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REVIEW ARTICLE

Application of Cytokines of the Bone Morphogenetic Protein (BMP) Family in Spinal Fusion - Effects on the Bone, Intervertebral Disc and Mesenchymal Stromal Cells

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Abstract: Low back pain is a prevalent socio-economic burden and is often associated with damaged or degenerated intervertebral discs (IVDs). When conservative therapy fails, removal of the IVD (discectomy), followed by intersomatic spinal fusion, is currently the standard practice in clinics. The remaining space is filled with an intersomatic device (cage) and with bone substitutes to achieve disc height compensation and bone fusion. As a complication, in up to 30% of cases, spinal non-fusions result in a painful pseudoarthrosis. Bone morphogenetic proteins (BMPs) have been clinically applied with varied outcomes. Several members of the BMP family, such as BMP2, BMP4, BMP6, BMP7, and BMP9, are known to induce osteogenesis. Questions remain on why hyper-physiological doses of BMPs do not show beneficial effects in certain patients. In this respect, BMP antagonists secreted by mesenchymal cells, which might interfere with or block the action of BMPs, have drawn research attention as possible targets for the enhancement of spinal fusion or the prevention of non-unions. Examples of these antagonists are noggin, gremlin1 and 2, chordin, follistatin, BMP3, and twisted gastrulation. In this review, we discuss current evidence of the osteogenic effects of several members of the BMP family on osteoblasts, IVD cells, and mesenchymal stromal cells. We consider *in vitro* and *in vivo* studies performed in human, mouse, rat, and rabbit related to BMP and BMP antagonists in the last two decades. We give insights into the effects that BMP have on the ossification of the spine. Furthermore, the benefits, pitfalls, and possible safety concerns using these cytokines for the improvement of spinal fusion are discussed.

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1. INTRODUCTION

Spinal fusion is currently a widely applied surgical intervention to treat painful discs, but its use involves intricacies, like non-union, in some cases. Low back pain (LBP) is a significant health problem in today's society. It is estimated that up to 80% of the population suffers from LBP symptoms at least once during their lifetime [1, 2]. When LBP fails to be treated with first-line conservative treatments, such as pharmacological and physical therapy, invasive procedures are required [3]. One of the current surgical interventions to treat degenerated or damaged discs is intersomatic spinal fusion. After removal of the intervertebral disc (IVD), a cage is implanted into the created space. The cage is usually filled with bone graft or bone substitutes. For further mechanical stability, the involved segments are frequently fixed with additional pedicle screws.

The primary source of autologous bone grafts (ABGs) is the iliac crest; other sources are the proximal tibia, the fibula, the ribs, and the vertebral body [4]. In case no ABGs are available, bone formation is promoted by non-autologous materials, such as allograft, cancellous chips, demineralized bone matrix, ceramics, tricalcium phosphate, and hydroxyapatite [5]. Although this surgical procedure is widely used, it can lead to failure in bone formation and/or [6] pseu-

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doarthrosis, which occurs in 5% to 35% of treated patients [7-10].

Bone morphogenetic proteins (BMPs) are used in clinics as osteoinductive factors, but they cause side effects. In the last decades, BMP and BMP antagonists have been found to promote fracture healing and bone regeneration [11-15].

Bone morphogenetic protein 2 (BMP2) and bone morphogenetic protein 7 (BMP7) are clinically approved to treat fracture non-unions [16-18]. In 2002, the US Food and Drug Administration (FDA) approved the use of recombinant BMP2 in clinics for the promotion of bone fusion in anterior lumbar interbody fusion [19, 20]. However, the use of recombinant BMP2 has been questioned: it is not cost-effective, and safety concerns have also been raised with both lumbar and cervical fusion procedures [21, 22]. Moreover, the application of BMPs did not result in better outcomes compared with the use of ABGs [23]. Supraphysiological doses of BMPs in the order of milligram are required to induce sufficient bone formation. This may potentially cause adverse events, such as overgrowth and abnormal bone formation, osteoclast activity and vertebral osteolysis, and local problems (*e.g*., inflammation, edema, and wound problems). Negative effects on the exposed dura and nerves, graft subsidence, graft migration, formation of neutralizing antibodies against BMPs, and carcinogenicity have also been reported [24-28]. In the field of spinal applications, several meta-analyses have been performed to quantify the success of BMP2 administration in spinal fusion. Carragee *et al*. (2011) [29] and Simmonds *et al.* (2013) [30] reported on the outcomes of industrial-sponsored BMP2 studies that claim no BMP2 side effects. Furthermore, Carragee *et al.* [29] compared conclusions about safety and related efficacy in industry-sponsored BMP2 studies with subsequently available FDA data summaries. They suggested the occurrence of adverse effects associated with rhBMP2 after spinal fusion surgery, and these ranged from 10% to 50% depending on the approach used. These studies questioned whether BMP2 had any positive effects on pain relief, and they explored whether cases of cancer could be connected to its application [30]. In light of these neutral and adverse outcomes, it seems evident that the biology and underlying pathways of BMPs are not yet understood, resulting in its low efficacy and poor results in clinics. To date, however, no systemic effects caused by the local application of BMP2 have been reported. Recombinant BMP7 is the second member of the BMP family, which is FDA approved and available for clinical use. An essential property of BMPs is their nature of distribution. When administered in buffer only, BMP2 has a half-life of 7 minutes in non-human primates [31]; BMP4 also has a rapid initial clearance rate. Conversely, BMP7 possesses an extended terminal half-life, which results in low and more permanent circulating levels of the protein. As an important fact for clinical use, it also has to be considered, that BMPs are pleiotropic proteins. In the case of BMP7, the pleiotropic nature seems to play a role, as it was found that when administered systemically, BMP7 protects the kidney by preventing tubulointerstitial fibrosis and preserving renal function [32].

Currently, only little is known about the expression pattern of BMP antagonists during spinal fusion. However, the physiological imbalance between BMP and BMP antagonists may be the reason for spinal non-union [6, 13]. The question on whether an insufficient bone formation is caused by a suboptimal BMP expression, an increase in local levels of BMP antagonists, or both remains unanswered [33]. This BMP imbalance and the failure of bone formation could further be discussed for IVDs. Clinical observations indicate that partial IVD removal often leads to spinal non-union [34, 35]. The central question is whether IVD cells can influence the BMP signaling pathway by expressing BMP antagonists. Recent studies already indicated the expression of BMP antagonists in IVD cells [6, 35]. Another question is how IVD cells react upon stimulation with BMPs. Earlier studies showed the anabolic effect of BMP2 stimulation of IVD cells. In a newer report, it is even hypothesized that IVD cells might shift toward an osteogenic phenotype [6].

In this review, we summarize current knowledge on the molecular pathways of BMP signaling with relevance to the bone and the spine. The effects of BMPs and BMP antagonists in spinal fusion and bone healing for *in vitro* and *in vivo* studies are discussed. We also present an overview of the latest research on BMP2 in bone healing or spinal fusion, which was the primary focus in the past, as well as new directions (*i.e*., involving BMP antagonists and other members of the TGF-β pathway). The main focus is on how these cytokines affect the respective target cells, namely osteoblasts (OBs), mesenchymal stem cells (MSCs), and IVD cells, which could play a role in spinal fusion. The expression levels of BMPs and BMP antagonists in spinal fusion models are also described.

2. DISCOVERY OF BMPs

The bone matrix consists of organic and inorganic components. BMPs were actually discovered through the study of these components. Approximately after two weeks when the osteoid (uncalcified matrix) is formed, the osteoid starts to mineralize; 70% of the final amount is reached very quickly, whereas the remaining 30% needs several months until deposition is completed. Seventy percent of the final bone-dry weight consists of hydroxyapatite mineral; the remaining 30% consists of organic materials, such as collagens, glycoproteins, proteoglycans, and sialoproteins [36]. In 1965, the orthopedic surgeon Marshall R. Urist studied the concept of induced bone formation for the first time by transplanting a specifically prepared allogenic bone matrix, called the demineralized bone matrix (DMBM), into muscle tissue and found the material to induce ectopic bone formation [37]. This discovery was a breakthrough in biologic bone graft substitute technology. DMBM is an osteoinductive scaffold produced by acidic treatment of allograft bone [38]. It is a mixture of non-collagenous proteins, osteoinductive growth factors, and collagen. Primarily, collagen type 1 (COL1) is present, but other types of collagen are also present, albeit at a lower proportion than that in soft tissue connections. Because of the enhanced bioavailability of growth factors and allografts, DMBM showed better osteoinductive potential than allograft [39]. Urist (1965) [37] discovered in his studies that the extracellular matrix of the bone has materials that can induce bone formation; he called these substances BMPs. Although Urist spent the next decades isolating and purifying BMPs, they were first cloned only in 1988

by John Wozney [40]. The research team of Wozney and Rosen (1988) [41] proved that BMPs are members of the transforming growth factor beta (TGF-β) superfamily. In 1988, the first studies on the effect of BMPs on spinal fusion were conducted by Johnson *et al*. (1988) [42]. Since then, many studies have shown the ability of BMPs to induce differentiation from MSCs into bone.

3. BMPs IN DEVELOPMENTAL PROCESSES

As described above, BMPs were initially known to induce bone formation. They play an important role in adult tissue homeostasis, such as sustaining joint integrity, initiating fracture repair, and remodeling the vasculature [43-45]. Today, it is well known that BMPs also have key functions in the development of all organ systems. BMP signaling is involved in cell growth, differentiation, survival, activation, and apoptosis during developmental processes [46, 47]. Besides embryogenesis and its function in the skeletal system, BMP signaling is important in the muscle [48], gastrointestinal [49], cardiovascular and pulmonary [50], urinary [51], neurological and ophthalmic [52], and reproductive systems [53], as well as in adipogenesis [54]. In embryogenesis, BMPs are especially essential for mesoderm and cardiac formation [55]. Both BMP2 and BMP4 knock-out mice experience embryonic lethality during early gastrulation because of the failure of mesoderm induction [50, 56]. BMP2-deficient mice have malformation of the amnion/chorion, which is caused by failure of proamniotic canal closing. They also have defects in cardiac development. With BMP4 deficiency, mice lack mesoderm differentiation [57]. Conversely, the knockout of BMP1, BMP7 (also called osteogenic protein 1 or OP-1), or BMP11 leads to death after birth [58-60]. A lack of BMP1 leads to failure in ventral body closure [58]. Furthermore, mice with BMP7 null mutations die shortly after birth because of renal failure. They are also characterized by eye defects and mild skeletal changes [61].

4. THE BMP SIGNALING PATHWAY

Two major pathways have particularly strong effects on bone homeostasis, and these are i) BMP signaling and ii) Wnt/β-catenin signaling cascade [62, 63]. A crosstalk has also been identified in several cell types between these two signaling pathways. One example is the activation of Wnt3a or the overexpression of β-catenin/TCF4, which both activate BMP2 expression in OBs [64].

BMPs are members of the TGF-β superfamily and play an essential role in skeletal tissue formation, as they induce the commitment of MSCs toward OBs. Hence, BMPs are involved in cartilage and bone formation during embryonic development, postnatal bone metabolism, and fracture healing [65].

Today, more than 22 members of the BMP family have been identified [14]. Several BMPs, such as BMP2, BMP4, BMP6, BMP7, and BMP9, have shown their potential for the induction of the osteogenic differentiation of MSCs toward osteoblastic lineage cells *in vitro* and *in vivo* [66]. BMPs bind as dimers to BMP type I (BMPRI) and type II (BMPRII) serine/threonine kinase receptors. Type I receptors are divided into the following three subtypes: BMPRIA (aka activin receptor-like kinase 3 (ALK3)), BMPRIB (ALK6), and activin receptor type-1 ActRI (ALK2)) [67]. BMP receptors are localized as heterodimers or homodimers in a caveolar structure on the cell surface [68]. The heterotetrameric signaling complex can vary, depending on which BMP binds to the receptors. BMP6 and BMP7 interact with type II receptors and activate type I receptors, whereas BMP2 and BMP4 mainly bind to BMP type I receptors and activate BMP type II receptors [69]. Through binding BMPs to their cognate receptors, BMPRII form a heterodimer with BMPRI. The kinase activity of BMPRII then activates BMPRI by phosphorylation and mediates signal cascade by initiating single mothers against decapentaplegic homolog (Smad) signaling. The signaling, however, can also proceed through the mitogen-activated protein (MAP) kinase pathway or possibly also other pathways [68]. The following three classes of Smad have been described: i) receptor-regulated Smad (R-Smad), which can be activated by BMP (Smad1/5/8) or TGF-β (Smad2 and 3), ii) common BMP- and TGF-β-mediated Smad (co-Smad: Smad3 and 4), and iii) inhibitory Smad (I-Smad: Smad 6 and 7). After BMP-activated BMP receptors, Smad1/5/8 are carboxy-terminally phosphorylated and build heteromers with Smad4, the whole complex translocates into the nucleus, where transcription is activated or inhibited [67, 70] (Fig. **1**). To be more specific, the complex binds in the nucleus DNA sequences or interacts with transcription factors. The three key transcription factors for bone formation, stimulated by the BMP signaling pathway, are distal-less homeobox protein 5 (*DLX5*), Osterix (*SP7*), and the runt-related transcription factor 2 (*RUNX2*) [67, 71]. *SP7* and *RUNX2* are regulators for many OB-specific genes, including osteopontin (*SPP1*), osteocalcin (*BGLAP)*, *COL1*, and bone sialoprotein (*BSP)*. *SP7* acts in conjunction with *RUNX2*; however, the levels of *RUNX2* remain unaffected by *SP7* gene absence, which indicates that *SP7* may act downstream or independently of *RUNX2* [72]. Furthermore, it was reported that the absence of *RUNX2* (but with BMP2 stimulation) had no effect on *SP7* expression, which indicates further *SP7* independence. However, the gene *DLX5* seems to play an important role in *SP7* expression, as the inactivation of *DLX5* leads to the suppression of *SP7* [73]. Through these factors, OBs undergo terminal differentiation; the matrix mineralizes, and, in the last step, it undergoes apoptosis [74].

Most BMP expressions in OBs are regulated by transcriptional auto-regulation, which can act as a negative feedback loop. BMP signaling can be regulated at different levels in the cell, for example, at the intracellular level by inhibitory Smads, miRNA, and methylation, as well as at the extracellular level by pseudoreceptors or BMP antagonists [75]. The BMP signaling pathway is influenced by BMP antagonists, which block BMP signal transduction at multiple levels. The members of BMP antagonists, including noggin (NOG), chordin (CHRD), gremlin (GREM1 and GREM2), twisted gastrulation (TWSG), and sclerostin (SOST), negatively regulate BMP signal transduction by competing with BMP ligands [76-78].

5. BMPs AND THEIR ANTAGONISTS

Bone-inducing BMPs can be categorized into three different groups based on the homology of their amino acid

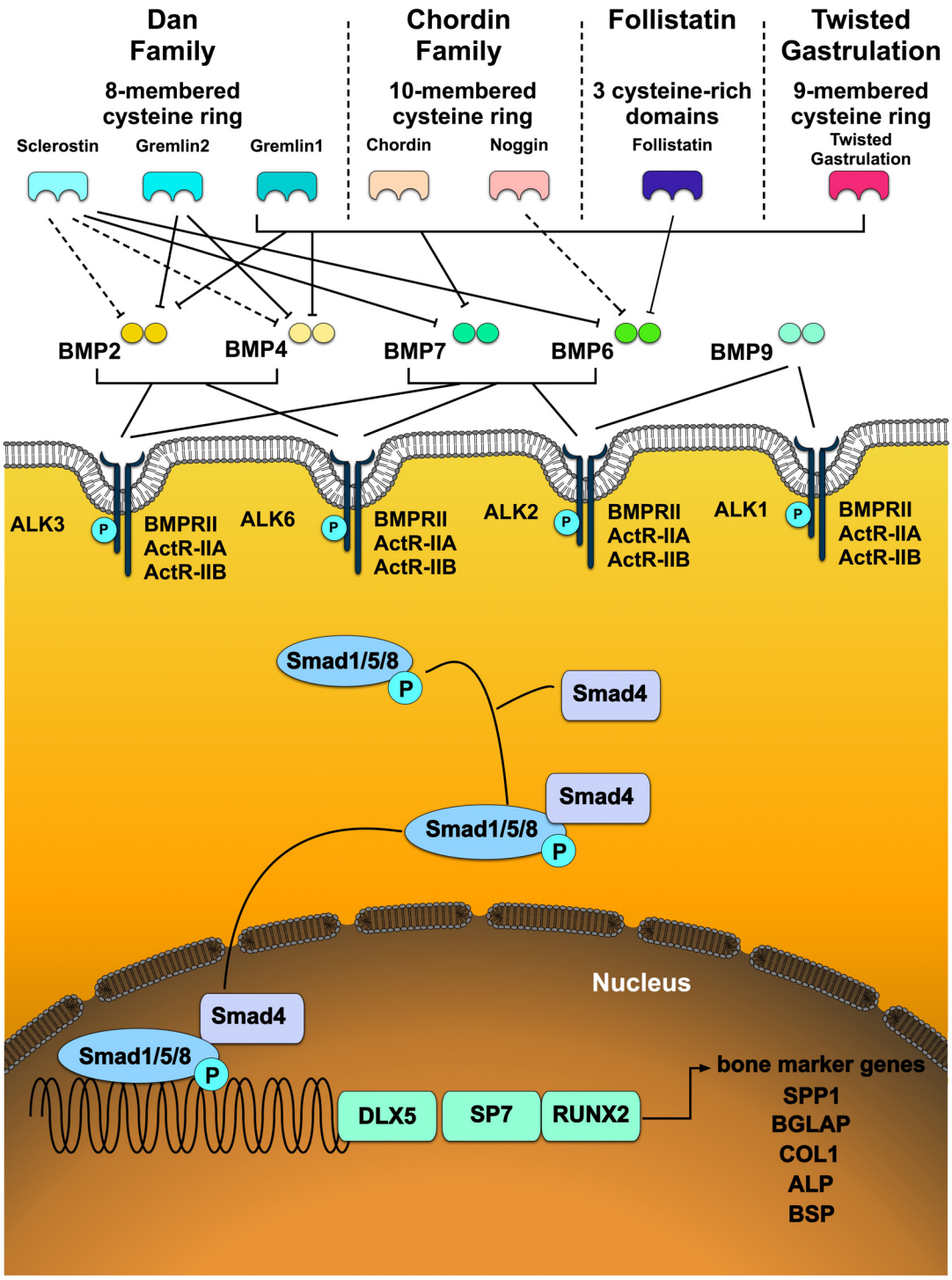

Fig. (1). Schematic overview of bone morphogenetic protein (BMP) signaling pathways. BMPs, such as BMP2, BMP4, BMP6, BMP7, and BMP9, which are discussed in this review and known for their potential to induce osteogenic differentiation, are presented. BMPs bind as dimers to BMP type I (BMPRI) and type II (BMPRII) serine/threonine receptors. BMPRs are localized in caveolar structures on the cell surface. Through the binding of BMPs with the receptor, Smad signaling is initiated. BMPs activate Smad1/5/8 and build heteromers with Smad4. After translocation into the nucleus, transcription is either activated or inhibited. The complex binds to DNA sequences or interacts with transcription factors as runt-related transcription factor 2 (*RUNX2*) directly. This plays an important role in the regulation of various osteoblast (OB)-specific genes, such as osteocalcin (*BGLAP*), osteopontin (*SPP1*), collagen type 1 (*COL1*), bone sialoprotein (*BSP*), and alkaline phosphatase (*ALP*). Furthermore, Osterix (*SP7*) acts as a master regulator for multiple OB-specific genes, including *SSP1*, *COL1*, and *BSP*. The gene distal-less homeobox protein 5 (*DLX5*) seems to play an important role in *SP7* expression. ALK2 = ActR-I, ALK3 = BMPR1A, ALK6 = BMPR1B. Dotted line: weak binding of BMP antagonists, continuous line: strong binding of BMP antagonists to their respective BMP [12, 63, 79-92]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

sequences [93]. BMP2 and BMP4 comprise the first subgroup; BMP5, BMP6, BMP7, and BMP8 comprise the second group; and BMP9 and BMP10, comprise the third group [94]. BMP1, which is not a member of the TGF-β superfamily, is a metalloprotease; it plays a role in collagen maturation as a procollagen C-proteinase and also induces bone and cartilage development [95]. But BMPs are not only inducers of bone formation but are also inhibitors, such as BMP3, which reduces bone density, and BMP13, which inhibits bone formation [96]. In bone, BMPs are mainly produced by osteoprogenitor cells, OBs, chondrocytes, and platelets; upon secretion, they are integrated in the extracellular matrix [97, 98].

Previous studies showed that BMP2 turns out to be the most osteoinductive member of the BMP family because of its biological activity throughout most stages of bone healing [20]. BMP2 could induce the differentiation of MSCs toward cells of osteoblastic lineage and enhance the differentiated function of OBs [99, 100]. BMP2 interacts primarily with ALK6 [82] and also has a higher binding affinity for ALK3. As already mentioned above it has lower binding affinity to BMPRII, ActR-IIB and ActR-IIB [101]. BMP2 can be up- and downregulated by other BMPs. Interestingly, promoters of BMP2 and BMP4 contain binding sequences for *RUNX2*, which may indicate regulation by a positive feedback loop [102].

Another BMP often discussed is BMP4, which is structurally highly related to BMP2 and interacts mostly with ALK3 and very likely also with ALK6 in some cell types [82]. Furthermore, BMP6 forms another subclass together with BMP5, BMP7, and BMP8, as BMP6 has a high degree of homology at the amino acid sequence level to BMP7. Both proteins BMP6 and BMP7 can bind to ALK2 and to the same receptors as BMP2 and BMP4 but conversely with higher affinity to type II than type I receptor [82, 103]. BMP9 is most likely inducing osteogenic differentiation by interacting with ALK1 and ALK2 [104].

The following selection of BMP antagonists belongs to extracellular BMP antagonists and includes secreted proteins, binding with different affinities to BMPs and thus preventing their interaction with BMP receptors. They can be grouped into the following three subfamilies based on the size of their cysteine knot: i) differential screening-selected gene aberrative in neuroblastoma (DAN) family (which includes GREM1 and 2 and cerberus) (an eight-membered cysteine ring), ii) TWSG (a nine-membered cysteine ring), and iii) CHRD family (in which NOG is included) (a 10-membered cysteine ring) [105]. Follistatin (FLST), which contains three cysteine-rich homologous domains, forms another group.

NOG plays a major role in the inhibition of osteogenesis, particularly in bone and joint formation [106]. NOG is secreted as a glycosylated homodimer of 64 kilodaltons (kDa) and is an antagonist to BMPs. It antagonizes through direct binding to BMP2, BMP4, BMP6, and BMP7, with a higher affinity to BMP2 and BMP4. Furthermore, NOG gene expression is upregulated in osteogenic cells upon exposure to BMPs, and it acts as a negative regulator to suppress BMP-induced bone formation [107]. In OBs, for example, NOG is upregulated in response to BMP2, BMP4, or BMP6, which suggests negative feedback to limit the excessive exposure of cells to BMP signaling [108]. These studies indicate that the effect of NOG varies between mouse and human. Rifas *et al.* (2007) [109] suggested that in the case of human MSCs (hMSCs), NOG might either balance the impact of BMPs or act, in their absence, as a ligand for BMP receptors to induce differentiation. Another BMP antagonist is CHRD, which is secreted as a glycosylated homodimer of 120 kDa. The expression of CHRD in OB lineage cells is limited, but it is highly expressed in undifferentiated chondrocytes [108, 110]. GREM1 and 2 are also antagonists of BMP. GREM1, also called downregulated by v-mos (DRM), is structurally characterized by an eight-cysteine carboxy-terminal ring domain [105]; it was first isolated from *Xenopus* embryos [111]. It has a molecular weight of 27 kDa and belongs to the DAN family; GREM1 is primarily present on the external surface of expressing cells, but it can also be found in small amounts in the endoplasmic reticulum-Golgi complex [112]. It exists in both secreted and cell-associated forms [113]. Generally, GREM1 shows the highest affinity for BMP2, followed by BMP4 and then BMP7 [78]. GREM2, which is also called the protein related to DAN and cerberus (PRDC), was initially found in embryonic stem cells and also belongs to the DAN family [81, 114]. Whereas GREM1 is expressed in the cartilage and bone of the skeleton, GREM2 is expressed in OBs during *in vivo* skeletogenesis [115, 116]. Another less-investigated BMP antagonist is FLST. Null mutation of the antagonist in mice leads to deficiencies in multiple tissues, including the skeleton; they are also unable to breathe, and they die just some hours after birth [117]. FLST expression is downregulated by BMPs and induced by TGF-β [118]. Another BMP antagonist that has been studied less is TWSG, with a molecular weight of 23.5 kDa and was initially identified in *Drosophila sp*. [119].

6. L51P AS A GENERAL INHIBITOR

L51P is a BMP2 analog that has one amino acid substituted at position 51 with leucine to proline. The proline variant loses one central hydrogen bond because of this substitution. With this, the dissociation constant between L51P and BMPRI goes about 8,000 times up compared with wild-type BMP2 binding; this leads to a lower affinity of L51P to the ligand binding domain of BMPRI [120, 121]. However, the binding affinity of L51P to BMPRII is comparable to that of the wild type. While the affinity for BMPRI is lower, L51P retains the wild-type affinity to BMP antagonists, such as NOG and GREM [121].

It was found that the application of L51P in combination with BMP2 induces improved bone formation in a rat animal large bone defect model and rat calvaria cells [122-124]. However, if L51P was applied alone, no osteoinduction could be observed in primary murine OBs, MC3T3-E1, ATCDC5, and pro-myoblasts [122, 123, 125]. These experimental data further indicate that L51P seemed to act as a general inhibitor of BMP antagonists and does not solely block the NOG-specific pathway. In a recent study by Hauser *et al.* (2018) [126] ovariectomized Wistar Crl:WI (Han) rats (female, retired breeders, 8–10 months) were treated with alendronate (at eight weeks postsurgery), which belongs to the group of bisphosphonates. At 14 weeks postsurgery, diaphyseal femoral defects were applied, followed by stabilization with a rigid osteosynthesis system.

The defects were then filled with β-tricalcium phosphate ceramics, which were loaded with BMP2, L51P, or a combination of the two cytokines. In this study, Hauser *et al.* (2018) [126] confirmed improved bone formation through the combined application of BMP2 and L51P by using μCT and histology.

The application of L51P to improve spinal fusion could be a promising approach, as the inhibition of NOG seems to result in a more efficient and physiological bone formation than the application of BMP [121].

7. BMPS AND BMP ANTAGONISTS AND THEIR IMPORTANCE IN SPINAL FUSION

Bone formation is driven by two different types of ossification - the direct intramembranous process, in which bone is formed directly into the primitive connective tissue, or the indirect endochondral process, in which cartilage is formed as a precursor before bone formation. As already discussed BMPs act, depending on their concentration gradient as differentiating factors, but they can also attract various cell types and function further as chemotactic and mitogenic agents [127]. Besides affecting the proliferation of cartilage- and bone-forming cells, they induce, as already described above, the differentiation of MSC toward chondroblasts and OBs. Therefore, BMPs most probably influence both direct and indirect bone formation [128].

Several studies in the last two decades have been conducted to investigate the effect of various BMPs or BMP antagonists in spinal fusion models. These studies showed the feasibility of using growth factors for the improvement of fusion rates. Table **1** provides an overview of studies focusing on spinal fusion, bone healing, or, in general, OB and osteoblastic cell line culture with the application of BMPs or BMP antagonists.

Previous studies that investigated BMP2 particularly focused on spinal fusion models. Several *in vivo* rat studies have demonstrated the beneficial effect of BMP2 on bone healing and spinal fusion [129-131]. Zhu *et al.* (2017) [129] showed early and large bone formation with transplanted DMBM and collagen binding BMP2 in a posterolateral rat spinal fusion model. The study of Alden *et al.* (1999) [130], which is based on the classic work of Urist *et al.* (1965) [37], focused on viral gene therapy and observed paraspinal endochondral ossification after injections in the paraspinal musculature of gene constructs. However, in a recent study by Koerner *et al.* (2018) [131], the application of BMP2 (10 or 100 μg) in a spinal fusion rat model demonstrated enhanced inflammatory reactions and inflammatory cytokine expression. Furthermore, growth factors, such as vascular endothelial growth factor, insulin-like growth factor 1, platelet-derived growth factor, and TGF-β, appeared to be repressed in the early stage of BMP2 treatment. This study showed that BMP2 leads to inflammatory reactions, but it may also contribute to enhancing the fusion process [131]. As in the study of Zhu *et al.* (2017) [129], rats were approximately eight weeks in age, and spinal fusion was processed at the same level (L4–L5), but the rat subspecies was not the same. Besides rat, rabbit and mouse studies have been conducted to investigate the effect of cytokines on spinal fusion. There are, of course, species-specific differences in the metabolism and age of the animals. In a study by Minamide *et al*. (2001) [132], rabbits underwent single-level process fusion (L4-L5). The animals were implanted with sintered bovine bone true bone ceramics, coated with a type 1 collagen sheet supplemented with BMP2 (rhBMP2) or just the collagen sheet with rhBMP2. Again, the use of rhBMP2 resulted in a higher fusion rate [132].

Besides BMP2, other BMPs have been investigated in relation to spinal fusion. Previously, we discussed the study of Alden *et al.* (1999) [130], which showed that BMP2 has the ability to induce bone formation in a tight musculature. In a similar study, Helm *et al.* (2000) [133] applied BMPs in the paraspinal region of 16-week-old rats and showed the ability of BMP9 to induce bone formation in rodents by gene therapy.

Not only BMPs but also BMP antagonists have gradually been a part of spinal fusion studies in recent years. Furthermore, studies that focused not only on BMPs but also on BMPs in combination with their antagonists or on antagonists alone have been conducted. Research on the BMP antagonist NOG were particularly of interest, as NOG seems to play an important role in bone and cartilage formation. For example, Abe *et al.* (2000) [134] examined the effect of NOG with BMP6. Although the BMP antagonist NOG can bind to BMP6, Abe *et al.* (2000) [134] showed that NOG does not antagonize BMP6 activity, as the ALP activity measured from BMP6 and NOG stimulated mouse C2C12 cells. Furthermore, in more recent studies by Song *et al.* (2010) [27] in which different cell lines were stimulated with different BMPs in the presence or absence of NOG, BMP7 has been shown to be more potent than BMP6 as a negative regulator. NOG expression was also more potently induced by BMP7 than by BMP6. BMP6 was more potent for OB differentiation promotion *in vitro* and bone regeneration *in vivo* [27]. Furthermore, Takayama *et al.* (2009) [107] showed that NOG mRNA was upregulated in C2C12 in response to rhBMP2. In their study, the silencing of NOG resulted in the acceleration of BMP-induced osteoblastic differentiation [107]. Wan *et al.* (2007) [135] conducted an *in vitro* and *in vivo* mouse study of the effect of NOG suppression in OBs. The inhibition of NOG was found to results in enhanced BMP signaling and *in vivo* bone formation [135]. However, Klineberg *et al.* (2014) [136] could not find a significant improvement in overall fusion rates compared with that of the controls when NOG was downregulated by electroporation of siRNA targeting NOG in the paraspinal muscle of a posterolateral intertransverse rabbit lumbar fusion model. When NOG is overexpressed, such as in the *in vivo* mouse study of Devlin *et al.*, (2003) [137] the animals developed decreased bone volume and osteopenia.

In a mouse study by Okamoto *et al.* (2006) [138], the overexpression of BMP4 and NOG was investigated in bone. The animals overexpressing BMP4 developed severe osteopenia, and the animals overexpressing NOG showed an increased bone volume associated with decreased bone formation [138]. In a study by Tsuji *et al.* (2008) [139], it was revealed that BMP4 is not necessary for successful skeletal growth and bone healing, which was investigated in mice with floxed BMP4 alleles bred with Prx1-cre transgenic mice to establish the limb-specific removal of BMP4.

Table 1. Application of BMP and BMP antagonists in spinal fusion and bone healing.

Author, Year	Study Type, Species	Cytokine	Study	Conclusion
Boden, 2002 [141]	*In vivo,* human	BMP2	Lumbar arthrodesis of patients (N = 25) by autograft/Texas Scottish Rite Hospital (TSRH) pedicle screw instrumentation (N = 5), rhBMP2/TSRH (N = 11) or rhBMP2 only without internal fixation (N = 9) (20 mg/mL of rgBMP2 in hydroxyapatite/tricalcium phosphate carrier)	Radiographic fusion rate of the TSRH group was 40%, both groups treated with rhBMP2 showed 100% fusion rate
Govender, 2002 [25]	*In vivo,* human	BMP2	Open tibial fractures patients (N = 450) received standard treatment with an implant containing 0.75 mg/mL (total 6 mg) of rhBMP2 or an implant of 1.5 mg/mL (total 12 mg)	Significantly faster fracture healing with 1.5 mg/mL over the current standard of care
Friedlaender, 2001 [142]	*In vivo,* human	BMP7	Tibial non-union patients (N = 124) were treated by an intramedullary rod, accompanied by BMP7 in a COL1 carrier or by fresh bone autograft	After 9 months, 75% of patients in the BMP7 treated group had healed fractures (evaluated by radiographic criteria)
Klineberg, 2014 [136]	*In vivo*, rabbit	NOG	SiRNA against NOG was electroporated in paraspinal muscle of bilateral, posterolateral intertransverse lumbar fusion in skeletally mature New Zealand White rabbits (L5-L6)	NOG protein was knocked down *in vivo* for seven days and detectable by six weeks No significantly improvement of overall fusion rates compared to controls
Minamide, 2001 [132]	*In vivo*, rabbit	BMP2	Japanese white rabbits, underwent single-level bilateral posterolateral intertransverse process fusion (L4-L5) Animals was implanted sintered bovine bone TBC coated with COL1 infiltrated ± 100 μg of rhBMP2 or COL1 sheet ± 100 μg of rhBMP2	TBC showed to be a more efficient carrier for rhBMP2 compared to collagen sheet, the process of spinal arthrosis showed a faster and stronger fusion The use of rhBMP2 resulted in a higher fusion rate (inTBC and collagen group)
Koerner, 2018 [131]	*In vivo*, rat	BMP2	Adult Wistar rats (age approximately 8 weeks) underwent postlateral intraverse fusion with DBM (L4-L5) 10 or 100 μg of rhBMP2 were added on an allograft collagen sponge. Animals were sacrificed at time points up to four weeks	Enhanced inflammatory reaction and expression of inflammatory cytokines in the early time points (1 hour, 6 hours) because of rhBMP2 Growth factor (VEGF, IGF1, PDGF, TGF-β) expression appears first suppressed followed by a peak at 24 hours and 7 days TNFα showed a lower expression in rhBMP2 treated groups at days 1, 2 and 4.
Zhu, 2017 [129]	*In vivo*, rat	BMP2	Mature male Sprague Dawley rats (8 weeks) undergoing posterolateral spinal fusion (L4-L5) were implanted with (A) demineralised bone matrix (DBM), (B) with a combination of DBM and BMP2 or (C) with DBM and a combination of collagen binding bone morphogenetic protein 2 (CBD-BMP2)	CBD-BMP2 showed a higher affinity to the scaffold than commercial BMP2. Bone formation in group C was observed to be earlier and larger, compared to the other groups Better trabecular bone microarchitecture assessment and statistically larger bone mineral density.
Song, 2010 [27]	*In vitro*, rat osteosarcoma cell line, C2C12 myoblasts	BMP2, BMP4, BMP5, BMP6, BMP7, GDF5, GDF6, NOG	Stimulation of cells with different BMP in the presence or absence of NOG Induction of NOG knockdown in C2C12 cells and exogenous stimulation of BMP6 and BMP7 (each 50 ng/mL)	Shallower slopes of dose-response curves for ALP activity for BMP2 and BMP4 compared to BMP5, BMP6, and BMP7 (suggesting a more negative regulatory mechanism for BMP2 and BMP4) More NOG induction by BMP7 than BMP6 But BMP6 is more resistant to NOG inhibition than BMP7

Table (1) contd….

Author, Year	Study Type, Species	Cytokine	Study	Conclusion
Helm, 2000 [133]	*In vivo*, rat	BMP9	Injection of BMP9 adenoviral vextor in 16-week old athymic male rats in the lumbar paraspinal musculature (sacrificed after 16 weeks)	Induction of massive bone at the injection sites, leading to solid spinal arthrodesis No evidence of pseudarthroses, nerve root compression, or systemic side effects
Alden, 1999 [130]	*In vivo*, rat	BMP2	Recombinant, replication-defective type 5 adenovirus with cytomegalovirus (CMV) promoter and BMP2 gene injection bilaterally or on the right side, percutaneously and paraspinally at the lumbosacral junction in athymic nude rats	Expression of BMP2 leads to endochondral bone formation in the paraspinal region Detection of cartilaginous tissue after three months postinjection at the injection site
Suzuki, 2012 [140]	*In vitro*, C2C12	BMP2, GREM1, GREM2	Performance of microarray analysis on mRNA extracted from C2C12 cells, stimulated with different concentrations of BMP2 (0-400 ng/mL). siRNA was used to down-regulate GREM1 and GREM2	GREM1 and GREM2 were differently regulated by BMP2; GREM1 was downregulated, whereas GREM2 was upregulated after stimulation in a dose- and time-dependent manner Groups treated with siRNA and stimulated with BMP2 showed a significantly enhanced ALP activity compared to control groups
Ideno, 2009 [115]	*In vitro*, mouse	GREM2	GREM2 expression was upregulated by adenovirus or downregulated by siRNA in pre-osteoblasts of embryonic day 18.5 mouse calvariae	Upregulated expression suppressed exogenous BMP activity and endogenous levels of phosphorylated Smad1/5/8 (pSmad1/5/8) protein Downregulation elevated ALP activity, increased endogenous levels of pSmad1/5/8 protein and induced matrix mineralisation.
Takayama, 2009 [107]	*In vitro*, C2C12 (myoblastic cell line), *In vivo*, mouse	NOG	NOG-siRNA silencing in C2C12 in rhBMP2 (0-300 ng/mL) stimulated cells NOG silencing in sites of exogenous rhBMP2-induced (5 µg/mL) ectopic ossicles in the muscle of six week old male ICR mice, by electroporation	NOG mRNA expression was upregulated in response to rhBMP2 in C2C12 cells, in a dose- and time-dependent way Silencing of NOG expression by transfection of NOG siRNA, suppressed BMP2-stimulated NOG expression, resulting in acceleration of BMP2-induced osteoblastic differentiation. No enhancement of BMP2 induced new bone formation, in sites where NOG expression was silenced But increase of radiological density of NOG-targeted siRNA transfected and rhBMP2 stimulated ossicles.
Tsuji, 2008 [139]	*In vivo*, mouse	BMP4	Mice with floxed BMP4 alleles were bred with Prx1-cre transgenic mice to establish limb-specific removal of BMP4	Limb skeletogenesis usually occurs in absence of BMP4, so postnatal skeletal growth was unaffected with removal of BMP4 Mice lacking BMP4 were able to mount a successful healing response
Wan, 2007 [135]	*In vitro*, *in vivo* mouse	NOG	NOG was downregulated in MC3T3-E1 preosteoblast and primary mouse calvarial osteoblasts, from 5-day-old CD-1 mice, by using siRNA or by adeno-CMV-Cre infection of floxed NOG osteoblasts. Treatment of critical-size calvarial defects by osteoblasts expressing NOG-specific siRNA constructs	Both cell types expressed enhanced osteogenic differentiation markers and showed more bone nodule deposition The removal of NOG leading to an increased signalling activity of endogenously produced BMP (enhanced levels of pSmads). Acceleration of early reossification of defects two or four weeks following injury

Table (1) contd….

Author, Year	Study Type, Species	Cytokine	Study	Conclusion
Okamoto, 2006 [138]	*In vivo*, mouse	BMP4, NOG	Mice overexpressing BMP4 or NOG in osteoblasts under the control of the COL1 alpha 1 (COL1A1) promoter sequence were generated by microinjecting of the respective insert into the pronuclei of fertilized eggs from F1 hybrid mice (C57BL/6 x DBA)	Mice overexpressing BMP4 developed severe osteopenia, associated with increased numbers of osteoclasts Mice overexpressing NOG showed an increase in bone volume but decreased bone formation rate and reduced osteoclast number.
Devlin 2003 [137]	*In vivo*, mouse	NOG	Fertilized oocytes were taken from CD-1 outbred albino mice and transfer of microinjected embryos into pseudopregnant mice Transgenic mice overexpressing NOG under the control of the osteoblastic specific osteocalcin promoter	Mice overexpressing NOG developed decreased bone volume and osteopenia Bone mineral content, osteopenia and poor healing of fractures were persistent for 6 months OB activity seemed to be reduced, osteoclast (OC) number was not increased, and neither was bone resorption affected
Abe, 2000 [134]	*In vitro*, mouse	BMP2, BMP6, NOG	C2C12 were cultured with 100 ng/mL BMP2, BMP6 and/or 10-600 ng/mL NOG for three days.	BMP2 induced ALP activity was inhibited by human recombinant NOG in a dose-dependent manner The effect of BMP6 was not affected

Moreover, a study by Suzuki *et al.* (2012) [140] showed that both BMP antagonists GREM1 and 2 were differentially regulated by BMP2 in the C2C12 cell line. GREM1 expression was found to be downregulated by BMP2, while expression levels of GREM2 were dose-dependently increased upon treatment with BMP2 [140]. Furthermore, Ideno *et al.* (2009) [115] investigated GREM2 in the pre-OBs of embryonic day 18.5 mouse calvariae, and they confirmed its negative feedback behavior on BMP signaling.

Finally, BMP2 and BMP7 were also investigated in clinical studies. Boden *et al.* (2002) [141] and Govender *et al.* (2002) [25] demonstrated the beneficial effect of BMP2 on bone formation in an open tibial fracture or spinal fusion in clinical studies. Friedlaender *et al*. (2001) [142] conducted a study in 2001, in which the effect of BMP7 was investigated in tibial non-unions. BMP7 was found to be a safe and effective treatment for tibial non-unions, and its output its comparable to that which could be achieved with bone autografts.

Besides this application, the expression level of BMP family members has been the focus of several past studies, such as those listed in Table **2**. In a posterolateral intertransverse fusion model in rabbit, Tang *et al.* (2001) [143] investigated the expression of BMP2, BMP4, BMP7, NOG, and CHRD. They distinguished between the outer (over transverse processes) and inner (between transverse processes) parts, as well as the surrounding muscles. Interestingly, the outer part showed the highest expression of BMP and early bone maturation. During the reparative and remodeling phase, NOG activity was decreased, whereas BMP expression was significantly increased in the outer and inner parts. However, CHRD expression in these two zones increased [143]. This finding suggests that these two BMP antagonists regulate BMP activity through a different mechanism. Furthermore, in the surrounding muscles, BMP expression could be detected, which may indicate that muscle contributes to spinal fusion.

Kwong *et al.* (2009) [144] determined the regional and cellular distribution in human healing bone fractures. All four proteins investigated, BMP2, the growth and differentiation factor (GDF) 5, CHRD, and NOG, were expressed stronger in cartilage formation and to a lesser extent in areas of bone formation. In a similar study, the same group compared the same cytokines in biopsies from human fractures that either healed normally or resulted in non-unions. BMP2 and GDF5 were found found to be expressed at lower levels in the non-union group compared with the case in which fractures normally healed. These findings of Kwong *et al.* (2009) [144] correspond with those of Kloen *et al*. (2012) [33], who conducted a similar study three years later on comparisons between regular healing fractures and non-unions. Similar to Kwong *et al.* (2009) [144], they detected decreased BMP2 and an almost absent BMP7 expression in the chondrocytes of non-unions. They agreed with the presence of a different balance between BMP and BMP antagonists [144]. In another highly similar study by Fajardo *et al.* (2009) [145], gene expressions in non-union and healing bone specimens were compared by qPCR. These authors observed an upregulation of BMP7 in the healing bone, whereas NOG, CHRD, and FLST were upregulated in non-union tissue [145]. Furthermore, Niikura *et al.* (2006) [146] investigated gene expression from several members of the BMP family in callus standard healing fractures and fibrous tissues of non-union. Similar to Kwong *et al.* (2009) [144], these authors detected a significantly lower gene expression of several BMPs (BMP2, BMP4, BMP6, BMP7, GDF5, and GDF7) and, surprisingly, even BMP antagonists [146]. Dean *et al.* (2010) [147] made different observations regarding the

Table 2. Expression of BMP and BMP antagonists in spinal fusion and bone healing.

Author, Year	Study Type, Species	Cytokine	Study	Conclusion
Kloen, 2012 [33]	*In vitro*, human	BMP2, BMP3, BMP7, pSmad1/5/8, NOG, GREM1, CHRD, BMABI	Expression of endogenous BMP ligands and BMP inhibitors in non-union compared to normal fracture healing	Decreased BMP2 and almost absent BMP7 expression in chondrocytes in non-unions Expression of BMP antagonists, *i.e*., NOG and GREM1 nearly the same in osteoblasts, chondrocytes, and fibroblasts of both fracture callus and non-unions Generally, expression of BMP antagonists was stronger than BMP
Fajardo, 2009 [145]	*In vitro*, human	BMP2, BMP4, BMP5, BMP7, BMP8, CHRD, NOG, GREM1, FLST	Two tissue samples from non-union patients: fibrous tissue from the non-union site and healing bone from the surrounding region	BMP4 and investigated BMP antagonists were upregulated in non-union compared to fracture callus Healing bone showed upregulation of BMP7, and in non-union tissue, an increased expression of BMP4, GREM1, FLST, and NOG but not of CHRD
Kwong, 2009a [144]	*In vitro*, human	NOG, CHRD, BMP2, GDF5	Determination of regional distribution of NOG, CHRD, BMP2, and GDF5 in tissue samples of patients undergoing surgery for failure of conservative management, or failure of the original surgical fixation to maintain alignment of their fracture and who were subsequently found to have fracture union on follow-up by immunohistochemistry Fracture biopsies were taken from extra-articular sites of the humerus, clavicle, femur, tibia, fibula, and acetabulum	Expression of NOG and CHRD in areas of cartilage formation Detection of NOG in active osteoblasts in areas of bone formation, in endothelial cells, and pericytes of the newly formed blood vessels of the fracture callus GDF5 staining revealed the strongest in fractures, expression, in parts of cartilage formation, it was detected in chondrocytes, osteoblasts, and fibroblastic cells BMP2 expression was the strongest in areas of endochondral ossification in hypertrophic chondrocytes and also in lower extent in osteoblasts, osteocytes, and osteoclasts
Kwong, 2009b [148]	*In vitro,* human	NOG, CHRD, BMP2, GDF5	Investigation of expression of NOG, CHRD, BMP2, and GDF5 in human biopsy samples from fractures, which heal normally or became non-unions	Biopsies from patients with non-union turned out to have a reduction in BMP and GDF5 expression No differences in expression level for NOG and CHRD between non-union and control group
Tang, 2011 [143]	*In vivo*, rabbit	BMP2, BMP4, BMP7, NOG, CHRD	Posterolateral intertransverse spinal fusion with autogenous bone graft Investigation of BMP2, BMP4, BMP7, NOG, CHRD, sex determining region Y-box 9 (SOX9), and RUNX2 from specimens collected from the outer zone over the transverse process, in the inner zone between the transverse process, the muscle surrounding bone grafts and the transverse process	BMP2, BMP4, and BMP7, NOG, and CHRD were co-localized in outer osteoblasts, osteoclasts, and chondrocytes The muscle around bone grafts showed significantly higher BMP expression and RUNX2 activity.
Dudarić, 2013 [77]	*In vitro*, rat	BMP2, BMP4, BMP7, CHRD, NOG, FLST	Investigation of expression levels of several BMP and BMP antagonists in induced ectopic bone formation in rats	Increased level of BMP2, BMP4, NOG, and FLST at day 14 of osteogenesis Increased level of GREM1 and CHRD in the later phase, which indicates their role in the regulation of the osteogenesis initiation

Table (2) contd….

Author, Year	Study Type, Species	Cytokine	Study	Conclusion
Niikura, 2006 [146]	*In vitro*, rat	BMP2, BMP3, BMP3B, BMP4, BMP6, BMP7, GDF5, GDF7, NOG, GREM, SOST, BAMBI	Creation of atrophic non-unions in rat femurs, by periosteal cauterisation at the fracture site Measurement of BMP and BMP antagonist's expression in RNA extracted from the callus of standard healing fracture and fibrosus tissue of non-union	Gene expression of BMP2, BMP3B, BMP4, BMP6, BMP7, GDF5, GDF7, NOG, DRM, SOST, and BMP and activin membrane-bound inhibitor (BAMBI) were significantly lowered in non-unions compared to normal healing fractures at multiple time points
Dean, 2010 [147]	*In vivo*, mouse	BMP2, BMP4, BMP7, BMPRIA, BMPRII, PRDC, SOST, Smad7, GREM1	Controlled femoral fractures of 40 mice, the tissue samples at the fracture sites were harvested at days 1, 3, 7, 14, and 21 after the intervention and quantified for the expression of BMP and BMP antagonists.	Upregulation of BMP2, BMP4 and BMP7 during frature healing, whereas expressions of GREM2, SOST, Smad7, GREM1, and CER were generally downregulated Significantly upregulation of NOG in the first week after fracture Seven days after the fracture other BMP antagonists such as DAN, CHRD, Smad6, and BAMBI showed increased expression.
Yoshimura, 2001 [149]	*In vivo,* mouse	NOG, BMP4	Temporal and spatial expression of NOG and BMP4 in a repair model of fracture in adult mice	Localisation of BMP4 and NOG were similar in cells within the proliferating periosteal layer, cells lining the newly formed bone (osteoblasts), cartilage tissue including differentiating chondrocytes, and hypertrophic chondrocytes

time point of BMP antagonist expression levels during bone healing. They suggested that BMP antagonists should be divided into functional groups - those that are suppressed for the initiation of osteogenesis and those that are upregulated to induce bone remodeling [147].

8. APPLICATION OF BMPs AND BMP ANTAGONISTS IN SPINAL FUSION AND BONE HEALING BY USING MSCs

BMPs exhibit broad spectra of biological activities not only during embryogenesis but also throughout life by influencing various tissues, such as bone, cartilage, blood vessels, heart, kidneys, neurons, liver, and lungs. As previously mentioned, BMPs were first identified for their ability to induce bone formation and hence their coined name. Later, it was also discovered that they act as pleiotropic players [150].

Proper bone formation requires the process of BMP signaling, which leads MSCs to differentiate toward OBs. Besides the studies conducted on spinal fusion models and OB cultures, much research has investigated the effect of BMP family cytokines in MSCs. Because of the plasticity of MSCs, they are useful tools for tissue engineering, gene therapy, or a combination of these two approaches. In this section, the different effects of BMP family cytokines on MSCs are discussed. An overview of the studies belonging to this can be found in Table **3**.

Several studies have described the upregulation of NOG expression when MSCs are stimulated with BMP2 [151]. Surprisingly, Chen *et al.* (2012) [151] detected a decrease in osteoblastic genes when NOG was downregulated in hMSCs. Rifas *et al.* (2007) [109] made a similar observation when they stimulated hMSCs with NOG. hMSCs underwent differentiation toward the osteoblastic lineage induced by NOG. Furthermore, NOG did not inhibit matrix mineralization as expected, but it rather synergized with dexamethasone (DEX) to increase calcium deposition. However, Kwong *et al.* (2008) [152] showed the upregulation of ALP activity when CHRD was downregulated in hMSCs. They also investigated the expression of BMP2 and CHRD when MSCs were stimulated with standard osteogenic differentiation media, in which an upregulation of BMP2 with the induction of osteogenic differentiation was confirmed; surprisingly, however, no CHRD expression could be detected [152]. Wang *et al.* (2017) [81] investigated the knock-down of GREM2 in hMSCs and an upregulation in bone-specific markers, such as *BGLAP*, *SPP1*, and *ALP*; higher calcium deposition was detected. The reverse was true when cells were treated with a GREM2 expression plasmid, in which a decrease in osteoblastic genes, ALP activity, and calcium deposits was observed [81]. Recently Wang *et al*. (2018) [153] performed NOG, GREM1 and CHRD knockdown by using siRNA in human bone mesenchymal stromal cells (hBMSCs). They detected that the knockdown of CHRD induced a stronger osteogenic response than did the knockdown of GREM1 and NOG [153].

Lately, gene therapy studies with MSCs have been conducted to induce bone formation. Hasharoni *et al.* (2005) [154] implanted hMSCs overexpressing BMP2 in the

Table 3. Application of BMP and BMP antagonists in spinal fusion and bone healing by using mesenchymal stromal cells.

Author, Year	Study Type, Species	Cytokine	Study	Conclusion
Wang, 2018 [153]	*In vitro*, human	NOG, GREM1, CHRD	Knock down of CHRD, NOG and GREM in hBMSCs from patient with normal bone healing and with nonunion Measurement of expression of BMPs and BMP antagonists were measured in hBMSCs of those patients	hBMSCs, treated with CHRD siRNA had a higher expression of *SP7*, *BGLAP* and *COLIA1* than hBMSCs, treated with GREM1 siRNA Knock down of NOG decreased the expression of the before mentioned genes Higher expression of NOG CHRD and in hBMSCs from patients with nonunion than from patients with normal fracture healing
Hu, 2017 [164]	*In vitro*, human	BMP2, GREM1	GREM1 was downregulated in hMSCs by using siRNA	GREM1 suppression significantly increased DNA content, cell metabolism, and enzymatic ALP activity
Chen, 2012 [151]	*In vitro*, human	NOG, BMP2	Expression of NOG in hMSCs, when stimulated with BMP2, in a dose- and time-depended manner NOG expression was knocked down in MSCs by using siRNA and stimulated with osteogenic medium supplemented with 0.1 µg/mL BMP2	NOG induction was enhanced by BMP2 at concentrations from 0.01 to 1 µg/mL, the induction decreased at higher concentrations (1 to 50 µg/mL) Osteoblastic genes (ALP, BSP2, Msh homeobox 2 (MSX2), BGLAP, SPP1, and RUNX2) were significantly decreased in MSC with NOG knockdown
Ramasubramanian, 2011 [165]	*In vitro*, human	BMP2, NOG	Human adipose-derived stromal cells (hADSC) were treated with varying doses of BMP2 DNA and/or siRNA of guanine nucleotide binding protein alpha stimulating activity polypeptide (GNAS) and NOG	No increase in matrix mineralization in hADSC treated with BMP2, while co-delivery of BMP2 with siGNAS or siNOG led to more intense mineralisation Groups treated with BMP2 showed a decrease in calcium deposits and ALP activity compared to siRNA groups Increase in BMP2 expression level in hADSC suppressing NOG Co-delivery of siNOG and BMP2 DNA reduced NOG knockdown and accelerated the differentiation towards osteogenic phenotype marked with increase bone marker expression and mineralisation
Kwong, 2008 [152]	*In vitro*, human	BMP2, CHRD	Measurement of BMP2 and CHRD expression in hMSCs during stimulation with osteogenic medium CHRD knock down was induced using RNA interference during osteogenic stimulation	Osteogenic differentiation was associated with an increase in BMP2 expression CHRD expression was not detectable with conventional qPCR Knock down of CHRD resulted in a significant increase of ALP activity and deposition of extracellular mineral
Rifas, 2007 [109]	*In vitro*, human	NOG	hMSCs were stimulated with NOG in addition to DEX, BMP (BMP2, BMP6 or BMP7) or inflammatory cytokines.	NOG induced an anabolic effect and induced hMSCs into a committed osteoblast lineage NOG showed no inhibition of DEX-induced ALP activity but rather acting in an additive manner. NOG does not lead to an inhibition of mineralisation induced by BMP; it even synergised with DEX to increase mineralisation NOG induced BMP2 and BGLAP but not RUNX2

Table (3) contd….

Author, Year	Study Type, Species	Cytokine	Study	Conclusion
Friedman, 2006 [161]	*In vitro*, human	BMP2, BMP4, BMP6, BMP7, GDF5	Osteoinductive effects of hMSCs were examined when stimulated with different BMP	BMP6 showed the most potent, donor-independent osteoinductive effects BMP6 was upregulated by DEX treatment and additional exogenous BMP6 induced hMSCs to differentiate towards an osteoblast phenotype When investigating BMP combinations, only co-treatment with BMP6 lead to robust mineralisation BMP6 leads to expression of *SP7, DLX5, BGLAP, SPP1* and *BSP* but no change in *RUNX2* and *COL1* expression.
Dragoo, 2003 [158]	*In vitro,* human	BMP2	Stimulation of pluripotent mesenchymal progenitor cells from liposuction aspirates, and bone marrow aspirate with BMP2 or exposition to adenovirus containing BMP2 Comparison to hOBs or cells cultured in osteogenic media	Pluripotent mesenchymal progenitor cells were positively transduced with BMP2 gene and transform towards an osteogenic phenotype, comparable when cells were stimulated with exogenous rhBMP or hOBs.
Wang, 2016 [81]	*In vitro*, human *In vivo*, mouse	GREM2, BMP2	Bone-marrow-derived hMSC were stimulated with BMP2 and *GREM2* mRNA expression was investigated in dose-response (0-50 µg/mL BMP2) and time-course studies (0.1 µg/mL BMP2) hMSC were transfected with siRNA targeting GREM2 or transfection with GREM2 expression plasmid Creation of 0.8 mm in diameter segmental bone defect of the left femur in male BALB/C nude mice followed by transplantation of hMSCs resuspended in medium and Matrigel (BD Bioscience) Partial were MSCs infected before with siGREM2 or Lentivirus (LV)-GREM2.	Higher concentration of BMP2 increased GREM2 expression GREM2 siRNA MSC showed significant suppression of *GREM2* expression and a significant increase in *COL1A1*, *BGLAP, SPP1*, and *ALP* Increase of ALP activity after 14 days of induction and measurement of more calcium deposits *BMP2/Smad/RUNX2* was activated, as levels of *BMPRII, RUNX2* and *pSmad1/5/8* were higher in the siGREM2 group compared to control group *GREM2* expression in LV-GREM2 transfected cells was significantly increased, as well as protein level Osteoblastic genes, ALP activity and calcium deposits were significantly decreased Callus size in mice femoral bone defect model was considerably larger in siGREM2 groups
Hasharoni, 2005 [154]	*In vitro*, human *In vivo*, mouse	BMP2	Genetically engineered MSCs, expressing rhBMP2 were implanted into the paraspinal muscles of mice	At 4 weeks postinjection genetically engineered MSCs induce active osteogenesis at the site of implantation Seven days of BMP2 induction was sufficient to form new bone tissue
Fan, 2013 [166]	*In vitro*, mouse	NOG, BMP2	Downregulation of NOG in adipose-derived stem cells using short hairpin technology (shRNA) Seeding of cells in chitosan or chondroitin sulphate scaffolds loaded with 15 µg/mL BMP2 and culturing in osteogenic medium	Osteogenic differentiation was significantly higher in NOG shRNA treated cells compared to control cells (both stimulated with BMP2)

Table (3) contd….

Author, Year	Study Type, Species	Cytokine	Study	Conclusion
Hannallah, 2004 [162]	*In vivo*, mouse	BMP4, NOG	Implantation of muscle-derived stromal cells (MDSC) transduced with BMP4 into both hind limbs of SCID mice with 0.1, 0.5 or 1 Mio of NOG expressing MDSC (mice were sacrificed after 4 weeks) Human DMBM was implanted into the hind limbs with 0.1, 0.5 or 1 Mio MDSC (mice were sacrificed after 8 weeks)	Varying doses of NOG expressing MDSC induced a reduction in heterotopic ossification in a dose-dependent manner Each of the three varying doses of NOG expressing MDSC significantly inhibited the heterotopic ossification
Sheyn, 2008 [160]	*In vitro*, porcine *In vivo*, mouse	BMP6	Primary porcine adipose-tissue-derived stem cells were nucleofected *ex vivo* with a plasmid containing rhBMP6. Cells were then injected into the lumbar paravertebral muscle in immunodeficient mice	Cells induced functional bone tissue formation and efficient spinal fusion.
Wang, 2010 [159]	*In vitro*, canine *In vivo*, rat	BMP2	Stimulation of beagle MSC with different concentrations of BMP2 (0, 25, 50, 100, or 200 ng/mL) or a combination of BMP2 with basic fibroblast growth factor (bFGF) in different ratios Calcium phosphate cement (CPC) was seeded with BMSCs in medium containing 100 ng/mL BMP2, 50 ng/mL bFGF or a combination of BMP2 and bFGF, subsequently cells were subcutaneously implanted in four sites in nude rats (rats were sacrificed four or 12 weeks postoperatively)	rhBMP2 was a more potent stimulator of BMSC differentiation than bFGF, proliferation was more stimulated with bFGF than with BMP2 CPC demonstrated to be a good bone scaffold, it induces rapid deposition of new bone at the cement surface interface *In vitro* was the bone formation the highest in groups stimulated with BMP2/bFGF treatment.
Wang, 2003 [155]	*In vivo*, rat	BMP2	Intertransverse spinal arthrodesis (L4 - L5) was attempted in Lewis rats with BMP2-producing rat bone marrow cells (Ad-BMP2 cells), created through adenoviral gene transfer with guanidine hydrochloride-extracted DBM as a carrier or Ad-BMP2 cells on a collagen sponge carrier Ten μg of recombinant BMP2 (rhBMP2) in a guanidine hydrochloride-extracted DBM carrier or 10 μg of rhBMP2 in a collagen sponge carrier.	Spines were fused four weeks postoperatively Spines that had received BMP2-producing bone marrow cells were filled with coarse trabecular bone postoperatively Spines receiving rhBMP2 were filled with thin, lace-like trabecular bone
Cheng, 2001 [157]	*In vitro*, *in vivo,* rabbit	BMP2	Transfuction of rabbit MSCs with an adenoviral vector carrying human BMP2 gene Transduced MSCs were implanted autologously into the intertransverse process space (L5 and L6 of donor rabbits)	Cells differentiated into an osteoprogenitor line, bone formation *in vitro* was induced by increased ALP activity and expression of *COL1, SPP1*, and *BGLAP* and induction of matrix mineralisation After four weeks, new bone formation could be demonstrated *in vivo*
Riew, 1998 [156]	*In vitro*, *in vivo*, rabbit	BMP2	MSCs derived from rabbits were transduced with an adenoviral vector carrying the human BMP2 Transduced cells were then autologously reimplanted into donor rabbits	MSCs transduced with adenovirus carrying the BMP2 gene, overproduce the BMP2 protein Only one of five rabbits, where the transduce MSCs were reimplanted produced radiographically and histologically evident bone

paraspinal muscle of mice and induced this is the hallmark of BMP activity, induction of ectopic bone formation osteogenesis at the site of implantation. Wang *et al*. (2017) [81] compared the different effects of rat MSCs expressing BMP2 through adenoviral transfer with those of BMP2. Both were applied either with DMBM or with a collagen sponge in a rat spinal fusion model (intertransverse spinal arthrodesis between L4 and L5). Here, as in comparable studies, all spines from all groups were fused. However, differences in the manner of bone production could be observed. Spines that received BMP2-producing bone marrow cells were filled with a coarse trabecular bone, whereas spines that received rhBMP2 were filled with a thin, lace-like trabecular bone [155]. An early but similar study by Riew *et al*. (1998) [156] investigated the effect of MSCs in an *in vivo* rabbit model. They reimplanted MSCs into donor rabbits and transduced them with an adenovirus, leading to BMP2 overexpression. Surprisingly, compared with the results of other studies, no significant effects were observed, as only one of the five rabbits produced an evident bone because of the reimplantation of transduced MSC. In another study by the same group of Cheng *et al.* (2001) [157], rabbit MSCs were transduced with an adenoviral vector carrying the human BMP2 gene and were implanted autologously into the intertransverse process space between L5 and L6 of the donor rabbits. This study could confirm bone formation *in vitro* and *in vivo*. Another human study by Dragoo *et al.* (2003) [158] demonstrated successful gene therapy with pluripotent mesenchymal progenitor cells, which were isolated from liposuction aspirates. Here, the cells were transfected with an adenovirus containing BMP2, which led to BMP2 overexpression and, finally, to a shift in mesenchymal progenitor cells toward an osteogenic phenotype [158]. Dragoo *et al.* (2010) [158] and Wang *et al*. (2010) [159] also demonstrated the beneficial effects of MSCs stimulated exogenously with BMP2.

In another gene therapy study by Sheyn *et al.* (2008) [160], MSCs overexpressing BMP6 were injected into the lumbar paravertebral muscle in immune-deficient mice. An induction of spinal fusion was observed here [160]. Friedman *et al.* (2006) [161] examined the same by testing the effect of several BMPs on hMSCs. The results clearly showed that BMP6 had the most potent osteoinductive effects. In the case of BMP4, the *in vivo* mouse study of Hannallah *et al.* (2004) [162] demonstrated that NOG inhibits heterotopic ossification caused by BMP4. However, BMP stimulation had adverse effects. A study by Diefenderfer *et al*. (2003) [163] showed that stimulation with BMP2, BMP4, or BMP7 on hMSCs after six days does not lead to an upregulation of ALP activity but to an upregulation of the NOG mRNA level. These results may suggest that MSCs use more than one system for transcriptional activation. Several *in vitro* studies have investigated if silencing of GREM1 or NOG could induce a stronger ossification using hMSCs [164-167], see Table **3**.

9. BMPs AND BMP ANTAGONISTS AND THE IVD

LBP, which redundancy is currently one of the most prevalent health problems worldwide, is often associated with damaged or degenerated discs [2]. Because of the avascular nature of IVD, its regeneration capacity is only limited. So far, no therapies are available for IVD regeneration. Painful discs, which cannot be treated anymore with painkillers or physical therapies, are removed, followed by the placement of a structural spacer, internal fixation, and fusion of the degenerated segment through natural bone growth, which is often supported by the application of osteoinductive growth factors. However, different treatment strategies to regenerate a damaged IVD are under investigation, with one approach being the application of growth factors. The most widely used therapy during spinal fusion, particularly in clinics, is the application of BMP2. BMP2 has been examined not only in multiple *in vivo* studies on spinal fusion but also in several *ex vivo* and *in vivo* IVD models, and promising results for IVD regeneration have been obtained. In recent studies, a new aspect that might influence the field in the future was investigated. Whether IVD can undergo ossification because of the cytokines of the BMP family and whether this might be an alternative treatment for spinal fusion patients have been discussed.

For the most widely used therapies - those that involve BMP2 and BMP7 - anabolic effects have been found in different studies (Table **4**). An upregulation of aggrecan (*ACAN*) and *COL2* in IVD cells because of BMP2 or BMP7 administration could be confirmed in different species, such as canine, rabbit, rat, bovine, and human [167-171]. However, the effect on *COL1* and *BGLAP* expression remains inconclusive. No change in *COL1* expression was observed in the studies of Li *et al*. (2004) [169] after the stimulation of rat AFC *in vitro or* after BMP2/BMP7 stimulation of bovine nucleus pulposus cells (NPCs) in a 3D fibrin-hyaluronan culture (2017) [170], whereas Kim *et al.* (2003) [171] showed an upregulation of *COL1* in human IVD cells in 3D alginate beads stimulated with BMP2. Besides *COL1*, Kim *et al.* (2003) [171] also showed an upregulation of *ACAN* and *COL2* expression. However, their study found no change in *BGLAP*, whereas Brown *et al.* (2018) [6] was able to observe an upregulation of *BGLAP* in a human NPC monolayer stimulated with BMP2. Furthermore, Lee *et al*. (2012) [172] could not detect an osteogenic effect on rabbit NPCs after stimulation with exogenous BMP2, as they did not observe any *BGLAP* expression. In the study of Kim *et al.* (2003) [171], cells cultured in a monolayer showed no osteogenic marker gene expression, ALP activity, or calcium deposition at the given dose of BMP2 [171].

However, studies concerning the osteogenic potential of IVD contradict themselves. While some authors were able to detect ossification in the outer AF [6, 168], other investigations did not show any evidence of osteogenic effects in the tissue [170, 173]. Interestingly, in a study by Willems *et al.* [167], an extensive ventral extradiscal bone formation after the injection of rhBMP7 in the NP (T13-S1) of canine discs could be observed. Furthermore, in some diseases, an ossification of the outer AF could be detected, as in diffuse idiopathic skeletal hyperostosis or ankylosing spondylitis [174, 175].

Moreover, the studies of Karamouzian *et al.* (2010) [176] and Brown *et al*. (2018*)* [6] showed that herniated discs possess a higher ability to calcify compared with normal or degenerated discs. One possible reason for this inequality in calcification ability among IVDs may be the difference in expression of BMP antagonists, such as NOG, GREM1, or CHRD in IVDs, which has already been shown in several studies [6, 35]. This endogenous expression of BMP antagonists might also be a possible reason for spinal fusion failure [34, 35, 177].

Table 4. Application of BMP and BMP antagonists in intervertebral disc regeneration or ossification.

Author, Year	Study Type, Species	Cytokine	Study	Conclusion
Brown, 2018 [6]	*In vitro*, *in vivo*, human	BMP2, BMP7	Stimulation of NPCs from hIVDs in monolayer with osteogenic medium containing 1.25-dihydroxyvitamin D3 (VitD3), parathyroid hormone (PTH) and BMP2/BMP7 Explant cultures of IVDs in osteogenic medium ± prior exposure to VitD3 and BMP2	Upregulation of *RUNX2*, *BGLAP*, and *SPP1* No osteogenic differentiation after application of BMP. One culture explant showed regions of calcification after stimulation with VitD3 and BMP2, another one after the stimulation with osteogenic medium
Imai, 2007 [178]	*In vitro,* human	BMP7	NPCs and AFCs from four cadaveric discs and one surgical specimen were cultured in 3D alginate beads for 21 days and stimulated with 0, 100 or 200 ng/mL BMP7 and 10% fetal bovine serum	Significant upregulation of proteoglycan synthesis in BMP7 treated NPC and AFC beads compared to control beads Cell proliferation was stimulated because of BMP7
Wei, 2008 [173]	*In vitro,* human	BMP7	hNPC were stimulated with rhBMP7 and inducers of apoptosis (tumour necrosis factor-alpha (TNF-α) or hydrogen peroxide (H_2O_2))	BMP7 had a positive effect on extracellular matrix production, which was reduced because of TNF-α and H_2O_2 stimulation BMP7 showed antiapoptotic effects, but no evidence of bone formation induction
Kim, 2003 [171]	*In vitro,* human	BMP2	hIVD cells were cultured in 3D alginate beads and stimulated with different concentrations of BMP2 (0-2000 ng/mL) for 21 days.	*ACAN, COL1* and *COL2* expression, as well as proteoglycan synthesis, were upregulated in stimulated cells, *BGLAP* showed no expression upon stimulation
Li, 2017 [170]	*In vitro,* bovine	BMP2/7	Regenerative effect of BMP2/7 heterodimer was investigated in bovine *in vitro* and in organ culture NPCs were cultured in a fibrin-hyaluronan hydrogel for 14 days. For in organ culture, a BMP2/7 heterodimer was delivered into the nucleotomized region	*COL2* and *ACAN* expression and glycosaminoglycan (GAG) content were upregulated in NPC Remaining NP tissue showed an increase in proteoglycan synthesis after stimulation with BMP2/7, *COL1*expression and ALP activity were not affected No observation of fibroblastic or osteogenic effects in the disc tissue
Willems, 2015 [167]	*In vitro*, *in vivo*, canine	BMP7	Stimulation of canine NPC *in vitro* with 100 ng/mL rhBMP7 to assess anabolic effects Injection of different dosages of rhBMP7 (2.5 µg, 25 µg, 250 µg) into early degenerated IVDs of canines (evaluation after six months or *post-mortem*)	Gene expression of *ACAN* and *COL2A1* was upregulated after the stimulation of canine NPC *in vitro*. *In vivo*, no regenerative effects, but extensive extradiscal bone formation after the intradiscal injection with 25 µg and 250 µg of rhBMP7
Haschtmann, 2012 [168]	*In vitro*, rabbit	BMP2, TGF-β3	Stimulation of rabbit IVD explants with 1 µg/mL BMP2 or TGF-β3 for 21 days (NP and AF were analysed separately)	Upregulation of *COL1, COL2*, and *ACAN* because of stimulation with BMP2 and TGF-β3 in AF, but matrix metallopeptidase genes were inhibited In NP, BMP2 stimulation leads to decreased *COL2* expression Induction of ossification in AF tissue (shown by histology)
Lee, 2012 [172]	*In vitro,* rabbit	BMP2, TGF-β1	Rabbit NPCs were cultured in antelocollagen type 1 and 2 scaffolds and stimulated with exogenous BMP2 (100 ng/mL) and TGF-β1 (10 ng/mL).	Significant increase in proteoglycan production in cells in antelocollagen 2 scaffold and TGF-β1 stimulation or co-treatment Groups of both scaffolds with BMP2 or/and TGF-β1 had increased *COL1*, *COL2*, and *ACAN* expression, but no expression of *BGLAP* Treatment groups exhibited a significantly increased cellular proliferation, but no additive or synergistic effects could be detected for the two cytokines, neither in proliferation nor matrix synthesis.

Table (4) contd….

Author, Year	Study Type, Species	Cytokine	Study	Conclusion
Leckie, 2012 [179]	*In vitro,* rabbit	BMP2	Rabbit discs (L2-L3, L3-L4, and L4-L5) were punctured and then treated with adeno-associated virus serotype 2 carrying BMP2 gene (analysis after 0, 6 and 12 weeks, rabbits were sacrificed after 12 weeks)	Delay of degenerative changes of the disc confirmed by MRI, histology, serum biochemical, and biomechanical criteria analysis
Huang, 2007 [180]	*In vitro,* rabbit	BMP2	Injection of 1 mg/ rhBMP2 ± coral grafts (L2-L3, L3-L4, and L4-L5) in rabbits after receiving annular tears	More degeneration in groups treated with BMP2 than control groups (treated with a physiological saline solution onl). BMP2 induced hypervascularity and fibroblast proliferation after an annular tear
Masuda, 2006 [181]	*In vivo,* rabbit	BMP7	Injection of BMP7 into the NP of rabbits, which received an annular puncture with an 18-gauge needle four weeks prior (after 2, 4, 8, 12 and 24 weeks, rabbits were sacrificed)	Single injection of BMP7 into the NP of the punctured rabbit discs lead to a restoring effect of the IVDs Disc height was sustained for up to 24 weeks.
An, 2005 [182]	*In vivo,* rabbit	BMP7	Injection in consecutive rabbit discs, 2 µg BMP7 in the NP by using a 28-gauge needle (radiographically analysis after 2, 4 and 8 weeks) in control group, discs were treated with physiological saline solution	Mean disc height was greater and proteoglycan content higher in groups treated with BMP7 compared to control group Higher DNA content in AF in BMP7 treated groups, no differences in NP
Masuda, 2003 [183]	*In vitro,* rabbit	BMP7	Rabbit NPCs and AFCs were cultured in 3D alginate beads and stimulated with 0, 50, 100 or 200 ng/mL BMP7	Proteoglycans and collagens were upregulated in a dose-related manner (increase in *COL2* and *ACAN* mRNA level) DNA content was increased after BMP7 stimulation compared to control groups.
Li, 2004 [169]	*In vitro,* rat	BMP2	Rat AFCs and cells from the transition zone were stimulated with 200 ng/mL BMP2	Increased production and expression of COL2 and ACAN after three days of stimulation with BMP2 but COL1 stayed unaffected Significant increase of BMP7 expression by BMP2 stimulation
Yoon, 2003 [184]	*In vitro,* rat	BMP2	Rat AFC and cells of the transition zones of lumbar IVDs were treated with different concentrations of rhBMP2 (0, 10, 100, 1000 ng/mL).	Upregulation in GAG content in dose depending manner after seven days Upregulation of *COL2, ACAN, SOX9*, and *BGLAP* in cells treated with a higher dosage of rhBMP2 (100 and 1000 ng/mL) but no change in *COL1* expression Overall rhBMP2 treatment increased disc cell proliferation.

Lately, Chan *et al.* (2015) [34] found evidence of the inhibitory effects of IVD cells if they are co-cultured with bone-marrow-derived MSCs undergoing ossification. These effects could also be found in direct contact culture of fresh human disc tissue samples on MSC monolayers, as detected histologically with alizarin red staining (Fig. **2**). For this, either NP tissue (NPT), annulus fibrosus (AFT), or cartilaginous endplate tissue (CEPT) were placed on an hMSC monolayer and stimulated for 21 days with an osteogenic medium. After 21 days, a lower deposition of the mineralized matrix was detected in areas surrounding the NPT, AFT, or CEPT compared with the remaining MSC monolayer. In another experiment, these results could also be statistically confirmed by a higher donor number. Furthermore, some of the co-cultures were additionally supplemented with L51P [177]. In these supplemented co-cultures, osteogenic differentiation was significantly less affected by the presence of IVD cells. These *in vitro* results are promising, because the action of L51P seemed to unblock the inhibitory effects of the secretome of IVD cells.

However, when the experiment is repeated but adult OBs isolated from human femur instead of bone-marrow-derived MSCs are the focus, the inhibitory effects could not be observed to the same extent [35].

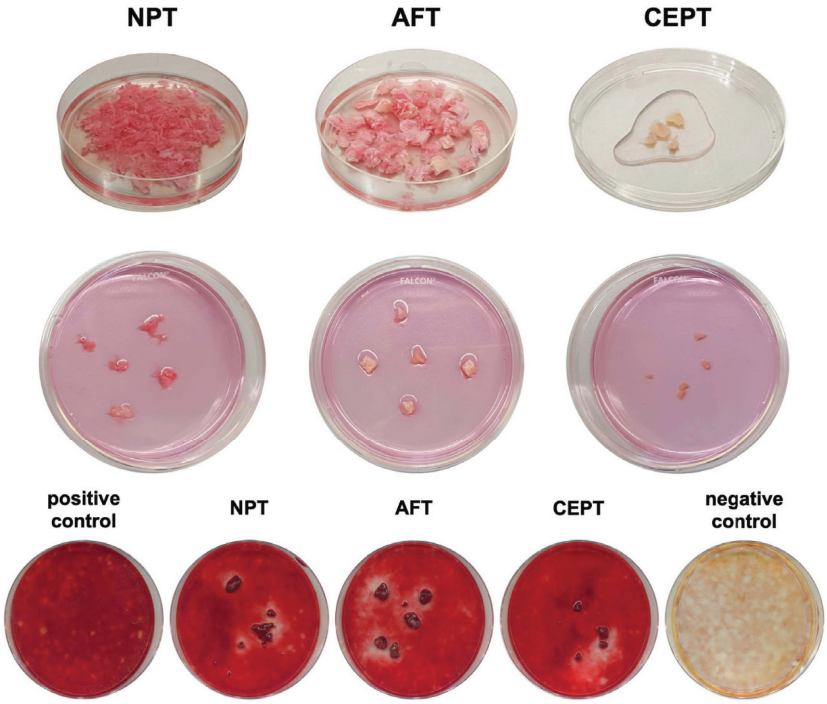

Fig. (2). Human mesenchymal stromal cells (hMSCs) were seeded in 100 mm Petri dishes and cultured until they reached 90% confluency. Human intervertebral disc (IVD) explants (tissue of the nucleus pulposus (NPT), annulus fibrosus (AFT), or cartilaginous endplate (CEPT), 2 - 5 mm^3) were cultured in direct contact with the hMSCs in an osteogenic medium (lacking bone morphogenetic protein 2). Top row: Preparation of the tissue. Middle row: Contribution of human NPT, AFT, and CEPT on top of the hMSC monolayer. Bottom row: Alizarin red staining of direct culture after stimulation for 21 days with an osteogenic medium (except negative control) and co-cultured with NPT, AFT, and CEPT. Proof-of-concept of inhibitory effects (N = 1). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

CONCLUSION

The aim of this review is to provide an overview on the specific roles of various BMP and BMP antagonists in orthopedic surgeries. We discussed studies from the last two decades, in which BMP and BMP antagonists were investigated in spinal fusion and bone healing models and in MSC or IVD cells.

In clinics, BMPs are leading to robust bone formation but frequently also to adverse effects. In pre-clinical testing, however, Zara *et al.* (2011) [185] described the side effects of a high dose of BMP2 (up to 45 µg in the rat femoral segmental defect model) by observing tissue inflammatory infiltrates and cyst-like bone formation after application. Furthermore, there are other problems in the application of growth factors, such as their short-term bioavailability when they are directly applied, the need for a complex biological carrier, or the control of growth factor release over time; these issues need to be addressed [133]. The BMP variant L51P could be a possible solution to decrease the high amounts of exogenous BMP2 that have been used [29]. Some *in vitro* studies have already investigated the effects of L51P. However, *in vivo* studies are still needed.

In general, a higher fusion potential was concluded in studies investigating any of the above-mentioned BMPs as summarised in Table **1**. BMP antagonists, such as GREM1 and GREM2, showed inhibitory effects on bone formation, whereas the effect of NOG remains inconlusive. The same was true regarding the expression pattern during bone formation; the expression, particularly of BMP antagonists, remains uncertain. Tang *et al.* (2011) [143] detected an increased NOG but a decreased CHRD expression during the reparative phase after posterolateral spine fusion in rabbit. It has been proven that BMP expressions differ between non-unions and regular healing fractures.

In multiple mouse and rat studies, it has been shown that BMPs promote OB differentiation [134], whereas the effect of BMPs in hMSCs has not been conclusive [163, 186, 187]. hMSCs seem to show differences in the regulation of BMPs. Friedmann *et al.* (2006) [161] did not find a significant increase in ALP activity in hMSCs with the application of BMP2 under serum-free conditions. However, they demonstrated a beneficial synergistic effect when different BMPs were applied together (BMP6 and BMP7). One possible explanation for the lack of hMSC response to BMP2 and BMP4 could be the high affinity of BMP antagonists for BMP2 and BMP4, whereas BMP6 and BMP7 have a much lower affinity [161]. Fan *et al.* (2006) [166] discovered that adipose-derived stem cells with an NOG knock-down and stimulated with BMP2 showed enhanced osteogenic differentiation compared with control cells, which indicated that NOG suppression can enhance the activity of exogenous BMP. Co-delivery of siNOG and BMP2 DNA in human ADSC reduced NOG knockdown. Overexpression of BMP2 might stimulate the expression of NOG through a negative feedback mechanism (Fig. **3**) [165].

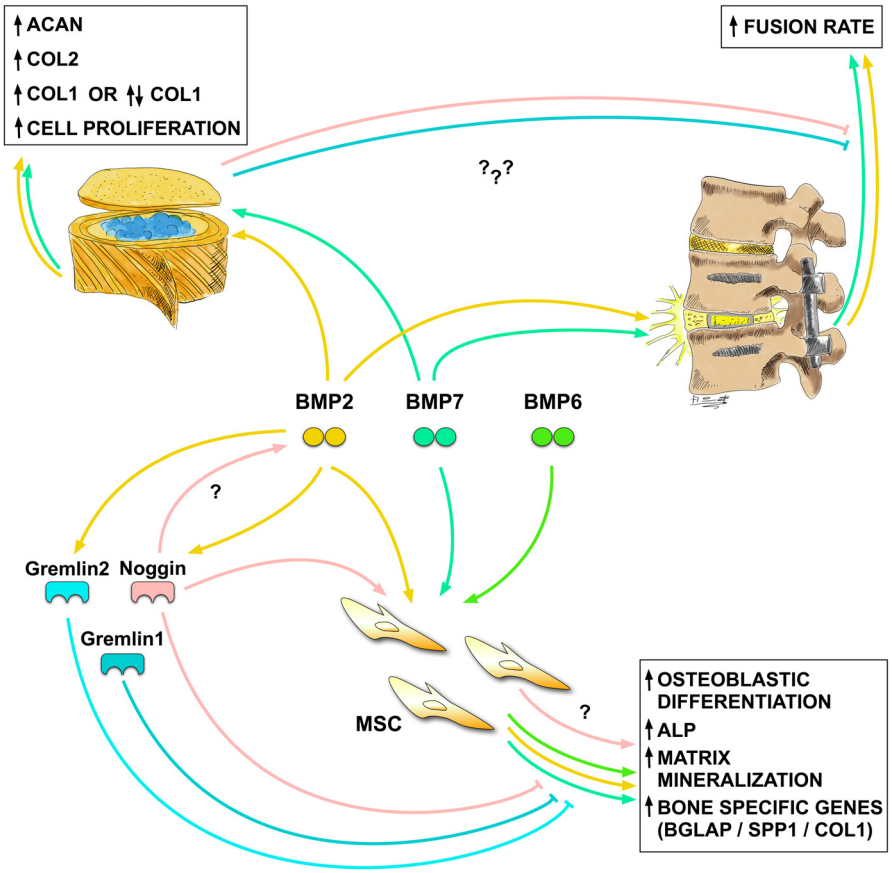

Fig. (3). Summary of the different effects of the main BMPs and BMP antagonists discussed in this review. This overview provides the main findings and how BMPs and BMP antagonists affect the bone in spinal fusion studies, the intervertebral disc (IVD), and mesenchymal stromal cells (MSCs). Effects that have already been found but thus far not confirmed are labeled with a question mark. BMP2 and BMP7 have a beneficial effect on spinal fusion, whereas BMP antagonists (probably also expressed in IVD) have been shown to inhibit bone formation after spinal fusion. In IVD, BMP2 and BMP7 induce upregulation of aggrecan (*ACAN*) and collagen type 2 (*COL2*) and in some studies, as well, collagen type 1 (*COL1*); however, *COL1* also seems to be unaffected in some cases. Regarding the effect on MSCs, it could be shown in several studies that BMPs, as well as noggin (NOG) in some cases, increase osteoblastic differentiation by up-regulating alkaline phosphatase (*ALP*) activity, matrix mineralization, and bone-specific genes as osteopontin (*SPP1*), osteocalcin (*BGLAP*), and *COL1*. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

With regard to how BMP antagonists influence MSCs, there is no consensus in the literature. Dean *et al*. (2010) [147] explained that BMP antagonists related to bone healing can be divided into two functional groups: one that is downregulated and is essential for the initiation of osteogenesis and another that is upregulated and thus functions in the remodeling of newly formed bones [147].

Current studies also disagree on the interaction of BMPs in IVD cells. Overall, the conclusion is that whether BMP has a chondrogenic or an osteogenic effect on IVD cells remains unclear. In most of the studies in which IVD cells were stimulated with BMPs, an upregulation of *ACAN* and *COL2* was observed, whereas the exact effect on *COL1* seems to be inconclusive. In several studies, however, even an ossification of the AF could be detected.

The clinical application of cytokines seems to be a good option to improve fusion rates, but the correct dose, the ideal conditions, and the exact mechanism involved remain unclear. Despite the many studies investigating the effect of BMPs and their antagonists, additional research is necessary to shed light on their effects on degenerated IVDs.

LIST OF ABBREVIATIONS

ABG	=	Autologous Bone Graft
ActRI	=	Activin Receptor Type-1
ADSC	=	Human Adipose-Derived Stem Cell
AF	=	Annulus Fibrosus
AFC	=	AF Cell
AFT	=	AF Tissue
ACAN	=	Aggrecan
ALK3	=	Activin Receptor-Like Kinase 3
ALK6	=	Activin Receptor-Like Kinase 6
ALP	=	Alkaline Phosphatase
BAMBI	=	BMP and Activin Membrane-Bound Inhibitor
bFGF	=	Basic Fibroblast Growth Factor
BGLAP	=	Osteocalcin

bMSC	=	Bone Mesenchymal Stromal Cell
BMP	=	Bone Morphogenetic Protein
BMP2	=	Bone Morphogenetic Protein 2
BMPRI	=	BMP Receptor I
BMPRII	=	BMP Receptor II
BSP	=	Bone Sialoprotein
CBD-BMP2	=	Collagen Binding BMP2
CEPT	=	Cartilaginous end Plate Tissue
CHRD	=	Chordin
CMV	=	Cytomegalovirus
COL1	=	Collagen Type 1
COL2	=	Collagen Type 2
CPC	=	Calcium Phosphate Cement
DAN	=	Differential Screening-Selected Gene Aberrative in Neuroblastoma
DMBM	=	Demineralized Bone Matrix
DLX5	=	Distal-Less Homeobox Protein 5
DRM	=	Downregulated by V-Mos
FLST	=	Follistatin
GAG	=	Glycosaminoglycan
GDF	=	Growth and Differentiation Factor
GNAS	=	Guanine Nucleotide Binding Protein Alpha Stimulating Activity Polypeptide
GREM1	=	Gremlin 1
GREM2	=	Gremlin 2
H_2O_2	=	Hydrogen Peroxide
hMSC	=	Human MSC
IGF1	=	Insulin-Like Growth Factor 1
IVD	=	Intervertebral Disc
kDA	=	Kilodalton
LBP	=	Low Back Pain
MDSC	=	Muscle-Derived Stromal Cell
MSC	=	Mesenchymal Stromal Cell
MSX2	=	Msh Homeobox 2
NOG	=	Noggin
NP	=	Nucleus Pulposus
NPC	=	NP Cell
NPT	=	NP Tissue
PDGF	=	Platelet-Derived Growth Factor
PRDC	=	Protein related to DAN and Cerberus
pSmad	=	Phosphorylated Smad
PTH	=	Parathyroid Hormone
rhBMP2	=	Recombinant Human BMP2
RUNX2	=	Runt-Related Transcription Factor 2
shRNA	=	Small Hairpin RNA
siRNA	=	Small Interfiering RNA
Smad	=	Single Mothers Against Decapentaplegic Homolog
SOST	=	Sclerostin
SOX9	=	Sex-Determining Region Y-box 9
SP7	=	Osterix
SPP1	=	Osteopontin
TBC	=	True Bone Ceramics
TGF-β	=	Transforming Growth Factor Beta
TGF-β1	=	Transforming Growth Factor Beta 1
TGF-β3	=	Transforming Growth Factor Beta 3
TNF-α	=	Tumor Necrosis Factor-Alpha
TSRH	=	Texas Scottish Rite Hospital
TWSG	=	Twisted Gastrulation
VEGF	=	Vascular Endothelial Growth Factor
VitD3	=	1.25-dihydroxyvitamin D3

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

SELECTION CRITERIA FOR INCLUDED STUDIES

For this review, we performed a search on PubMed using the keywords “spinal fusion” or “osteoblast,” “intervertebral disc,” “mesenchymal stem cell,” AND “BMP” or “BMP antagonist.”

We included studies published in 1998 to 2019 that were considered relevant to the subject. The search exceptions are important historical publications on BMPs.

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