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BMP-6 EXERTS ITS OSTEOINDUCTIVE EFFECT THROUGH ACTIVATION OF IGF-I AND EGF PATHWAYS

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Abstract

We have recently shown that human recombinant BMP-6 (rhBMP-6), given systematically, can restore bone in animal models of osteoporosis. To further elucidate the underlying mechanisms of new bone formation following systemic application of BMPs we conducted gene expression profiling experiments using bone samples of ovariectomized mice treated with BMP-6. Gene Set Enrichment Analysis revealed enrichment of insulin-like growth factor-I and epidermal growth factor related pathways in animals treated with BMP-6. Significant upregulation of IGF-I and EGF expression in bones of BMP-6 treated mice was confirmed by quantitative PCR. To develop an in vitro model for evaluation of the effects of BMP-6 on cells of human origin, we cultured primary human osteoblasts. Treatment with rhBMP-6 accelerated cell differentiation as indicated by the formation of mineralized nodules by day 18 of culture versus 28 to 30 days in vehicle treated cultures. In addition, alkaline phosphatase gene expression and activity were dramatically increased upon BMP-6 treatment. IGF-I and EGF expression was upregulated in human osteoblast cells treated with BMP-6. These results collectively indicate that BMP-6 exerts its osteoinductive effect at least in part through IGF-I and EGF pathways, which can be observed both in a murine model of osteopenia and in human osteoblasts.

Introduction

Bone morphogenetic proteins (BMP) are potent local factors that have been shown to have specific functions during organogenesis and embryogenesis, regulate the differentiation of mesenchymal progenitor cells and promote bone and cartilage formation in bone and cartilage defects, fracture repair and periodontal diseases (3, 5, 11, 15-17). BMPs are members of the transforming growth factor-β (TGF-β) superfamily and were first identified by their ability to induce ectopic bone formation in vivo (11).

The function of individual BMPs have been evaluated *in vitro* using various cell lines, e.g., multipotent progenitor cells, osteoprogenitor cells, osteoblasts, chondroblasts, and osteosarcoma cells (22). BMPs were shown to be important in osteoblast differentiation (23, 24) and bone formation *in vivo,* where they, in concert with other cytokines and matrix components, induce sequential cascade events for chondro-osteogenesis, consisting of chemotaxis, mesenchymal proliferation and differentiation, angiogenesis, and controlled synthesis of extracellular matrix (11, 18, 24)

Recently, we have shown that human recombinant BMP-6 (rhBMP-6), administered systematically, induces new bone formation in both osteoporotic rats and mice (19, 20). In order to elucidate the underlying mechanism of new bone formation following systemic application of BMP-6 we conducted gene expression profiling in bone tissue of ovariectomized (OVX) mice following BMP-6 therapy. Primary human osteoblasts served as an *in vitro* model for evaluation of the *in vivo* effect of BMP-6 human bone-derived cells.

Materials and Methods

Animals

Wild type C57BL/6J mice were purchased from Charles River, Budapest, Hungary. Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals (14). All protocols were approved by Institutional Ethics Committee and Ministry of Science Grant Review Committee. At three months of age in sham mice ovaries were exteriorized but replaced intact, while bilateral OVX with dorsal approach was performed in the remaining mice. Animals were left untreated for three weeks following surgery to await the development of osteopenia and then divided into the following groups: (1) sham (n=10), (2) OVX (n=10), (3) OVX + BMP-6 (10 μg/kg intravenously, 3 times x week) (n=10). Human mature BMP-6 was produced in CHO cells and purified as previously described (6, 20). Therapy started 3 weeks following OVX and continued for the next nine weeks for bone densitometry measurements (n=15) or four weeks for gene expression analysis (n=15) when the animals were euthanized by cervical dislocation.

Bone mineral density (BMD) measurements

To determine the anabolic effect of BMP-6 on bone tissue, ex vivo BMD measurements of lumbar spine, femurs and tibiae were performed by a PIXImus apparatus (Norland, Medizintechnik, Pforzheim, Germany) according to manufacturer's instructions (using the mouse-specific software - version 1.43).

Cell Culture

Normal human osteoblasts were purchased from Cambrex Bioscience (Cambrex, Walkersville, MD, USA). Cells were plated in 12-well dishes at a density of 5000 cell/cm^2 in Osteoblast Basal Growth Medium (OBGM) containing 10% Fetal Bovine Serum, ascorbic acid (100 μg/ml) and antibiotics. Media were replaced every two days until cells were confluent and then media were replaced every two days until the completion of experiment with Differentiation Media (OBGM additionally supplemented with 1 μM dexamethasone and 10 mM β-glycerolphosphate). BMP-6 was added at plating and at every feeding in concentration of 100 ng/ml.

Cell number, alkaline phosphatase activity and in vitro mineralization

Cells were plated and cultured ± BMP-6 and at subsequent time points (1, 2, 3, 6, 10, 18, 28 and 37 days) were trypsinized, collected, rinsed with PBS and counted on a hemacytometer. Alkaline phosphatase activity was measured over a 28 day period at specific time points (6, 10, 14, 19, 23 and 28 days) as previously described (22) and expressed as product produced/min/μg protein. Adherent cell cultures from day 19 through day 28 were stained for the presence of mineral using the method of Von Kossa as previously described (4). Mineralized nodules appeared dark brown and were photographed using a Spot digital camera (Diagnostic Instruments, Inc., Sterling Heights, MN, USA).

RNA isolation and reverse transcription

Total RNA was isolated from the whole murine femur samples using both TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and RNeasy Mini Kit (Qiagen, Hilden, Germany) and from the primary human osteoblasts using only RNeasy Mini Kit. The quality of RNA was analyzed on a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). One-Step quantitative RT-PCR was performed on 2.5μg (bone samples) or 1 μg (cell culture) of total RNA using Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA).

Microarray and Gene Set Enrichment Analysis

Microarray analysis was performed using Mouse Genome 430 2.0 GeneChips (Affymetrix, Santa Clara, CA, USA), which contains 45 000 25-mer probe sets. The RNA from murine samples was processed using the standard Affymetrix protocol. Usual quality measures and normalization for the Affymetrix GeneChip were used in the experiments. All arrays were run in the same core facility at the Center for Functional Genomics, School of Medicine, University of Zagreb.

In an effort to test the functional correlation of gene sets that might be systemically altered in mice treated with BMP-6, we applied a statistical method called Gene Set Enrichment Analysis (21). Using gene expression profiles of OVX mice treated with BMP-6 we calculated the enrichment scores (ES) of 472 predefined gene sets (MSigDB1, Broad Institute, Boston, USA) containing genes involved in specific metabolic and signaling pathways and 50 sets containing genes coregulated in response to genetic and chemical stimuli. Estimated statistical significance (nominal *P* value) of the ES was also calculated by using an empirical phenotype-based permutation test procedure that preserves the complex correlation structure of the gene expression data. Nominal P value of $P<0.05$ was taken as a cut-off value in the analysis.

Gene expression analysis by quantitative PCR

For experiments performed on murine samples, gene expression of interest was measured using LightCycler FastStart DNA Master SYBR Green kit (Roche Diagnostics, Mannheim, Germany) in the LightCycler instrument (Roche Diagnostics, Mannheim, Germany). The comparative C_T method ($\Delta\Delta C_T$) was used for relative quantification of gene expression (10). Expression of four housekeeping genes was analyzed and glyceraldehyde-3-phosphate dehydrogenase was chosen as the most stable housekeeping gene. Results are represented as fold change of comparative expression level to OVX group of animals. Sequence of primers is shown in Table 1.

Total RNA from human osteoblast cultures, isolated at various time points (1, 2, 6, 10, 14, 18, 23, 28 days), was analyzed using different q-PCR method to compensate for lower RNA yield and accordingly weaker gene expression. Human primer/probe sets (assay numbers available on demand) were purchased as a 20X assay-on-demand mix from Applied Biosystems (Foster City, CA, USA). Data were normalized to the expression of Acidic Ribosomal Binding Protein which showed no change in mRNA expression with BMP-6 treatment.

Statistical analysis

Densitometric data measurement and changes in gene expression in mice were analyzed with One way ANOVA with one-sided Dunett-t post hoc test against OVX animals test ($p<0.05$). Changes in gene expression in the cell culture were analyzed with Student T-test ($p<0.05$).

Results

BMP-6 induces new bone formation in OVX mice and enhances differentiation of human osteoblasts

Bone mineral density (BMD) measurements showed that systemic administration of BMP-6 for a period of nine weeks restored bone volume of both the peripheral and the axial skeleton of OVX mice. BMD values of tibiae, femurs and vertebrae were increased for 12.8%, 10% and 7.4%, respectively, in mice treated with BMP-6 (Figure 1).

To develop an in vitro model for evaluation of the effect of BMP-6 on the cells of human origin, we used the primary human osteoblasts (HOB). After intensive proliferation period (first ten days of culture), cell number remained relatively stable between day 10 and day 28 of culture. No statistically significant difference was seen between BMP-6 and untreated cultures indicating that BMP-6 does not effect the cell growth (Figure 2a). In order to determine the effect of BMP-6 treatment on the differentiation of HOBs, alkaline phosphatase activity was measured, and increased from day 14 to day 23 by 2-2.7 fold in BMP-6 treated cultures when compared to untreated cells (Figure 2b). Since numerous osteoblastic cell lines spontaneously form mineralized nodules during the process of cell differentiation and matrix mineralization, we tested whether BMP-6 enhances the nodule formation in the primary human osteoblasts. We found that following BMP-6 treatment the cells formed mineralized nodules between day 18 and 23 of culture (Figure 2c). Cell cultures not exposed to BMP-6 did not show the formation of mineralized nodules until day 30 of culture (data not shown).

Effect of BMP-6 on markers of osteogenic activity

Gene expression analysis of common markers of osteogenic activity (AP, osteocalcin, collagen I and BMPs) confirmed that BMP-6 induces osteoblast differentiation both *in vivo* and *in vitro*. In mice, 4 weeks of BMP-6 therapy induced expression of AP above the sham value (Figure 3a). No change in alkaline phosphatase gene expression was observed when the comparison was made between BMP-6 treated cultures and untreated cultures until approximately day 19 of culture where the difference was 3.2-fold higher in the BMP-6 treated cultures as compared to control cultures. By day 23 of culture, the difference in alkaline phosphatase mRNA levels had risen to 4.3-fold and to 6-fold by day 28 of culture (Figure 3f).

In mice osteocalcin and collagen type I expression was increased upon BMP-6 treatment (Figure 3b and c). On the contrary expression of these common markers of differentiated osteoblasts showed no difference in the expression levels when untreated and BMP-6 treated cultures were compared (Figure 3g and h). In mice, BMP-6 expression was not changed following OVX, but was reduced 4 weeks after BMP-6 therapy (Figure 3d). Endogenous BMP-6 expression increased upon differentiation of HOBs, and addition of exogenous BMP-6 resulted in a decrease of BMP-6 transcripts expression (Figure 3i) indicating the ability of BMP-6 to auto-regulate its own expression level. Expression of BMP-4 was reduced in OVX animals and significantly increased after BMP-6 therapy (Figure 3e). On the contrary, in HOBs no change in the expression of BMP-4 was observed in either treated or untreated cells during the period of 30 days (Figure 3j). We observed no change in the BMP-2 expression in OVX and BMP-6 treated mice (data not shown). Similarly, in HOBs there was an analogous pattern of increased BMP-2 expression with BMP-6 treatment as compared to control cultures (Figure 3j).

Activation of IGF-I and EGF pathways upon BMP-6 treatment

To elucidate the underlying mechanisms of the new bone formation following systemic application of BMP-6 we analyzed the gene expression profile in OVX mice receiving BMP-6. In order to ascertain whether BMP-6 can activate formation related pathways in OVX animals, we performed BMP-6>OVX analysis using GSEA (Table 2). BMP-6 treatment specifically enriched IGF-I related pathways, including IGF-I MTOR pathway and insulin signaling. Epidermal growth factor (EGF) pathway was also highly enriched, indicating that BMP-6 might at least in part achieve its effects through the activation of IGF-I and EGF. OVX>BMP-6 comparison revealed increased enrichment of interleukin-1 receptor pathway, which indicates that BMP-6 treatment reduced the cytokine production in OVX animals. This could also be substantiated by an increased enrichment of cell adhesion molecule activity pathway in OVX, which includes known effectors of immune response, such as intercellular adhesion molecule 1. WNT signaling was also highly enriched, specifically the WNT beta catenin and WNT signaling pathways.

Effect of BMP-6 on expression of IGF-I and EGF was subsequently determined by quantitative PCR. Analysis of the gene expression in murine bones confirmed the results obtained by microarray. It was shown that IGF-I, IGF-II binding protein 3 and EGF expression was reduced following OVX (Figure 4 a-c) and that BMP-6 therapy corrected altered gene expression levels. In human osteoblasts it was confirmed that BMP-6 induces expression of both EGF and IGF-I. The peak of the IGF-I expression was observed in the beginning of the treatment (day 7), followed by decline, but the expression was still elevated compared to vehicle cultures. Expression of EGF was also increased upon BMP-6 treatment, and again on day 12 and 23 of the culture period (Figure 4 d and e).

Discussion

In this study we explored the mechanisms by which BMP-6 induces new bone formation in OVX mice and effects the differentiation of human osteoblasts. It was shown that BMP-6 induced enrichment of IGF-1 and EGF related pathways in both experimental models.

The activities of bone formation and resorption are regulated by systemic hormones and local factors produced in bone (2). IGFs are among the most important regulators of bone cell function because of their abundance and their proven anabolic effects on the skeleton (1, 12, 13). IGF-I regulates bone formation and remodeling by induction of early osteoblast gene expression in the differentiation of mature osteoblastic and preosteoblastic cells (8). It was shown that BMP-7 can act locally by modulating the IGF regulatory system, suggesting that the mitogenic/differentiative effect of BMP-7 on human bone cells may in part be mediated via IGF-II (7). Activation of IGF-I and markers of early osteoblastic differentiation by BMP-6 in both mice and cell cultures of human osteoblasts indicates that BMP-6 might act on bone at least partially via the IGF-I-related pathways and that this mechanisms might be regulated by mutual interactions.

In addition, BMP-6 upregulation of EGF in bones and osteoblasts has not been previously demonstrated and serves as an additional collaboration pathway for transmitting its anabolic bone effect. Recently, importance of EGF and EGF-like ligands in regulating bone growth and modeling has been discovered (25), including the fact that EGF stimulates the differentiation of human mesenchymal stem cells into bone-forming cells as a single molecule picked by a full scale proteomic analysis (9). This finding might explain how BMP-6 forms bone in the bone marrow of OVX mice lacking Bmp-6 gene (19). Thus, EGF might contribute to the BMP-6 anabolic effect on bone *in vivo*, which has been confirmed in this study also on human osteoblasts.

These results collectively indicate that BMP-6 exerts its osteoinductive effect at least in part through the IGF-1 and EGF pathways, which was observed both in a murine model of osteopenia and in human osteoblastic cell line and could serve as a basis for developing new bone-forming drugs based on BMP-6 biology.

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Figure legends

Figure 1. Ex vivo BMD values of sham, OVX and BMP-6 treated mice as analyzed by PIXImus. Within 9 weeks of therapy BMP-6 restored bone volume of tibiae (a), femurs (b) and lumbar spine (c). *, significantly different from OVX control mice ($p<0.05$, ANOVA, Dunnett test).

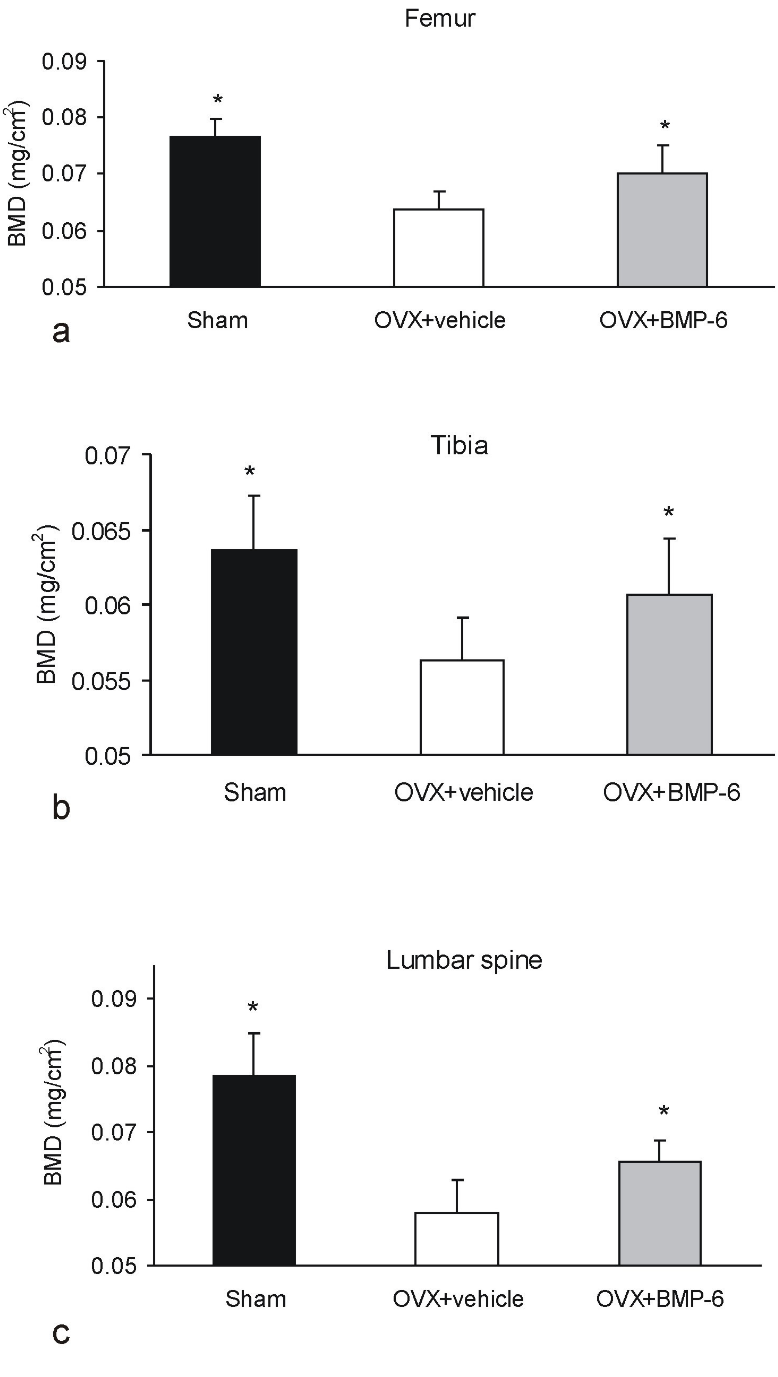

Figure 2. Primary human osteoblasts upon BMP-6 treatment. a) Cell number during the culture period. BMP-6 did not significantly affect cell growth when compared with untreated cultures. b) An increase in alkaline phosphatase activity is seen beginning at day 10 of culture in BMP-6 treated cultures. This activity appeared to peak at day 23 of culture. *, significantly different from untreated cell cultures ($p<0.05$, Student T-test) .c) *In vitro* human osteoblast cultures were Von Kossa stained. High magnification views represent BMP-6 treated human osteoblasts at days 19, 23 and 28 days of culture. Areas of mineralization appear brown. Mineralization usually appeared at approximately day 19 of culture.

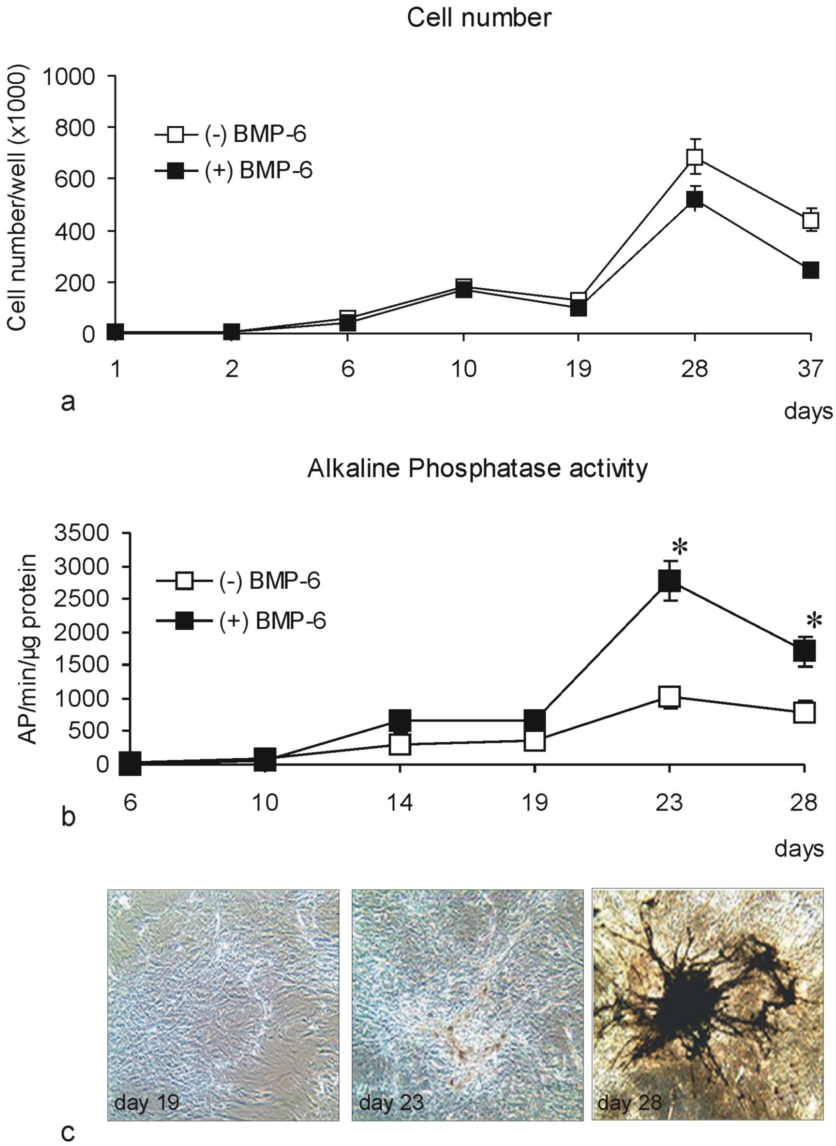

Figure 3. Gene expression of osteogenic markers in mice and human primary osteoblast treated by BMP-6. Total RNA was isolated from femurs of mice (n=15) (a-e) following 4 weeks of BMP-6 therapy and from human osteoblasts at certain time points (day 2,6, 10, 14, 19, 23 and 28) of culture (f-j). Results are represented as fold change of comparative expression level to OVX group of animals (a-e) or as fold change as compared to the first day of culture period (f-j). *, significantly different from OVX control mice (p<0.05, ANOVA, Dunnett test).

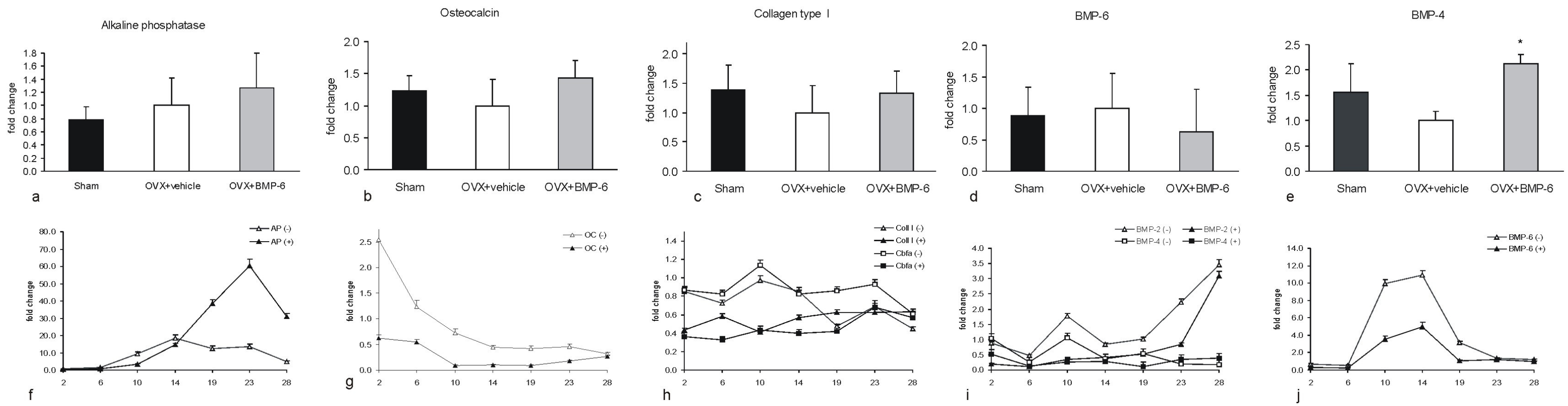

Figure 4. Gene expression of IGF-I (a), EGF (b) and IGFbp3 (c) in mice and IGF-I (d) and EGF (e) in human primary osteoblast treated with BMP-6. Total RNA was isolated from femurs of mice (n=15) (a-c) following 4 weeks of BMP-6 therapy and from human osteoblasts at certain culturing time points (day 7, 12, 17, 23) (d, e) Results are represented as fold change of comparative expression level to OVX group of animals (a-c) or as fold change as compared to the first day of culture period (d, e). *, significantly different from OVX control mice ($p<0.05$, ANOVA, Dunnett test).

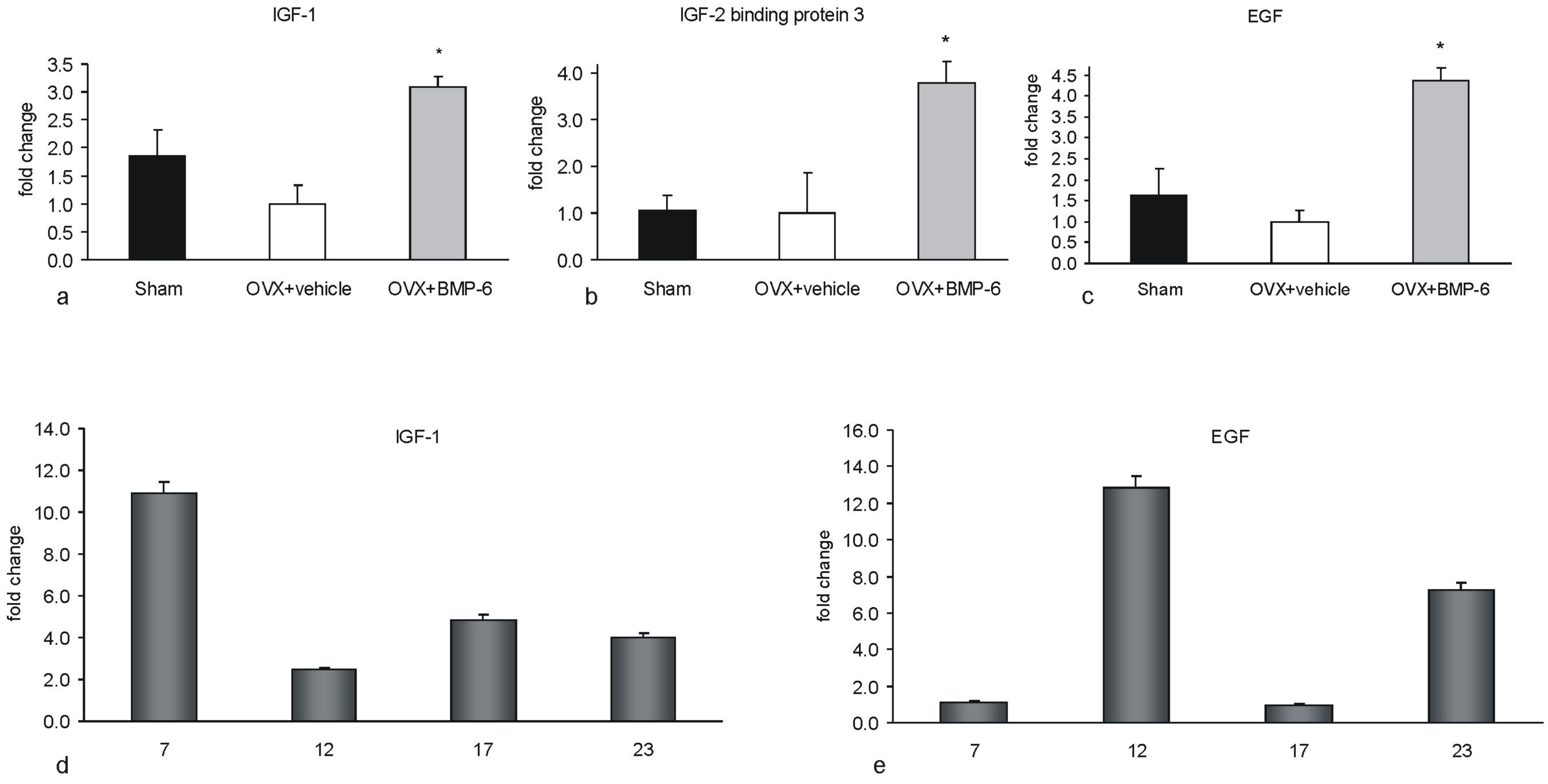

Table 1. List of primers used in quantitative PCR reactions for gene expression analysis in murine femurs

Gene	Forward Primer	Reverse primer
AP	5'-CGGACATCATGAGGGTAAGG-3'	5'-GAG ACA TTT TCC CGT TCACC-3'
BMP 4	5'-GACTTCGAGGCGACACTTCTA-3'	5'-GCCGGTAAAGATCCCTCATGTAA-3'
BMP 6	5'-CAACGCCCTGTCCAATGAC-3'	5'-ACTCTTGCGGTTCAAGGAGTG-3'
EEF1	5'-ACCGCACCCCTGAATTTCTC-3'	5'-CTGGCGTACTTCCTCGCAG-3'
EGF	5'-TTGGTATGCAAGGATGTGTC-3'	5'-CCACTTTGCGAAGTAACTTGGTA-3'
GAPDH	5'-AGGTCGGTGTGAACGGATTTG-3'	5'-TGTAGACCATGTAGTTGAGGTCA-3'
IGF2bp3	5'-GTTGAGCACTCGGTCCCTAAA-3'	5'-CCGTTTCCGAATCCGTGTT-3'
IGF-I	5'-GACCGAGGGGCTTTTACTTCA-3'	5'-GGACGGGGACTTCTGAGTCTT-3'
Osteocalcin	5'-CTGACCTCACAGATCCCAAGC-3'	5'-TGGTCTGATAGCTCGTCACAAG-3'
Collagen type I	5'-TGTGTGCGATGACGTGCAAT-3'	5'-GGGTCCCTCGACTCCTACA-3'
β-2-microglobulin	5'-TTCTGGTGCTTGTCTCACTGA-3'	5'-CAGTATGTTCGGCTTCCCATTC-3'
β-actin	5'-GTGGGCCGCTCTAGGCACCAA-3'	5'-CTCTTTGATGTCACGCACGATTTC-3'

Abbreviations used in the table: AP = Alkaline phosphatase; BMP = Bone morphogenetic protein; EE1G = Eukaryotic translation elongation factor 1 gamma; GAPDH = Glyceraldehyde-3-phosphate dehydrogenase; IGF = Insulin growth factor; bp = binding protein

Table 2. Functional sets of genes specifically altered in BMP-6 treated mice

	Pathway name	ES
OVX vs. BMP-6	Wnt_Signaling	0,348
	il1rPathway	0,330
	ST_Wnt_beta_catenin_Pathway	0,322
	p38mapkPathway	0,310
	cell_adhesion	0,306
	lairPathway	0,300
	FRASOR_ER_UP	0,239
BMP-6 vs. OVX	carm-erPathway	0,399
	igf1mtorPathway	0,239
	etsPathway	0,216
	egfPathway	0,170
	igf1rPathway	0,147

GSEA results of functional sets of genes altered following BMP-6 therapy. n=5 per group. ES=enrichment score. Estimated statistical significance of $p<0.05$ was taken as a cut-off value in the analysis.