

Bone morphogenetic proteins in tissue engineering: the road from the laboratory to the clinic, part I (basic concepts)

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Abstract

Discovered in 1965, bone morphogenetic proteins (BMPs) are a group of cytokines from the transforming growth factor- β (TGF β) superfamily with significant roles in bone and cartilage formation. BMPs are used as powerful osteoinductive components of diverse tissue-engineering products for the healing of bone. Several BMPs with different physiological roles have been identified in humans. The purpose of this review is to cover the biological function of the main members of BMP family, the latest research on BMPs signalling pathways and advances in the production of recombinant BMPs for tissue engineering purposes. Copyright © 2008 John Wiley & Sons, Ltd.

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1. Body morphogenetic proteins

Research on bone regeneration began decades ago as a result of intensive studies on bone growth and healing. Bone has been recognised, among the many tissues in human body, as one with the highest potential for regeneration. As early as 1889 Senn noticed that decalcified bone could induce healing of bone defects (Senn, 1889). Later Lavender provided the first evidence of ectopic bone formation after injecting bone crude extracts into muscle (Levander, 1934, 1938). In 1965 the pioneering work by Urist marked a landmark on the research in bone regeneration. Urist discovered that the active compound responsible for bone regeneration was a mix of proteins and named these as *bone morphogenetic proteins* (BMPs; Urist, 1965). In the years that followed Sampath and Reddi created a crude but highly reproducible bioassay for BMP for ectopic bone

formation (Sampath and Reddi, 1981). The assay was based on the activity of the enzyme alkaline phosphatase enzyme and the content of calcium in the newly formed bone. Reddi proposed that BMPs are responsible for the initiation of a cascade of developmental events, in which progenitor cells in the bone marrow were induced by these factors to produce bone cells leading to bone regeneration (Reddi and Huggins, 1972; Reddi, 1981).

During the decades of 80s and 90s the BMP genes were cloned and the recombinant proteins were shown to be biologically potent (Wozney *et al.*, 1988). Much work followed with the use of recombinant BMPs (rhBMPs) for clinical applications such as spinal fusion, fracture healing and dental tissue engineering (Nakashima and Reddi, 2003; Seeherman and Wozney, 2005). Human BMPs are now produced in larger amounts by recombinant technology. In 2002 FDA gave approval of two products containing rhBMP-2 and rhBMP-7 in absorbable collagen carriers for spinal fusion and long bone non-unions (McKay *et al.*, 2007; White *et al.*, 2007). In a recent review Reddi proposed naming BMPs also as *body morphogenetic proteins*, due to their extensive roles in various tissues and organs beyond the bone (Reddi, 2005).

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2. Members of the BMP family

In humans several members are under the designation of BMPs, from BMP-2 to BMP-18 (Table 1). BMP-1 is not a member of the BMP family. It is a misidentified protein with chordinase and procollagen proteinase activities, implicated in embryonic patterning (Kessler *et al.*, 1996). In fact, since BMPs were named by homology-based cDNA cloning, there is confusion over BMP designations and there is a need to develop a more appropriate nomenclature for the BMP family.

The biological functions of BMPs are mainly related to bone and cartilage formation (Reddi, 2005), although BMP-8b, -10 and -15 do not have known roles in bone or cartilage. BMP-10 is involved in cardiac development and BMP-15 in ovarian physiology (Chen *et al.*, 2004; Knight and Glister, 2006). BMP-8b is involved in reproductive cells (Zhao *et al.*, 1996). Usually only BMP-2 to BMP-11 are considered to be BMPs. BMP-12, -13 and -14 are named cartilage-derived morphogenetic proteins (CDMPs), as these induce chondrogenic phenotypes rather than osteogenesis (Reddi, 2003). BMPs are also involved in embryonic patterning (Kishigami and Mishina,

2005), in skeletal formation (Tsumaki and Yoshikawa, 2005) and in organogenesis of other tissues behind bone. For example, BMP-2 plays a role in heart morphogenesis (Callis *et al.*, 2005) and in neural stem cells (White *et al.*, 2001), BMP-7 in kidney formation (Simic and Vukicevic, 2005) and diverse BMPs have unique roles in reproductive organs (Shimasaki *et al.*, 2004; Tsumaki and Yoshikawa, 2005). During the events of early embryogenesis, BMPs are involved in the dorsal–ventral patterning with different roles, e.g. BMP-2 and -4 induce embryo ventral differentiation, while BMP-3 and -3b oppose the ventralizing effect by inducing tail and head formation, respectively (Hino *et al.*, 2004). During gastrulation a BMP gradient in signalling established by BMP antagonists is responsible for directing cells into forming organs such as bone, cartilage, kidney or heart, depending on the levels of BMP activity and of other cytokines (Yamamoto and Oelgeschlager, 2004). The pleiotropic effect of the different BMPs is of important consideration for tissue engineering. For instance, besides being used for differentiation of cartilage and bone BMP-4 is being studied for a role in keeping the undifferentiated state in embryonic stem cells (Qi *et al.*, 2004). BMPs

Table 1. BMP members in humans and their main physiological roles

BMP	Nomenclature	Main physiological roles	References
<i>Bone morphogenetic proteins</i>			
<u>BMP-2</u>	BMP-2a	Cartilage and bone morphogenesis/heart formation	Wang <i>et al.</i> , 1990a; Kang <i>et al.</i> , 2004; Callis <i>et al.</i> , 2005
BMP-3	Osteogenin	Negative regulator of bone morphogenesis	Hino <i>et al.</i> , 2004
BMP-3b	GDF-10	Negative regulator of bone morphogenesis	Hino <i>et al.</i> , 2004
<u>BMP-4</u>	BMP-2b	Cartilage and bone morphogenesis/kidney formation	Luyten <i>et al.</i> , 1994; Kubler <i>et al.</i> , 1998a; Oxburgh <i>et al.</i> , 2005
BMP-5	–	Limb development/bone morphogenesis	Cho <i>et al.</i> , 2002; Zuzarte-Luis <i>et al.</i> , 2004
<u>BMP-6</u>	Vrg1, Dvr6	Hypertrophy of cartilage/bone morphogenesis/oestrogen mediation	Gitelman <i>et al.</i> , 1994; Rickard <i>et al.</i> , 1998; Kang <i>et al.</i> , 2004
<u>BMP-7</u>	OP-1	Cartilage and bone morphogenesis/kidney formation	Reddi, 1998; Kang <i>et al.</i> , 2004; Simic and Vukicevic, 2005
BMP-8	OP-2	Bone morphogenesis/spermatogenesis	Ozkaynak <i>et al.</i> , 1992; Zhao <i>et al.</i> , 1996; Cho <i>et al.</i> , 2002
<u>BMP-9</u>	GDF-2	Bone morphogenesis/development of cholinergic neurons/glucose metabolism	Chen <i>et al.</i> , 2003; Kang <i>et al.</i> , 2004; Lopez-Coviella <i>et al.</i> , 2006
BMP-11	GDF-11	Axial skeleton patterning/eye development/pancreas development/kidney formation	Esquela and Lee, 2003; Harmon <i>et al.</i> , 2004; Kim <i>et al.</i> , 2005; Andersson <i>et al.</i> , 2006
<i>Cartilage-derived morphogenetic proteins</i>			
BMP-12	CDMP-3, GDF-7	Ligament and tendon development/development of sensory neurons	Reddi, 2003; Lo <i>et al.</i> , 2005
BMP-13	CDMP-2, GDF-6	Cartilage development and hypertrophy	Reddi, 2003
BMP-14	CDMP-1, GDF-5	Chondrogenesis/angiogenesis	Yamashita <i>et al.</i> , 1997; Reddi, 2003; Zeng <i>et al.</i> , 2007
<i>Others</i>			
BMP-8b	OP-3	Spermatogenesis	Zhao <i>et al.</i> , 1996
BMP-10	–	Heart morphogenesis	Chen <i>et al.</i> , 2004
BMP-15	GDF-9b	Ovary physiology	Knight and Glister, 2006
BMP-16	Nodal	Embryonic patterning	Celeste and Murray, 1999
BMP-17	Lefty	Embryonic patterning	Celeste and Murray, 2000
BMP-18	Lefty	Embryonic patterning	Celeste and Murray, 2000

BMPs known to induce complete bone morphogenesis are underlined.

clearly possess pivotal roles in controlling the proliferation and differentiation fates of cells. In bone formation a clear bone-inducing role is observed for BMP-2, -4, -6, -7 and -9. These BMPs have been shown to induce bone mineralisation and increase in osteocalcin levels in C2C12 cells (Chen *et al.*, 2003) and orthotopic ossification in mice (Kang *et al.*, 2004). BMP-3 and -3b are possibly inhibitors or negative regulators of osteogenesis, as these downregulate the expression of ALP in bone cells (Hino *et al.*, 2004). During fracture healing different BMPs follow specific defined temporal sequences. BMP-2 appears to be an early factor peaking at day 1 after fracture while BMP-14 peaks at day 7 during cartilage formation and BMP-3, -4, -7 and -8 are expressed mainly after 2 weeks (Cho *et al.*, 2002). In analogy different BMPs are also expressed in site-specific patterns during the formation of bone (Zoricic *et al.*, 2003).

2.1. Subgroups within the BMP family

BMPs belong to TGF β superfamily, which includes several other growth factors, such as activins, inhibins or TGF β s. The members of the BMP family may be subdivided into different subgroups based in their gene homology and similarity in protein structure (Wozney and Rosen, 1998; Kishigami and Mishina, 2005; Reddi, 2005; see Table 2). The proteins within the BMP-2/4 group, osteogenic protein-1 (OP-1) group, BMP-9/10 group and BMP-12/13/14 group share sequence similarities of more than 50%. BMP-11 and BMP-15 are more distant members, similar to growth differentiation factors -8 and -9, respectively. Interestingly, the morphogens named as growth and differentiation factors (GDFs) have similarity to some BMPs and could therefore be included in the BMP family. However, these are not described in this review since their biological roles are beyond bone induction, except for GDF-3 a BMP inhibitor, and GDF-8, a negative regulator of bone and muscle mass (Levine and Brivanlou, 2006; Hamrick *et al.*, 2007). Additionally, under the term BMP are BMP-16 to -18. These were given disclosure in patent applications (Celeste and Murray, 1999, 2000). BMP-16 is a human homologue to murine Nodal and BMP-17/18 are related to Lefty. Both have important roles during embryonic patterning, mainly by antagonising the effect of BMP signalling in mesoderm formation (Nodal) or for the establishment of left-right embryonic asymmetry (Lefty) (Meno *et al.*, 1997; Thisse *et al.*, 2000). Since BMP-16 to -18 are more distantly related to BMPs than to other TGF β superfamily members these should conceivably form a different group in the TGF β superfamily, distinct from BMPs.

3. The structure of BMPs

BMPs as all members of the TGF β superfamily, are homo or heterodimers linked via disulphide bridges. These

Table 2. Suggested subgroups within BMP family (grouped by similarity to OP-1)

Subgroup	Members of TGF β superfamily
OP-1 (BMP-7)	BMP-5, -6, -7, -8, -8b
BMP-2/4	BMP-2, -4
CDMP1/2/3	BMP-12, -13, -14 (GDF-5, 6, 7)
BMP-9/10	BMP-9 (GDF-2), BMP-10
BMP-3/3b	BMP-3, BMP-3b (GDF-10)
BMP-11/GDF-8	BMP-11 (GDF-11), GDF-8
BMP-15/GDF-9	BMP-15 (GDF-9b), GDF-9

proteins are expressed as large precursor polypeptide chains containing a hydrophobic signal sequence, a long and poorly conserved N-terminal pro-region sequence, a mature domain with a highly conserved C-terminal region and an N-terminal region that varies among the different BMPs.

BMPs are biologically active in homodimer and heterodimer conformation (Israel *et al.*, 1996). BMPs form a conserved motif of seven cysteines, which is involved in the formation of six intrachain disulphide bonds and a single interchain bond, necessary to dimer formation. Heterodimers in cell cultures have been observed to induce much higher yields of osteogenic marker alkaline phosphatase (ALP) (Aono *et al.*, 1995) than homodimeric BMP. Moreover, BMP-4/7 heterodimers have also been reported for their very potent mesoderm inducing activity in *Xenopus* (Suzuki *et al.*, 1997). A role has been attributed to heterodimers of BMP-4/8b in primordial germ cell formation (Ying *et al.*, 2001). In humans, there is evidence that BMP heterodimers may be also involved in specific biological processes (Butler and Dodd, 2003), challenging researchers to look for working with heterodimers besides the widely documented investigation with BMP homodimers.

Intracellularly BMPs are produced as precursors that dissociate after proteolytical cleavage by subtilisin-like proprotein convertases to form mature proteins (Ge *et al.*, 2005). However, in some cases the pro-region remains associated after secretion to the extracellular space, inhibiting the binding of BMPs to cell receptors. Importantly, propeptide forms of mature BMPs have been linked to specific physiological roles, such as in synovial rheumatoid arthritis for proBMP-2 and proBMP-6 (Lories *et al.*, 2007), and in the stabilisation of BMPs in the cases of proBMP-4 (Degnin *et al.*, 2004) and proBMP-9 (Brown *et al.*, 2005). It is possible that the half-life of mature BMPs may be influenced by the identity of their prosequences, and that prodomains serve for other cellular effects (Gregory *et al.*, 2005) which by itself is an important consideration for tissue-engineering approaches involving the use of these morphogens.

3.1. Wrist and knuckle epitopes

During the last years crystallographic studies have provided insights into the structure of BMPs and their interaction with receptors upon binding. Crystallographic

studies have been reported for BMP-7 (Griffith *et al.*, 1996), BMP-2 (Scheufler *et al.*, 1999) BMP-9 (Scheufler *et al.*, 1999), BMP-9 (Brown *et al.*, 2005) and BMP-14 (Schreuder *et al.*, 2005). These studies have revealed a common polypeptide core for BMPs and TGF β . The differences in hydrophobic core amongst these BMPs explain the different affinities for the various cell receptors and possibly for its different physiological roles (Allendorph *et al.*, 2006). In BMP-2 two symmetrical pairs of juxtaposed epitopes were described: the wrist epitope, with affinity to BMP receptor I (BmprI), containing residues from both monomers; and the knuckle epitope, which includes residues from only one monomer, with low affinity for BMP receptor II (BmprII; Nickel *et al.*, 2001). Mutations in epitope II lead to loss of activation of both BMP/Smad pathway and induction of ALP, or even complete inhibition of its function (Kirsch *et al.*, 2000). Variants with antagonistic properties are exclusively generated by mutations in the knuckle epitope of BMP-2. A synthetic knuckle peptide alone was also sufficient to induce ALP, osteocalcin and ectopic bone formation in a rat model (Saito *et al.*, 2003). Alterations in epitope I lead only to a reduction of ALP activity while activation of the BMP–Smad pathway is maintained (Knaus and Sebald, 2001). From these findings it was possible to conclude that the activation of the BMP–Smad pathway and the induction of early osteogenic marker ALP were triggered by distinct BMP–receptor complexes. Different pathways are probably triggered in consequence to the interaction between the different epitopes of BMPs and cell receptors (Hassel *et al.*, 2003).

3.2. N-terminal of BMP

Another particular feature of the structure of BMP-2 is a heparin-binding domain in the N-terminal region of the mature polypeptide, which modifies its biological activity (Ruppert *et al.*, 1996). Ruppert and colleagues demonstrated that the presence of the N-terminus of BMP-2 reduced its specific activity by interacting with heparinic sites in the extracellular matrix. A variant of recombinant BMP-2 was produced without this domain (EHBMP-2), and biological activity increased five-fold in a limb bud assay when compared to normal BMP-2 and was not affected by the presence of heparin. However, other studies have demonstrated that the presence of heparin binding site increased region increased retention times at the site of injury, and that this was necessary for higher osteoinductive effects (Wurzler *et al.*, 2004). In a recent work a basic core of only three amino acids in the N-terminal region of BMP-4 was demonstrated to be required for its site restriction to the non-neural ectoderm (Ohkawara *et al.*, 2002). The authors suggested that heparan sulphate proteoglycans bind to this basic core and thus play a role in trapping BMP-4. This study was the first to identify a critical domain responsible for the interaction between the BMP and the extracellular environment that restricts its diffusion *in vivo*. Interestingly, heparan

sulphates were also observed to be required for BMP-7 signalling (Irie *et al.*, 2003) and recently these have been observed to greatly influence the chondrogenic activity of BMPs (Fisher *et al.*, 2006) and the transcription of several osteogenic genes in response to BMP (Manton *et al.*, 2007). For these reasons heparin has been included in several different tissue engineering constructs using BMPs (Jeon *et al.*, 2007; Lin *et al.*, 2008).

3.3. BMP antagonists

There are diverse BMP-binding proteins that act as modulators of BMP activity or as antagonists, such as Noggin (Zimmerman *et al.*, 1996), Chordin (Blader *et al.*, 1997), Follistatin (Fainsod *et al.*, 1997) and Gremlin (Hsu *et al.*, 1998). These are extracellular regulator proteins that prevent the BMP assembly to cell receptors and in this way modulate the effect of BMP signalling during the formation of tissues. Crystallographic studies showed that the binding of antagonists, such as Noggin, to BMPs inhibited its signalling through blocking the binding of BMP epitopes for type I and type II receptors to which BMPs bind (Groppe *et al.*, 2002). Recently, two new cofactors that regulate BMP signalling, Betaglycan and crypto, were described (Dale *et al.*, 2004). Betaglycan formed a complex with inhibins which binds to activin type II receptors, thus preventing BMP signalling (Wiater and Vale, 2003). Because of the association of these proteins with various diseases and with additional biological roles, the generation of antagonists of TGF β superfamily members might generate potent tools for basic research and therapeutic approaches (Gazzerro and Canalis, 2006).

4. BMP signalling – from cell receptor to gene activation

BMPs, and all members of the TGF β superfamily, bind to serine–threonine kinase receptors on the cell surface, triggering specific intracellular pathways that activate and influence gene transcription, having precise effects in cell proliferation and differentiation (Figure 1). There are types I, II and III receptors for TGF β superfamily members. Only types I and II appear to play significant roles in BMP binding and signalling, both of which are required for signal transduction (Heldin *et al.*, 1997; Shi and Massague, 2003). For the BMP family, significantly more ligands than receptors are known. To the best of our knowledge the main receptors to which most BMPs bind are type I receptors activin receptor Ia (ActR-I or Alk2), BmprIa (Alk3) and BmprIb (Alk6) and the type II receptors BMP receptor II (BmprII), activin type II receptor (ActR-II) and activin type IIB receptor (ActR-IIB) (Derynck and Zhang, 2003). However, type III TGF β receptors have been shown to serve as cell receptors for BMP signalling, mediating epithelial to mesenchymal cell conversion (Kirkbride *et al.* 2008).

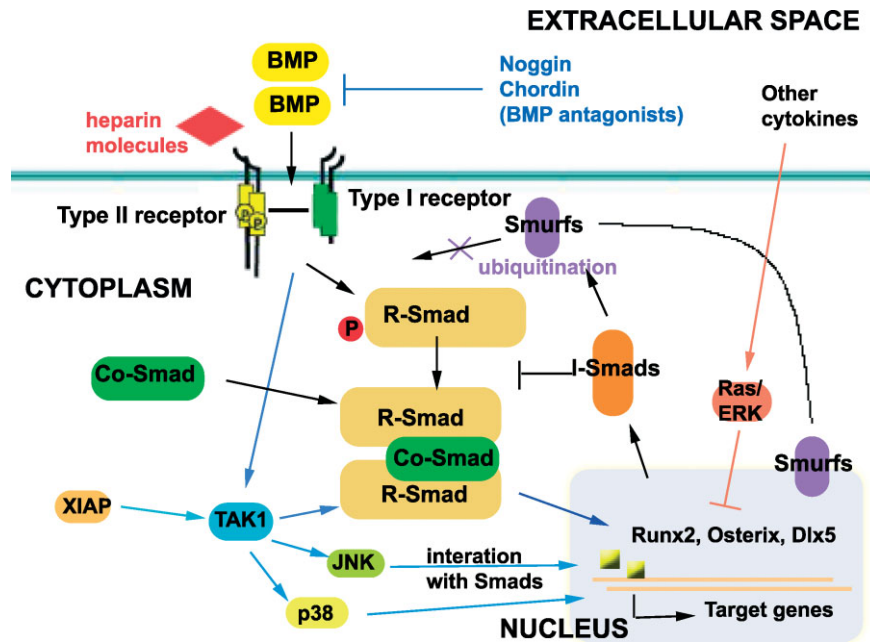


Figure 1. BMP signalling pathways: schematic representation of Smad-dependent and -independent pathways and their main mechanisms of modulation

Upon BMP binding low-affinity type II receptors are constitutively activated and type I receptors are activated by the transphosphorylation of the glycine-serine (GS)-rich domain of these receptors (Heldin *et al.*, 1997). Therefore, the specificity of intracellular signals is mainly determined by type I receptors (Miyazono *et al.*, 2005). BMP2-binding to preformed receptor complexes induces the SMAD pathway whereas signalling complexes sequentially assembled by BMP2 result in the activation of p38-mitogen-activated protein kinase (MAPK; Hassel *et al.*, 2003). Different combinations of type I and type II receptors are the key to providing different and specific signals which result in different cell effects (Sebald *et al.*, 2004). In fact, a recent study showed that Alk3 (type Ia) and Alk6 (type Ib) BMP receptors may transmit different signals during the specification and differentiation of mesenchymal lineages (Kaps *et al.*, 2004). BMP-2 and BMP-4 bind preferentially to Alk3 and Alk6, whereas the BMP-7 group binds more to Alk2 and Alk6. BMP-14 (GDF5) binds to Alk6, but not efficiently to other receptors (Miyazono and Miyazawa, 2002), and the same is noted for BMP-15 (Shimasaki *et al.*, 2004; Tsumaki and Yoshikawa, 2005). BMP-13 (GDF6) binds to Alk3 (Kishigami and Mishina, 2005). All these BMPs activate Smad-1, -5 and -8 signalling, whereas for TGF β , Nodal and activins, Smad-2 and -3 are mainly involved (Shimasaki *et al.*, 2004; Tsumaki and Yoshikawa, 2005). Differently, BMP-11 binds to Alk4, -5 and -7 to activate Smad-3 signalling (Andersson *et al.*, 2006). It has been shown that BMP-3 binds to Alk4 type I receptor and that it may block BMP signalling by competing with ActR-IIB and reducing Smad-1, -5, -8 or Smad-2, -3 phosphorylation (Gamer *et al.*, 2005). Finally, activin receptor-like kinase 1 (Alk1) was identified as a potential receptor for BMP-9 (Brown *et al.*, 2005) acting through Smad-1 and -5

signalling (Lopez-Coviella *et al.*, 2006). Mutations in the BMP receptors have been described to cause diseases. Mutations in the *Bmpr11* gene have been found in patients with primary pulmonary hypertension (PPH; Lane *et al.*, 2000); those in the *Bmpr1A* gene have been found in some patients with juvenile polyposis (Howe *et al.*, 2001). Furthermore, proteomic studies have revealed that the C-terminal tail of BMP receptors type II is also involved in the regulation of cytoskeletal proteins (Hassel *et al.*, 2004). Targeting BMP cell receptors may then be an alternative way to mediate bone-inducing stimuli in tissue-engineering approaches.

4.1. BMP/Smad signalling

Smads are the main signal transducers of serine-threonine receptors (Derynck and Zhang, 2003). Upon BMP binding, type I receptors phosphorylate receptor-regulated Smads (R-Smads). Different R-Smads were identified in mammals: Smad1, Smad5, Smad8 and possibly Smad9 (Xu *et al.*, 2003). Activated R-Smads form a complex with common-partner Smad (Co-Smad) (Xu *et al.*, 2003). There is only one described Co-Smad, Smad4. Two phosphorylated R-Smads form a heterotrimeric complex with one Co-Smad, which is translocated into the nucleus and modulates gene transcription with cooperation with other transcription factors (Massague and Wotton, 2000; Derynck and Zhang, 2003; Vincent *et al.*, 2003). All R-Smads are activated by BMP-2 or -4, whereas BMP-6, -7 and -9 only efficiently induce Smad1 and -5 but not Smad8 (Aoki *et al.*, 2001; Brown *et al.*, 2005). Negative regulation of BMP-induced Smad signal transduction is modulated by inhibitory Smads (I-Smads). I-Smads act by inhibiting the activation of type I

receptors upon BMP binding (Hanyu *et al.*, 2001). Smad6 and -7 act as I-Smads. Smad6 preferentially inhibits BMP signalling while Smad7 inhibits both TGF β -activin and BMP signalling. I-Smads also interact with R-Smads, preventing the formation of the complex with Smad4 (Murakami *et al.*, 2003) inhibiting the transcription of specific genes (Edlund *et al.*, 2003). BMP signalling is also modulated by ubiquitin-dependent protein degradation. Smad ubiquitin regulatory factors (Smurfs) induce the ubiquitination and degradation of Smads thus controlling the signal of BMP. Smurfs form a complex with inhibitory Smad7, which associates with type I receptors, promoting the ubiquitination and degradation of these receptors (Murakami *et al.*, 2003). Interestingly, Smurfs also enhance the responsiveness to Smad2, which mediates TGF β signalling and has as opposite effect to that of BMPs (Zhu *et al.*, 1999; Zhang *et al.*, 2001). The Smad signalling pathway has been also investigated in cancer treatment (Leivonen and Kahari, 2007; ten Dijke and Arthur, 2007; Katoh, 2007). By controlling the different Smad signalling and thus specific cell responses, researchers could in the near future not only achieve a better knowledge of how BMPs but also modulate the subtle biological roles inherent to the diverse TGF- β and BMP members (Schmierer and Hill, 2007).

4.2. BMP/MAPK signalling

Mitogen-activated protein kinase (MAPK) pathways are alternative pathways that are also implicated in the signal transduction of BMPs (Derynck and Zhang, 2003; Massague, 2003). There are three characterised MAPK pathways in mammalian cells: the extracellular-signal regulated kinases (ERKs); the c-Jun-NH2-terminal kinases (JNKs); and the p38 MAPK pathways. These pathways are triggered either by cytokines such as BMPs or by environmental stress (Tibbles and Woodgett, 1999). Thus it may be of importance for biomedical purposes that MAPK pathways are considered since these are connected to a wide range of cellular responses. Cooperative interactions have been reported between Smads and transcription factors activated by MAPK pathways. The X-linked inhibitor of apoptosis links the BMP receptor signalling to TGF β 1-activated tyrosine kinase 1 (TAK1) and activates p38 and JNK kinases and nuclear factor-kappaB (NF- κ B) (Silverman *et al.*, 2003; Takaesu *et al.*, 2003). In osteoprogenitor cells TAK1 interacts with Smads, thus interfering with the BMP signals and osteogenic differentiation (Hoffmann *et al.*, 2005). MAPK pathways interact with other pathways mediated by different cytokines such as epithelial growth factor (EGF), insulin-like growth factor (IGF) or fibroblast growth factor (FGF) (Massague, 2003). These act by extracellular signal-regulated kinases which inhibit the BMP-Smad signalling through Ras/Raf/Mek (Aubin *et al.*, 2004). It was reported that during embryonic differentiation activation of these pathways inhibit the BMP signalling thus favouring neural differentiation

(Pera *et al.*, 2003). It appears that MAPK pathways modulate BMP-Smad signalling through interaction with two conserved globular domains present in Smads, the MH1 and MH2 domains. The MH1 domain is involved in DNA binding and the MH2 domain in binding to cytoplasmic retention factors, activated receptors, nucleoporins in the nuclear pore, and DNA-binding cofactors, coactivators and corepressors in the nucleus (Yamamoto *et al.*, 2003). Recently, it has been shown that MAPK pathways promoted the termination of Smad-mediated BMP signalling, in *Xenopus* (Fuentelba *et al.*, 2007). The simultaneously application of BMPs and other cytokines in therapeutical situations may therefore have specific antagonistic or synergistic effects depending on the interactions occurring between the signalling pathways triggered by each growth factor.

4.3. BMP gene modulation

When translocated to the nucleus, Smads regulate gene transcription by interacting and binding to specific DNA sequences and DNA-binding proteins, such as transcriptional factors (Miyazono *et al.*, 2000). BMP-activated Smads bind preferentially to the GCCGnCGC sequence of target genes and only weakly to AGAC or GTCT gene sequences, which are preferentially bound by TGF β - or activin-activated Smads (Kusanagi *et al.*, 2000). Smads also interact with transcriptional factors and transcriptional coactivators and corepressors.

Runx-related transcription factor (Runx) is one the most studied transcription factors for BMP signalling, regulating processes such as bone formation and haematopoiesis (Ito and Miyazono, 2003). Three isoforms have been identified in mammals: Runx1, Runx2 (also known as Cbfa1, core binding factor A1) and Runx3. The three are reported to interact with R-Smads. Runx2 cooperatively regulates transcription of genes leading to the differentiation of mesenchymal progenitor cells into osteoblasts (Zhang *et al.*, 2000; Miyazono *et al.*, 2004), and for this reason is widely screened as a marker for commitment of cells into the osteochondral lineage and differentiation into bone. Expression levels of Runx2 are low in mesenchymal cells and are induced upon BMP signalling (Maeda *et al.*, 2004). The induction of Runx2 is mediated by a balance between two transcription factors, Dlx5 and Msx2, that responding to BMP signalling, triggering the activation of Runx2 (Miyama *et al.*, 1999; Lee *et al.*, 2003, 2005). Diverse osteogenic markers are then induced by Runx2, such as ALP, osteocalcin, osteopontin and others (Kumar *et al.*, 2005; Takahashi *et al.*, 2005), which are closely related to the process of endochondral calcification.

Osterix (OSX) is another transcription factor that is mediated by BMP/Smad signals and probably by MAPK signalling and other pathways (Celil *et al.*, 2005). Osterix, together with Runx2, are the most studied transcription factors specific for BMP signalling, involved in the differentiation of mesenchymal stem cells into

bone cells (Satiya *et al.*, 2007). Smad signalling also interacts with other proteins such as ATF4, TAZ, and NFATc1 transcriptional factors (Deng *et al.*, 2008), Hoxc-8 (Shi *et al.*, 1999), MyoD (Liu *et al.*, 2001), OAZ (Hata *et al.*, 2000), Msx1 (Yamamoto *et al.*, 2000), and SIP1 (Verschueren *et al.*, 1999). Menin is another required factor that regulates Runx2-induced transcription of genes during the early commitment phase of osteoblast differentiation (Sowa *et al.*, 2004). Among the several BMP target genes, Id (inhibitor of differentiation or inhibitor of DNA binding) proteins are some of the most important ones (Ogata *et al.*, 1993). Id proteins function as negative regulators of cell differentiation and as positive regulators of cell proliferation (Yokota, 2001). Recent advances in microarray analysis enable the identification of many BMP target genes in diverse cell types (Clancy *et al.*, 2003; Garcia *et al.*, 2002; Korchynskyi *et al.*, 2003; Kowanzetz *et al.*, 2004) and allow a better understanding of the physiological effects of BMPs.

4.4. Cross-talk with other pathways

BMP signalling is modulated by cross-talk with several other pathways in a very elaborate network of interacting molecules. BMP and TGF β -activin pathways compete with each other, as they share similar signalling molecules, such as Smad-4 (Candia *et al.*, 1997). Inhibitory Smads are also modulated by several other cytokines and the presence of shear stress in cells (Miyazono *et al.*, 2000). Expression of endogenous TGF β in preosteoblastic differentiation cells induces I-Smads, which regulate a faster or slower BMP-induced differentiating effect (Maeda *et al.*, 2004). The BMP-Smad pathway was also observed to interact in either synergistic or antagonistic ways with other signalling pathways, such as the Notch pathway (Dahlqvist *et al.*, 2003), the EGF pathway (Kretzschmar *et al.*, 1997), the STAT/LIF pathway (Nakashima *et al.*, 1999), the Wnt pathway (Nishita *et al.*, 2000), IGF and FGF signalling (Pera

et al., 2003) and in convergence/antagonistic effect with the MAP kinase pathway (Aubin *et al.*, 2004). MAPK pathway has also been shown to enhance BMP signalling in response to collagen stimulus through activation of the Ras-ERK pathway (Suzawa *et al.*, 2002). These molecular pathways have been involved in modulating BMP signalling in diverse cellular effects and processes, such as osteoblast maturation (Zamurovic *et al.*, 2004), migration of endothelial cells (Itoh *et al.*, 2004), epithelial-to-mesenchymal cell transition (Zavadil *et al.*, 2004) and inhibition of myogenic differentiation (Dahlqvist *et al.*, 2003).

4.5. Recombinant BMPs for tissue engineering

After Urist's pioneering experiments in 1965, BMPs were extracted and purified from bone of several different species including rabbit (Urist *et al.*, 1979), cow (Wang *et al.*, 1988) and human (Urist *et al.*, 1983). Isolation of native BMP from bone results in very low yields around 1–2 $\mu\text{g}/\text{kg}$ cortical bone. The need for obtaining larger quantities of BMPs for tissue engineering prompted researchers to produce and purify these growth factors by DNA recombinant technology (Table 3).

Nowadays, recombinant BMPs are produced mainly by two expression systems: in mammalian cells or in bacteria. With mammalian cells the recombinant protein is obtained active but yields are usually very low. With bacteria the protein is obtained in much larger yields but usually in non-soluble inactive inclusion bodies which are later solubilised and refolded to yield bioactive protein. A common disadvantage is that the protein is not glycosylated as is normal in the human body and thus may present reduced stability or biological activity.

Recombinant human BMP-2 was first obtained in mammalian cell cultures from Chinese hamster ovary (CHO) cells (Wang *et al.*, 1990a). The rhBMP-2 induced ectopic bone and cartilage formation when implanted

Table 3. Selected works reporting the production of recombinant BMPs in diverse expression systems

BMP	Expression system	Novelty	References
BMP2	CHO cells	Bone formation, characterisation of expressed BMP-2	Wang <i>et al.</i> , 1990b; Israel <i>et al.</i> , 1992
BMP2	Insect cells	Alternative expression system	Maruoka <i>et al.</i> , 1995
BMP2	<i>E. coli</i>	The heparin binding domain reduces ALP and specific <i>in vitro</i> biological activity	Ruppert <i>et al.</i> , 1996
BMP2	<i>E. coli</i>	High density expression in bacteria	Li <i>et al.</i> , 1998
BMP2	<i>E. coli</i> , pCYTEXP3	Optimisation of refolding conditions for BMP-2	Vallejo <i>et al.</i> , 2002; Vallejo and Rinas, 2004
BMP2	<i>E. coli</i>	Additional heparin binding domains enhance <i>in vivo</i> bone formation	Wurzler <i>et al.</i> , 2004.
BMP2	<i>E. coli</i> , pET-11a	Comparison with BMP-2 propeptide	Hillger <i>et al.</i> , 2005
BMP2	<i>E. coli</i> , pET-21a	Use of different refolding buffers	Long <i>et al.</i> , 2006
BMP2	<i>E. coli</i> , pET-25b	Bioactivity in human stem cells	Bessa <i>et al.</i> , 2008
BMP4	<i>E. coli</i>	Bone formation in mice	Kubler <i>et al.</i> , 1998a
BMP4	<i>E. coli</i> , pET-11a	Stability in physiological buffer	Klosch <i>et al.</i> , 2005
BMP6	<i>E. coli</i> , pET-15b	Induction of several osteogenic markers	Yang <i>et al.</i> , 2003
BMP7	CHO cells	Bioactivity in rats	Sampath <i>et al.</i> , 1992
BMP2/7, 4/7	CHO cells	Heterodimer expression	Israel <i>et al.</i> , 1996

in rats after 2 weeks. Characterization of rhBMP-2 produced in CHO cells led to the conclusion that the protein was secreted in three forms, a propeptide of 40–45 kDa, a mature homodimer of 30 kDa and a small portion of uncleaved precursor of 60 kDa (Israel *et al.*, 1992). The mature peptide possessed N-glycosylation. Sampath *et al.* confirmed the bioactivity of rhBMP-7 from CHO cells by analysis of bone formation and specific osteogenic markers, such as osteocalcin (Sampath *et al.*, 1992). Koenig *et al.* (1994) noticed a strong tendency of recombinant BMP-2 to adhere non-specifically to culture dishes or to the negatively charged extracellular matrix via its positively charged N-terminal. They modified the BMP by a mild trypsin digestion, removing the first seven to nine residues, and named it digit-removed BMP-2, that still binds to the BMP receptor. Nowadays, commercial recombinant human BMP-2 lack this N-terminal heparin-binding end, since the region decreases the specific bioactivity of the BMP (Ruppert *et al.*, 1996).

In 1996, various combinations of rhBMPs were co-expressed in CHO cells to test for possible heterodimer formation (Israel *et al.*, 1996). Transient co-expression between the different rhBMPs resulted in more bioactivity than expression of any single rhBMP. The rhBMP-2/7 heterodimer yielded 20-fold higher activity in the *in vivo* ALP assay. In 1998 Kubler and colleagues produced rhBMP-2 and -4 in *Escherichia coli* and rhBMPs were tested in a collagen carrier and induced bone formation in rats after 4 weeks (Kubler *et al.*, 1998a, 1998b). Recombinant human BMP-6 was later expressed and purified in *E. coli* (Yang *et al.*, 2003), using pET-15b vector. The protein was obtained in inclusion bodies, refolded and induced ALP activity in C3H10T1/2 cells and other osteogenic markers, as observed by RT-PCR.

During the last years, Vallejo and colleagues reported interesting works regarding the production of rhBMP-2 in inclusion bodies, with optimized protein refolding procedures (Vallejo *et al.*, 2002; Vallejo and Rinas, 2004). The group found that refolding of BMP-2 was particularly sensitive to pH, temperature and concentration, but not so much to ionic strength or redox conditions. The rhBMP-2 was refolded in buffer with L-arginine or CHES at pH 8.5 to a yield of 750 mg dimer per litre of bacteria growing media.

In 2005, mature rhBMP-2, unprocessed rhBMP-2 and propeptide of rhBMP-2 were produced in *E. coli* (Hillger *et al.*, 2005), using a pET-11a expression vector. This group reported bioactivity from both the propeptide and an N-terminal truncated BMP-2 form. Curiously, in 2004, Wurzler and co-workers used mutants of rhBMP-2 with enhanced heparin binding sites (in the N-terminus) and a variant with no heparin binding site. The variant with no binding site showed great reduction in osteoinduction, while the mutants with additional heparin binding sites led to enhanced bone formation, due to longer retention times of the BMP-2 in the extracellular matrix (Wurzler *et al.*, 2004; Depprich *et al.*, 2005). This fact could be of great interest for drug-delivery approaches providing recombinant proteins with increased affinity for binding

to the extracellular matrix itself *in vivo*. In 2005, rat BMP-4 has been expressed and purified as inclusion bodies in *Escherichia coli* that are stable in a physiological compatible buffer showing bioactivity in MC3T3-E1 cells (Klosch *et al.*, 2005).

A report on the production of rhBMP-2 was published in 2006, using pET-21a in *E. coli*. The protein, obtained in inclusion bodies, was refolded in different folding buffers (Long *et al.*, 2006). RhBMP-2 was best refolded at low concentrations (0.1 mg/ml), 4°C and in the presence of L-arginine. Recently, soluble rhBMP-2 was obtained upon production with the use of pET25b *E. coli* vector. The rhBMP-2 showed bioactivity in primary cultures of human fat-derived stem cells and an increase in levels of osteogenic markers Runx2, Osterix and Smad-1 and -5 in the C2C12 cell line (Bessa *et al.*, 2008).

5.1. From manufacture of BMPs to tissue-engineering products

Since their discovery in 1965, BMPs have revealed a promising future in the field of tissue engineering as powerful components of biomedical products for the regeneration of body parts of human patients, namely bone and cartilage. At present, the state of the art of regenerative medicine envisages the use of growth factors, loaded into scaffolding materials and using precursor or stem cells, possibly from the patient him/herself. It is expected that future advances in fundamental biology will allow researchers to design novel tissue-engineering strategies that make use of combinations of different BMPs and other growth factors. The use of these growth factor cocktails will certainly present a challenging approach in tissue engineering since much is still poorly understood about the orchestration of molecular cascades underlying the regeneration of tissues.

Currently, there are two products approved by the Food and Drug Administration (FDA) consisting of recombinant BMP-2 and BMP-7 loaded into absorbable collagen sponges for clinical use for fusion of spinal vertebrae and treatment of long bone fractures (McKay *et al.*, 2007; White *et al.*, 2007). However, a whole set of other less understood BMPs will probably make its way into the clinic, such as with the case of cartilage-derived morphogenetic proteins (BMP-12, -13 and -14) for cartilage or tendon reconstruction, or BMP-9 that has stronger osteogenic potencies compared to other BMPs (Kang *et al.* 2004). Recombinant BMPs used in clinics are derived from mammalian expression cells, which elevates much of the cost of clinical products containing rhBMPs. It is expected that advances in recombinant technology namely those involving the large-scale production of BMPs in bacteria will allow researchers to obtain and use these proteins at a much lower cost.

At present, recombinant technology is also a useful tool to achieve molecular modifications of BMPs to improve their bioactivity, stability and affinity to both the tissue-engineering scaffold or the extracellular

Table 4. Chemical or genetic engineering modifications for improving particular characteristics of BMPs for use in tissue engineering applications

Modification process	Advantages	References
Enzymatic cleavage of BMP heparin binding domain	Reduces non-specific interactions with extracellular matrix, leading to increased bioactivity <i>in vitro</i>	Koenig <i>et al.</i> , 1994; Uludag <i>et al.</i> , 2001
Plasmin enzymatic cleavage of BMP	Reduces non-specific interactions with extracellular matrix, leading to increased bioactivity <i>in vitro</i>	Israel <i>et al.</i> , 1992; Hollinger <i>et al.</i> , 1998
Adding additional heparin binding domains	Increases retention to extracellular matrix or affinity to biomaterial, leading to increased activity <i>in vivo</i>	Wurzler <i>et al.</i> , 2004; Depprich <i>et al.</i> , 2005
Immobilisation of synthetic peptides of partial BMP sequences	Improves the stability and specific activity of the BMP and reduces the initial burst release	Suzuki <i>et al.</i> , 2000; Saito <i>et al.</i> , 2003, 2004, 2005; Saito <i>et al.</i> , 2006; Seol <i>et al.</i> , 2006; Bergeron <i>et al.</i> , 2007; Park <i>et al.</i> , 2007
Fusion protein BMP with domain to bind a specific biomaterial	Increases the retention of BMP on carrier, accelerating tissue regeneration; increases stability and activity of the BMP	Han <i>et al.</i> , 2002; Schmoekel <i>et al.</i> , 2005
Fusion protein BMP with RGD for enhanced cell attachment or domain for other specific biological function	Enhances attachment of cells, tissue regeneration and possibly the biocompatibility of the material	Not yet reported
Chemical modification of BMP, such as with succinylation, acetylation or biotinylation, changes the isoelectric point	Enhances the affinity for binding to the biomaterial by changing the peptide solubility, to suit a desired release profile	Hollinger <i>et al.</i> , 1998; Uludag <i>et al.</i> , 1999a; Uludag <i>et al.</i> , 1999b; Uludag <i>et al.</i> , 2000; Uludag <i>et al.</i> , 2001
Formation of heterodimers between different BMPs	The increased bioactivity of BMP heterodimers leads to lower dose	Zhu <i>et al.</i> , 2006 Aono <i>et al.</i> , 1995; Israel <i>et al.</i> , 1996

matrix and cells (see Table 4). For example, by adding additional heparin binding domains, there is a significantly different bioactivity and bioavailability of the rhBMP, decreasing its specific activity *in vitro* but increasing bone formation *in vivo* (Depprich *et al.*, 2005). Currently, most recombinant BMP-2 and BMP-7 are produced with no heparin binding domain and thus may present reduced bioactivity when used for clinical uses. Another approach may consist of producing recombinant peptides containing only the binding sites to the cell receptors thus achieving specific biological effects with no problems derived from loss of bioactivity or stability which occur during the incorporation into the scaffold (Suzuki *et al.*, 2000; Saito *et al.*, 2003, 2004, 2005, 2006). Designing chimeric proteins is a further powerful approach which may include the addition of other domains of biological importance such as immune modulatory agents, RGD for increased cell attachment, GFP for *in vivo* localization, or domains for specific binding to biomaterials such as for example to collagen (Han *et al.*, 2002) or to fibrin (Schmoekel *et al.*, 2005). Naturally, the chimeric rhBMPs would have different refolding characteristics as compared to native proteins. These could have altered stability, solubility, surface binding and bioactivity/biospecificity thus making an appealing approach of producing specific-tissue target proteins and with increased bioactivity (Oppermann *et al.*, 2005). Chemical modification also improves binding, stability, bioactivity and bioavailability of BMPs (Luginbuehl *et al.*, 2004). Thus, by chemically or recombinantly modifying the isoelectric point of the BMP the affinity of the BMPs to the delivery matrices could be greatly enhanced that allows the achievement of a desired release profile. Chemically modified rhBMP-2

with enhanced affinity to carriers has been reported by succinylation (Hollinger *et al.*, 1998), acetylation (Uludag *et al.*, 2000) and biotinylation (Uludag *et al.*, 1999b). Finally, the expression of recombinant BMP heterodimers is another way of obtaining these growth factors with much increased bioactivity (Aono *et al.*, 1995; Israel *et al.*, 1996).

Despite being promising recombinant technology is still at an early stage, since there is a need to bypass some fundamental limitations, such as optimising the stability and bioactivity of recombinant BMPs, obtaining glycosylation patterns identical to that of native BMP, and reducing the possibility of triggering immunogenic responses when used in clinical situations. In the next years, the combination of advances of the BMP field with that of biomaterial science (Mano *et al.*, 2007) will surely herald a revealing future for the use of these morphogens for biomedical and tissue-engineering applications.

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