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120 Abstract

Presence of the pituitary adenylate cyclase-activating polypeptide (PACAP) signalling has been proved in various peripheral tissues. PACAP can activate protein kinase A (PKA) signalling via binding to pituitary adenylate cyclase-activating polypeptide type I receptor (PAC1), vasoactive intestinal polypeptide receptor (VPAC) 1 or VPAC2 receptors. Since little is known about the role of this regulatory mechanism in bone formation, we aimed to investigate the effect of PACAP on osteogenesis of UMR-106 cells. PACAP 1-38 as an agonist and PACAP 6-38 as an antagonist of PAC1 were added to the culture medium. Surprisingly, both substances enhanced protein expressions of collagen type I, osterix and alkaline phosphatase, along with higher cell proliferation rate and an augmented mineralisation. Although expression of PKA was elevated, no alterations were detected in the expression, phosphorylation and nuclear presence of CREB, but increased

		nuclear appearance of Runx2, the key transcription factor of osteoblast differentiation, was shown. Both PACAPs increased the expressions of bone morphogenetic proteins (BMPs) 2, 4, 6, 7 and Smad1 proteins, as well as that of sonic hedgehog, PATCH1 and Gli1. Data of our experiments indicate that activation of PACAP pathway enhances bone formation of UMR-106 cells and PKA, BMP and Hedgehog signalling pathways became activated. We also found that PACAP 6-38 did not act as an antagonist of PACAP signalling in UMR-106 cells.
121	Keywords separated by ' - '	PKA - CREB - Sonic hedgehog - PACAP 6-38 - Mineralisation - Cellular proliferation
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Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) Signalling Enhances Osteogenesis in UMR-106 Cell Line

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Abstract Presence of the pituitary adenylate cyclase-activating polypeptide (PACAP) signalling has been proved in various peripheral tissues. PACAP can activate protein kinase A (PKA) signalling via binding to pituitary adenylate cyclase-activating polypeptide type I receptor (PAC1), vasoactive intestinal polypeptide receptor (VPAC) 1 or VPAC2 receptors. Since little is known about the role of this regulatory mechanism in bone formation, we aimed to investigate the effect of PACAP on osteogenesis of UMR-106 cells. PACAP 1-38 as an agonist and PACAP 6-38 as an antagonist of PAC1 were added to the culture medium. Surprisingly, both substances enhanced protein expressions of collagen type I, osterix and alkaline phosphatase, along with higher cell proliferation rate and an augmented mineralisation. Although expression of PKA was elevated, no alterations were detected in the expression, phosphorylation and nuclear presence of CREB, but increased nuclear appearance of Runx2, the key transcription factor of osteoblast differentiation, was shown. Both PACAPs increased the expressions of bone morphogenetic proteins (BMPs) 2, 4, 6, 7 and Smad1 proteins, as well as that of sonic hedgehog, PATCH1 and Gli1. Data of our experiments indicate that activation of PACAP pathway enhances bone formation of UMR-106 cells and PKA, BMP and Hedgehog signalling pathways became activated. We also found that PACAP 6-38 did not act as an antagonist of PACAP signalling in UMR-106 cells.

Keywords PKA · CREB · Sonic hedgehog · PACAP 6-38 · Mineralisation · Cellular proliferation

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Abbreviations

ALP	Alkaline phosphatase
BMP	Bone morphogenetic protein
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CREB	cAMP response element-binding protein
DMEM	Dulbecco's modified Eagle's medium
dNTP	Deoxynucleotide triphosphate
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
HH	Hedgehog
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
hMSC	Human mesenchymal stem cell
IHH	Indian hedgehog
MAPK	Mitogen-activated protein kinase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PAC1	Pituitary adenylate cyclase-activating polypeptide type I receptor
PACAP	Pituitary adenylate cyclase-activating polypeptide
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline supplemented with 1 % Tween 20
PLC	Phospholipase C
PKA	Protein kinase A
PKC	Protein kinase C
PTHrP	Parathyroid hormone-related peptide
RT-PCR	Reverse transcription followed by polymerase chain reaction
Runx2	Runt-related transcription factor 2
SHH	Sonic hedgehog
TBE	Tris-boric acid-EDTA
TGFβ	Transforming growth factor-β
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal polypeptide
VPAC	Vasoactive intestinal polypeptide receptor

Introduction

Bone formation and regeneration are well-organised processes orchestrated by several signalling pathways. Initially, osteoprogenitor cells undergo a rapid proliferation and then differentiate to early osteoblasts secreting organic bone matrix. In consecutive steps, activity of late osteoblasts results in an intensive extracellular matrix (ECM) mineralisation and ultimately osteocytes are formed. Differentiation processes of osteogenic cells are induced by a few fundamental regulatory pathways; activation of bone morphogenetic protein (BMP) (Chen et al. 2012), WNT (Kim et al. 2013), fibroblast growth factor (FGF) (Marie 2012) and Hedgehog (HH) (Pan et al. 2013) regulated signalling cascades lead to proper bone formation. BMPs, related to the transforming growth factor-β superfamily (TGF-β), are generally considered as cytokines regulating various events during embryonic development including physiological osteogenesis but also play role in ectopic bone formation (Bae et al. 2013). Via the initiation of the activation of several genes, BMPs are key regulators of ECM production during bone and cartilage formation both in vitro and in vivo (Chen et al. 2012; Perrier-Groult et al. 2013; Zouani et al. 2013). The activation of BMP receptors through Smads may induce elevated expression of alkaline phosphatase (ALP) or collagen type I; moreover, it can activate the expression of bone-specific transcription factors such as osterix (Wang et al. 2013). Some of these cytokines, including BMPs 2, 4, 5, 6 and 7 have been identified as markers of proper osteogenic differentiation, although experimental evidence suggested that a combined expression of these morphogenes was more essential than the single presence of any of them (Lavery et al. 2008). One mechanism which may result in transcriptional activation of BMP encoding genes is the increased activity of protein kinase A (PKA). This kinase can phosphorylate cAMP response element-binding protein (CREB) transcription factor which subsequently translocates into the nucleus and can induce messenger RNA (mRNA) expression of BMPs (Zhang et al. 2011). The complexity of this regulatory system is hallmarked by the fact that activation of genes encoding BMPs can also be regulated by HH signalling pathway, e.g. via a negative feedback loop of sonic hedgehog (SHH) signalling (Bastida et al. 2009; Jiang et al. 2013). The members of HH family are fundamental regulators of various embryonic developmental processes, e.g. neuronal differentiation, tooth and limb development (Ehlen et al. 2006; Hu et al. 2013; Vazin et al. 2014). The well-balanced spatio-temporal expression of HH molecules is crucial during endochondral ossification both for survival of chondrocytes and for induction of their physiological apoptotic program (St-Jacques et al. 1999). The elevated expression of Indian hedgehog (IHH) can regulate the expression of parathyroid hormone-related peptide (PTHrP) which in turn may upregulate the activation of Runx2 transcription factor, a key player of bone formation (Ochiai et al. 2010; St-Jacques et al. 1999).

Pituitary adenylate cyclase-activating polypetide (PACAP), a member of the VIP–secretin–GHRH–glucagon superfamily, was originally isolated from extract of rat hypothalamus (Miyata et al. 1989). The expression of the neuropeptide has been demonstrated in various peripheral organs, such as gonads (Reglodi et al. 2012), intestinal tract (Pirone et al. 2011) and urinary systems (Gonkowski and Całka 2012), and the presence of PACAP has also been verified in human milk and blood plasma (Borzsei et al. 2009). The posttranslationally modified active form of the neuropeptide consists of 38 amino acids, and a shorter 27 amino acid-long biologically active variant also exists (Miyata et al. 1989). Several in vitro and in vivo data demonstrated the importance of PACAP during neuronal differentiation and its general role in embryonic development (Ago et al. 2011; Falluel-Morel et al. 2008; Ohta et al. 2006). Trophic effect of PACAP has been demonstrated in oxidative stress, under ischaemic, toxic or traumatic conditions (Horvath et al. 2011; Sanchez et al. 2008; Shioda et al. 2006; Tamas et al. 2012; Wada et al. 2013). The neuropeptide is generally expressed by neurons or released in autonomic nerve endings (Braas et al. 1998; Inglott et al. 2012), but several nonneuronal cell types such as developing germ cells of testis (Shioda et al. 1994), intestinal tissue (Pirone et al. 2011) and endothelial cells (Seeliger et al. 2010) have also been found to release PACAP. It can bind to three specific receptors (pituitary adenylate cyclase-activating polypeptide type I receptor (PAC1), vasoactive intestinal polypeptide receptor (VPAC) 1 and VPAC2 (Jolivel et al. 2009)), from which the last two can be activated by both PACAP and VIP with equal efficiency, while PAC1 receptor has 100-fold

greater affinity to PACAP than VIP (Gourlet et al. 1997). Besides these well-characterised members of PACAP signalling pathway, recent data indicated the existence of a novel PACAP receptor or a novel PACAP receptor-mediated pathway (Jansen-Olesen et al. 2014). The canonical PACAP signalling pathway operates via activation of PAC1 receptor leading to the elevation of intracellular cAMP concentration and consequent activation of PKA signalling (Vaudry et al. 2009). The truncated form of the neuropeptide, PACAP 6-38, having the first five amino acids cleaved down, is regarded as an antagonist of PAC1 receptor (Vandermeers et al. 1992), although its antagonistic effect seems to be tissue and cell type dependent (Reglodi et al. 2008).

Presence of the members of PACAP signalling system has already been demonstrated in different osteogenic cells such as MC3T3 (Nagata et al. 2009) and UMR-106 cells (Kovacs et al. 1996). It has also been shown that PACAP binding can elevate cAMP concentration of UMR-106 cells (Kovacs et al. 1996), and sporadic data prove the importance of the neuropeptide in osteogenesis or bone fracture healing. UMR-106 cell line was originally isolated from rat osteosarcoma. Cells of this cell line can differentiate into osteoblasts after serum withdrawal and show the signs of regular bone formation along with the expression of osteogenic markers and secretion of both organic and inorganic components of bone ECM (Midura et al. 1990; Forrest et al. 1985).

In this report, we demonstrate that addition of PACAP neuropeptides enhances bone formation along with elevated nuclear presence of Runx2. PACAPs activate expression of various BMPs and increase the nuclear signal of their downstream target Smad1. Moreover, elevated expression of the components of HH signalling pathways and an enhanced nuclear presence of Gli1 transcription factor is also detected. These observations suggest a multifactorial and dominantly noncanonical PACAP signalling in UMR-106 osteoblastic cells.

Materials and Methods

Cell Culturing

Rat osteosarcoma osteoblast-like cell line, UMR-106 (ATCC® CRL-1661™), was used to monitor osteogenic differentiation (Forrest et al. 1985). Cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) (PAA Laboratories, Pasching, Austria) supplemented with 10 % foetal bovine serum (FBS) (PAA Laboratories) at 37 °C in the presence of 5 % CO_2 and 80 % humidity in a CO_2 incubator. At 70 % confluence, normal medium was changed to DMEM without FBS for inducing osteogenic differentiation. This day was considered as day 0.

Administration of PACAP Polypeptides

PACAP 1-38 at 100 nM (stock solution 100 μM, dissolved in sterile distilled water) was used as agonist of PAC1 receptor; as an antagonist, PACAP 6-38 at 10 μM (stock solution 10 mM, dissolved in sterile distilled water) was applied continuously from day 1. PACAPs were synthesised as previously described (Jozsa et al. 2005).

Staining Procedures for Light Microscopical Analysis

UMR cells of different experimental groups were cultured on round coverslips (Menzel-Gläser, Menzel GmbH, Braunschweig, Germany) placed into Petri dishes (PAA Laboratories). On day 4 or 8, cells were fixed in a 4:1 mixture of absolute ethanol and 40 % formaldehyde. For morphological analysis, cells were stained with haematoxylin-eosin (HE, Sigma-Aldrich, St. Louis, MO, USA); for visualisation of collagen accumulation, Picrosirius red (Sigma-Aldrich) was used; calcium-rich deposits were evaluated with Alizarin red (Sigma-Aldrich); and von Kossa method (Millipore, Billerica, MA, USA) was used to demonstrate appearance of calcium phosphate in cell cultures. All staining protocols were carried out according to the instructions of manufacturer. Photomicrographs were taken using an Olympus DP72 camera on a Nikon Eclipse E800 microscope (Nikon Corporation, Tokyo, Japan).

Monitoring of Cell Proliferation with ^{3}H-thymidine Incorporation, Mitochondrial Activity with MTT Assay

DMEM medium containing 1 μCi/mL ^{3}H-thymidine (diluted from thymidine [6-^{3}H] 20–30 Ci/mmol; 0.74–1.11 TBq/mmol), American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) was added to cell cultures for 16 h on day 4 of culturing. Cells were fixed with ice-cold 5 % trichloroacetic solution for 20 min and were harvested into wells of special opaque 96-well microtitre plates (Wallac, PerkinElmer Life and Analytical Sciences, Shelton, CT, USA). Samples were air-dried for 1 week, and radioactivity was counted by Chameleon liquid scintillation counter (Chameleon, Hidex, Turku, Finland).

For investigation of general viability or mitochondrial activity, 25 μL 3-[4,5-dimethylthiazolyl-2]-2,5-diphenyltetrazolium bromide (MTT) reagent (25 mg MTT/5 mL PBS) was pipetted into each Petri dish on day 4 of culturing. Cells were incubated for 2 h at 37 °C, followed by addition of 500 μL MTT solubilising solution; absorption of samples was measured at 570 nm (Chameleon, Hidex).

RT-PCR Analysis

Cells of UMR-106 cell line were dissolved in TRIzol (Applied Biosystems, Foster City, CA, USA), and after the addition of

t1.1 **Table 1** Nucleotide sequences, amplification sites, GenBank accession numbers, amplimer sizes and PCR reaction conditions for each primer pair are shown

t1.2	Gene	Primer	Nucleotide sequence (5′→3′)	GenBank ID	Annealing temperature	Amplimer size (bp)
t1.3	Alkaline phosphatise (Alpl)	Sense	GAA GTC CGT GGG CAT CGT (474–491)	NM_013059	59 °C	347
t1.4		Antisense	CAG TGC GGT TCC AGA CAT AG (801–820)			
t1.5	BMP2 (Bmp2)	Sense	AAG CCA GGT GTC TCC AAG (697–714)	NM_017178.1	53 °C	209
t1.6		Antisense	AAG TCC ACA TAC AAA GGG TG (886–905)			
t1.7	BMP4 (Bmp4)	Sense	TAG TCC CAA GCA TCA CCC (876–893)	NM_012827.2	53 °C	294
t1.8		Antisense	TCG TAC TCG TCC AGA TAC AAC (1,149–1,169)			
t1.9	BMP6 (Bmp6)	Sense	CCC AGA TTC CTG AGG GTG A (936–954)	NM_013107.1	56 °C	248
t1.10		Antisense	CAT GTT GTG CTG CGG TGT (1,166–1,183)			
t1.11	BMP7 (Bmp7)	Sense	AGG GAG TCC GAC CTC TTC T (607–625)	NM_001191856.1	54 °C	297
t1.12		Antisense	GTT CTG GCT GCG TTG TTT (886–903)			
t1.13	BMPR1 (Bmpr1a)	Sense	CCA TTG CTT TGC CAT TAT (240–257)	NM_009758.4	47 °C	487
t1.14		Antisense	TTT ACC AAC CTG CCG AAC (709–726)			
t1.15	Collagen type I (Col1a1)	Sense	GGG CGA GTG CTG TGC TTT (348–365)	NM_007742.3	60 °C	388
t1.16		Antisense	GGG ACC CAT TGG ACC TGA A (717–735)			
t1.17	CREB (Creb1)	Sense	AGA TTG CCA CAT TAG CCC (95–112)	NM_031017.1	52 °C	441
t1.18		Antisense	GCT GTA TTG CTC CTC CCT (518–535)			
t1.19	GAPDH (Gapdh)	Sense	TGG CAA AGT GGA GAT TGT TG (69–88)	NM_008084.2	59 °C	486
t1.20		Antisense	GTC TTC TGG GTG GCA GTG AT (535–554)			
t1.21	Gli1 (Gli1)	Sense	CCA CCC TAC CTC TGT CTA TTC G (2,201–2,222)	NM_010296.2	49 °C	423
t1.22		Antisense	CAC CCT TGT TCT GGT TTT ACC (2,603–2,623)			
t1.23	IHH (Ihh)	Sense	CCA ACT ACA ATC CCG ACA TCA (248–268)	NM_053384.1	58 °C	477
t1.24		Antisense	GTC TTC ATC CCA GCC TTC C (390–408)			
t1.25	Osterix (Sp7)	Sense	GCC TAC TTA CCC GTC TGA CTT T (525–543)	NM_001037632.1	56 °C	131
t1.26		Antisense	GCC CAC TAT TGC CAA CTG C (634–652)			
t1.27	PACAP (ADCYAP1)	Sense	GAA GAC GAG GCT TAC GAC CA (314–333)	NM_001001291	56 °C	288
t1.28		Antisense	GTC CGA GTG GCG TTT GGT (584–601)			
t1.29	PAC1 (ADCYAP1R1)	Sense	CTA CGC CCT TTA CTA CCC AG (210–229)	NM_016989.2	49 °C	247
t1.30		Antisense	GTA TTT CTT GAC AGC CAT TTG T (435–456)			
t1.31	PKA (Prkaca)	Sense	GCA AAG GCT ACA ACA AGG C (847–865)	NM_008854	53 °C	280
t1.32		Antisense	ATG GCA ATC CAG TCA ATC G (1,109–1,126)			
t1.33	PKCα (Prkca)	Sense	AGG GAT GAA ATG CGA CAC C (652–670)	NM_001105713.1	55 °C	408
t1.34		Antisense	GAG ACG CCG AAG GAA AGG (1,042–1,059)			
t1.35	PTCH1 (Ptch1)	Sense	TGC TAC AAA TCA GGG GAA CTT (565–585)	NM_053566.1	56 °C	310
t1.36		Antisense	CAG GGC AAT CTG GGT CGG (854–874)			
t1.37	PTHrP (Pthlh)	Sense	CAG ACG ACG AGG GCA GAT (290–307)	NM_012636.1	58 °C	145
t1.38		Antisense	GAC CGA GTC CTT CGC TTT (417–434)			
t1.39	Runx2 (Runx2)	Sense	GGA CGA GGC AAG AGT TTC A (598–616)	NM_001278483.1	55 °C	249
t1.40		Antisense	TGG TGC AGA GTT CAG GGA G (828–846)			
t1.41	SHH (Shh)	Sense	TCG TGC TAC GCA GTC ATC G (1,042–1,060)	NM_017221.1	56 °C	156
t1.42		Antisense	CCT CGC TTC CGC TAC AGA (1,180–1,197)			
t1.43	Smad1 (Smad1)	Sense	AGC ACC TAC CCT CAC TCC C (935–953)	NM_013130.2	56 °C	306
t1.44		Antisense	GAA ACC ATC CAC CAA CAC G (1,222–1,240)			
t1.45	VEGF (Vegfa)	Sense	GCT ACT GCC GTC CGA TTG (1,167–1,184)	NM_001025250.3	54 °C	267
t1.46		Antisense	GCT TTG TTC TGT CTT TCT TTG G (1,412–1,433)			
t1.47	VPAC1 (VIPR1)	Sense	GTT CTA TGG CAC GGT CAA (376–393)	NM_001097523	52 °C	216
t1.48		Antisense	AGC AAT GTT CGG GTT CTC (573–590)			
t1.49	VPAC2 (VIPR2)	Sense	TCG GAA CTA CAT CCA TCT (477–497)	NM_001014970	48 °C	177

t1.50 **Table 1** (continued)

Gene	Primer	Nucleotide sequence (5′→3′)	GenBank ID	Annealing temperature	Amplimer size (bp)
t1.51	Antisense	TTT GCC ATA ACA CCA TAC (636–653)			

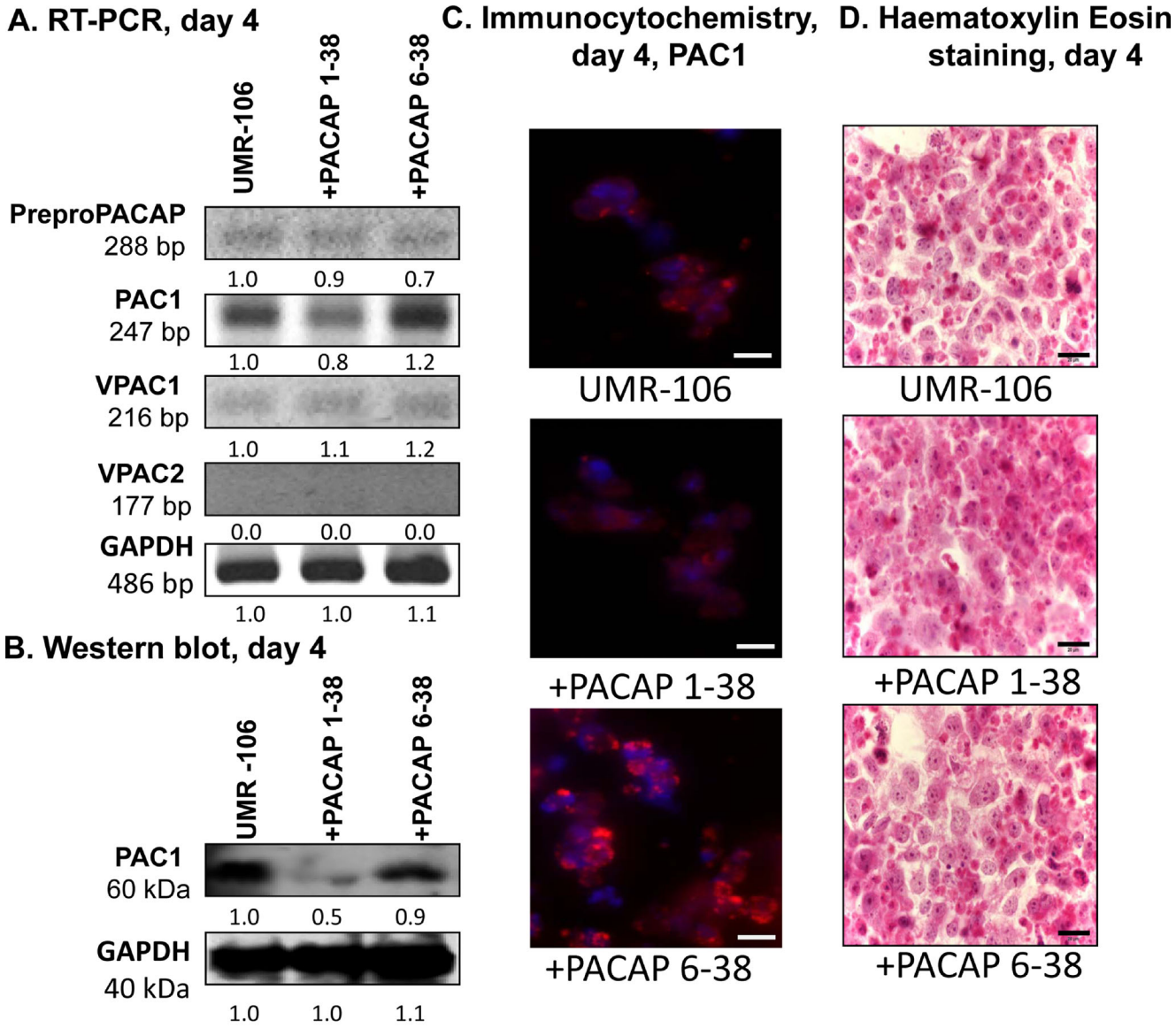
20 % RNase-free chloroform, samples were centrifuged at 4 °C at 10,000×*g* for 15 min. Samples were incubated in 500 μL of RNase-free isopropanol at −20 °C for 1 h; then, total RNA was harvested in RNase-free water and stored at −20 °C. The assay mixture for reverse transcriptase reaction contained 2 μg RNA, 0.112 μM oligo(dT), 0.5 mM deoxynucleotide triphosphate (dNTP), 200 units of High Capacity RT (Applied Bio-Systems) in 1× RT buffer. For the sequences of primer pairs and further details of polymerase chain reactions, see Table 1. Amplifications were performed in a thermal cycler (Labnet MultiGene™ 96-well Gradient Thermal Cycler; Labnet International, Edison, NJ, USA) in a final volume of 21 μL (containing 1 μL forward and reverse primers [0.4 μM], 0.5 μL dNTP [200 μM], and 5 units of Promega GoTaq® DNA polymerase in 1× reaction buffer) as follows: 95 °C, 2 min, followed by 35 cycles (denaturation, 94 °C, 1 min; annealing at optimised temperatures as given in Table 1 for 1 min; extension, 72 °C, 90 s) and then 72 °C, 10 min. PCR products were analysed by electrophoresis in 1.2 % agarose gel containing ethidium bromide. GAPDH was used as internal control. Optical density of signals was measured by using ImageJ 1.40 g freeware, and results were normalised to the optical density of untreated control cultures.

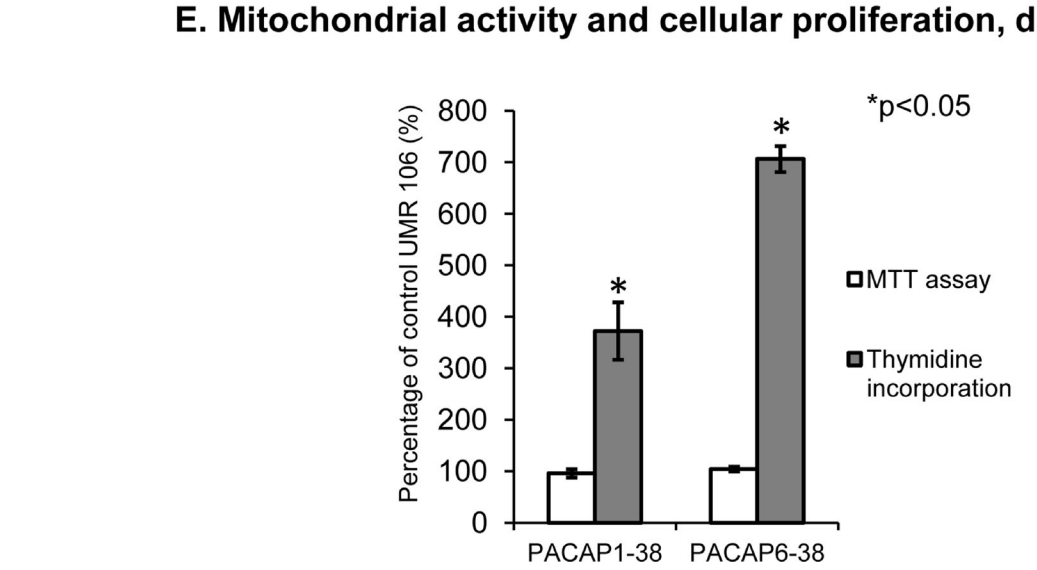
Western Blot Analysis

Cells were washed in physiological NaCl solution and were harvested. After centrifugation, cell pellets were suspended in 100 μL of homogenisation radio immunoprecipitation assay (RIPA) buffer (150 mM sodium chloride; 1.0 % NP_40, 0.5 % sodium deoxycholate; 50 mM Tris, pH 8.0) containing protease inhibitors (aprotinin (10 μg/mL), 5 mM benzamidine, leupeptin (10 ug/mL), trypsine inhibitor (10 ug/mL), 1 mM PMSF, 5 mM EDTA, 1 mM EGTA, 8 mM Na fluoride, 1 mM Na orthovanadate). Samples were stored at −70 °C. Suspensions were sonicated by pulsing burst for 30 s at 40 A (Cole-Parmer, IL, USA). For Western blotting, total cell lysates were used. Samples for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were prepared by the addition of Laemmli electrophoresis sample buffer (4 % SDS, 10 % 2-mercaptoehtanol, 20 % glycerol, 0.004 % bromophenol blue, 0.125 M Tris–HCl pH 6.8) to cell lysates

t2.1 t2.2 **Table 2** Tables of antibodies used in the experiments

Antibody	Host animal	Dilution	Distributor
t2.3 Anti-PAC1	Rabbit, polyclonal	1:600	Sigma-Aldrich, St. Louis, MO, USA
t2.4 Anti-PKA	Rabbit, polyclonal	1:600	Cell Signaling, Danvers, MA, USA
t2.5 Anti-CREB	Rabbit, polyclonal	1:800	Millipore, Billerica, MA, USA
t2.6 Anti-p-CREB	Rabbit, polyclonal	1:800	Millipore, Billerica, MA, USA
t2.7 Anti-Coll. I.	Mouse, monoclonal	1:1,000	Sigma-Aldrich, St. Louis, MO, USA
t2.8 Anti-osterix	Goat, polyclonal	1:200	Santa Cruz Biotechnology, Dallas, TX, USA
t2.9 Anti-ALP	Rabbit, polyclonal	1:600	Abcam, Cambridge, UK
t2.10 Anti-Runx2	Rabbit, polyclonal	1:600	Cell Signaling, Danvers, MA, USA
t2.11 Anti-PKCα	Rabbit, polyclonal	1:600	Cell Signaling, Danvers, MA, USA
t2.12 Anti-BMP2	Rabbit, polyclonal	1:400	Abcam, Cambridge, UK
t2.13 Anti-BMP4	Rabbit, polyclonal	1:600	Cell Signaling, Danvers, MA, USA
t2.14 Anti-BMP6	Goat, polyclonal	1:200	Santa Cruz Biotechnology, Dallas, TX, USA
t2.15 Anti-BMP7	Rabbit, polyclonal	1:600	Abcam, Cambridge, UK
t2.16 Anti-BMPR1	Mouse, monoclonal	1:600	Abcam, Cambridge, UK
t2.17 Anti-Smad1	Rabbit, polyclonal	1:600	Cell Signaling, Danvers, MA, USA
t2.18 Anti-SHH	Rabbit, polyclonal	1:600	Cell Signaling, Danvers, MA, USA
t2.19 Anti-IHH	Rabbit, polyclonal	1:600	Millipore, Billerica, MA, USA
t2.20 Anti-PTHrP	Mouse, monoclonal	1:300	R&D Systems, Minneapolis, MN, USA
t2.21 Anti-PTCH1	Rabbit, polyclonal	1:600	Abcam, Cambridge, UK
t2.22 Anti-Gli1	Rabbit, polyclonal	1:600	Cell Signaling, Danvers, MA, USA
t2.23 Anti-GAPDH	Rabbit, polyclonal	1:1,000	Abcam, Cambridge, UK


A. RT-PCR, day 4
UMR-106
+PACAP 1-38
+PACAP 6-38
PreproPACAP
288 bp
1.0 0.9 0.7
PAC1
247 bp
1.0 0.8 1.2
VPAC1
216 bp
1.0 1.1 1.2
VPAC2
177 bp
0.0 0.0 0.0
GAPDH
486 bp
1.0 1.0 1.1
B. Western blot, day 4
UMR -106
+PACAP 1-38
+PACAP 6-38
PAC1
60 kDa
1.0 0.5 0.9
GAPDH
40 kDa
1.0 1.0 1.1
C. Immunocytochemistry, day 4, PAC1
D. Haematoxylin Eosin staining, day 4
UMR-106
+PACAP 1-38
+PACAP 6-38
UMR-106
+PACAP 1-38
+PACAP 6-38


E. Mitochondrial activity and cellular proliferation, day 4
*p<0.05
Percentage of control UMR 106 (%)
800
700
600
500
400
300
200
100
0
*
*
MTT assay
Thymidine incorporation
PACAP1-38
PACAP6-38

◀ **Fig. 1** Effects of PACAP on receptor expression, morphology, mitochondrial or proliferation activity of UMR-106 cells. mRNA (**a**) and protein (**b**) expression of preproPACAP and PACAP receptors in UMR-106 cell line. For RT-PCR and Western blot reactions, GAPDH was used as controls. Integrated optical densities of signals were determined by ImageJ software, and the results were normalised to the optical density of control cultures. Representative data of three independent experiments are shown. **c** Immuncytochemistry of PAC1 receptor in UMR-106 cells on day 4 of culturing. Original magnification was×60. *Scale bar* 10 μm. **d** Morphology of 4-day-old UMR-106 cells was visualised with haematoxylin-eosin (HE) staining. Original magnification was×40. *Scale bar* 20 μm. **e** Effects of PACAP administration on mitochondrial metabolic activity (MTT) and cellular proliferation (^{3}H-thymidine incorporation) in UMR-106 cell line on culturing day 4. *Asterisks* indicate significant (*$p<0.05$) alteration of cell proliferation as compared to the respective control

to set equal protein concentration of samples, and boiled for 10 min. About 40 μg of protein was separated by 7.5 % SDS-PAGE gel for detection of PAC1, PKA, GAPDH, CREB, p-CREB, Coll. I, osterix, ALP, Runx2, PKCα, BMP2, BMP4, BMP6, BMP7, BMPR1, Smad1, PTHrP, SHH, IHH, PTCH1 and Gli1. Proteins were transferred electrophoretically to nitrocellulose membranes. After blocking with 5 % nonfat dry milk in phosphate-buffered saline with 0.1 % Tween 20 (PBST), membranes were washed and exposed to the primary antibodies overnight at 4 °C in the dilution as given in Table 2. After washing for 30 min in PBST, membranes were incubated with anti-rabbit IgG (Bio-Rad Laboratories, CA, USA) in 1:1,500, anti-goat IgG (Sigma) in 1:2,000 and anti-mouse IgG (Bio-Rad Laboratories) in 1:1,500 dilution. Signals were detected by enhanced chemiluminescence (Pierce) according to the instructions of the manufacturer. Signals were manually developed on X-ray films (Agfa-Gevaert Group, Mortsel, Belgium). Optical density of Western blot signals was measured by using ImageJ 1.40 g freeware, and results were normalised to that of untreated control cultures.

Immunocytochemistry

On day 4, immunocytochemistry was performed on cells cultured on the surface of coverslips to visualise the intracellular localisation of PAC1, p-CREB, Runx2, Smad1 and Gli1. Extracellular organisation of collagen type I was also monitored by immunhistochemical staining. Cells were fixed in Saint-Marie's fixative (99 % ethanol and 1 % anhydrous acetic acid) and washed in 70 % ethanol. After rinsing in PBS (pH 7.4), nonspecific binding sites were blocked with PBST supplemented with 1 % bovine serum albumin (Amresco LLC, Solon, OH, USA); then, cultures were incubated with polyclonal anti-PAC1 antibody (Sigma), polyclonal Runx2 (Cell Signaling), Gli1 (Cell Signaling), Smad1 (Cell Signalling) and p-CREB (Millipore) antibodies at a dilution of 1:400 and monoclonal anti-Coll. I. (Sigma) antibody at a dilution of 1:800 at 4 °C overnight. Primary antibodies were visualised with anti-rabbit Alexa555 or anti-mouse Alexa555 secondary antibodies (Life Technologies Corporation, Carlsbad, CA, USA) at a dilution of 1:1,000. Specificity of antibodies was confirmed by applying control peptides that were identical to antigens against which the antibodies were raised; in these experiments, no specific signals were detected (data not shown). Cultures were mounted in Vectashield mounting medium (Vector Laboratories, Peterborough, England) containing DAPI for nuclear DNA staining. Photomicrographs of the cultures were taken using an Olympus DP72 camera on a Nikon Eclipse E800 microscope (Nikon Corporation, Tokyo, Japan). Images were acquired using cellSense Entry 1.5 software (Olympus, Shinjuku, Tokyo, Japan) using constant camera settings to allow comparison of fluorescent signal intensities. For investigation of subcellular localisation of p-CREB, Runx2, Gli1 and PAC1, fluorescent images were also taken with an Olympus FV1000S confocal microscope (Olympus Co., Tokyo, Japan) using×60 oil immersion objective (NA: 1.3). For excitation, laser line of 543 nm was used. The average pixel time was 4 μs. Z image series of 1-μm optical thickness were recorded in sequential scan mode. Images of Alexa555 and DAPI were overlaid using Adobe Photoshop version 10.0 software. Optical density of fluorescent signals was measured by using ImageJ 1.40 g freeware, and the data were compared to that of untreated control cultures. Integrated optical density of nuclei of 30 independent cells in randomly chosen field of view was calculated.

PKC Activity Assay

Cells were washed in physiological NaCl solution and were harvested. After centrifugation, cell pellets were suspended in 100 μL of homogenisation RIPA buffer containing protease inhibitors mentioned above. Suspensions were sonicated by pulsing burst for 3×10 s at 40 A (Cole-Parmer) on ice. After centrifugation at 10,000×g for 10 min at 4 °C, supernatants of samples were used for in vitro enzyme activity measurements. Untreated cultures were used as controls. Gö 6976 was added as classical PKC inhibitor, the activity decrease of all PKC isotypes considered as classical PKC activity. Measurements were performed according to Huang and Huang (1991).

Determination of Cytosolic Free Ca^{2+} Concentration

Measurements were performed on day 2 on cultures seeded onto 30-mm round coverslips using the calcium-dependent fluorescent dye Fura-2 as described previously (Matta et al. 2008). Fura-2-loaded cells were placed on the stage of an inverted fluorescent microscope (Diaphot, Nikon, Kowasaki, Japan) and viewed using a×40 oil immersion objective. Measurements were carried out in Tyrode's salt solution (containing 1.8 mM Ca^{2+}; composition 137 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 11.8 mM HEPES, 1 g/L glucose; pH 7.4) in a perfusion chamber using a dual wavelength monochromator equipment (DeltaScan, Photon

Technologies International, Lawrenceville, KY, USA) at room temperature. Excitation wavelength was altered between 340 and 380 nm at 50 Hz, and emission wavelength was detected at 510 nm. Data acquisition frequency was 10 Hz. Ratios of emitted fluorescence intensities (detected at alternating exCitation wavelengths; F_{340}/F_{380}) were measured as previously described (Matta et al. 2008). Basal cytosolic Ca^{2+} concentration was determined on day 2 directly after PACAP administration in three independent experiments, measuring 10 cells in each case.

Statistical Analysis

All data are representative of at least three different experiments. Where applicable, data are expressed as mean±SEM. Statistical analysis was performed by Student's *t* test where statistical method reported significant differences among the groups at $p<0.05$.

Results

PACAP Neuropeptides Act on PAC1 Receptor and Increase Proliferation of UMR-106 Cells

Weak signals of preproPACAP mRNA were detected in UMR-106 cells without any significant alteration when PACAP treatments were applied. Out of the three PACAP receptors, obvious mRNA expression of PAC1 and weaker signals of VPAC1 mRNA were found, while expression of VPAC2 mRNA remained undetectable. PACAP treatments did not cause any significant alterations of these signals (Fig. 1a). Expression of PAC1 protein was detected on Western blot and treatment with PACAP 1-38 gave rise to a moderate decrease of its signal, while PACAP 6-38 did not have any significant influence on the PAC1 protein level (Fig. 1b). Presence of PAC1 receptor was also demonstrated with immunocytochemistry and stronger fluorescent signals appeared by the addition of PACAP 6-38 (Fig. 1c).

Morphology and viability of UMR-106 cells were not altered by the addition of PACAP neuropeptides to the cell cultures (Fig. 1d, e), but significantly increased proliferation was observed. Continuous application of PACAP 1-38 at 100 nM for 4 days resulted in an approximately 400 % elevation of proliferation rate and surprisingly PACAP 6-38, accepted as an antagonist of PAC1 receptor, exerted an even stronger stimulatory effect on cell proliferation (Fig. 1e).

Osteogenic Differentiation Was Enhanced After PACAP Addition

In the next step of our experiments, we aimed to investigate the effect of PACAP treatments on bone formation. To this end, we performed RT-PCRs and Western blots of various osteogenesis markers. Administration of PACAPs did not cause any significant change in the mRNA and protein expression profile of Runx2 (Fig. 2a, b), although the nuclear appearance of this osteogenic transcription factor became more pronounced under the effect of PACAP neuropeptides, as it was revealed by immunocytochemistry (Fig. 2c). Nuclear activity of Runx2 may result in an elevation of bone-specific extracellular matrix production. Indeed, an elevated protein level of collagen type I, the major organic component of bone matrix, was detected with Western blots (Fig. 2b). Accumulation of collagen in extracellular space was confirmed with Picrosirius red staining (Fig. 2d) and immunocytochemistry of collagen type I (Fig. 2e) as the result of PACAP treatment. Osterix is another key transcription factor during osteogenesis, characteristic for more advanced stages of bone formation. mRNA and protein expression of osterix showed approximately a 2-fold elevation at the presence of PACAPs (Fig. 2a, b). Later stages of bone formation are characterised by beginning of matrix mineralisation, and indeed, elevated mRNA and protein expression of ALP and an increased mRNA expression of VEGF were found (Fig. 2a, b).

Mineralisation of bone matrix begins with accumulation of Ca^{2+} salts and continues with deposition of phosphates in the extracellular space. In order to detect these inorganic bone matrix constituents, we performed Alizarine red staining to demonstrate calcification and von Kossa reactions to investigate deposition of phosphates. Both PACAPs elevated the extracellular calcium deposits in cell cultures' UMR-106 cell line investigated on day 4 of culturing, and this effect was more pronounced in case of the application of PACAP 6-38 (Fig. 2f). No positive signals were detected with von Kossa staining on day 4 of culturing (data are not shown), but both neuropeptides elevated the extracellular phosphate accumulation comparing with the untreated control by day 8 of culturing; effect of PACAP 6-38 was stronger again, compared to that of PACAP 1-38 (Fig. 2g). As a very surprising and unexpected result of these experiments, although PACAP 6-38 is believed as an antagonist of PAC1 receptor, it exerted positive effects on osteogenesis, similar to the application of the PAC1 receptor agonist, PACAP 1-38.

Canonical PKA-Mediated Downstream Signalling Pathway Showed Only Partial Activation Under the Effect of PACAPs

In further steps of our experiments, we aimed to clarify downstream signalling mechanisms evoked by PACAPs and resulting in enhanced bone formation. The canonical downstream pathway of PAC1 receptor activation is the cAMP-dependent PKA signalling via CREB phosphorylation. In line with the previously described osteogenesis-promoting effect, both PACAP neuropeptides were found to induce significant

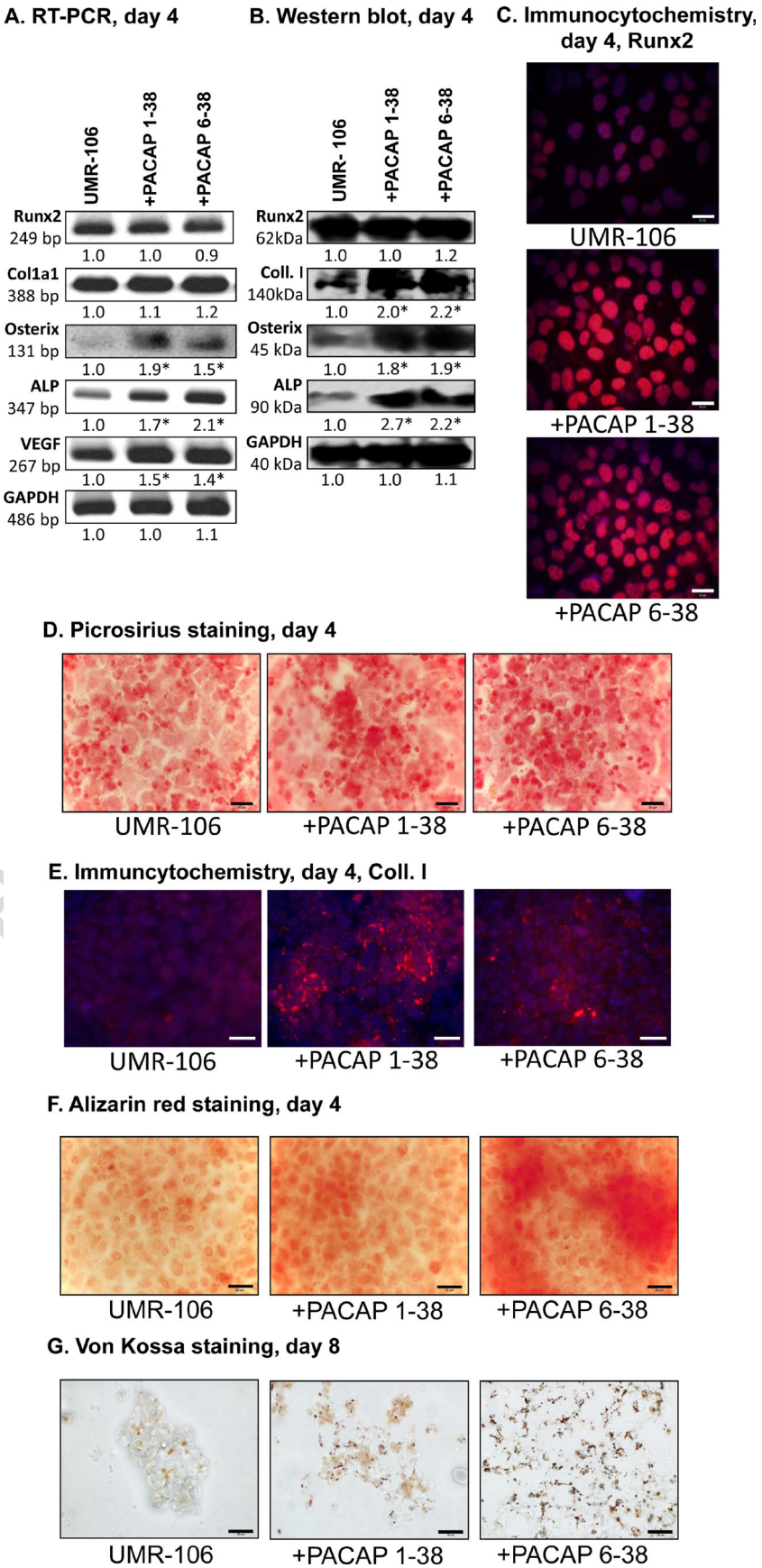
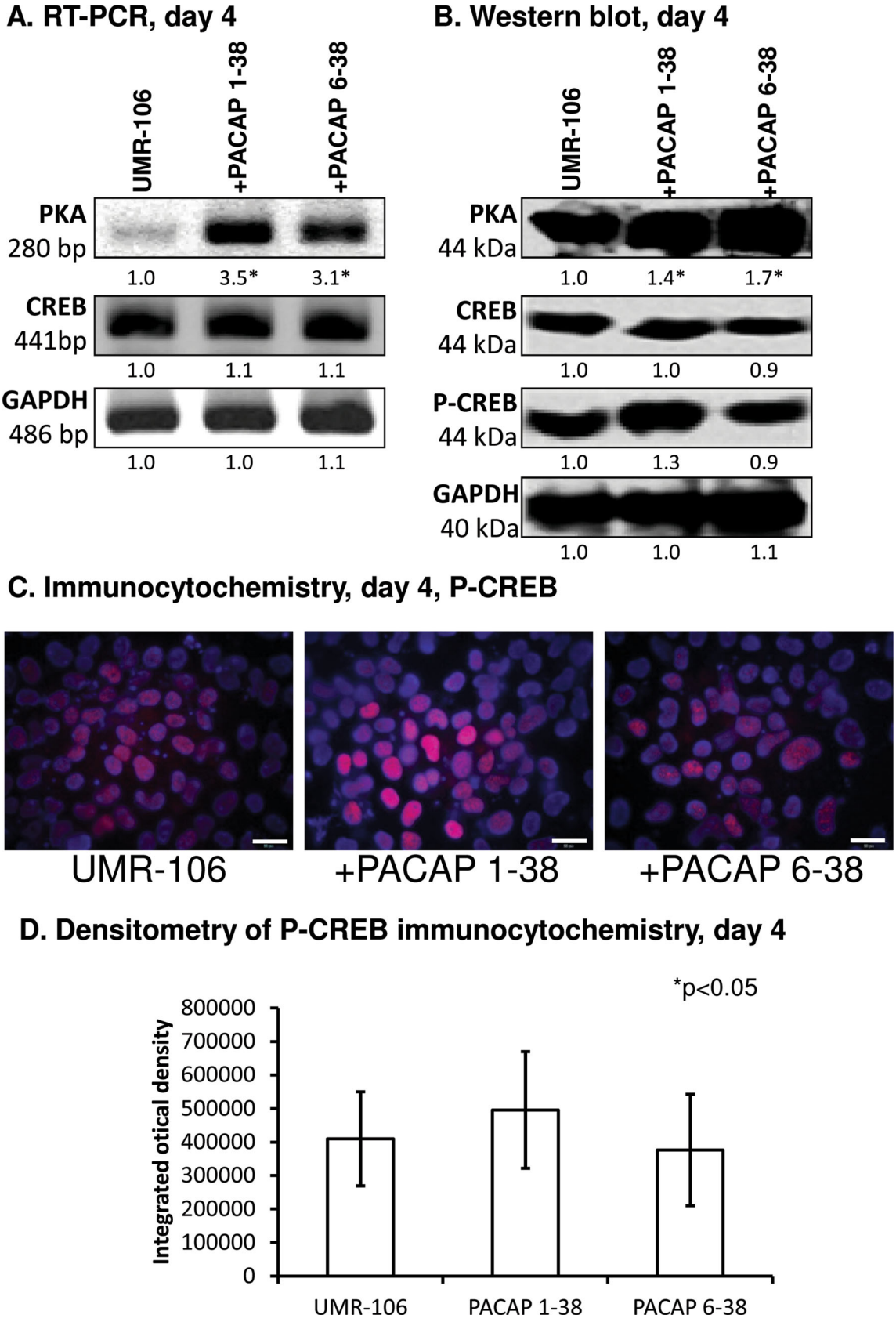


Fig. 2 PACAP influence Runx2, Coll. I, osterix, ALP and VEGF expression in UMR-106 cells. mRNA (**a**) and protein (**b**) expression of Runx2, Coll. I, osterix, ALP and VEGF in UMR-106 cells on day 4 of culturing. For RT-PCR and Western blot reactions, GAPDH was used as controls. Optical density of signals was measured, and the results were normalised to the optical density of controls. **a**, **b**, *Numbers below signals* represent integrated densities of signals determined by ImageJ software. **c** Immunocytochemistry of Runx2 in UMR-106 cells on day 4 of culturing. Original magnification was×60. *Scale bar* 20 μm. **d** Collagen in 4-day-old UMR-106 cell culture was visualised with Picrosirius staining. Original magnification was×40. *Scale bar* 20 μm. **e** Immunocytochemistry of collagen type I in 4-day-old UMR-106 cell cultures. Original magnification was×100. *Scale bar* 20 μm. **f** Extracellular Ca^{2+} deposits of 4-day-old UMR-106 cells were visualised with Alizarin red staining. Original magnification was×40. *Scale bar* 20 μm. **g** Extracellular Ca^{2+} phosphate crystals were detected with von Kossa method on day 8 of culturing. Original magnification was×40. *Scale bar* 20 μm. *Asterisks* indicate significant (*$p<0.05$) alteration of expression as compared to the respective control. Representative data of three independent experiments are shown



elevation either mRNA or protein levels of PKA (Fig. 3a, b). CREB transcription factor is the major downstream effector of PKA signalling; thus, we investigated the possible changes of its expression and phosphorylation. Although elevation of

◀ **Fig. 3** PACAP augments PKA expression without affecting CREB phosphorylation in UMR-106 cells. mRNA (**a**) and protein (**b**) expression of PKA, CREB and p-CREB in UMR-106 cells on day 4 of culturing. For RT-PCR and Western blot reactions, GAPDH was used as controls. Optical density of signals was measured, and the results were normalised to the optical density of controls. **a**, **b** *Numbers below signals* represent integrated densities of signals determined by ImageJ software. **c** Immunocytochemistry of p-CREB in UMR-106 cells on day 4 of culturing. Original magnification was×60. *Scale bar* 20 μm. **d** Integrated density of nuclei of 30 independent cells in randomly selected field of view was measured. Analysis of fluorescent signal of nuclei of PACAP treated and 30 control cells of three independent experiments was performed, respectively. *Asterisks* indicate significant (*$p<0.05$) alteration of expression as compared to the respective control. Representative data of three independent experiments are shown

expression and/or phosphorylation of CREB could be anticipated, we failed to detect any significant change of these parameters under the effect of PACAPs (Fig. 3a, b). To confirm these unexpected results, we performed immunocytochemistry, and indeed, we did not observe any significant change in the nuclear signal of p-CREB in PACAP-treated cells as it was demonstrated with densitometry of immunofluorescent signals detected at nuclear area of cells (Fig. 3c, d).

Ca^{2+}-Induced PKC-Mediated PACAP Signalling Pathway Was Not Activated

After being unable to prove any significant activation of PACAP-related CREB phosphorylation, we turned our attention to explore whether the Ca^{2+}-dependent effector mechanisms responded to the presence of the neuropeptides. PAC1 receptor may signal towards PLC pathway activation, through which it can regulate IP_3 operated release of Ca^{2+} from internal stores and in turn can activate PKC (Osipenko et al. 2000). Resting intracellular Ca^{2+} concentration of UMR-106 cells was monitored first, and we found that PACAP treatment did not result in any significant change of this parameter (Fig. 4a). No alterations were detected either in the mRNA or protein expression of PKCα (Fig. 4b, c); furthermore, we also failed to detect any significant change in the activity of classical PKCs following PACAP administration (Fig. 4d).

Regulation of BMP Expression is Involved in PACAP Downstream Signalling Pathways

As we failed to detect any significant response of CREB and cPKC as a result of PACAP treatments, we tried to identify other osteogenesis-related signalling mechanisms which could be responsible for the elevated expression of ALP and osterix observed after PACAP treatments. As BMPs can stimulate osteoblast differentiation and bone formation, we investigated the responsiveness of the members of this signalling pathway to PACAP treatments. Elevated protein expressions of BMPs 2, 4, 6 and 7 were detected along with inconsistent alterations of mRNA expressions under the effect of PACAP treatments (Fig. 5a, b). BMPs 2, 4, 6 and 7 can exert their biological effects via binding primarily to BMPR1, and this interaction results in activation of members of R-Smad transcription factor family. We found that UMR-106 cells express mRNA and protein of BMPR1, and either expression remained constant under the effect of PACAP treatments (Fig. 5a, b). Smad1 is one of the downstream targets of BMP signalling. Expression either of Smad1 mRNA or protein became significantly elevated (Fig. 5a, b). We also detected enhanced immunofluorescent signals of Smad1 following of PACAP treatments, when nuclear presence of this transcription factor was investigated with immunocytochemistry (Fig. 5c). We also compared the nuclear intensity of Smad1 immunofluorescent signals detected in PACAP-treated cells with that of the untreated control cells, and a significantly elevated nuclear presence of Smad1 transcription factor was proved (Fig. 5d).

HH Signalling Pathways Were Activated During PACAP Administration

Despite having an intimate cross talk between Runx2 and BMP pathways, osteogenesis is also characterised by the connection of these signalling mechanisms with other morphogenes. One of the major candidates of this link is the HH signalling pathway, which can regulate BMP expression and/or proliferation of cells. The balance between the activity of IHH and PTHrP is a key factor of bone formation. In UMR-106 cells, the mRNA and protein expression of IHH remained at a constant level after PACAP administration while both of the mRNA and protein expression of PTHrP elevated significantly (Fig. 6a, b). mRNA and protein expression of SHH was also detected in UMR-106 cells, and either signals showed strong elevation after treatments with PACAPs (Fig. 6a, b). The mRNA expression of PTCH1, the receptor of SHH and/or IHH, was not altered by PACAPs, but its protein expression became elevated by the neuropeptide treatments (Fig. 6a, b). Ligand binding of PTCH1 ultimately induces activation of Gli1 transcription factor; therefore, we investigated the presence and subcellular localisation of this signalling molecule. In line with the elevation of SHH protein level, stronger bands for Gli1 protein were detected in Western blots (Fig. 6a, b) and enhanced nuclear signals were observed with immunocytochemistry (Fig. 6c) upon PACAP treatments. This elevation proved to be significant with densitometry of nuclear Gli1fluorescent signals (Fig. 6d).

Discussion

PACAP neuropeptide is a well-known regulator of neurogenic differentiation and/or migration; therefore, its presence is essential for proper central nervous system formation (Toriyama

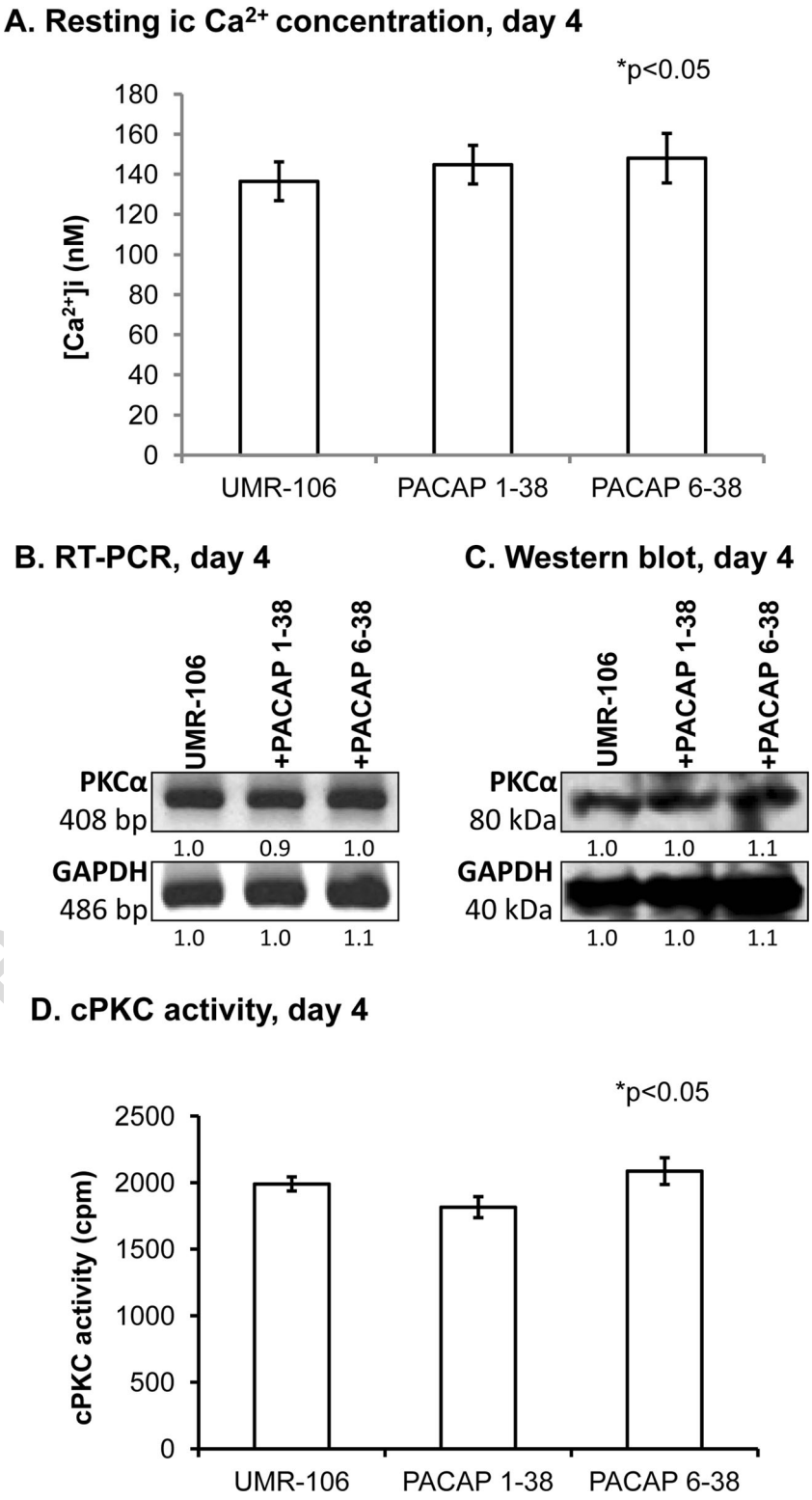
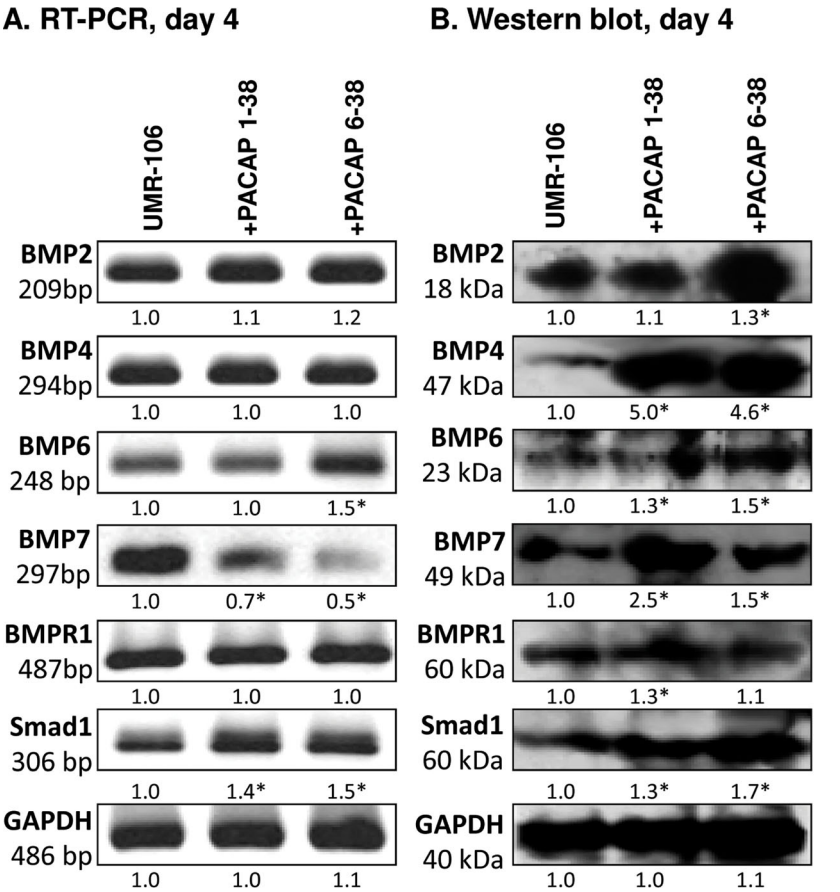
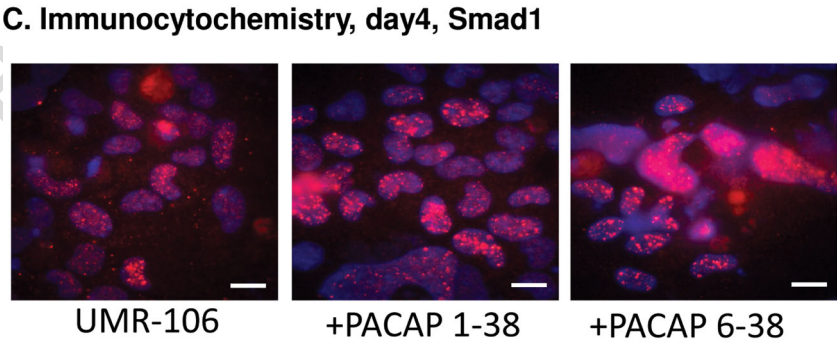


Fig. 4 Effects of PACAPs on intracellular Ca^{2+} of UMR-106 cells. **a** Basal cytosolic Ca^{2+} concentration in Fura-2-loaded cells on day 4 of culturing. Measurements were carried out in untreated control cultures and during PACAP treatments. Data shown are mean values of ten cells in each experimental group. **b** mRNA and **c** protein expression of PKCα in UMR-106 cells on day 4 of culturing. GAPDH was used as a control. *Numbers below signals* represent integrated densities of signals determined by ImageJ software. **d** Enzyme activity of classical PKC in UMR-106 cells on day 4. *Asterisks* indicate significant (*$p<0.05$) increase of expression or activity as compared to the respective control. Representative data of three independent experiments are shown

et al. 2012; Vaudry et al. 2009; Watanabe et al. 2007). In the last decade, increasing number of experiments have been performed proving presence of the neuropeptide in nonneuronal organs and tissues, such as intestinal tract (Pirone et al. 2011), gonads (Shpakov et al. 2011) or even in blood (Reglodi et al. 2010). Although a substantial amount of





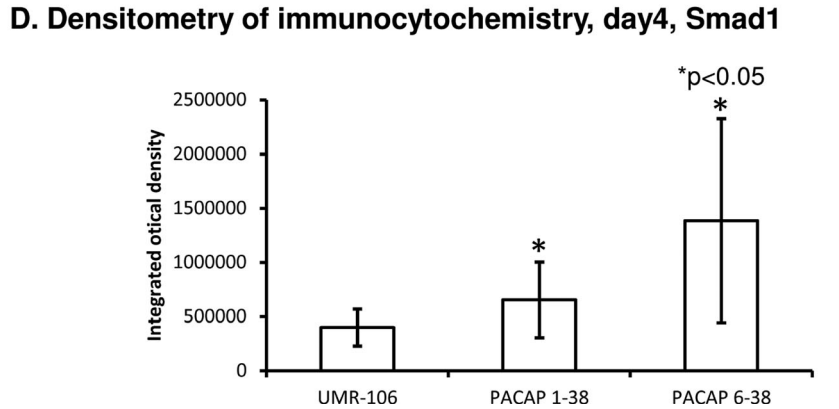


Fig. 5 Administration of PACAPs activates BMP signalling of UMR-106 cells. mRNA (**a**) and protein (**b**) expression of BMP2, BMP4, BMP6, BMP7, BMPR1 and Smad1 in UMR-106 cells on day 4 of culturing. For RT-PCR and Western blot reactions, GAPDH was used as control. Optical density of signals was measured, and the results were normalised to the optical density of controls. **a**, **b** *Numbers below signals* represent integrated densities of signals determined by ImageJ software. **c** Immunocytochemistry of Smad1 in UMR-106 cells on day 4 of culturing. Original magnification was×60. *Scale bar* 5 μm. **d** Integrated density of nuclei of 30 independent cells in randomly selected field of view was measured. Analysis of fluorescent signal of nuclei of PACAP treated and 30 control cells of three independent experiments was performed, respectively. *Asterisks* indicate significant (*$p<0.05$) alteration of expression as compared to the respective control. Representative results of three independent experiments are shown

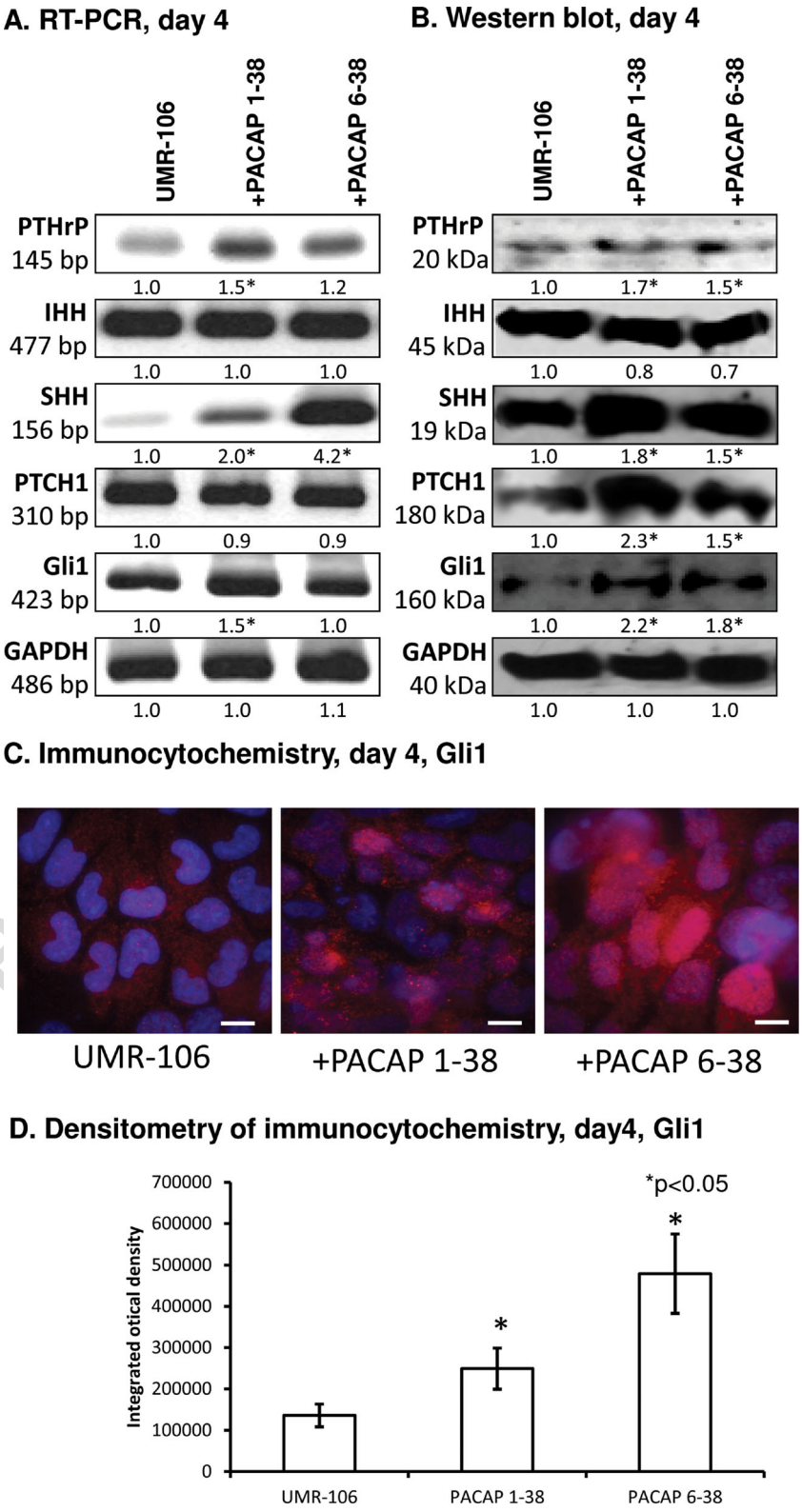


Fig. 6 Effects of PACAPs on HH signalling of UMR-106 cells. PACAP 1-38 at 100 nM and PACAP 6-38 at 10 μM were administrated continuously from day 1. mRNA (**a**) and protein (**b**) expression of IHH, PTHrP, SHH, PTCH1 and Gli1 in UMR-106 cells on day 4 of culturing. For RT-PCR and Western blot reactions, GAPDH was used as controls. Optical density of signals was measured, and the results were normalised to the optical density of controls. **a**, **b** *Numbers below signals* represent integrated densities of signals determined by ImageJ software. **c** Immunocytochemistry of Gli1 in UMR-106 cells on day 4 of culturing. Original magnification was×60. *Scale bar* 5 μm. **d** Integrated density of nuclei of 30 independent cells in randomly selected field of view was measured. Analysis of fluorescent signal of nuclei of PACAP treated and 30 control cells of three independent experiments was performed, respectively. *Asterisks* indicate significant (*$p<0.05$) alteration of expression as compared to the respective control. Representative data of three independent experiments are shown

data providing evidence on function of PACAP as a neurohormone in CNS has been reported, only sporadic data prove its origin and role in development of skeletal elements as in cartilage or bone (Juhász et al. 2014; Nagata et al. 2009; Strange-Vognsen et al. 1997). Several results confirm the essential function of PACAP/VIP system in the differentiation or activation of osteoclasts (Nagata et al. 2009; Persson and Lerner 2005, 2011), and it has also been shown that PACAP/

VIP has vital role in bone absorption (Jones et al. 2004). In our experiments, low expressional level of PACAP mRNA was demonstrated in UMR-106 cell line, suggesting the ability of endogenous PACAP release by osteogenic cells. Under physiological circumstances in living bone, PACAP release from nerve endings in bone marrow or in periosteum is also possible, as it has been shown earlier (Braas et al. 2007; Strange-Vognsen et al. 1997).

Calvaria-derived MC3T3 cells were shown to express VPAC2 receptors (Nagata et al. 2009), and addition of PACAP to the medium of UMR-106 cells induced cAMP production (Kovacs et al. 1996). In our experiments, UMR-106 cells were found to express PAC1 receptor dominantly. As PAC1 has 100-fold greater affinity to bind PACAP than VIP, we administrated PACAP 1-38 as an agonist and PACAP 6-38 as an antagonist to the medium of osteoblast cells. Without any alteration on morphology or mitochondrial activity of the cells, both neuropeptides increased cellular proliferation of UMR-106 cells. In fact, PACAP 1-38 is known to influence proliferation in a tissue- and/or cell-dependent manner: it elevated the proliferation of astrocytes (Nakamachi et al. 2011) and neuronal progenitor cells (Nishimoto et al. 2007), but it inhibited the proliferation of neuroblastoma (Waschek et al. 2000), endothelial (Castorina et al. 2010) or Leydig cells (Matsumoto et al. 2008). PACAP 6-38 was reported as a potent antagonist of PAC1 receptor originally (Bergstrom et al. 2003), but recent data suggested its agonistic behaviour in certain conditions such as in sensory nerve terminals or in cytotrophoblast cells (Reglodi et al. 2008) and in glia cells (Walker et al. 2013). Recently, we also reported a dominantly agonistic effect of this compound in a chicken chondrogenesis model (Juhász et al. 2014). One can hypothesise that PACAP 6-38 may have unknown effect on different isoforms of PAC1 receptor or it may act on a yet unidentified PACAP receptor (Jansen-Olesen et al. 2014) which can activate variable signalling pathways of cells (Holighaus et al. 2011).

As cAMP accumulation by PACAP of UMR-106 cells has already been published (Kovacs et al. 1996), it could be the question of interest which downstream target molecules of PAC1 receptors may activate osteoblast differentiation. PKA, as it is activated by the increased concentration of cAMP, is one of the canonical downstream signalling targets of PACAP binding (Vaudry et al. 2009), and PACAP was supposed to be a positive regulator of osteogenesis via this mechanism in hMSC (Siddappa et al. 2008) or in osteoblasts (Lo et al. 2012). CREB, a downstream target of PKA, can directly bind to the promoter region of osteogenic morphogen BMP2 (Zhang et al. 2011). Although the expression of PKA was elevated in UMR-106 cells, we were not able to detect any significant alteration in the expression or in the phosphorylation of CREB transcription factor. Furthermore, the nuclear presence of p-CREB did not show any significant change at the presence of PACAPs. These results suggested an alternative, CREB-independent initiation of osteogenic differentiation. As a matter of fact, Runx2, the master transcription factor of osteogenesis, can be phosphorylated by PKA and ultimately can be translocated into the nucleus (Jonason et al. 2009; Franceschi and Xiao 2003). Although we did not find any alteration of Runx2 expression after PACAP addition, its nuclear localisation became pronouncedly elevated, suggesting that it became activated by ligand binding of PAC1 receptor and may initiate expression of osteogenic genes. Indeed, we detected elevated levels of alkaline phosphatase (ALP) and collagen type I mRNA and proteins following PACAP treatments. It is also known that Runx2 can directly regulate osterix expression in osteoblasts (Jonason et al. 2009). In line with this observation, we found a significant elevation in osterix expression under the effect of PACAPs. Both of ALP and osterix can be activated by other upstream signalling elements, such as Dlx5 and Smads; moreover, Runx2 can cross talk with BMP signalling, and in turn, BMPs can also regulate the expression of the above osteogenesis markers (Li and Xiao 2007). Another proof of enhanced osteoblast differentiation was the increased VEGF expression in our experimental model. This growth factor is crucial for initiation of vascularisation of developing bone during endochondral ossification and was capable to enhance bone formation in tissue-engineered bone in animal experiments (Wu et al. 2013). In line with our data, it was also demonstrated that VEGF expression can be regulated by the increased activity of PKA (Yang et al. 2013).

The activation of ALP and osterix can indirectly stimulate Ca^{2+} accumulation and mineralisation process in bone development both in vitro and in vivo (Yan et al. 2014). There is evidence in neurons that PACAP binding can activate receptors or ion channels, generating action potential on the postsynaptic cell (Aoyagi and Takahashi 2001) by changing the Ca^{2+} release of cells. The vesicular transport of Ca^{2+} or the Ca^{2+} release induced by PACAP has not been characterised in nonexcitable cells yet, although it can be hypothesised that NMDA receptors, present on osteoblast cells, may have an indirect connection with PAC1 receptor activation through which they can regulate Ca^{2+} inflow and/or outflow of cells (MacDonald et al. 2007). Nevertheless, our results suggest that PACAP binding may have an indirect effect on Ca^{2+} transport of osteoblasts (Morita et al. 2002) and increase the mineralisation of bone matrix. PAC1 receptor activation can also result in the induction of PLC signalling pathway, which subsequently may regulate IP_3-dependent intracellular Ca^{2+} release of cells as it has been demonstrated in several neuronal models (Osipenko et al. 2000; Payet et al. 2003). The concentration changes of free cytosolic Ca^{2+} ions can regulate the activation of classical PKC (Hodges et al. 2006), as well as the extracellular vesicular Ca^{2+} transport of cells. In UMR-106 cells, we were not able to detect significant alterations of resting intracellular Ca^{2+} concentration by PACAPs, and the

Ca^{2+}-dependent PKCα was not activated, suggesting an uninfluenced PLC pathway of UMR-106 cells. These results may further support the hypothesis that developmental stage-dependent expression of different isoforms of PAC1 receptor results in the activation of Ca^{2+}-related or unrelated downstream pathways in PACAP signalling (Yan et al. 2013).

One of the main regulators of bone formation is the proper coexpression or sequential expression of several BMPs, including BMPs 2, 4, 6 and 7 (Lavery et al. 2008). According to our data, PACAP addition induced the elevation of protein expression of these cytokines in UMR-106 cells. Moreover, the elevated expression of BMP2 and BMP4, characterised by the highest osteogenic capacity, can be responsible for the induction of collagen type I expression or indirectly the increase of mineralisation processes (Chen et al. 2012; Zouani et al. 2013). Furthermore, the increase of BMP7 can be partly responsible for the activation of ALP gene expression (Bei et al. 2012), and indeed, we found elevated ALP mRNA and protein levels after PACAP addition in our experiments. BMP6 has a crucial role in regulation of osteoblast differentiation through osterix activation (Zhu et al. 2012). Consistent with this finding, BMP6 and osterix expressions both were elevated following PACAP applications in our experiments. The canonical pathways of BMPRI activation lead to the regulation of Smad1/5/8 transcription factors and which can activate expression of various bone-specific genes (Chen et al. 2012). We found elevated expression and nuclear presence of Smad1, strongly suggesting that activation of PACAP signalling results in the increased activity of BMP signalling in UMR-106 cells, and besides activation of Runx2 by PKA, this pathway also plays role in proosteogenic effect of PACAPs. Moreover, activated Smad1 can cooperate with Runx2 transcription factor on the promoter region of genes responsible for bone formation (Drissi et al. 2003).

Another group of crucial osteogenesis-regulating morphogens is the HH family consisting of three members: SHH), IHH and Desert hedgehog (DHH) (Pan et al. 2013). Although the functions of IHH in endochondral ossification or in cranial skeleton development have been demonstrated and its presence in osteoblasts has also been detected (St-Jacques et al. 1999; Tu et al. 2012), we did not find any alteration in its expression after PACAP administration. SHH can be responsible for proper bone formation beyond its crucial role in regulation of several tissue or even cancer development (Han et al. 2013; Hu et al. 2013; Kiuru et al. 2009). HHs can bind to Patched1 (PTCH1) receptor which releases the membrane associated Smoothened leading to the activation of the Gli transcription factors, which ultimately translocate to the nucleus and activate target genes (James et al. 2010; Pan et al. 2013). Gli1 can regulate early osteogenic differentiation by the activation of Runx2 gene expression (Hojo et al. 2012). SHH was also shown to have an important function in neurogenic development where its connection with PACAP signalling system has been published (Waschek et al. 2000, 2006). Addition of PACAPs resulted in a pronounced elevation of SHH, PTCH1, Gli1, mRNA and protein levels; moreover, nuclear signal of Gli1 also became stronger. Taken together, PKA and SHH pathways both were found activated by PACAPs in UMR-106 cells. Nonetheless, others reported antagonistic relationship of cAMP-activated PKA and HH signalling during Drosophila development (Waschek et al. 2006) and in bone formation (Regard et al. 2013). Moreover, PACAP was shown to inhibit the *gli1* gene expression during the proliferation of medulloblastoma cells or antagonise the SHH signalling pathways of motoneuron formation in embryonic stem cell cultures (Waschek et al. 2000). However, evidence about GPCR-induced SHH activation has also been reported under certain circumstances and a possible involvement of noncanonical SHH regulation can also complicate the picture (Brennan et al. 2012). Although the antagonistic communication of PKA-SHH signalling pathways is convincingly proved in neuronal tissues or cell types (Niewiadomski et al. 2013), the universality of this way of signalling cross talk is not widely demonstrated. Another possible reason which may cause the contradictory expression pattern and signalling communication of PKA and SHH in UMR-106 cells is the fact that this cell line originates from an osteosarcoma, and HH pathways are frequently overactivated in this type of tumour (Hirotsu et al. 2010).

In conclusion, this study shows that PACAP signalling plays pro-osteogenic role in consecutive steps of in vitro bone tissue formation via activation of various signalling pathways in UMR-106 cells. This observation raises the opportunity that exogenously administered PACAP may enhance bone formation in case of hampered fracture healings or during therapy of larger bone defects in the distant future.

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