Brief communication

# Inhibition of endogenous antagonists with an engineered BMP-2 variant increases BMP-2 efficacy in rat femoral defect healing

Hans-Jörg Sebald [a], Frank M. Klenke [a,*], Mark Siegrist [b], Christoph E. Albers [b], Walter Sebald [c], Wilhelm Hofstetter [b]

[a] Department of Orthopedic Surgery, Inselspital, Bern University Hospital, CH-3010 Bern, Switzerland
[b] Department of Clinical Research, Group for Bone Biology and Orthopedic Research, University of Bern, CH-3010 Bern, Switzerland
[c] Department of Physiological Chemistry II, Biocenter, University of Würzburg, D-97074 Würzburg, Germany

## ARTICLE INFO

## ABSTRACT

Bone morphogenetic proteins (BMP) have been used successfully by orthopedic clinicians to augment bone healing. However, these osteoinductive proteins must be applied at high concentrations to induce bone formation. The limited therapeutic efficacy may be due to the local expression of BMP antagonists such as Noggin that neutralize exogenous and endogenous BMPs. If so, inhibiting BMP antagonists may provide an attractive option to augment BMP induced bone formation. The engineered BMP-2 variant L51P is deficient in BMP receptor type I binding, but maintains its affinity for BMP receptor type II and BMP antagonists including Noggin, Chordin and Gremlin. This modification makes L51P a BMP receptor-inactive inhibitor of BMP antagonists. We implanted β-tricalcium phosphate ceramics loaded with BMP-2 and/or L51P into a critical size defect model in the rat femur to investigate whether the inhibition of BMP antagonist with L51P enhances the therapeutic efficacy of exogenous BMP-2. Our study reveals that L51P reduces the demand of exogenous BMP-2 to induce bone healing markedly, without promoting bone formation directly when applied alone.

## 1. Introduction

Recombinant bone morphogenetic proteins such as BMP-2 and BMP-7 have been approved for clinical use to promote bone healing in tibia fractures, long bone non-unions and spinal fusions [1,2]. The growth factors, however, must be administered at concentrations that exceed those of naturally occurring BMPs present in 1000 kg of bone tissue [3]. This may be due to the *in vivo* regulation of the bioavailability of endogenous BMP by antagonistic proteins such as Noggin and members of the Dan and Chordin families [4,5]. Furthermore, BMPs themselves modulate the expression of various BMP regulatory proteins, including Noggin, Gremlin and Crossveinless-2 [6–8].

Expression of BMP antagonists increases during fracture healing and distraction osteogenesis [9–11]. Therapeutic requirements for administering high BMP concentrations to induce bone formation *in vivo* may result from significant neutralization of the growth factor by BMP inhibitory proteins. Thus, for clinical applications, the inhibition of BMP antagonists, rather than the direct application of BMPs, may present a preferred future strategy. L51P is an *in vitro* engineered BMP-2 variant with a leucine-to-proline substitution at codon 51. The modified protein is deficient in BMP type I receptor activation, but maintains its affinity for BMP type II receptors and inhibitory proteins such as Noggin, Gremlin and Chordin [12]. At equimolar concentrations, L51P blocks the inhibitory action of Noggin and restores BMP-2 dependent induction of alkaline phosphatase *in vitro* [12]. However, when added alone, L51P does not induce the differentiation of ATDC5 and C2C12-cells. Therefore, L51P represents a tool to enhance BMP-2-mediated bone formation *in vivo* through inhibition of endogenous BMP antagonists. Here, we determined whether the amount of BMP-2 required to induce bone formation could be reduced in the presence of L51P in a critical-size segmental defect in rat femur.

## 2. Methods

Ceramic β-tricalcium phosphate (β-TCP) cylinders (5 mm in diameter, 6 mm in length, 75% porosity) were provided by the Robert Mathys Foundation (RMS), Bettlach, Switzerland. Wistar rats (female, retired breeders, 350–450 g), purchased from Charles River (Sulzfeld, Germany), were housed in the Central Animal Facility of the Medical Faculty, University of Bern, Switzerland, in compliance with the Swiss Federal Government guidelines for care and use of experimental animals. The Bernese State Committee for the Control of Animal Experimentation approved our study

* Corresponding author. Tel.: +41 31 632 2222; fax: +41 31 632 3600.
E-mail address: frank.klenke@dkf.unibe.ch (F.M. Klenke).

(approval number 001/08 to WH). The BMP-2 and L51P proteins were expressed in *Escherichia coli*, refolded and purified as described previously [12–14].

For the measurement of the *in vitro* release of L51P and BMP-2 from the ceramic, 1 or 10 μg of BMP-2 or L51P was dissolved in 25 μl of deionized water and adsorbed to β-TCP carriers. After 24 h of drying, the loaded ceramics were incubated in 1.5 ml culture medium (alpha-minimum essential medium, 10% fetal bovine serum, 1% penicillin/streptomycin) for 19 days. The medium was changed after 24 h, and 4, 7, 10, 13 and 16 days. The amounts of BMP-2 and L51P released into the culture medium were quantified using an ELISA kit for the detection of BMP-2 (Human BMP-2 ELISA development kit, Peprotech, Rocky Hill, USA). This kit is suitable for the detection of L51P as well, as the structures of L51P and BMP-2 are identical except for the site of mutation.

For the *in vivo* experiments, BMP-2 and L51P were dissolved in 25 μl of deionized water and adsorbed to β-TCP carriers (Table 1). The loaded carriers were air-dried overnight and implanted into 6 mm critical-size segmental diaphyseal bone defects in rat femurs (seven groups, $n = 6$ per group). The defects were stabilized with the RatFix® (AO Foundation, Davos, Switzerland) osteosynthesis system. Migration of the implants, failure of the osteosyntheses and bone formation were monitored by X-ray imaging immediately after surgery and after 1, 4, 8 and 12 weeks. Implants were harvested together with the surrounding bone from animals sacrificed after 12 weeks. Bone formation was determined by quantitative histomorphometry on six serial 300 μm McNeal tetrachrome-stained ground sections (cross-sections) per implant. Data were analyzed statistically by one-way analysis of variance and the Sidak post-hoc test using SPSS® software for Mac (Version 16, SPSS Inc., Chicago, IL, USA).

## 3. Results

Evaluation of the *in vitro* protein release from β-TCP cylinders showed no difference in release kinetics between BMP-2 and L51P coatings. Furthermore, there was no difference in the relative protein release whether 1 or 10 μg of BMP-2/L51P was adsorbed to the ceramics. Adsorption of either 1 or 10 μg of both proteins resulted in a burst release of approximately 60% of the total protein amount within the first 24 h (Fig. 1).

Twelve weeks after implantation, no bone formation was observed when unloaded β-TCP cylinders (without proteins) or carriers adsorbed with 1 μg BMP-2 were implanted into the 6 mm rat femur defects (Figs. 2 and 3A). Ten micrograms of BMP-2 induced a significant increase in bone formation as compared to the unloaded carriers (bone volume/total volume (BV/TV): 10 μg BMP-2: 34.0 ± 1.9% vs. unloaded: 3.1 ± 1.4%, $p < 0.001$, Figs. 2 and 3B), resulting in the preservation of the original volume of the ceramic implant as seen in high resolution radiographs (Fig. 4B). Ten micrograms of L51P did not stimulate bone formation (Figs. 2 and 3C). When added to 1 μg BMP-2, L51P promoted bone formation in a dose-dependent manner. With 3 μg of L51P added to 1 μg BMP-2, a significant induction of bone formation over those carriers loaded with 1 μg BMP-2 alone was found (BV/TV: 3 μg L51P/1 μg BMP-2: 16.3 ± 6.1% vs. 1 μg BMP-2: 3.4 ± 0.7%, $p < 0.001$). Carriers loaded with 10 μg L51P and 1 μg BMP-2 induced bone formation
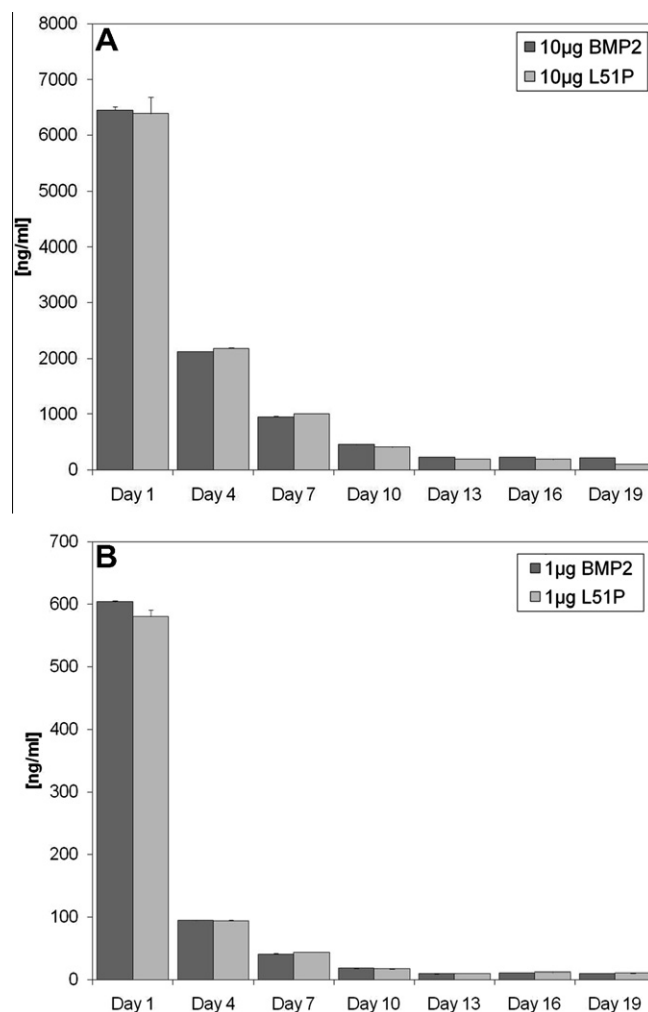


**Fig. 1.** Passive release kinetics of L51P and BMP-2 from β-TCP ceramics. BMP-2 or L51P (1 or 10 μg) was dissolved in 25 μl of deionized water and adsorbed to β-TCP cylinders. The ceramics were incubated in culture medium, and the BMP-2 and L51P release was measured after 24 h and after 4, 7, 10, 13, 16 and 19 days. (A) 10 μg L51P and BMP-2, (B) 1 μg L51P and BMP-2. Mean values are represented ± standard deviation ($n = 6$ for each group).

equivalently to carriers loaded with 10 μg BMP-2 (BV/TV: 10 μg L51P/1 μg BMP-2: 32.9 ± 6.7% vs. 10 μg BMP-2: 34.0 ± 1.9%). Bone formation was associated with degradation of the implanted carrier materials. Approximately 60% of the ceramic material degraded after 12 weeks in carriers loaded with 10 μg BMP-2. In carriers loaded with combinations of 1 μg BMP-2 and L51P, increasing the concentration of L51P induced β-TCP-degradation in a dose-dependent manner. Addition of 10 μg L51P to 1 μg BMP-2 resulted in similar material turnover as compared to materials loaded with 10 μg BMP-2 (material volume/total volume: 3 μg L51P/1 μg BMP-2 27.7 ± 6.9, 10 μg L51P/1 μg BMP-2: 17.6 ± 3.0% vs. 10 μg BMP-2: 13.5 ± 2.0%). Histologic transversal sections showed that the formation of new bone and the degradation of the carriers progressed from the center to the periphery of

**Table 1**
BMP-2 and L51P loading of β-TCP carriers.

| Experimental groups | | | | | | |
|---|---|---|---|---|---|---|
| I | II | III | IV | V | VI | VII |
| 1 μg BMP-2 | 10 μg BMP-2 | 10 μg L51P | 1 μg BMP-2<br>1 μg L51P | 1 μg BMP-2<br>3 μg L51P | 1 μg BMP-2<br>10 μg L51P | Unloaded |
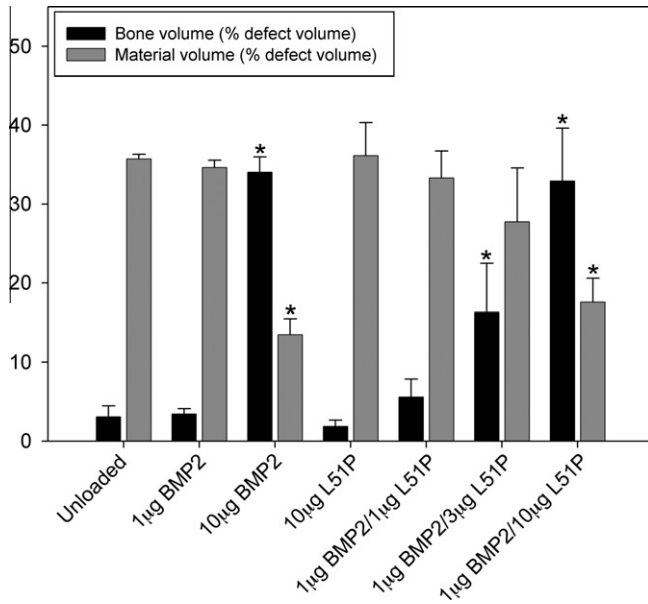
**Fig. 2.** Bone formation and material degradation were quantified by histomorphometry after 12 weeks. The values are stated as the volume percentages of newly formed bone/total defect volume and β-TCP volume/total defect volume. Mean ± standard deviation. *$p < 0.05$ vs. empty β-TCP ceramics and ceramics loaded with 1 μg BMP-2 or 10 μg L51P.

the materials. There was no bone formation found in the peripheral areas of the implants (Fig. 3E, F). After 12 weeks, high-resolution X-rays showed that the volume of the β-TCP ceramic implant upon adsorption with 1 μg BMP-2 and 10 μg L51P was preserved and that an interface between the host bone and the β-TCP ceramics

was formed (Fig. 4F), similar to carriers loaded with 10 μg BMP-2 (Fig. 4B). Despite significant induction of bone formation, the defect volume was not maintained in carriers loaded with 1 μg BMP-2 and 3 μg L51P, and there was no formation of an interface between the implant and the host bone observed (Fig. 4E).
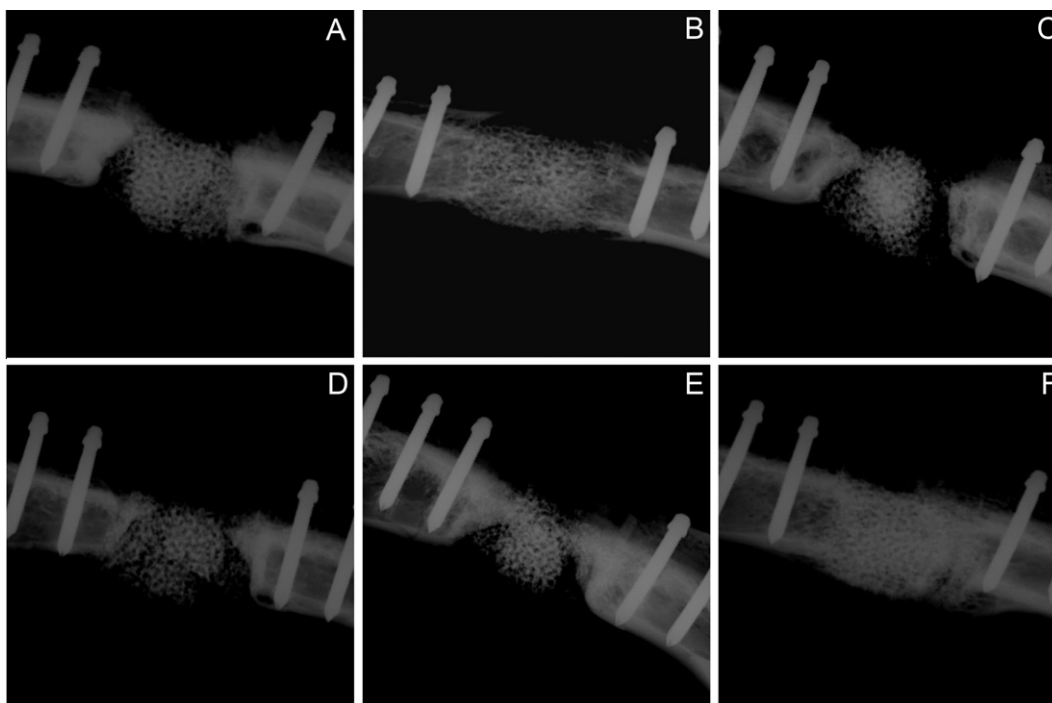
## 4. Discussion

Blocking the biological activities of BMP antagonists such as Noggin represents a promising strategy to stimulate bone formation [15]. Noggin levels in unstimulated osteoblasts are low but are strongly up-regulated by BMP-2 in vitro [6]. Expression of Noggin, Chordin, Gremlin and Dan was shown to be elevated during fracture healing in mice [9] and distraction osteogenesis in rats [10]. Noggin activity or expression has been successfully inhibited using cognate antibodies [8] and siRNA [16,17]. Furthermore, growth factor variants with reduced binding affinity to Noggin have been developed to enhance the osteogenic activity of BMPs [18,19]. Several groups were able to show that blocking Noggin [17] and Chordin [20] enhanced osteogenic differentiation by human mesenchymal stem cells and induced bone formation in vivo [17]. Our study did not aim at the identification of specific BMP antagonists limiting the activity of BMP-2. However, our results strongly suggest that endogenous BMP inhibitors limit exogenous BMP-2 activity in vivo, because L51P reconstituted the osteoinductive activity of otherwise ineffective BMP-2 concentrations.

L51P binds Noggin, Chordin, Crossveinless-2 and Gremlin, and possesses the same binding site for BMP-2 inhibitors as BMP-2 [12], suggesting that L51P binds and inhibits all known BMP-2 antagonists. In the present study, L51P promoted bone formation in critical size femur defects in rats by enhancing the activity of exogenously added BMP-2. L51P lacked osteoinductive activity when added alone, suggesting that the expression of endogenous
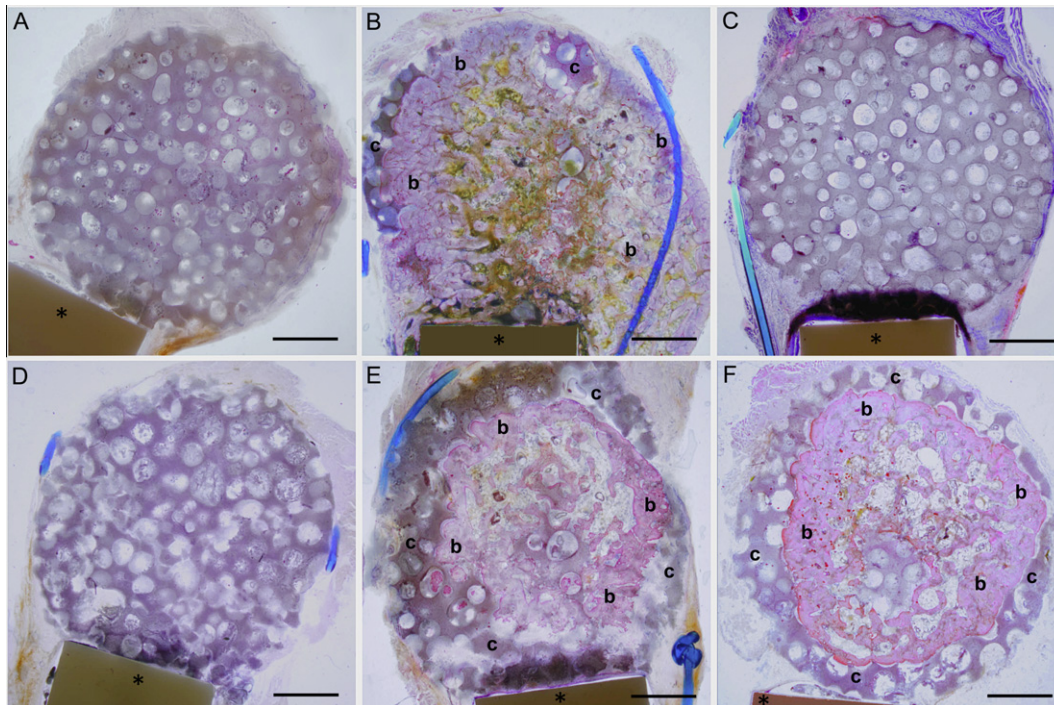


**Fig. 3.** McNeal tetrachrome stained histologies of representative tissue sections. Twelve weeks after implantation of β-TCP carriers with (A) 1 μg BMP-2, (B) 10 μg BMP-2, (C) 10 μg L51P, (D) 1 μg BMP-2 + 1 μg L51P, (E) 1 μg BMP-2 + 3 μg L51P, and (F) 1 μg BMP-2 + 10 μg L51P, the ceramics were embedded in methyl methacrylate (MMA). The images show the center cross-sections of the serial implant cross-sectioning. No bone formation or material degradation was identified when ceramics were loaded with 1 μg BMP-2, 10 μg L51P or 1 μg BMP-2 + 1 μg L51P (A, C, D). In ceramic loaded with 10 μg BMP-2, 1 μg BMP-2 + 3 μg L51P or 1 μg BMP-2 + 10 μg L51P large parts of the carrier material (c) were substituted by bone (b) (B, E, F). *Cross-sectioned poly(ether-ether-ketone) osteosynthesis plate. The brown tissue in panel B is blood (hemosiderin). Scale bars = 1 mm.

**Fig. 4.** Representative X-rays taken 12 weeks after implantation of β-TCP carriers with (A) 1 μg BMP-2, (B) 10 μg BMP-2, (C) 10 μg L51P, (D) 1 μg BMP-2 + 1 μg L51P, (E) 1 μg BMP-2 + 3 μg L51P and (F) 1 μg BMP-2 + 10 μg L51P.

BMPs were not up-regulated beyond a critical threshold level necessary during the healing process of the applied critical-size defect model to induce the healing cascade.

Relatively large amounts (3–10 μg) of L51P were necessary to rescue the osteogenic response to 1 μg BMP-2. The necessity to add high amounts of L51P is most likely due to high local concentrations of BMP-2 inhibitors. Exogenous BMP-2 results in a strong induction of Noggin and Chordin mRNA expression in osteoblasts [6,7]. We therefore suggest that the concentrations of BMP antagonists at the healing site surmount the concentration of BMP-2 by a molar ratio of greater than 3 to 10. Furthermore, the access of L51P to the local environment of the target cells may be limited and the availability of L51P restricted due to rapid degradation of the protein following release from the carrier.

## 5. Conclusion

This study shows that the efficacy of exogenous BMP-2 during *in vivo* bone defect healing – which by itself does not exert a biological effect when applied at 1 μg per implant – is improved by delivering an inhibitor of endogenous BMP antagonists. Furthermore, this study provides evidence that endogenous BMP antagonists play a major role in regulating BMP actions during bone repair. It is preferable to introduce an inhibitor of BMP antagonists into a bone defect site instead of active BMP-2, since exogenous BMP-2 is not controlled by cellular mechanisms and may induce side effects such as heterotopic ossification if the protein cannot be contained at the repair side. In contrast, improving the bioavailability of BMP-2 and increasing its efficacy by blocking BMP antagonists with L51P allows BMP-2 levels and activities to be regulated by the cellular environment. The protein delivery-based approach with β-tricalcium phosphate ceramics reported here will allow for the use of smaller doses of exogenous BMP-2 to augment bone regeneration and may lead to an easy-to-handle "off-the-shelf" clinical product not requiring intraoperative implant preparation. Further long-term studies with biomechanical testing are warranted to investigate whether the combination of small BMP-2

doses and L51P does induce complete substitution of β-TCP ceramics by authentic bone and whether this results in mechanically competent defect bridging.

## References

[1] Axelrad TW, Einhorn TA. Bone morphogenetic proteins in orthopaedic surgery. Cytokine Growth Factor Rev 2009;20:481–8.
[2] Jones AL, Bucholz RW, Bosse MJ, Mirza SK, Lyon TR, Webb LX, et al. Recombinant human BMP-2 and allograft compared with autogenous bone graft for reconstruction of diaphyseal tibial fractures with cortical defects. A randomized, controlled trial. J Bone Joint Surg Am 2006;88:1431–41.
[3] Urist MR, Mikulski A, Lietze A. Solubilized and insolubilized bone morphogenetic protein. Proc Natl Acad Sci USA 1979;76:1828–32.
[4] Canalis E, Economides AN, Gazzerro E. Bone morphogenetic proteins, their antagonists, and the skeleton. Endocr Rev 2003;24:218–35.
[5] Walsh DW, Godson C, Brazil DP, Martin F. Extracellular BMP-antagonist regulation in development and disease: tied up in knots. Trends Cell Biol 2010;20:244–56.
[6] Gazzerro E, Gangji V, Canalis E. Bone morphogenetic proteins induce the expression of noggin, which limits their activity in cultured rat osteoblasts. J Clin Invest 1998;102:2106–14.
[7] Pereira RC, Economides AN, Canalis E. Bone morphogenetic proteins induce gremlin, a protein that limits their activity in osteoblasts. Endocrinology 2000;141:4558–63.
[8] Tsialogiannis E, Polyzois I, Oak Tang Q, Pavlou G, Tsiridis E, Heliotis M. Targeting bone morphogenetic protein antagonists: in vitro and in vivo evidence of their role in bone metabolism. Expert Opin Ther Targets 2009;13:123–37.
[9] Dean DB, Watson JT, Jin W, Peters C, Enders JT, Chen A, et al. Distinct functionalities of bone morphogenetic protein antagonists during fracture healing in mice. J Anat 2010;216:625–30.
[10] Haque T, Hamade F, Alam N, Kotsiopriftis M, Lauzier D, St-Arnaud R, et al. Characterizing the BMP pathway in a wild type mouse model of distraction osteogenesis. Bone 2008;42:1144–53.
[11] Kloen P, Doty SB, Gordon E, Rubel IF, Goumans MJ, Helfet DL. Expression and activation of the BMP-signaling components in human fracture nonunions. J Bone Joint Surg Am 2002;84-A:1909–18.

[12] Keller S, Nickel J, Zhang JL, Sebald W, Mueller TD. Molecular recognition of BMP-2 and BMP receptor IA. Nat Struct Mol Biol 2004;11:481–8.

[13] Kirsch T, Nickel J, Sebald W. Isolation of recombinant BMP receptor IA ectodomain and its 2:1 complex with BMP-2. FEBS Lett 2000;468:215–9.

[14] Ruppert R, Hoffmann E, Sebald W. Human bone morphogenetic protein 2 contains a heparin-binding site which modifies its biological activity. Eur J Biochem 1996;237:295–302.

[15] Heliotis M, Tsiridis E. Suppression of bone morphogenetic protein inhibitors promotes osteogenic differentiation: therapeutic implications. Arthritis Res Ther 2008;10:115.

[16] Takayama K, Suzuki A, Manaka T, Taguchi S, Hashimoto Y, Imai Y, et al. RNA interference for noggin enhances the biological activity of bone morphogenetic proteins in vivo and in vitro. J Bone Miner Metab 2009;27:402–11.

[17] Wan DC, Pomerantz JH, Brunet LJ, Kim JB, Chou YF, Wu BM, et al. Noggin suppression enhances in vitro osteogenesis and accelerates in vivo bone formation. J Biol Chem 2007;282:26450–9.

[18] Seemann P, Brehm A, Konig J, Reissner C, Stricker S, Kuss P, et al. Mutations in GDF5 reveal a key residue mediating BMP inhibition by NOGGIN. PLoS Genet 2009;5:e1000747.

[19] Song K, Krause C, Shi S, Patterson M, Suto R, Grgurevic L, et al. Identification of a key residue mediating bone morphogenetic protein (BMP)-6 resistance to noggin inhibition allows for engineered BMPs with superior agonist activity. J Biol Chem 2010;285:12169–80.

[20] Kwong FN, Richardson SM, Evans CH. Chordin knockdown enhances the osteogenic differentiation of human mesenchymal stem cells. Arthritis Res Ther 2008;10:R65.