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REVIEW

Bone morphogenetic proteins and their antagonists: current and emerging clinical uses

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Bone morphogenetic proteins (BMPs) are members of the TGFβ superfamily of secreted cysteine knot proteins that includes TGFβ1, nodal, activins and inhibins. BMPs were first discovered by Urist in the 1960s when he showed that implantation of demineralized bone into intramuscular tissue of rabbits induced bone and cartilage formation. Since this seminal discovery, BMPs have also been shown to play key roles in several other biological processes, including limb, kidney, skin, hair and neuronal development, as well as maintaining vascular homeostasis. The multifunctional effects of BMPs make them attractive targets for the treatment of several pathologies, including bone disorders, kidney and lung fibrosis, and cancer. This review will summarize current knowledge on the BMP signalling pathway and critically evaluate the potential of recombinant BMPs as pharmacological agents for the treatment of bone repair and tissue fibrosis in patients.

Abbreviations

ALK, activin receptor-like kinase; BMP, bone morphogenetic protein; DN, diabetic nephropathy; EMT, epithelial-mesenchymal transition; Smad, sma and mothers against decapentaplegic

Introduction

Bone morphogenetic proteins (BMPs) are glycosylated, secreted extracellular matrix-associated molecules that regulate a wide variety of biological processes (Walsh *et al.*, 2010). BMPs are members of the TGFβ superfamily of proteins (for nomenclature see Alexander *et al.*, 2013), and they have been shown to regulate limb and digit formation, kidney development, cancer, angiogenesis and tissue fibrosis. The importance of BMP signalling during development is underpinned by the elaborate regulatory mechanisms controlling BMP signalling intra- and extracellularly. These processes range from epigenetic methylation and miRNA-mediated RNA regulation, protein ubiquitination, pseudo-receptors and secreted extracellular antagonists that bind to BMPs, preventing their engagement with their cognate receptors (Walsh *et al.*, 2010). Dysregulation of the BMP signalling pathway can have drastic consequences during mammalian development. Mutations in BMP receptors are implicated in vascular conditions, such as pulmonary artery hypertension (PAH), skeletal abnormalities, such as brachydactyly, and polyp formation in the colon (reviewed in Miyazono *et al.*, 2010). These data are supported by a wide variety of skeletal and other phenotypes in BMP pathway transgenic and knockout mice. Herein, we will summarize current knowledge on BMP signalling in cells, how BMPs are processed and secreted, and focus on the role of BMPs in bone and cartilage formation. In particular, we will discuss current and potential uses for recombinant human BMPs for the treatment of compound bone fractures and fibrotic diseases of the kidney and lung. Finally, we will briefly discuss new data demonstrating the therapeutic potential of targeting BMP antagonists in PAH.

Intracellular BMP processing and secretion

There are approximately 20 members of the BMP family, and these have been expertly described by colleagues elsewhere (Balemans and Van Hul, 2002; Weiskirchen and Meurer, 2013). BMPs are synthesized as large inactive precursor proteins that contain a signal peptide at the N-terminus and a mature polypeptide at the C-terminus, connected by a pro-domain that regulates proper folding (Xiao *et al.*, 2007). In the case of BMP-4, the precursor protein is cleaved by the proprotein convertase furin at two sites within the prodomain (S1 and S2), a process thought to occur within the Golgi network (Figure 1). Cleavage at the S1 (RXKR) site alone results in a non-covalently associated ligand–prodomain complex that is relatively unstable and targeted for degradation by the proteasome. The mature active BMP peptide is generated by initial cleavage at the S1 site, which is then trafficked to a compartment within the post-trans-Golgi network, where the acidic environment makes the accessibility and subsequent cleavage at the S2 site (RXXR) site possible. This liberates the mature BMP peptide from the prodomain to yield the stable, active, mature BMP protein (Nelsen and Christian, 2009). These active, mature BMP monomers contain seven cysteines, six of which form three intramolecular disulphide bonds, also known as cysteine knots. The remaining seventh cysteine amino acid facilitates the dimerization with another BMP monomer by forming a covalent disulfide bond, establishing a biologically active dimeric ligand for BMP receptor activation (Bragdon *et al.*, 2011). BMP homodimers are the dominant signalling form of each BMP, and these are bound by homodimeric BMP antagonists such as noggin and gremlin, which restrict their activity (discussed below) (Israel *et al.*, 1996; Zhu *et al.*, 2006; Guo and Wu, 2012). A role for BMP-2/7 and BMP-4/7 heterodimers in mesoderm induction and differentiation of bone marrow cells has also been described (Suzuki *et al.*, 1997; Yuan *et al.*, 2011).

BMP signalling

BMPs elicit their effects through two classes of transmembrane serine/threonine kinase receptors known as type I (BMPR-I) and type II receptors (BMPR-II). (Rosenzweig *et al.*, 1995). There are three type II receptors that BMPs bind to: BMP type II receptor (BMPR2), activin A receptor type II (ActR2) and activin A receptor type IIB (ActR2B). There are also three type I receptors that BMPs preferentially bind to: activin receptor-like kinase (ALK)2, ALK3 (BMPRIA) and ALK6 (BMPRIB) (Nohe *et al.*, 2004). Certain BMPs have been shown to have a higher affinity for certain type I receptors. For example, BMP-4 preferentially binds to ALK3 and ALK6, whereas BMP-6 and -7 preferentially bind to ALK2, but can

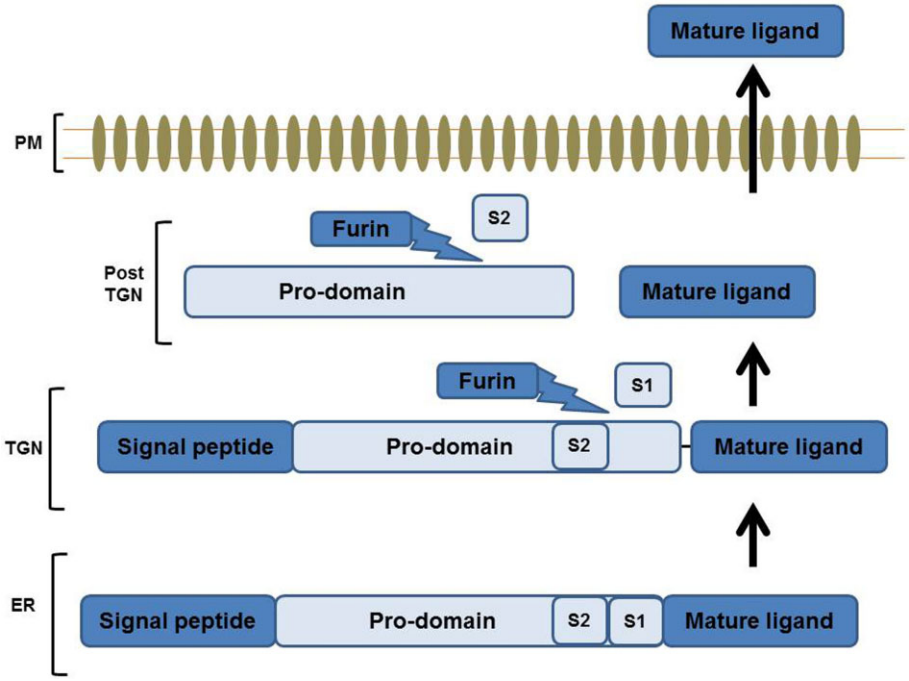

Figure 1

Model of BMP synthesis and site-specific cleavage by proprotein convertases. BMPs are synthesized within the cell as large, inactive dimeric precursor proteins within the ER that require site-specific cleavage by proprotein convertases to produce stable, active dimers. This occurs at two sites located within its pro-domain. Initial cleavage at S1 site is thought to occur in the TGN and results in a pro-domain–ligand complex. This pro-domain–ligand complex is further cleaved at S2 site, which is rendered accessible due to the acidic enviroment within the post-TGN. This releases the mature ligand from the pro-domain, yielding a stable biologically active BMP monomer. ER, endoplasmic reticulum; TGN, trans-Golgi network; CM, cell membrane.

also engage with ALK3 (Aoki *et al.*, 2001). Once bound, this ligand/receptor complex recruits the constitutively active type II receptor, which phosphorylates the type I receptor on its cytoplasmic domain that is rich in glycine and serine residues (GS domain) (Miyazono *et al.*, 2010). Upon ligand binding, the BMP signal is transmitted from the cell membrane to the nucleus via the canonical sma and mothers against decapentaplegic (Smad)-dependent and/or non-canonical Smad-independent pathways (e.g. MAPK and Akt pathways).

Smad proteins: mediators of BMP signalling

Smad proteins are the vertebrate homologues of the *Drosophila melanogaster* mothers against decapentaplegic and related *Caenorhabditis elegans* Sma gene (Riggins *et al.*, 1996). The Smad proteins are grouped based on their activators and functions. Smad1/5/8 are termed as receptor Smads (R-Smads) and are downstream targets of BMP ligands via BMPR1 activation. There is a second class of R-Smads called Smad2/3, which mainly regulates downstream targets of TGFβ signalling through TGFβ2 and ALK5 type I receptors (Massague *et al.*, 2005; Murakami *et al.*, 2009). Recent data suggest that TGFβ can also phosphorylate Smad1/5 in endothelial cells (Liu *et al.*, 2009). This 'switch' in Smad protein engagement by TGFβ1 is facilitated by the loss of BMP and activin membrane bound inhibitor (BAMBI) expression and may contribute to endothelial homeostasis (Guillot *et al.*, 2012). Structurally, BAMBI is similar to the type I receptor; however, it lacks an intracellular kinase domain. BMP signalling can also be regulated through BAMBI (Figure 2). As BAMBI is structurally similar to the type I BMP receptor, it competes with the type I receptors for BMP ligand interaction, subsequently trapping BMP ligands and inhibiting signalling (Onichtchouk *et al.*, 1999). BMP-2 has been shown to phosphorylate Smad2/3 in B16 melanoma cells (Murakami *et al.*, 2009), thus demonstrating that the previous model of specific activation of R-Smads by specific TGFβ family members is not as restricted as previously thought.

Once the R-Smads have undergone phosphorylation, interaction with their respective anchor proteins [endofin for R-Smad1/5/8 (Shi *et al.*, 2007) and SARA for R-Smad2/3 (Tsukazaki *et al.*, 1998)] is destabilized, facilitating R-Smad interaction with activated type I receptors to form a heteromeric complex with the common co-mediator Smad, Smad4 (co-Smad) (Xu *et al.*, 2000; Shi *et al.*, 2007). This interaction takes place via the MH2 domain of the R-Smads (Chacko *et al.*, 2001). The MH2 domain is conserved among all types of Smads, including the inhibitory Smads (I-Smads), Smad6/7, which are endogenous inhibitors of this pathway. In contrast, the MH1 domain is conserved in the R-Smads and co-Smad4 only (Miyazono *et al.*, 2010). The MH1 domain is responsible for Smad-mediated DNA binding, interacting with certain DNA binding proteins and antagonizing MH2 functions. The MH2 domain is responsible for BMP receptor recognition [via their Ser-Ser-*X*-Ser (SS*X*S) sequence motif], interaction with other Smads, nuclear translocation and DNA binding (Wrana, 2000). Unphosphorylated R-Smads are inactive, as their MH1 and MH2 domains interact with each other, therefore suppressing the functions of each other. Upon receptor activation, Smad-receptor binding occurs, the MH1/MH2 interaction is disrupted, and the Smad is phosphorylated. The Smad nuclear import signal is exposed, which increases R-Smad affinity for co-Smad4, facilitating the formation of an R-Smad/co-Smad complex. This complex then translocates to the nucleus and regulates the transcription of various target genes such as *Smad6*, *ID* genes, *BAMBI* and *Smad7* through interaction with either Smad-binding elements (SBEs) or GC-rich sequences found in the promoters of *BMP* target genes (Figure 2; Miyazono *et al.*, 2010). The affinity between the Smad complex and SBE motif is relatively weak due to promoters of target genes containing only one or more SBEs (Shi *et al.*, 1998; Massague *et al.*, 2005). *In vitro*, this can be overcome by using concatemers with multiple SBE repeats, which increase the binding affinity for transcriptional activation (Zawel *et al.*, 1998). Physiologically, concatemers rarely occur, so increasing the affinity of Smad binding to target DNA requires the association of Smads with DNA binding cofactors (Massague *et al.*, 2005). One example of R-Smad/co-Smad complex DNA binding cofactor is the forkhead family member, FoxH1. The R-Smad/co-Smad complex binds to FoxH1 to regulate the transcription of *Mix2* (Chen *et al.*, 1997). There are, however, a number of other DNA binding cofactors for BMP signalling, including Runx, HOXc8 and CREB-binding protein (Li and Cao, 2006; Miyazono *et al.*, 2010).

Regulation of BMP signalling

The BMP/Smad signalling cascade is tightly regulated by both intra- and extracellular processes: intracellular processes include proteasome-mediated degradation via Smurf proteins, for example, Smurf1, which has been shown to target R-Smad1/5 for degradation (Zhu *et al.*, 1999), and also inhibition via I-Smad6 and 7 action, which act at different points in the pathway. Smad7 resides within the nucleus, and upon BMP/TGFβ receptor activation, it is released into the cytoplasm (Itoh *et al.*, 1998). It has been shown that Smurf1/2 interacts with Smad7 to facilitate inhibition of the Smad pathway via binding to the activated receptor, thereby competitively antagonizing Smad1/5/8 binding (Suzuki *et al.*, 2002). Smad6 specifically inhibits the BMP pathway by interacting with activated R-Smads, preventing the formation of the R-Smad/co-Smad complex, thus inhibiting signal transduction (Hata *et al.*, 1998).

Protein phosphorylation/dephosphorylation is also important in the tight regulation of BMP cell signalling. Protein phosphatase-1 (PP1) regulates BMP signalling by dephosphorylation of the TGFβ/BMP receptor. TGFβ/BMP type 1 receptor stimulation leads to Smad7 interaction with growth arrest and DNA damage protein (GADD34), which is a regulatory/targeting subunit of the type 1 receptor. This then triggers the recruitment of the catalytic subunit of PP1 (PP1c) and subsequent dephosphorylation of the TGFβ type 1 receptor, thereby reducing TGFβ receptor activation (Shi *et al.*, 2004). Dephosphorylation of R-Smads can also occur through the metal ion-dependent protein phosphatases 1A (PPM1A). PPM1A directly interacts with phosphorylated

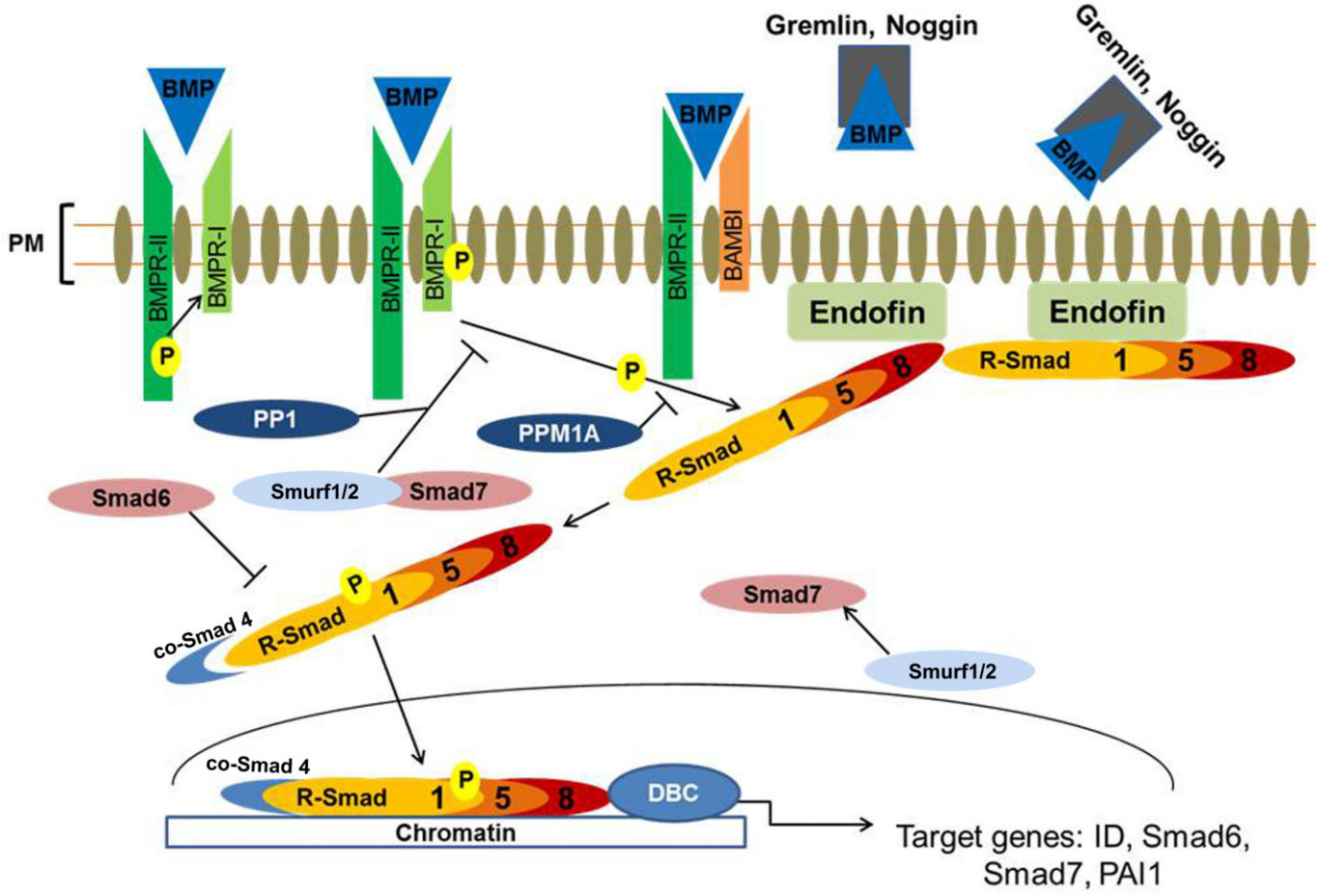

Figure 2

Activation and regulation of BMP/Smad-dependent signalling. BMP ligands bind to and activate type I and II serine/threonine kinase receptors (BMPR-I and BMPR-II, respectively), which triggers phosphorylation of the R-Smad1/5/8. Phosphorylated R-Smads form a heteromeric complex with the co-Smad 4, enabling its subsequent translocation to the nucleus where it binds to specific GC rich sequences within the promoters of several target genes in concert with various DNA binding co-factors. This cascade is tightly regulated, through pseudo-receptors such as BAMBI, which quench BMP ligands thereby limiting their availability to interact with their receptors. Extracellular regulation occurs via direct interaction of BMPs with their secreted antagonists, thereby preventing ligand receptor interaction. Intracellularly, this pathway is regulated by the I-Smads, Smad6 and 7. Smad6 prevents the formation of R-Smad and co-Smad complex formation. Smad7 regulates this pathway by forming a complex with Smurf1/2 and competing with R-Smad for type I receptor-mediated activation effectively antagonizing BMP/Smad pathway activation. This pathway is also regulated by phosphatases such as PP1, which dephosphorylates the BMP type I receptor, and PPM1A, which dephosphorylates R-Smads. R-Smad, receptor regulated Smads; I-Smad, inhibitory Smads; co-Smad, co-mediator Smad; ID, inhibitor of differentiation; PAI1, plasminogen activator inhibitor-1; PP1, protein phosphatase 1; PPM1A, metal ion-dependent protein phosphatases 1A; DBC, DNA binding co-factors; PM, plasma membrane.

R-Smads, resulting in their inactivation through dephosphorylation (X Lin *et al.*, 2006b). Together, both PP1c and PPM1A act as effective 'off' switches for TGFβ/BMP signalling.

The BMP/Smad signalling pathway is also regulated by a family of secreted extracellular antagonists, that directly bind to the BMP ligand preventing their interaction with BMP receptors. These antagonists have been extensively reviewed previously (Balemans and Van Hul, 2002; Walsh *et al.*, 2010; Nakamura and Yanagita, 2012). Briefly, antagonists of BMPs include proteins such as noggin, chordin, gremlin, crossveinless, USAG-1 and follistatin. Most of these proteins are expressed in a highly regulated temporospatial manner during development. The key role of these antagonists and their BMP targets is highlighted by the often lethal developmental defects displayed by mice lacking one or more of these proteins (reviewed in Walsh *et al.*, 2010). Noggin is a potent antagonist of BMP signalling, showing high affinity for BMP-2 and -4, although it can also antagonize BMP-7. The co-crystal of homodimeric noggin bound to homodimeric BMP-7 has provided the field with critical information regarding the structural basis of BMP-BMP antagonist action (Zimmerman *et al.*, 1996; Groppe *et al.*, 2002).

Chordin is expressed in the brains of adult rats, where high levels of BMP-2/4 have also been reported (Mikawa and Sato, 2013). Gremlin was originally identified as a protein capable of inducing secondary axis formation in *Xenopus laevis* embryos (Hsu *et al.*, 1998). Gremlin has been shown to play a critical role in kidney development, with *grem1*

homozygous knockout mice born without kidneys as a result of excessive BMP/Smad signalling (Michos *et al.*, 2007). This phenotype was rescued by reducing BMP-4 signalling 'volume' in *grem1–/–;bmp-4+/–* mice (Michos *et al.*, 2007). Like noggin and chordin, gremlin regulates BMP signalling through direct interaction with BMP ligands, thereby blocking ligand–receptor interaction (Wordinger *et al.*, 2008). Gremlin can also regulate BMP signalling intracellularly through intracellular interaction of gremlin with BMP-4 precursor protein, inhibiting the formation and secretion of the mature and active BMP ligand (Sun *et al.*, 2006). Levels of gremlin are increased in fibrotic disease, and pharmacological strategies to inhibit gremlin action will be discussed later in this review.

BMP signalling during bone development

The key role of BMPs in skeletal development has been expertly summarized by others elsewhere. We provide a brief summary of the area below. The vertebrate skeleton consists of bone and cartilage, and contains three main cell types: chondrocytes, osteoblasts and osteoclasts (Karsenty, 2003). Bone formation in vertebrates involves both membranous ossification (involving osteoblast differentiation) and endochondral ossification (involving chondrocyte differentiation, review in Karsenty, 2003; Nishimura *et al.*, 2012). Both of these processes are regulated by BMPs, and BMP-2 and BMP-4 are powerful inducers of osteoblast and chondrocyte differentiation, which induces bone and cartilage formation (Nishimura *et al.*, 2012). BMPs were originally isolated from bone by Marshall Urist in 1965, when he showed that soluble extracts from bone extracellular matrix could stimulate bone formation when injected into rodents (Urist, 1965).

So how do BMPs such as BMP-2 and BMP-4 drive bone formation in vertebrates? The mechanism of BMP activation of their type I/II receptors and the R-Smad1/5/8 canonical signalling pathway is described earlier. This pathway is active in both chondrocytes and osteoblasts, and is highly regulated by a series of intracellular and extracellular mechanisms (reviewed in Walsh *et al.*, 2010). Mature BMPs are secreted from osteoblasts and may either (i) activate their membrane receptors; (ii) be bound and inhibited by one or more of their secreted antagonists; or (iii) bind to extracellular matrix proteins such as collagen and act as a 'reservoir' of BMP for neighbouring cells (Miyazono *et al.*, 2010). A series of transcription factors critical to bone and cartilage formation have been identified (reviewed in Nishimura *et al.*, 2012). Runx2, Osterix and Sox9 are key transcription factors that regulate downstream BMP signalling in osteoblasts (Nishimura *et al.*, 2008). The conservation of BMP action and the bone formation process in vertebrates give added value to the interpretation of data from the phenotypes of mouse models displaying manipulations in BMP or BMP antagonist function (Karsenty, 2003). Patients with mutations in the *Runx2* gene develop cleidocranial dysplasia, characterized by short stature and defective clavicle (collar bone) formation (Otto *et al.*, 2002), and *runx2–/–* mice die shortly after birth due to a severe defect in osteogenesis (Komori *et al.*, 1997; Otto *et al.*, 1997).

Apart from the extracellular cysteine-knot containing antagonists such as gremlin and noggin discussed earlier, some BMP proteins themselves act as competitive antagonists that bind to the type I/II receptors. BMP-3 is secreted from osteoblasts and is found in the bone extracellular matrix. BMP-3 binds to the ActR type II and inhibits BMP-2/4 binding (Daluiski *et al.*, 2001; Gamer *et al.*, 2005). Mice lacking BMP-3 display an increased bone mass (Daluiski *et al.*, 2001), whereas transgenic mice overexpressing BMP-3 develop spontaneous fractures, highlighting a key role for BMP-3 in bone development (Gamer *et al.*, 2009). Recent data have shown that BMP-3 can repress osteoblast differentiation from progenitor cells (Kokabu *et al.*, 2012). Inhibin is another circulating antagonist of BMPR2 that also blocks BMP-2/4 action in osteoblasts (Rosen, 2006). Negative feedback for the BMP signal is provided by intracellular molecules such as Smad6 and Tob. Smad6 is a BMP-2 target gene, and increases in Smad6 levels inhibit osteoblast differentiation by triggering Smurf1-dependent proteasomal degradation of the BMP receptor and Smad proteins (Murakami *et al.*, 2003; Horiki *et al.*, 2004; Massague *et al.*, 2005). Another inhibitor of osteoblast function, called Tob, blocks BMP action by binding and inhibiting R-Smads, thus preventing BMP signalling (Yoshida *et al.*, 2000). Other molecules such as CRIM1, BAMBI and endoglin regulate BMP signalling in bone and other tissues (Onichtchouk *et al.*, 1999; Wilkinson *et al.*, 2003; Ishibashi *et al.*, 2010; Pardali *et al.*, 2011). CRIM1 inhibits BMP action via regulation of intracellular BMP processing, whereas BAMBI acts a pseudo-receptor to reduce active BMP binding to type I/II receptors (Onichtchouk *et al.*, 1999; Wilkinson *et al.*, 2003). In contrast, endoglin (also known as CD105) is sometimes referred to as the type III BMP receptor and acts to enhance BMP action at the plasma membrane (Ishibashi *et al.*, 2010).

The highly complex nature of BMP action and regulation emphasizes the critical nature of this pathway in bone (and other tissue) development. The effect of changes in the levels or function of proteins in the BMP pathway can be seen in the wide array of phenotypes observed in patients. Many of these phenotypes have been reviewed elsewhere (Rosen, 2006; Walsh *et al.*, 2010). Mutations in the BMP antagonists noggin and sclerostin cause brachydactyly (lack of digits; Lehmann *et al.*, 2007), and sclerosteosis characterized by fused digits and excessive bone growth (Brunkow *et al.*, 2001). Increases in the level of several BMP antagonists including noggin, chordin and follistatin have been implicated in osteoarthritis (Tardif *et al.*, 2009). Mice overexpressing Grem1 in bone develop osteopenia and increased bone fractures (Gazzerro *et al.*, 2005), whereas mice with bone-specific deletion of Grem1 develop increased bone formation and density (Gazzerro *et al.*, 2007). Changes in the levels of other BMP antagonists such as noggin and twisted gastrulation have also been implicated in bone density changes in mouse models (Wu *et al.*, 2003; Sotillo Rodriguez *et al.*, 2009). All of these data and more underline the principle that tight temporospatial regulation of BMP action is critical for normal bone and cartilage formation in mammals. The 'volume' of BMP signalling is a fundamental parameter in determining correct bone formation during development. With this in mind, the next section of this review will discuss the uses of BMPs in modulating bone formation in patients with a range of bone injuries.

Therapeutic uses of BMPs for bone repair

Between 5 and 10% of fractures have impaired healing due to non-union of bone (Gautschi *et al.*, 2007). This can greatly increase patient morbidity due to an extended hospital stay, infection rates concomitant healthcare costs. The gold standard of therapy for non-union fractures is bone autograft, where bone fragments are harvested from the patient's iliac crest and used to heal the fracture (Cook *et al.*, 1994). BMPs have been shown in pre-clinical models to stimulate bone formation (osteoinduction; Einhorn *et al.*, 2003) and angiogenesis (Zhang *et al.*, 2009). Both BMP-2 and BMP-7 are now clinically approved as adjunct therapies for the treatment of non-union fractures (Gautschi *et al.*, 2007). Recombinant human BMP-2 is available from Medtronic (Minneapolis, MN, USA) as InFUSE®, and rhBMP-7 is available from Stryker (Kalamazoo, MI, USA) as OP-1. The efficacy of BMPs has been tested in a number of clinical trials. The BESTT study evaluated the effect of rhBMP-2 on open tibia fractures, as a supplement to standard surgical intervention (Nauth *et al.*, 2009). In this single blind trial, all patients underwent the standard irrigation/mechanical fixation of the fracture, followed by placebo or rhBMP-2 treatment (Govender *et al.*, 2002). RhBMP-2 was delivered via an absorbable type I collagen sponge, and the primary outcome of secondary surgical intervention due to non-union of the fracture was assessed. The study showed that patients treated with rhBMP-2 had accelerated fracture and wound healing, as well as lower infection rates, with few safety concerns (Govender *et al.*, 2002). A follow-up study by Swiontkowski *et al.* (2006) looking at the effect of rhBMP-2 on open tibial fractures supported the beneficial effects of rhBMP-2 seen above. Others have assessed the effect of rhBMP-2 in parallel with bone allografting and shown benefit in several cases (e.g. Jones *et al.*, 2006, summarized in Nauth *et al.*, 2009). Alternatives to the absorbable collagen sponge carrier have been developed. Boerckel *et al.* (2011) suggested that a hybrid nanofibre mesh/alginate delivery system yielded greater bone connectivity compared with the collagen sponge. Novel approaches such as using mesenchymal stem cells transfected with BMPs for the treatment of non-union fracture are being explored (Liebergall *et al.*, 2013).

Some caution should be noted regarding the use of BMPs in bone repair. A Cochrane review by Garrison and colleagues collated data from 11 clinical trials and suggested that a lack of robust data on the use of rhBMPs in fracture healing exists. Many of the clinical trials have been limited in terms of unconscious bias risk due to a lack of double blinding, and there has been considerable industry involvement to date. Garrison *et al.* (2010) concluded that the high cost of rhBMP treatment may be justified only in patients with the most severe fractures. Others have also examined the economics of rhBMP treatment. A single dose of rhBMP costs approximately $5000 in the USA (Nauth *et al.*, 2009). The cost of BMP treatment was weighed against potential savings from a societal/healthcare perspective, with groups in the USA and UK suggesting that rhBMP treatment was only cost-effective when used in severe open tibia fractures and in high-risk patients such as smokers (Jones *et al.*, 2006; Garrison *et al.*, 2007; Ziran *et al.*, 2007). rhBMP-7 is currently used 'off-label' for the treatment of non-union fractures (Nauth *et al.*, 2009). Similar to rhBMP-2, studies have shown that rhBMP-7 can accelerate the healing time and reduce the number of secondary interventions when used in conjunction with surgical repair of tibial fractures (Ristiniemi *et al.*, 2007).

BMP-7 may also be useful in cases of osteoarthritis, where BMP-7 induction of extracellular matrix collagen production may oppose the degradation of the articular cartilage via stimulation of chondrocyte function (Fan *et al.*, 2004; Nishida *et al.*, 2004). BMP-7 may also provide a benefit in this situation by reducing pro-inflammatory cytokine release (e.g. IL-1 and IL-6), thereby inhibiting MMP-1 and MMP-13 matrix metalloproteinase expression (Huch *et al.*, 1997; Koepp *et al.*, 1999; Im *et al.*, 2003; Boon *et al.*, 2011). In human patients, rhBMP-7 has entered a Stryker-sponsored phase 2 double-blind randomized dose-finding trial for the treatment of osteoarthritis of the knee. Doses of rhBMP-7 used in this trial ranged from 0.03 to 0.3 $mg \cdot mL^{-1}$, with appropriate placebo comparators (http://clinicaltrials.gov/ct2/show/NCT01111045). Phase 1 studies by this company suggested limited toxicity to report with rhBMP-7 treatment of age and sex-appropriate (60 years, female) control subjects (Hunter *et al.*, 2010). Results on the phase 2 study for rhBMP-7 are pending. rhBMP-7 was approved by the Food and Drug Administration for use in lumbar spinal fusion in patients where bone autograft was not feasible or likely to be successful, such as in smokers or diabetic patients. Many adverse events identified as a result of rhBMP-7 treatment, such as haematoma, swelling, neurological defects and retrograde ejaculation, may have been underreported. A review of these trials by Dr Nancy Epstein concluded that there is mounting evidence that the use of rhBMP-7 in spinal fusion surgery contributes to major perioperative and post-operative morbidity (Epstein, 2013).

What should we conclude from the wealth of conflicting data reporting on the therapeutic benefit versus side effects of rhBMPs in the treatment of non-union fractures, spinal fusions, and other bone and cartilage-related diseases? Nauth *et al.* (2009) concluded that the data on the use of rhBMPs have been disappointing so far due to the high doses required, unresolved issues with the carrier molecules and the limitations of the clinical trials completed to date. When the safety concerns identified by Epstein (2013) are also considered, it is clear that more data from well-designed double-blind, placebo controlled, multiple dose and carrier formulation clinical trials are required to allow clinicians to proceed with confidence when utilizing rhBMPs in orthopaedic surgeries and other procedures.

Role of BMPs in tissue fibrosis

BMPs have been implicated in mammalian development, cancer, and fibrosis or tissue scarring. Roles for BMP-2 in heart development, BMP-4 and 7 in neural crest cell maturation and BMP-7 in kidney formation have been demonstrated (summarized in McCormack and O'Dea, 2013). Fibrosis or scarring occurs in organs such as kidney, lung and heart as a result of damage caused by hyperglycaemia, hypoxia and ischaemic insult. Fibrosis is characterized by an increase in the number of fibroblast cells that secrete extracellular matrix

proteins such as collagen and fibronectin, which contribute to the damage experienced by the organ or tissue. Scar-forming fibroblasts may derive from the tissue itself (resident fibroblasts), from activation of quiescent cells in the circulation (fibrocytes) or from injured epithelial or endothelial cells that undergo an epithelial/endothelial-mesenchymal transition (EMT/EndMT) to form collagen-secreting myofibroblasts (LeBleu *et al.*, 2013). EMT was originally proposed as a source of myofibroblasts in kidney injury by Iwano *et al.* (2002), who suggested that approximately one-third of all myofibroblasts in the fibrotic kidney arise from EMT. More recent data using fate mapping suggest that in the fibrotic kidney, 50% of myofibroblasts arise from proliferation of local resident fibroblasts, 35% arise from infiltration of bone marrow-derived cells, 10% arise from endothelial cells via EndMT and 5% arise from epithelial cells via EMT (LeBleu *et al.*, 2013). However, there is a large body of evidence suggesting that EMT does not contribute to renal fibrosis, and that vascular pericytes are the source of the myofibroblast in the kidney (reviewed in Grgic *et al.*, 2012). Why are these details important? Firstly, the degree of tubulointerstitial fibrosis is inversely correlated to renal function and is a good predictor of renal function going forward (Bohle *et al.*, 1994). Secondly, the development of therapeutics to delay, halt or reverse renal fibrosis is at the forefront of many research programmes in both academia and industry. It has been hypothesized that the numbers of scar-forming fibroblasts in a fibrotic tissue can be reduced by pharmacological approaches that aim to reverse EMT or otherwise reduce fibroblast burden in the affected tissue. Manipulation of BMP signalling is at the forefront of many of these strategies, and these data will be summarized below.

Manipulation of BMPs as a strategy to reverse tissue fibrosis

TGFβ1 was identified as the primary fibrotic cytokine that induces EMT in multiple organs including kidney, heart, skin, lung (Okada *et al.*, 1997; Zeisberg *et al.*, 2003b). Exposure of epithelial cells to TGFβ1 leads to a decrease in adherens junction proteins such as E-cadherin and ZO-1, and an increase in α-smooth muscle actin and collagen IV. Regardless of the cellular context, the key inducers of EMT are a series of transcription factors called Snai1/2 and Zeb1, which orchestrate the wave of gene expression changes critical to EMT progression (Peinado *et al.*, 2007). There are a limited number of conflicting reports on the role of BMPs in EMT in different cell types. BMP-4 has been convincingly shown to have a pro-EMT, pro-fibrotic effect in different epithelial cells. BMP-4 induces EMT and enhanced cell migration in airway epithelium (Molloy *et al.*, 2008; McCormack *et al.*, 2013), as well as EMT and invasion in squamous cell carcinoma and other cancer cells (Hamada *et al.*, 2007; Theriault *et al.*, 2007; Xu *et al.*, 2011). BMP-4 also contributes to cardiac hypertrophy in models of pressure overload (Sun *et al.*, 2013). Transgenic overexpression of BMP-4 induced glomerular damage and proteinuria in mice, a phenotype similar to that seen in diabetic nephropathy (DN) (Tominaga *et al.*, 2011). BMP-2 has been suggested to enhance EMT in human skin wounds and in human lung epithelial cells (Yan *et al.*, 2010; McCormack *et al.*, 2013). BMP-2 can also increase the expression of α-SMA in hepatic stellate cells, which are thought to undergo EMT during liver fibrosis (Shen *et al.*, 2003). Others have suggested that BMP-2 possesses anti-fibrotic activity by suppressing TGFβ1-induced EMT in an *in vivo* model of renal fibrosis (Yang *et al.*, 2009). These authors subsequently showed that BMP-2 attenuated Snail expression, thus reversing TGFβ1-induced EMT (Yang *et al.*, 2011).

In terms of EMT and fibrosis, most of the interest has surrounded BMP-7, as it is this member of the BMP family that appears to have strong anti-fibrotic activity. In the heart, delivery of rhBMP-7 reduced both EndMT and associated cardiac fibrosis induced by pressure overload in mice (Zeisberg *et al.*, 2007). The same report also demonstrated that rhBMP-7 reduced fibrosis in a heart transplant mouse model of chronic organ rejection (Zeisberg *et al.*, 2007). Kang *et al.* (2010) showed that s.c. delivery of rhBMP-7 reduced vascular calcification in the aorta as a result of vitamin D overload (Kang *et al.*, 2010). Significantly, these authors demonstrated that pretreatment with rhBMP-7 for 7 days could prevent vitamin D-induced vascular calcification (Kang *et al.*, 2010). In an asbestos model of pulmonary fibrosis, administration of rhBMP-7 reduced the severity of fibrosis in these mice (Myllarniemi *et al.*, 2008).

In the liver, BMPs have been implicated in the wound healing response to carbon tetrachloride (CCl_4)-induced fibrosis, and *Bmpr1a*+/– mice displayed retarded healing post-CCl_4 treatment (Oumi *et al.*, 2012). Oral administration of adeno-associated virus-rhBMP-7 also suppressed CCl_4-induced liver fibrosis in mice (Hao *et al.*, 2012). Intraperitoneal injection of BMP-7 was similarly effective in liver fibrosis in rats (Zhong *et al.*, 2013). The mechanism of BMP-7-mediated repair is suggested to be inhibition of TGFβ1 signalling in hepatic stellate cells due to reductions of extracellular matrix collagen I and III deposition and enhanced hepatocyte regeneration (Hao *et al.*, 2012; Yang *et al.*, 2012). In models of inflammatory bowel disease and colitis, i.v. administration of BMP-7 reduced the expression of TGFβ1 and pro-inflammatory cytokines such as IL-6, causing an attenuation of colitis severity (Maric *et al.*, 2003). Further data from this group identified that BMP-7 administration increased BMP-2 levels and decreased the levels of the BMP antagonist noggin, leading to an overall recovery of BMP signalling that reversed the inflammatory bowel disease phenotype (Maric *et al.*, 2012). These data suggest that BMP-2 may also have a pro-resolution rather than a pro-fibrosis role during fibrosis-associated inflammation in the gut and other tissues.

In the kidney, BMP-7 antagonizes TGFβ1 actions in renal mesangial and tubular epithelial cells. Significantly, loss of BMP-7 is associated with fibrosis associated with DN (Wang *et al.*, 2001; Wang and Hirschberg, 2003; 2004). Importantly, the Hirschberg group also showed that overexpression of BMP-7 in glomerular podocytes attenuated renal fibrosis and improved renal function (Wang *et al.*, 2006). Previous data had shown that the administration of recombinant BMP-7 (OP-1) reduced the severity of ischaemic acute renal injury in mice (Vukicevic *et al.*, 1998). The Kalluri group demonstrated the benefit of BMP-7 administration in a chronic model of nephrotoxic serum nephritis (Zeisberg *et al.*, 2003b), as well as genetic models of renal fibrosis (Zeisberg *et al.*, 2003a) and

DN (Sugimoto *et al.*, 2007). These authors also demonstrated a BMP-7-mediated reversal of EMT in their model system, as well as in adult renal fibroblasts, facilitating regeneration of injured kidney (Zeisberg *et al.*, 2005). BMP-7 gene transfer using gold nanoparticles into rabbit keratocytes also inhibits corneal fibrosis *in vivo* (Tandon *et al.*, 2013).

In contrast, a group from Centocor, Inc., showed that BMP-7 failed to reverse TGFβ1-induced EMT in human proximal tubular epithelial cells (Dudas *et al.*, 2009). Similarly, BMP-7 did not reverse EMT or protect against fibrosis in a mouse model of lung or skin fibrosis (Murray *et al.*, 2008). These groups suggested that recombinant BMP-7 may not be an optimal anti-fibrotic agent for human disease treatment. Despite these disappointing results, the potential for anti-fibrotic therapy based on BMP-7 is still strong. A small peptide mimetic of BMP-7 called THR123, which activates the BMP ALK3 receptor, has shown remarkable activity in reversing renal fibrosis from a diverse range of mouse models of acute and chronic kidney disease (Sugimoto *et al.*, 2012). Some authors in the field have expressed some concerns about the interpretation of these data. For example, issues were raised with the signalling properties of THR123 versus BMP-7, and also whether oral delivery of a peptide such as THR123 can be expected to deliver an effective therapeutic dose *in vivo* (Whitman *et al.*, 2013). The Kalluri group have responded to these questions and discussions are ongoing as to the therapeutic potential of the THR123 peptide for human kidney disease (Sugimoto *et al.*, 2013).

A small molecule called dorsomorphin was identified by Yu *et al.* (2008b) as the first small molecular inhibitor of BMP signalling. Using dorsomorphin, the authors identified a role for BMP signalling in iron metabolism in the zebrafish liver (Yu *et al.*, 2008b). Mice expressing a constitutively activated form of the ALK2 receptor develop ectopic endochondral bone formation, mimicking a disease called fibrodysplasia ossificans progressiva (FOP) in humans. Treatment of these mice with a dorsomorphin derivative (LDN-193189) inhibited BMP signalling in C2C12 cells and reduced the severity of the FOP phenotype in mice (Yu *et al.*, 2008a; Boergermann *et al.*, 2010). A recent paper by Sanvitale *et al.* (2013) described a new class of ALK2 inhibitor, the lead compound of which is called K02288. K02288 inhibits BMP-stimulated Smad1/5/8 phosphorylation, without affecting TGFβ1 signalling, suggesting an impressive degree of specificity (Sanvitale *et al.*, 2013). Both dorsomorphin and its analogues, together with K02288, offer exciting tools for the development of specific, small-molecule inhibitors of BMP signalling in human disease.

Another small molecule called tilerone has been shown to reduce the severity of pulmonary fibrosis in mice, by increasing the expression of BMP-7 (Lepparanta *et al.*, 2013). A secreted molecule called kielin/chordin-like protein (KCP-1) can bind to and inhibit TGFβ1, while enhancing BMP-7 signalling (J Lin *et al.*, 2005; 2006a). Transgenic expression of KCP-1 can attenuate both acute and chronic renal injury in mice (Soofi *et al.*, 2013), and the authors speculate that KCP-1 may have potential as a therapeutic agent if administered in the correct context (Soofi *et al.*, 2013). Therefore, despite the difficulty in translating the anti-fibrotic potential of BMP-7 in animal models to patients, alternative strategies that boost BMP-7 signalling such as THR123 or KCP-1 may provide an indirect route to utilize the anti-fibrotic potential of BMP-7 for the treatment of fibrotic disease in patients.

Targeting BMP antagonists for the treatment of fibrotic disease

One of the proposed mechanisms by which BMP-7 reduced liver fibrosis is by decreasing the expression of the secreted BMP antagonist gremlin (Yang *et al.*, 2012). Increased levels of Grem1 are associated with fibrotic conditions in the kidney, lung, heart liver and eye (Dolan *et al.*, 2005; Lee *et al.*, 2007; Mezzano *et al.*, 2007; Carvajal *et al.*, 2008; Costello *et al.*, 2008; Walsh *et al.*, 2008; Rodrigues-Diez *et al.*, 2012; Yang *et al.*, 2012; Mueller *et al.*, 2013). In parallel with data showing that reduced BMP-7 signalling is associated with diabetic kidney disease, mutations in the BMP receptor type II are implicated in >70% of heritable cases of PAH (Li *et al.*, 2010). These data identify the targeting of Grem1 as a novel therapeutic modality for the treatment of fibrosis *in vivo*. Supporting this hypothesis, mice lacking one copy of the Grem1 gene (*grem1*+/–) are partially protected from the early sequelae of DN (Roxburgh *et al.*, 2009). In addition, *in vivo* delivery of siRNA-mediated targeting of Grem1 demonstrated therapeutic potential for the treatment of DN by restoring BMP-7 levels (Zhang *et al.*, 2010). In the lung, Grem1 is overexpressed in idiopathic pulmonary fibrosis (Koli *et al.*, 2006). Transient adenovirus-mediated overexpression of Grem1 in lung led to epithelial cell activation and a reversible lung fibrosis (Farkas *et al.*, 2011). Grem1 levels are also elevated in response to hypoxia in models of PAH, and haplodeficiency of *grem1* increased BMP signalling and reduced vascular remodelling associated with PAH (Costello *et al.*, 2008; Cahill *et al.*, 2012). Given the large body of data demonstrating that elevated levels of Grem1 contribute to tissue fibrosis, pharmacological strategies designed to inhibit Grem1 function *in vivo* have been tested in a range of disease models. Data from a group in Novartis have shown for the first time that an anti-Grem1 antibody ameliorated PAH in a mouse model (Ciuclan *et al.*, 2013). Pretreatment with the anti-Grem1 monoclonal antibody reduced pulmonary vascular remodelling and right ventricle hypertrophy, and increased BMP signalling in the lung (Ciuclan *et al.*, 2013). These data provide pharmacological proof-of-principle that this approach of targeting Grem1 as a means of increasing BMP signalling is now being explored in other fibrotic conditions of the kidney and other tissues. Of course, other BMP antagonists may also represent important bona fide targets in fibrotic disease. Consistent with this idea, a recent paper from the Yanagita group demonstrated that twisted gastrulation exacerbates podocyte injury via inhibition of BMP-7 signalling (Yamada *et al.*, 2014).

Concluding remarks

This review has summarized a wealth of data suggesting that pharmacological manipulation of the BMP pathway holds great potential for the treatment of human diseases of bone, kidney fibrosis, cancer, etc. The identification of small mol-

ecules that specifically target the BMP pathway creates the potential for screening these compounds in a range of *in vitro* and *in vivo* models of disease where BMP actions are implicated. The natural progression of this work is the drive towards clinical trials for the small-molecule inhibitors of BMP signalling in various diseases. We look forward to monitoring the evolution of this exciting field, which will hopefully generate improved targeted therapies for patients suffering from bone disorders as well as fibrosis in the kidney, lung and other tissues.

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Conflict of interest

D.B. collaborates with scientists in Astra Zeneca, Mölndal, Gothenburg, Sweden, on a BBSRC CASE PhD studentship.

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