

## Effects of bioactive peptides isoleucine-proline-proline (IPP), valine-proline-proline (VPP) and leucine-lysine-proline (LKP) on gene expression of osteoblasts differentiated from human mesenchymal stem cells

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Food-derived bioactive peptides are reported to express a variety of functions *in vivo*. We studied the *in vitro* effect of three bioactive tripeptides, isoleucine-proline-proline (IPP), valine-proline-proline (VPP) and leucine-lysine-proline (LKP), on osteoblast proliferation and gene expression. We used UMR-106 osteosarcoma cells, human marrow-derived mesenchymal stem cells (hMSC) and osteoblasts differentiated from hMSC. Treatment with 50 µM-IPP increased UMR-106 cell and hMSC proliferation. The gene expression of hMSC-differentiated osteoblasts was analysed by the microarray method. Microarray analysis revealed that IPP up-regulated 270 genes and down-regulated 100 genes. VPP and LKP, by contrast, had a very modest influence on osteoblast gene expression. Real-time PCR confirmed that IPP up-regulated PTHrP, BMP-5 and CREB-5 and down-regulated VDR and caspase-8. IPP possesses potential to increase osteoblast proliferation, differentiation and signalling. Agents that increase the number and function of osteoblasts could improve bone mass and structure, and decrease fracture risk.

### IPP: Osteoblast proliferation: Dietary protein: Bioactive peptide

Protein accounts for one-third of bone mass, making bone one of the most protein-dense tissues of the body. Many epidemiological studies report high dietary protein intake to have a positive association with bone mineral mass<sup>1–4</sup> and fracture repair<sup>5–7</sup>. Dietary protein plays an important role in bone strength<sup>4,8</sup> as it is essential for bone turnover and matrix formation<sup>8,9</sup>. Moreover, dietary protein increases intestinal calcium absorption<sup>10</sup>, enhances insulin-like growth factor-I production and action<sup>11</sup>, and serves as a source of bioactive compound formation.

Bioactive peptides can be present as such in dietary protein or incorporated in the primary sequence of proteins. Active peptides are released when proteins are broken down in the gastrointestinal tract by proteolytic enzymes and in the bacterial fermentation processes. Active peptides can be formed from protein of animal and plant origin<sup>12</sup>. Good candidate proteins for peptide formation come from eggs, meat, fish, soya and wheat, but by far the most from milk-based products<sup>13</sup>. Naturally occurring bioactive peptides are found in traditional foods such as ripened cheese and lactic acid fermented sour milk. During microbial fermentation bioactive peptides isoleucine-proline-proline (IPP) and valine-proline-proline (VPP) are formed from milk caseins. Leucine-lysine-proline (LKP), another active peptide, is found in traditional Japanese fish meal containing processed dried bonito<sup>14</sup>. The commercially

available functional foods containing bioactive peptides include soft drinks (caseinophosphopeptide), milk drink (VPP, IPP), soup (LKP) and sour milk (VPP, IPP)<sup>13</sup>. Bioactive peptides are also used in non-food matrices for health effects, e.g. toothpaste, mouth rinse and chewing gum<sup>13</sup>. Bioactive peptides have a wide range of activities. Some peptides express activity in the intestine<sup>15</sup>, others after absorption<sup>16</sup>. Their actions vary from anticariogenic and antimicrobial functions to opioid-like neuronal effects and angiotensin-converting enzyme inhibitory effects<sup>17–19</sup>. Peptides remaining functional after absorption are typically two to five amino acids in length and have a proline residue in their C-terminus<sup>20</sup>. This structure is common among food-derived tripeptides such as IPP, VPP and LKP.

The *in vivo* effects of IPP and VPP on calcium and bone metabolism have been studied in rats and man. *Lactobacillus helveticus*-fermented milk containing IPP and VPP increased bone mineral density in growing rats<sup>21</sup> and acute calcium absorption in postmenopausal women<sup>22</sup>. These results suggest bioactive peptides can aid bone mass development and maintenance.

The aim of the present *in vitro* study was to investigate the effects of IPP, VPP and LKP on osteoblast proliferation and to clarify their function at the gene level. The results contribute to a better understanding of how bioactive peptides affect

**Abbreviations:** BMP, bone morphogenetic protein; CREB, cAMP response element binding protein; hMSC, human mesenchymal stem cells; IPP, isoleucine-proline-proline; LKP, leucine-lysine-proline; PTH, parathyroid hormone; qRT-PCR, quantitative real-time PCR; VDR, vitamin D receptor; VPP, valine-proline-proline.

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osteoblasts and human mesenchymal stem cells and shed light on the mechanisms by which dietary protein supports bone health at the cellular level.

## Materials and methods

### Cell culture

UMR-106 rat osteosarcoma cells (American Type Culture Collection) were used for preliminary screening of suitable peptide concentration and treatment time. UMR-106 cells were used because they grow fast and share a number of phenotypic properties with mature osteoblasts<sup>23</sup>. UMR-106 were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 50 IU penicillin and 50 µg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> on ninety-six-well plates. The cells were seeded at 1 × 10<sup>4</sup> cells/cm<sup>2</sup>, cultured for 24 h and thereafter the medium was changed to fresh medium containing various concentrations of IPP, VPP or LKP.

A cell line prepared from tumour tissue may not correctly reflect osteogenic changes in normal human mesenchymal lineages, hence we used human mesenchymal stem cells (hMSC; Poietics, Cambrex Bio Science, Walkersville, MD, USA) purified from bone marrow and osteoblasts differentiated from them. The hMSC cells were seeded at 3100 cells/cm<sup>2</sup> and cultured in Poietics mesenchymal stem cell growth medium with mesenchymal cell growth supplements (Cambrex Bio Science) at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> on ninety-six-well plates or 10 ml plastic dishes. Differentiation of the hMSC into osteoblasts was induced when the cultures were approximately 50% confluent. The medium was then substituted with either a fresh growth medium (α-modified Eagle's medium, 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin) or a differentiation medium (α-modified Eagle's medium, 10% fetal bovine serum, 10<sup>-8</sup> M-dexamethasone, 50 µg/ml L-ascorbic acid and 10 mM-β-glycerophosphate (GP), 100 U/ml penicillin and 100 U/ml streptomycin). After 12–14 d of differentiation osteoblastic phenotype was confirmed by the

amplification of osteocalcin with quantitative real-time PCR (qRT-PCR; primers in Table 1), by alkaline phosphatase staining (Sigma Histological Alkaline Phosphatase Kit No. 86; Sigma, St Louis, MO, USA) and by assessing mineralization with Alizarin Red S staining<sup>24</sup>. There was no visible alkaline phosphatase staining or mineralization in the undifferentiated cells (Fig. 1). Osteoblastic phenotype was confirmed before each experiment.

### Isoleucine-proline-proline, valine-proline-proline and leucine-lysine-proline tripeptides

The peptides were synthesized by 9-fluorenylmethyloxycarbonyl chemistry and purified by HPLC reverse-phase columns. Peptides were synthesized in the Core Facility for Synthetic Peptides, at the Division of Biochemistry, Department of Biological and Environmental Sciences, University of Helsinki, Finland. Peptides were dissolved in sterile water prior to administration.

### Proliferation assay

A proliferation experiment was carried out with 5, 50 and 500 µM-peptide concentrations and with treatment times of 6, 24 and 48 h. DNA synthesis activity was assessed by a Cell Proliferation ELISA kit (Roche Diagnostics GmbH, Mannheim, Germany). The 5-bromo-2'-deoxyuridine (BrdU) binds to the newly synthesized cellular DNA. The 5-bromo-2'-deoxyuridine incorporation was measured with a spectroscopic plate reader at 450 nm (Multiskan Ex; Thermo Labsystems, Helsinki, Finland). The assay was performed twice on eight replicate observations per experiment.

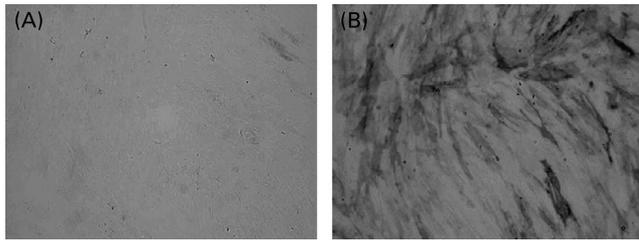
### Total RNA isolation

Total RNA from the hMSC-differentiated osteoblasts was extracted from two separate experiments using RNeasy Protect mini kit (Qiagen GmbH, Hilden, Germany). The RNA concentration was measured at 260 nm by a spectrophotometer (SmartSpec™ 3000; Bio-Rad, Hercules, CA, USA). Integrity of RNA was confirmed by formaldehyde agarose gel (1.2%) electrophoresis.

**Table 1.** Primer sequences for quantitative real-time PCR\*

Primer	Sequence	Product size (bp)	Reference no.
β-Actin	Forward: AGGCCAACCGCGAGAAGATGACC Reverse: GAAGTCCAGGGCGACGTAGCAC	350	43
BMP-2	Forward: GGAGAAGGAGGAGGCAAG Reverse: GACACGTCCATTGAAAGAGC	100	44
BMP-5	Forward: AAGAGGACAAGAAGGACTAAAAATAT Reverse: GTAGAGATCCAGCATAAAGAGAGGT	303	43
Caspase-8	Forward: AGGAGGAGATGAAAAGGGAACCT Reverse: ACCTCAATTCTGATCTGCTCACTTCT	108	45
CREB-5	Forward: GCTTTGGTGCTTTTCTCCAG Reverse: GGTGACACCACAGCACAAAC	245	–
OCN	Forward: ATGAGAGCCCTCACACTCCTCG Reverse: GTCAGCCAACTCGTCACAGTCC	255	46
PTHrP	Forward: GTCTCAGCCGCCGCTCAA Reverse: GGAAGAATCGTCGCCGTA	93	47
VDR	Forward: CCAGTTCGTGTGAATGATGG Reverse: CCTTTTGGATGCTGTAACCTG	256	–

\* All primers were designed as described previously, except primer pairs for VDR and CREB-5, which were designed for the present study with Primer 3 software (v. 0.3.0; Whitehead Institute for Biomedical Research ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi))).



**Fig. 1.** Alkaline phosphatase (ALP) staining of undifferentiated human mesenchymal stem cells (hMSC) (A) and hMSC differentiated into osteoblasts (B). Cultures of undifferentiated cells showed no ALP staining, whereas differentiated cells showed strong staining for ALP.

### Microarray and data processing

The Hum16-K cDNA microarrays were printed on glass slides in the Finnish DNA Microarray Center at Turku Centre for Biotechnology. The chips contain approximately 16 000 annotated genes selected from the Finnish DNA Microarray Centre's Research Genetics clone library spotted in duplicate on a glass slide. The array design accession number is A-MEXP-557 (<http://www.ebi.ac.uk/arrayexpress/>). The expression profiles of osteoblasts differentiated from hMSC were compared after a 24 h treatment with 50  $\mu\text{M}$ -IPP, VPP or LKP. Samples were labelled with FluoroLink Cy-3-dUTP and Cy-5-dUTP (Amersham Biosciences, Uppsala, Sweden) using 20  $\mu\text{g}$  of total RNA for direct labelling and hybridized using augmented reference design<sup>25</sup>. Discrete images for Cy-3 and Cy-5 dyes were obtained using a Scan Express laser-scanning microscope (Packard BioSciences, Meriden, CT, USA) and gene transcript levels were determined from the fluorescence intensities of scanned data image files with the QuantArray Microarray Analysis software (Packard BioSciences). Gene expression profiles were analysed by Kensington Discovery Edition 2.0 software (Inforsense, Cambridge, MA, USA). Replicates were analysed by *t* test to define normal versus changed gene expression within treatments. Results were considered significant at the 95 % significance level ( $P < 0.05$ ). At a *P* value of 0.05 the fold difference in gene expression was 1.8 and it was used in control/treatment comparison to show up- and down-regulated genes. The cDNA microarray experiment is available at accession number E-MEXP-885 (<http://www.ebi.ac.uk/arrayexpress/>).

### Quantitative real-time PCR

The cDNA microarray results were confirmed by quantitative real-time reverse transcription PCR for  $\beta$ -actin, BMP-2, BMP-5, caspase-8, CREB-5, PTHrP and VDR. The RNA preparations (two biological replicates) were used for cDNA synthesis. cDNA was synthesized from 1.5  $\mu\text{g}$  of total RNA by the method described earlier<sup>26</sup>. Each PCR amplification was performed in a qRT-PCR (Mx3000P; Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions using Brilliant SYBR Green QPCR Master mix kit (Stratagene) and cDNA as templates in a 25  $\mu\text{l}$  reaction volume containing 200 nM of the gene-specific primer (Table 1). Fluorescence data were collected during annealing step and analysed with Mx3000P software. Amplification was obtained by denaturing at 95°C for 10 min, followed by forty cycles of denaturing at 95°C for 30 s, annealing at 58°C for 1 min, and

extension at 72°C for 30 s. For dissociation measurement, the amplification cycle was followed by 1 min at 95°C and 30 s at 55°C, after which the temperature was returned to 95°C.

A dilution series of cDNA from human brain total RNA (BD, Biosciences, Clontech) was used as a calibration standard, and data were normalized by the amplification results of  $\beta$ -actin. All reactions were run in triplicate with five dilution concentrations, and the mean value was used to calculate the ratio of target gene/ $\beta$ -actin expression in each sample. Using the ratio in an untreated sample as a standard (100), the relative ratio of the treated sample was presented as the relative expression level of the target gene.

### Statistical analysis

Statistical analyses were performed with GraphPad Prism software version 3.03 (GraphPad Prism, San Diego, CA, USA) and SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Results are expressed as means and their standard errors of at least two independent experiments. Data were analysed using either Student's unpaired *t* test or ANOVA and followed by Tukey's *post hoc* test. The microarray gene expression data for IPP-treated osteoblasts was analysed by principal component analysis to determine the main components affected. Results were considered significant at the 95 % significance level ( $P < 0.05$ ).

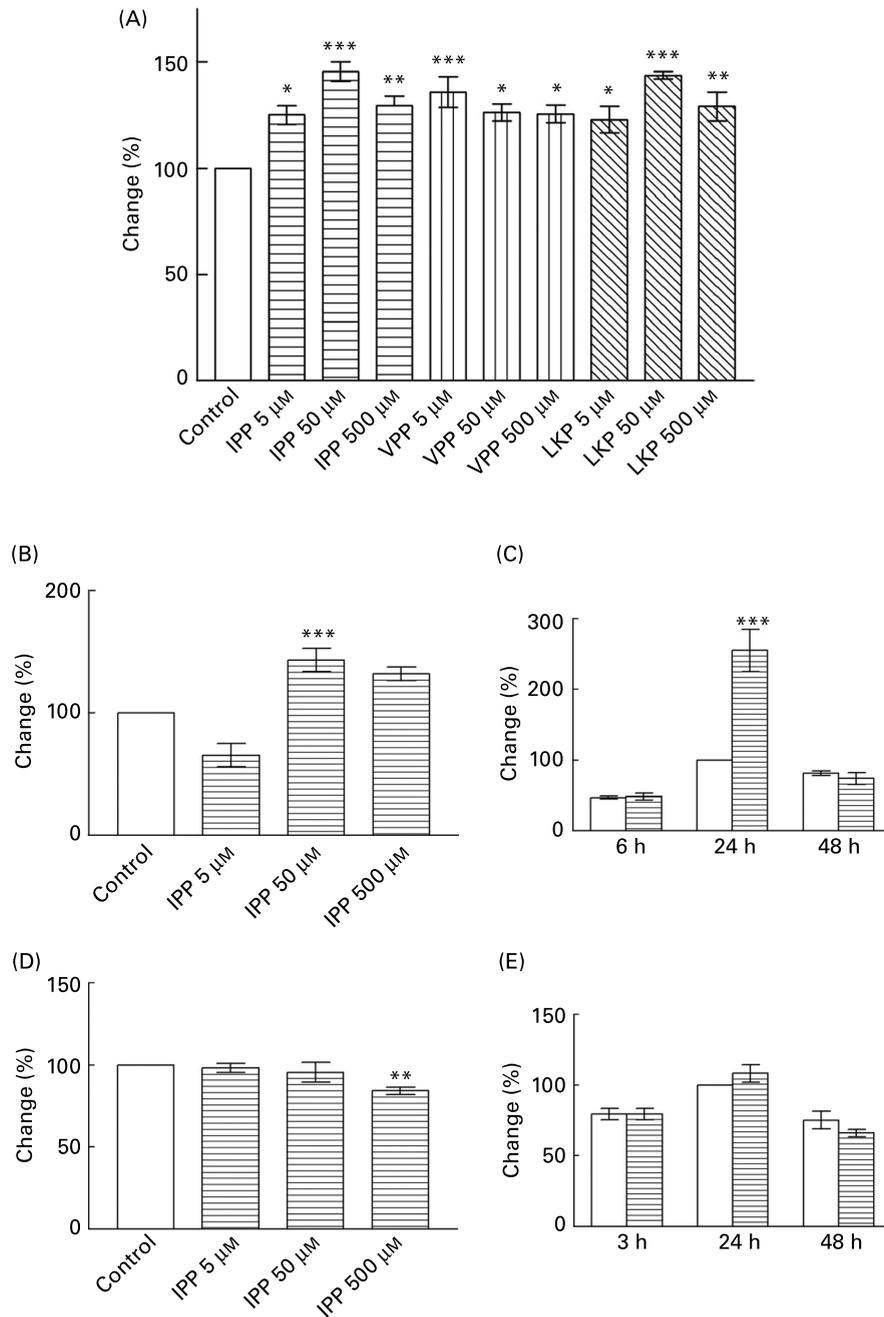
## Results

### Cell proliferation

The preliminary screening of the peptides with UMR-106 cell culture model revealed that all of the tripeptides studied increased cell proliferation as compared with the control cells (Fig. 2(A)). After a 24 h treatment, 50  $\mu\text{M}$ -IPP increased hMSC proliferation (Fig. 2(B, C)). In a dose-response study with mature osteoblasts, IPP decreased proliferation at a concentration of 500  $\mu\text{M}$  (Fig. 2(D)). In a time-response study with 50  $\mu\text{M}$ -IPP, no change was seen in osteoblast proliferation at 3, 24 or 48 h (Fig. 2(E)).

### Microarray

Expression profiling was performed by cDNA microarray. Microarray data analysis revealed IPP to regulate more genes in osteoblasts than VPP or LKP. IPP up-regulated 270 genes and down-regulated 100 genes. The respective numbers for VPP were twenty-five and ten and for LKP they were sixteen and fourteen. IPP did not only regulate a larger number of genes than VPP or LKP, but IPP was also the only tripeptide to up-regulate osteogenic differentiation factors (Table 2). The principal component analysis included 245/270 up-regulated genes and 47/100 down-regulated genes into the different components. The principal component analysis revealed 45 % of the variation in the up-regulated genes from the IPP-treated osteoblasts could be addressed to the first principal component. The second principal component explained 25.3 % and the third 22.6 % of the variation. The first component (115 genes) contained had heavy positive loadings on differentiation-, receptor-, mitochondria-, apoptosis-, enzyme-,



**Fig. 2.** Cell proliferation: results are shown as the relative amounts of 5-bromo-2'-deoxyuridine (BrdU) incorporation. The mean amount of BrdU incorporation in the control cells after 24 h treatment has been given the value 100. Values are means with their standard errors depicted by vertical bars. (A), UMR-106 cell proliferation was increased by 24 h peptide treatment. Mean values were significantly different from those of the controls (ANOVA and Tukey's *post hoc* test): \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . (B), Dose-response effect of isoleucine-proline-proline (IPP) on human mesenchymal stem cells (hMSC) after 24 h treatment showed 50 μM increased cell proliferation. Mean values were significantly different from those of the controls (ANOVA and Tukey's *post hoc* test): \*\*\* $P < 0.001$ . (C), The time-response effect of 50 μM-IPP (▨) was increased hMSC proliferation after 24 h treatment (□, control). Mean values were significantly different from those of the controls (unpaired Student's *t* test): \*\*\* $P < 0.001$ . (D), Osteoblast proliferation was decreased with 500 μM-IPP (24 h). Mean values were significantly different from those of the controls (ANOVA and Tukey's *post hoc* test): \*\* $P < 0.01$ . (E), IPP (50 μM; ▨) had no effect on osteoblast proliferation at 3, 24 or 48 h (□, control). LKP, leucine-lysine-proline; VPP, valine-proline-proline.

signal transduction-, membrane architecture-, function- and transport-related genes. Thus we named it 'Up-regulated cell differentiation'. The second component (forty-seven genes) had heavy positive loadings on cell adhesion-, osteogenic differentiation-, and cell growth and proliferation-related genes. This component was named 'Up-regulated cell

growth'. The third component (eighty-three genes) had heavy positive loadings on transcription-, splicing-, translation-, expressed sequence tag- and 'unknown functions'-related genes, hence it was named 'Up-regulated cell transcription'. Table 3 presents a set of interesting up-regulated genes with a high fold change.

**Table 2.** Summary of the microarray analysis: number of up-regulated (Up) and down-regulated (Down) genes by isoleucine-proline-proline (IPP), valine-proline-proline (VPP) and leucine-lysine-proline (LKP) in osteoblasts differentiated from human mesenchymal stem cells and the average fold change

Functional group	IPP						VPP						LKP					
	Up	Average	SD	Down	Average	SD	Up	Average	SD	Down	Average	SD	Up	Average	SD	Down	Average	SD
Adhesion, cytoskeleton	13	3.2	0.73	8	-2.5	0.24	2	1.6	0.07	1	-1.2	0.00	1	2.0	0.00			
Antiapoptosis	3	2.8	0.31	1	-2.6	0.00							1	2.0	0.00			
Apoptosis	5	3.2	0.87	3	-2.3	0.06	2	1.9	0.43									
Cell cycle, growth, proliferation	25	2.9	0.63	4	-2.2	0.05	2	2.1	0.04	2	-1.6	0.04				2	-1.9	0.12
Oncogenes	4	3.0	0.98	3	-2.5	0.15	1	1.7	0.00									
Suppressor genes	1	2.8	0.00	2	-2.4	0.07				1	-3.9	0.00				1	-2.3	0.00
Differentiation	15	2.8	0.31	4	-2.6	0.54	2	2.8	0.04	2	-1.5	0.01				3	-2.0	0.44
Osteogenic factors	7	3.0	0.60															
Enzymes, metabolism	23	2.7	0.46	7	-2.5	0.28	2	1.8	0.01				3	3.4	2.81	1	-1.9	0.00
EST and unknown functions	46	2.9	0.54	11	-2.6	0.44	5	1.9	0.57	2	-2.0	0.29	4	1.9	0.67	1	-2.3	0.00
Extracellular matrix, cell migration	4	2.9	0.11	6	-2.5	0.33				1	-1.7	0.00						
Hormones, hormone- and cytokine-related proteins	4	2.9	0.47	1	-2.2	0.00												
Immunosystem	5	3.4	0.94	2	-3.1	1.13												
Membrane architecture, function, transport	27	2.8	0.46	13	-2.4	0.18	1	1.6	0.00				1	4.1	0.00			
Mitochondria	7	2.8	0.54	4	-2.4	0.28										1	-1.8	0.00
Receptors and their regulators	9	2.8	0.58	4	-2.2	0.19	3	1.7	0.17							1	-4.9	0.00
Signal transduction	29	2.8	0.52	8	-2.6	0.54	3	2.1	0.69	1	-1.4	0.00	4	2.0	0.22	3	-3.1	2.44
Transcription, splicing, translation	37	2.9	0.54	15	-2.4	0.34							2	1.8	0.10	1	-1.5	0.00
Zing finger proteins	6	2.7	0.53	4	-2.5	0.8	2	1.7	0.05									
Total	270	2.9	0.55	100	-2.5	0.37	25	1.9	0.38	10	-1.9	0.76	16	2.3	1.28	14	-2.4	1.32

EST, expressed sequence tags.

**Table 3.** Effect of isoleucine-proline-proline (50  $\mu\text{M}$ , 24 h) on microarray-analysed gene expression in human osteoblasts (a set of selected up-regulated genes divided into three principal component analysis categories)

Principal component/Accession no.	Gene/protein	Fold change
<b>Up-regulated cell differentiation</b>		
Differentiation		
AA011062	Bone morphogenetic protein 2	2.1
AA779457	Bone morphogenetic protein 5	2.4
Receptors and their regulators		
R51015	Thyroid-stimulating hormone receptor	2.7
R43817	Neuropeptide Y receptor Y1	3.9
Mitochondria		
AA176581	Myoglobin	3.7
	$\beta$ -Polypeptide cytochrome <i>b</i> -245	2.2
Apoptosis		
AA620708	Apoptosis regulator BCL-G	1.9
R49650	Fas apoptotic inhibitory molecule 2	1.8
Enzymes and metabolism		
H87471	Kynureninase (L-kynurenine hydrolase)	3.0
N51496	EF hand calcium binding protein 1	2.5
Signal transduction		
R22790	cAMP responsive element binding protein 5	2.3
	Guanine nucleotide binding protein (G protein)	2.3
Membrane architecture, function and transport		
	Laminin $\gamma$ -3	2.7
AA621725	Nucleoporin 43 kDa	2.8
<b>Up-regulated cell growth</b>		
Cell cycle, growth and proliferation		
A1538525	Mitogen-activated protein kinase kinase kinase 1	3.1
AA402447	Mitogen-activated protein kinase kinase kinase 4	2.3
Adhesion and cytoskeleton		
A1652836	CD96 antigen	4.6
N30615	Contactin 6	3.0
Osteogenic differentiation		
N69672	Glycosylphosphatidylinositol-specific phospholipase D1	4.2
A1272878	Fibroblast growth factor 20	3.2
<b>Up-regulated cell transcription</b>		
Transcription, splicing and translation		
AA857407	Transcription factor 20 (AR1)	2.3
AA975221	Glucocorticoid modulatory element binding protein 2	3.0
AA609862	RNA binding protein with multiple splicing	3.9
EST or unknown function		
AA608709	Vasculin	3.2
AA620709	Hypothetical protein KIAA1434	4.1
AA448002	CD209 antigen-like	2.3

EST, expressed sequence tags.

Principal component analysis on the down-regulated genes in IPP-treated cells showed 73.5% of the variation to be addressed to the first principal component (twenty genes) and the rest of the variation (26.5%) could be addressed to the second component (twenty-seven genes). The first component had heavy positive loadings on cell differentiation-, apoptosis-, enzyme- and cell migration-related genes, hence we named it 'Down-regulated cell differentiation'. The second component captured the variance of cell cycle-, growth-, proliferation-, adhesion- and transcription related genes, hence we named it 'Down-regulated cell growth'. Table 4 presents a set of selected down-regulated genes divided into principal components.

#### Quantitative real-time PCR

A set of IPP-regulated genes was further analysed by qRT-PCR. Microarray analysis had revealed IPP-treated osteoblasts to express more differentiation genes (BMP-2, BMP-5),

transcription factor genes (CREB-5) and hormone-related genes (PTHrP) than control cells. Apoptosis-related caspase-8 and vitamin D receptor gene expression was decreased due to IPP treatment. The qRT-PCR results for these genes were in accordance with the microarray analysis and showed IPP to increase CREB-5, BMP-5 and PTHrP expression, whereas VDR and caspase-8 expression was decreased (Fig. 3). The effect of IPP on BMP-2 was not statistically significant.

#### Discussion

To our knowledge, the present study is the first to describe bioactive peptide IPP, VPP and LKP effects on osteoblast gene expression. IPP regulated more genes in hMSC-differentiated osteoblasts than VPP or LKP. The up-regulated genes could be categorized into three principal components: up-regulated cell differentiation genes, up-regulated cell growth genes and up-regulated cell transcription genes. The up-regulation of these genes indicates IPP enhancing osteoblast

**Table 4.** Effect of isoleucine-proline-proline (50  $\mu$ M, 24 h) on microarray-analysed gene expression in human osteoblasts (a set of selected down-regulated genes divided into two principal component analysis categories)

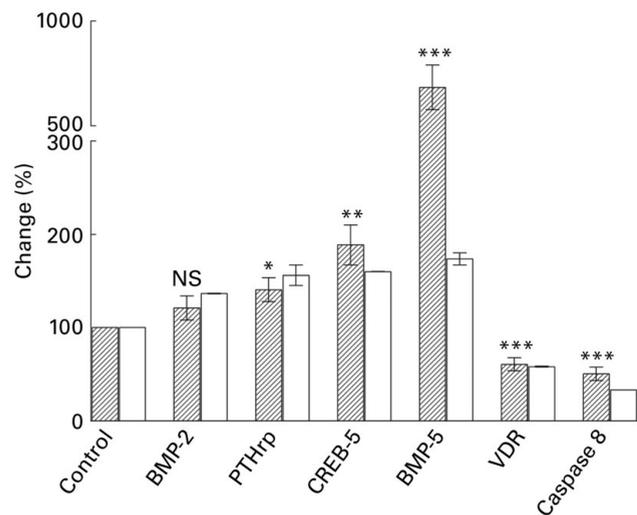
Principal component/Accession no.	Gene/protein	Fold change
<b>Down-regulated cell differentiation</b>		
Differentiation		
AA085749	ATP binding protein associated with cell differentiation	-2.1
	Protein tyrosine phosphatase receptor type	-2.2
Extracellular matrix and cell migration		
	Collagen type II	-2.2
	Fibrinogen $\gamma$ -polypeptide	-2.9
Enzymes and metabolism		
H53275	Histamine <i>N</i> -methyltransferase	-2.9
	Glutamate-cysteine ligase modifier subunit	-2.5
Apoptosis		
	Caspase-8	-1.9
	Protocadherin $\gamma$ -subfamily C	-2.3
<b>Down-regulated cell growth</b>		
Cell cycle, growth and proliferation		
N47966	Cell cycle progression 8 protein	-2.2
	Fanconi anaemia complementation group L	-2.1
Adhesion and cytoskeleton		
	Non-voltage-gated 1 $\alpha$ sodium channel	-2.8
	Tubulin $\gamma$ -2	-2.6
Transcription, splicing and translation		
	Interleukin enhancer binding factor 3	-2.3
N57921	HBxAg transactivated protein 2	-2.2

proliferation and differentiation. The present results also show IPP increasing UMR-106 and hMSC proliferation, but not the proliferation of mature osteoblasts. A previous study by Amedee *et al.*<sup>27</sup> showed mature osteoblast proliferation becoming limited as the state of differentiation increased, hence the present result is in line with their study. Biomaterial studies conducted with Arg-Gly-Asp-containing peptide

sequence also show increased primary calvarial osteoblast cell proliferation in response to the peptide<sup>28,29</sup>.

In the present study 24h treatment with IPP increased UMR-106 and hMSC proliferation and up-regulated PTHrP and CREB-5 genes. Parathyroid hormone (PTH) exerts anabolic and catabolic effects *in vivo* on bone<sup>30</sup>. *In vitro* studies on osteoblasts have shown continuous treatment with very low concentration of PTH stimulates UMR-106-01 and primary osteoblast proliferation<sup>30,31</sup>. PTHrP is a polypeptide hormone sharing a common sequence with PTH. Both hormones can function with the PTH/PTHrP receptor<sup>32</sup>. PTHrP produced by osteoblasts is thought to function locally. Within the skeletal microenvironment, PTHrP propels pluripotential bone marrow stromal cells towards the osteogenic lineage and also exerts antiapoptotic effects<sup>33</sup>. The ability of PTH to drive changes in gene expression is dependent upon activation of transcription factors such as the cAMP response element binding protein (CREB)<sup>34</sup>. In response to PTH, CREB is phosphorylated and in turn activates transcription. The increased proliferation in IPP-treated hMSC could be due to increased PTHrP and CREB. Because cell proliferation and maturation are sequential processes, increased numbers of pluripotent cells and their augmented maturation into osteoblasts could increase the total number of osteoblasts.

Osteoblast differentiation can be divided into commitment of undifferentiated mesenchymal cells to osteoblast progenitors and their maturation into osteoblasts<sup>35</sup>. Bone morphogenetic proteins (BMP) are multifunctional regulators of cells of the osteoblastic lineage. In osteoblasts BMP regulate growth, differentiation and apoptosis. A fundamental function of BMP is to induce the differentiation of mesenchymal cells towards cells of the osteoblastic lineage<sup>36</sup>. As a result, the pool of mature osteoblastic cells increases<sup>36</sup>. The BMP family can be divided into several subgroups based on the BMP gene sequence identity. In this family tree, BMP-5 and



**Fig. 3.** The gene expression of isoleucine-proline-proline-treated osteoblasts analysed by a cDNA microarray method (□) and quantitative real-time PCR (qRT-PCR; ▨). At least 1.8-fold difference was used in control *v.* treatment comparison in microarray data, and the ratio in the untreated sample was used as a control (100). qRT-PCR results were normalized by amplification of the result of  $\beta$ -actin, and the untreated sample has been given the value 100. Values are means, with their standard errors depicted by vertical bars, of triplicate determinations, where all amplified genes have their own controls. Mean values were significantly different from those of the controls (unpaired Student's *t* test): \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

-6 belong to the same subgroup. BMP-6 induces osteoblast differentiation and maturation<sup>35</sup> and BMP-5 has similar osteoinductive properties<sup>37</sup>. BMP also play a role in fracture healing<sup>36</sup>, especially BMP-5, as one characteristic of BMP-5 null mice is impaired fracture repair. The ability of IPP to induce BMP-5 production can increase osteoblast differentiation and augment fracture repair, a feature associated with a protein-rich diet<sup>5-7</sup>. Here, the microarray results showed that IPP up-regulated BMP-2. However, qRT-PCR results revealed no statistical difference in BMP-2 expression between control and IPP-treated cells. As BMP-2 functions early in the cell differentiation pathway<sup>36</sup>, an analysis at an earlier stage might have produced a more significant effect.

Vitamin D receptor (VDR) attenuates osteoblast differentiation *in vitro*<sup>38</sup>. Osteoblasts lacking VDR have enhanced differentiation, as shown by increased alkaline phosphatase activity and increased formation of mineralized matrix. An *in vivo* experiment with VDR knock-out mice revealed increased bone volume and density compared with wild-type mice<sup>39</sup>. The present study showed that IPP decreased VDR, which could lead to an increase in differentiation and bone formation. A previous *in vitro* study on mouse bone marrow-derived osteoblast precursors has shown IPP and VPP activating osteoblast bone formation<sup>40</sup>.

The down-regulated genes in IPP-treated osteoblasts were divided into two principal components: cell differentiation genes and cell growth-related genes. Apoptosis-related genes were located in the 'Down-regulated cell differentiation' component. Apoptosis is the major cause of osteoblast cell death. Jilka *et al.*<sup>41</sup> have shown osteoblast apoptosis to be under the regulatory control of factors produced in the bone microenvironment. Caspases are essential apoptosis effectors, exerting their effects in a cascade involving receptor- and mitochondria-dependent pathways<sup>42</sup>. Caspase-8 is a receptor-mediated caspase which in turn activates caspase-3, the true executioner leading to cell apoptosis. In the present study, caspase-8 was down-regulated and PTHrP up-regulated, suggesting that IPP exerts antiapoptotic effects on osteoblasts. Alterations in the timing and extent of osteoblast apoptosis could have a significant impact on the rate of bone formation<sup>41</sup>. Furthermore, the number of osteoblasts increases if cell proliferation and differentiation is increased and cell apoptosis is decreased.

The present results at the cellular and mRNA levels show tripeptide IPP increasing cell proliferation. Genes associated with cell differentiation were up-regulated and apoptosis inducers were down-regulated. The present findings indicate that IPP enhances gene expression in a way that would increase the differentiation of hMSC into osteoblasts and prolong their viability. Agents that increase the number and function of osteoblasts improve bone mass and structure, thereby decreasing the risk of fracture. Bioactive peptides, such as IPP, might well contribute to the positive effects dietary protein has on bone mineral mass and decreased fracture risk.

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