STC1 interference on calcitonin family of receptors signaling during osteoblastogenesis via adenylate cyclase inhibition

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A R T I C L E I N F O

A B S T R A C T

Stanniocalcin 1 (STC1) and calcitonin gene-related peptide (CGRP) are involved in bone formation/remodeling. Here we investigate the effects of STC1 on functional heterodimer complex CALCRL/RAMP1, expression and activity during osteoblastogenesis. STC1 did not modify *CALCRL* and *ramp1* gene expression during osteoblastogenesis when compared to controls. However, plasma membrane spatial distribution of CALCRL/RAMP1 was modified in 7-day pre-osteoblasts exposed to either CGRP or STC1, and both peptides induced CALCRL and RAMP1 assembly. CGRP, but not STC1 stimulated cAMP accumulation in 7-day osteoblasts and in CALCRL/RAMP1 transfected HEK293 cells. Furthermore, STC1 inhibited forskolin stimulated cAMP accumulation of HEK293 cells, but not in CALCRL/RAMP1 transfected HEK293 cells. However, STC1 inhibited cAMP accumulation in calcitonin receptor (CTR) HEK293 transfected cells stimulated by calcitonin. In conclusion, STC1 signals through inhibitory G-protein modulates CGRP receptor spatial localization during osteoblastogenesis and may function as a regulatory factor interacting with calcitonin peptide members during bone formation.

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1. Introduction

In vertebrates, bone homeostasis is a dynamic process which includes a balanced calcium and phosphate metabolism, essential to maintain a healthy bone mass. Bone formation and remodeling rely on coordinated functions of bone forming (osteoblasts), resident (osteocytes) and resorbing cells (osteoclasts) that are regulated by several local and endocrine factors that act on calcium and phosphate homeostasis (Bellido et al., 2013; Henriksen et al., 2009; Nakahama, 2010; Stenbeck et al., 2012). Most of these calciotropic hormones mediate their action on osteoblasts regulating the osteoclastic differentiation and activity (Cao, 2011; Costa et al., 2011; Kohli and Kohli, 2011; Naot and Cornish, 2008; Stenbeck et al., 2012).

Stanniocalcin 1 (STC1) is a glycosylated 50 kDa disulfide-linked homodimeric protein (Trindade et al., 2009) that not only acts on bone metabolism, but also is highly abundant in other tissues such as kidney, heart, ovary and brain (Basini et al., 2010; Jiang et al., 2000; Westberg et al., 2007; Worthington et al., 1999; Yoshiko et al., 2002). During embryonic development, STC1 is highly expressed in muscular and skeletal tissues, exhibiting calciotropic effects on osteogenesis by stimulating the major constituents of the mineral phase of bone (Jiang et al., 2000; Stasko and Wagner, 2001; Wu et al., 2006; Yoshiko et al., 2002). The STC1 calciotropic effect is associated with osteoblastic differentiation (Johnston et al., 2010; Yeung et al., 2012; Yoshiko and Aubin, 2004; Yoshiko et al., 2003) and chondrogenic growth inhibition (Wu et al., 2006). Moreover, transgenic studies on mice have shown that human (h) STC1 ectopic expression decreases bone length (Varghese et al., 2002) leading to dwarfism (Filvaroff et al., 2002). The STC1 induced-delay in bone development is associated with its effect on the regulation of growth plate

chondrogenesis, acting as a paracrine/autocrine growth factor (Wu et al., 2006). Furthermore, STC1 is also involved in angiogenesis (He et al., 2011). Recently, both STC1 mRNA and protein were shown to be up-regulated during synovial membrane inflammation in patients with osteoarthritis and was considered to be a key mediator of synovium neovascularization in osteoarthritis synovitis (Lambert et al., 2014).

Similar to STC, the calcitonin (CT) family members, a group of small peptide hormones, are also involved in calcium homeostasis, vertebrate osteogenesis and osteoblast function (Naot and Cornish, 2008). CT members share moderate sequence similarity but their secondary structures are strongly correlated. They are characterized by the presence of two conserved cysteine residue positions that form a ring structure at the N-terminus crucial for biological activity and receptor activation (Feyen et al., 1992; Naot and Cornish, 2008; Pozvek et al., 1997). In mammals, six CT family member peptides have been identified, which include calcitonin gene-related peptide alpha and beta (αCGRP and βCGRP), amylin (AMY), adrenomedullin 1 (ADM1), adrenomedullin 2 (ADM2 or intermedin) and calcitonin receptor-stimulating peptide (CRSP) (Granholm et al., 2011; Naot and Cornish, 2008) with distinct effects on bone cells.

Calcitonin gene-related peptide (CGRP) is a small neuroendocrine peptide (Amara et al., 1982; Goodman and Iversen, 1986) that, similar to STC1, has an important role in osteogenesis and osteoblast activity. CGRP is abundantly expressed in skeletal sensory neurons and is involved on bone formation and inhibition of bone resorption (Granholm et al., 2011; Schinke et al., 2004; Villa et al., 2006; Wang et al., 2010). Similar to CT, and different from ADM1, the members of this peptide family have a predominant anabolic action and inhibit bone resorption and osteoclast formation (Granholm et al., 2011; Naot and Cornish, 2008; Wang et al., 2010). In mammals, CGRP immunoreactivity is detected in bone tissue – in the periosteum, bone marrow and the epiphyseal trabecular bone, and the presence of CGRP in osteoblast cultures was found to increase cell proliferation and the synthesis of cytokines, growth factors, and collagen (Chang and Hsu, 2013; Vignery and McCarthy, 1996; Villa et al., 2006). An important stimulatory effect of CGRP during early stages of the osteoblastic differentiation has been also reported (Kawase et al., 2003; Wang et al., 2010). The receptor for CT (CTR) is also detected in osteoblasts. However the effect of CT in osteoblasts bone formation is not consensual (Monier-Faugere et al., 1996; Villa et al., 2003).

CGRP activates a specific receptor, CALCRL, a member of the class 2 B1 GPCR family which forms a dimer complex with a transmembrane accessory protein, the receptor activity-modifying protein 1 (RAMP1) (Born et al., 2002; Conner et al., 2002). CALCRL and RAMP1 have been detected in pre- and mature osteoblasts and, in the presence of CGRP, the protein complex signals via an increase in intracellular cAMP, calcium and insulin-like growth factors (Naot and Cornish, 2008; Togari et al., 1997; Uzan et al., 2004; Villa et al., 2006).

In contrast to CGRP, the molecular mechanisms that regulate STC1 action remain largely unknown. A specific STC1 receptor has not yet been identified, which makes the functional characterization of the protein more difficult. Based on the fact that calcitonin family peptides and STC1 act on bone metabolism, we hypothesize that their signaling pathways may interact with each other. Recently in the zebrafish embryo model, it was demonstrated that overproduction of CT peptide increased by 3-fold the expression of STC1 suggesting a feedback mechanism between the two hormones (Lafont et al., 2011). Thus, the present study was designed to better elucidate the role of STC1 in human bone formation/remodeling by investigating the potential role of STC1 on CGRP signaling during the differentiation of osteoblast, and to determine whether STC1 modulates CTR and the CGRP functional receptor. The abundance of CALCRL/RAMP1 transcript, the modulation of the receptor complex in cell plasma membrane and the influence of STC1 on CGRP intracellular signaling were studied using human pre-osteoblasts adipose derived stem cells and receptor transfected human embryonic kidney cell lines.

2. Materials and methods

2.1. Cell lines and culture maintenance

Commercially available Poietics™ human adipose derived stem cells (hADSC) isolated from lipoaspirates were purchased from Lonza (Lonza Group, Switzerland). Cells were maintained as adherent cultures in complete Dulbecco's modified Eagle's medium (DMEM, Sigma, Spain) supplemented with 10% fetal bovine serum (FBS), 0.1% penicillin:streptomycin antibiotic mix (10.000 U:10 mg/ml, Sigma, Spain) and 250 µg/ml sterile filtered 1:100 amphotericin B solution (Sigma, Spain) at 37 °C/5% CO_2. Media were changed every 3 days, and the cells were split when they reached 80–90% confluence. Cells were used at early passage (4–8 passages) for all experiments. Human Embryonic Kidney (HEK) 293 cells (ECACC collection, UK) were maintained at 37 °C/5% CO_2 in complete DMEM supplemented with FBS as previously described and divided every 3–4 days 80–90% confluence was reached.

2.2. hADSCs osteoblastic differentiation

hADSC were differentiated to pre-osteoblast for 7, 14, and 21 days, using osteoblastogenic differentiation medium consisting of DMEM supplemented with 10% FBS, 50 µM ascorbate-2-phosphate, 100 mM beta-glycerophosphate, and 0.1 µM dexamethasone, according to previously published techniques (Zuk et al., 2002). To confirm the osteogenic phenotype, differentiated cells were fixed in 4% PFA, rinsed with PBS and stained with Alizarin Red S for 5 minutes and imaged with light microscopy.

2.3. ATP assay

ATP concentration is used as a marker for cell viability because its concentration rapidly declines when cells undergo necrosis or apoptosis. hADSCs were differentiated to pre-osteoblast cells using the osteoblastogenic cocktail supplemented with increased doses of human STC1 (5.0, 50 and 2000 pM; hSTC1, Biovendor, Czech Republic) (Basini et al., 2009) to eliminate the hypothesis that STC1 could induce cell death during osteoblastogenesis. The effects of hSTC1 on the intracellular ATP pool were measured on day 7 after the beginning of the osteoblastic induction. After the experimental period, cells were collected and immediately frozen in liquid nitrogen prior to homogenization in lysis buffer (TCA 6%, NaF 20 mM, Gelatin 0.4 g.l^{-1}). ATP was determined using the bioluminescent luciferin kit (Invitrogen, USA) according to the manufacturer's protocol. Results obtained were expressed as intracellular ATP concentration (pM).

2.4. Isolation and cloning of CALCRL and ramp1

The human CALCRL and ramp1 mRNAs were isolated from hADSCs differentiated to osteoblast cells. The human CALCRL full-length transcript was amplified from cDNA (see discussion later) using the primers forward 5′ atggagaaaaagtgtaccctgaat and reverse 5′ tcaattatataaattttctggtttttaag (annealing temperature 54 °C, 35 cycles) and ramp1 with primers forward 5′ atggcccgggccctgtgccgcct and reverse 5′ ctacacaatgccctcagtgcgcttg (annealing temperature 64 °C, 40 cycles). All PCR reactions were performed using the Taq DNA Polimerase High Fidelity (Invitrogen, USA) for a final reaction of 50 µl according to the manufacturer's protocol. PCR products obtained were run on 1% agarose/1× TBE gels and products of the correct size

were cloned in pGEM T-easy vector (Promega) and sequenced to confirm identity. The human *CALCRL* and *ramp1* transcripts were amplified from the cloning vector using the iProof DNA polymerase (BioRad, Portugal) according to the previously described conditions and the PCR products obtained were purified and subcloned into the expression pcDNA3.1 vector (Directional TOPO Expression Kit, Invitrogen, USA). The ligation product was used to transform DH5-alpha *E. coli* bacteria. Bacteria containing the correct construct were selected and recombinant plasmids were extracted using the GeneJet TM plasmid Miniprep kit (Fermentas, Portugal). All purified plasmids were used to transfect human HEK293 cells for intracellular signaling assays.

The human calcitonin receptor (CTR; *CALCR gene*) clone in pcDNA 3.1 expression vector was purchased from Missouri S&T cDNA resource Center, USA (catalog number CALCR 00000) and used in the transfection assays (as described earlier) as a model of comparison on cAMP assays (see discussion later).

2.5. RNA extraction, cDNA synthesis and quantitative expression

To analyze the effect of hSTC1 on *CALCRL* and *ramp1* mRNA expression during human osteoblastogenesis, hADSCs cells were differentiated to osteoblasts for up to 21 days using the osteoblastogenic cocktail supplemented with 2 nM hSTC1. Cells were collected at days 7, 14 and 21. Briefly, semi-confluent cultures of undifferentiated hADSCs and pre-osteoblast cells were washed three times with $1\times$ PBS and harvested by centrifugation. Total RNA was extracted with Trizol (Invitrogen, USA) according to the manufacturer's instructions. RNA quantity was assessed by absorbance in the BioPhotometer plus (Eppendorf, USA) and quality was checked by electrophoresis on 1.5% agarose gels.

cDNA was synthesized with 2 μg of total RNA in a reaction mixture containing 5 mM of DTT, 0.5 mM dNTPs mixture (Ludwig Biotech, Brazil), 1.0 μg of random hexamers (pd(N)6), 10 U rRNAsin RNase inhibitor (Promega, USA), 50 U MMuLV reverse transcriptase (Thermo Scientific, USA) for a 20 μl final reaction volume complemented with sterile distilled water. The cDNA synthesis reaction was performed as follows: 10 min at 25 °C followed by 120 min at 37 °C and finally 10 min at 65 °C to inactivate enzyme activity.

Semi quantitative real-time reverse-transcriptase PCR (RT-PCR) analyses were run using specific primer pairs for *CALCRL* and *ramp1*. Primer sequences for RT-PCR were designed using the IDT Design Software (Integrated DNA Technologies Inc., USA) to distinguish exons. Primer sequences were assessed for specificity using non-redundant basic local alignment search tools (www.ncbi .nlm.nih.gov/BLAST) (Altschul et al., 1990) and target-specific sequence alignment programs (Espinosa et al., 2013). *Tbp* was used as reference gene and the results presented relative to *Tbp* expression. Primer pairs were: *CALCRL* forward 5′ gatttccactgattcctgcttg (exon 11); reverse 5′ ggcaccaagataagagtagctc (exon 13) (amplicon size: 218pb; NM_005795.4); *ramp1* forward 5′ atcacctcttcatgaccactg (exon 2); reverse 5′ cctgtccacctctgcatt (exon 3) (amplicon size: 218pb; NM_005855.2). RT-PCR reactions were performed with 0.1 μM of each specific primer, 0.5 ng/μl cDNA and Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen, USA). The thermal cycling conditions used were: 2 min at 50 °C, 2 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s. Under these conditions, RT-PCR produced a single product at the melting temperature for each analyzed gene. Control reactions (cDNA synthesis reaction without reverse transcriptase) were performed to verify genomic DNA contamination. The mean Ct values from triplicate measurements were used to calculate expression of the target gene, with normalization to internal control (*Tbp*) using the $2^{-\Delta\Delta Ct}$ formula (Schmittgen and Livak, 2008). All gene expression results are based on 3 independent experiments.

2.6. Laser-scanning confocal microscopy

To quantify the CALCRL and RAMP1 protein expression on plasma membrane of hADSCs and 7-days differentiated pre-osteoblasts and to verify the effects of hSTC1 peptide on the distribution pattern of the CGRP heterodimeric receptor complex, an immunocytochemistry assay was performed and confocal microscopy analysis was carried out. Briefly, hADSC were cultured at the aforementioned conditions on 4-chamber glass bottom dishes and were differentiated to pre-osteoblasts during 7 days without hSTC1. Sequentially, hADSCs and pre-osteoblasts were exposed to 2.0 nM of hSTC1 or 2.0 nM of human CGRP for 30 min at 4 °C (hCGRP, cat. number C 0167; Sigma-Aldrich, São Paulo, Brazil). A number of studies that describe the biological effects of STC1 *in vitro* used STC1 at 0.1–15 nM, however, the STC1 effects at doses ranging from 1 nM to 10 nM were found to be very similar (Sazonova et al., 2008; Zlot et al., 2003) . Thus, the concentrations of STC1 and hCGRP used in the experiments (2 nM) were chosen based on studies of Granholm et al. (2011), Zlot et al. (2003), Yoshiko et al. (2003) and Wang et al. (2010). The hCGRP peptide was used as a positive control since it is the native ligand of the heterodimeric CALCRL/RAMP1 receptor complex (Bailey et al., 2012; Hay et al., 2005; McLatchie et al., 1998; Naot and Cornish, 2008).Unexposed cells were used as control. After hSTC1 or hCGRP exposure, cells were washed and incubated with rabbit polyclonal antibody against human CALCRL (clone H-42, cat. no. SC30028) and goat polyclonal antibody against human RAMP1 (clone N-20, cat. no. SC8050) (both from Santa Cruz Biotechnology, USA) diluted in a PBS/blockage solution with 5% of FBS plus 1% of bovine serum albumin (BSA) at 4 °C for 1 h to reduce background staining. Further, cells were washed, fixed with 4% paraformaldehyde in PBS for 20 min before incubation at 4 °C with anti-rabbit antibody conjugated with the green fluorescent Alexa Fluor® 488 and anti-goat antibody conjugated with red fluorescent Alexa Fluor® 555 (both from Life Technologies, USA), which were used to label the primary antibodies for CALCRL and RAMP1, respectively (Vilcaes et al., 2011). Ten single confocal sections of 0.7 μm were taken parallel to the coverslip (*xy* sections) on the Olympus FV1000 laser-scanning confocal microscopy equipped with an ×60 (numeric aperture 1.35) oil-immersion objective (Olympus, U plan-super-apochromat, UPLSAPO60XO). The CALCRL fluorescence was acquired after excitation by a 488 nm laser beam and the emission scan collected at 520 nm. The RAMP1 fluorescence was acquired after laser excitation at 555 nm and emission collected at 647 nm. Imaging analysis is based on 3 independent experiments. Images of three fields of each sample were acquired with Olympus FluoView FV1000 software. Imaging pre-processing (iterative deconvolution) and fluorescence intensity measurement were performed using the public domain Java Image processing software imageJ (http://rsb.info .nih.gov/ij/). Fluorescence emission for CALCRL and RAMP1 was measured by establishing multiple regions of interest (ROI) that surrounded cell boundary thus intending to reduce background interference in all acquired images. To confirm the viewed proximity between CALCRL and RAMP1, co-localization analysis was performed in all images using the JACoP plugin as previously described (Bolte and Cordelieres, 2006; Dunn et al., 2011; Zinchuk et al., 2007). Pearson's coefficients were calculated considering Costes's automatic threshold that seeks to reduce nonspecific correlations (Costes et al., 2004).

2.7. Mammalian cell transfections with the CGRP heterodimeric receptor complex CALCRL/RAMP1

On the day prior to transfection, $2–3 \times 10^5$ cells were seeded in 6-well plates (Sarstedt, Portugal) and cells were transiently transfected with Fugene 6 transfection reagent (1:6 DNA:Fugene, Roche; USA) according to the manufacturer's protocol. Briefly, the

DNA transfection complex was incubated for 40 min at room temperature before adding to the cells. Cells with the transfection complex were incubated with 500 µl of DMEM medium for 6 h prior to addition of complete medium to a final volume of 2 ml per well.

Human *CALCRL* was transfected in a human RAMP1 stable HEK293 cell line. The stable RAMP1 cell line was generated using the linearized expression construct and 72 h after incubation, cells were selected by supplementing the complete medium with 800 µg/ml of the antibiotic geneticin (G418 sulphate, Gibco® Life Technologies, USA). The human RAMP1-pcDNA3 expression plasmid was constructed using a methodology similar to that previously described for the receptors. Prior to transfection, 5 µg of the plasmid was linearized using the NotI enzyme (Thermo Scientific, Fermentas, Portugal) and purified using the standard phenol:chloroform method. Cell recovery was monitored daily by constant changes of medium until no cell death was observed. RT-PCR using *ramp1* specific primers confirmed gene integration and transcript expression. The efficiencies of the cell transfections were obtained by performing a simultaneous transfection using the pCMV-EGFP vector (Clontech, USA). The human CTR was transiently transfected on wild HEK293 cells using the same protocol described earlier and used for comparisons with *CALCRL*. The receptor pharmacological responses to the hSTC1, hGCRP or human CT (human calcitonin cat no. T3535, Sigma, Spain) peptides to activate the human CALCRL-RAMP1 and CTR on cAMP-signaling pathway were assayed 72 h post transfections.

2.8. cAMP-signaling pathway

The capacity of the human STC1, CGRP and CT peptides to activate transfected CALCRL/RAMP1 and CTR HEK293 transfected cells and human 7 days pre-osteoblast cultures was measured by quantification of intracellular cAMP using the cAMP dynamic 2 kit (Cisbio, France) following the manufacturer's protocol. Approximately 15,000 of transfected cells/well were assayed and peptide incubations were performed in white 384 well small Volume™ HiBase Polystyrene microplates (Greiner, Germany). The osteoblast cell line was also accessed for cAMP production in the presence of the hSTC1 and hCGRP peptides using cells that derived from a 100% confluent culture on a T25 bottle due to their slow growth and difficulty to dislodge from the plastic.

Cell assays were performed for a final reaction volume of 20 µl. Prior to the assay, cells were re-suspended in 1× PBS with 1 mM of 3-isobutyl-1-methylxantine (IBMX, Sigma, Spain) and incubated for 5 min at 37 °C. Peptides diluted to a final concentration of 1–0.1 µM in 1× PBS/1 mM IBMX were added to the cells and incubated for 30 min at 37 °C in the CO_2 incubator. Non-transfected HEK293 cells were used as negative control and were stimulate with the highest STC1, CGRP and CT peptide concentration (1 µM). Forskolin (5 µM, Sigma, Spain) was used as positive control to assess cellular responsiveness of the cAMP pathway. For the calculations, two additional assay controls were also performed according to the manufacturer's instructions: (a) a negative background control without cAMP-d2 and (b) a non-stimulated control (no peptide added). Plates were read using a Biotek Synergy 4 plate reader (Biotek, USA) and results of cAMP stimulation were normalized according to the manufacturer's recommendations for data analysis. To test the peptides capacity to stimulate cell cAMP production, assays were initially performed using 1 µM and 0.1 µM of the hSTC1 and hCGRP on CGRP receptor complex and 1 µM and 0.1 µM of the hSTC1 and 0.1 µM of CT on the CTR transfected cells. The doses selected for the assays were based on the cell responsiveness to peptide stimulation and previous studies (Granholm et al., 2011; Wang et al., 2011).

To assess the effect of hSTC1 on the inhibition of cAMP production, native HEK293 cells were initially stimulated with 5 µM of forskolin for 30 min to achieve maximum cAMP production and then incubated for an additional 30 min with 0.1 µM or 1 µM of hSTC1. Maximum cAMP production of the receptor transfected cells was also stimulated by initially incubating CALCRL/RAMP1 and CTR HEK293 transfected cells for 30 min with 0.1 µM of the hCGRP and CT peptides, respectively followed by 30 min with 0.1 µM or 1 µM of hSTC1. To access if hSTC1 utilizes the CALCRL/RAMP1 complex and inhibits CGRP binding, transfected cells were also initially stimulated with 0.1 µM of hSTC1 for 30 min followed by 0.1 µM or 1 µM of hCGRP peptide for 30 min. Similar assays for the CTR were also performed using 0.1 µM or 1 µM of hCT peptide for 30 min. The amount of cAMP produced by non-transfected receptor cells was subtracted and assay data were normalized as percentage of cAMP accumulation. The effect of the empty receptor vector in cell signaling was also tested and values were equivalent to the peptide assays using non-transfected cells.

2.9. Intracellular calcium release

The effect of hSTC1 to stimulate intracellular Ca^{2+} (iCa^{2+}) release (RFU) was measured using the Ca^{2+} sensitive fluorescent dye Fluo-4 NW (Molecular Probes, Invitrogen, USA) according to the manufacturer's instructions on hADSCs, 7 days pre-osteoblast cultures, receptor transfected cell lines CALCRL/RAMP1 and CTR. Prior to the assay, plates (96 well black/plates, µClear bottom, Greiner, Germany) were coated with sterile poly-L-lysine (0.1 mg/ml, Sigma, Spain) to avoid cell release. Approximately 50,000 cells re-suspended in 100 µl of complete DMEM medium were plated per well and were allowed to attach overnight at 37 °C in a humid 5% CO2 incubator. Prior to the assay, growth medium was removed and cells were washed twice with 1× PBS and incubated for 30 min at 37 °C with 100 µl of Fluo-4 NW dye followed by an additional 30 min incubation period at RT. The background RFU of each well was measured prior to addition of the peptide and receptor response was carried out using 0.1 µM of hSTC1 peptide diluted in assay buffer. Calcium mobilization provoked by the presence of the peptide was measured every 10 s over a total period of 2 min on the plate reader. Positive control assays were performed with 100 nM Carbachol (Sigma-Aldrich, Spain) and negative control assays were performed using non-transfected HEK293 cells stimulated with 0.1 µM of hSTC1. Background fluorescence was measured in each assay by adding 10 µl of assay buffer. Maximal RFU values were used for calculations, which entailed subtraction of background values and data normalization before plotting RFU values against peptide concentration.

2.10. Statistical analysis

Statistical analysis was performed using SigmaStat (v. 3.50, Systat Software, Inc, San Jose, CA, USA). Gene expression and cAMP data were evaluated using one-way or two-way ANOVA when appropriated, followed by the Bonferroni multiple comparison procedures. Before applying the ANOVA test, data were checked for normality (Kolmogorof–Smirnov test) and homogeneity (Levene test). In the absence of homogeneity, data were log-transformed prior to the ANOVA test. Protein expression was analyzed by Student's *t*-test (see Supplementary material). Data are presented as the mean ± standard error of the mean (SEM) and the level of statistical significance was $P < 0.05$. Each experiment was repeated three to six times.

3. Results

3.1. Effect of hSTC1 on osteoblasts ATP levels

Increased concentrations of hSTC1 (5.0, 50 and 2000 pM) during osteoblastogenesis did not modify the intracellular levels of ATP, indicating that hSTC1 peptide incubations did not affect human osteoblast cell viability (see Supplementary material, Fig. S1).

3.2. Effect of hSTC1 on CGRP signaling

3.2.1. Effect on CGRP receptor complex expression

CALCRL gene expression was low in undifferentiated hADSC and did not change over time. However, in differentiating cells and hSTC1-treated cells, the expression of CALCRL was significantly elevated at 7 days compared to undifferentiated hADSC and by 21 days levels were similar between the three groups of cells (see Supplementary material, Fig. S2, A). Ramp1 gene expression was generally low and only elevated in untreated differentiated cells at 7 and 14 days (see Supplementary material, Fig. S2, B). Ramp1 transcript expression was not affected by STC1 peptide treatment.

CALCRL and RAMP1 proteins were present at the plasma membrane cell surface of hADSCs and 7-day pre-osteoblast cells, as revealed by immunofluorescence (Figs. 1 and 2 and Fig. S3). CALCRL expression was highest at day 7 compared to non-differentiated hADSC, in agreement to the mRNA expression results. There was no statistical difference in RAMP1 protein expression during the same time period (see Supplementary material, Fig. S3).

3.2.2. Effect of hSTC1 or hCGRP on the CGRP heterodimeric receptor complex CALCRL/RAMP1
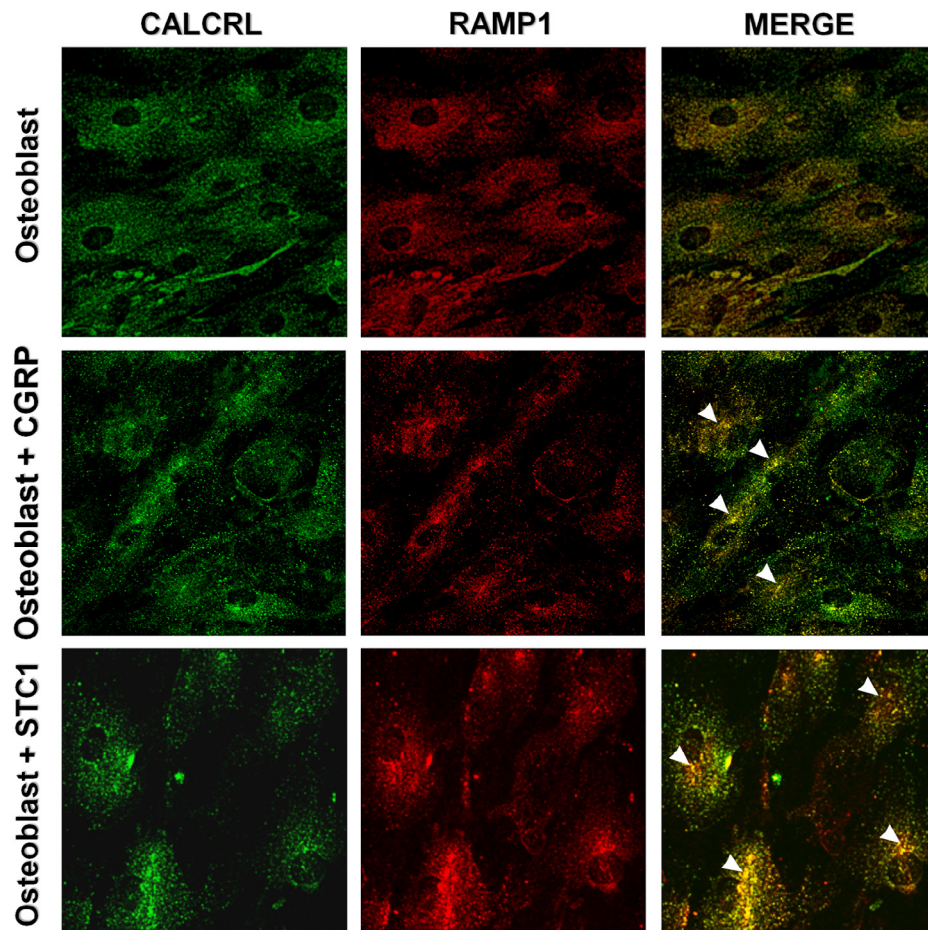
The expression of the CGRP heterodimeric receptor complex on the cell membrane surface was evaluated in hADSCs (Fig. 1) and in 7-day pre-osteoblast cells (Fig. 2) by confocal microscopy in the absence and presence of hSTC1 and hCGRP peptides. The overall co-localization between the CALCRL/RAMP1 monomers was confirmed by calculating Pearson's coefficient and can be observed in orange in the merged images that were obtained by superimposition of the green- and red-labeled fluorescent images (Figs 1 and 2). Incubation with 2 nM of hSTC1 or hCGRP for 30 min modified the spatial localization of CALCRL and RAMP1, eliciting a cluster of the receptor complex in specific areas at the surface of both types of cells, indicating a similar effect of both hSTC1 and hCGRP peptides.

3.2.3. Effect of hSTC1 or hCGRP on cAMP and iCa^{2+} cell signaling

Incubation of 7-day differentiated human osteoblast cells with 0.1 μM hCGRP, but not hSTC1, promoted cAMP accumulation. This indicates that CGRP receptor complex is expressed and functional at the osteoblast cell plasma membrane and suggests that the effect
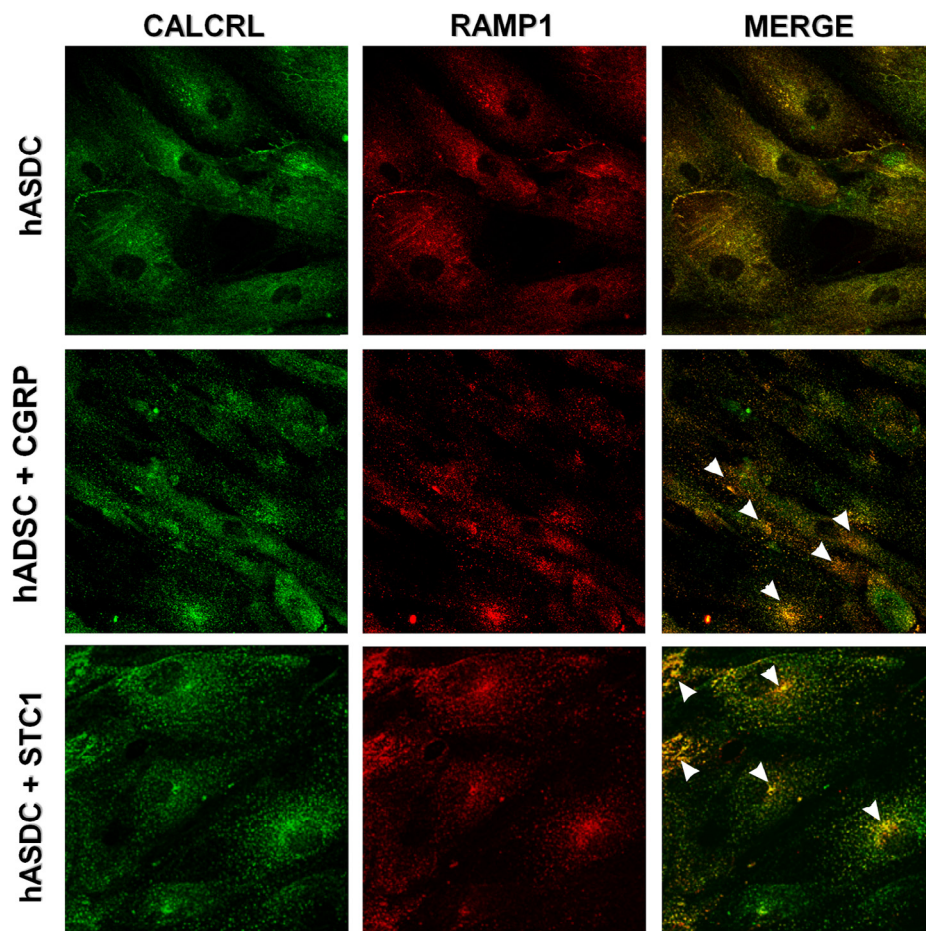
Fig. 1. Spatial distribution of CGRP heterodimeric receptor in the presence of hCGRP and hSTC1 on the cell surface of human adipose derived stem cells (hADSCs) demonstrated by immunocytochemistry. Green fluorescence indicates the presence of CALCRL receptors; red fluorescence indicates RAMP1 accessory protein. Overlapping of CALCRL and RAMP1 images (MERGE) revealed that both proteins are in the same cellular microcompartment. hADSCs were grown on 4-chamber glass bottom dishes, then exposed to hCGRP (2 nM) or hSTC1 (2 nM) diluted in DMEM for 30 min. Unexposed cells were used as negative control (hASDC). Cells were immunostained for CALCRL and RAMP1 as indicated under Section 2. Both proteins seem to be close to each other at the cell surface of unexposed hADSCs. Incubation with STC1 or CGRP modified the spatial localization of CALCRL and RAMP1, eliciting a concentration of the receptor complex in specific areas at the cell surface (white arrows head). Pearson's coefficients of correlation for all the overlapping CALCRL and RAMP1 images were: 0.65 ± 0.02 for control; 0.64 ± 0.01 for hCGRP and 0.55 ± 0.03 for hSTC1. The fluorescence images are representative of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Spatial distribution of CGRP heterodimeric receptor in the presence of hCGRP and hSTC1 on the cell surface of pre-osteoblasts demonstrated by immunocytochemistry. Green fluorescence indicates the presence of CALCRL receptors; red fluorescence indicates RAMP1 accessory protein. Overlapping of CALCRL and RAMP1 images (MERGE) revealed that both proteins are in the same cellular microcompartment. hADSCs were differentiated to 7-days pre-osteoblasts on 4-chamber glass bottom dishes, then exposed to hCGRP (2 nM) or hSTC1 (2 nM) diluted in osteoblastogenic cocktail for 30 min. Unexposed 7-days pre-osteoblasts were used as negative control. Cells were immunostained for CALCRL and RAMP1 as indicated under Section 2. Both proteins seem to be close to each other at the cell surface of unexposed 7-days pre-osteoblasts. Incubation with hSTC1 or hCGRP modified the spatial localization of CALCRL and RAMP1, eliciting a concentration of the receptor complex in specific areas at the cell surface (white arrows head). Pearson's coefficients of correlation were 0.64 ± 0.02 for control pre-osteoblasts, 0.64 ± 0.02 for the hCGRP peptide incubations and 0.71 ± 0.01 for hSTC1 peptide incubations, suggesting that the presence of hSTC1 favored the assembly of the CALCRL and RAMP1 complex. The fluorescence images are representative of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

CGRP in osteoblasts involves the cAMP pathway (Fig. 3A) but the signaling of STC1 remains unknown.

In vitro studies with HEK293 cells were used to clarify the potential interference of hSTC1 in hCGRP signaling. The hSTC1 failed to increase the cAMP levels in native HEK293 cells (Fig. 3B). However, in HEK293 cells first stimulated with forskolin to induce cell maximal cAMP accumulation, hSTC1 decreased cAMP levels in a dose-dependent manner (Fig. 3C). In contrast incubation with CGRP failed to induce cAMP accumulation (not shown), confirming that HEK293 do not express the CGRP functional complex (Atwood et al., 2011). These results indicate that a putative STC1 receptor is naturally expressed in HEK293 cells, suggesting that the observed hSTC1 signaling is not via CGRP functional receptor complex (Fig. 3C).

hCGRP was also able to activate cAMP accumulation in HEK293 CALCRL/RAMP1 transfected cells and an increase of cAMP levels was obtained when 1.0 µM of hCGRP was used in comparison to 0.1 µM (Fig. 4A). In contrast, no signal was obtained when hSTC1 was incubated with HEK293 CALCRL/RAMP1 transfected cell lines. *In vitro* cell assays using HEK293 expressing the CTR were also performed to compare the effect of STC1 in CGRP functional complex. The hCT peptide also stimulated cAMP in CTR transfected cells (Fig. 4B) but not in non-transfected HEK293 (not shown) conforming that HEK293

also do not express the CT receptor (Atwood et al., 2011). Similar to CALCRL/RAMP1, incubations using hSTC1 in HEK293 CTR transfected cells primed with hCT also induced a decrease of the cell cAMP levels (Fig. 4B).

CALCRL/RAMP1 and CTR transfected cells were also stimulated using different combinations of hCGRP/hCT and hSTC1 peptides to investigate the existence of potential peptide interactions by accessing the receptor response on cAMP production. In cells pre-incubated with 0.1 µM of hCGRP, addition of 0.1 µM and 1.0 µM of hSTC1 does not seem to modify cAMP accumulation. However, a dose-dependent decrease in cAMP accumulation was observed in hCT stimulated cells when hSTC1 was added (Fig. 4A and B). Similarly when receptor transfected cells were pre-incubated with hSTC1 (0.1 µM), addition of hCGRP and hCT also show different responses on the cell levels of cAMP. The presence of 1.0 µM of hCGRP significantly increased the cell cAMP ($p < 0.05$) to levels higher than 1.0 µM hCGRP alone (Fig. 4A) but a significant reduction ($p < 0.05$) of cAMP was observed in cells first primed with hSTC1 and subsequently with hCT when compared to hCT at 0.1 µM and 1.0 µM (Fig. 4B).

We also investigated the receptor response to intracellular calcium mobilization in the presence of hSTC1. The 7-day differentiated

A

B

C
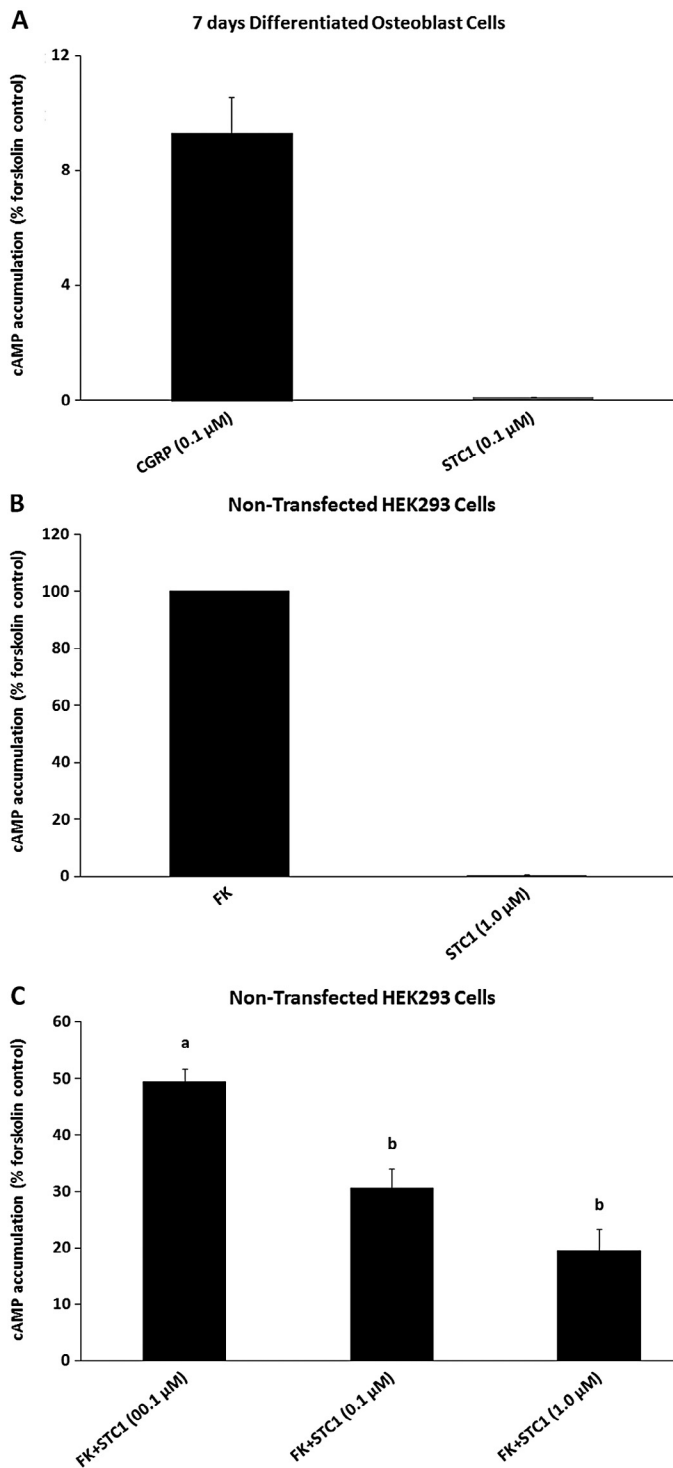
Fig. 3. Intracellular cAMP induced by hCGRP and hSTC1 peptides. (A) cAMP accumulation in pre-osteoblast cells differentiated from human adipose derived stem cells (hADSCs) after incubation with hCGRP (0.1 μM) and hSTC1 (0.1 μM). cAMP was measured after treatment with forskolin (FK, 5 μM), CGRP or STC1 for 30 min. (B) cAMP accumulation in non-transfected HEK293 cell lines stimulated by FK (5 μM) and hSTC1. (C) FK treated cells were subsequently incubated with STC1 (0.01 μM, 0.1 μM and 1.0 μM) for 30 min. Statistical differences were estimated using one-way ANOVA and different letters above bars indicate statistical significance of the 5% level.

human osteoblasts, native and transfected HEK293 cells expressing the human CGRP heterodimeric complex receptor or CTR receptor incubated with 0.1 μM of hSTC1 failed to mobilize calcium.

4. Discussion

The present study demonstrates that during the early period of osteoblastic differentiation, the hADSCs pre-osteoblast derived cells express the highest levels of the CALCRL/RAMP1 heterodimer complex. Furthermore, it shows that hSTC1 is able to elicit clustering of the CALCRL/RAMP1 heterodimer complex receptor in specific areas at the cell membrane. STC1 signal via inhibition of cAMP levels and the presence of the STC1 do not seem to affect cAMP levels induced by hCGRP, but inhibit the action of hCT via CTR.

Several lines of evidence indicate that STC1 (Yoshiko et al., 2002, 2003) and CGRP (Schinke et al., 2004; Wang et al., 2010) are associated with the same processes in osteoblast cells. In the absence of a cognate STC1 receptor we focus on the potential interaction between STC1 and CGRP signaling during osteoblastic differentiation.

The CGRP receptor is a dimeric complex of CALCRL and RAMP1, needing the presence of both for peptide activation (Aiyar et al., 1996; McLatchie et al., 1998; Roh et al., 2004; Smillie and Brain, 2011). The CALCRL/RAMP1 complex was detected in several osteoblastic lineages, including mouse MC3T3-E1, MG63 osteoblastic cell lines, rat primary calvarial osteoblasts and human primary osteoblast cell cultures (Schinke et al., 2004; Togari et al., 1997; Uzan et al., 2004; Villa et al., 2006). The effects of CGRP in osteoprogenitor cells are predominant in the earlier stages of the differentiation process (in the first 2 weeks) prior the mineralization onset (Naot and Cornish, 2008; Wang et al., 2010).

When osteoblast cells are stimulated by CGRP, the CALCRL/RAMP1 heterodimeric complex increases adenylate cyclase (AC) activity and, consequently, intracellular cAMP levels (Chang and Hsu, 2013; Kawase et al., 2003; Villa et al., 2006). In contrast, in the present study, hSTC1 alone had no effect on *CALCRL* and *Ramp1* gene expression and iCa^{2+} release in any of the cell types studied (undifferentiated, differentiating and HEK293 cells). Moreover, it inhibited cAMP buildup in HEK293 cells primed by forskolin but does not seem to modify cAMP levels on CALCRL/RAMP-transfected cells when hCGRP is present. This indicates that HEK293 cells express a functional STC1 receptor and that the presence of STC1 does not seem to modify CGRP functional complex response to CGRP. In contrast, STC1 inhibits CT induced intracellular cAMP formation in CTR over-expressing HEK293 cells. In fact, the results obtained in our study provide evidence that hSTC1 seems to interfere with hCT potency for cAMP production. Moreover we were able to demonstrate that the presence of STC1 inhibits CT peptide function by decreasing the peptide potency to produce cAMP in cells in both combinatory *in vitro* peptide assays performed. The distinct effect of hSTC1 on cAMP levels elicited by hCGRP and hCT peptides may be related with the different capacities that both peptides had on cAMP production *in vitro* and that hCT was more potent than hCGRP in the HEK293 cell assay. Cross regulation of CT and STC has previously been suggested and an increase of CT peptide production in zebrafish eggs was shown to stimulate the increase expression of STC1 transcript resulting in a decrease of total calcium (Lafont et al., 2011). The fish data may indicate that the action of CT and STC1 may be coordinated in tetrapods and that STC1 may inhibit the role of CT *in vivo*, as suggested by our cAMP *in vitro* assays.

Since intracellular cAMP accumulation and calcium mobilization were not detected in response to STC1, it is unlikely that STC1 signaling occurs through a Gαs or Gαq coupled-GPCR in these cell lines. Despite previous suggestions of STC1 activation and signaling via cAMP–PKA pathway in fish tubular proximal cells (Lu et al., 1994), or improved calcium waves evoked by extracellular ATP (Block et al., 2010). Our results indicate that STC1 inhibits cAMP accumulation possibly through a Gαi coupled-receptor signaling or through unrelated GPCR receptor(s). The inhibition of cAMP synthesis by Gαi results in decreased activity of cAMP-dependent protein kinase (Skalhegg and Tasken, 2000). It appears that STC1 may bind to a Gαi
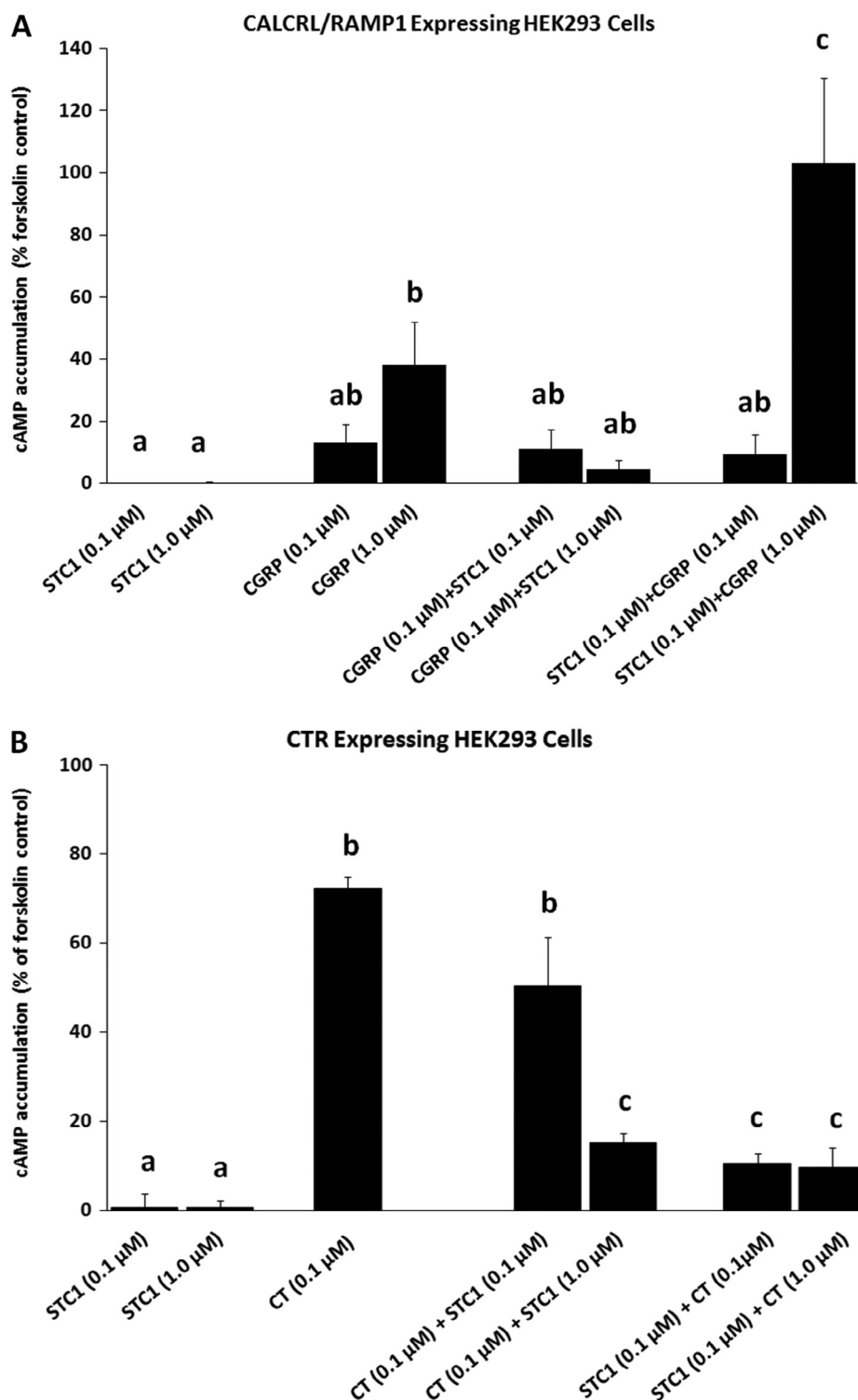
Fig. 4. cAMP accumulation in CALCRL/RAMP1 and CTR expressing HEK293 cell lines stimulated by hSTC1, hCGRP and hCT. (A) CALCRL/RAMP1 transfected cells were treated with hSTC1 (0.1 μM and 1.0 μM) or hCGRP (0.1 μM and 1.0 μM) for 30 min before cAMP measure. To examine if hSTC interferes with hCGRP cAMP production, cells were pre-treated with hCGRP (0.1 μM) or with hSTC1 (0.1 μM) for 30 min and subsequently with hSTC1 (0.1 μM and 1.0 μM) or hCGRP (0.1 μM and 1.0 μM), respectively for 30 min. (B) CTR transfected cells were treated with hSTC1 (0.1 μM and 1.0 μM) or hCT (0.1 μM) for 30 min before cAMP measure. To examine if hSTC interferes with hCT cAMP production, cells were pre-treated with hCT (0.1 μM) or with hSTC1 (0.1 μM) for 30 min and subsequently with hSTC1 (0.1 μM and 1.0 μM) or hCT (0.1 μM and 1.0 μM), respectively for 30 min. Bars (mean ± SEM) indicate the percentage of cAMP levels in relation to maximal FK stimulation of at least three independent experiments performed in triplicate. Statistical differences were estimated using two-way ANOVA and different letters above bars indicate statistical significance of the 5% level.

coupled-receptor (Gαi coupled-GPCR), which in turn blocks AC activity inside the cell with subsequent decrease in intracellular cAMP formation. This hypothesis is also supported by two other features: firstly, ERK1/2 (Nguyen et al., 2009) and cAMP/CREB pathways (Lu et al., 1994; Yeung et al., 2012; Yoshiko et al., 1996) are involved in STC1 intracellular signaling; secondly, Gαi coupled-GPCR stimulation can activate the ERK1/2 pathway through a downstream phosphorylation cascade, which activates RAS, RAF and

MEK, culminating in migration of the transcription factors ELK1 and/ or CREB to the nucleus (Avlani et al., 2013; Goldsmith and Dhanasekaran, 2007).

Similar inhibitory results on cAMP have been obtained using rat osteosarcoma cell lines incubated with human parathyroid hormone (PTH) plus a synthetic teleost *N*-terminal STC1 truncated peptide (Yoshiko et al., 1996). The fish *N*-terminal STC1 had a suppressing effect on cAMP accumulation evoked by PTH. Furthermore, in assays in which granulosa cells were incubated with 10 ng/ml of FSH, or hCG, plus 10 nM of STC1, a significant reduction in cAMP was also detected (Luo et al., 2004). Thus, it seems that STC1 activates similar signaling pathways independently of the cell line used, via inhibition of cAMP accumulation. Moreover, it is unlikely that STC1 inhibits intracellular cAMP levels by stimulating phosphodiesterase (PDE) activity, given that PDE activity was blocked by IBMX before the beginning of the cAMP assays. The measurements of intracellular ATP during osteoblastogenesis indicated that ATP levels are not affected by long-term treatment with hSTC1. Therefore, it is also unlikely that hSTC1 may reduce intracellular ATP, which must be available as a substrate for cAMP formation. Although HEK293 cell viability when incubated with STC1 was not measured, it is unlikely that the peptide affects cell function since vertebrate kidney cell lines normally express high levels of STC1 (Sazonova et al., 2008) and commercially available recombinant STC1 is produced in HEK cells.

Surprisingly, when CALCRL/RAMP1 transfected cells were preincubated with hSTC1 and subsequently with CGRP, the hCGRP stimulation of cAMP accumulation was more efficient than hCGRP alone. The increase of cAMP production in the presence of CGRP is intriguing and further studies are required. However, it is most likely to be an example of a paradoxical enhancement or sensitization of AC activity that results in increased cAMP accumulation when the action of the inhibitory receptor is ended (Watts and Neve, 2005). This has been interpreted as an adaptive response to the chronic inhibition. Moreover, confocal immunocytochemistry analysis also suggests that STC1 may interfere in the CGRP receptor localization in differentiated 7-day pre-osteoblasts by an unknown interaction and/or translocation mechanism at the plasma membrane level. However, how this modifies receptor signaling response remains to be established.

Taken together, the present results indicate an interaction of STC1 with the CGRP heterodimeric receptor complex CALCRL/RAMP1 with a possible specific cross-talk between STC1 and CGRP signaling during osteoblastogenesis. Further evaluation is required in order to establish the possible mechanism for this interaction. STC1 was also able to inhibit the CT signaling on CTR transfected cells and since CTR is also expressed on osteoblasts (Monier-Faugere et al., 1996; Villa et al., 2003) this results point out a possible important role of STC1 on the inhibition of the CT signaling during the osteoblastogenesis process.

Although binding assays have not been performed, evidence indicates that STC1 does not seem to share the same receptors than CGRP and CT; nonetheless the presence of STC1 seems to interfere with peptide signaling. Furthermore, future studies are necessary to clarify our new data about STC1 intracellular cAMP inhibition, such as the use of pertussis toxin that inhibit Gi proteins, coprecipitation assays as well as activation and phosphorylation of intracellular signaling molecules including MAP kinase and ERK.

5. Conclusions

To the best of our knowledge, this is the first investigation of a possible link between STC1 and CGRP and CT signaling during osteoblastogenesis. Our study indicates a cross-talk between CGRP and STC1 at the earlier stages of the osteoblast differentiation process. In addition to the previously reported direct effects on osteogenesis, our study shows that STC1 may regulate CGRP and CT signaling during osteoblastogenesis although the physiological consequences of these remain to be elucidated.

Author contributions

Conceived and designed the experiments: SRT, RCF, JCRC, FCRG, VS. Performed the experiments: SRT, JCRC, LAMM, RCF, VS. Analyzed the data: SRT, JCRC, LAMM, RCF, FCRG, VS, AVMC. Wrote the paper: JCRC, LAMM, FCRG, AVMC, VS. Supervisor: VS. Performed the experiments on RT-PCR, immunocytochemistry and ATP measures: SRT, LAMM, VS. Provided reagents and performed the experiments on transfection cells: JCRC, RCF, VS. Provided reagents and financial support on cell experiments: DOGS, AVMC, FRCG, VS.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.mce.2015.01.010.

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