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# Parathyroid hormone-related protein exhibits antioxidant features in osteoblastic cells through its N-terminal and osteostatin domains

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## Objectives

Oxidative stress plays a major role in the onset and progression of involutional osteoporosis. However, classical antioxidants fail to restore osteoblast function. Interestingly, the bone anabolism of parathyroid hormone (PTH) has been shown to be associated with its ability to counteract oxidative stress in osteoblasts. The PTH counterpart in bone, which is the PTH-related protein (PTHrP), displays osteogenic actions through both its N-terminal PTH-like region and the C-terminal domain.

## Methods

We examined and compared the antioxidant capacity of PTHrP (1-37) with the C-terminal PTHrP domain comprising the 107-111 epitope (osteostatin) in both murine osteoblastic MC3T3-E1 cells and primary human osteoblastic cells.

## Results

We showed that both N- and C-terminal PTHrP peptides at 100 nM decreased reactive oxygen species production and forkhead box protein O activation following hydrogen peroxide ($H_2O_2$)-induced oxidation, which was related to decreased lipid oxidative damage and caspase-3 activation in these cells. This was associated with their ability to restore the deleterious effects of $H_2O_2$ on cell growth and alkaline phosphatase activity, as well as on the expression of various osteoblast differentiation genes. The addition of Rp-cyclic 3′,5′-hydrogen phosphorothioate adenosine triethylammonium salt (a cyclic 3',5'-adenosine monophosphate antagonist) and calphostin C (a protein kinase C inhibitor), or a PTH type 1 receptor antagonist, abrogated the effects of N-terminal PTHrP, whereas protein phosphatase 1 (an Src kinase activity inhibitor), SU1498 (a vascular endothelial growth factor receptor 2 inhibitor), or an anti osteostatin antiserum, inhibited the effects of C-terminal PTHrP.

## Conclusion

These findings indicate that the antioxidant properties of PTHrP act through its N- and C-terminal domains and provide novel insights into the osteogenic action of PTHrP.

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**Keywords:** Parathyroid hormone-related protein, Osteostatin, Antioxidant activity

## Article focus

- Oxidative stress is associated with bone loss.
- We assessed *in vitro* the putative antioxidant properties of the osteogenic parathyroid hormone-related protein (PTHrP) and their ability to modulate osteoblastic function.

## Key messages

- PTHrP displays antioxidative stress activity in mouse and human osteoblastic cells.
- PTHrP (1-37) and PTHrP (107-111) (osteostatin) counteract the decrease of osteoblastic function triggered by oxidative stress.
- N- and C-terminal domains of PTHrP exhibit similar antioxidative stress features through different signalling mechanisms in osteoblastic cells.

## Strengths and limitations

- PTHrP preserves osteoblastic function in an *in vitro* oxidative environment that further supports the putative use of PTHrP peptides as osteoporosis therapy.
- Caution should be taken when translating these *in vitro* results to an *in vivo* scenario.

## Introduction

It is widely accepted that bone mass in humans starts to decline between the second and third decades of life, and this has been attributed to an increase in oxidative stress.[1,2] Compelling evidence indicates that oxidative stress is a major cause of the ageing process, and that it occurs as the result of an imbalance between the production of reactive oxygen species (ROS) and the molecular defence mechanisms that control ROS, such as antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and antioxidant compounds (glutathione, among others).[3,4] It has been reported that age-related conditions that are associated with bone derangement, such as post-menopausal oestrogen depletion and diabetes mellitus, produce an increase of ROS.[5,6] The expression of ROS inhibiting molecules, namely the enzymes SOD[7] and CAT,[8] as well as factors that induce cell cycle arrest in response to excessive oxidative stress (such as growth arrest DNA damage 45 protein),[9] can be regulated by forkhead box protein O (FOXO) transcription factors, which are essential to bone integrity.[10] In fact, it has recently been demonstrated that knocking down SOD results in bone deterioration in mice.[11] In humans, an augmented oxidative stress has been traced to decreased bone mineral density.[12,13] The mechanisms by which oxidative stress may impair bone formation are complex and involve DNA damage,[14] lipid peroxidation,[15] protein carbonylation,[16] inhibition of Wnt pathway activation,[17] decreased osteoblast proliferation[18] and differentiation,[19,20] and increased osteoblast apoptosis.[21]

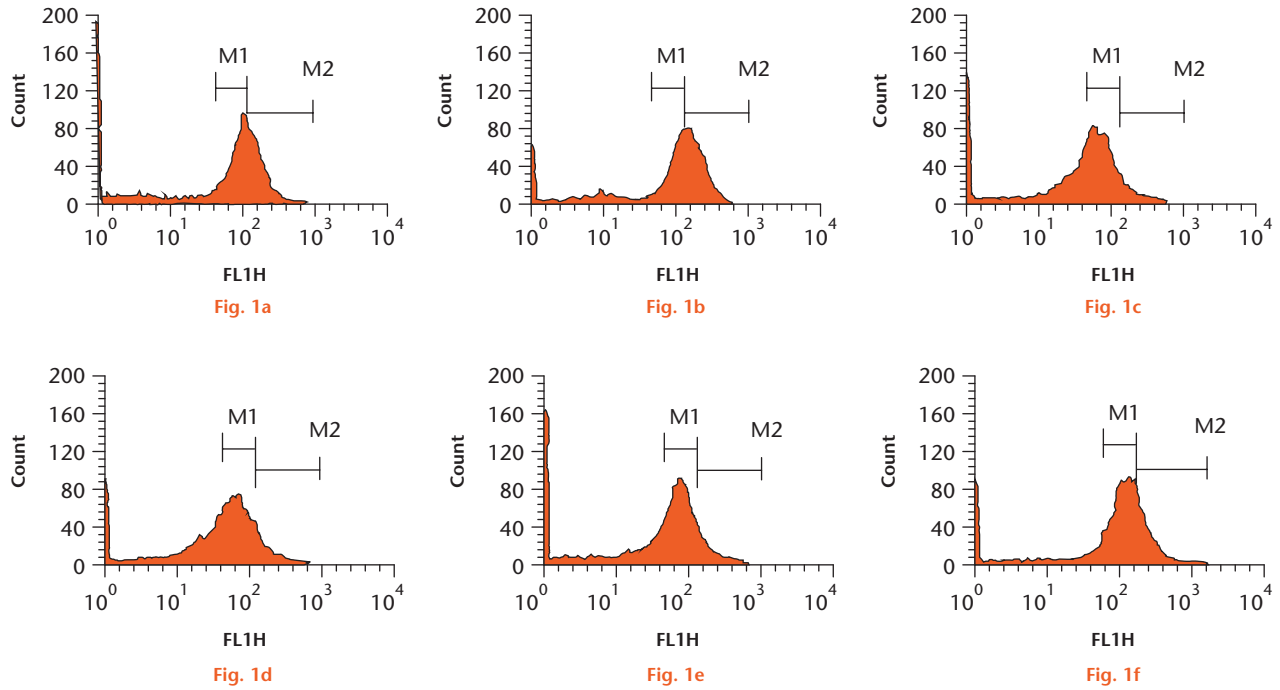
Parathyroid hormone (PTH)-related protein (PTHrP) is abundantly expressed in bone, where it performs an important role in bone formation and remodelling; similarly to PTH, intermittent administration of PTHrP displays anabolic actions in rodents and/or humans.[22] In fact, the N-terminal region of PTHrP shows homology with the same region of PTH and interacts with the common PTH type 1 receptor (PTH1R), which is a class II G protein-coupled receptor. PTH binding to PTH1R produces the activation of protein kinase A (PKA) by cyclic 3',5'-adenosine monophosphate (cAMP) and phospholipase C.[23] On the other hand, the PTH-unrelated C-terminal tail of PTHrP contains the 107-111 epitope (named osteostatin), which does not interact with the parathyroid hormone 1 receptor (PTHR1) but can apparently signal through opening voltage-sensitive calcium channels and protein kinase C (PKC) activation in osteoblastic cells.[24] It has recently been shown that osteostatin may transactivate vascular endothelial growth factor receptor 2 (VEGFR2) to promote osteoblastic function through an Src kinase-mediated mechanism.[25]

Recent data have disclosed the impact of the antioxidative stress properties of PTH on its bone anabolic action in aged mice.[26] In this regard, we recently reported that PTHrP (1-36) and PTHrP (107-139) were able to prevent the inhibitory effect of oxidative stress on the Wnt pathway activation in mouse mesenchymal C3H10T1/2 cells, suggesting their antioxidant potential.[27] The aim of the present study was to determine the antioxidant properties of both N- and C-terminal domains of PTHrP in murine osteoblastic MC3T3-E1 cells and human osteoblastic cells (hOBs). Regarding the latter domain, we tested both the native fragment PTHrP (107-139) and osteostatin, both of which have a simple structure that would make them attractive agents for promoting bone formation and bone regeneration.[28-32] The impact of the antioxidant properties of these PTHrP peptides on their ability to affect murine and hOB function has also been explored in this study.

## Materials and Methods

**Cells and reagents.** Previously well-characterized osteoblastic MC3T3-E1-subclon 4 cells (CRL-2593; American Type Culture Collection, Manassas, Virginia), showing high mineralization capacity under differentiation conditions, were used. These cells have been used in previous studies evaluating several osteogenic actions of PTH and PTHrP.[28,29,33,34] Primary hOBs were obtained from trabecular bone explants from two osteoarthritic patients undergoing total knee arthroplasty surgery, as previously reported.[22,28,29,35-37] Briefly, hOBs were cultured from bone slices with Dulbecco's modified Eagle medium (MEM; Thermo Fisher Scientific, Waltham, Massachusetts) containing 20% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine and 100 U/ml of penicillin/streptomycin, and experiments were carried out using cells at the third passage. The Ethics Committee for Clinical Research at Instituto de Investigación Sanitaria-Fundación Jiménez Díaz, Madrid, Spain approved the protocol, and written informed consent from the patients was granted.

MC3T3-E1 cells were grown in α-MEM with 10% FBS and antibiotics were made up of 1% penicillin/streptomycin (standard medium) in a humidified atmosphere of 5% $CO_2$ at 37°C. When present, PTH (1-34) (Sigma-Aldrich, St Louis, Missouri), PTHrP (1-37), PTHrP (107-139), and osteostatin (Bachem AG, Bubendorf, Switzerland), depending on the type of experiment (detailed in each case), were added at 100 nM to the cell culture. This dose has demonstrated osteogenic capacity in previous studies using PTHrP (1-37) and osteostatin.[27,29,34,38] In order to evaluate gene expression changes as described below, the PTH1R antagonist (Asn10, Leu11, D-Trp12) PTHrP (7-34) amide (PTHrP 7-34), at 100 nM, or neutralizing rabbit polyclonal antiserum C7 recognizing the osteostatin epitope in the entire PTHrP molecule, at 1:100 dilution,[39] were added together with PTHrP (1-37) or osteostatin, respectively. CAT (Sigma-Aldrich), at 200 U/ml, was used as an antioxidant control. In alkaline phosphatase (ALP) activity and viability assays, we used the PKA inhibitor, adenosine 3',5'-cyclic monophosphorothioate, and Rp-isomer (Rp-cyclic 3',5'-hydrogen phosphorothioate adenosine triethylammonium salt (Rp-cAMPs)) (Santa Cruz Biotechnology Inc., Santa Cruz, California), at 25 μM; the PKC inhibitor, calphostin C (CalpC) (Calbiochem Corp., La




Oxidative stress induced by hydrogen peroxide ($H_2O_2$) can be prevented by N- and C-terminal parathyroid hormone (PTH) and PTH-related protein (PTHrP) peptides in osteoblastic cells. Cells were exposed to 200 µM $H_2O_2$ following pretreatment with each PTHrP peptide or PTH (1-34) (100 nM) or control. Reactive oxygen species levels were measured by 2',7'dichloro fluorescein diacetate (DFC) fluorescence. Representative flow cytometry images for a) control, b) $H_2O_2$, c) PTH + $H_2O_2$, d) PTHrP (1-37) + $H_2O_2$, e) PTHrP (107-139) + $H_2O_2$, and f) osteostatin + $H_2O_2$. M1 and M2 denote the two areas used to quantify increment in fluorescence.

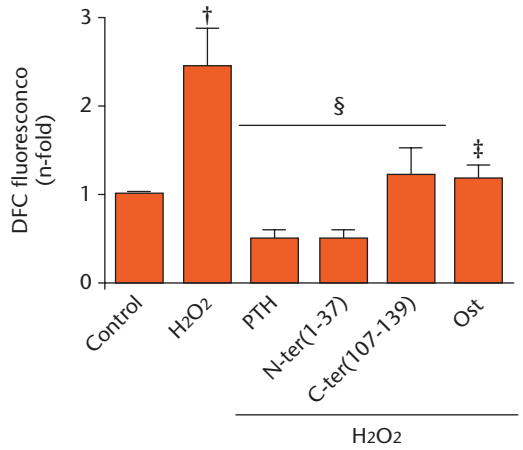



Fig. 2

Representative flow cytometry quantification. †p < 0.01 *versus* control; ‡p < 0.05 *versus* $H_2O_2$ treatment; §p < 0.01 *versus* $H_2O_2$ treatment. Data are represented by mean ± standard error of the mean (SEM). Kruskal–Wallis test followed by Dunn's *post hoc* test. N-ter(1-37), C-ter(107-139), and Ost denote parathyroid hormone-related protein (1-37), parathyroid hormone-related protein (107-139), and osteostatin, respectively.

Jolla, California), at 250 nM; or the Src kinase inhibitor PP-1 (Calbiochem Corp.) and VEGFR2 phosphorylation inhibitor SU1498 (Sigma-Aldrich), each at 10 µM, all of them added 30 minutes prior to the tested peptides.

**ROS level determination.** ROS were measured by evaluating the fluorescence increment of 2',7'-dichlorofluorescein diacetate (DCF) dye reacting with strong oxidants such as cell peroxides, as previously described.[40,41] Briefly, a final concentration of 10 µM DCF was added to subconfluent cells (10 000 cells/cm$^2$ to 20 000 cells/cm$^2$) in a FBS-depleted medium for 15 minutes at 37°C. After extensive washing with phosphate-buffered saline (PBS), pH 7.4, cells were incubated for another 15 minutes, followed by treatment with the tested peptides at 100 nM for ten minutes in standard medium. After removing this medium, cells were washed with PBS followed by an addition of 200 µM hydrogen peroxide ($H_2O_2$) for ten minutes in a FBS-depleted medium and then tripsinized, measuring the increment of fluorescence in a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, New Jersey).

Confocal experiments were also performed following the same protocol described above for DCF loading and peptide and $H_2O_2$ treatments. Cells were subsequently fixed with methanol including the DNA fluorescent dye 4',6-diamidino-2-phenylindole dihydrochloride (1 mg/ml) for ten minutes, mounted in FluorSave reagent (Calbiochem Corp.) and examined using a Leica DM IRB confocal microscope (Leica, Wetzlar, Germany).

**FOXO-luciferase (luc) assay.** Subconfluent MC3T3-E1 cells were transfected with a mixture of 1 µg of FOXO-luc reporter plasmid and 5 ng of a renilla coding plasmid (Promega Corp., Fitchburg, Wisconsin) (transfection control) using X-tremeGENE 9 DNA Transfection Reagent (Roche Diagnostics, Indianapolis, Indiana), following the manufacturer's instructions. After transfection, cells were treated with the peptides (each at 100 nM) for one hour in standard medium and then washed with PBS before the addition of 100 µM $H_2O_2$, followed by an overnight incubation in 1% FBS-containing medium at 37°C.

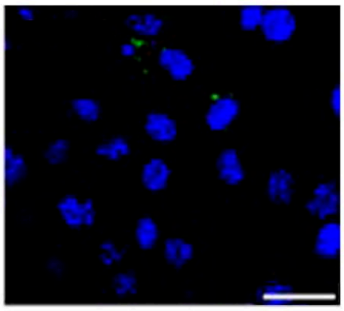


Fig. 3a

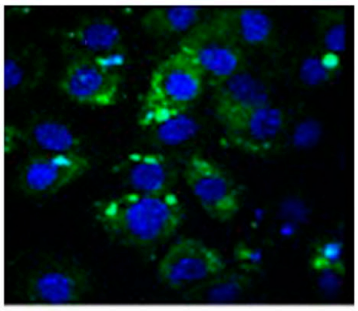


Fig. 3b

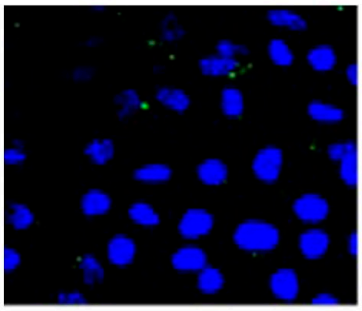


Fig. 3c

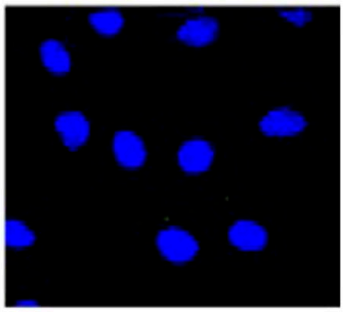


Fig. 3d

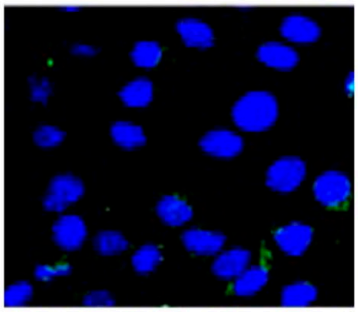


Fig. 3e

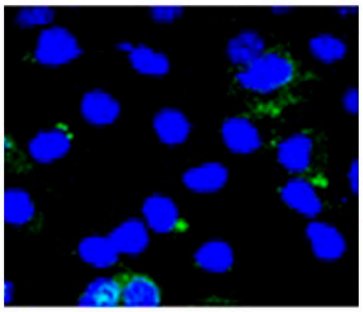


Fig. 3f

Confocal microscopy images of MC3T3-E1 cells for a) control, b) $H_2O_2$, c) parathyroid hormone (PTH) + $H_2O_2$, d) PTH-related protein (1-37) + $H_2O_2$, e) PTH-related protein (107-139) + $H_2O_2$, and f) osteostatin + $H_2O$. The white bar denotes 75 µm.

Thereafter, cells were homogenized with a lysis buffer, and luciferase and renilla activities were quantified with a dual luciferase kit (Promega Corp.), following the manufacturer's instructions, using the FB12 tube luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). Renilla activity values were used to normalize the luciferase values.

**Malondialdehyde (MDA) assay.** Lipid peroxidation levels were determined by measuring the formation of MDA using a commercial kit (BioVision, Inc., Mountain View, California) per the manufacturer's instructions. Briefly, MC3T3-E1 cells and hOBs ($2 \times 10^6$ cells) were pretreated with PTHrP (1-37), PTHrP (107-139), osteostatin or PTH (1-34), each at 100 nM, for ten minutes in a serum-depleted medium. Cells were then washed three times with PBS and treated with 200 µM $H_2O_2$ for 24 hours, followed by centrifugation at 500 ×g for five minutes. The pellets were then washed three times with PBS. Pellets were subsequently resuspended in 300 µl of lysis buffer, as supplied by the kit, with 3 µl butylated hydroxytoluene (0.1 mM), sonicated, and centrifuged (13 000 ×g for ten minutes). The supernatants (200 µl) from each sample were added to 600 µl of thiobarbituric acid and incubated in a water bath at 95°C for 60 minutes. Samples were then ice-cooled for ten minutes and 200 µl (from each 800 µl of reaction mixture) was placed into a 96-well microplate for measuring absorbance at 532 nm. The MDA supplied in the kit was used as standard and MDA levels were determined by comparing the absorbance of samples with that of the standard controls. The protein concentration of the samples was measured with bicinchoninic acid protein assay (Sigma-Aldrich), using bovine serum albumin as standard. Results were expressed as nmols of MDA per mg of protein.

**Caspase-3 assay.** Subconfluent cells were serum-depleted overnight prior to incubation with the different peptides tested (each at 100 nM) for one hour in FBS-depleted medium. After washing the cells with PBS, 100 µM $H_2O_2$ was added for two, six, and 24 hours in the latter medium. Caspase-3 activity was analyzed using the CaspACE Assay System (Promega) following the manufacturer's instructions. In brief, cell extracts obtained in lysis buffer were submitted to three freeze-and-thaw cycles and then centrifuged at 15 000 g at 4°C. Supernatants were then incubated with the substrate Ac-DEVD-p-nitroaniline (Sigma-Aldrich) for four hours at 37°C, and caspase-3 activity was determined by measuring absorbance at 405 nm. Caspase activity was expressed as pmol p-nitroaniline/h/µg protein (measured as described above).

**Cell proliferation.** Cells were seeded at 3000 cells/cm$^2$ in 96-well plates. After 24 hours, cells were treated with each tested peptide, at 100 nM, or CAT (an antioxidant control) at 200 U/ml, for one hour in standard medium. After washing with PBS, 200 µM $H_2O_2$ was added for one hour in FBS-depleted medium. The medium was then replaced with the standard medium. This procedure was repeated after 48 hours and 24 hours after this repetition we proceeded to measure cell proliferation (a total five days since assay onset). At this time, cell proliferation was determined by the addition of resazurin salt solution[42] (Sigma-Aldrich), at 10 µg/ml. After four hours, absorbance was measured at both 540 nm and 630 nm.

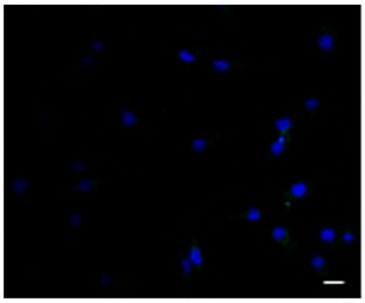


Fig. 4a

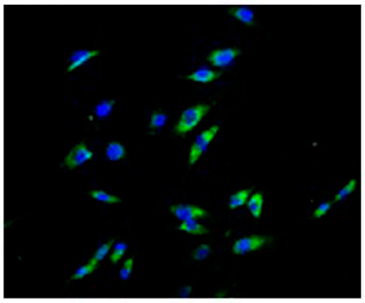


Fig. 4b

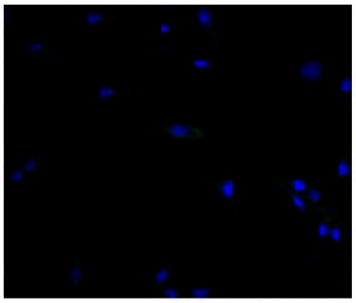


Fig. 4c

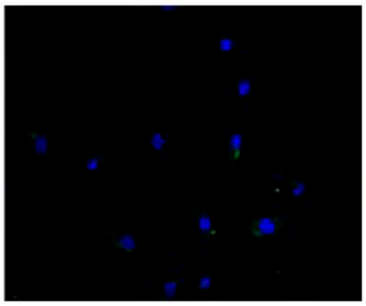


Fig. 4d

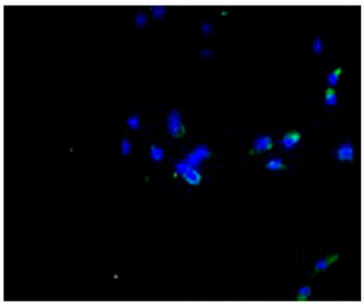


Fig. 4e

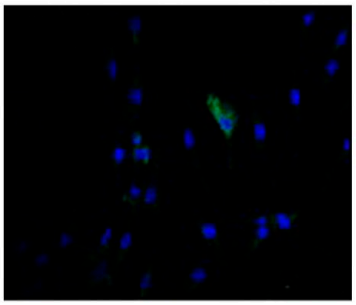


Fig. 4f

Confocal microscopy images of human osteoblastic cells for a) control, b) $H_2O_2$, c) parathyroid hormone (PTH) + $H_2O_2$, d) PTH-related protein (1-37) + $H_2O_2$, e) PTH-related protein (107-139) + $H_2O_2$, and f) osteostatin + $H_2O$. The white bar denotes 20 µm.

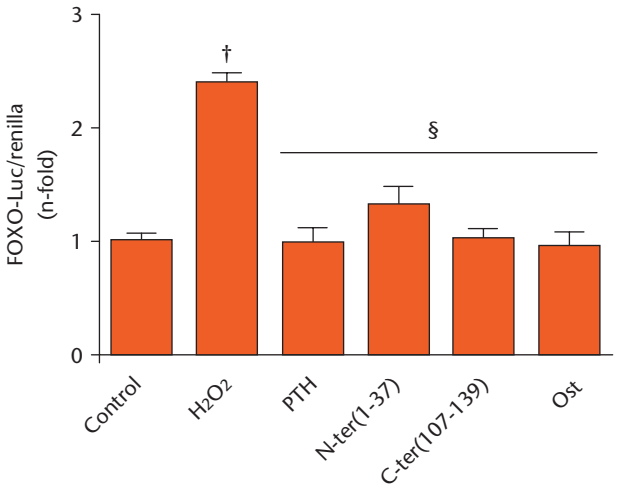



Fig. 5

Forkhead box protein O (FOXO) activity in MC3T3-E1 cells was measured with a FOXO-luciferase plasmid. Bars represent the mean ± standard error of the mean (SEM) of three independent experiments performed in triplicate. †$p < 0.01$ *versus* control; §$p < 0.01$ *versus* $H_2O_2$ treatment. Kruskal–Wallis test followed by Dunn's *post hoc* test. N-ter(1-37), C-ter(107-139), and Ost denote parathyroid hormone-related protein (1-37), parathyroid hormone-related protein (107-139), and osteostatin, respectively.

The percentage difference in cell growth between treated and control cells was calculated using the following equation: [(O2 × A1) - (O1 × A2) / (O2 × P1) - (O1 × P2)] × 100, where O1 and O2 are the molar extinction coefficients of oxidized resazurin salt at 540 nm and 630 nm, respectively; A1 and A2 are the absorbance of treated cells at 540 nm and 630 nm, respectively; and P1 and P2 correspond to the absorbance of control cells (cells without treatment) at 540 nm and 630 nm, respectively.

**Cell viability.** Subclonfluent MC3T3-E1 cells were pretreated for 30 minutes with the inhibitors prior to the addition of PTHrP peptides for 30 minutes in standard medium. Controls were not pretreated. The cells were then washed with PBS and incubated with 200 µM $H_2O_2$ for six hours in either serum-depleted or standard medium (to control for endogenous growth factors in FBS). After treatment, nonadherent cells were collected and pooled with adherent cells (following trypsinization), and cell viability was assessed by trypan blue exclusion, as previously described.[36,43] Nonviable cells exhibiting intracellular trypan blue staining and total cell numbers were counted to calculate the percentage of cell viability in each case.

**ALP activity assay.** Subconfluent MC3T3-E1 cells and hOBs were preincubated with the different inhibitors for 30 minutes, subsequently followed by the addition of the tested PTHrP peptides, each at 100 nM for one hour in standard medium and 200 µM of $H_2O_2$ for two hours in FBS-depleted medium. The FBS-depleted medium was then replaced with standard medium and cells were incubated for a further 24 hours. ALP activity was measured in cell extracts obtained with PBS and 0.1% Triton X-100 (Sigma-Aldrich) using p-nitrophenyl phosphate as substrate, as previously described.[28] ALP activity was normalized to cell protein content determined as described in MDA and Caspase assays.

**Quantitative real-time polymerase chain reaction (PCR).** Subconfluent MC3T3-E1 cells were treated as described for the ALP activity assay. Following treatment, cell total RNA was extracted with TRIzol (Thermo Fisher Scientific,

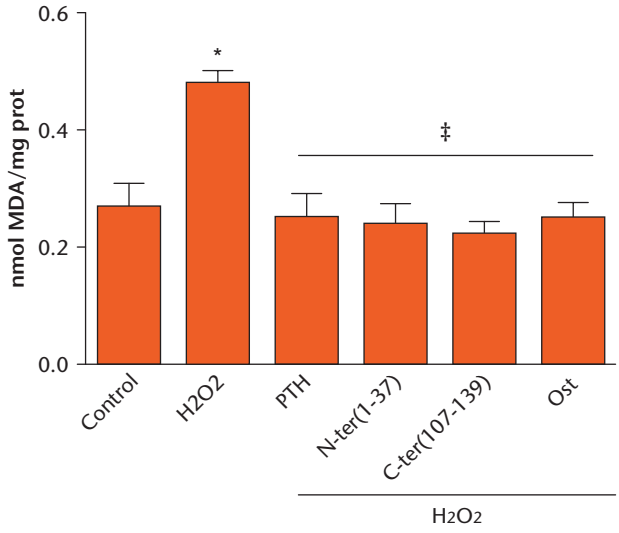



Fig. 6a

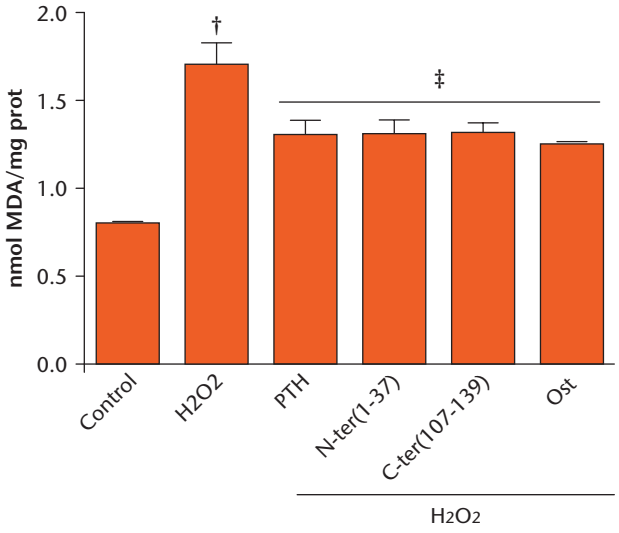



Fig. 6b

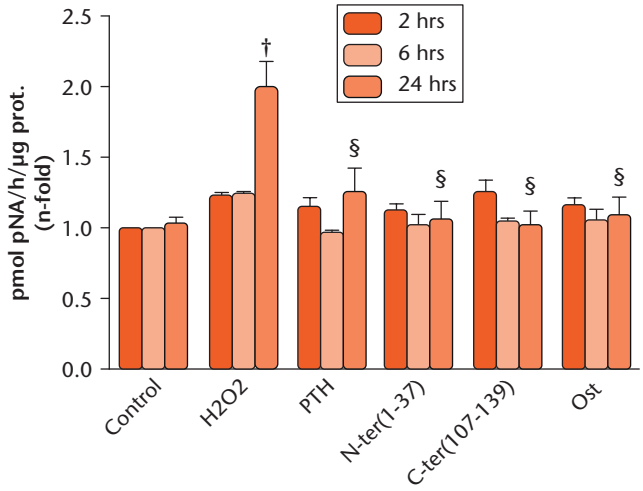



Fig. 6c

Parathyroid hormone (PTH) and both N- and C-terminal PTH-related protein (PTHrP) peptides prevented the increase in malondialdehyde (MDA) levels and caspase-3 activation induced by $H_2O_2$ in osteoblastic cells. Cells were pretreated with the different peptides at 100 nM followed by $H_2O_2$ (200 µM) for a) and b) ten minutes, or c) two to 24 hours. Changes in MDA levels in a) MC3T3-E1 cells and b) human osteoblastic cells, and in c) caspase-3 activity in MC3T3-E1 cells, are shown. Data are mean ± standard error of the mean (SEM) of three independent experiments performed at least in triplicate. *$p < 0.05$ *versus* corresponding time control; †$p < 0.01$ *versus* corresponding time control; ‡$p < 0.05$ *versus* $H_2O_2$ at the corresponding time; §$p < 0.01$ *versus* $H_2O_2$ at the corresponding time. Kruskal–Wallis test followed by Dunn's *post hoc* test. N-ter(1-37), C-ter(107-139), and Ost denote PTHrP (1-37), PTHrP (107-139), and osteostatin, respectively.

Waltham, Massachusetts). Synthesis of complementary DNA (cDNA) was performed using the high-capacity cDNA reverse transcription kit following the manufacturer's instructions (Applied Biosystems, Grand Island, New York). A quantitative real-time PCR was performed in an ABI PRISM 7500 system (Applied Biosystems). TaqMan minor groove binder probes (Applied Biosystems, Assay-by-Design) were obtained for amplification of the following genes: runt-related transcription factor 2 (Runx2), osterix (Osx) and ALP using Premix ex-Taq polymerase (Takara Bio Inc., Otsu, Japan). The messenger RNA (mRNA) copy numbers were calculated for each sample using the cycle threshold value and normalized against 18S ribosomal RNA, as previously reported.[44,45] Results were expressed as n-fold mRNA values *versus* corresponding values in non-treated control cells.

**Statistical analysis.** Results are expressed as mean ± standard error of the mean (SEM) throughout the text. Differences among groups were analyzed by the Kruskal-Wallis test followed by Dunn's *post hoc* test and $p < 0.05$ was considered significant. Statistical analysis was performed with SPSS v21 software (IBM Corp., Armonk, New York).

## Results

**Both N- and C-terminal PTHrP peptides counteract $H_2O_2$-induced oxidative stress in osteoblastic cells.** First, we aimed to explore whether N- and C-terminal PTHrP peptides might be able to prevent ROS production by the

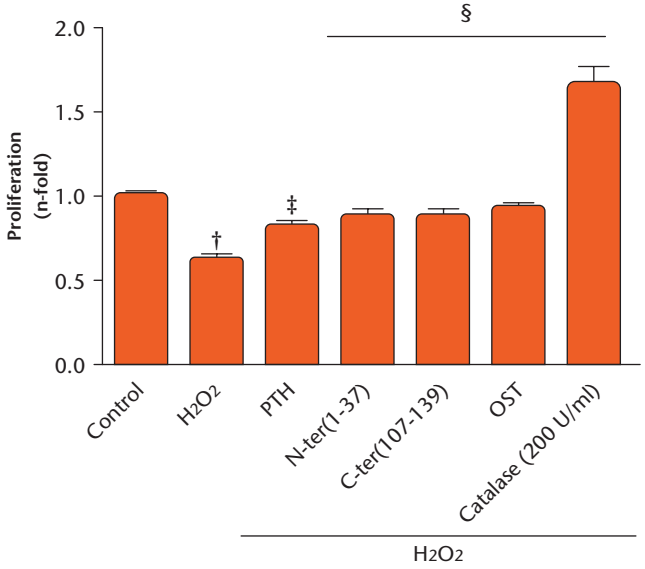



Fig. 7a

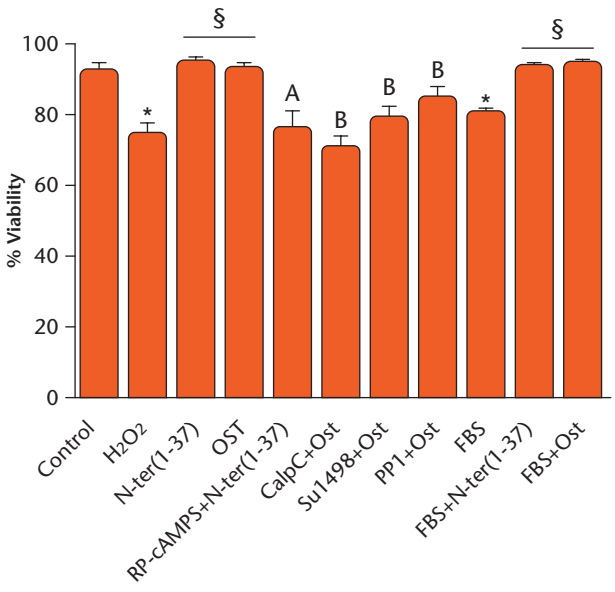



Fig. 7b

Parathyroid hormone (PTH) and both N- and C-terminal PTH-related protein (PTHrP) peptides counteract the deleterious effects of $H_2O_2$ on MC3T3-E1 cell growth. Cells were preincubated with different inhibitors for 30 minutes: Rp-cyclic 3',5'-hydrogen phosphorothioate adenosine triethylammonium salt (Rp-cAMPS; 25 μM), protein phosphatase 1 and Su1498 (5 μM) or calphostin C (CalpC) (250 nM), and then treated with PTHrP peptides (100 nM) before exposure to $H_2O_2$. Control cells were not treated. a) For calculation of cell proliferation, cell growth percentage values in treated samples were expressed as n-fold *versus* control (normalized to 1). Catalase (200 U/ml) was used as an antioxidant control. b) Cell viability was expressed as the percentage of viable cells over total cells. Data are mean ± standard error of the mean (SEM) of three independent experiments performed at least in triplicate. *$p < 0.05$ *versus* control; †$p < 0.01$ *versus* control; ‡$p < 0.05$ *versus* $H_2O_2$; §$p < 0.01$ *versus* $H_2O_2$; probability associated with comparison A ($^A p$) $< 0.05$ *versus* $H_2O_2$ + N-ter (1-37); probability associated with comparison B ($^B p$) $< 0.05$ *versus* $H_2O_2$ + Ost. Kruskal–Wallis test followed by Dunn's *post hoc* test. N-ter(1-37), C-ter(107-139) and Ost denote PTHrP (1-37), PTHrP (107-139), and osteostatin, respectively. FBS, fetal bovine serum.

prominent oxidative stress-related agent $H_2O_2$ in osteoblastic cells. Short exposure (ten minutes) of MC3T3-E1 cells to 200 μM $H_2O_2$ produced a significant ($p < 0.01$) increase in intracellular ROS (Figs 1 and 2). However, this increase was prevented by each N- or C-terminal PTHrP peptide tested or PTH (1-34) (a positive control) (each at 100 nM). Further confirmation of the capacity of these peptides to impair oxidative stress was obtained by using confocal microscopy to assess $H_2O_2$-induced DCF fluorescence changes in MC3T3-E1 cells and in hOBs (Figs 3 and 4).

It has been demonstrated that increased cell ROS lead to an augmented FOXO transcriptional activity.[46,47] Thus, we next examined the changes in this activity elicited by $H_2O_2$ and the possible modulatory effects of the different peptides evaluated in MC3T3-E1 cells. We found that preincubation with either PTHrP peptide or PTH (1-34) abrogated the oxidant-related effect of $H_2O_2$ in these cells (Fig. 5).

Oxidative stress induces different kinds of cellular damage, which impairs regular cell function.[1] Thus, we next analyzed the changes in MDA levels induced by $H_2O_2$ as an oxidative stress damage marker and the possible modulatory action of the different PTHrP peptides. As expected, this oxidative agent increased MDA levels, which were normalized by pretreating MC3T3-E1 cells and hOBS with the PTHrP peptides or PTH (1-34) (Figs 6a and b). It has been demonstrated that oxidative stress triggers an apoptotic cascade resulting in caspase activation.[21] We found that caspase-3 activity increased significantly after 24 hours of exposure to $H_2O_2$, and all of the peptides tested were similarly effective in normalizing this activity in these cells (Fig. 6c).

**Protective effects of both N- and C-terminal PTHrP peptides against the deleterious effects of $H_2O_2$ on osteoblastic cell function.** Osteoblast growth has been shown to be altered negatively by oxidative stress.[18,19] This prompted us to examine whether PTHrP would prevent the negative effects of a short exposure (one hour) to $H_2O_2$ during MC3T3-E1 cell growth for five days. Indeed, we showed a diminished cell proliferation with $H_2O_2$ treatment, which was similarly compensated for by both N- and C-terminal PTHrP peptides (Fig. 7a). Of note, CAT (used as an antioxidant control) produced a dramatic stimulation for cell proliferation, suggesting that basal $H_2O_2$ levels keep proliferation under control in these cells. In addition, we found that either the N- or C-terminal PTHrP domain could counteract the inhibitory effect of acute exposure of MC3T3-E1 cells to $H_2O_2$ in the presence or absence of FBS on cell viability (Fig. 7b).

We also aimed to explore the putative changes elicited by short exposure to $H_2O_2$ on various osteoblast differentiation markers and the modulatory effect of PTHrP peptides in MC3T3-E1 cells and hOBs. This oxidant was found to reduce ALP activity in both osteoblastic cell

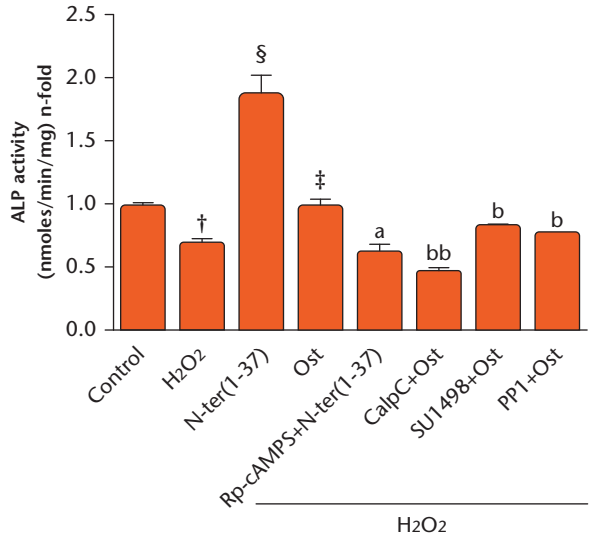



Fig. 8a

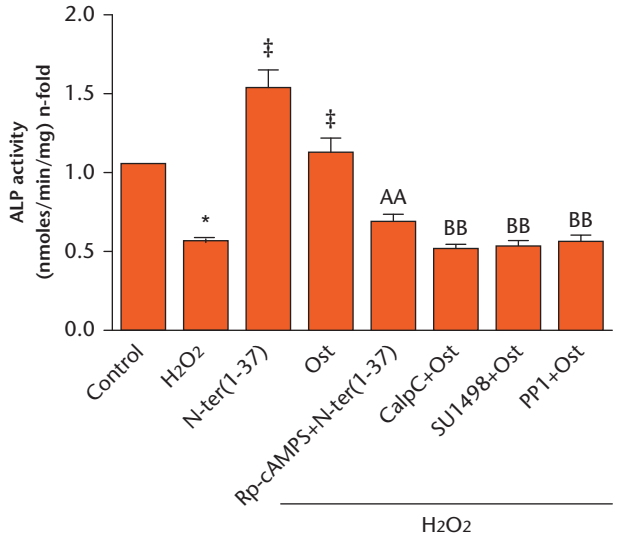



Fig. 8b

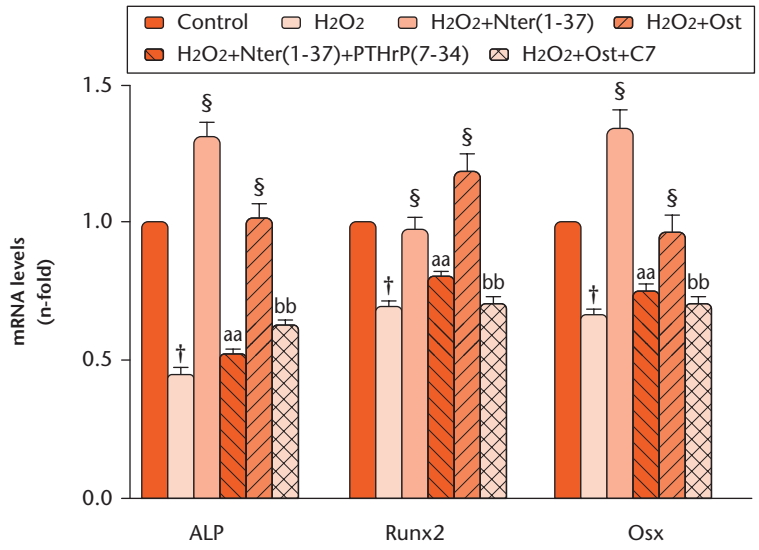



Fig. 8c

Parathyroid hormone (PTH) and both N- and C-terminal PTH-related protein (PTHrP) peptides counteract the negative effects of $H_2O_2$ on osteoblastic cell differentiation. a) MC3T3-E1 cells and b) human osteoblastic cells were preincubated or not with different inhibitors, as described in the legend for Figure 7, and then treated or not (control) with the PTHrP peptides (100 nM) before exposure to $H_2O_2$. Alkaline phosphatase (ALP) activity was expressed as n-fold over control (mean ± standard error of the mean (SEM): 2.6 nmoles/min/mg (SEM 0.5) and 4.3 nmoles/min/mg (SEM 0.22) protein for MC3T3-E1 cells and human osteoblastic cells, respectively). c) MC3T3-E1 cells were pre treated with the PTHrP peptides in the presence or absence of the antagonist PTHrP (7-34) or the anti-osteostatin antiserum C7 before addition of $H_2O_2$. Changes in gene expression of ALP, runt-related transcription factor 2 (Runx2) and osterix (Osx) were analyzed in cell total RNA isolates by quantitative polymerase chain reaction. †$p < 0.01$ *versus* control; ‡$p < 0.05$ *versus* $H_2O_2$;§$p < 0.01$ *versus* $H_2O_2$; probability associated with comparison A ($^{A}p$) < 0.05; probability associated with comparison AA ($^{AA}p$) < 0.01 *versus* $H_2O_2$ + N-ter(1-37); probability associated with comparison B ($^{B}p$) < 0.05; comparison associated with comparison BB ($^{BB}p$) < 0.01 *versus* $H_2O_2$ + Ost. N-ter(1-37), C-ter(107-139) and Ost denote PTHrP (1-37), PTHrP (107-139) and osteostatin, respectively. Rp-cAMPS, Rp-cyclic 3',5'-hydrogen phosphorothioate adenosine triethylammonium salt; CalpC, calphostin C; mRNA, messenger RNA.

types (Figs 8a and b), as well as the gene expression of ALP, Runx2, and Osx in MC3T3-E1 cells (Fig. 8c); these deleterious effects were prevented by pretreating these cells with each PTHrP peptide tested (Figs 8a to c).

As a specificity control of each PTHrP peptide action in this setting, we used several inhibitors or antagonists. Thus, we found that Rp-cAMPS (a PKA inhibitor) inhibited the ability of PTHrP (1-37) to preserve cell viability and ALP activity upon exposure to $H_2O_2$ in MC3T3-E1 cells, whereas CalpC (a PKC inhibitor), protein phosphatase 1 (Src activation inhibitor) or SU1498 (VEGFR2 activation inhibitor) abrogated such ability of osteostatin to restore ALP activity upon oxidant treatment in these cells (Figs 7b, 8a, and 8b). In addition, PTHrP (7-34) (a PTHR1 antagonist) and anti-osteostatin antiserum C7 inhibited the positive effects of PTHrP (1-37) and osteostatin, respectively, on osteoblast differentiation-related genes in these cells following $H_2O_2$ treatment (Fig. 8c).

## Discussion

Oestrogen depletion has traditionally been considered the hallmark of the pathogenesis of involutional osteoporosis,[48] but age-related factors intrinsic to bone, mainly oxidative stress, appear also to contribute to bone loss in this condition.[1,2] In this regard, the recent finding that intermittent PTH, currently the only bone anabolic therapy, may function, as least partially, through reducing oxidative stress in aged bone[26] provides insight into the mechanisms and possible treatment options for bone loss. However, the use of antioxidants alone or in combination with PTH presents several drawbacks. Thus, common antioxidants such as N-acetyl cysteine and CAT produce a decrease in ROS levels, but can also decrease osteoblastogenesis by blocking Wnt pathway activation,[49] thereby inhibiting β-catenin-dependent transcription of osteogenic genes.[26] In addition, antioxidants have anti-osteoclastic features that would inhibit bone remodelling.[26]

Previous studies have shown the ability of N- and C-terminal PTHrP peptides to promote cell growth and osteoblast differentiation in MC3T3-E1 cells and other osteoblastic cell preparations. Studies on these proteins have also reported their osteogenic properties *in vivo* in high oxidative stress-related conditions, namely diabetes mellitus and ovariectomized mice.[24,25,27-29,32,34-36,39] Very recently, the osteoregenerative features of both PTHrP (1-37) and osteostatin have been reported *in vivo* in an ageing rat model with diabetes mellitus, although only indirect evidence for the antioxidant capacities of these peptides was provided.[50]

In this *in vitro* study, we disclose the antioxidant capacity of both N- and C-terminal domains of PTHrP in well-characterized osteoblastic cells. To induce oxidative stress, we used $H_2O_2$ at concentrations widely used in previous studies.[18,38,51] ROS elevation can impair bone formation by interaction with the Wnt/β-catenin pathway.[17] Thus, ROS-mediated activation of FOXO transcription factors diverts β-catenin from the promoter regions of genes involved in proliferation and differentiation of cells of the osteoblastic lineage, impairing these cellular programmes.[52] Our data show that these PTHrP peptides were able to prevent the $H_2O_2$-induced generation of cell ROS in both murine osteoblastic cells and hOBs, and accordingly, the increase in FOXO transcriptional activity as demonstrated by a FOXO-luc reporter assay in the former cells. This is consistent with recent results showing the ability of PTHrP to increase Wnt/β-catenin signalling in osteoblastic cells exposed to high glucose concentrations, another oxidative stress-related scenario.[33] Oxidative stress also triggers cellular damage and apoptosis through caspase activation.[21,53] Here we show that pretreatment with these peptides decreased lipid peroxidation and blocked caspase-3 activation, thus protecting MC3T3-E1 cells from apoptosis in both osteoblastic cell types used.

It has been reported previously that oxidative stress impairs osteoblast function.[5,17-19,21,51] In the present study, we report that short exposure (one to six hours) of MC3T3-E1 cells to $H_2O_2$ significantly decreased cell proliferation and viability by approximately 30%. This decrease is moderate compared with previous data in the same osteoblast cell line, although previous studies used prolonged exposure to the oxidant in a FBS-containing medium and/or higher oxidant concentration than that used herein.[18,19] In addition, ALP enzymatic activity and mRNA levels, as well as gene expression of other well-known osteoblast differentiation markers, were all diminished by acute exposure to $H_2O_2$, and this was also prevented by pretreatment with both PTHrP peptides in MC3T3-E1 cells and hOBs.

Previous reports have shown the important role of the cAMP/PKA pathway in the anabolic actions elicited by the homologous N-terminal domains of PTH and PTHrP.[22,24,25,35,36] This fact was confirmed by using a PKA inhibitor in the present oxidative stress scenario. In addition, specific inhibitors of PKC and Src-dependent VEGFR2 activation inhibited the protective/restoring role of osteostatin in viability and ALP activity, supporting the notion that both pathways play a major role in its osteogenic actions.[24,25,36] Our results thus further emphasize the involvement of distinct signalling pathways triggered by both the N- and C-terminal PTHrP domains in osteoblasts.

Taken together, the present data indicate that both N- and C-terminal domains of PTHrP display various antioxidant features in both murine osteoblastic cells and hOBs. However, we understand that this is an *in vitro* study and caution should be taken when translating our results to an *in vivo* scenario. Even so, these findings provide novel insights that add support to the generally accepted use of PTHrP peptides as osteoporosis therapy.

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**Author Contribution**

- S. Portal-Núñez: Designing the study, Data acquisition, Statistical analysis, Writing the manuscript, Final approval of the manuscript.
- J. A. Ardura: Designing the study, Data acquisition, Writing the manuscript, Statistical analysis.
- D. Lozano: Data acquisition, Writing the manucript, Statistical analysis.
- I. Martínez de Toda: Data acquisition.
- M. De la Fuente: Designing the study, Revising the manuscript, Final approval of the manuscript.
- G. Herrero-Beaumont: Revising the manuscript, Financial support.
- R. Largo: Revising the manuscript, Financial support.
- P. Esbrit: Study direction, Designing the study, Writing and revising the manuscript, Financial support.

**Conflicts of Interest Statement**

- None declared