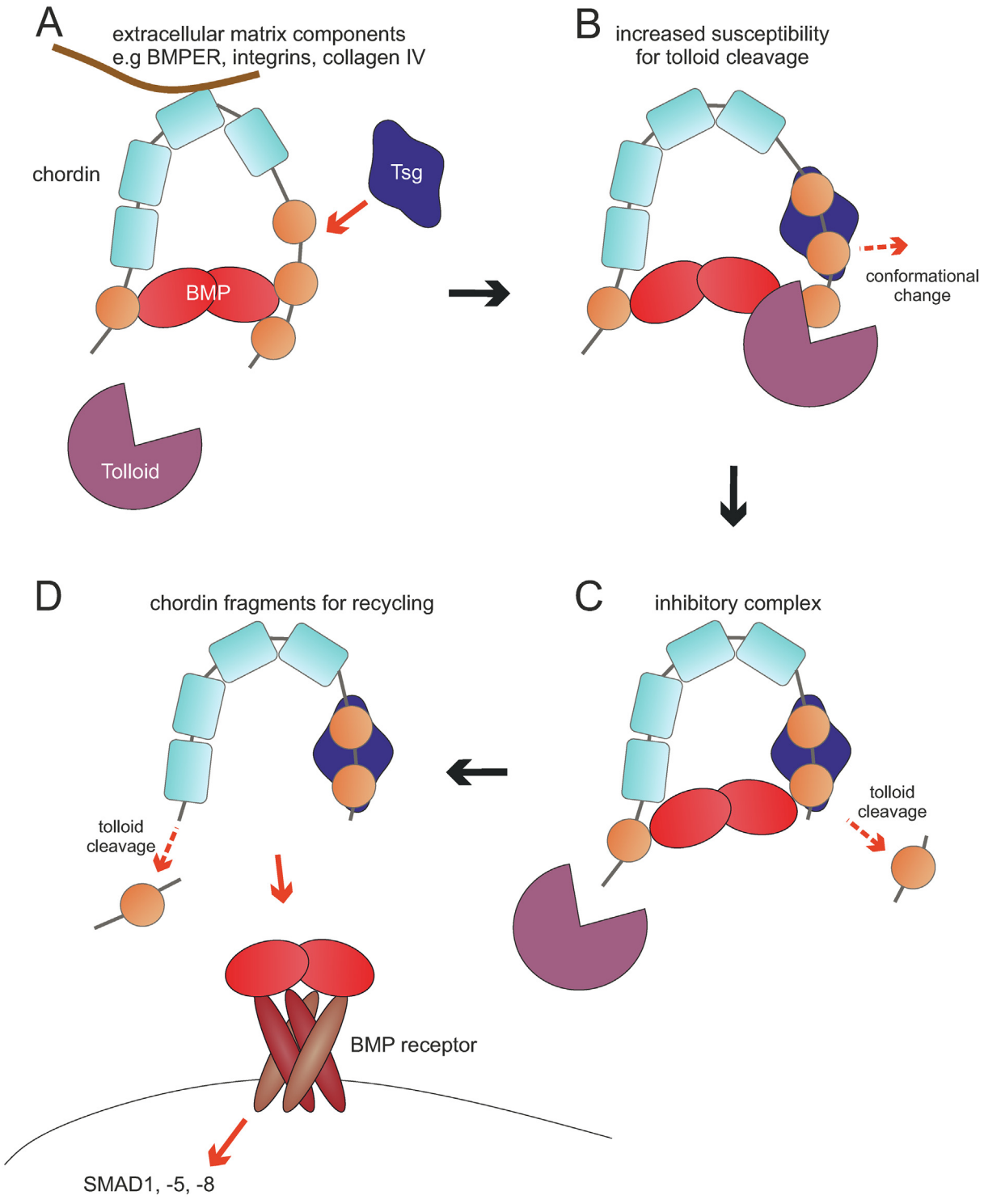

Fig. 3. Mechanism of BMP inhibition and release by chordin. **A:** Chordin binds BMP via its first and third von Willebrand factor type C (vWC) domains (orange). Likely, chordin is bound to matrix components such as BMPER, integrins or collagen IV (brown) in this process. **B:** Upon binding of twisted gastrulation (Tsg) to chordin, a conformational change is induced, making chordin more susceptible to cleavage by tolloid proteases. **C:** Tolloid cleavage of the fourth vWC domain leaves the inhibitory function of chordin intact. Tsg strengthens the remaining inhibitory complex. **D:** Tolloid cleavage of the first vWC domain mediates release of the BMP growth factor, which in turn can bind to a BMP receptor, activating intracellular BMP signalling. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sclerostin bind to heparin and HS via their cystine knot domain and exposed basic amino acid side chains within the second β-strand finger loop [89–91]. However, DAN/NBL1 does not bind heparin / heparan sulphate [90]. Interestingly, for gremlin-2 it was found that heparin binding interferes with antagonism of BMP-2 [21].

Noggin binds heparin via a basic-rich site N-terminal to the cystine-knot domain [92]. Follistatin binds to cell surfaces by interacting with HS [93], displaying a higher binding affinity if it forms a complex with GDF-8 (myostatin) [94] or activin A [95]. Chordin and Cv2/BMPER also bind heparin/HS [25,39] with a yet unknown binding site. Binding of chordin and noggin to HS and biglycan was demonstrated to increase their ability to inhibit signalling in vivo [96–98]. Moreover, the complexes formed by BMPs and antagonists to some extent have an increased affinity for heparin compared to uncomplexed proteins [16].

Sclerostin is an atypical member of the cystine-knot family, it does not form homo- or heterodimers and contains long flexible N- and C-termini which are not present in other family members [91,99]. SOST exhibits a linear extended basic surface on the convex side of the protein that is present in known heparin binding sites of other proteins and was confirmed to bind heparin through nuclear magnetic resonance experiments [91]. For binding, a network of hydrogen bonds supports the predominant stabilisation by electrostatic interactions between negatively charged sulphate groups on heparin and positively charged arginine and lysine residues on SOST; neither of which undergoes structural changes upon binding [91]. The high affinity binding to heparin ($K_D \sim 36–77$ n M) is chain length dependent, with preferred binding to full length heparin or large oligosaccharides (octadecasaccharide) and is enhanced by higher sulphation levels [100]. SOST acts as both as inhibitor of BMP [101] and Wnt/ β-catenin [102] signal transduction, but its interaction with heparin/ HS may facilitate a connection between both pathways. For instance, it was found that osteoblastic HS controls bone remodelling by regulating Wnt signalling and the crosstalk between bone surface and marrow cells [103]. Experimental data suggest that the interaction between SOST and heparin might result in spatial concentration of SOST near the cell surface of responsive cells, which could regulate its inhibitory effect on Wnt/β-catenin signalling. However, only a minor effect on Wnt signalling inhibition by SOST was observed upon impaired heparin binding [91]. An explanation might be the potential interaction site for Wnt/β-catenin inhibition, which is suggested to reside within the semi-flexible solvent exposed loop 2 region. Antibody targeting of this region blocks the protein inhibition of the Wnt/β-catenin signalling pathways and does not overlap with the heparin interaction site [91].

Interactions of gremlin with fibrillin microfibrils

Fibrillin microfibrils (FMF) form supramolecular networks in all connective tissues [104] and are known to be integrators of BMP signal transduction [15]. Fibrillins (fibrillin-1 and −2) are large (350 kDa) cysteine-rich glycoproteins that assemble into small diameter (10–12 nm) extracellular “microfibrils” with a characteristic “beads-on-a-string”-like appearance by electron microscopy that are ubiquitously found in bundles in association with basement membranes, and in all elastic fibres [104–108]. Fibrillin deficiency leads to connective tissue disorders with opposing features (termed “fibrillinopathies”) characterised by tall versus short stature, and arachnodactyly versus brachydactyly, hyperflexible versus stiff joints, hypo- versus hypermuscularity, and thin, hyperelastic, and translucent to thick, stiff, and hard skin [109], which has recently been reviewed by Sakai and colleagues [110]. This clearly suggests that FMF control developmental and homeostatic events most likely by regulating the activity of extracellular growth factors or their inhibitors [15]. Targeting of BMPs to fibrillin-1 and fibrillin-2 is mediated through specific and high-affinity interactions with their prodomains [111,112]. Biochemical investigations with recombinantly expressed proteins also showed that direct binding of BMPs to fibrillin-1 changed their activation status from bioactive to latent [113].

In addition to controlling BMP bioavailability directly via prodomain-mediated interactions, FMF may further modulate BMP signalling by targeting BMP antagonists. In a first report, it was shown that gremlin-1 and fibrillin-2 are overexpressed and co-localise in the microenvironment of malignant mesotheliomas, aggressive tumours, which originate from the mesothelial surface cells lining the serous body cavities such as the pleura, peritoneum, or pericardium with strong linkage to asbestos exposure [114]. In this study, a direct interaction of gremlin-1 to FMF building blocks was demonstrated in protein–protein interaction assays. Gremlin-1 showed a strong interaction to the N-terminal regions of fibrillin-1 and −2 with molecular affinities in a low nanomolar range ($K_D \sim 7.55$ and $K_D \sim 9.05$, respectively) [114]. As FMF serve as extracellular platforms for the integration of TGF-β and BMP signalling pathways, targeting of BMP antagonists to FMF may have the purpose to suppress BMP signalling and thereby promoting TGF-β signalling in certain disease conditions. Spatial concentration of BMP antagonists such as gremlin-1 may counteract BMP signalling in fibrotic reactions where gremlin was shown to be upregulated, e.g. in pulmonary fibrosis [39]. Similar mechanism may be at play in the microenvironment of mesotheliomas, but also of basal cell

carcinomas (BCCs) where gremilin-1 was also found to be upregulated [66]. Rare fibrillin-1 mutations resulting in fibrotic reactions do not lead to overall FMF deficiency but to functional inactivation of certain domains [115,116]. It remains to be investigated whether deletion or mutational inactivation of these domains affects gremlin-1 binding.

New research directions

BMP antagonists are ECM proteins that serve as potent regulators of BMP growth factor signal transduction. However, currently it is not understood how interactions of BMP antagonists with the dynamic tissue specific ECM microenvironment modulate their bioavailability in a spatio-temporal manner. Investigations in this direction may provide a better understanding of disease pathways resulting in ECM destruction or defective remodelling as not only observed in rare connective tissues disorders but also in more common chronic diseases such as fibrosis and cancer.

Our current research aims at identifying new interactions between BMP antagonists and FMF. These studies will also shed new light on the underlying molecular mechanisms leading to skeletal malformations of the fibrillinopathies (e.g. craniofacial abnormalities: narrow palate, underdeveloped jaws, malformation and misalignment of teeth) which show a significant clinical overlap with the skeletal dysplasias caused by mutations in BMP antagonists. In addition, it may also clarify why FMF deficiency leads to dysregulated growth factor signalling events that are tissue specific. For instance, prevalent fibrillin-1 mutations result in increased TGF-β signalling correlating with aortic wall destruction and aneurysm formation in Marfan syndrome (MFS: OMIM#154700). However, rare fibrillin-1 mutations result in stiff skin syndrome (SSkS: OMIM#184900) characterised by severe fibrosis and no aneurysm development [115]. MFS patients do not suffer from skin fibrosis suggesting the presence of extracellular pathways counteracting BMP or TGF-β dysregulation in a tissue-specific manner. Currently we do not know by which mechanisms BMP antagonists may be targeted to the cellular microenvironment in active or latent conformation. It is possible that spatial concentration of BMP antagonists via ECM targeting may not only potentiate their inhibitory function under certain cellular circumstances, but also increase their cellular uptake and clearing in another physiological context. Moreover, ECM targeting of BMP antagonists may be essential to establish crosstalk with other signalling axes such as the Wnt pathway.

Conclusions

BMP antagonists are diverse and highly important for tightly regulated BMP signalling during development and postnatal life. To ensure a regulated BMP response, interactions between growth factors and their respective inhibitors are fine-tuned by interactions with other regulators and the ECM. So far, due to the complexity of the extracellular mechanisms controlling BMP signal transduction, the understanding of the functional role of the ECM in this context remains limited. For example, chordin cleavage likely takes place in close proximity to matrix components such as BMPER, integrins or collagen IV, since the chordin inhibitory complex can bind these components, facilitating BMP storage as inactive complex. The influence and participation of these matrix components needs to be further investigated. Therefore, for a better understanding of disease mechanisms underlying the pathogenesis of skeletal dysplasias resulting from BMP antagonist mutations more research is essential.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations:
ActR, activin receptor; ALK3, anaplastic lymphoma kinase 3; ATF2, activating transcription factor 2; BDB2,

brachydactyly type B2; BISC, BMP-induced signalling complex; BMPER, BMP binding endothelial regulator; BMPs, bone morphogenetic proteins; CAN, cerberus and DAN; CDD, craniodiaphyseal dysplasia; CHRD domain, chordin specific domain; CUB domain, for complement C1r/C1s, Uegf, Bmp1 domain; Cv2, crossveinless-2; DAN, differential screening selected gene aberrative in neuroblastoma; Dpp, decapentaplegic; DSD, diaphanospondylodysostosis; ECM, extracellular matrix; ERK, extracellular signal-regulated kinases; FMF, fibrillin microfibrils; HS, heparan sulphate; HSPGs, heparan sulphate proteoglycans; MAPKs, mitogen-activated protein kinases; MGC1, megalocornea 1; PRDC, protein related to DAN and Cerberus; PI3K, phosphoinositide 3-kinase; Scw, screw; Sog, short gastrulation; SOST, sclerostin; SYNS1, multiple synostoses syndrome 1; TCC, tarsal-carpal coalition syndrome; TGF-β, transforming growth factor- β; Tld, tolloid; Tsg, twisted gastrulation; VBCH, Van Buchem disease; vWC, von Willebrand factor type C; vWD, von Willebrand factor type D; Xlr/Tll, xolloid-related metalloprotease

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