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ORIGINAL ARTICLE

Would three BMPs at low concentration be better than one at high concentration? An experimental study with rat osteoprogenitor cells

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ABSTRACT

Background: Bone morphogenetic proteins (BMPs) are used in clinical practice for stimulation of bone formation, but often evoke serious complications. Recent studies demonstrated that BMPs involved in early stages of bone formation are species specific. In cattle dominate BMP7, growth differentiation factor 5 (GDF5) and NEL-like protein 1 (NELL1) while in rats BMP2, BMP5 and BMP6. The purpose of the study was to compare the action of the species specific BMPs on the osteoprogenitor cells. Thus, rat osteoprogenitor cells were exposed to one BMP in a high dose and three of them at 1/3 of the former.

Materials and methods: Isolated rat osteoprogenitor cells were treated in culture with different concentrations of BMP2, BMP5 and BMP6 or with lower concentration of combinations of these cytokines. Activity of alkaline phosphatase, calcium deposition and mRNA level for transcription factor SP7 (osterix) and tissue non-specific alkaline phosphatase (TNAP) served as indicators of BMPs effect.

Results: BMPs stimulated all studied parameters in comparison with control cultures, but no statistically significant differences were observed between the action of a large dose of one cytokine and a combination of cytokines given at lower concentrations.

Conclusions: Three BMPs used in a low dose exert similar effect as the one used at high dose. Since the BMPs stimulate different receptors and activate different signaling pathways the use of the mixture of properly chosen BMPs at low concentration may give better results than the single one at high concentration and may avoid untoward effects.

Keywords: BMP, rat osteoprogenitor cells, alkaline phosphatase expression, calcium deposition

INTRODUCTION

Treatment of bone disorders represents serious clinical problem, particularly in cases of delayed healing of fractures or the need for substitution of dissected fragments of bone with neoplastic changes. Thus, discovery of bone morphogenetic proteins (BMPs) and their production by genetic engineering raised hopes for opening a new era in orthopedic surgery [57]. Recombinant BMP2 and BMP7 were approved for human applications in several orthopedic and oral and maxillofacial applications. (reviewed by Lowery and Rosen [34]). Application of BMPs was, however, frequently disappointing and evoked several serious side effects such as ectopic bone formation, osteoclast-mediated bone resorption, or, in extreme form, the life-threatening spine swelling [20, 25, 27, 29, 36]. Courvoisier et al. [7] in a review based on the PubMed database concluded, however, that it seems safe and efficient to use BMP for treatment of long bone nonunions.

Discovery of bone morphogenetic factors led to recognition that the BMP signaling is required for normal skeletal development (reviewed by Salazar et al. [44]). Numerous extensive reviews survey function of cell types participating in bone formation as well as BMPs structure and signaling pathways [10, 28, 34, 46, 61, 64]. It is recognized that different BMPs exert distinct but overlapping biological functions [61]. Thus, it is important to understand which BMPs have a leading role in particular biological process. During physiological bone formation growth factors produced by chondrocytes are stored in zone of provisional calcification of epiphyseal cartilage and then used for stimulation of bone formation in metaphysis (reviewed in Hyc [22]). In calcified cartilage from calf ribs costochondral junction (an equivalent of epiphyseal cartilage of long bones) quantitatively dominated BMP7, growth differentiation factor 5 (GDF5 also called BMP14) and NEL-like protein 1 [24]. The latter factor does not belong to the BMPs family but is highly specific to the osteochondral lineage and can promote bone formation [26, 49, 62]. Since in case of other species accumulation of the sufficient amount of calcified cartilage for quantitative growth factors determination would be difficult, and therefore indirect approach for detection of factors participating in initial bone formation was used. Chondrocytes were isolated from rat ribs costochondral junction and mRNA level for selected growth factors was determined [21]. Among bone inducing factors predominated BMP2, 5, 6 and 7, GDF5 was less prominent and

NELL1 barely detectable. These observations led to the postulation that the expression of different BMPs during early stages of bone formation is species specific [22].

Discovery that rat chondrocytes from costochondral junctions express several BMPs in roughly similar intensity, raises a question about participation of these factors in bone formation. In rat ectopic assay BMP2 and 5 induce bone formation [9, 45, 56]. BMP6 stimulates also bone formation in rats [47], The question arises whether simultaneous application of all these BMPs for the physiological bone formation would offer some advantage over the use of a single factor. Thus, the aim of this work was to find out whether one factor used at high concentration would act similarly as three factors used simultaneously at one/third concentration of the former. Factors were applied to the cultured osteoprogenitor cells isolated from young rat calvariae. As the indicators of osteogenic differentiation served determination of activity of alkaline phosphatase, evaluation of calcium deposition [13] and determining mRNA level for alkaline phosphatase and SP7 genes. SP7 is transcriptional factor (osterix) crucial for formation of osteoblasts in both endochondrally and intramembranously formed bones [19, 39]. While the response in both experimental groups was similar, the use of three factors could have some advantage due to the minimizing side effects.

MATERIALS AND METHODS

Animals, cell isolation and culture system

According to the opinion of the Medical University of Warsaw Local Ethical Committee, euthanasia of animals is not considered a procedure and does not require special consent. Calvaria of 2-5-day-old Wistar rats of both sexes were excised and inspected under dissecting microscope. Remnants of chondrocranium were cut off, but the periosteum was left intact. The cells were isolated by 0.13% collagenase type I digestion (Merck, Darmstadt, Germany) [18, 59]. The liberated cells were suspended in RPMI containing 10% calf serum and 1 % antibiotic antimycotic solution (Merck). The yield of cells estimated in the Bürker's chamber was about 40–50 $\times 10^6$ cells. The culture schedule and exposition to BMPs is presented in Figure 1 and Table 1. The following recombinant BMPs were used: BMP2, BMP5 and BMP6 (R&D Systems, Minneapolis, Canada).

Histochemical staining

Cultures were started on 12 mm round cover slips placed in 24 well culture plates (Sigma). For alkaline phosphatase visualization cultures were fixed in 4% formaldehyde for 10 min and procedure described in Vector Blue alkaline phosphatase kit (Vector Laboratories,

Inc. 30 Ingold Road, Burlingame, CA 94010, USA) was used. Incubation lasted for 20 min at room temperature. After careful rinsing the cultures were mounted in glycerol gelatine (Sigma). Calcium deposits were visualized by 2% Alizarin S (Sigma) adjusted to pH to 4.1~4.3 with 10% ammonium hydroxide. Staining lasted for 5 min. Cultures were dehydrated in acetone and mounted in a synthetic medium.

Alkaline phosphatase (ALP) activity determination

Cells in particular wells were extracted with 20 mM TBS buffer (Trisha HCl - 42mM, Trisha base 8 mM, NaCl — 150 mM) (Merck), pH 7.4, containing 1% Triton X-100 (Merck) and stored at –20° C until the assay [3]. ALP activity was determined with Pierce™ PNPP Substrate Kit (Thermos Fisher Scientific, Waltham, MA, USA). Activity was determined from the standard curve prepared from the diluted alkaline phosphatase from bovine intestinal mucosa — in DEA units/mg protein (Merck). Thus, values of osteoblastic phosphatase activity read from the curve corresponded to the activity of intestinal ALP expressed in DEA units.

Calcium determination

Calvarial osteoblasts cultures were washed with PBS and fixed with 4% (v/v) formaldehyde for 30 minutes (Merck). After washing twice with dH_2O, 0.7 g Alizarin Red S (Merck), dissolved in 50 ml dH_2O at pH 4.2 was added for 30 min (staining solution). Both fixation and staining were done at room temperature. Alizarin was eluted with 150 all per well of 96 plate in 10% (w/v) methylpyridinium chloride (Merck) in an aqueous phosphate buffer 0.01M Na_2HPO_4/NaH_2PO_4 (Merck) at pH 7,0 for 1 h. Serial dilution of 20 mM of Alizarine red S in 10% methylpyridinium chloride served for standard curve preparation. As a blank was used 10% methylpyridinium chloride in phosphate buffer. The intensity of staining was quantified by measuring the absorbance at 550 nm in spectrophotometer (FLU Ostar Omega, BMG LABTECH, Rotenberg, Germany) [12, 15, 38, 63]. The amount of the absorbed dye served as an indicator of the amount of deposited calcium.

Gene expression analysis

Total RNA isolation. RNA was isolated with Nucleoporin II kit (Macherey-Nagel, Duren, Germany), according to manufacturer’s protocol. The quantity and quality of the isolated total RNA was evaluated spectrophotometrically using ND-2000-Spectrophotometer Nanodrop 2000 with software for analysis of nucleic acids (Thermos Fisher Scientific).

Reverse transcription. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription kit (Thermos Fisher Scientific) according to the manufacturer's protocol in Eppendorf Master cycler gradient (10 min at 25°C, 120 min at 37°C and 5 sec. at 85°C). Two μL of 10× RT buffer, 0.8 μL of 25× dNTP Mix, 2 μL of 10× Random Primers, 1 μL of Multiscribe Reverse Transcriptase 4.2 μL of nuclease-free water and 10 μL of mRNA (1 μg) were used for one reaction. cDNA samples were stored at –20°C.

Real-time PCR. Real-time PCR was performed in the ABIPRISM 7500 (Thermo Fisher Scientific) using 96-well optical plates. Each sample was run in triplicate and was supplied with an endogenous control – eukaryotic translation initiation factor 2B subunit alpha alpha (EIF2B1). For gene expression analysis, proper TaqMan expression assays were used: Rn00596951_m1 for EIF2B1, Rn01516028_m1 for alkaline phosphatase and Rn01761789_m1 for SP7. All probes were stained with FAM (Thermo Fisher Scientific). Reactions were run in 25 μL TaqMan Universal Master Mix (Thermo Fisher Scientific), appropriate primer set, MGB probe and 50 ng of cDNA template. Universal thermal conditions, 10 min at 95°C, 40 cycles of 15 sec at 95°C and 1 min at 60°C were used. Data analysis was done with sequence detection software version 1.2 (Thermo Fisher Scientific). Relative expression was calculated against the reference gene EIF2B1 [1]. Analysis was conducted as a ΔCT values using sequence detection software ver. 1.2 (Thermo Fisher Scientific).

Statistical analysis. Differences between the control and experimental groups were evaluated by the Wilcoxon matched-pairs test.

RESULTS

The morphological appearance of cultured cells is presented in Figure 2. Both in alizarin and phosphatase preparations cells either grew in monolayer or formed small groups. The latter were distinctly stained in both types of culture and presumably corresponded to osteoprogenitors differentiating into osteoblasts. Part of monolayer cells showed strong ALP staining.

The activity of produced ALP and amount of calcium deposits in cultures of rat osteoprogenitor cells were similar in all experiments with one BMP used at 6 or 30 ng and three BPMs used together at one/third of the first value. Generally, BPMPs stimulated the activity of alkaline phosphatase and calcium deposition. Only in cultured cells treated with BMP5 used at 1.2 vs 3 × 0.4 ng the ALP activity was not stimulated (Fig. 3, 4). The expression of ALP and SP7 genes raised in cultures stimulated by BMPs (2, 5, and 6 at 30 and

3 × 10 ng concentration) in relation to controls; but did not differ between cultures exposed to one BMP in high dose and three BMPs on 1/3 of the high value (Fig. 5).

DISCUSSION

Mesenchymal stem cell [23, 61] or osteoblastic cell lines [2, 52] usually serve as a convenient material for studies on bone formation. The purpose of this work was the study of the influence of the rat species specific BMPs on cells of the same species. Thus, freshly isolated rat calvarial cells were used. Since isolated cells represented osteoprogenitors at various stages of differentiation, addition of BMPs gave the overall survey of their action on ALP production and calcium deposition considered, respectively, as early and late ossification effects [13]. The disadvantage of the method are the differences in the magnitude of response to BMPs of cultures. It probably reflects differences in the dissection of calvariae, what is difficult to standardize even for the experienced operator. Nevertheless, the system was sufficiently sensitive for detection of difference between activity and expression of ALP as well as calcium deposition and SP7/osterix expression in control and BMP stimulated cultures. The oligonucleotide used for ALP expression evaluation is specific for the tissue-nonspecific isozyme of ALP (TNAP). This enzyme is strongly expressed in bone, liver and kidney and plays a key function in the calcification of bones [53]. Sp7 works as an osteoblast determinant critical for osteoblast differentiation and mineralization of cartilage and bone [19, 39]. Thus, while in the whole organism these genes are expressed in various tissues, in cultures of calvaria cells there are only osteoprogenitor cells in which they can be expressed.

Synergistic use of BMP5, BMP6, BMP7 and BMP2 for bone regeneration in humans was already suggested by Celeste et al. [5]. In cultures of mouse bone marrow cells BMP2 alone and the combination of BMP2 and BMP5 significantly enhanced osteoclastogenesis while BMP2, BMP5, and BMP6 used jointly did not exert additional effects. On the other hand, the same agents used together stimulated matrix mineralization and SP7 expression [58]. In studies with mouse pluripotential mesenchymal precursor and preosteoblastic cell lines, Cheng et al., [6] proposed hierarchical model of BMPs action with BMP2, 6, and 9, inducing differentiation of mesenchymal stem cells into osteoblast, while BMP2, 4, 6, 7, and 9 stimulating osteogenesis. Luu et al. [35] based on mouse cell lines study and *in vivo* mouse intramuscular transplants showed that besides BMP2 and BMP7, BMP6 and BMP9 from all BMPs have the highest osteogenic activity and suggested that they may be used for formulation of synergistic pairs for successful bone regeneration in humans. Friedman et al. [14] found that in cultures of human mesenchymal stem cells exposed to BMP2, 4, 6, and 7

the BMP6 most efficiently stimulated osteoblast differentiation. When BMPs were used jointly, only formulations containing BMP6 stimulated mineralization. BMP6 has, according to Vukicevic and Grgurevic [54], unique structural and functional properties being a powerful regulator of MSC differentiation into osteoblasts, more efficient than BMP2 and BMP7. Moreover, it is released by osteoclasts and recruits osteoblasts to the resorption site serving as a key factor coupling bone formation to bone resorption.

At the onset of bone formation in rats predominated BMP5, 6, and 2 [22], what considered together with the results of this *in vitro* study, suggests that these BMPs would have comparable effects during bone formation in adult life. Nevertheless, in the *in vivo* situation action of each of them could be differentially influenced by other factors. Numerous studies indicate synergistic action of VEGF and BMPs (reviewed by Li et al., [31]). For example, Peng et al. [42] demonstrated in mice that exogenous vascular endothelial growth factor (VEGF) enhanced BMP2-induced bone formation at least partially by stimulation of angiogenesis.

Bone morphogenetic protein receptors (BMPRs) are transmembrane receptors with the activity of serine-threonine kinase and are divided into type I and II. To type I belong ACVR1 (activin A receptor, type I or ALK2 activin receptor-like kinase-2), ACVR1B (ALK4), ACVR1C (ALK7), ACVRL1 (ALK1), BMPR1A (bone morphogenetic protein receptor, type IA or ALK3), BMPR1B (bone morphogenetic protein receptor type-IB or ALK6) and TGFR1 (TGF beta receptor I or ALK5). Type II BMP receptors include BMPR2 (bone morphogenetic protein receptor type II), ACVR2A (activin receptor type-2A), ACVR2B (activin receptor type-2B), TGFBR2 (TGF receptor II), and AMHR2 (anti-Mullerian hormone receptor type 2). BMPs may bind to type I receptors in the absence of type II receptors, but their binding affinities increase dramatically when both type I and type II receptors are present (reviewed by Chen et al. [6], Katagiri and Watabe [28], Lowery and Rosen [34], Nickel and Mueller [41]. Inside the cell, the activity of BMPs is controlled through the combination of signal-transducing Smad proteins and inhibitory Smad proteins (reviewed by Ebara and Nakayama [10], Sanches-Duffues et al. [46]). Based on the structural homology, the BMP family members can be classified into several subgroups, including i.e., the BMP2/4 and BMP5/6/7/8 group, (reviewed by Katagiri and Watabe [28], and Liu et al. [33]). The specificities of the BMPs binding to type I receptors depend on the identities of the interacting type II receptors and cell types [60]. The three BMPs used in this work differ in their binding abilities (Fig. 6). BMP2 binds to ALK2, 3, 6 whereas BMP6 binds weakly to ALK6 and strongly to ALK2 [11].

The receptor(s) for BMP5 is, as yet, not determined [28, 33, 37], but since it belongs to the same subgroup as BMP6 it probably also binds to ALK2 and ALK6.

BMPs, after binding to the receptor. mediate signals for osteoblastic differentiation through Smad-dependent and Smad-independent pathways [37]. BMP2 signals through Smad1, 5, and 8 [4], BMP5 through Smad1 and 8 [65], and BMP6 through Smad1 and Smad5 [2] (Figure 6) . BMP receptor signaling is also regulated by the localization of receptors in specific membrane domains, such as caveolae, clathrin coated pits or lipid rafts. Their localization can determine which signaling pathways are activated (reviewed by Halloran et al. [16]).

Several BMPs (2, 4, 5, 6, 7, 9) have the unique property of inducing osteoinduction by themselves [6, 51]. In rats, at the initial stages of bone formation, predominate BMP2, BMP5, BMP6 and BMP7 [22]. These BMPs could cooperate in bone formation acting through different receptors and signaling pathways. The multitude of participating factors expressed roughly at the similar level instead of one acting at high concentration may offer additional advantage. BMPs signaling is, for example, essential for limb bud development [43, 55]. BMP2, BMP4, BMP5 and BMP7 participate in the control of limb programmed cell death [65]. Thus, BMP produced at high concentration in cartilage and bone compartment could diffuse and adversely affect limb development while BMP from other limb compartments could interfere with proper epiphyseal cartilage and bone formation. Similar situation could also exist during therapeutic BMP application — the use of several of them at low concentration could possibly prevent untoward effects by limiting their spreading.

The lack of recognition of species specificity [22] makes evaluation of reports concerning human tissues BMPs difficult, since some important factor could be missed. For example, in calves, during initial bone formation, occurs NELL1 which is also expressed in human tissues [62] and could have a role in early bone formation.

Takemoto et al. [48] determined expression of BMPs in human bone marrow from the iliac crest, the proximal humerus, and the proximal tibia representing typical autogenous bone graft harvesting sites and found no statistically significant differences in the mRNA levels of BMP2 4, 5, 6, 7, 8, and 9. Liu et al. [32] found that BMP9 has high therapeutic potential in oral and maxillofacial tissue engineering. Haubruck et al. [17] compared effectiveness of BMP2 and BMP7 for treatment of lower limb nonunions and found that patients who received rhBMP2 had a significantly higher rate of healing compared to patients treated with rhBMP7.

It is evident that BMPs action on cells in comfortable tissue culture conditions may not be comparable to clinical situation when blood vessels and inflammatory cells are present. In

such case one BMP may offer particular advantages and the others may take second place. As long, however, as the species specificity of human BMPs in bone formation is not established, the possible advantages offered by simultaneous use of several BMPs acting on different receptors (see Fig. 6) deserves attention.

Ethical and practical problems involved in determination of the set of BMPs specific for early bone formation were discussed in the recent paper [22]. Based on the existing data seems that the joint use of BMP2 and BMP7 would be better than any of them applied singly. BMP2 binds to ALK3 and signals through Smad1, 5, and 8 [4]. BMP7 binds to ALK2 and ALK6 efficiently, and to ALK3 less efficiently [8, 50] and also signals through Smad1, 5, and 8 [40]. While both BMPs stimulate the same Smads, they nevertheless utilize different cell surface receptors to induce osteoblastic differentiation [30], thus they joint use could be profitable.

CONCLUSIONS

The panel of BMPs acting during early stages of bone formation is species specific. Here, we demonstrate that the three species specific BMPs used at low concentration stimulate differentiation of the rat osteoprogenitor cells similarly as one BMP at high concentration. Since the BMPs act through different receptors and activate different signaling pathways the use of species specific BMPs at low concentration could decrease untoward effects observed during their clinical use. The species specificity of human BMPs remains to be determine.

Article information and declarations

Data availability statement

Raw data is available on request.

Ethics statement

According to Polish and European law, euthanasia of animals is not considered a procedure and does not require the consent of the ethics committee.

Author contributions

A.H. evaluation of gene expression, A. O-I. evaluation of ALP activity and calcium deposition, A. H. and A.O-I. reviewed, edited, and wrote the manuscript. S.M. reviewed the

literature, conceptualized, reviewed, edited, and wrote the manuscript. All authors have read and agreed to publish this version of the manuscript**.**

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Conflict of interest: The authors declare no conflict of interest.

Table 1. BMPs concentration given into rat calvarial osteoblast cultures (ng)

	BMP given individually, concentration, (ng)	Three BMPs given together, each at the same concentration, (ng)
ALP activity	**BMP2** — 6	**BMP2/5/6** — 2
	BMP5 — 30	**BMP2/5/6** — 10
	BMP5 — 6	**BMP2/5/6** — 2
	BMP5 — 1.2	**BMP2/5/6** — 0.4
	BMP6 — 6	**BMP2/5/6** — 2
Calcium deposition	**BMP2** — 30	**BMP2/5/6**— 10
	BMP5 — 1.2	**BMP2/5/6** — 0.4
	BMP6 — 30	**BMP2/5/6** — 10
ALP and SP7 mRNA level	**BMP2** — 30	**BMP2/5/6** —10
	BMP5 — 30	**BMP2/5/6** —10
	BMP6 — 30	**BMP2/5/6** —10

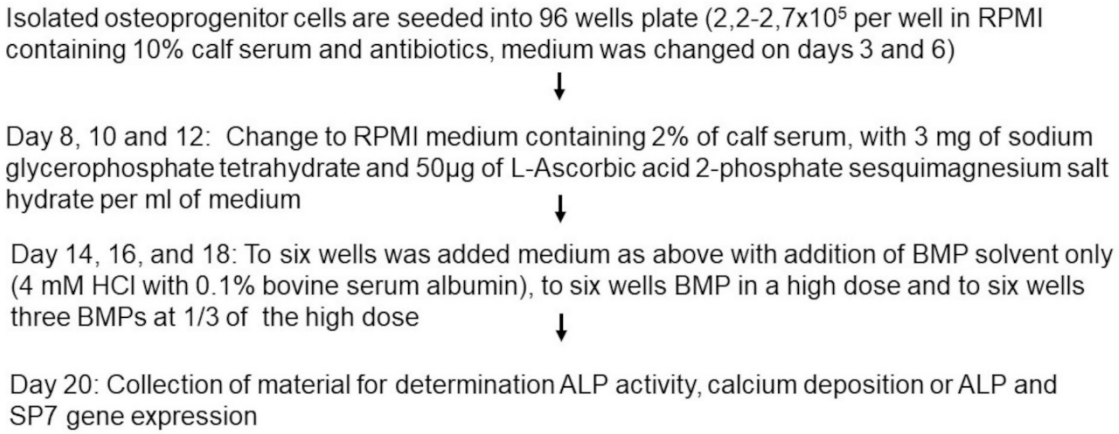

Figure 1. The rat calvarial cell cultures schedule

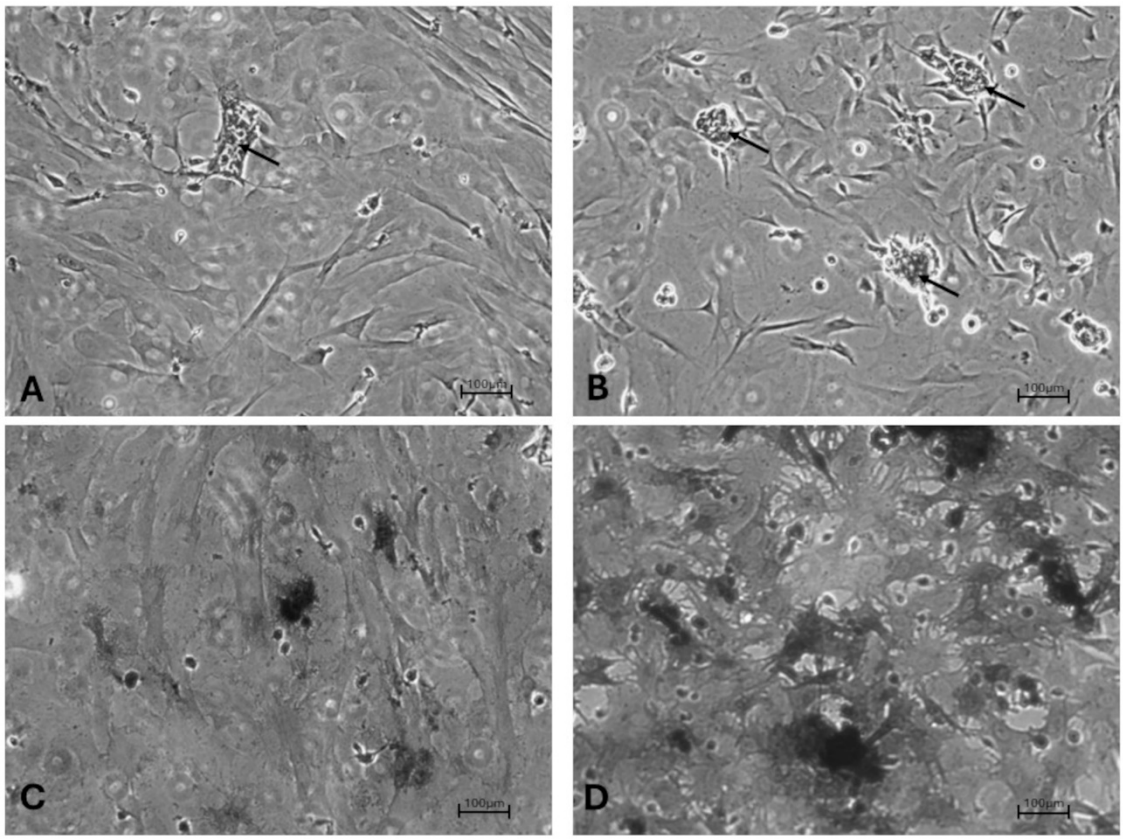

Figure 2. Control (**A, C**) and BMPs (**B, D**) treated cultures of rat calvarial cells. Cultures in A and B were stained with alizarin, in C, D demonstrated ALP. In A and B arrows indicate

groups of cells with deposited calcium. In C and D ALP was demonstrated in groups of cells and also in some cells in monolayer.

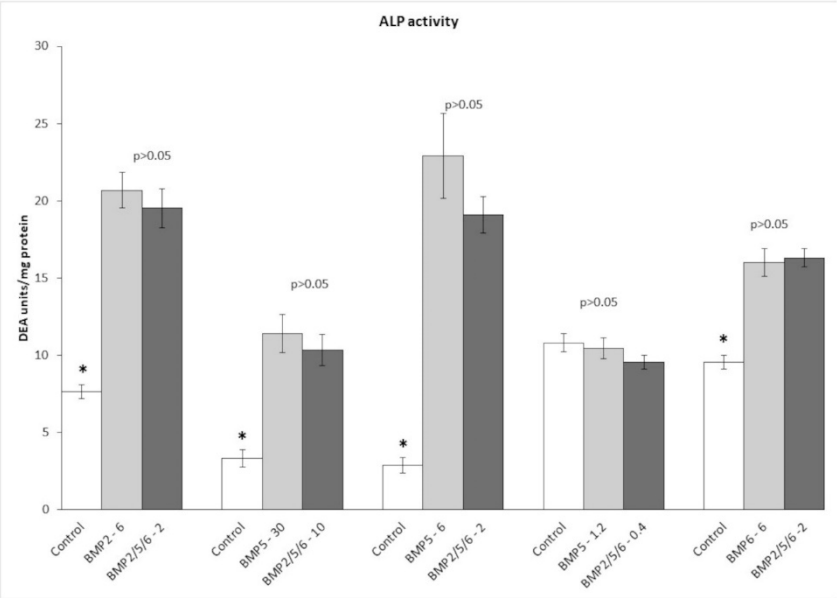

Figure 3. The activity of alkaline phosphatase in control and BMPs treated cultures of rat calvarial cells. Activity was determined from the standard curve prepared from the diluted alkaline phosphatase from the bovine intestinal mucosa and is presented in DEA units/mg protein (n = 6). Statistical analysis was preformed using Wilcoxon matched-pair test. Differences between groups were significant at $p < 0.05$. Statistically significant differences are marked by asterisks.

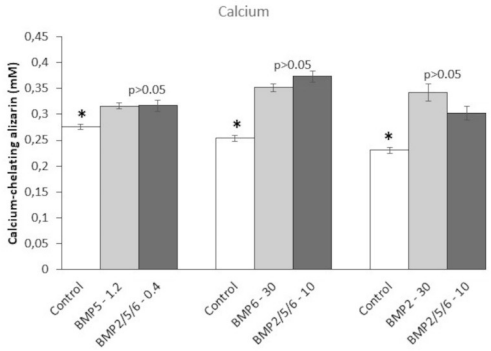

Figure 4. Calcium deposits determination in control and BMPs treated cultures of rat calvarial cells. Serial dilution of 20 mM of Alizarine red S in 10% cetylopirydinium chloride served for standard curve preparation. As a blank was used 10% cetylopirydinium chloride in

phosphate buffer. The intensity of staining was quantified by measuring the absorbance at 550 nm in spectrophotometer. The amount of the absorbed dye served as an indicator of the amount of deposited calcium (n = 6). Differences between groups were significant at $p < 0.05$. Statistically significant differences are marked by asterisks.

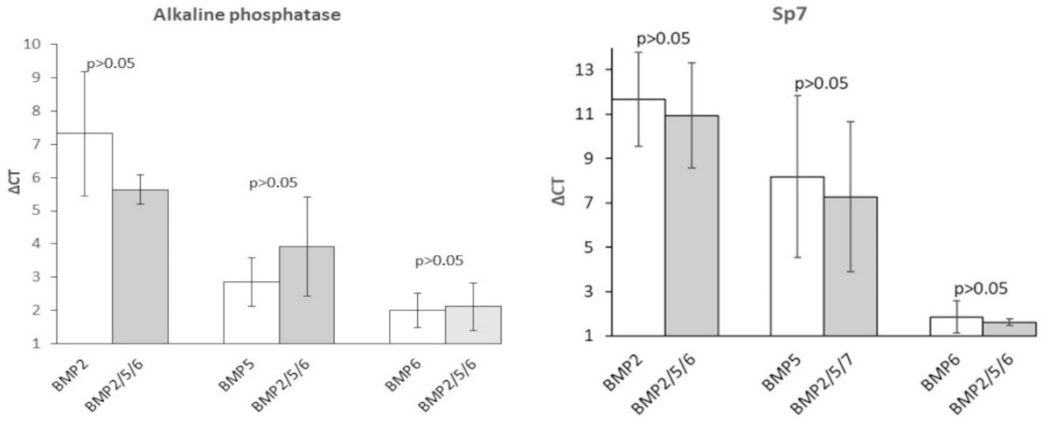

Figure 5. Influence of BMPs on alkaline phosphatase and SP7 expression in cultured calvarial osteoblasts determined by real-time PCR. Results are shown as an average ΔCT values (± SE). n = 6. Relative expression was calculated against the reference gene, EIF2B1. Analysis was conducted as a relative quantification study, using control cultured rat calvarial cells gene expression as a calibrator (value 1). Statistical analysis was preformed using Wilcoxon matched-pair test. The expression of ALP and SP7 genes raised in cultures stimulated by in relation to controls ($p < 0.05$) but did not differ between BMP treated cultures ($p > 0.05$).

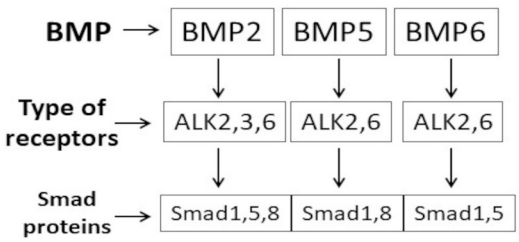

Figure 6. Schema of receptors and transcription factors stimulated by BMP2/5/6

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