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Bone morphogenetic protein (BMP)1-3 enhances bone repair

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Running head: Circulating BMP1-3 regulates bone and kidney function

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Members of the astacin family of metalloproteinases such as human bone morphogenetic protein 1 (BMP-1) regulate morphogenesis by processing precursors to mature functional extracellular matrix (ECM) proteins and several growth factors including TGFβ, BMP2, BMP4 and GFD8. We have recently discovered that BMP1-3 isoform of the *Bmp-1* gene circulates in the human plasma and is significantly increased in patients with acute bone fracture. We hypothesized that circulating BMP1-3 might have an important role in bone repair and serve as a novel bone biomarker. When administered systemically to rats with a long bone fracture and locally to rabbits with a critical size defect of the ulna, recombinant human BMP1-3 enhanced bone healing. In contrast, neutralization of the endogenous BMP1-3 by a specific polyclonal antibody delayed the bone union. *In vitro* BMP1-3 increased the expression of collagen type I

and osteocalcin in MC3T3-E_1 osteoblast like cells, and enhanced the formation of mineralized bone nodules from bone marrow mesenchymal stem cells. We suggest that BMP1-3 is a novel systemic regulator of bone repair.

In purified plasma samples from healthy volunteers using liquid chromatography–mass spectrometry (LC-MS), we identified BMP1-3 isoform of the *Bmp1* gene [1]. BMP1 (procollagen C proteinase-1; BMP1) and mammalian tolloid (mTLD/BMP1-3) are alternatively spliced products of the *Bmp1* gene. BMP1 was originally isolated from bone with other BMPs due to its affinity for heparin; however, it is not an authentic member of the BMP protein family and belongs to the astacin/BMP1/tolloid (TLD)-like family of zinc metalloproteinases which are fundamental in the development and formation of the extracellular matrix (ECM) [2-5]. The BMP1-like proteases share a common domain structure that includes an amino-terminal prodomain that is cleaved to yield the mature protease. Mature BMP1 consists of the metalloprotease domain followed by EGF-like motifs and three complement domains (CUB), thought to be involved in protein-protein interaction. Mature BMP1-3 has two more CUB domains (CUB IV and CUB V).The C-terminal domains have been shown to control and restrict the BMP1 substrate specificity, so difference between BMP1 and BMP1-3 in number of CUB domains maybe important for differences in the activity and specificity of these molecules particularly connected with organ and tissue targeting. BMP1-1 isoform is involved in the biosynthetic processing of a range of ECM precursors, including major and minor fibrillar collagens, the collagen and elastin cross-linking enzyme prolysyl oxidase [6], cellular anchoring proteins prolaminin-5 [7], and procollagen VII, and the small leucine-rich proteoglycans osteoglycin and probiglycan [8]. BMP1-1 also releases several TGFβ superfamily members, including BMP2 and BMP4, growth and differentiation factors (GDF) 8/11, and TGFβ1 from their corresponding latent complexes [5, 9-11]. These dual roles have fuelled speculation that BMP1/TLD proteinases orchestrate the ECM assembly by means of signaling by TGFβ-like proteins.

The structural components of bone consist of extracellular matrix, collagen and cells. The noncollagenous protein and enzymes present in the bone matrix might influence its matrix and regulate bone formation. Circulating BMP1-3 seems to be well suited as a biological control point for the regulation of collagen deposition

into the callus formation and bone regeneration. In this paper we therefore explored whether circulating BMP1-3 influences osteoblast function and bone fracture repair.

MATERIALS AND METHODS

Antibodies- Polyclonal antibodies against mature BMP1-3 and BMP1-1 were generated in rabbits immunized with specific synthetic peptides: BMP1-3 (aa759-772; CTSPNWPDKYPSKKE) and BMP1-1 (aa705-717. Peptide specific antibodies were affinity purified (Multiple Peptide Systems), and their cross-reactivity and neutralizing effect were tested (data not shown).

Cloning and expression of BMP1 isoforms- Human BMP1-3 cDNA was synthesized from the human adult normal placenta total RNA using the SuperScript^{TM}III First-Strand Synthesis System (Invitrogen). To generate the pcDNA^{TM}3.2/V5-DEST expression clone, the sequence confirmed by pENTR221/BMP1-3 was recombined with the pcDNA^{TM}3.2/V5-DEST vector in an LR recombination reaction (Invitrogen Gareway® Technology). The mammalian expression plasmid pSecTag2 *Bmp1-3* was transfected into FreeStyle™ CHO cells using the FreeStyle™ MAX transfection reagent. The protein was affinity-purified via a BMP1-3 specific antibody column.

Gene expression analysis- Total RNA was isolated from MC3T3 cells using TRIzol (Invitrogen). cDNA was synthesized and amplified from 1 μg of total RNA using Super Script III First-Strand Synthesis System (Invitrogen). Gene expression of interest was measured by using a LightCycler FastStart DNA Master SYBR Green kit in a LightCycler instrument (Roche Diagnostics), as described [12]. *Gapdh* was used as normalized gene. Results are represented as a fold change of the comparative expression level. The list of primers used is shown in Table 1.

Human tissue, immunohistochemistry and morphometric analysis- Human embryos and fetuses ranging from 5-14 weeks of gestation [10-52 mm crown-rump length (CRL)] used in the present study were collected at the University of Zagreb Medical School as previously described [13-15] and controlled by the Internal Review Board of the Ethics Committee at the University of Zagreb Medical School [16]. Immunocytochemistry was

performed using the immunoperoxidase detection system (Zymed) and photographs were taken by Olympus Provis microscope.

Rat femoral fracture model- An osteotomy in the proximal third of the femur was made in male Sprague-Dawley rats (250-300 g) using a circular saw. The fragments were repositioned under general anesthesia and fixed by an intramedullary Kirschner pin. The rats were randomly assigned to one of the groups (n=8): A/ control, only osteotomy; B/ control with non-immune rat IgG; C/ osteotomy receiving rhBMP1-3 iv, 3x/week (3 μg); D/ osteotomy receiving BMP1-3 antibody iv, 1x/week (50 μg). The effect of the treatment was monitored by radiographs every two weeks up to the 6th week when the animals were killed.

Critical size defect of rabbit ulna- An ulnar critical size defect model was used to evaluate the efficacy of BMP1-3 in bone healing of adult male New Zealand White rabbits (3 to 4 kg weight), as previously described [17]. Shortly, blood was collected from rabbit marginal ear veins into tubes without any anticoagulant substance. BMP1-3 (20 μg) with or without BMP7 (100 μg) was added into the blood supplemented with 10 mM $CaCl_2$ .The coagulum whole blood containing device (WBCD) was modified to provide a rigid and flexible carrier and used for the treatment [18,19]. Animals were divided into four groups (n=8): A/ control, defect filled with WBCD only; B/ defect filled with WBCD and rhBMP1-3 (20 μg); C/ defect filled with WBCD and rhBMP7 (100 μg); D/ defect filled with WBCD and BMP1-3 (20 μg) plus BMP7 (100 μg). The protocol was approved by the Institutional Ethics and Animal Committees.

MicroCT- The microcomputerized tomography machine and the analyzing software were obtained from SkyScan [20, 21]. Rabbit ulna analysis started just above the site of the critical size defect and continued in the dorsoventral direction embracing the entire ulnar defect and the healthy radius. However, only new trabecular bone in the ulnar defect was depicted and analyzed in three dimensions. Rat femur analysis started by determining the connection of proximal growth plates as a reference point from which 100 slices distally were set as offset and 200 slices were analyzed for trabecular bone and new bone formation [21].

Bone nodule formation assay- Bone marrow cells were harvested from femurs and tibias of four 8-week-old WT mice, pooled and plated at 7 x 10^6/well on 6 well plates in αMEM, containing 10% fetal calf serum (FCS). Half the volume of the medium was changed on day 3. Differentiation medium containing αMEM, 10% FCS, 8

mM β-glycerophosphate, 50 μg/ml ascorbic acid and 10^{-8} M dexamethasone was added on day 7. The medium was subsequently changed every two days until the culture was terminated. BMP1-3 (150 ng/ml) and BMP1-3 antibody (1 μg/ml) were added to the medium at every feeding. At day 21 of cell culture, the von Kossa stain was used to determine the mineralized matrix formation. Quantification of the mineralized area was performed using SForm software and expressed as percentage of the mineralized area.

MC3T3-E1 cells - The mouse osteoblast-like MC3T3-E1 cell line, derived from newborn mouse calvaria as described [22]. Cultured cells were divided into two groups: control and cells treated every second day with BMP1-3 (50 ng/ml). After treating the cells 3 times, total RNA was extracted with TRIzol (Gibco BRL, Grand Island, NY, USA).

Id-1 reporter assay for measuring BMP activity- C2C12-BRE-Luc cell line stably transfected with a reporter plasmid consisting of BMP response element (BRE) from the *Id-1* promoter fused to a luciferase reporter gene was provided by courtesy of Gareth J. Inman (The Beatson Institute for Cancer Research, Glasgow, UK). Cells were plated at $2{,}5x10^4$ cells / well in 48-well plates. After 24 hours the medium was changed to serum-free medium for 7 hours [23]. Cells were then treated for 17 hours in DMEM/F-12 with BMP7 [24], washed with chilled PBS (Invitrogen) and lysed in reporter lysis buffer (Promega). Luciferase activity was measured using the Promega luciferase assay reagent on Victor Wallack luminometer. BMP1-3, BMP7 and a control peptide with mixed amino acids from BMP heparin binding site sequence (piChem) were used in the assay.

Statistics- All data are presented as mean ± SD. One way analysis of variance (ANOVA) was performed to determine the effect of BMP1-3 and BMP1-3 antibody treatment efficacy on the bone and kidney. Statistical evaluation of the survival rate was carried out by the Petö-Wilcoxon test. The results were considered significant when P was < 0.05.

RESULTS

Circulating BMP1-3 supports fracture repair in rats- To examine whether the circulating BMP1-3 is physiologically relevant for bone modeling in rats we chose a model of fracture repair. Administration of a

neutralizing polyclonal BMP1-3 antibody, intravenously (iv) to rats with a fractured femur resulted in significantly delayed bone union (Fig. 1D). In contrast, systemic administration of the BMP1-3 protein for a period of 6 weeks (3 µg/3x week) increased the bone volume and accelerated the fracture repair (Fig.1C). Ex vivo microCT analyses of femurs showed that the bone volume (BV) of rats treated with rhBMP1-3 (3 µg, 3x/week) was 20% increased and was 55% higher as compared to rats treated with the BMP1-3 antibody (50 µg, 1x/week) (Fig.1).

Expression of BMP1-3 in human bone development- To assess the potential colocalization of BMP1-3 with other bone morphogenetic proteins we investigated its expression during the human development. Up to 8 weeks of gestation both BMP1-3 and BMP1-1 were localized in the epithelial ectoderm of the growing limb (Fig.2 A-C). Following the hypertrophy of chondrocytes, prior to vascular invasion, they were expressed in the periosteal bone collar, in the surrounding ECM and in the cells of osteoblastic lineage (Fig. 2D-H). Fetuses at 10-14 weeks of gestation showed BMP1-3 restricted to the epithelial ectoderm (Fig.2B), where it co-localized with BMP7 (data not shown). BMP1-3 isoforms were not localized in the areas of intramembranous bone formation (Fig. 2B).

Locally administered BMP1-3 accelerates and enhances bone regeneration- Based on the localization of BMP1-3 in the human development where it co-localizes with BMP7, we next tested their individual and combined local efficacy in rabbits with a critical size bone defect of the ulna. BMP1-3 (Fig. 3B) individually stimulated bone formation. The combination of BMP1-3 with BMP7 had an additive effect on bone repair, which at 6 weeks resulted in the rebridgement of the cortical bone and advanced remodeling of the trabecular bone (Fig. 3D). In contrast, 6 weeks following surgery, individual therapy with BMP1-3 or BMP7 (Fig. 3B, C) did not result in the rebridgement of the ulnar defect with newly formed and mineralized bone. MicroCT analysis of the critical size defect area showed a 28% increased BV/TV in rabbits treated with the combination of BMP1-3 and BMP7 (Fig.3D). Histomorphometry of Goldner stained histology sections confirmed the µCT results, showing a high percentage of newly formed bone (38%) with a well established medullary canal in the same group (Fig. 3E). However, at 6 weeks following therapy, bone defects treated with BMP1-3 alone stimulated in particular the

trabecular bone formation, without a clearly formed cortical bridge and without remodeling of the trabecular bone (Fig. 3B, histology panel). Similarly, BMP7 treated bone defects were filled with newly formed trabecular bone without signs of advanced remodeling and cortical rebridgement (Fig. 3C, histology panel).

BMP1-3 affects osteoblast differentiation in vitro- The exogenous BMP1-3 enhanced the differentiation of bone marrow-derived MSC towards osteoblasts as evidenced by increased mineralized bone nodule formation as compared to control cultures after staining with van Kossa (Fig. 4 B, D). Treatment of MSC with the BMP1-3 antibody had an opposite effect on mineralized bone nodule formation (Fig. 4 C, D). Gene expression analysis of MC3T3E1 pre-osteoblasts treated with BMP1-3 protein indicated an increased expression of *procollagen*, *BMP1-3* and *osteocalcin*, and decreased expression of Bmp6 and *Bmp7* (Fig.4 E), suggesting that the enhanced maturation of MSC into osteoblasts was not mediated via BMPs, while increased *BMP1-3* expression level might have supported the accelerated fracture healing in rats and rabbits via processing of ECM (Fig.1, 3). BMP1-3 antibody added to MC3T3E1 cell cultures increased the expression of *Bmp6* and *Bmp7 (date not shown)*

BMP1-3 enhances activity of BMP7 in vitro- When added to C2C12 cells stably transfected with the BMP response element (BRE) from the *Id-1* gene promoter fused to a luciferase reporter gene, BMP1-3 had no effect on luciferase induction. However, in combination with BMP7 it significantly enhanced the BMP7 activity (Fig.4 F). We suggest that BMP1-3 acts as a coactivator of BMP7, but the exact mechanism has to be established.

DISCUSSION

The role of BMP-1 in promoting bone regeneration has not been previously evidenced. In the original discovery by Wozney at al. [25] it was suggested that recombinant human BMP-1 implanted with a bovine collagen in a rat subcutaneous assay induced new bone formation. However, this was an artifact of the rat growth plate chondrocytes isolated together with the demineralized bone matrix preparation [26]. Since the bone fracture healing process is thought to recapitulate skeletal development, and since BMP1-3 is highly expressed in the limb development we hypothesized that BMP1-3 plays an important role in fracture healing. Utilizing a rat femur fracture model and the osteoblast differentiation culture, the present studies demonstrates that the inhibition of BMP1-3 leads to a significant delay in the fracture healing process, and that BMP1-3 plays a role in the osteoblast

differentiation and bone formation. Following the localization of BMP1 in human development we assumed that BMP1-3 in circulation might systemically influence the acceleration of ECM deposition.

For the proper bone formation functional relationships between proteinases and growth factor and bone cells must be achieved, because they are all necessary for normal ossification and bone ECM remodeling [27]. For cells to migrate through matrix during the healing process, the matrix degradation surrounding the migrating cells is required. The initial step of angiogenesis includes the migration of endothelial cells and also involves proteolytic degradation of ECM.

BMP1-3 enhanced the bone formation when implanted locally in combination with BMP7. The combination potentially associates two independent, but synergistic functions essential to the healing process, stimulation of cells proliferation and differentiation by BMP7, and facilitating cell migration by clearing the extracellular matrix components by BMP1-3. This combination may be used for supporting bone healing and the regeneration process [28]. In addition, BMP1-3 enhanced the *Id* gene expression activity of BMP7 in C_2C_{12} myoblasts *in vitro*, which might *in vivo* contribute to successful bone repair. BMP1-3 eventually contributes to the bone regeneration via stimulating the synthesis and processing of ECM proteins and via increased formation of osteoblasts from MSC**.** BMP1-3 also increased bone nodule formation and osteoblast differentiation. It is well known that proteinases (MMPs) safeguard osteoblasts from apoptosis and maintain osteocyte viability. During bone development, ECM remodeling is required for osteoblast differentiation and the process is largely mediated by the proteolitic activity of MMPs which play a fundamental role in osteoblast migration. Expression of a collagen type I in the murine calvarial osteoblast cell line MC3T3-E1 after treatment with BMP1-3 may result in deposition of a type I collagen matrix which is specific for a period of rapid cell division. Exposure to BMP1-3 increases the collagen type I and osteocalcin expression. Osteoblast, nuclear matrix, DNA-binding proteins, with a capacity for bending DNA, recognize elements on the osteocalcin and type I collagen promoters. This establishes a physical link between the regulation regions of osteoblast ECM genes and cell structure [29] The stimulation of osteocalcin gene expression in the presence of a rapid matrix turnover suggests a more complex mechanism for its regulation. Osteocalcin may be regulated by factors released from osteoblast matrix undergoing high rates of

turnover. Collagen type I may act directly on cellular receptors or it might be merely required as a latticework for other matrix proteins which themselves interact directly with cells. The precise mechanism of BMP1-3 action must be therefore further explored.

Systemic administration of the specific BMP1-3 antibody delayed the bone healing confirming that the amount of circulating BMP1-3 is relevant for bone repair. This was accompanied with a more than 50% decreased circulating level of TGF-β1 [1] , which might have contributed as well to the delayed bone formation due to its known stimulatory effect on osteoblast proliferation. Also delayed fracture healing or nonunion formation may be associated with alternations in the MMP system. Measurement of BMP1-3 in plasma in patients with fracture might be a precious performance indicator for the bone healing process.

In conclusion, this study provides *in vivo* and *in vitro* evidence for the critical role of BMP1-3 in bone fracture repair.

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REFERENCES:

[1] L. Grgurevic, B. Macek, D. R. Healy, A. L. Brault, I. Erjavec, A. Cipcic , I. Grgurevic, D. Rogic, K. Galesic, J. Brkljacic, R. Stern-Padovan, V. M. Paralkar, S. Vukicevic , Circulating bone morphogenetic protein 1-3 isoform increases renal fibrosis J. Am. Soc. Nephrol. 22 (2011) 681-692.

[2] J.M. Wozney, V. Rosen, A.J. Celeste, L.M. Mitsock, M.J. Whitters, R.W. Kriz, R.M. Hewick, E.A. Wang, Novel regulators of bone formation: molecular clones and activities, Science. 242(1988) 1528-1534.

[3] G. Pfleiderer, R. Zwilling, H.H. Sonneborn, On the evolution of endopeptidases, 3. A protease of molecular weight 11,000 and a trypsin-like fraction from Astacus fluviatilis fabr. Hoppe Seylers Z Physiol Chem. 348 (1967)1319-1331.

[4] G. Ge, D.S. Greenspan, Developmental roles of the BMP1/TLD metalloproteinases, Birth Defects Res C Embryo Today. 78(2006) 47-68.

[5] D.R. Hopkins, S. Keles, D.S. Greenspan, The bone morphogenetic protein 1/Tolloid-like metalloproteinases, Matrix Biol. 26(2007) 508-523.

[6] M.V. Panchenko, W.G. Stetler-Stevenson, O.V. Trubetskoy, S.N. Gacheru, H.M. Kagan, Metalloproteinase activity secreted by fibrogenic cells in the processing of prolysyl oxidase. Potential role of procollagen C-proteinase, J Biol Chem. 271(1996) 7113-7119.

[7] S. Amano, I.C. Scott, K. Takahara, M. Koch, M.F. Champliaud, D.R. Gerecke, D.R. Keene, D.L. Hudson, T. Nishiyama, S. Lee, D.S. Greenspan, R.E. Burgeson, Bone morphogenetic protein 1 is an extracellular processing enzyme of the laminin 5 gamma 2 chain, J Biol Chem. 275(2000) 22728-22735.

[8] I.C. Scott, Y. Imamura, W.N. Pappano, J.M. Troedel, A.D. Recklies, P.J. Roughley, D.S. Greenspan, Bone morphogenetic protein-1 processes probiglycan, J Biol Chem. 275(2000)30504-30511.

[9] L. Garrigue-Antar, C. Barker, K.E. Kadler, Identification of amino acid residues in bone morphogenetic protein-1 important for procollagen C-proteinase activity, J Biol Chem. 276 (2001)26237-26242.

[10] R. Jasuja, G. Ge, N.G. Voss, J. Lyman-Gingerich, A.M. Branam, F.J. Pelegri, D.S. Greenspan, Bone morphogenetic protein 1 prodomain specifically binds and regulates signaling by bone morphogenetic proteins 2 and 4, J Biol Chem. 282(2007) 9053-9062.

[11] N. Suzuki, P.A. Labosky, Y. Furuta, L. Hargett, R. Dunn, A.B. Fogo, K. Takahara, D.M. Peters, D.S. Greenspan, B.L. Hogan, Failure of ventral body wall closure in mouse embryos lacking a procollagen C-proteinase encoded by Bmp1, a mammalian gene related to Drosophila tolloid, Development. 122 (1996) 3587-3595.

[12] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, *Nucleic Acid. Research* 29 (2001) 45.

[13] S. Vukicevic, J. B. Kopp, F. P. Luyten, T. K. Sampath, Induction of nephrogenic mesenchyme by osteogenic protein 1 (bone morphogenetic protein 7), Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 9021-9026.

[14] S. C. Chang, B. Hoang, J.T. Thomas, S. Vukicevic, F.P. Luyten., N. J. Ryba, C.A. Kozak, A. H. Reddi, M. Jr. Moos, Cartilage-derived morphogenetic proteins. New members of the transforming growth factor-beta superfamily predominantly expressed in long bones during human embryonic development. J. Biol. Chem. 269 (1994), 28227-28234.

[15] M. N. Helder, E. Ozkaynak, K. T. Sampath, F. P. Luyten, V. Latin, H. Oppermann, S. Vukicevic, Developing human lung and kidney are major sites for synthesis of bone morphogenetic protein-3 (osteogenin). J. Histochem. Cytochem. 43 (1995), 1035-1044.

[16] S. Vukicevic, V. Latin, P. Chen, R. Batorsky, A. H. Reddi, T. K. Sampath, Localization of osteogenic protein-1 (bone morphogenetic protein-7) during human embryonic development: high affinity binding to basement membranes, Biochem. Biophys. Res. Commun. 198 (1994), 693-700.

[17] S. Vukicevic, L. Grgurevic, B. Macek. BMP-1 procollagen C-proteinase for diagnosis and treatment of bone and soft tissue defects and disorders. USPTO No. 7850964

[18] S. D. Cook, G.C. Baffes, M. W. Wolfe, T. K.Sampath, D. C. Rueger, Recombinant human bone morphogenetic protein-7 induces healing in a canine long-bone segmental defect model, Clin. Orthop. Relat. Res. 301 (1994), 302-312.

[19] V. M. Paralkar, F. Borovecki, H. Z. Ke, K. O. Cameron, B. Lefker, W. A. Grasser, T. A. Owen, M. Li, P. DaSilva-Jardine, M. Zhou, R. L. Dunn, F. Dumont, R. Korsmeyer, P. Krasney, T. A. Brown, D. Plowchalk, S.

Vukicevic, D. D. Thompson, An EP2 receptor-selective prostaglandin E2 agonist induces bone healing, Proc. Natl. Acad. Sci. U.S.A. 100 (2003), 6736-6740.

[20] P. Ruegsegger, B. Koller, R. Mullar, A microtomographic system for the nondestructive evaluation of bone architecture, Calcif. Tissue Int. 58 (1996), 24-29.

[21] T. Hildebrand, A. Laib, R. Muller, J. Dequeker, P. Ruegsegger, Direct three-dimensional morphometric analysis of human cancellous bone: microstructural data from spine, femur, iliac crest, and calcaneus, J. Bone Miner. Res. 14 (1999), 1167-1174.

[22] S. Vukicevic, F. P. Luyten, A. H. Reddi. Stimulation of the expression of osteogenic and chondrogenic phenotypes in vitro by osteogenin. Proc. Natl. Acad. Sci. U. S. A. 86 (1989), 8793-8797.

[23] G.J. Inman, , B. Herrera, A rapid and sensitive bioassay for the simultaneous measurement of multiple bone morphogenetic proteins. Identification and quantification of BMP4, BMP6 and BMP9 in bovine and human serum, BMC Cell Biology. 10 (2009), 20

[24] P. Simic, J.B. Culej, I. Orlic, L. Grgurevic, N. Draca, R. Spaventi, S. Vukicevic, Systemically administered bone morphogenetic protein-6 restores bone in aged ovariectomized rats by increasing bone formation and suppressing bone resorption, J. Biol. Chem. 281 (2006), 25509-25521

[25] J. M. Wozney, V. Rosen, A. J. Celeste, L. M. Mitsock, M. J. Whitters, R. W. Kriz, R. M. Hewick, E. A. Wang, Novel regulators of bone formation: molecular clones and activities, Science. 242 (1988), 1528-1534.

[26] A. H. Reddi, BMP-1: resurrection as procollagen C-proteinase, Science. 271 (1996), 463.

[27] N. Ortega, K. Wang,N. Ferrara, Z. Werb, T.H. Vu, Complementary interplay between matrix metalloproteinase-9, vascular endothelial growth factor and osteoclast function drives endochondral bone formation. Disease Models & Mechanisms. 3 (2010), 224-235.

[28] D.D. Kakagia, K.J. Kazakos, K.C. Xarchas, M. Karanikas, G.S. Georgiadis, G. Tripsiannis, C. Manolas. Synergistic action of protease-modulating matrix and autologous growth factors in healing of diabetic foot ulcers. A prospective randomized trial. Journal of Diabetes and Its Complications. 21 (2007) 387-391.

[29] J. P. Bidwell, M. Alvarez, H. Feister, J.Onyia, J. Hock, Nuclear Matrix Proteins and Osteoblast Gene Expression, J. Bone Miner. Res.13 (1998) 155-167.

FOOTNOTES

Abbreviations used: BMP – bone morphogenetic protein, CHO - Chinese hamster ovary, DMP - dentin matrix protein, ECM – extracellular matrix, EGF - epidermal growth factor, GDF - growth differentiation factor, LC-MS - liquid chromatography-mass spectrometry, mTLD - mammalian Tolloid, mTld - mammalian tolloid gene, mTLL – mammalian Tolloid-like, MW - molecular weight, SDS - sodium dodecyl sulfate, TGF – transforming growth factor, TLD – drosophila tolloid**.**

FIGURE LEGENDS

Figure 1. MicroCT imaging of rat femurs *ex vivo*. The rats were randomly assigned to one of the groups as described in Matherials and Methods: A/ control; B/ control with non-immune rat IgG; C/ BMP1-3 iv therapy; D/ BMP1-3 antibody iv therapy. Bone volume (BV); [a] $P<0.05$ vs control animals; [b] $P<0.05$ vs control animals treated with non-immune IgG rat antibody;[c] $P<0.05$ vs BMP1-3 (ANOVA; Dunnett test).

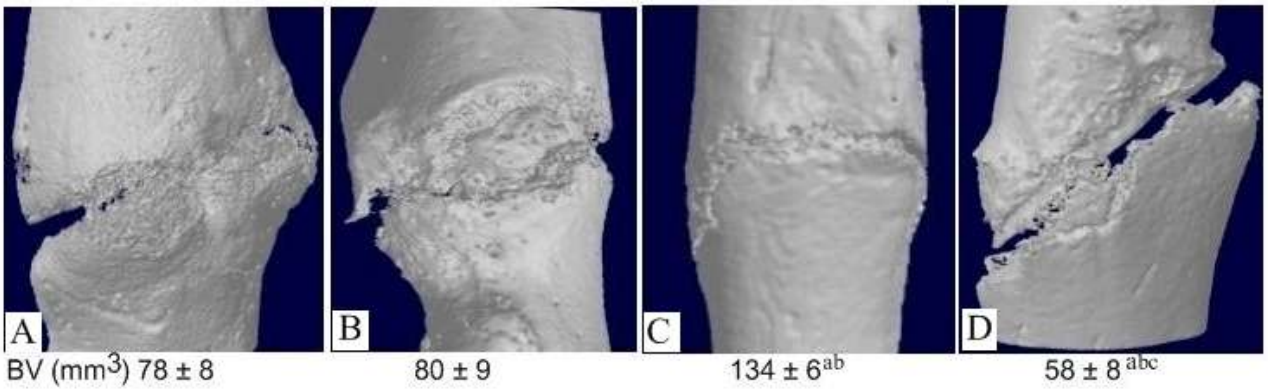


Figure 2. Localization of BMP-1 isoforms in human development.

At 10 weeks of gestation (B, C) BMP-1-3 was expressed in the ectodermal epithelium (e), in cells of the progression zone (pz), in the basement membrane (bm), and in the intermittent layer of the future skin (il) (B arrows, C asterisk); (A) control antibody.(A) and (B) magnification x10; (C) x40. BMP1-3 was absent in the mesenchyme (m) and the area of intramembranous ossification above the distal phalanx. During endochondral ossification only BMP-1-1 was present in periosteum (arrow; E), osteocytes (stars; E,F) and osteocytic canalicular system (arrow; F),pre-osteoblasts and osteoblasts (arrows; H) of the periosteal collar. (D) and (G) control antibody. (D) and (E) magnification x20; (F, G, H) x40.

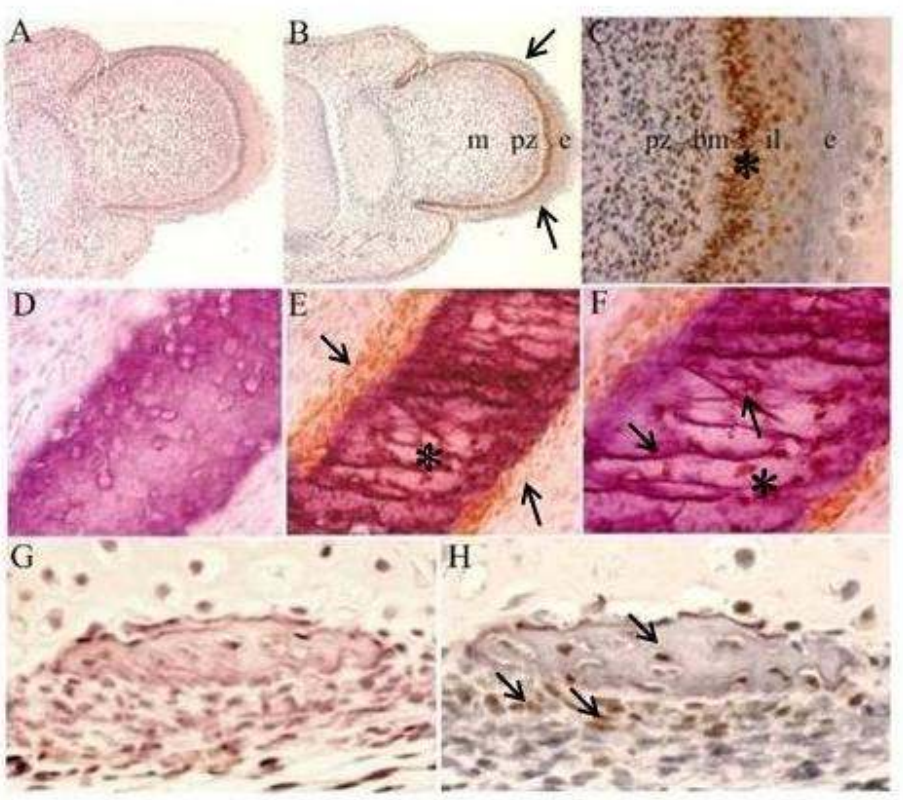
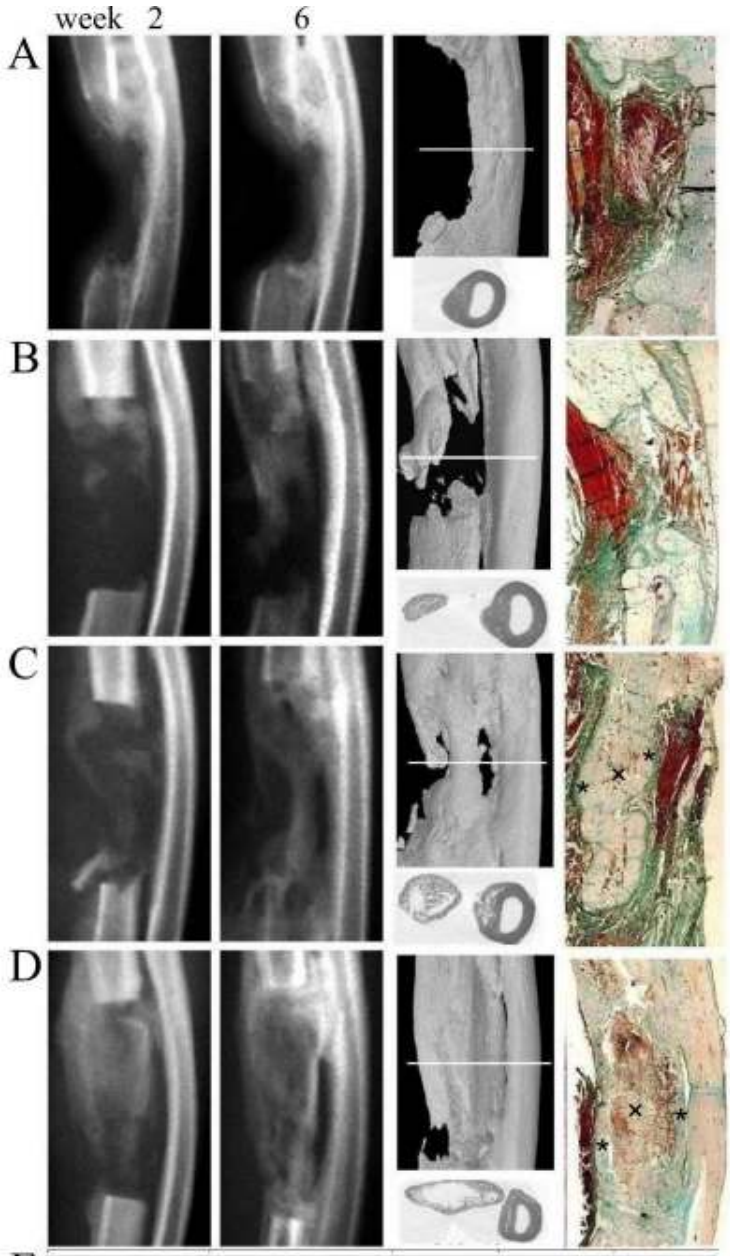


Figure 3. X-ray of critical size defects of rabbit ulnae treated with a control modified whole blood coagulum alone (WBDC) (A) or with BMP1-3 (B), WBCD with BMP7 (C) and BMP1-3 + BMP7 (D) *in vivo* for 2 and 6 weeks after surgery. MicroCT and histological analyses (Goldner stain) of critical size defects were performed *in vivo* at 6 weeks after surgery, *cortical bone; ×trabecular bone. (E) Table presents healing efficacy analyzed by radiographic grading scores (0-6) as previously described [33, 34], volume of the newly formed bone, the area of medullar cavity (Sform softwer) and BV/TV values determinate by μCT. [a] $P<0.05$ vs control; [b] $P<0.05$ vs BMP1-3+ BMP7 (ANOVA; Dunnett test).



E

Therapy	Week / X-ray score		Volume of newly formed bone (%)	Medullary canal (%)	µCT BV/TV (%)
	2	6			
Control	0	0	2.9 ± 1.5	0	0
rh BMP-1-3	1 ± 0.3	3 ± 0.3[ab]	7.3 ± 5.6[ab]	0[ab]	12.3±5.5[ab]
BMP-7	3 ± 0.5	5 ± 0.4[ab]	28.4 ± 12.3[ab]	13.5 ± 7.4[ab]	16.4±5.2[ab]
rh BMP-1-3 + BMP-7	4 ± 0.4	6 ± 0.4[a]	37.7 ± 14.5[a]	38.2 ± 15.7[a]	28±6.7[a]

Figure 4. BMP1-3 effects bone nodule formation from bone marrow (BM) mesenchymal progenitor cells (MSC). MSC were grown in the medium with: (A) control; (B) BMP1-3 (150 ng/ml), 8 mM β-glycerophosphate, and 50 μg/ml ascorbic acid (C) BMP1-3 antibody (1 μg/ml) , 2x and 20x magnification. (D) Quantitation of von Kossa stained nodules using an image analysis; (E) MC3T3 mouse preosteblasts, relative gene expression after treatment with 50 ng of BMP1-3* P<0.05 *vs* control; #P<0.05 *vs* BMP1-3 therapy.(F) Effect of BMP1-3 enhancement on BMP7 mediated *Id-1* promoter activation. The cells were treated for 17 h with: BMP7, BMP1-3 and BMP1-3 + BMP7 . In combination with BMP7, BMP1-3 was preincubated for 3h on 37°C, and then BMP7 was added. Control peptide was used as control. Medium with 0,1% FBS was used as negative control, n = 4. Representative data from one of three separate experiments are shown. * p<0,05 vs control; ** p<0,05 vs BMP7.

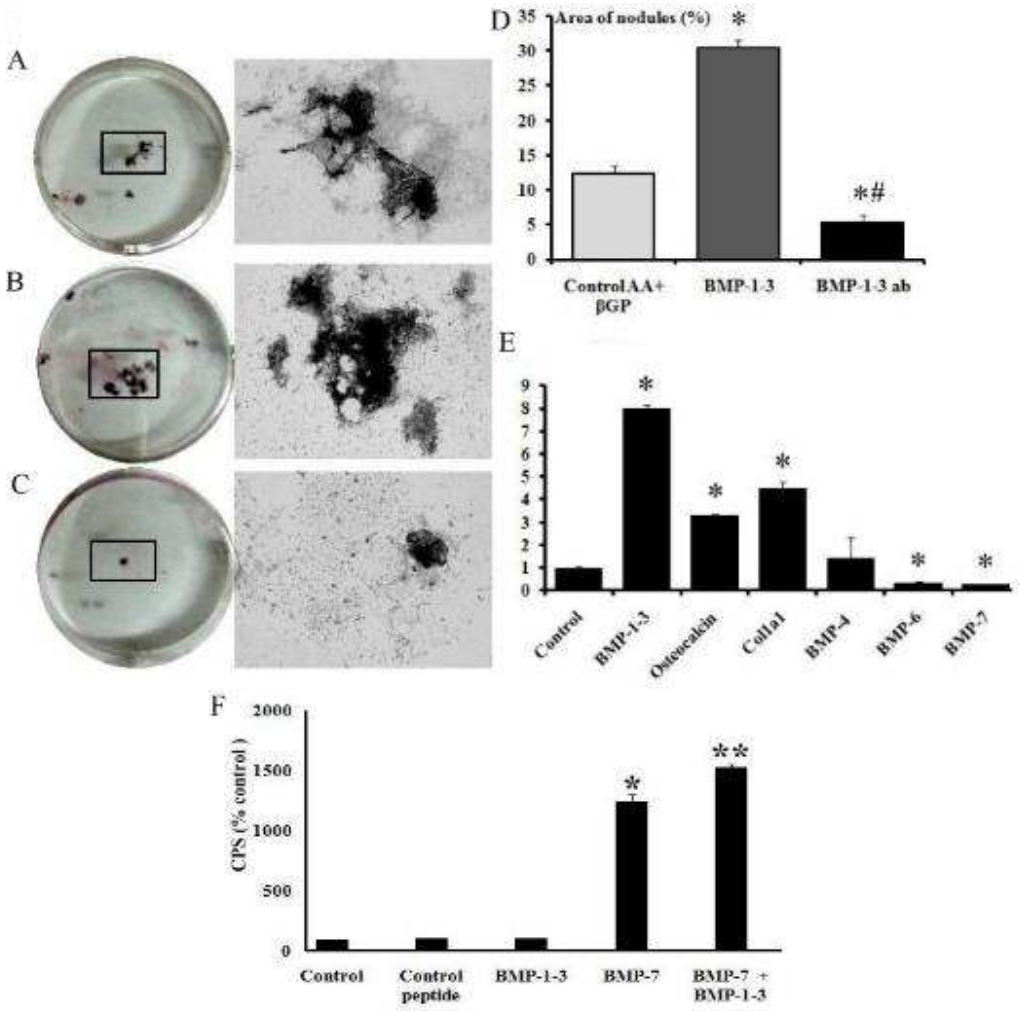

A
B
C
D
Area of nodules (%)
35
30
25
20
15
10
5
0
*
*#
Control AA+ βGP
BMP-1-3
BMP-1-3 ab
E
9
8
7
6
5
4
3
2
1
0
Control
BMP-1-3
Osteocalcin
Col1a1
BMP-4
BMP-6
BMP-7
F
CPS (% control)
2000
1500
1000
500
0
**
Control
Control peptide
BMP-1-3
BMP-7
BMP-7 + BMP-1-3

Table1. Sequences of primers used for gene expression analysis.

Target gene	Forward 5'-3'	Reverse 5'-3'
Bmp-1-3	CCCTGAGTATCCCAATGGCTA	CCACATAGTCATACCAGCACAG
Bmp-4	GACTTCGAGGCGGACACTTCTA	GCCGGTAAAGATCCCTCATGTAA
Bmp-6	CAACGCCCTGTCCAATGAC	ACTCTTGCGGTTCAAGGAGTG
Bmp-7	ACGGACAGGGCTTCTCCTAC	ATGGTGGTATCGAGGGTGGAA
Col1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
Bglap	TGACCTCACAGATCCCAAGG	GTCTGATAGCTCGTCACAAGG
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA