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The Influence of RAMP1 Overexpression on CGRP-Induced Osteogenic Differentiation in MG-63 Cells In Vitro: An Experimental Study

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ABSTRACT

The aim of this study was to elucidate the influence of receptor activity modifying protein 1 (RAMP1) overexpression on the expression and distribution of calcitonin receptor-like receptor (CRLR) in MG-63 cells. Our research also focused on whether RAMP1 overexpression enhanced the promoting effect of exogenous CGRP on osteogenic differentiation in MG-63 cells. We first constructed a eukaryotic expression vector containing human RAMP1 and stably transfected it into MG-63 cells. Real-time PCR and Western blotting were used to determine the expression levels of RAMP1 and CRLR mRNA and protein, respectively. Immunofluorescence analysis was employed to compare the distribution of CRLR in transfected cells. After treatment with CGRP, the extent of osteogenic differentiation was evaluated by simultaneous monitoring of alkaline phosphatase activity, the expression patterns of osteoblastic markers and mineralisation staining. We found that RAMP1 was more highly expressed in the transfected group compared with the control groups ($P < 0.01$). The CRLR expression was significantly higher than that in the control groups ($P < 0.05$). In addition, after 7 days of CGRP treatment to induce osteogenic differentiation, the expression of collagen I mRNA was markedly increased in the transfected group ($P < 0.05$). The transfected group exhibited more granular precipitation in the cytoplasm with alkaline phosphatase staining after 7 and 14 days of differentiation. When stained with Alizarin Red, cells overexpressing RAMP1 were darker and formed many mineralised nodules with clear boundaries and calcium deposition typical of mineralised bone matrix structures at 28 days post-induction of differentiation. The CGRP-induced ALP activity in the RAMP1 overexpression group was significantly higher 3, 6 and 9 days after induction than that in the two control groups ($P < 0.05$). RAMP1 overexpression promotes CRLR expression, localisation on the cell membrane and enhanced CGRP-mediated differentiation of MG-63 cells. This study contributes to a better understanding of the molecular mechanisms governing CGRP-induced MG-63 differentiation. J. Cell. Biochem. 114: 314–322, 2013.

KEY WORDS: RAMP1; CRLR; CGRP; MG-63 CELLS; OSTEOGENIC DIFFERENTIATION

Calcitonin gene-related peptide (CGRP) has a broad distribution and expression pattern in vivo. It is transcribed from the same gene locus as calcitonin (CT). CGRP plays a significant pathophysiological role in the regulation of bone metabolism, the cardiovascular system, the gastrointestinal system and pain [Naot and Cornish, 2008]. During bone wound healing, CGRP and other neuropeptide substances are secreted by nerve endings in the bony callus and promote bone metabolism and reconstruction [Lerner and

Abbreviations used: RAMP1, receptor activity modifying protein 1; CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor-like receptor; RCP, receptor component protein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ALP, alkaline phosphatase; pNPP, *p*-nitrophenyl phosphate; min, minutes.

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Persson, 2008]. CGRP is one of the most widely distributed sensory neuropeptides in bone tissue and is largely present in areas undergoing active bone metabolism. It can promote the proliferation and differentiation of osteoblasts [Irie et al., 2002; Kawase et al., 2005]. Our previous experiments have shown its importance in jaw wound healing [Li et al., 2009]. CGRP has also been demonstrated to control nitric oxide production in osteoblasts in vitro [Li et al., 2011]. Our previous experiments have also confirmed that CGRP can significantly stimulate the proliferation and differentiation of rat osteoblasts in vitro [Xu et al., 2005]. We have also explored the proteomic differential expression of osteoblasts in the process of bone wound healing participated by CGRP [Wu et al., 2010].

The CGRP receptor is usually situated in cell membranes and consists of a complex comprising calcitonin receptor-like receptor (CRLR), receptor activity modifying proteins (RAMPs) and receptor component protein (RCP) [Recober and Goadsby, 2010]. The CGRP receptor belongs to the G protein-coupled receptor family, which includes calcitonin receptor (CTR) and CRLR [Lagerström and Schiöth, 2008]. RAMPs regulate the plasticity of the CGRPR. RAMPs can regulate both CRLR and CGRP. Upon binding to the receptor, different RAMPs can modulate the specificity of CRLR for various ligands [Wang et al., 2010]. The RAMP family has three members: RAMP1, RAMP2 and RAMP3. RAMPs determine the ligand binding capabilities and strength signalling of CRLR [Hay et al., 2006]. It is not yet clear how RAMP1 expression correlates with CGRP-mediated promotion of osteoblast differentiation.

McLatchie et al. [1998] have shown that RAMPs and CGRP can transport CRLR to the cell membrane and thus can determine the glycosylation status of CRLR and the ability of CRLR to convey signals to the cell. There are pathophysiological studies of RAMPs that mainly focus on the cardiovascular system, with only limited research targeting other systems [Hay et al., 2005]. CGRP can regulate bone metabolism in bone tissues and thus can affect bone wound healing. RAMPs, as components of the receptor, will certainly participate in that process. In addition, the wide distribution of RAMPs emphasises the importance of RAMP-related research. The current literature does not fully explore the influence of RAMP1 over-expression on CRLR's distribution in an osteoblast-like cell membrane, nor does it discuss in detail whether RAMP1 expression can influence the ability of CGRP to promote osteoblast differentiation.

MG-63 cells were cultured from a human osteosarcoma and immortalised to generate an osteoblast-like cell line. Bone wound healing studies similar to ours often use MG-63 cells as an experimental model [Saracino et al., 2009; Kim et al., 2010]. In our study, we first constructed a eukaryotic RAMP1 expression vector and transfected it into MG-63 cells. Then, we observed the influence of RAMP1 overexpression on CRLR expression and distribution on the cell membrane and on the CGRP-mediated promotion of MG-63 cell differentiation.

MATERIALS AND METHODS

MATERIALS

Human CGRP was purchased from Sigma–Aldrich Corp. (Sigma, St. Louis, MO). CGRP was dissolved in distilled water to a stock concentration of 100 μM and stored in 100 μl aliquots at −80°C. The following reagents were purchased from Invitrogen Corp. (Invitrogen, Carlsbad, CA): pcDNA3.1 (+), PureLink™ HiPure Plasmid Miniprep Kit, Lipofectamine™ 2000 Transfection Reagent, Opti-MEM® Reduced Serum Medium and TRIzol® Reagent. RAMP1 (FL-148) and CRLR (V-20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The Reverse Transcription System was purchased from Promega Corp. (Promega, Madison, WI).

CELL CULTURE

MG-63 human osteoblast-like cells were obtained from ATCC (America Type Culture Collection). Cells were cultured in DMEM (Dulbecco's modified Eagle's medium; Gibco, Carlsbad, CA) supplemented with 10% foetal bovine serum (GIBCO, Grand Island, NY), 100 U/ml penicillin and 0.1 mg/ml streptomycin. Cells were subcultured every 72 h in a humidified 5% CO_2 incubator. Cells from passages 4–6 were used in the experiments.

TRANSFECTION AND G418 SELECTION

We retrieved the RAMP1 gene sequence from GeneBank and designed the following primers: RAMP1-F: 5′-GAC ACA AGC TTA TGG CCC GGG CCC TGT GCC G-3′ and RAMP1-R: 5′-GTG TCG GAT CCC TAC ACA ATG CCC TCA GTG CGC TTG-3′. The eukaryotic expression plasmid pcDNA3.1 (+) was digested with restriction endonucleases *Bam*HI and *Hin*dIII. The RAMP1 insert and linearised plasmid were joined by T4-DNA ligase to construct the plasmid pcDNA3.1 (+)-RAMP1. The recombinant plasmid pcDNA3.1 (+)-RAMP1 was characterised by PCR, restriction endonuclease digest and sequencing analysis.

The MG-63 cells were divided into negative control, empty vector control and overexpression groups. Untransfected cells were used as the negative control. Transfection was carried out using Lipofectamine™ 2000 according to the manufacturer's instructions.

To obtain stably transfected cells, at 24 h post-transfection, G418 was added to a final concentration of 0.8 mg/ml and antibiotic selection was continued for 10 days. Cells stably expressing RAMP1 were continuously passaged in DMEM medium supplemented with 10% foetal bovine serum, penicillin (100 U/ml), streptomycin (10 g/ml) and G418 (0.2 mg/ml). G418-resistant single cell clones were screened and amplified for the following experiments. Control cells were mock-transfected with empty vector and selected in the same manner described above. The distribution and expression of RAMP1 and CRLR on MG-63 cell membranes was observed on a confocal laser scanning microscope and detected by the use of Western blots and real-time PCR.

WESTERN BLOTTING

Western blots were performed to detect the expression of RAMP1 and CRLR at the protein level. After transfection and G418 selection, cells were washed three times with ice cold PBS and then incubated with lysis buffer (20 mM HEPES, pH 7.5, 200 mM NaCl, 0.2 mM EDTA–Na_2, 1% Triton X-100, 0.05% SDS, 0.5 mM DTT, 1 mM Na_3VO_4, 20 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin) at 4°C for 30 min and centrifuged at 12,000*g* for 5 min; the supernatant was stored at −80°C. The amount of total protein was determined using a bicinchoninic acid (BCA) assay (Pierce, Rockford, IL). Equal amounts

(20 μg) of total protein from each sample were separated on 12% Bis–Tris gels following the manufacturer's protocol and transferred to PVDF membranes. Membranes were blocked overnight in TBST/5% fat free milk. After incubating with appropriate antibodies, immunoreactive bands were visualised with enhanced chemiluminescence (ECL) detection reagents (Amersham Biosciences, NJ) on autoradiography films, as described previously [Lindemann et al., 2007]. The intensity of immunoreactive bands was quantified by image analysis software (ImageJ_1.32J, NIH).

RNA EXTRACTION AND REAL-TIME PCR

Cells were washed two to four times with PBS prior to RNA isolation. Total cellular RNAs were isolated with Trizol reagent and used for first strand cDNA synthesis with the Reverse Transcription System. Quantification of gene transcripts was performed in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) using Power SYBR® Green (Applied Biosystems). The primer sequences of the genes, including RAMP1, CRLR and collagen I, are listed in Table I. PCR conditions were one cycle of 94°C for 2 min followed by 50 cycles of 94°C for 10 s, a specified annealing temperature for 15 s and 72°C for 15 s. Amplification was followed by melting curve analysis, which used the following program: one cycle at 65°C for 1 s, 94°C for 2 s and 37°C for 5 s. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference gene to which the expression of other genes was normalised using the comparative cycle of threshold value.

IMMUNOFLUORESCENCE

After transfection and G418 selection, MG-63 cells were seeded at 1×10^6 cells/well in 6-well plates (Nunc). The cells were cultured to 70–90% confluency, and then the media in the 6-well plate was removed. The cells were washed three times with PBS, fixed with 4% paraformaldehyde for 10 min at 37°C, and then washed with PBS (pH 7.4) three times. MG-63 cells were labelled with RAMP1 (FL-148, Santa Cruz Biotechnology) and CRLR (V-20, Santa Cruz Biotechnology) primary antibodies and incubated overnight at 4°C. After several washes with PBS (pH 7.4), cells were incubated with appropriate Cy3- and FITC-conjugated secondary antibodies for 1 h in the dark at room temperature. To counterstain the nuclei, 4′6-diamidino-2-phenylindole dihydrochloride (DAPI; 0.5 μg/ml in PBS; Molecular Probes) was applied after the secondary antibody. Images were acquired on a fluorescence microscope (AxioImager Z1) equipped with a charged-coupled-device camera (AxioCam MRm) and processed with Axiovision software (Carl Zeiss MicroImaging).

ALP ACTIVITY ASSAY

MG-63 cells were seeded in 6-well plates, and the alkaline phosphatase (ALP) activity was determined by staining with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate on the 7th and 14th days after osteogenic differentiation.

For quantification of ALP activity, cells seeded in 6-well plates were rinsed two times with PBS, followed by trypsinisation and scraping in ddH_2O. Then, three cycles of freezing and thawing were performed. ALP activity was determined at 405 nm using *p*-nitrophenyl phosphate (pNPP) as the substrate. A 50 μl sample was mixed with 50 μl pNPP (1 mg/ml in 1 M diethanolamine buffer containing 0.5 mM $MgCl_2$ (pH 9.8)) and incubated at 37°C for 15 min on a bench shaker. The reaction was stopped by adding 25 μl of 3 M NaOH/100 μl of reaction mixture. Enzyme activities were quantified by absorbance measurements at 405 nm. Total protein contents were determined with the bicinchoninic acid method using the Pierce (Thermo Fisher Scientific, Rockford, IL) protein assay kit in aliquots of the same samples, which were read at 562 nm and calculated against a series of bovine serum albumin (BSA) standards. ALP levels were normalised to the total protein amount at the end of the experiment. The alkaline phosphatase assay was conducted in triplicate and repeated in five cultures.

ALIZARIN RED STAINING

The presence of calcium in the cell cultures was determined by Alizarin Red (Sigma–Aldrich) staining after 4 weeks of osteogenic induction. Briefly, the cells were rinsed with cold PBS and then fixed in cold 70% ethanol for 1 h. Following a rinse with distilled water, the cells were stained with 40 mM Alizarin Red (pH 4.2) for 10 min at room temperature. This experiment was repeated three times.

STATISTICAL ANALYSIS

The data were analysed with the software SPSS, Version 12.0 (SPSS Inc., Chicago, IL). All values are expressed as the mean ± standard deviation. The data were analysed using a one-way analysis of variance (ANOVA) and Newman–Keuls Student's *t*-test. A tied *P*-value of <0.05 was considered statistically significant.

RESULTS

PLASMID CONSTRUCTION AND TRANSFECTION

The pcDNA3.1 (+) plasmid was used to construct the transgene vector pcDNA3.1 (+)-RAMP1 in this study. The final pcDNA3.1 (+)-RAMP1 plasmid was extracted via an alkaline lysis method. The plasmid was identified by *Hin*dIII and *Bam*HI digestion and gel

TABLE I. Primers for Real-Time PCR

Definition	Sequence (forward/reverse)	GenBank accession
GAPDH	Forward primer: 5′-CAATGACCCCTTCATTGACC-3′ Reverse primer: 5′-TTGATTTTGGAGGGATCTCG-3′	NM_002046
RAMP1	Forward primer: 5′-AGGTTCTTCCTGGCAGTG-3′ Reverse primer: 5′-ACCACCAGTGCCGTCAC-3′	AY 265457.1
CRLR	Forward primer: 5′-CCAGTTCATTCATCTTTACCT-3′ Reverse primer: 5′-AGCCAAGAAAATAATACCAC-3′	NM_005795.4
Collagen I	Forward primer: 5′-AACAGTGTCAAAGGTCCAG-3′ Reverse primer: 5′-CACTGTGCTAGTTTCCATTC-3′	NM_000088.3

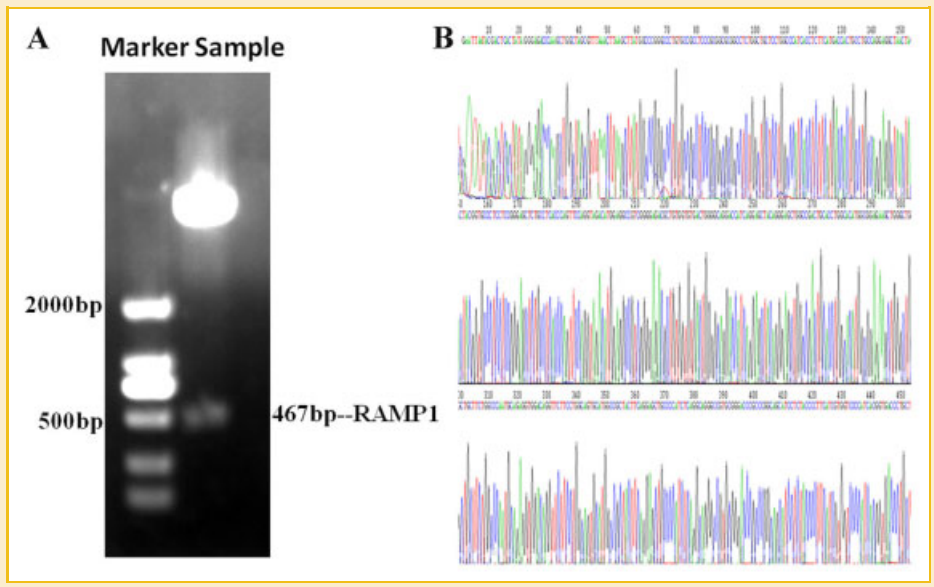

Fig. 1. A: Enzyme digestion analysis of RAMP1 transgene vector. (Marker) DI2000 (Marker) and (Sample) fragments of pcDNA3.1(+)-RAMP1 plasmid digested by *Hin*dIII and *Bam*HI (B) Gene sequencing analysis. This image depicts the gene sequence of the *Homo sapiens* RAMP1 from GenBank (Serial Number: AY265457.1).

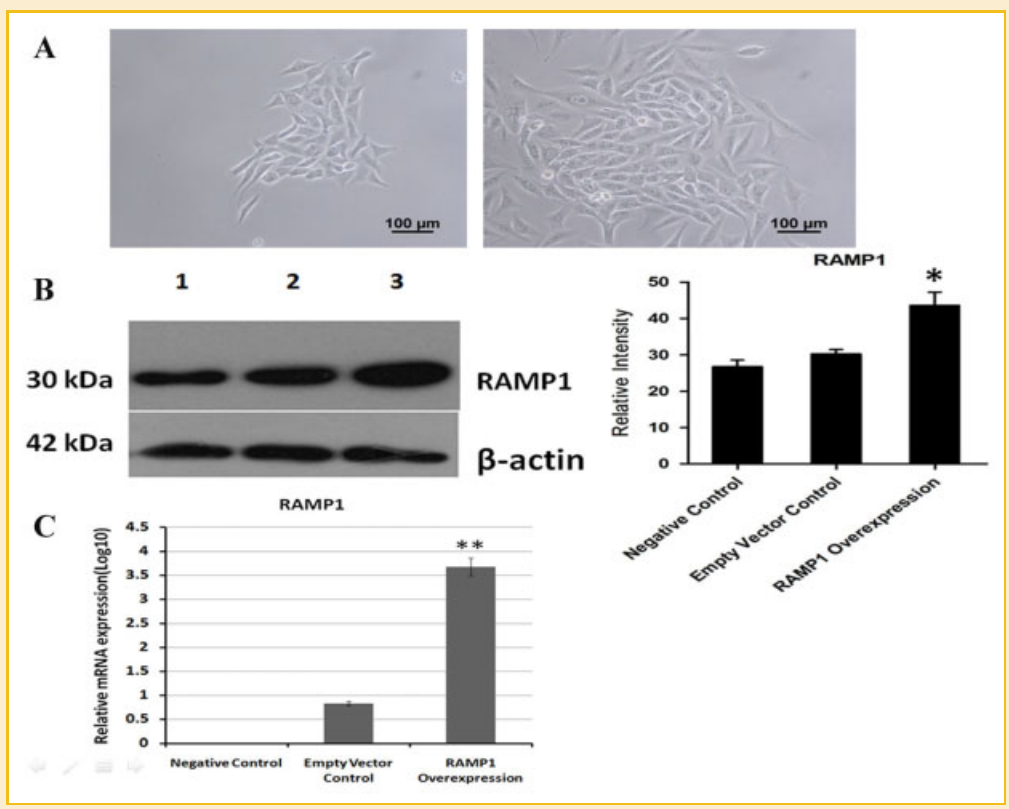

Fig. 2. A: G418-resistant MG-63 cell clone selection. Small colonies were formed under the presence of G418 on the 14th day of selection (left), whereas significantly larger colonies appeared after 20 days of selection (right). B: Expression profiles of RAMP1 protein in MG-63 cells transfected with pcDNA3.1 (+)-RAMP1.The expression profiles of RAMP1 and β-actin proteins in the negative control group (Lane 1), empty vector control group (Lane 2) and RAMP1 overexpression group (Lane 3). Cells were analysed as described in the text. The molecular mass of RAMP1 (30 kDa) and β-actin (42 kDa) are indicated to the left. The intensity of protein bands was quantified by image analysis software (ImageJ_1.32J, NIH). The data are shown as the mean ± SD from three independent experiments. Statistical analysis was conducted using ANOVA with Newman–Keuls Student's *t*-test. Significant differences between transfection with RAMP1 gene and transfection with vector alone or control are indicated ($^{*}P < 0.05$ negative control vs. RAMP1 overexpression and the empty vector control). C: Expression of the RAMP1 gene. Significant differences between transfection with RAMP1 gene and transfection with vector alone or control are indicated ($^{**}P < 0.01$, negative control vs. empty vector control and RAMP1 overexpression). All data are normalised to GAPDH and calibrated on the negative control, whose expression is considered 1 for all genes. Y-axis is on a logarithmic scale.

electrophoresis. The product of this digestion of pcDNA3.1 (+)-RAMP1 is shown in Figure 1. The enzymes *Hin*dIII and *Bam*HI specifically recognised and digested the desired sites on pcDNA3.1 (+)-RAMP1. One fragment of approximately 500 bp was observed after digestion, which is the RAMP1 gene. Meanwhile, the pcDNA3.1 (+)-RAMP1 recombinant plasmid was isolated from bacteria and analysed by Invitrogen Biotechnology Co. Ltd. (Branch, Shanghai, China). The sequence was identical to the GenBank (Serial Number: AY265457.1). These results confirmed that the RAMP1 gene fragment was successfully inserted into the pcDNA3.1 (+) plasmid. The recombinant plasmid was then used in the following experiments (Fig. 1).

GENE TRANSFECTION AND OVEREXPRESSION OF RAMP1

To study the biological functions of RAMP1, the recombinant plasmid encoding RAMP1 was constructed and transfected into MG-63 cells. Forty-eight hours after transfection, the cells were selected by G418 for 20 days, and G418-resistant clones were propagated and screened for RAMP1 expression. Stably transduced cells were analysed individually to determine the expression level of RAMP1 using Western blots and real-time PCR. This analysis confirmed that the MG-63 cells transfected with pcDNA3.1(+)-RAMP1 expressed higher levels of RAMP1 at the mRNA and protein levels when compared with the negative control and empty vector control groups (Fig. 2).

RAMP1 OVEREXPRESSION ENHANCED CRLR EXPRESSION

Our Western blots and ImageJ grey scan analysis demonstrate that stably transfected MG-63 cells overexpressing RAMP1 exhibit higher CRLR protein expression compared with the control groups.

The real-time PCR results show that the RAMP1 overexpression group has significantly higher CRLR mRNA expression compared with that of the controls, indicating that the recombinant cells have a higher transcriptional level of this gene (Fig. 3).

A triple-immunofluorescence staining was conducted to identify CRLR, RAMP1 and DAPI in MG-63 cells to elucidate the direct contribution of RAMP1 gene overexpression to the expression and distribution of CRLR. The CRLR staining on the cell membranes observed in MG-63 cells was brighter in pcDNA3.1(+)-RAMP1-transfected cells compared with the control cells. In fact, CRLR staining was barely detectable in the cells that were untransfected or transfected with the empty vector. No significant differences in the DAPI signal were observed between the three groups. From this immunofluorescence staining, we found that in the RAMP1

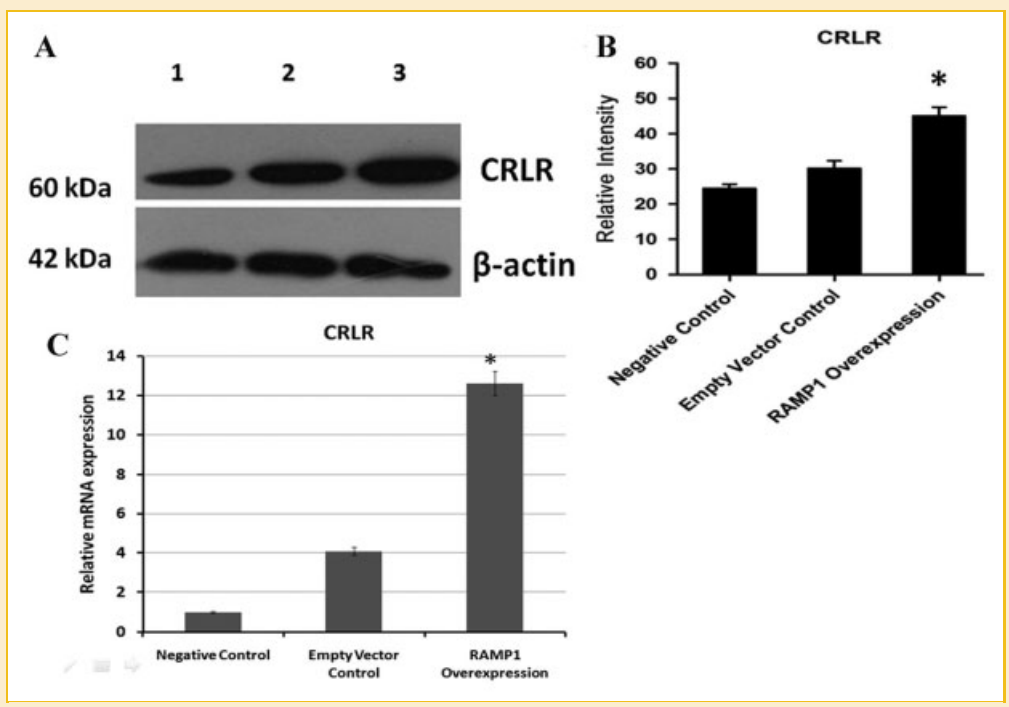

Fig. 3. A,B: The expression profiles of CRLR protein are modulated by RAMP1 overexpression in MG-63 cells. Equal amounts of cell lysates (containing 50 μg protein) were detected by specific antibodies as described in Materials and Methods Section. β-actin was used as a loading control. The figure is representative of three separate experiments. The intensity of the protein bands was quantified by image analysis software (ImageJ_1.32J, NIH). The data are shown as the mean ± SD from three independent experiments. Statistical analysis was conducted using ANOVA with Newman–Keuls Student's *t*-test. A significant difference between transfection with pcDNA3.1 (+)-RAMP1 and transfection with vector alone or control are shown ($^*P < 0.05$ negative control vs. empty vector control and RAMP1 overexpression). C: The influence of RAMP1 on CRLR gene expression. Significant differences in CRLR expression between cells transfected with pcDNA (+)-RAMP1, empty vector or nothing are shown ($^*P < 0.05$ negative control vs. RAMP1 overexpression and empty vector control). All data are normalised to GAPDH and calibrated based on the negative control, whose expression was considered 1 for all genes. The Y-axis is on a logarithmic scale.

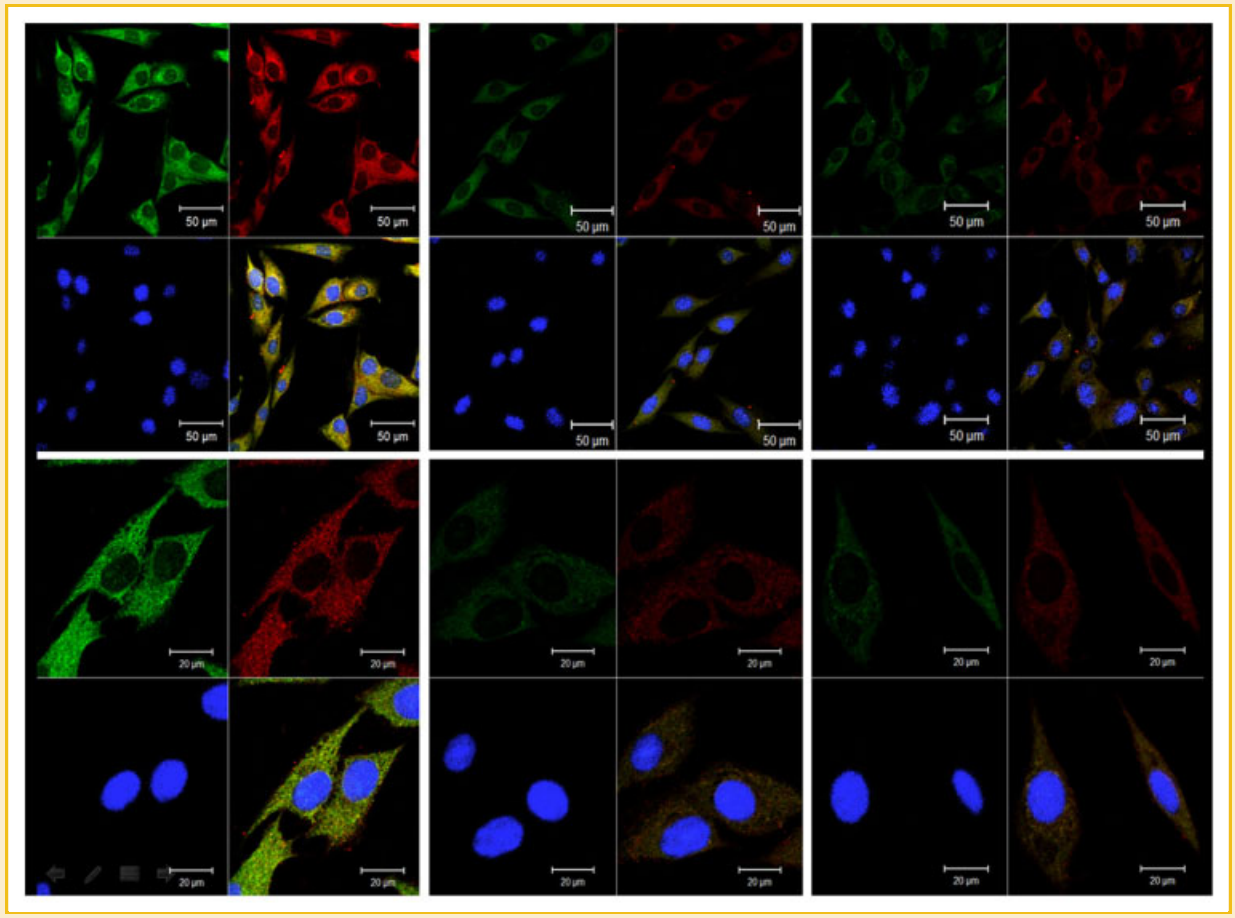

Fig. 4. Immunofluorescent images of MG-63 cells transfected with pcDNA3.1 (+)-RAMP1 (left), empty vector (middle) and nothing (right) are shown. Single colour and merged images for RAMP1 (green), CRLR (red) and nucleus (blue) staining are shown. The scale bar represents 50 μm (top row) and 20 μm (bottom row). Representative photomicrographs are shown for three independent experiments.

overexpression group, CRLR is more highly expressed and more widely distributed than that in the other two groups (Fig. 4).

OSTEOGENIC DIFFERENTIATION

ALP and alizarin red staining. Alkaline phosphatase expression was used to assess early osteogenic differentiation of cells osteogenic induction cultured under the same CGRP concentration (10^{-8} mol/L). Light micrographs were taken of culture wells containing cells that were stained blue to qualitatively visualise ALP expression. Cells in the RAMP1 overexpression, empty plasmid control and negative control groups were stained 7 or 14 days after induction of osteogenic differentiation. On the 7th day, the microscopy images revealed that cells in the RAMP1 overexpression group were strongly positive for ALP expression, while the other two groups were weakly positive. The ALP expression status is indicated by varying intensities of purple staining. We observed dark purple granules in the cytoplasm. The RAMP1 overexpression group exhibited larger precipitation areas with darker colours, which indicated that there was more ALP expression than that in the other two groups. We observed no obvious difference between the empty vector and untransfected control groups in terms of positive staining. When stained on the 14th day, all groups exhibited weakly positive staining for ALP expression, with a fairly even distribution within the culture wells. Overall, we observed more dark purple granular precipitation than that on day 7. The staining in the RAMP1 overexpression group was darker than that in the other two groups, which indicated stronger ALP expression. We observed no significant difference between the empty vector group and the untransfected control group (Fig. 5).

Calcium content was used to assess late osteogenic differentiation of cells that had been undergoing osteogenic differentiation for 28 days. Light micrographs were taken of culture wells containing RAMP1-overexpressing cells or control cells stained red to qualitatively visualise the calcium deposition. All cultures showed some calcium deposition with varying intensities of red staining. Furthermore, the distribution of calcium deposits within the culture wells varied overall. The cells in all the groups decreased in size, and the gaps between the cells narrowed or disappeared compared with the earlier cultures. Cells in the RAMP1 overexpression group were darker in colour and formed many mineralised nodules with clear boundaries, which are typical patterns of calcium deposition in mineralised bone matrix structures. These mineralised nodules can be detected under optical microscope, but it is difficult to distinguish individual cells within the nodules. The empty vector control and

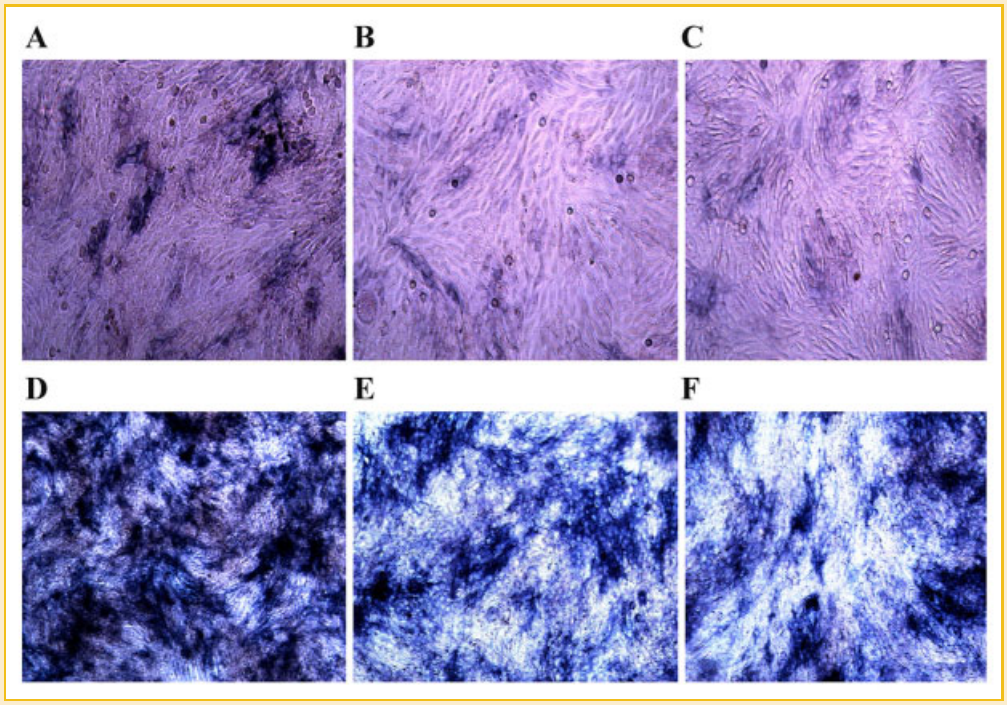

Fig. 5. Alkaline phosphatase staining of cells after 7 and 14 days of culture with CGRP. A–C are representative images of cells stained for ALP 7 days after induction of osteogenic differentiation, representing each of the three groups: the RAMP1 overexpression group, empty vector group and untransfected control group, respectively. D–F are representative images of cells stained for ALP 14 days after induction of osteogenic differentiation, representing each of the three groups: the RAMP1 overexpression group, empty vector group and untransfected control group, respectively. All images are magnified 100 times.

negative control groups showed a significant difference in calcium content compared with the overexpression group. Blank regions lacking calcium deposits were the most apparent for the empty vector control and negative control groups, whereas calcium deposition appeared more evenly distributed in the RAMP1 overexpression cultures. Microscopy images revealed that the blank regions indeed had cells growing that did not stain red for calcium deposits. We observed no significant difference between the empty vector group and the blank control group (Fig. 6).

COLLAGEN I

We used real-time PCR to detect collagen I mRNA expression in the three groups. We used real-time PCR to detect collagen I mRNA expression in the three groups after they had been treated with CGRP for 7 days to induce osteogenic differentiation. Quantification of the osteogenic marker collagen I revealed a remarkable up-regulation of this gene in the RAMP1 overexpression group compared with controls ($P < 0.05$).

ALP ACTIVITY

The alkaline phosphatase activity was also measured for each group and normalised to the total protein content to quantitate the amount of early osteogenic differentiation. ALP activity of the three groups was detected after CGRP induction for 3, 6 or 9 days. ALP activity of MG-63 cells had increased after 3 days of induction compared with that of untreated cells. The CGRP-induced ALP activity of the overexpression group was significantly higher 3, 6 and 9 days after induction than that in both control groups ($P < 0.05$). ALP activity increased over time and reached a maximum on the 9th day. There was no significant difference in the ALP activity between the negative control and the empty vector control groups ($P > 0.05$) (Fig. 7).

DISCUSSION

In our experiments, we constructed a eukaryotic RAMP1 expression vector and stably transfected it into MG-63 cells. As expected, this construct clearly promoted RAMP1 expression. Our experiments show that the experimental group has higher RAMP1 mRNA and protein expression than the empty vector and untransfected control groups, indicating that we successfully overexpressed the target gene in MG-63 cells. RAMP1 mRNA and protein expression did not differ between the empty vector and untransfected control groups, which demonstrates that transfection of the pcDNA3.1 (+) vector alone does not affect RAMP1 expression in MG-63 cells.

During bone metabolism, CGRP regulates osteoblast proliferation and differentiation mainly through phosphorylation of CRLR, which then regulates the intracellular Ca^{2+} concentration, cAMP levels and protein kinase C (PKC) [Li et al., 2009]. RAMP1 is the rate-limiting protein for this receptor and determines the ligand binding capabilities and signalling strength of CRLR [Hay et al., 2006]. Our data show that cells overexpressing RAMP1 display higher levels of CRLR mRNA and protein compared with those in either

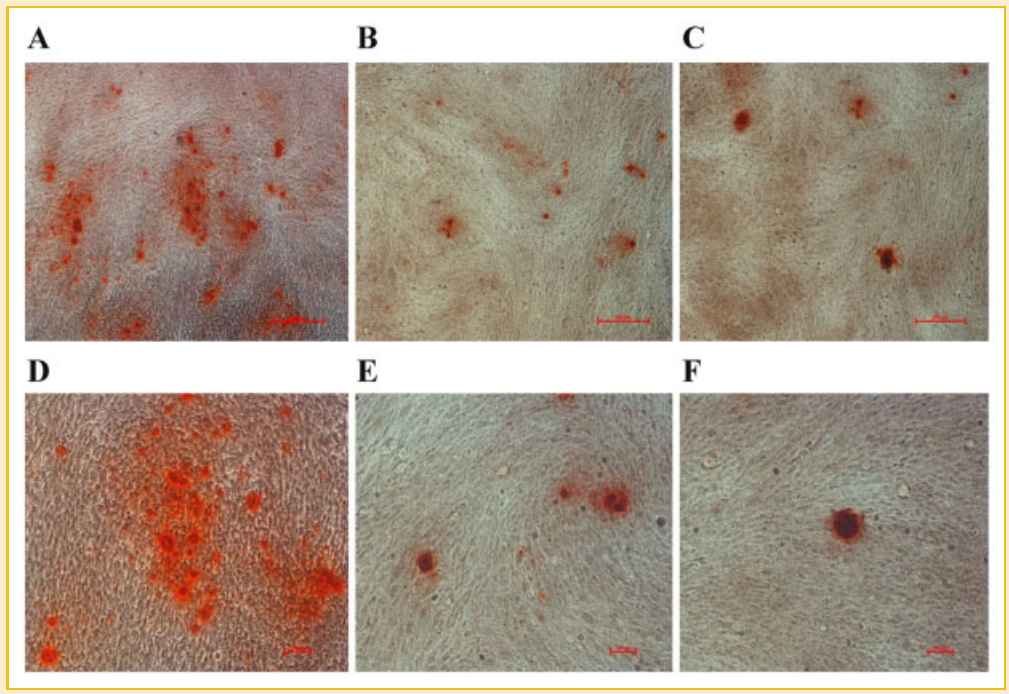

Fig. 6. Alizarin Red staining of cells after 28 days of culture with CGRP. A–C are images of cells stained with Alizarin Red after 28 days of osteogenic differentiation, representing the RAMP1 overexpression group, empty vector group and untransfected control group, respectively. D–F are the same images at a higher magnification. The scale bar represents 500 μm for A–C and 100 μm for D–F.

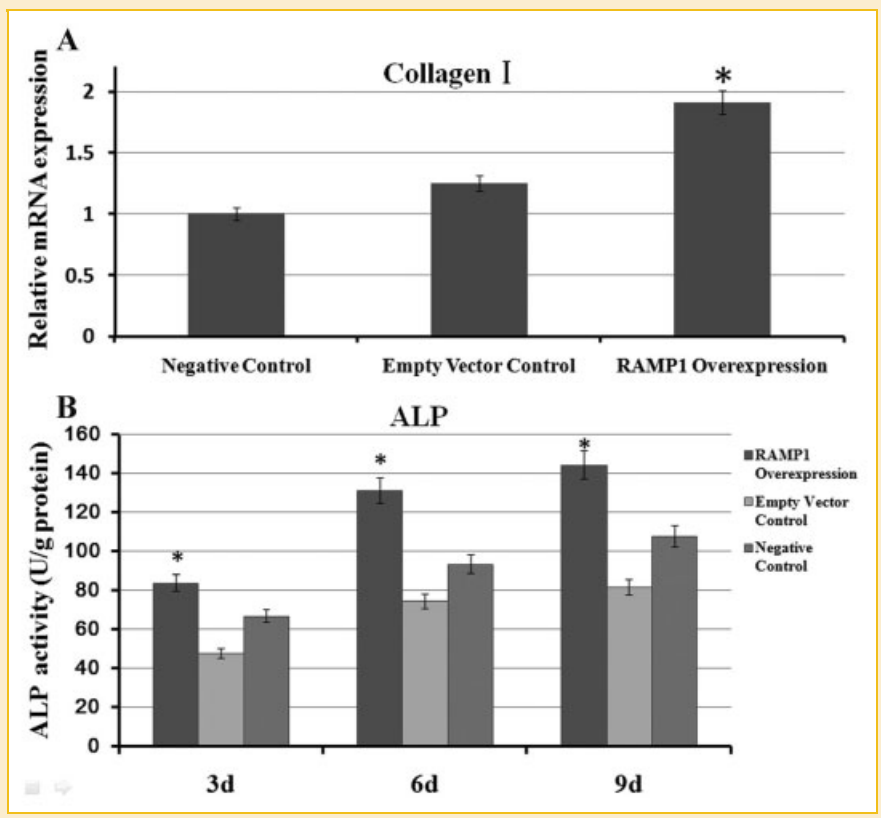

Fig. 7. A: Expression of Collagen I mRNA was examined using real-time PCR. Osteogenic differentiation led to a considerable increase in the expression of collagen I mRNA. Real-time PCR analysis of MG-63 cells that had undergone osteogenic differentiation demonstrated that cells in the overexpression group had higher collagen I levels compared with control groups. The RAMP1-overexpressing cells show a remarkable up-regulation of collagen I. All data are normalised to GAPDH and calibrated on the negative control, whose expression is considered 1 for all genes. The Y-axis is on a logarithmic scale. A significant difference between transfection with pcDNA3.1 (+)-RAMP1 and transfection with vector alone or control is shown ($^{*}P < 0.05$ negative control vs. the empty vector control and RAMP1 overexpression). B: Relative activities of ALP of different groups at day 3, 6 and 9 after osteogenic differentiation. Error bars represent the mean ± SD for n = 3. $^{*}P < 0.05$ versus the negative control group.

control group. We did not detect any difference between the empty vector and untransfected control groups. These results indicate that RAMP1 overexpression promotes CRLR protein expression and contributes to its transport from the cytoplasm to the cell membrane. This relocalisation increases the amount of CRLR on the cell membrane and increases the binding sites available to CGRP. RAMP1 overexpression clearly increases osteoblasts' sensitivity to CGRP, receptor signalling and more efficiently promotes signalling into the cell. Kristiansen have previously shown that RAMP1 can efficiently transport CRLR from the cytoplasm to the cell membrane, and our results are consistent with this conclusion [Kristiansen and Edvinsson, 2010].

We detected collagen I mRNA and ALP activity in cells overexpressing RAMP1 after CGRP treatment. Compared with the two control groups, the experimental group showed greater differentiation as a result of the CGRP treatment. This result indirectly supports that RAMP1 overexpression can increase the sensitivity of MG-63 cells to CGRP. Similar phenomena were also observed in the ALP and osteocalcin stainings. After ALP staining, compared with the controls, the cells overexpressing RAMP1 exhibited larger areas of precipitation and their staining was darker. After the alizarin red staining, the RAMP1-overexpressing cells showed more dark mineralised nodules compared with the controls, which are a typical sign of osteogenic differentiation.

In our study, we overexpressed RAMP1 in MG-63 cells and found increased CRLR localisation on the cell membrane and increased sensitivity to CGRP. Our preliminary results provide evidence that RAMP1 overexpression enhances the promoting effect that exogenous CGRP has on osteogenic differentiation in MG-63 cells. However, in the bone microenvironment, the reciprocal osteoblast–osteoclast communication and the corresponding coordinated bone formation and resorption events are highly complex and regulated processes responsible for the life-long bone remodelling in order to ensure a healthy tissue [Matsuo and Irie, 2008]. The reciprocal modulation between MG-63 and osteoclastic cells might influence bone metabolism [Costa-Rodrigues et al., 2011]. We did not thoroughly explore many other aspects of this pathway, such as ligand binding sites, receptor activation, desensitisation and transportation. Further study of CGRP's role in regulating the activation of osteoblasts and osteoclasts during bone wound healing and in promoting growth factors associated with bone trauma will help to more clearly understand the role of neural peptides in this process [Mao et al., 2009]. We will continue to examine these aspects of osteogenic differentiation in our future research.

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