Research Communication

Mid-region parathyroid hormone-related protein is a genome-wide chromatin-binding factor that promotes growth and differentiation of HB2 epithelial cells from the human breast

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Abstract

Human parathyroid hormone-related protein (PTHrP) is a polyhormone that undergoes proteolytic cleavage producing smaller peptides which exert diversified biological effects. PTHrP signalization is prominently involved in breast development and physiology, but the studies have been focused onto either N-terminal species or full-length protein introduced by gene transfer techniques. Our present work investigates for the first time the effect of the mid-region PTHrP secretory form, that is, the fragment [38–94], on HB2 non-tumoral breast epithelial cells. We examined viability/proliferation of cells grown in PTHrP-containing media supplemented with different serum concentration and on different substrates, extending our investigation to check whether (a) by analogy with MDA-MB231 cells, also HB2 cell chromatin possesses genome-wide binding sites for mid-region PTHrP, and (b) the peptide is endowed with modulating properties toward the expression of proliferation/differentiation signatures by HB2 cells. Our results indicate that mid-region PTHrP acts as a cell growth/differentiation stimulator in routine and "nutrient stress" culture conditions, accordingly reprogramming gene expression, and is able to bind to cytogenetic preparations from HB2 cells. This supports the concept that the physiological mechanisms involving PTHrP during breast development may include mature processed forms of the protein different from the N-terminal fragment. © 2018 BioFactors, 45(2):279–288, 2019

Keywords: *PTHrP; breast cells; cell growth; gene expression; chromosome decoration*

Abbreviations: Bcl-2, B-cell lymphoma 2; Bcl-xl, B-cell lymphoma-extra large; BMP, bone morphogenetic protein; CERBB2, c-Erb-B2 receptor tyrosine kinase 2; DABCO, 1,4-diazabicyclo[2.2.2]octane; DMEM, Dulbecco's modified Eagle medium; dNTP, deoxyribonucleotide triphosphate; dT, doubling time; ETS, E26 transformation-specific; FCS, foetal calf serum; HGF, hepatocyte growth factor; ITGA1, integrin subunit α1; ITGB1, integrin subunit β1; MAPK, mitogen-activated protein kinase; MSX-1, MSH homeobox 1; MUC1, mucin-1; NLS, nuclear localization signal; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; PTH, parathyroid hormone; PTHR1, type 1 parathyroid hormone receptor

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1. Introduction

Human parathyroid hormone-related protein (PTHrP) is a polyhormone whose unprocessed translation products are three isoforms of either 139, 141, or 173 aminoacids differing in the C-terminal end and endowed with sequence homology with PTH at its N-terminal end which is thus able to recognize the type 1 parathyroid hormone receptor (PTHR1). Differently from PTH, PTHrP are almost ubiquitous and in normal conditions it is not found in the circulation but rather acts as a local tissue factor. It is known that the parental molecule undergoes proteolytic cleavage producing smaller peptides which may exert biological effects different from those related to the regulation of calcemia (*e.g.*, [1,2]). Early literature articles [3–5] demonstrated that the mature mid-region secretory form of PTHrP, originated by aminoacid cleavage at Arg37, was [38–94]-amide. Subsequent characterization of the biological role played by this peptide indicated that it may be active in the regulation of transplacental calcium flux in sheep, rodents and humans,

promoting phospholipase C activation without any involvement by cAMP [6], and in the impairment of viability, proliferation, and invasiveness of different breast cancer cell lines [7,8], while promoting the survival of myeloma plasma cells [9]. To date, the cognate surface receptor for this fragment has not been identified yet. On the other hand, a nuclear localization signal (NLS) situated between aminoacids 84 and 93 has been proven to mediate importin β/GTP Ran-dependent intra-nuclear shuttling of the peptide which may thus be regarded also as an intracrine factor able, inter alia, to bind chromatin and play a putative transcription-factor-like role [10,11]. In this context, Luparello and coworkers have published a number of papers demonstrating that PTHrP [38–94]-amide actively modulates gene expression of the triple negative breast cancer cells MDA-MB231 and is able to "decorate" their chromosomes in denatured metaphase spreads, and also to bind a specific DNA sequence *in vitro* [12–14].

It is known that PTHrP signalization via PTHR1 is prominently involved in breast development and physiology. Epithelial cells of the fetal mammary bud produce PTHrP which activates Wnt and BMP pathways in the surrounding PTHR1-exposing mesenchymal cells, thereby establishing a molecular cross talk which is instrumental for the outgrowth of the embryonic ducts. Nipple formation and sexual dimorphism are other developmental events controlled by the correct timing and location of PTHrP production. In the adult, PTHrP is expressed at high level by mammary epithelial cells when activated for lactation. Part of this amount is released into the milk and an aliquot exceptionally gets into the bloodstream where it triggers the mobilization of skeletal calcium to be delivered to the breast for milk production [9,15]. Li et al. [16] have recently reported that overexpression of PTHrP via recombinant adenovirus transduction promotes proliferation and restrain apoptosis of goat mammary epithelial cells by regulating the expression of key genes such as cyclin-D1, PCNA, Bcl-2, and Bcl-xl, demonstrating that this factor plays an important role in the control of the survival of this cytotype.

It must be emphasized that studies examining the role of PTHrP on the biological behavior of mammary epithelial cells generally have been focused onto either N-terminal species or full-length protein introduced by gene transfer techniques. Our present work investigates for the first time the effect of the mid-region PTHrP secretory form on a non-tumoral breast epithelial cell line, HB2, derived from sub-cloning, and SV40-mediated immortalization of MTSV1-7 mammary luminal epithelial cells isolated from milk. As reported in the initial article describing their establishment in culture [17], these cells, which grow strictly connected in scattered islands on tissue plastics in normal medium, are able to develop branching structures from spheroids when cultured in collagen gels in the presence of hepatocyte growth factor (HGF). These cells, although cannot be considered "genetically normal" for a number of genomic alterations produced and stabilized during the immortalization process, largely recapitulate the morphology and phenotype of normal luminal epithelium due to the high expression of associated gene signatures and the absence or low expression of mesenchymal marker genes. [18–20].

In this study, we explored the effect of mid-region PTHrP specifically examining viability/proliferation of cells grown in media supplemented with different serum concentration and on different substrates, extending our investigation to check whether (a) by analogy with MDA-MB231 cells, also HB2 cell chromatin possesses genome-wide binding sites for mid-region PTHrP analyzing the "decoration" pattern in cytogenetic preparations, and (b) the peptide is endowed with modulating properties toward the expression of proliferation/differentiation signatures by HB2 cells.

2. Experimental procedures

2.1. Cells and mid-region PTHrP

The HB2 breast epithelial cell line (courtesy of Cancer Research, UK) was routinely grown in high glucose–DMEM medium plus 10% foetal calf serum (FCS; ThermoFisher, Waltham MA, USA), 5 μg hydrocortisone/ml (Sigma, St. Louis, MO, USA), 10 μg bovine insulin (Sigma) and antibiotic/antimycotic mixture (100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 mg/l amphotericin B; ThermoFisher), at 37 °C in a 5% CO_2 atmosphere.

MDA-MB231 breast tumor cells, taken from laboratory stocks, were maintained in RPMI 1640 medium plus 10% FCS and antibiotic/antimycotic mixture, at 37 °C in a 5% CO_2 atmosphere.

PTHrP [38–94]-amide (courtesy of Prof. A.F. Stewart, University of Pittsburgh School of Medicine, USA) was synthesized using solid phase methods and subjected to aminoacid composition, mass spectroscopy and analytical reversed-phase HPLC analyses for confirmation of the structure, purity, and peptide content of the preparation [5].

2.2. Proliferation assay

HB2 cells were plated in 4-well plates at 1×10^4 cells/well in either 1, 5, or 10% FCS-containing medium supplemented with mid-region PTHrP at different concentrations. After 48, 72, or 96 h of culture, cells were trypsinized and counted with a hemocytometer. According to Luparello et al. [21], growth parameters were calculated as follows:

$$Growth\ rate\ (\alpha) = (lnC - lnC_0)/(t - t_0)$$

where C is the initial cell concentration at time t and C_0 is the number of cells seeded initially (*i.e.*, at time t_0)

$$Doubling\ time\ (dT) = ln2/\alpha$$

Control assays were performed in the absence of PTHrP [38–94]-amide.

2.3. Cell cultures in collagen gels

A collagen solution was prepared by mixing Collagen S (Roche, Manheim, Germany), 1% FCS/DMEM, FCS-free DMEM and 1 M HEPES (7:1:1:1) and kept in ice. Trypsinized HB2 cells were washed twice in FCS-free DMEM, resuspended in 1% FCS/DMEM and mixed with the cold collagen solution at a final concentration of 1×10^5 cells/ml. Aliquots of 60 μl of the collagen-containing cell suspension were dispensed in 96-well plates, allowed to solidify at 37 °C for 2 h and cultured for 4 days in the incubator with daily changes of 60 μl of 1% FCS/DMEM. Subsequently, the cells were grown in the presence of either 1, 10, or 100 pM PTHrP-containing medium. Due to its stimulating effect on viability and branching morphogenesis of HB2 cells [17], parallel experiments were performed with the further addition of 10 ng HGF (Sigma)/ml to the cultures.

2.4. RNA isolation and reverse transcription

Isolation of total RNA from trypsinized HB2 cells was carried out with TRI reagent (Sigma), according to manufacturer's instruction. In the case of cells grown within collagen gels, they were obtained in suspension after lysis of the gel with collagenase type IA (Sigma). Before the reverse transcription, the total RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) and its quality and integrity checked through agarose gel electrophoresis in denaturing conditions. The cDNAs were synthesized using SuperScript II reverse transcriptase (ThermoFisher) in the presence of 100 ng random 6-mer primers (Sigma), 50 U RNase inhibitor (ThermoFisher), and 0.5 mM each of dNTPs; reverse transcription was carried out for 60 min at 42 °C, followed by treatment with 2 U Ribonuclease H (ThermoFisher) for 20 min at 37 °C. The quality of the cDNA was checked by amplification of "housekeeping" 18S cDNA.

2.5. Conventional and real-time PCR

PCR analysis was performed using 2.5 μM of appropriate sense and antisense primers (Table 1), 1 U RedTaq DNA polymerase (Sigma)/μl, 200 μM each of dNTPs and 1 μl of the cDNA template obtained from total RNA. The latter one was omitted in negative controls. The thermal cycle used was a denaturation step of 95 °C for 3 min, followed by 30–35 cycles of 95 °C for 30 sec, the appropriate annealing temperature for 1 min, and 72 °C for 1 min. A final extension of the product was performed for 4 min at 72 °C. The PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining under UV light.

Messenger RNA expression levels were evaluated by quantitative real-time PCR using Power SYBR Green PCR Master Mix (Applied Biosystems, Philadelphia, PA, USA) as previously described [26] in a 7300 Real Time PCR system (Applied Biosystems) in the presence of 2.5 μM of the primers. The PCR data obtained were automatically analyzed by the Relative Quantification Study Software (Applied Biosystem) and expressed as target/reference ratio.

2.6. Immunolocalization of PTHrP [38–94]-amide on denatured metaphase spreads

Immunolocalization experiments were performed as reported by [12,27]. Essentially, both HB2 and MDA-MB231 cells were cultured on plasticware, trypsinized and processed for preparation of cytospin slides, fixed with Carnoy's solution at room temperature and denatured through exposition to UV light. After

TABLE 1 *Primers used for PCR amplification*

Gene *(primer)*	*Sequence (5′-3′)*	*Fragment (bp) and reference*
18S (sense) *18S* (antisense)	GGACCAGAGGCAAAGCATTTGCC TCAATCTCGGGTGGCTGAACGC	495 [14]
ITGA2 (sense) *ITGA2* (antisense)	CTTTGGCAACCTTCCTCCTCCCTT GGTGGTGGTTTCCAGTGCCTCAT	371 [22]
ITGB1 (sense) *ITGB1*(antisense)	AACGAGGTCATGGTTCATGTTGTG GCATTCAGTGTTGTGGGATTTGC	278 [22]
ESX (sense) *ESX* (antisense)	CTCGGAGCTCCCACTCCTCAGA GCTCTTCTTGCCCTCGAGACAGT	188 [23]
CERBB2 (sense) *CERBB1* (antisense)	GGCTGCTGGACATTGACGAG GGGGCTGGGGCAGCCGCTC	231 [24]
MUC1 (sense) *MUC1* (antisense)	GGTACCTCCTCTCACCTCCTCCAA CGTCGTGGACATTGATGGTACC	203 [25]
HOX7 (sense) *HOX7* (antisense)	CTCCCTGAGTTCACTCTCCGAA TCAGTTTCCCCATCTTTAACTCGAG	113 [13]

pretreatment with blocking solution, preparations were incubated with either 1 or 2 μg of PTHrP [38–94]-amide diluted in HEN buffer overnight at 37 °C in a humidified chamber. Then, the slides were washed and treated with the primary antibody (polyclonal anti-human PTHrP 34–53 Ab-2, Millipore, Billerica MA, USA) for 2 h at 37 °C, and with FITC-conjugated secondary antibody (Sigma) overnight at 37 °C, washed and mounted with DABCO antifade plus 0.2 μg/ml propidium iodide for chromosome counterstaining [28]. The observation of metaphases was performed under a Leitz Diavert inverted fluorescence microscope [29] and the photomicrographs were processed using Adobe Photoshop 3.0 LE software as reported by [30]. Negative control samples were UV-treated metaphase spreads submitted to immunolocalization in the absence of PTHrP.

3. Results

3.1. PTHrP [38–94]-amide promotes survival and growth of HB2 cells cultured on tissue plasticware

In a first set of experiments, the survival/proliferative behaviour of HB2 cells in "routine" culture condition, that is, in the presence of 10% FCS-supplemented medium, and in "FCS starvation" condition, that is, decreasing FCS concentration down to either 5 or 1%, were assayed. As expected, only cells cultured in 10% FCS-containing medium grew exponentially with a dT of approx. 38 h between 0–72 h of culture and approximately 30 h between 72–96 h of culture. Conversely, in the two other experimental conditions after a slight numerical increase within 72 h from seeding the cell number appeared to diminish with a similar trend, conceivably due to the lack of an appropriate amount of the essential nutriens and growth factors present in FCS (Fig. 1).

In light of the results obtained, we tested the effect of either 1, 10, or 100 pM mid-region PTHrP dissolved in either "routine" (10% FCS) or "starvation" (1% FCS) medium on HB2 cell proliferation. As shown in Figs. 2A and 2B, the supplement of 10 and 100 pM PTHrP to routine medium was able to increase cell number by 48 or 20%, respectively, compared to the control with a decrease of dT down to 15 h and 30 min between 72 and 96 h of culture with 10 pM PTHrP. The microscopic observation confirmed the cell count data, as in the presence of 10 pM mid-region PTHrP the cells appeared to form more expanded islets on the culture dish.

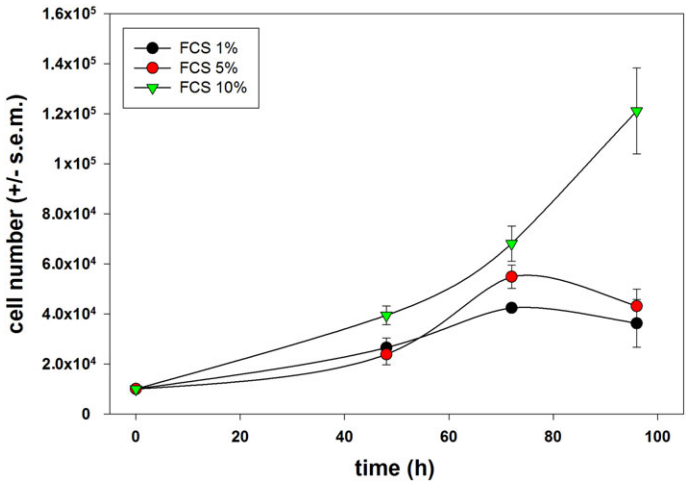

FIG 1 *Growth response of HB2 cells to FCS at different concentrations. Error bars correspond to SEM of three independent measurements.*

Noteworthy, as shown in Figs. 2Cand 2D, when HB2 cells where grown in FCS starvation condition the supplement of PTHrP [38–94]-amide solely at the concentration of 1 pM resulted in the abrogation of cell number decrease after 96 h of culture due to nutrient/growth factor depletion stress. Higher concentrations of the peptide were not able to exert such a positive control on cell culture. When submitted to the microscopic observation, the number of residual HB2 cells after 96 h of culture in control conditions and in the presence of either 10 or 100 pM PTHrP was small, and the cells appeared rounded and loosely-attached to the substrate, indicative of cell suffering and death. Conversely, HB2 cell appearance in the presence of 1 pM PTHrP was healthy, and they were able to form moderately-extended islets on the culture dish with well-spread, and thereby vital, individuals.

Thus, PTHrP [38–94]-amide appeared to have properties of a likely survival factor for mammary epithelial cells cultured on tissue plasticware.

3.2. PTHrP [38–94]-amide promotes survival and growth of HB2 cells cultured in collagen gels

In a second set of experiments, the effect of mid-region PTHrP on the survival/proliferative behaviour of HB2 cells was tested in the presence of a more physiological substrate for cell attachment than tissue plastics, such as three-dimensional type I collagen gel. As shown in Fig. 3A, after 21 days of culture in FCS-starvation medium the exposure to the peptide resulted in an increase of cell viability/growth of similar magnitude to that observed in the presence of HGF. The proliferation-promoting effect was even more pronounced when cells were coexposed to PTHrP and HGF for the same time lapse. When submitted to the microscopic analysis (Fig. 3B), cell increase in the presence of both PTHrP and PTHrP/HGF mixture compared to control was evident, although no development of branching colonies, as reported by Berdichevski et al. [17], could be observed in any experimental condition, likely due to nutrient/factor deprivation stress induced by culture in 1% FCS-supplemented medium.

3.3. PTHrP [38–94]-amide binds HB2 cell chromatin in denatured metaphase spreads

To explore whether the peptide proved effective in chromatin binding, parallel preparations of metaphase spreads from HB2 and MDA-MB231 cells, the latter as controls, where immunoprobed for mid-region PTHrP localization. Differently from what found with MDA-MB231 cell samples, no fluorescent spot could be visualized in HB2 metaphases after incubation with 1 μg of the peptide (Fig. 4, left); interphase nuclei, conversely, showed a genomic decoration (*e.g.*, arrow in Fig. 4, left). On the

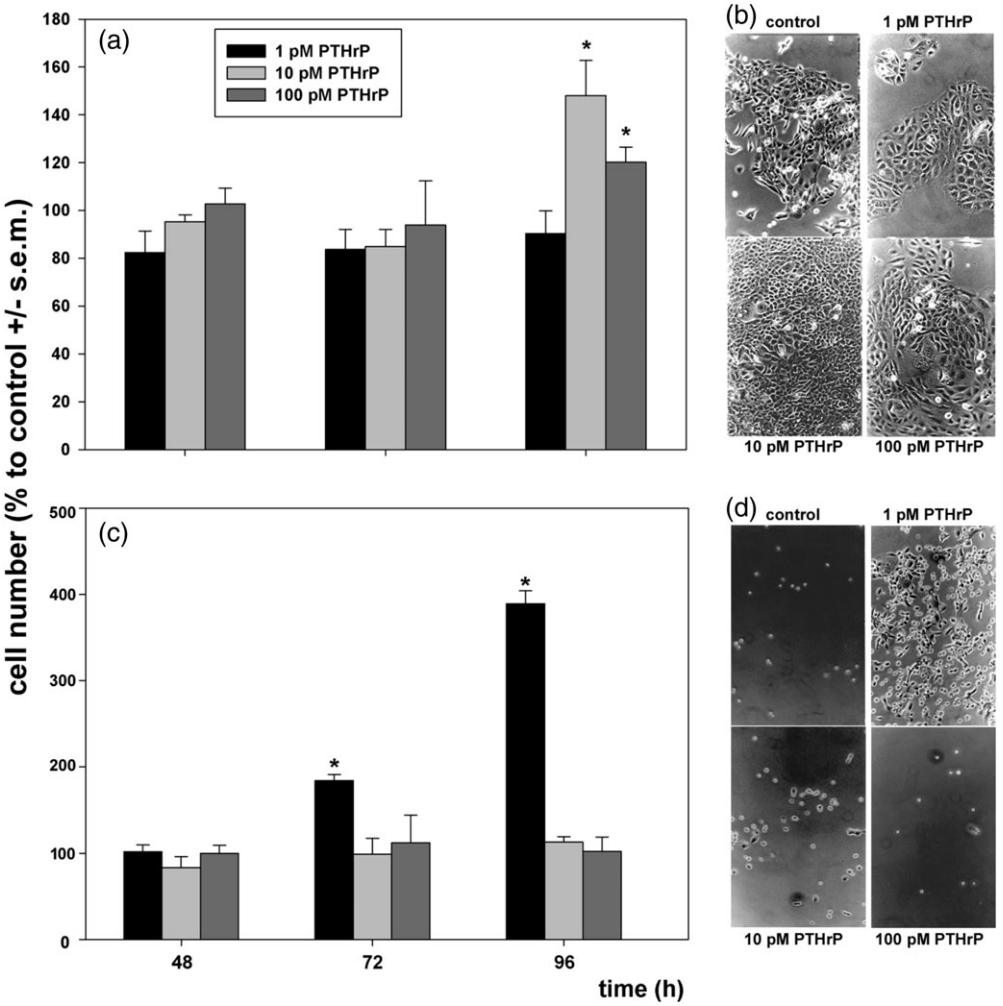

FIG 2

Histograms showing the percentage of growth of HB2 cells treated with mid-region PTHrP at different concentrations in either "routine" (a) or "starvation" medium (c) versus controls, and representative phase contrast micrographs of cells grown for 96 h in the same experimental conditions as above (b, d). The results are expressed as mean ± SEM of triplicate experiments. *P *< 0.05 with respect to control sample (ANOVA).*

other hand, decoration on metaphases appeared evident after increasing the in vitro amount of PTHrP up to 2 μg (Fig. 4, center), thus confirming the DNA/chromatin-binding ability of the peptide also in this in vitro system, although the amount of fluorescent signals was far below that observed in MDA-MB231 metaphase preparations (Fig. 4, right). No attempt was made to compare PTHrP immunolocalization on metaphases of HB2 cells grown within collagen gels, as no differences of accessibility of chromosomes induced by a growth environment are expected.

3.4. PTHrP [38–94]-amide influences differently the gene expression of HB2 cells grown on plastics and in collagen gels

In light of the obtained data demonstrating a positive effect of PTHrP [38–94]-amide on HB2 cell survival and proliferation in selected conditions and its ability to bind the chromosomal DNA, we examined whether the peptide, when supplemented at the concentrations able to sustain cell growth, might influence the expression level of some selected genes involved in the control of proliferation and differentiation of breast epithelial cells. To this purpose, we submitted cDNA preparations obtained from cells cultured in control conditions, in 10 pM PTHrP-containing 1%FCS/DMEM, or in 1 pM PTHrP-containing 10% FCS/DMEM to conventional and real time-PCR. As shown in the panel in Fig. 5A, the genes taken into consideration were expressed in all the experimental conditions tested. In particular, when gene expression levels was quantitated and compared via real time-PCR (Fig. 5B), CERBB2, ESX, and ITGA2 appeared to be upregulated about 13.5-folds, 4.6-folds, and 4.2-folds, respectively, in cells grown in 10 pM PTHrP-containing 1%FCS/DMEM, whereas only MUC1 showed an about 2.6-fold overexpression in cells cultured in 1 pM PTHrP-containing 10% FCS/DMEM. No difference was found for the expression levels of HOX7 and ITGB1 in all the experimental conditions tested.

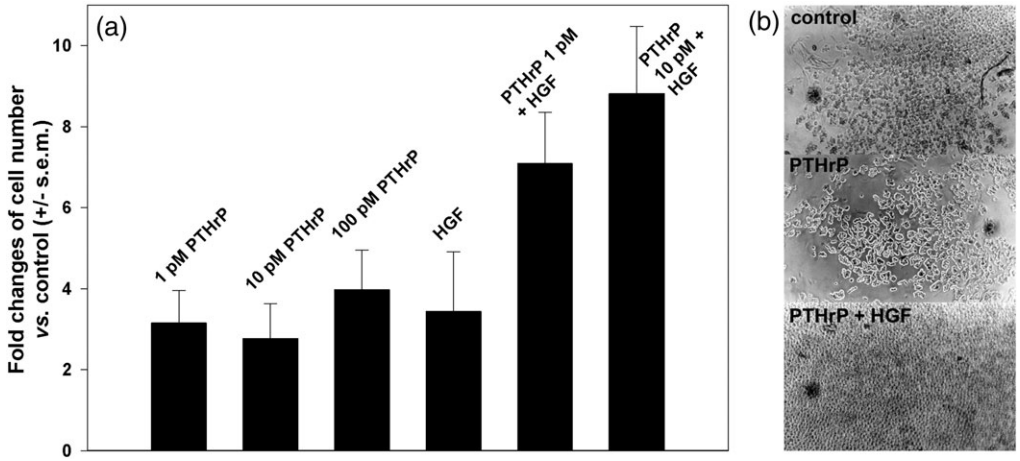

FIG 3 *(a) Histogram showing the average changes in cell number of HB2 cells cultured in collagen gels and exposed to mid-region PTHrP at different concentrations and/or HGF versus control. The results are expressed as mean ± SEM. of triplicate experiments. All the* P *values were < 0.05 (ANOVA). (b) Representative phase contrast micrographs of cells grown in control medium, in the presence of PTHrP at any concentration and in the copresence of PTHrP at any concentration and HGF. In particular, the images show cells cultured in the presence of 100 pM PTHrP and 10 pM PTHrP + HGF.*

In a second set of assays, cDNA preparations obtained from cells cultured in collagen gels in control conditions, in the presence of 100 pM mid-region PTHrP, or coexposed to 10 pM mid-region PTHrP and HGF were submitted to conventional and real-time PCR. Interestingly, not all genes examined appeared to be expressed in all the experimental conditions tested. As shown in the panel in Fig. 6A, no amplification signal could be observed for ITGA2 in the three samples, for HOX7 in the treated samples, and for ITGB1 in the control sample. The quantitative evaluation via real-time PCR (Fig. 6B) showed that MUC1 was the only upregulated gene in both treated samples (about 4.2-folds and 2.4-folds in the presence of 100 pM PTHrP and 10 pM PTHrP + HGF, respectively), whereas an about 5.5-fold overexpression of CERBB2 was found solely in the sample obtained after exposure to 100 pM PTHrP. No difference was observed for the expression levels of ESX in the three experimental conditions tested, and of ITGB1 in the two treated samples (the latter not shown).

4. Discussion

It is now widely demonstrated that PTHrP, initially discovered as the factor responsible of the humoral hypercalcemia of malignancy syndrome, exerts a variety of activities as local tissue factor involved in signal trasduction mechanisms controlling cell viability, growth, and differentiation. As an example, Miao et al. [31] demonstrated that PTHrP may trigger a protein kinase C-dependent activation of the Ras and MAPK signaling pathway, whereas Li et al. [32] provided experimental data of signal transducer and activator of transcription 5a (Stat5a)-mediated regulation of PTHrP expression. The present article reports the first evidence on the biological role played by mid-region PTHrP, the [38–94]-amide fragment, in an in vitro model of non-tumoral breast epithelial cells. Interestingly, the peptide appears to exert opposite effects to those observed when tested on different breast carcinoma cell lines [7]. In fact, when administered at lower concentrations (1 or 10 pM, as appropriate) than that active on

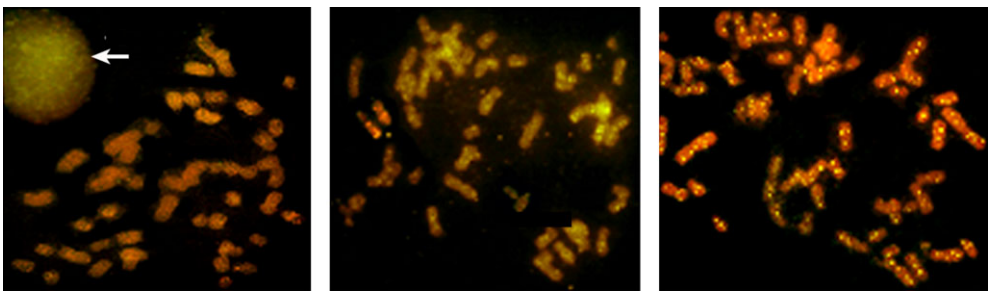

FIG 4 *Chromatin-binding property of mid-region PTHrP. Representative HB2 metaphase spread, immunoprobed with 1 μg of PTHrP [38–94]-amide, with no fluorescent spot in chromosomes (left); several signals are present in an interphase HB2 nucleus (left, arrow). Chromosomal decoration of a representative HB2 metaphase treated with 2 μg of the peptide (center). Chromosomal decoration of a representative MDA-MB231 metaphase treated with 1 μg of the peptide (right).*

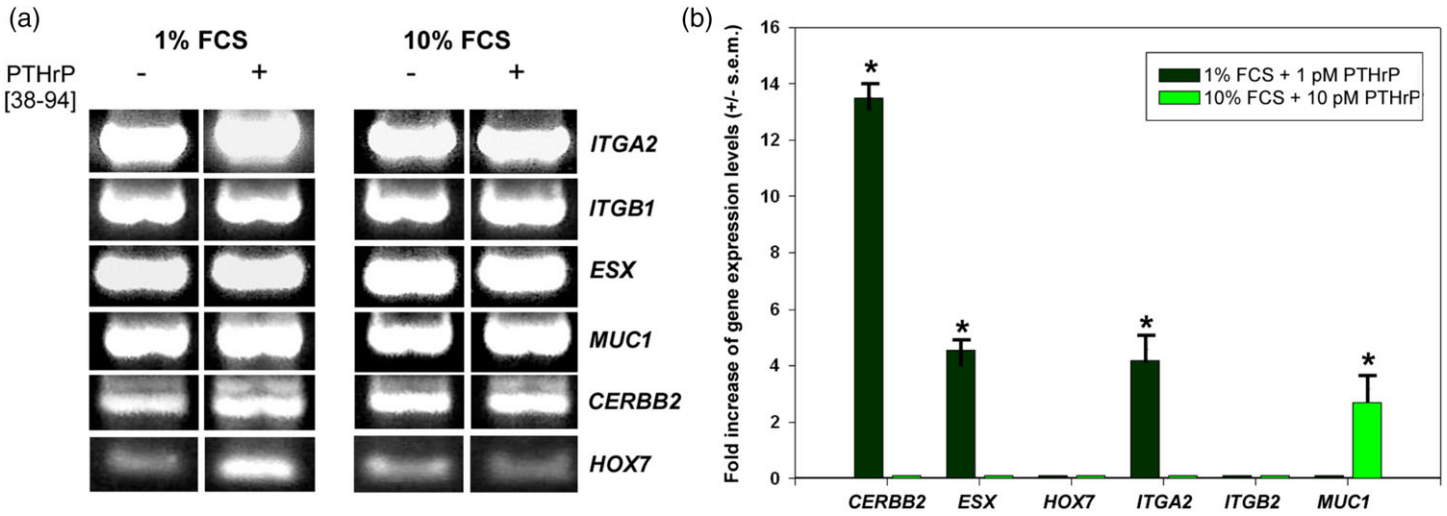

FIG 5 *(a) Conventional PCR analysis showing the presence of all the amplification products in cDNA preparations from HB2 cells cultured on tissue plastics in 10 pM PTHrP-containing 1%FCS/DMEM, or in 1 pM PTHrP-containing 10% FCS/DMEM. (b) Quantitative real-time PCR analysis of transcript levels of CERBB2, ESX, HOX7, ITGA2, ITGB1, and MUC1 in HB2 cells, treated as described above, versus controls. The results are expressed as mean ± SEM of triplicate experiments.* *P < 0.05 *(ANOVA).*

neoplastic cells (1 mM), mid-region PTHrP was found to stimulate cell proliferation in routine culture conditions, and, notably, to act as a cell survival-promoter in "starvation" culture that is, in nutrient stress conditions, inhibiting cell death and maintaining cell growth behaviour approximately constant. Also when cells were grown within collagen gels, PTHrP [38–94]-amide exerted an effect comparable to that of a powerful growth factor, that is, HGF, and, if administered in cotreatment with the latter, was able to enhance the positive effect on cell proliferation, also in this case in conditions of "stress" culture. Of note, HB2 cells were seeded in the presence of mid-region PTHrP and/or HGF also on another physiologic substrate, that is, the basement membrane extract Matrigel. In such experimental condition, cells did not survive when grown with 1%FCS/DMEM, whereas they survived and proliferate with 10%FCS/DMEM but no difference was found at every PTHrP concentration assayed and in cotreatment with HGF after 120 h of culture, the cell number being approximately twice that of control cells in all the

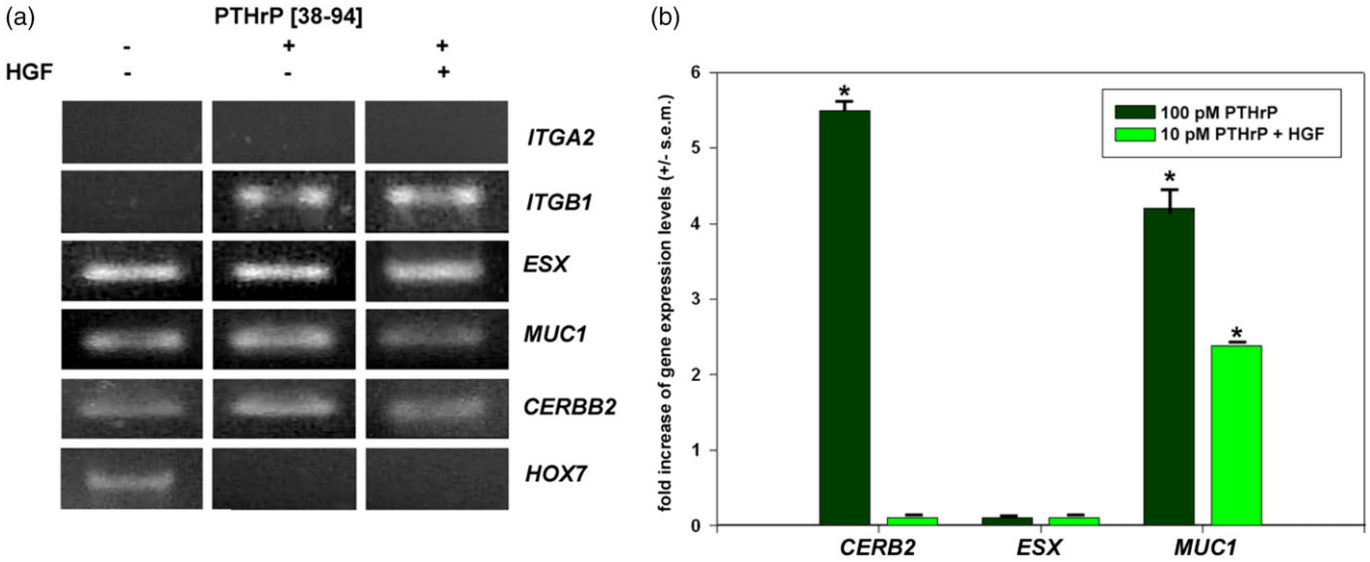

FIG 6 *(a) Conventional PCR analysis showing the amplification products in cDNA preparations from HB2 cells cultured in collagen gels either in plain control medium (1%FCS/DMEM), or supplemented with 100 pM PTHrP, or 10 pM PTHrP and 10 ng HGF/ml. (b) Quantitative real time-PCR analysis of transcript levels of CERBB2, ESX and MUC1 in HB2 cells, treated as described above, versus controls. The results are expressed as mean ± SEM of triplicate experiments.* *P < 0.05 *(ANOVA).*

conditions tested (Luparello, unpublished data). This suggests that the composition of the extracellular matrix may significantly modify cell response to peptide administration.

The obtained results showing an active role of PTHrP [38–94]-amide on the control of HB2 cell viability and proliferation prompted us to explore whether the peptide proved effective in chromatin binding, as already observed for MDA-MB231 breast cancer cells [12], and, in positive case, which pattern of "chromosomal decoration" would come out. The data obtained show that HB2 chromatin has several genome-wide redundant binding sites for the peptide, similarly but not identically to MDA-MB231 cells. In fact, by immunoprobing with 1 μg of the peptide, HB2 chromosomes didn't show any green/yellow signal; interestingly, the same amount of peptide is able to display several fluorescent grains in the interphase nuclei of the same cell line suggesting that the more relaxed interphase chromatin is able to receive even low amount of the peptide as immunoprobe. The confirmation of this hypothesis is that all those HB2 metaphases immunoprobed with 2 μg of the peptide show appreciable fluorescent grains similarly to those seen in MDA-MB231 metaphases treated with 1 μg of PTHrP [38–94]-amide. An explanation of these differences could be due to dissimilar epigenetic state between the two genomes. In fact, it is known that the crosstalk between DNA methlyation and histone modifications facilitates chromatin condensation [33] and consequently it is conceivable that the metaphase chromatin of non-tumoral HB2 cells is more methylated with respect to that of the tumoral MDA-MB231 cell line. Thus, the different accessibility of the two chromatins to the peptide balances only between relaxed interphase HB2 chromatin and hypomethylated/relaxed MDA-MB231 chromosome chromatin or in case of technical doubling amount of ligand in compacted HB2 chromosome chromatin.

We also produced some novel data on the effect of PTHrP [38–94]-amide and culture conditions on gene expression of HB2 cells, the most interesting being those obtained at low FCS concentration considering that in "routine" medium supplemented with 10% FCS the contribution of mid-region PTHrP to transcriptional regulation can be masked. The molecular signatures selected for the assay were associated to growth and differentiation of breast epithelial cells, and in particular they were: (a) ITGA2 and ITGB1 coding for two integrin subunits whose expression is related to the proliferative activity of HB2 cells [17]; (b) CERBB2 coding for an epidermal growth factor receptor a.k.a. HER2/neu involved in ductal outgrowth, cell differentiation, and milk protein production [34]; (c) ESX whose protein product is a member of the ETS family of transcription factors which was found necessary for survival and growth of nontrasfomed breast epithelial cells [35]; (d) MUC1 coding for a glycosylated transmembrane protein whose expression is a marker of lactation [36]; and, (e) HOX7 whose protein product is the MSX-1 transcription factor which is essential for the morphological development of the mammary gland [37].

The results obtained indicate that the only signature whose upregulation is associated with cell exposure to mid-region PTHrP in "routine" medium is the functional marker of luminal differentiation MUC1 [38], which was upregulated also in the presence of 100 pM PTHrP added to "starvation" medium and in collagen gel cultures with either PTHrP or PTHrP/HGF supplements, thus suggesting a rather generalized pro-differentiation role played by the peptide administered at the higher concentrations.

On the other hand, the cell proliferative behaviour observed in the presence of the lower (1 pM) concentration of PTHrP in "starvation" medium may be conceivably linked to the enhancement of CERBB2 and ESX transcriptional levels. It is known, in fact, that ESX is able to transactivate the CERBB2 promoter by binding at −26 position [35], thereby upregulating its expression. HB2 cells have low constitutive expression of CERBB2 [39] and therefore the increased exposure of the overexpressed receptor, activated upon homo- or heterodimerization with another member of the same family, may ultimately lead to a powerful stimulation of anti-apoptotic and growth pathways [40]. Of note, an upregulation of CERBB2 although to lesser extent, was found also in HB2 cells cultured within type I collagen gels in the presence of 100 pM mid-region PTHrP but this was not coupled to ESX overexpression. It is known that the stiffness of the microenvironment, such as that of collagenous matrices, triggers CERBB2 upregulation in MCF10 non-tumoral breast cells [41], and therefore the CERBB2-addressed signaling induced by PTHrP in HB2 cells cultured within collagen gels may include mechanotransduction pathways different from that ESX-mediated [42], such as those activated by integrin receptors.

It is generally acknowledged that the expression of integrins conditions the mitogenic and locomotory behaviour of HB2 cells [43]. On the other hand, Suzuki et al. [44] reported the decreased expression level of integrin subunit α2 in luminal cells of pregnant breast tissues undergoing differentiation and decreasing their growth rate. Therefore, it is conceivable that the upregulation of ITGA2 here observed contributes to the advance of the survival and proliferation program elicited by low PTHrP concentration in "starvation" medium. The α2β1 integrin receptor is the major collagen-binding complex on cell surfaces. When HB2 cells were grown within collagen gels a down-regulation of ITGA2 in all the conditions tested, being under the detection limit of PCR, and an upregulation of ITGB1 in the presence of HGF and/or PTHrP supplements were found. Literature data indicate that β1-containing integrins are involved in alveologenesis and lactogenesis in vivo and in vitro [45]. By consequence, the obtained data are in line with the hypothesis that in the latter experimental conditions HB2 cells undergo a proliferation program addressed to the formation of mammary acini endowed with differentiated secretory function.

The results obtained on HOX7 expression levels corroborate the previous statements. It is known that during early pregnancy its protein product, the transcription factor MSX-1, promote proliferation and prevent the secretory differentiation of lobular cells until the onset of lactation, and that pregnant transgenic mice overexpressing HOX7 in their mammary gland show inhibition of terminal epithelial differentiation linked to

over-accumulation of cyclin D1 [46,47]. Therefore, the drastic downregulation of HOX7 expression in HB2 cells seeded within collagen gels and exposed to HGF and/or PTHrP supplements (being under the detection limit of PCR) may be interpreted as an additional marker of lactogenic differentiation. Interestingly, some of us have previously demonstrated HOX7 downregulation as a molecular signature associated with the cytotoxic effect of mid-region PTHrP on MDA-MB231 breast carcinoma cells [13]. These collective findings suggest that HOX7 gene is a preferential target for PTHrP [38–94]-mediated transcriptional reprogramming in normal and neoplastic breast cells.

Although it is acknowledged that PTHrP [38–94] contains a NLS which may account for its ability to cross the nuclear pores and enter the nucleoplasm where it can bind to chromatin, less is known about the surface receptor responsible of the internalization of exogenous non-N-terminal PTHrP fragments. Aarts et al. [48] suggested that also cell surface adhesion and endocytosis of PTHrP may be mediated by an aminoacid motif embedded in the NLS. Grzesiak et al. [49] reported the binding of distinct PTHrP peptides to hsp70 chaperone protein exposed on the plasma membrane of different cancer cell lines. On the basis of their experimental data, Kumari et al. [50] have postulated the existence of a midregion receptor, unable to activate ERK1/2, which couple to calcium signaling without mediation of phospholipase C and production of inositol phosphate. Nevertheless, despite numerous efforts to isolate and characterize the extracellular midregion PTHrP receptor, the data available in the literature are still limited and fragmentary.

In conclusion, the data reported represent a starting point for a more detailed biochemical and molecular analysis of the intracellular signalization that confers to PTHrP [38–94]-amide a role of survival and differentiation-promoting factor toward non-tumoral breast epithelial cells, and also provide first evidence supporting the concept that the physiological mechanisms involving PTHrP during mammary gland development may include mature processed forms of the protein different from the N-terminal fragment as well as the whole molecule.

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Conflict of interest

The authors declare no conflict of interest.

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