

The N- and C-terminal domains of parathyroid hormone-related protein affect differently the osteogenic and adipogenic potential of human mesenchymal stem cells

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Abbreviations: ALP, alkaline phosphatase; LPL, lipoprotein lipase;
MSC, mesenchymal stem cell; PTH, parathyroid hormone; PTHrP,
parathyroid hormone-related protein

Abstract

Parathyroid hormone-related protein (PTHrP) is synthesized by diverse tissues, and its processing produces several fragments, each with apparently distinct autocrine and paracrine bioactivities. In bone, PTHrP appears to modulate bone formation in part through promoting osteoblast differentiation. The putative effect of PTH-like and PTH-unrelated fragments of PTHrP on human mesenchymal stem cell (MSCs) is not well known. Human MSCs were treated with PTHrP (1-36) or PTHrP (107-139) or both (each at 10 nM) in osteogenic or adipogenic medium, from the start or after 6 days of exposure to the corresponding medium, and the expression of several osteoblastogenic and adipogenic markers was analyzed. PTHrP (1-36) inhibited adipogenesis in MSCs and favoured the expression of osteogenic early markers. The opposite was observed with treatment of MSCs with PTHrP (107-139). Moreover, inhibition of the adipogenic differentiation by PTHrP (1-36) prevailed in the presence of PTHrP (107-139). The PTH/PTHrP type 1 receptor (PTH1R) gene expression was maximum in the earlier and later stages of osteogenesis and adipogenesis, respectively. While PTHrP (107-139) did not modify the PTH1R

overexpression during adipogenesis, PTHrP (1-36) did inhibit it; an effect which was partially affected by PTHrP (7-34), a PTH1R antagonist, at 1 μ M. These findings demonstrate that both PTHrP domains can exert varying effects on human MSCs differentiation. PTHrP (107-139) showed a tendency to favor adipogenesis, while PTHrP (1-36) induced a mild osteogenic effect in these cells, and inhibited their adipocytic commitment. This further supports the potential anabolic action of the latter peptide in humans.

Keywords: adipogenesis; bone; mesenchymal stem cells; osteogenesis; parathyroid hormone-related protein

Introduction

Parathyroid hormone-related protein (PTHrP) is the product of a single gene. This, through alternative splicing the multi-exonic PTHrP gene, gives rise to three initial translation products: PTHrP (1-139), PTHrP (1-141), and PTHrP (1-173) (Wysolmerski and Stewart, 1998). These three peptides are identical in positions 1-139 and differ only in their carboxy termini. The three initial PTHrP translation products undergo extensive posttranslational processing and give rise to a family of mature secretory forms of the peptide, each with its own physiological function (s) and each with its own receptor or receptors (Orloff *et al.*, 1994). For example, the amino-terminal PTHrP secretory form, PTHrP (1-36), shares N-terminal homology with PTH and acts through same PTH type 1 receptor (PTH1R) (Juppner *et al.*, 1991); thereby initiating multiple events (e.g. vasorelaxation, bone resorption, cellular proliferation). Mid-region PTHrP appears to have its own cognate receptor (Orloff *et al.*, 1996) and to have different effects, for example, on placental calcium transport. The carboxy-terminal PTHrP peptide, PTHrP (107-139), appears to act through a still different receptor and to have other effects such as inhibition of osteoclastic bone resorption (Fenton *et al.*, 1991).

PTH is considered a key hormone in the regulation of calcium homeostasis in the body, and in contrast to PTHrP, is only produced by the parathyroid glands. However, PTHrP, whose effects are

mainly autocrine and paracrine, is synthesized by diverse tissues including cartilage and bone (Strewler, 2000). In the latter, PTHrP seems to exert a modulating role on bone formation and remodeling both in the fetus as well as in adults (Miao *et al.*, 2004, 2005).

The anabolic effect of PTH in bone is now well established. Thus, while prolonged exposure to PTH induces bone resorption, its intermittent administration stimulates bone formation (Hock and Gera, 1992). *In vitro* studies suggest that PTH can affect osteoblast function differently apparently depending on the stage of osteoblast differentiation. In mouse osteoblastic cells and primary cultures of human osteoblast-like cells from bone explants grown in osteogenic medium, PTH induces mineralization when added in a later but not in an earlier time period (Schiller *et al.*, 1999). This anabolic effect of PTH appears to be complex and dependent on Runx2 transactivation (Fujita *et al.*, 2001). Interestingly, daily treatment with PTH (1-34) has been shown to reverse both the decreased expression of osteogenic genes and the increased levels of adipogenic genes, namely peroxisome proliferator-activated receptor (PPAR)- γ and lipoprotein lipase (LPL), as shown in the femoral metaphysis of ovariectomized rats (Kulkarni *et al.*, 2007). Moreover, a high PTH (1-34) dose (50 nM) added as a 1-h pulse, but not continuously, to human bone marrow mesenchymal cells was recently found to inhibit their adipogenic commitment (Rickard *et al.*, 2006). These recent data thus suggest that the anabolic effect of PTH could be related at least in part to its capacity to inhibit the adipocytic program in the precursor cells for both osteoblasts and adipocytes. Related to these results using PTH (1-34), in the pre-adipocyte cell line 3T3-L1, the expression of both PTHrP and the PTH1R decreased with adipocyte differentiation, and adipogenesis was inhibited in these PTHrP- overexpressing cells after transfection with a PTHrP cDNA plasmid (Chan *et al.*, 2001). Furthermore, PTHrP (1-34) has been shown to activate osteoblastogenesis and to inhibit adipogenesis in pluripotential mouse C3H10T1/2 cells (Chan *et al.*, 2003).

The C-terminal fragment of PTHrP containing the 107-111 epitope -named osteostatin- is a potent inhibitor of bone resorption *in vitro*, although its true effect on bone *in vivo* is unclear (Fenton *et al.*, 1991; Cornish *et al.*, 1997). This PTHrP domain is known to interact with an uncharacterized receptor, but different from the PTH1R in osteoblasts (Valin *et al.*, 1997, 2001; Alonso *et al.*, 2008). It has been previously demonstrated that PTHrP (107-139) can rapidly increase VEGF expression

in human osteoblastic cells (Esbrit *et al.*, 2000). In addition, recent studies have shown that its intermittent administration induces various osteogenic effects through interaction with VEGF receptor 2 in these cells (de Gortazar *et al.*, 2006; Alonso *et al.*, 2008). However, the putative effects of this C-terminal PTHrP fragment on human osteoprogenitors are currently unknown.

In the present study, we assessed and compared the effects of the N- and C-terminal domains of PTHrP on the commitment of human mesenchymal stem cells (MSCs) to either osteoblastic or adipocytic lineages.

Results

MSCs from human bone marrow can be induced to osteogenesis or adipogenesis

We first tested the differentiation capacity of our human MSC cultures to the osteogenic or adipogenic lineage. We found that these cell cultures originated from the three bone marrow donors showed a similar capacity to differentiate into osteoblasts or adipocytes in either osteogenic or adipogenic medium, respectively (Figure 1). In the presence of osteogenic medium, we found that early markers of osteogenesis, such as ALP activity as well as ALP and runx2 gene expression, showed higher values after 6 days than those at the later times evaluated (Figure 1A). On the other hand, this medium also promoted mineralization of the extracellular matrix, as observed after 24 days of culture by alizarin red staining (Figure 1B, upper panels). The adipogenic medium induced a sharp increase in the expression of both PPAR- γ 2 and LPL genes, whereas no expression of those genes was detected in control cultures (Figure 1C). Moreover, these cultures accumulated fat vesicles, as shown by oil red staining (Figure 1B, lower panels).

Effect of PTHrP (1-36) and PTHrP (107-139) on osteoblastogenesis in human MSCs

When human MSCs were treated with PTHrP (1-36) or PTHrP (107-139) at either the start or after 6 days of addition of osteogenic medium, ALP activity was significantly increased at day 6 in the cultures treated with PTHrP (1-36) and at day 18 when that peptide was added from days 6 to 18 of osteogenic induction (Figure 2A). Moreover, a significant increase in ALP mRNA levels was detected in the latter cells, compared with those in untreated cells at day 18 (Figure 2A). This was associated with a weak increase of runx2 gene expression

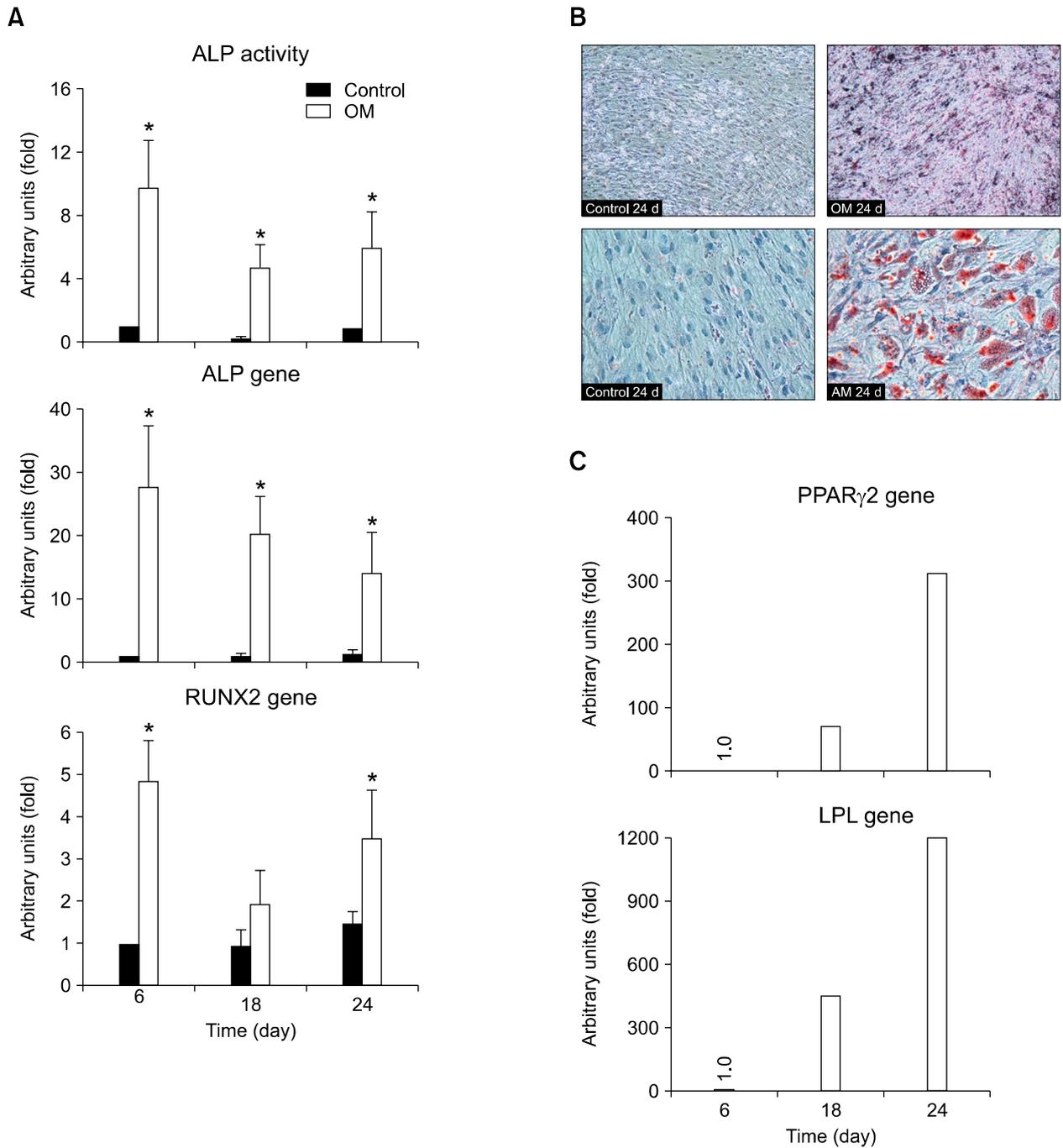


Figure 1. Expression of osteogenic and adipogenic markers in human MSCs grown for 24 days in osteogenic medium (OM) or adipogenic medium (AM). (A) ALP activity and gene expression of ALP and RUNX2 in control cell cultures and in those induced to osteoblasts at days 6, 18 and 24. (B) Representative alizarin red staining from one MSC culture grown in OM or basal medium (control) for 24 days (upper panels) and oil red staining showing fat vesicle formation in MSC cultures maintained in AM or basal medium (control) for 24 days (lower panels). (C) Gene expression of PPAR- γ 2 and LPL in MSCs grown in AM at days 6, 18 and 24. Data represent the mean increase over control at day 6 (value = 1) from 3 different human MSC preparations each one obtained from a different bone marrow sample (A, C). * $P < 0.05$ vs control.

induced by this PTHrP peptide within the same time period (Figure 2A). On the other hand, PTHrP (107-139) significantly reduced the gene expres-

sion of these early osteoblastic markers at day 18 of osteogenic induction in these cultures (Figure 2A). These changes were no longer observed at

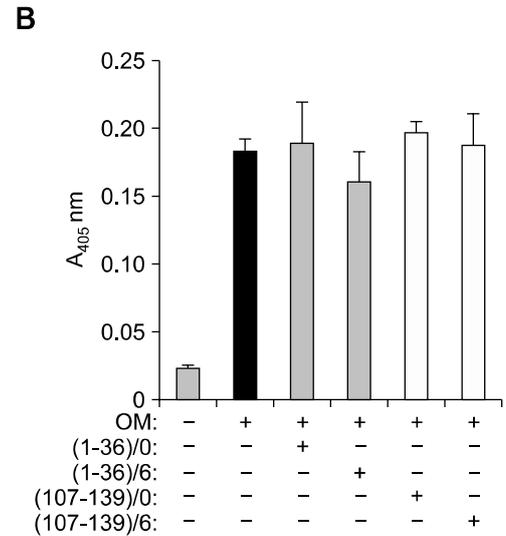
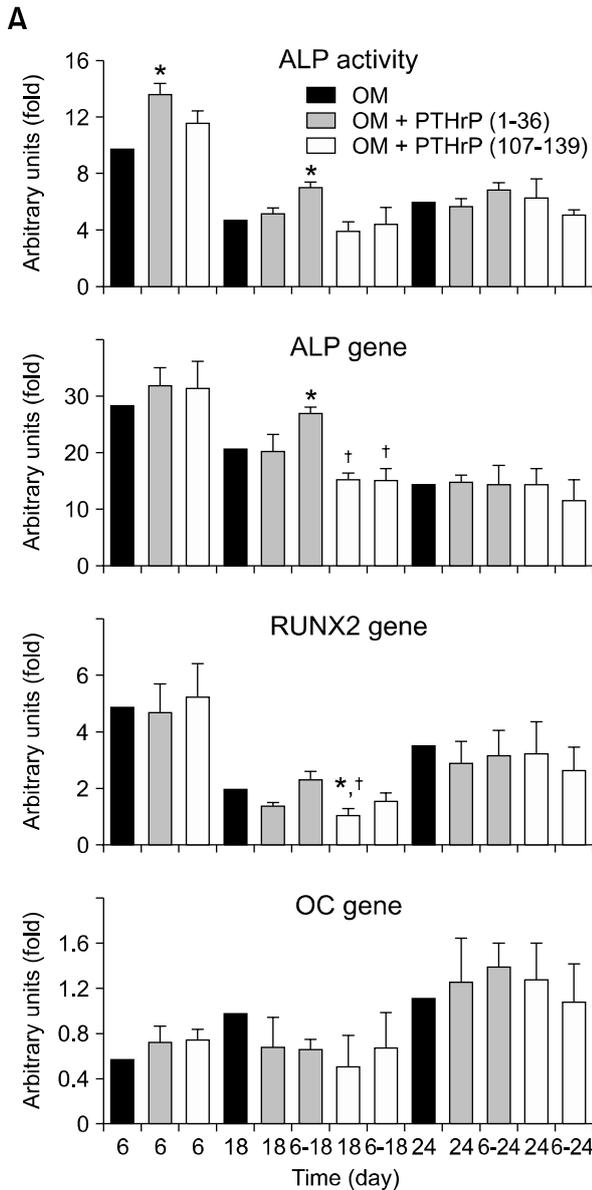


Figure 2. Effect of PTHrP (1-36) and PTHrP (107-139) on commitment of human MSCs to osteoblastic lineage. MSC cultures in osteogenic medium (OM) were exposed to 10 nM of PTHrP (1-36) or PTHrP (107-139) from either day one or day six. (A) ALP activity and gene expression of ALP, RUNX2 and OC at different times of cell exposure to each PTHrP peptide or vehicle. (B) Spectrophotometric quantification of alizarin red staining in MSCs at day 24 after osteogenic induction in the presence or absence of PTHrP (1-36) or PTHrP (107-139) from either day one (0) or day six (6). Values are mean \pm SEM of 3 different cultures, each from a different subject, analyzed in duplicate. ALP activity and gene expression values were expressed as a fold change relative to those in undifferentiated MSCs (maintained in basal medium) at day 6. * $P < 0.05$ vs corresponding peptide-untreated value. † $P < 0.05$ vs corresponding PTHrP (1-36)-treated value from day 6 to 18.

the end of the culture period (24 days). Moreover, OC gene expression, a late osteoblastic marker, was unaffected by any treatment pattern with these peptides throughout the period of study (Figure 2A). Moreover, these PTHrP peptides failed to produce any significant change in mineralization in human MSCs after 24 days of osteogenic induction (Figure 2B).

As both PTHrP peptides mainly affected early markers of the osteogenesis, the expression of runx2 gene and alkaline phosphates was then analysed, as well as the alkaline phosphate activity at days 2 and 4 of the treatment with each of the peptides. The alkaline phosphatase activity did not vary between these two times in the cultures

treated with PTHrP (1-36) or PTHrP (107-139) with respect to untreated cultures (Figure 3). However, the expression of the ALP gene at 4 days and that of the runx2 genes at 2 days increased significantly in the cultures treated with PTHrP (1-36). As far as the PTHrP (107-139) is concerned, it did not significantly affect the expression of either of these two genes.

Effect of PTHrP (1-36) and PTHrP (107-139) on adipogenesis in human MSCs

The expression of the adipogenesis-related genes PPAR γ -2 and LPL was found to decrease between 18 and 24 days of adipogenic induction in MSC

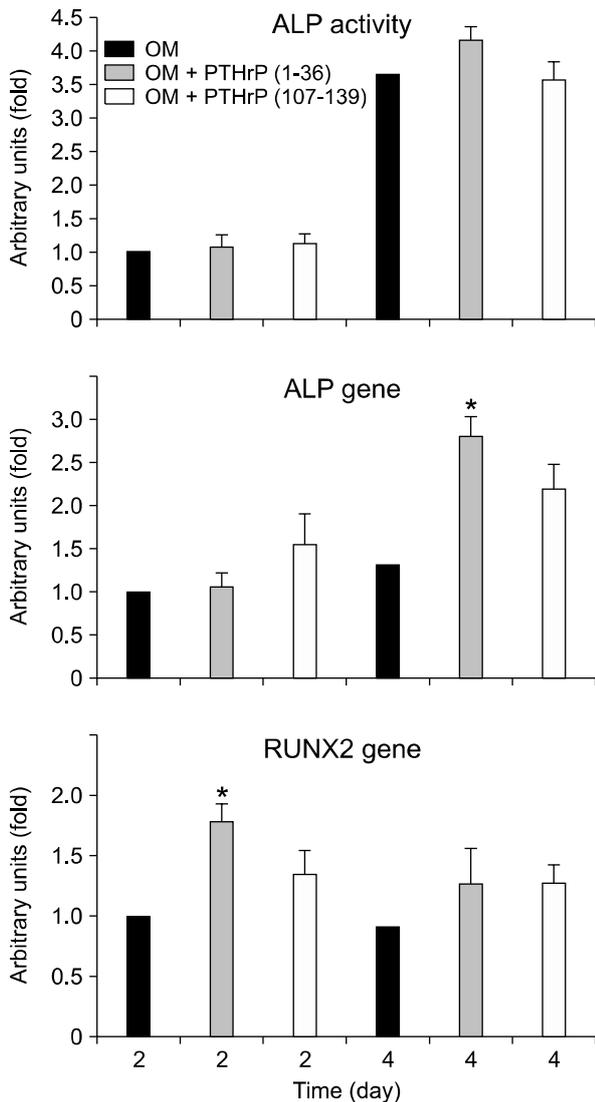


Figure 3. Effect of PTHrP (1-36) and PTHrP (107-139) on commitment of human MSCs to osteoblastic lineage. MSC cultures in osteogenic medium (OM) were exposed to 10 nM of PTHrP (1-36) or PTHrP (107-139) for two or four days, and ALP activity and gene expression of ALP and RUNX2 were measured. Values are mean \pm SEM of 3 different cultures, each from a different subject, analyzed in duplicate. ALP activity and gene expression values were expressed as a fold change relative to those in osteogenic medium untreated at day 2. * $P < 0.05$ vs corresponding peptide-untreated value.

cultures treated with PTHrP (1-36); whereas it showed a non-significant tendency to increase in those treated with PTHrP (107-139), compared to that in the untreated cells (Figure 4A). This different effect of these PTHrP peptides was observed even by delaying for 6 days their addition to MSC cultures after adipogenic induction (Figure 4A). This was also apparent by comparing the number of adipocytes and the content of fatty vesicles (as

the amount of oil red stain) in these differently treated cell cultures (Figures 4B and 4C). The increase of oil red stain in the cultures PTHrP (107-139) treated was significant (Figure 4B, down panel).

Given that PTHrP (1-36) exerts an inhibitory effect on adipogenesis, whilst PTHrP (107-139) tends to induce it, we set out to assess which effect would predominate if both PTHrP peptides were simultaneously added to MSCs in an adipogenic medium. With this aim, MSC cultures induced to adipocytes were treated as before from day 6 with either PTHrP peptide. Nine days later, PTHrP (107-139)-supplemented cell cultures did or did not receive the same concentration of PTHrP (1-36) for one more week. In those cultures treated with combined PTHrP (107-139) and PTHrP (1-36), PPAR γ 2 and LPL gene expression as well as the number of adipocytes and fat accumulation all decreased with respect to those treated only with PTHrP (107-139) (Figures 5A and 5B).

With the aim of analyzing the possible role of PTH1R in the inhibitory action of PTHrP (1-36) on adipogenesis, the PTH1R antagonist PTHrP (7-34) was used. Treatment of MSCs from day 6 up to day 22 with PTHrP (1-36) in the presence of this antagonist, at 1 μ M, in adipogenic medium increase significantly the PPAR γ 2 and LPL gene expression and the number of adipocytes (Figures 5A and 5B). But these values were still significantly smaller than those of untreated cultures.

Changes in the PTH1R gene expression induced by PTHrP (1-36) and PTHrP (107-139) in human MSCs induced to osteoblasts or adipocytes

We next examined the putative changes in the PTH1R gene expression induced by the different treatments in human MSCs during osteoblastogenesis or adipogenesis. PTH1R gene expression in these cell cultures showed a maximum at day 6 after induction to osteoblasts, followed by a later drop between 18 and 24 days. This pattern of expression was not significantly altered by the presence of either PTHrP fragment tested (Figure 6A). In those cultures induced to adipocytes, it is noteworthy that the PTH1R gene expression was considerably higher than that observed in the cells maintained in an osteogenic medium, mainly between 18-24 days of adipogenic induction. At this time period, PTHrP (1-36) inhibited this gene expression, whilst the C-terminal PTHrP peptide was inefficient in this regard (Figure 6A). Moreover, the inhibitory effect elicited by PTHrP (1-36) on PTH1R during adipogenesis was not significantly modified by the presence of PTHrP (107-139) in these cell

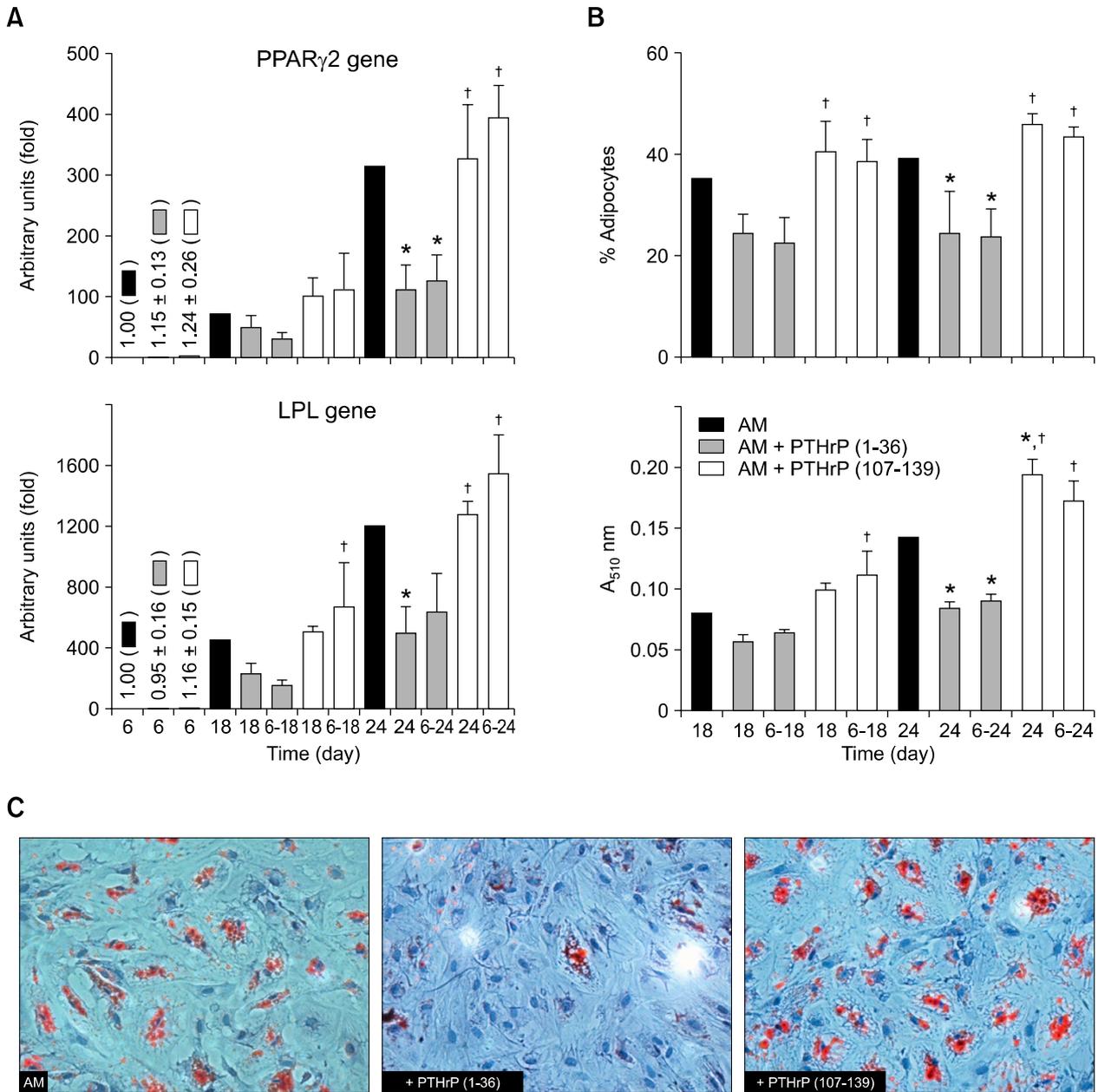


Figure 4. Effect of PTHrP (1-36) and PTHrP (107-139) on commitment of human MSCs to adipocytic lineage. MSC cultures in adipogenic medium (AM) were exposed to 10 nM of PTHrP (1-36) or PTHrP (107-139) from either day one or day six. (A) Gene expression of PPAR- γ and LPL at different times of cell exposure to each PTHrP peptide or vehicle. (B) Percentage of adipocytes and spectrophotometric quantification of oil red staining of fat vesicles in MSCs at day 24 after adipogenic induction. (C) Representative cultures induced to adipocytes and stained with oil red, treated or untreated with PTHrP (1-36) or PTHrP (107-139) are shown (magnification, \times 200). Values are mean \pm SEM of 3 different cultures, each from a different subject, analyzed in duplicate. The mRNA expression is expressed as a fold change relative to day 6 untreated MSC adipocyte induced as a reference (value = 1). * P < 0.05 vs corresponding peptide-untreated value. † P < 0.05 vs corresponding PTHrP (1-36)-treated value.

cultures (Figure 6B).

Discussion

In this work, we have analysed the effect on MSC

differentiation to osteoblasts and adipocytes of two PTHrP fragments, N- and C- terminal, that have different cellular receptors.

Recent studies indicate that endogenous PTHrP acts as a potent anabolic factor in fetal and adult bone (Strewler, 2000; Miao *et al.*, 2004, 2005). In

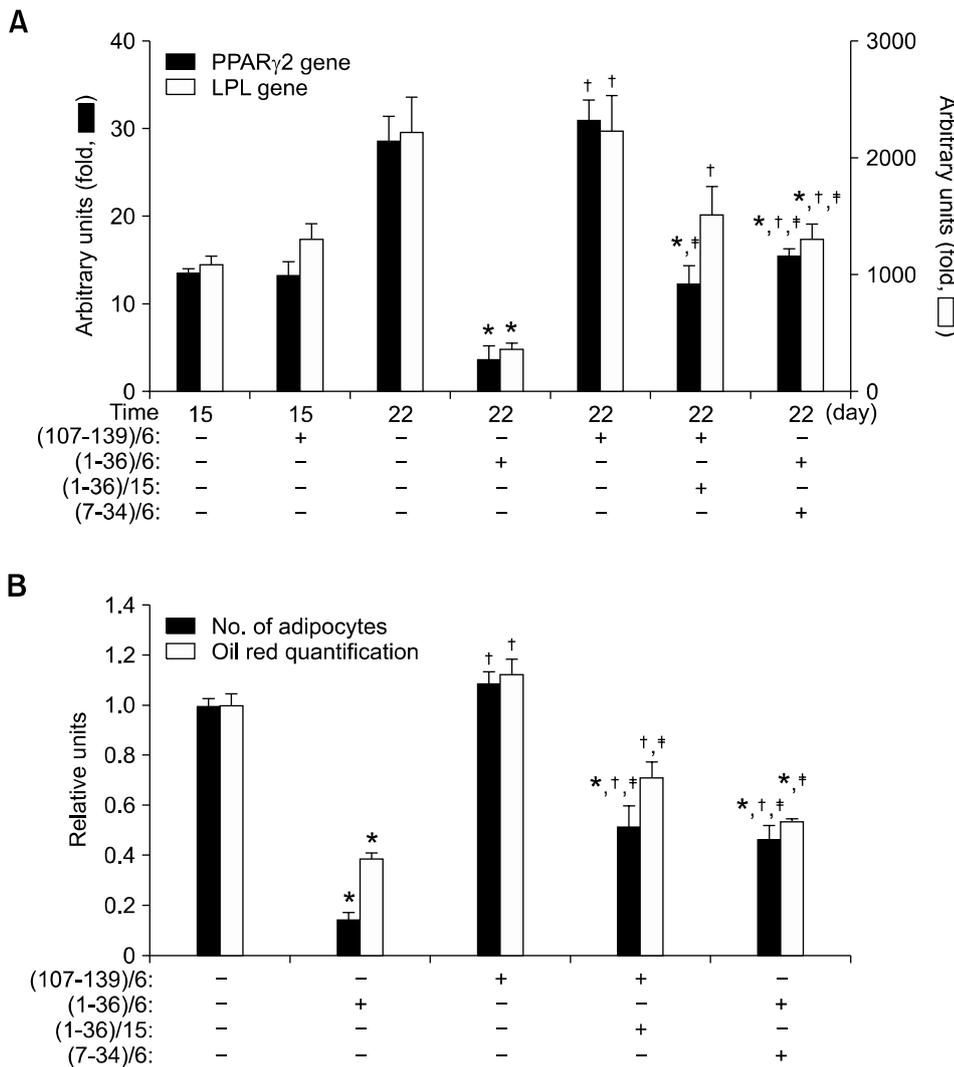


Figure 5. Effect of the PTH1R inhibitor PTHrP (7-34) and the pre-treatment with PTHrP (107-139) on the adipogenic inhibitory effect of PTHrP (1-36) in human MSCs. Cell cultures in adipogenic medium (AM) were exposed to PTHrP (1-36) or PTHrP (107-139) (at 10 nM), from day 6 up to day 22 ((1-36)/6, (107-139)/6). At day 15, the same concentration of PTHrP (1-36) was added to some cultures ((1-36)/15) which had been pre-treated with PTHrP (107-139) for one more week. Some PTHrP (1-36)-treated cultures were also treated with PTHrP (7-34), at 1 μ M added 30 min before, from day 6 ((7-34)/6). (A) Gene expression of PPAR- γ and LPL at different times of cell exposure to each PTHrP peptide or vehicle. Gene expression of both genes was expressed as a fold change relative to that of the house-keeping gene GAPDH. (B) Number of adipocytes and spectrophotometric quantification of oil red staining of fat vesicles in human MSCs at day 22 after adipogenic induction. The values are expressed as relatives to those in peptide-untreated cultures. Values are mean \pm SEM of 3 different cultures, each from a different subject. * $P < 0.05$ vs corresponding peptide-untreated value; $\dagger P < 0.05$ vs corresponding PTHrP (1-36)-treated value; $\ddagger P < 0.05$ vs corresponding value in cultures treated with PTHrP (107-139) only.

postmenopausal women with osteoporosis, daily PTHrP (1-36) administration increases trabecular bone mineral density, apparently at the expense of an increase in bone formation without significant changes in bone resorption (Plotkin *et al.*, 1998; Horwitz *et al.*, 2003). However, the true molecular mechanisms associated with the osteogenic action of this PTH-like fragment of PTHrP - in contrast to those of PTH (Goltzman, 2008) - have not been explored much. In the present study, exposure of human MSCs to PTHrP (1-36) in an osteogenic medium since day 6 increased the expression of some early osteoblastic markers. However, this effect was no longer present at the end of the study (day 24). This might be related to the current notion, based on studies in various osteoblastic cell preparations, that the anabolic effect of the N-terminal domain of PTH and PTHrP seems to depend on both exposure time and the cell

differentiation stage (Schiller *et al.*, 1999; Fujita *et al.*, 2001; Krishnan *et al.*, 2003; de Gortazar *et al.*, 2006). On the other hand, this effect of PTHrP (1-36) was not found to be elicited by PTHrP (107-139) in human MSCs; in fact, the latter peptide transiently decreased RUNX2 gene expression at day 18 in these cells. However, neither PTHrP (1-36) nor PTHrP (107-139) affected OC gene expression or mineralization in human MSCs. These findings thus suggest that these PTHrP peptides may affect the earlier stages of osteoblastogenesis in human MSCs in different ways. This is also supported by the fact that PTHrP (1-36) increased in expression of genes runx2 and ALP at days 2 and 4 of treatment, whilst PTHrP (107-139) did not affect the expression of either osteogenic markers.

A previous report has shown that continuous exposure of either rat osteosarcoma UMR-106

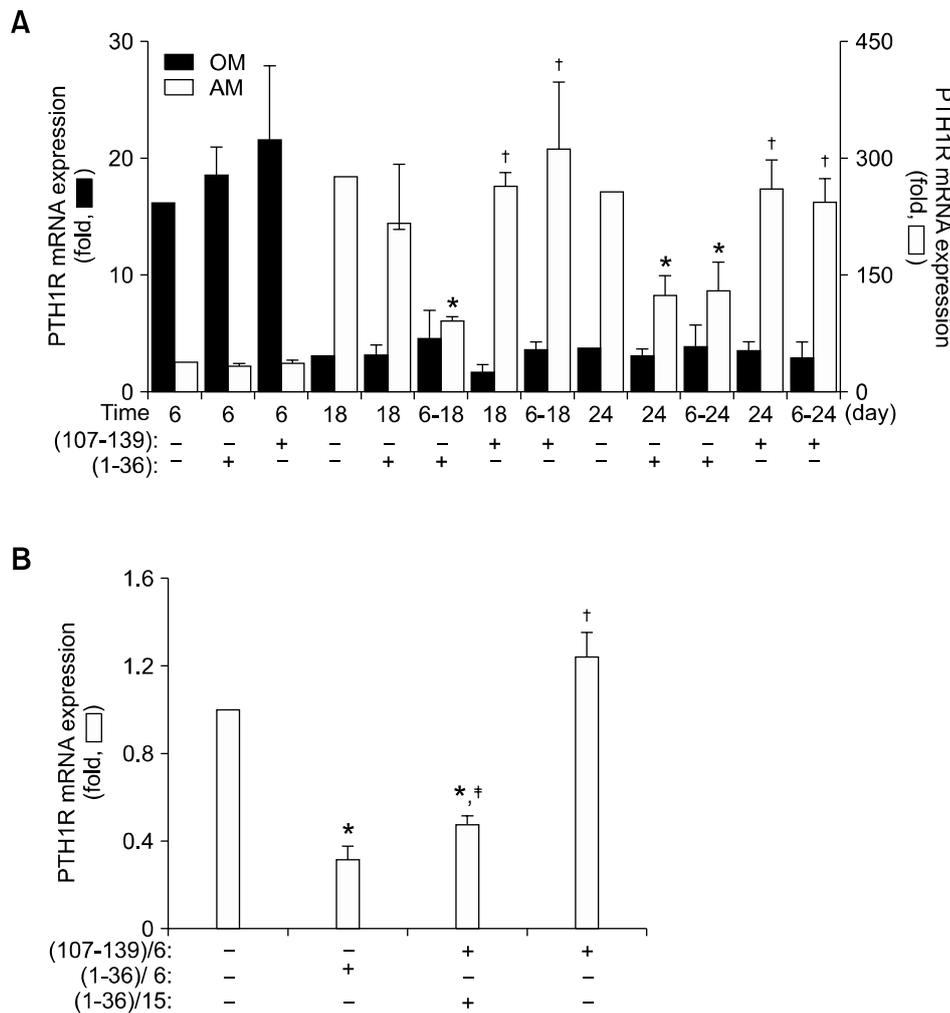


Figure 6. Relative expression of PTH1R gene in human MSCs during osteogenesis and adipogenesis. (A) Cells in osteogenic medium (OM) or adipogenic medium (AM) were exposed to 10 nM of PTHrP (1-36) or PTHrP (107-139) from either day one or day six. (B) PTH1R gene expression at 22 days in MSC cultures exposed to adipogenic medium (AM) that were exposed to PTHrP (1-36) or PTHrP (107-139) (at 10 nM), from day 6 up to day 22. At day 15, the same concentration of PTHrP (1-36) was added to some cultures which had been pre-treated with PTHrP (107-139) for one more week. Values are mean \pm SEM of 3 different cultures, each from a different subject. Gene expression was expressed as a fold change relative to that in undifferentiated MSCs (maintained in basal medium) at day 6 (A), or to that in peptide-untreated cultures in AM medium at day 22 (B). * $P < 0.05$ vs corresponding peptide-untreated value; † $P < 0.05$ vs corresponding PTHrP (1-36)-treated value; ‡ $P < 0.05$ vs corresponding value in cultures treated with PTHrP (107-139) only.

cells or human osteoblast-like cells from trabecular bone explants to PTHrP (107-139) for several days inhibited ALP activity (Martinez *et al.*, 1997; Valin *et al.*, 1999). In contrast, it has been recently shown that intermittent but not continuous exposure of these cells or human osteosarcoma MG-63 cells during cell growth to this peptide for the first 6 to 24 h of a 48-h cycle stimulates osteoblastic differentiation (de Gortazar *et al.*, 2006). Thus, different types of exposure to PTHrP (107-139) can stimulate or inhibit the osteogenesis.

Our findings demonstrate that the effects of PTHrP (1-36) and PTHrP (107-139) in human MSCs induced to adipocytes were still more different; the former peptide inhibited adipogenesis, but the latter showed a tendency to favor it. Moreover, this anti-adipogenic action of PTHrP (1-36) was somewhat attenuated but not abolished in the presence of PTHrP (107-139). Previous *in vitro* studies support an inverse relationship between PTHrP and adipogenesis. Thus, overexpression of

PTHrP or exogenous addition of its N-terminal fragment inhibit adipogenesis in 3T3-L1 preadipocytes and pluripotential C3H10T1/2 cells induced to adipocytes (Chan *et al.*, 2001, 2003). Moreover, a recent report has shown that transient (1 h) but not continuous exposure of human MSCs to 50 nM PTH (1-34) inhibited their differentiation to adipocytes (Rickard *et al.*, 2006). The finding that a short exposure to the latter peptide was as efficient as the continuous presence of PTHrP (1-36), as shown here, to inhibit adipogenesis in these cells might be related to their different capacity to bind stably to different conformations of the PTH1R (Dean *et al.*, 2008); considering that both peptides are likely to act through the PTH1R in human MSCs. Related to these *in vitro* data, a decrease in the expression of adipogenic genes in bone were found in osteopenic rats after daily administration of PTH (1-34) (Kulkarni *et al.*, 2007). Furthermore, mice with heterozygous inactivation of the PTHrP gene have been shown to display

low bone mass associated with increased bone marrow adipocytes (Amizuka *et al.*, 1996). However, this increased bone marrow adiposity was absent in osteopenic mice with osteoblast-specific targeted disruption of PTHrP by using Cre/lox technology, which was thought to be accounted for by the timing of PTHrP ablation relative to the osteogenic and adipocytic programs (Miao *et al.*, 2005). These *in vivo* results thus suggest that PTHrP could act on precursor cells before they are committed to either preosteoblasts or the adipocyte lineage. The present *in vitro* results in human MSC cultures further support the aforementioned *in vivo* findings, and provide a new rationale to explain the osteogenic effect of PTHrP (1-36) in osteoporotic postmenopausal women (Horwitz *et al.*, 2003).

The important role of the PTH1R to negatively modulate adipogenesis in bone was further supported by the fact that its overexpression in mice was associated with an almost total absence of fatty cells in the bone marrow (Kuznetsov *et al.*, 2004). In the present study, the PTH1R antagonist PTHrP (7-34) decreased the inhibitory effect of PTHrP (1-36) on adipogenesis, but was inefficient in completely re-establishing the PTHrP (1-36)-inhibited adipogenesis in human MSCs. Although we used a concentration of PTHrP (7-34) which was 100-fold higher than that of PTHrP (1-36), this might have been insufficient to block the PTH1R in these cells induced towards the adipocytic program. This is supported by the fact that in human MSCs, the PTH1R mRNA levels increased up to 300 times over those in non-induced control cell cultures by 18-24 days; possibly related to the high concentration of dexamethasone in the adipogenic medium, based on recent findings (Haramoto *et al.*, 2007; Ahlstrom *et al.*, 2009). In contrast, the PTH1R gene expression was found to increase in the first stages of osteoblastic differentiation -in agreement with previous data in primary mouse bone marrow cell cultures (Kuznetsov *et al.*, 2004)-, decreasing thereafter, which might be consistent with a putative role of this receptor to commit human MSCs to the osteogenic lineage (Kuznetsov *et al.*, 2004; Miao *et al.*, 2005). The mechanism(s) whereby the PTH1R expression was higher in more advanced stages of adipocyte differentiation in human MSCs are presently unknown. However, this might be due to the concomitant increase of the PPAR γ -2 gene which could inhibit PTHrP production (Lappas *et al.*, 2004), and this would in turn activate the expression of the PTH1R. In fact, ligand-dependent down-regulation of PTH1R is a well characterized event in osteoblasts and other cell types (Abou-Samra *et al.*, 1994; Du *et al.*, 2000; Song *et al.*,

2009), and it might also well occur in cells of the adipocytic lineage.

In conclusion, our results demonstrate for the first time different effects of the N- and C-terminal domains of PTHrP in the commitment of human MSCs. The continuous exposure of these cells to PTHrP (1-36) induced a mild osteogenic effect but inhibited adipogenesis, further supporting its potential use as a bone anabolic agent. On the other hand, a similar pattern of exposure to PTHrP (107-139) seems to favor adipogenesis over osteogenesis in these cell cultures.

Methods

Bone marrow donors

The bone marrow donors were recruited by the Bone Marrow Transplantation Program of the Hematology Service at the "Reina Sofia University Hospital" (Córdoba, Spain). The experiments were performed with bone marrow obtained from three donors, two females and a male, aged 18, 23 and 31 years, respectively. All subjects gave their written consent.

Bone marrow cell cultures

Human MSCs were isolated from the bone marrow, following an optimized procedure based on previously reported methodology (Kotobuki *et al.*, 2004; Casado-Diaz *et al.*, 2008). In short, the bone marrow from each donor was centrifuged at $140 \times g$ for 10 min at 4°C. The cellular fraction was seeded in two 75-cm² flasks (Nunc, Kamstrupvej, Denmark) in α -MEM (Cambrex, East Rutherford, NJ) with 15% FBS (Invitrogen, Carlsbad, CA), 2 mM UltraGlutamine (Cambrex), 100 U ampicillin, 0.1 mg streptomycin/ml and 1 ng/ml basic fibroblast growth factor (bFGF) (Sigma-Aldrich, St. Louis, MO) (culture medium), and maintained at 37°C in 5% CO₂. The medium was replaced every three to four days. When near-confluent cell colonies were observed, the cells were trypsinized and reseeded with the same culture medium at about 300 cells/cm² in 75-cm² flasks, using the same culture growing conditions previously described. These confluent cells were collected again by trypsinization. Between 8×10^5 to 10^6 cells/ml α -MEM with 30% FBS and 5% dimethylsulphoxide (Sigma-Aldrich) were collected in CryoTubes (Nunc), and stored in liquid nitrogen until their used.

Osteogenic and adipogenic differentiation

Cells were plated at 500 cells/cm² on 6- or 12-well plates (TPP, Trasadingen, Switzerland) containing culture medium supplemented with 10% FBS. When cells were 80% to 90% confluent, the medium was supplemented with either 10 nM dexamethasone, 0.2 mM ascorbic acid and 10 mM β -glycerophosphate to induce osteoblast differentiation, or

0.5 μ M dexamethasone, 0.5 mM isobutylmethylxanthine and 50 μ M indomethacin to induce adipogenesis. All these inducers were from Sigma-Aldrich.

PTHrP (1-36) and PTHrP (107-139) treatments

The human PTHrP (1-36) and human PTHrP (107-139) were synthetic peptides, kindly supplied by Pedro Esbrit (Laboratorio de Metabolismo Mineral y Óseo, Fundación Jiménez Díaz, Madrid), A.F. Stewart, M.D. (Division of Endocrinology and Metabolism, University of Pittsburgh School of Medicine, Pittsburgh, PA) and F. Roncal, Ph.D. (Proteomics Unit, Centro Nacional de Biotecnología, Madrid), respectively.

Human MSCs were induced to either osteoblasts or adipocytes, in the presence or absence (control) of each PTHrP peptide, at 10 nM. Six days later, some of these control cultures started to be treated with either PTHrP (1-36) or PTHrP (107-139), at 10 nM, in the presence or absence of the PTHrP antagonist, [Asn¹⁰,Leu¹¹,D-Trp¹²] PTHrP (7-34) amide (PTHrP (7-34); Bachem, Bubendorf, Switzerland), at 1 μ M. When the cells were treated with PTHrP antagonist, this was added 30 min before peptide PTHrP. Cells were grown for 24 days, with fresh medium replacement every 48 h. Some of those cultures which had previously been stimulated with PTHrP (107-139) for 15 days in adipogenic medium were subsequently exposed to the combination of both PTHrP peptides in the same medium up to the end of the study. Some cell cultures treated with the PTHrP peptides for 12 days starting at day 6 after induction of differentiation, based on initial experiments showing a response of osteoblastic makers at this time period (18 days).

Alkaline phosphatase (ALP) activity

Human MSCs maintained in osteogenic medium were lysed in PBS with 0.1% Triton \times 100 (Sigma-Aldrich), with the aid of a one-ml syringe plunger. Three to twenty μ l of the resulting extract were mixed with 200 μ l of the ALP substrate solution (p-Nitrophenyl Phosphate Liquid Substrate System ready-to-use, Sigma-Aldrich). The reaction mixture was incubated at 37°C for 30 min, and then halted with 50 μ l of 3 M NaOH. The reaction product was measured by absorbance at 405 nm on a GENios ELISA microplate reader (Tecan, Männedorf, Switzerland). The absorbance values were standardized for the total genomic DNA present in the extracts. The relative DNA amount of each sample was determined by quantitative real-time PCR (QRT-PCR), using GAPDH primers (Supplemental Data Table S1), with the QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany), using an Mx3005P QPCR System (Stratagene, La Jolla, CA).

Reverse-transcription and real time PCR

Total RNA was isolated with Tri-Reagent (Sigma-Aldrich) and treated with DNase I (Sigma-Aldrich). RNA quantitation was assessed at 260 nm with a ND-1000 spectrophotometer (NanoDrop, Wilmington, DE), and its quality (integrity) was checked on agarose gel electrophoresis. One μ g of total RNA was retrotranscribed to cDNA using

the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) in 20 μ l. Real-time PCR was carried out in a LightCycler II real-time PCR thermocycler (Roche, Mannheim, Germany) with 1 μ l of cDNA and 10 pmol of each primer corresponding to the tested genes (Supplemental Data Table S1), using the QuantiTect SYBR Green PCR Kit. The relative quantity of mRNA was calculated for each sample using the copy threshold (Ct) value, and normalized against GAPDH mRNA (housekeeping gene). The amplification profile included one cycle at 95°C for 15 min and 45 three-step cycles: 94°C for 15 s, 60°C for 20 s and 72°C for 20 s. The results were generated and analyzed with LightCycler Software V 3.5, using the second derived method for the Ct calculation.

Histochemical staining

Mineralization of human MSCs induced to osteoblasts was tested using alizarin red staining. Thus, cells were fixed with 3.7% formaldehyde in methanol for 10 min and stained for 45 min with a mixture made up of 10 ml of 1% (w/v in water) alizarin red plus one ml of 1% ammonium hydroxide. Then, the cells were washed with water, dried and visualized under a light microscope. The alizarin red precipitates were eluted with 10% acetic acid, neutralized with 10% ammonium hydroxide (all chemicals from Sigma-Aldrich), and the absorbance at 405 nm was measured, as previously described (Gregory *et al.*, 2004).

Fat accumulation in human MSC cultures induced to adipocytes was tested using oil red staining. Cells were fixed as described above, washed with water, and subsequently stained for 15-20 min with a mixture of 8.2 ml of 0.3% (w/v in isopropanol) of oil red plus 6.8 ml of distilled water. Cells were then washed with water, stained with hematoxylin and visualized under a light microscope (all chemicals from Sigma-Aldrich). The cells with lipid accumulation were counted in 10 different fields of each plate well, using contrast phase microscopy at 200 \times magnification. To quantify fat, oil red stain was extracted with isopropanol for 10 min at room temperature, and the absorbance of the resulting solution was measured at 510 nm.

Statistical analysis

The data throughout the text are means \pm standard error of the mean (SEM). All *P* values were calculated using ANOVA and Fisher's projected least significant difference (PLSD) test. A value of *P* < 0.05 was considered significant.

Supplemental data

Supplemental Data include a table and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-42-2-02.pdf.

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