# Parathyroid Hormone-Related Protein Is a Mitogenic and a Survival Factor of Mesangial Cells from Male Mice: Role of *Intracrine* and *Paracrine* Pathways

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Glomerulonephritis is characterized by the proliferation and apoptosis of mesangial cells (MC). The parathyroid-hormone related protein (PTHrP) is a locally active cytokine that affects these phenomena in many cell types, through either paracrine or intracrine pathways. The aim of this study was to evaluate the effect of both PTHrP pathways on MC proliferation and apoptosis. In vitro studies were based on MC from male transgenic mice allowing PTHrP-gene excision by a CreLoxP system. MC were also transfected with different PTHrP constructs: wild type PTHrP, PTHrP devoid of its signal peptide, or of its nuclear localization sequence. The results showed that PTHrP deletion in MC reduced their proliferation even in the presence of serum and increased their apoptosis when serum-deprived. PTH1R activation by PTHrP(1–36) or PTH(1–34) had no effect on proliferation but improved MC survival. Transfection of MC with PTHrP devoid of its signal peptide significantly increased their proliferation and minimally reduced their apoptosis. Overexpression of PTHrP devoid of its nuclear localization sequence protected cells from apoptosis without changing their proliferation. Wild type PTHrP transfection conferred both mitogenic and survival effects, which seem independent of midregion and C-terminal PTHrP fragments. PTHrP-induced MC proliferation was associated with $p27^{Kip1}$ down-regulation and c-Myc/E2F1 up-regulation. PTHrP increased MC survival through the activation of cAMP/protein kinase A and PI3-K/Akt pathways. These results reveal that PTHrP is a cytokine of multiple roles in MC, acting as a mitogenic factor only through an intracrine pathway, and reducing apoptosis mainly through the paracrine pathway. Thus, PTHrP appears as a probable actor in MC injuries. **(*Endocrinology* 154: 853–864, 2013)**

Mesangial cells (MC) play a key role in both physiological and pathological functioning in the glomerulus. In normal conditions there is little mesangial turnover. However, aberrant proliferation of MC occurs in immune-mediated glomerular diseases, including IgA nephropathy and lupus nephritis (1). MC apoptosis is another characteristic of human and experimental glomerulonephritis (2, 3). Experimental models of glomerulonephritis show initial mesangiolysis linked to excessive MC apoptosis (4). Later during glomerulonephritis, MC apoptosis tends to balance the excessive proliferation of MC. This ends in either homeostatic cicatrization or progression into glomerulosclerosis and loss of kidney function (5, 6).

The parathyroid hormone-related protein (PTHrP), originally identified as the factor responsible for malig-

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Abbreviations: αSMA, alpha smooth muscle actin; BrdU, bromodeoxyuridine; ΔNLS, devoid of its nuclear localization signal; ΔSP, devoid of its signal peptide; FBS, fetal bovine serum; 4-OH-Tam, 4-hydroxy-tamoxifen; MC, mesangial cells; PDGF-BB, platelet-derived growth factor-BB; PI3-K, phosphoinositide-3-kinase; PKA, protein kinase A; PKC, protein kinase C; PTHrP, parathyroid hormone-related protein; PTH1R, PTH/PTHrP receptor; TUNEL, terminal deoxynucleotidyltransferase dUTP nick end labeling; VSMC, vascular smooth muscle cells; WT, wild type.

nant hypercalcemia (7), was later found to be widely expressed in fetal and adult tissues, where it acts locally, rather than in an endocrine manner (8). PTHrP has a constitutive expression but may also be up-regulated by inflammatory cytokines (9, 10). Full-length PTHrP undergoes posttranslational processing, which gives rise to a family of mature secretory peptides (11). Peptides with the N-terminal PTH-like region, like PTHrP(1–36), bind to and activate the PTH/PTHrP receptor (PTH1R), whereas midregion and C-terminal peptides are functionally active through yet uncharacterized receptors (11). Cytosolic PTHrP can also use a bipartite multibasic nuclear localization signal to translocate to the nucleus and act through an intracrine pathway (12). PTHrP effects on proliferation have been described in many cell types. These effects are largely pathway- and cell-dependent. In vascular smooth muscle cells (VSMC), PTHrP has been shown to induce proliferation through the intracrine pathway, whereas it is antiproliferative through the paracrine one (12, 13). In renal epithelial and pancreatic $\beta$ cells, mitogenic effects of PTHrP have been reported through the paracrine pathway (14, 15). PTHrP has also been shown to regulate cell apoptosis. PTHrP is most often a survival factor, either through intracrine or paracrine pathways, as shown in chondrocytes and renal carcinoma cells, respectively (16, 17). Opposite effects of the two pathways were also observed in prostate cancer cells, where intracrine PTHrP increases and paracrine PTHrP decreases survival (18).

In different animal models of renal disease, overexpression of PTHrP has been reported in the glomerulus, suggesting a role of PTHrP in the initiation or evolution of the pathology (19–23). In vivo, PTHrP seems not to be expressed in MC under physiological conditions, but immunoreactive PTHrP was present in MC after protein overload in the rat and in diabetic nephropathy in the mouse and patients (19, 23). A role for PTHrP has been suggested in the pathogenesis of inflammatory or autoimmune diseases (24).

The effects of PTHrP on MC proliferation have been analyzed in few studies. An increase in [$^3$H]-thymidine incorporation was reported when exogenous PTHrP(1–36) was added to the culture media of rat or human MC (20, 25). However, the effect of intracrine PTHrP on MC proliferation has not yet been considered, and whether PTHrP has any effect on MC apoptosis remains unknown.

The aim of this work was to elucidate the effects of paracrine and intracrine PTHrP on MC proliferation and apoptosis. For this purpose, MC in primary culture were obtained from C57BL/6 mice kidneys ($MC^{Bl6}$) and from transgenic mice carrying a CreLoxP system for PTHrP ($MC^{CreloxP}$) (26, 27). Transgenic mice allow specific knockdown of PTHrP by 4-hydroxy-tamoxifen (4-OH-Tam) treatment in cells expressing alpha smooth muscle actin ($\alpha$SMA), which is the case in MC in culture. Stable transfection of MC was also performed with different constructs of human PTHrP: PTHrP devoid of its nuclear localization signal ($\Delta$NLS), so only active through paracrine pathway, PTHrP devoid of its signal peptide ($\Delta$SP) with only intracrine action, and wild type (WT) PTHrP (please see Supplemental Figure 1 published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Altogether, our results show that PTHrP induces MC proliferation through the intracrine pathway, while it reduces apoptosis through both paracrine and intracrine pathways.

## Materials and Methods

### Mouse strains

Homozygous PTHrP-floxed ($PTHrP^{L2/L2}$) mice were crossmated with $\alpha$SMA-Cre-$ER^{T2(tg/0)}$ mice (26, 27). Male $\alpha$SMA-Cre-$ER^{T2}$/$PTHrP^{L2/L2}$ ($PTHrP^{CreloxP}$) mice as well as male C57BL/6 mice (Janvier, Le Genest-Saint-Isle, France) were used to prepare MC in primary culture. Mice breeding, maintenance, and experiments were in compliance with guidelines of the European Community and the French government concerning the use of animals.

### MC culture

MC in primary culture were derived from glomeruli isolated from the $PTHrP^{CreloxP}$ mice ($MC^{CreloxP}$) and C57BL/6 mice ($MC^{Bl6}$). Briefly, kidneys obtained from 6-wk-old mice were subjected to sequential sieving (125, 71, and 53 $\mu$m) and sedimentation for extraction of glomeruli, which were set in culture in DMEM (Life Technologies, Saint Aubin, France) enriched with 30% fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin, Life Technologies). MC outgrew from the glomeruli in about 1 wk. Cells were further grown for 1 wk in DMEM with high D-valine content (2.5 mg/ml, Sigma-Aldrich, Saint Quentin Fallavier, France) for the elimination of fibroblasts. MC were then maintained in DMEM supplemented with 20% FBS and 1% antibiotics at 37 °C in a 10% $CO_2$ atmosphere. Serum-free media was supplemented with 0.1% BSA. MC were used between passages 4 and 16.

### Fluorescent immunocytochemistry

Cells growing in glass chamber slides (Lab-Tek II, Nalge Nunc, Rochester, New York) were used at 60% confluence. Cells were fixed in 4% paraformaldehyde in PBS for 15 min, washed with PBS and permeabilized with 0.1% Triton X-100 in 1 mM glycine-PBS for 10 min. After a 40-min exposure to blocking buffer (3% BSA in PBS), cells were incubated for 1 hour at room temperature with the specific primary antibodies diluted in blocking buffer, and then for 1 hour in the dark with the appropriate Alexa-conjugated IgG secondary antibodies (Life Technologies). Nuclei were stained by bisbenzimide H 33342 (HOE 33342, Sigma-Aldrich). After final washes, cells were mounted

with Moviol and visualized by fluorescence-microscopy (Eclipse E800, Nikon, Tokyo, Japan) using NIS-elements program (Nikon).

The characterization of MC was performed by positive staining to anti-Thy1.2 and anti-αSMA antibodies (Sigma-Aldrich), and negative staining for a marker of epithelial cells, antipan-cytokeratin (Abcam, Paris, France). HK-2 cells, a renal epithelial cell line (ATCC CRL-2190, LGC France, Illkirch, France), Mes-13, a MC line (ATCC CRL-1927), and murine VSMC in primary culture were used as positive/negative controls (Supplemental Figure 2). HK-2 and Mes-13 cells were grown in DMEM supplemented with 10% FBS.

PTHrP deletion in MC and transfection with the various human PTHrP-HA constructs were verified by immunocytochemistry, as detailed in the next two paragraphs. The activation of the phosphoinositide-3-kinase (PI3-K)/Akt pathway by PTHrP was visualized with antiphospho(S473)-Akt (Cell Signaling, Ozyme, Saint Quentin-en-Yvelines, France). Finally, the presence and distribution of $Na^+/H^+$ exchanger regulatory factors 1 and 2 (NHERF1 and NHERF2), as well as their colocalization with the PTH1R, were looked for with specific antibodies (NHERF1: Abcam; NHERF2 and PTH1R: Santa Cruz, TebuBio, Le Perray-en-Yvelines, France).

## PTHrP gene excision

$MC^{CreloxP}$ were seeded in 25-$cm^2$ flasks at low confluence (20%–30%) and treated for three alternative days with 4-OH-Tam (5 μM, Sigma-Aldrich). Control MC were treated with the solvent (1% ethanol final concentration). To verify PTHrP gene excision, DNA was extracted by the phenol/chloroform method, and recombination of PTHrP floxed alleles was verified by PCR using the strategy shown in Figure 1; primers are shown in Table 1. The presence of amplicons was verified by electrophoresis in 1.5% agarose gel. PTHrP deletion was also verified by RT-PCR (primers on Table 1) and immunofluorescence staining using monoclonal anti-PTHrP (34–53) antibody (PTH2E11, IGBMC, Strasbourg, France). The effect on PTH1R expression was verified by Western blot. Control and PTHrP-excised MC were used within four passages after excision.

## MC transfection

$MC^{CreloxP}$ were seeded in 6-well plaques, grown for 24 hours, and transfected with plasmids (2 μg) encoding for one of three HA-tagged human PTHrP(1–139) constructs (Supplemental Figure 1): PTHrP-ΔNLS, PTHrP-ΔSP, or the PTHrP-WT, as reported previously (12, 28). MC transfected with the empty plasmid vector (pcDNA3) were taken as control. Transfection was performed with Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. Stably transfected cells were selected by geneticin treatment (G418, 300 μg/ml, Life Technologies). Transfection efficacy was verified by immunostaining with an anti-HA antibody (Merck Millipore, Saint Quentin-en-Yvelines) and RT-PCR for the expression of the human PTHrP isoforms. The expression of PTH1R was verified by RT-PCR and Western blot. PTHrP-transfected MC were used within four passages after transfection.

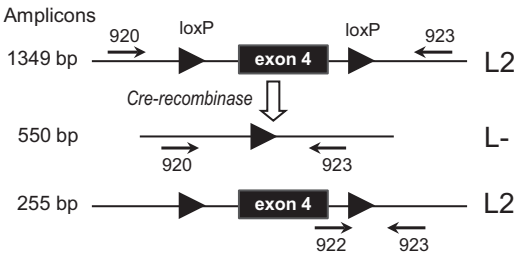


**Figure 1.** Genomic structure of the floxed allele of PTHrP (L2) and the deleted allele (L-) after 4-OH-Tam treatment. PCR strategy using the primer pairs 920–923 and 922–923 to analyze Cre-mediated excision of exon 4.

## Real time RT-PCR

Total RNA was extracted from MC using the RNeasy kit (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol. Five micrograms total RNA were reverse transcribed in a reaction buffer (Life Technologies) with nonspecific primer p(dT)15 (Roche Diagnostics, Meylan, France), at 37°C for 1 hour. Quantitative real-time PCR reaction was performed with the LightCycler-FastStart DNA Master SYBR Green kit (Roche Diagnostics). The sense and antisense primers are given in Table 1. Each sample was analyzed three times and quantified with the analysis software for LightCycler (Roche Diagnostics).

## Fluorescence-Activated cell sorting (FACS) flow cytometry

For the evaluation of proliferating cells, MC were seeded in 25-$cm^2$ flasks with DMEM + 20% FBS. At 60%–70% of confluence, cells were serum-deprived for 24 hours to render them quiescent. Medium was then changed for DMEM + 20% FBS or DMEM + 0.1% BSA. Some groups were treated with PTHrP(1–36) (100 nM daily treatment; NeoMPS, Illkirch, France) or with platelet-derived growth factor-BB (PDGF-BB, 10 ng/ml; Sigma-Aldrich). Studies were also performed on control and PTHrP-excised $MC^{CreloxP}$ and on the MC stably transfected by the PTHrP constructs. Cells proliferation was analyzed over 3 days by FACS. Briefly, cells were harvested, washed with PBS, fixed, and permeabilized by 70% ethanol for 1 hour at 4°C. Cells were then washed again with PBS, treated with RNAse (50 μg/ml) for 1 hour at 37°C, stained with propidium iodide (1 mg/ml, Sigma-Aldrich) for 10 min, and filtered through a 60-μm nylon mesh (Merck Millipore) before analysis using FACSort flow cytometer (BD Biosciences, Pont de Claix, France). The fraction of proliferating cells (cells in phase S+G2/M) was determined using FCS Express software (DeNovo Software, Los Angeles, California). All experiments were performed at least three times in triplicate.

For the evaluation of apoptotic cells, MC were seeded in 25-$cm^2$ flasks and rendered quiescent, by a 24-hour serum deprivation. Medium was then changed for DMEM + 20% FBS or DMEM + 0.1% BSA to induce apoptosis for 1 or 2 days. Here again, PTHrP-excised and PTHrP-transfected MC were used, and some groups were treated with exogenous PTHrP(1–36) (100 nM). FACS analysis of apoptotic cells was performed on both floating and adherent cells, as described previously (29). Briefly, cells were harvested, stained with Annexin V-FITC (Roche Diagnostics) and propidium iodide (5 μg/ml; Sigma-Aldrich) in a dark chamber at 4°C for 10 min and fixed in 1% formol before analysis using FACSort flow cytometer (BD Biosciences). The fraction of viable and apoptotic cells was determined using FCS Express software (DeNovo Software). All experiments were performed at least three times in triplicate.

**Table 1.** Nucleotide Sequences of the Primers Used for PCR or Real-time RT-PCR

| Mouse gene | Primer Sequence 5′ to 3′ | |
|---|---|---|
| | Forward | Reverse |
| *Semi-quantitative PCR* | | |
| PTHrP floxed | 920: TTTGGAGGG TGC TCA CTTAGAGCAA<br>922: GTCTTCCTCACAGCCAAGACTGACT | 923: CAGCAAACCAT GGTGAGGCTCATCA |
| myogenin | TTACGTCCATCGTGGACAGC | TGGGCTGGGTGTTAGCCTTA |
| *Real-time RT-PCR* | | |
| mouse PTHrP | CAGCCGAAATCAGAGCTACC | CTCCTGTTCTCTGCGTTTCC |
| human PTHrP | CAAGATTTACGGCGACGATT | GAGAGGGCTTGGAGTTAGGG |
| PTH1R | GGGCACAAGAAGTGGATCAT | GGCCATGAAGACGGTGTAGT |
| 18S | CATGGCCGTTCTTAGTTGGT | CGCTGAGCCAGTCAGTGTAG |

## Bromodeoxyuridine (BrdU) incorporation

MC were seeded in 96-well plate (20 000 cells/ml), grown for 24 hours before being rendered quiescent in serum-deprived medium for the next 24 hours. The medium was then changed for DMEM + 20% FBS or DMEM + 0.1% BSA for the following days (1–3 days). Some groups were treated with PTHrP(1–36) (1, 10, 100 nM daily treatment), PTH(1–34) (10 nM; NeoMPS), PDGF-BB (10 ng/ml), or the corresponding vehicle. In other studies, BrdU incorporation was analyzed in PTHrP-excised $MC^{CreloxP}$, in cells stably transfected with the PTHrP constructs (sometimes in the presence of the PTH1R selective antagonist ($Asn^{10}$, $Leu^{11}$, D-$Trp^{12}$)-PTHrP(7–34)amide, 1 $\mu$M; Bachem, Weil-am-Rhein, Germany), and finally in PTHrP-excised $MC^{CreloxP}$ exposed for 24 hours to conditioned medium obtained from pcDNA3, PTHrP-ΔSP, or PTHrP-WT-transfected MC. BrdU incorporation studies were performed according to the protocol of the manufacturer (Calbiochem, Merck KGaA, Darmstadt, Germany).

## Terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL)

For the evaluation of apoptotic cells by TUNEL, MC were seeded on glass slides (Lab-Tek II), maintained 24 hours in DMEM + 20% FBS and synchronized for 24 hours in DMEM + 0.1% BSA. Apoptosis was induced by serum deprivation, changing the medium for DMEM + 0.1% BSA with or without treatments: PTHrP(1–36) (1, 10, 100 nM), PTH(1–34) (1 and 10 nM), PTHrP(107–111) (100 nM; a gift from Dr P. Esbrit), ($Asn^{10}$, $Leu^{11}$, D-$Trp^{12}$)-PTHrP(7–34)amide (1 $\mu$M; Bachem), forskolin (1 $\mu$M; Sigma-Aldrich), protein kinase A (PKA) inhibitor (H89, 1 $\mu$M; Sigma-Aldrich), PI3-K inhibitor (LY294002, 20 $\mu$M; Sigma-Aldrich), or protein kinase C (PKC) inhibitor (Gö6983, 5 $\mu$M; Sigma-Aldrich), alone or in combination as indicated in the appropriate Figures. Treatments with the inhibitors began 1 hour before the addition of PTHrP(1–36). Apoptosis was also determined in PTHrP-excised $MC^{CreloxP}$ and in cells stably transfected with the PTHrP constructs, sometimes in the presence of the PTH1R selective antagonist. Apoptotic cells were detected by the commercial In Situ Cell Death Detection kit (Roche Diagnostics) following the manufacturer's specifications. Nuclei were stained by HOE 33342. Cells were subsequently analyzed by fluorescence-microscopy (Nikon Eclipse E800) using NIS-elements program. At least 10 successive images were taken in each condition. The percentage of apoptotic cells was calculated with the help of Image J program (National Institutes of Health, Bethesda, Maryland).

## Direct cyclic AMP measurements

MC were seeded in 25-$cm^2$ flasks. After 24 hours of growth, they were made quiescent by serum-deprivation for 24 hours. Cells were pretreated for 15 min by 3-isobutyl-1-methyl-xanthine (200 $\mu$M; Sigma-Aldrich) and sometimes by the PTH1R antagonist, ($Asn^{10}$, $Leu^{11}$, D-$Trp^{12}$)-PTHrP(7–34)amide (100 nM). Cells were then exposed for 15 min to PTHrP(1–36) (1–300 nM), PTH(1–34) (100 nM), forskolin (10 $\mu$M), or isoproterenol (100 nM; Sigma-Aldrich). The reaction was stopped by aspiration of the incubation medium, followed by the addition of 0.5 ml of 0.1 M HCl supplemented with 1% Triton-X100 for cell lysis during 20 min at 37°C. The lysed cells were scrapped and samples were centrifuged at 1,300*g* for 10 min at 4°C. Cyclic AMP concentrations were determined in the supernatants and in the incubation media, using a direct enzyme immunoassay kit (Assay Designs, Euromedex, Strasbourg, France). Protein concentration was determined in each sample by the Lowry method (30). Total cAMP levels were expressed as picomoles per milligram protein.

## Western blot

MC proteins were extracted at 4 °C with the lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40) supplemented with 0.5% proteases and phosphatases inhibitors (Sigma-Aldrich). The supernatants were collected after centrifugation at 12 000*g* at 4°C for 5 min. Protein concentration was determined by the Lowry method (30). Protein expression was analyzed by Western blot using the semidry iBlot (Life Technologies). The primary antibodies used were: anti-HA (1/6000, Roche Diagnostics), anti-Akt (1/200, Santa Cruz), antiphospho(Thr308)-Akt (1/200, Santa Cruz), antiphospho(S473)-Akt (1/500, Cell Signaling), anti-$p27^{Kip1}$ (1/500, Epitomics, CliniSciences, Nanterre, France), anti-c-Myc (1/5000, Sigma Aldrich), anti-E2F1 (1/200, Santa Cruz), anti-PTH1R (1/200, Santa Cruz), and antiglyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (1/1000, Merck Millipore). The appropriate horseradish peroxidase-conjugated secondary antibody was used. Chemiluminescence was detected (Immobilon Western kit, Merck Millipore) and visualized (Fusion Fx7, ViberLourmat, Marne-la-Vallée, France).

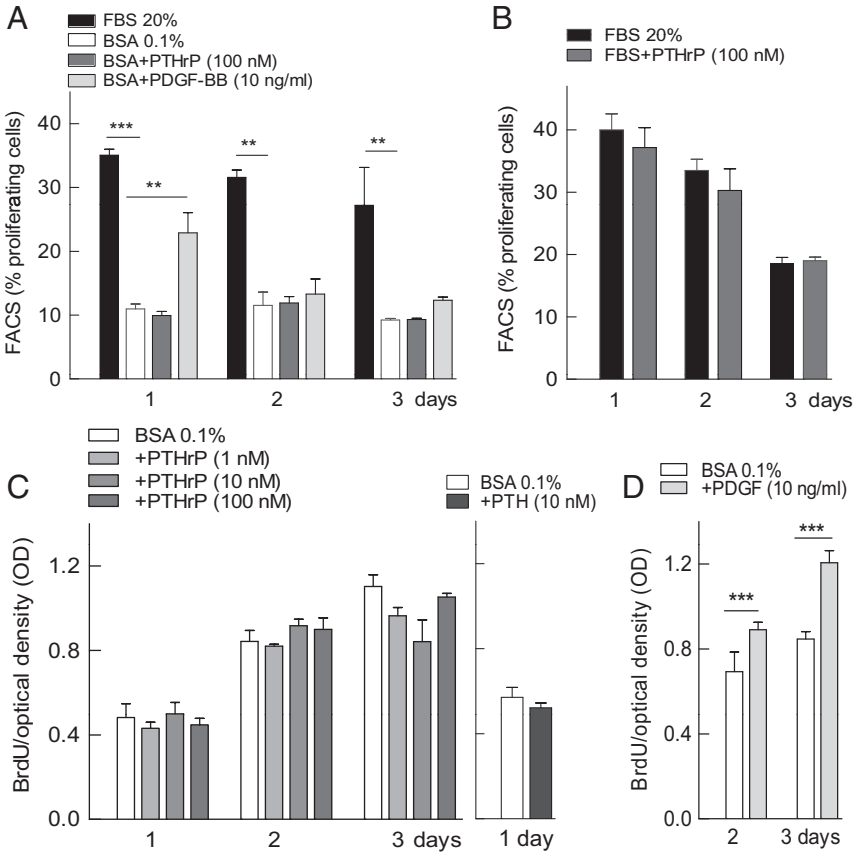


**Figure 2.** Effect of exogenous PTHrP(1–36) and PTH(1–34) on MC proliferation. The effect on MC proliferation has been studied in murine $MC^{Bl6}$ prepared from C57BL/6 mice kidneys. A, B, Proliferation of MC was analyzed by FACS on 3 days, in the presence (FBS 20%) or absence of serum (BSA 0.1%), with or without PTHrP(1–36) or PDGF-BB (n = 4–7 per group). Proliferation of MC was also assayed by BrdU incorporation in serum-deprived media after exposure to incrementing PTHrP(1–36) concentrations (1, 10, and 100 nM; n = 8) or 10 nM PTH(1–34) (n = 5) (C), or PDGF-BB (10 ng/ml) (n = 4) (D). Exogenous PTHrP(1–36) or PTH(1–34) were devoid of any effect on MC proliferation. Results are shown as means ± SEM. Tukey test, ** $P < .01$ and *** $P < .001$.

### Statistical analysis

Results are expressed as means ± SEM. Statistical analysis was performed when appropriate using Student *t* test, one-way or two-way ANOVA followed by Tukey multiple comparison test. Statistics were run with SigmaStat (SPSS Inc, Chicago, IL). Differences with $P < .05$ were considered statistically significant.

## Results

### Exogenous PTHrP(1–36) has no effect on MC proliferation

The effect of PTHrP(1–36) has been first studied on $MC^{Bl6}$. Quiescent cells were exposed to DMEM + 20% FBS or DMEM + 0.1% BSA at day 0, before FACS analysis over 3 days (Figure 2A). In the presence of serum, more than 30% of MC were proliferating, a value that decreased to 10% in serum-deprived cells. In the later cells, daily PTHrP treatment (100 nM) did not modify the proliferation level. However, PDGF-BB, a known mitogen for MC (10 ng/ml, added only on day 0), increased MC proliferation at day 1 before its effects faded later. To see if PTHrP(1–36) may conversely reduce MC proliferation, PTHrP(1–36) was added daily to MC grown in serum-supplemented media. Here again, no change in cell proliferation was observed (Figure 2B).

MC proliferation was also assessed by BrdU incorporation in serum-deprived media in the presence of incrementing PTHrP(1–36) concentrations (1–100 nM) (Figure 2C) or with PDGF-BB (Figure 2D). Here again, PTHrP showed no effect on cell proliferation over the 3-day experiment, whereas PDGF-BB increased BrdU incorporation ($P < .001$). PTH(1–34) (10 nM) also had no effect on BrdU incorporation (Figure 2C), confirming that the activation of the PTH1R was unable to induce changes in MC proliferation.

### Excision of PTHrP gene in $MC^{CreloxP}$ reduces MC proliferation

We verified PTHrP gene excision in terms of DNA, mRNA, and protein (Figure 3). PTHrP gene deletion was stable over 5 wk (Figure 3, A and B), resulted in a 90%–99% decrease in PTHrP mRNA (Figure 3C) and absence of PTHrP protein in all the cells (Figure 3D). PTH1R expression was maintained although at a lower level (Figure 3E).

To determine whether endogenous PTHrP has any effect on MC proliferation, PTHrP-excised $MC^{CreloxP}$ were compared to control cells by FACS analysis (Figure 4A). Results showed a decrease in MC proliferation in PTHrP-deleted cells even in the presence of serum ($P < .001$). Serum withdrawal reduced MC proliferation of about 30% in control $MC^{CreloxP}$ and 50% in PTHrP-deleted cells ($P < .001$). Finally, the addition of exogenous PTHrP(1–36) had no impact on cell proliferation, whether tested on control or PTHrP-excised cells. Similar results were obtained on BrdU incorporation (Figure 4B). These observations suggest that endogenous PTHrP significantly regulates MC proliferation but not through PTH1R activation.

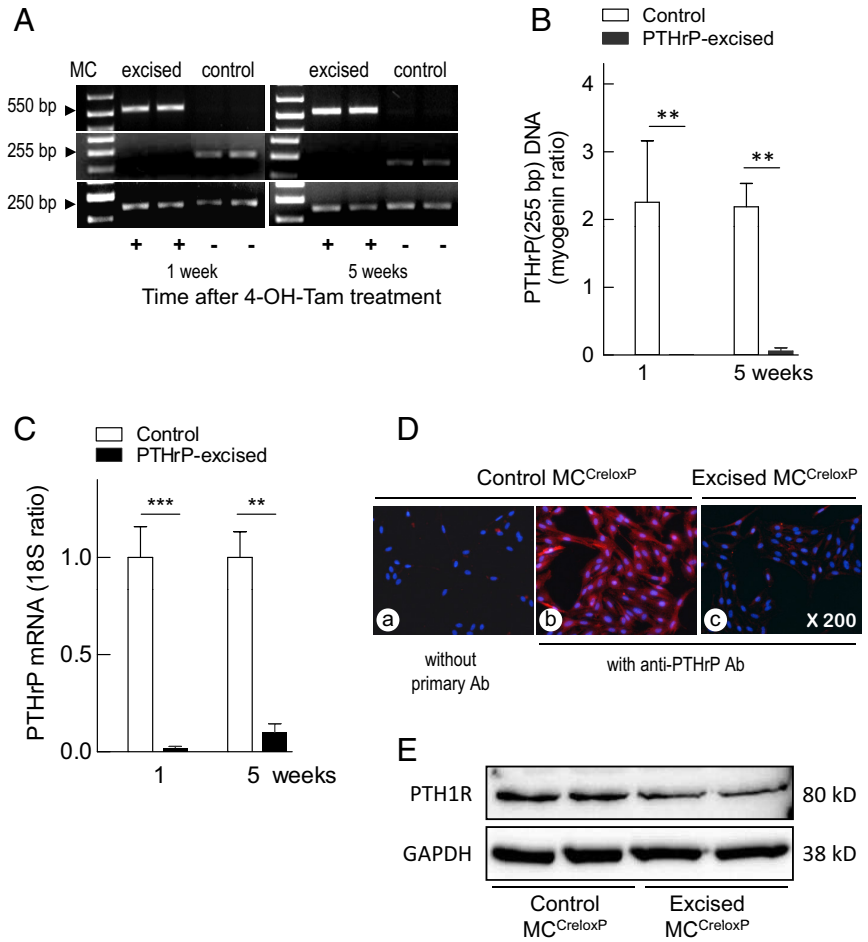


**Figure 3.** PTHrP gene excision in MC. MC were prepared from αSMA-Cre-ER$^{T2}$/PTHrP$^{L2/L2}$ mice kidneys (MC$^{CreloxP}$) and treated in vitro by 4-OH-Tam (5 μM) for three alternate days. A, PCR analysis on DNA showing the amplification of the 550 bp fragment from the deleted L-allele in PTHrP-excised MC while the band of 255 bp disappeared. Myogenin (250 bp) was used as a housekeeping gene. B, Expression of PTHrP DNA 255 bp fragment in MC$^{CreloxP}$ as a ratio vs myogenin, 1 and 5 wk after treatment with 4-OH-Tam. C, Expression of PTHrP mRNA as a ratio vs 18S mRNA, 1 and 5 wk after treatment with 4-OH-Tam. D, Immunofluorescent staining of PTHrP in control (b) and PTHrP-excised MC$^{CreloxP}$ (c), using a monoclonal anti-PTHrP(33–52) antibody. No PTHrP staining was observed in the MC after 4-OH-Tam treatment (excised MC$^{CreloxP}$), as was also the case in control MC$^{CreloxP}$ in the absence of the primary antibody (a) (initial magnification, ×200). E, Effects of PTHrP knockdown on PTH1R immunoblot. Results are given as means ± SEM, n = 5. Student *t* test, ** $P < .01$ and *** $P < .001$ vs control.

## PTHrP increases MC proliferation through intracrine pathway, via c-Myc, E2F1, and p27$^{Kip1}$

PTHrP has been shown to induce proliferation through the intracrine pathway in VSMC (12). To further investigate whether a similar effect is responsible for PTHrP-mediated proliferation in MC, cells were stably transfected with HA-tagged PTHrP-WT, -ΔNLS, or -ΔSP. HA immunostaining, as expected, showed only cytoplasmic localization of PTHrP-ΔNLS, whereas PTHrP-WT and PTHrP-ΔSP were seen in both nuclei and cytoplasm (Supplemental Figure 3). The transfected cells expressed high levels of the human PTHrP forms, at least 50-fold higher than the mouse endogenous isoform, and had no effect on PTH1R expression (Supplemental Figure 4).

BrdU incorporation analysis on day 1 (Figure 5A) showed that MC transfected with either PTHrP-WT or PTHrP-ΔSP proliferated more than those transfected with PTHrP-ΔNLS or empty plasmid (pcDNA3). This was particularly clear in the absence of serum ($P < .001$). The increase in proliferation rate was similar in PTHrP-WT and PTHrP-ΔSP transfected cells, whereas proliferation was not different between PTHrP-ΔNLS and pcDNA3 transfected cells. Moreover, the mitogenic effect of PTHrP-WT overexpressing cells was not affected by blocking the PTH1R with (Asn$^{10}$, Leu$^{11}$, D-Trp$^{12}$)-PTHrP(7–34) amide (Figure 5A). Finally, the conditioned medium from these cells, expected to contain the secreted fragments of PTHrP, even those acting via yet unknown receptors, was also inactive, supplemented or not with the antagonist (Figure 5B). These findings indicate that only intracrine PTHrP is mitogenic.

Next, to gain more insight into the possible mechanisms involved in the intracrine effects of PTHrP, we showed that conditioned medium from PTHrP-ΔSP–transfected MC did not increase BrdU incorporation in cells deleted from endogenous PTHrP (Figure 5B). This result excludes that some mitogenic factors were released into the medium by PTHrP-ΔSP overexpression. It has been described that nuclear PTHrP induces VSMC proliferation by enhancing c-Myc expression that targets p27$^{Kip1}$ to proteosomal degradation (31). Similar pathways seem to be induced in MC (Figure 5C). Indeed, c-Myc expression was enhanced in MC overexpressing PTHrP-WT or PTHrP-ΔSP, and decreased after knockdown of PTHrP in MC. One principal cell-cycle transcription factor, E2F1, displayed the same profile. Conversely, p27$^{Kip1}$ expression was decreased in MC overexpressing PTHrP-WT and enhanced in PTHrP-deleted MC.

## PTHrP is a survival factor for MC

MC apoptosis was tested in control and PTHrP-deleted MC$^{CreloxP}$ at day 2 by FACS (Figure 6A) and TUNEL

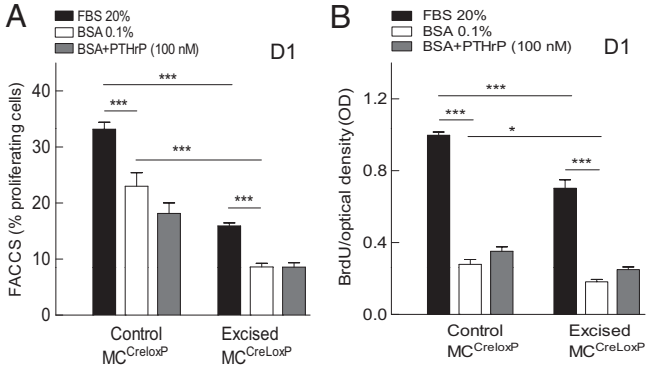


**Figure 4.** MC proliferation after deletion of PTHrP. PTHrP gene was excised (Excised $MC^{CreloxP}$) or not (Control $MC^{CreloxP}$) by 4-OH-Tam treatment. MC proliferation was evaluated by (A) FACS and (B) BrdU incorporation. MC were synchronized for 24 hours, before proliferation was analyzed at day 1 in the presence (FBS 20%) or absence of serum (BSA 0.1%). Serum-deprived MC were also treated with PTHrP(1–36) at 100 nM. PTHrP-excised MC showed significantly less proliferation than did control cells even in the presence of serum. The addition of exogenous PTHrP(1–36) had no effect on MC proliferation. Results are given as means ± SEM, n = 8 (FACS) and n = 4 (BrdU). Tukey test, * $P < .05$ and *** $P < .001$.

analysis (Figure 6B). Serum deprivation increased the number of apoptotic MC, and this increase was more important in MC after PTHrP knockdown ($P < .01$). No difference was seen between control and PTHrP-excised cells in the presence of serum. Exogenous PTHrP(1–36) rescued MC by reducing apoptosis in both control and PTHrP-deleted cells (Figure 6, A and B), and this effect was dose dependent (Figure 6C). A similar effect was observed with PTH(1–34) and forskolin, whereas PTHrP(107–111), the functional pentapeptide of osteostatin [PTHrP (107–139)], was inactive (Figure 6C). Moreover, the addition of a specific PTH1R antagonist to the culture media of serum-starved MC increased their apoptosis ($P < .01$) (Figure 6C), indicating that, under basal conditions, endogenously expressed PTHrP seems to confer resistance to MC apoptosis through PTH1R activation.

To further verify the pathway through which PTHrP elicits cell survival, quiescent MC stably transfected with one of the PTHrP constructs (WT, ΔNLS, ΔSP) or the empty vector were analyzed by FACS after 24 hours of serum deprivation. The results showed that overexpression of any of the three PTHrP forms conferred resistance to apoptosis, particularly with PTHrP-WT (39%, $P < .001$) and PTHrP-ΔNLS (31%, $P < .001$), and less with PTHrP-ΔSP (8.4%, $P < .05$) (Figure 6D). Moreover, PTHrP-WT–induced resistance to apoptosis was completely reversed by the PTH1R antagonist (Figure 6E). Altogether, these data indicate that PTHrP contributes to MC survival mainly by the autocrine/paracrine pathway.

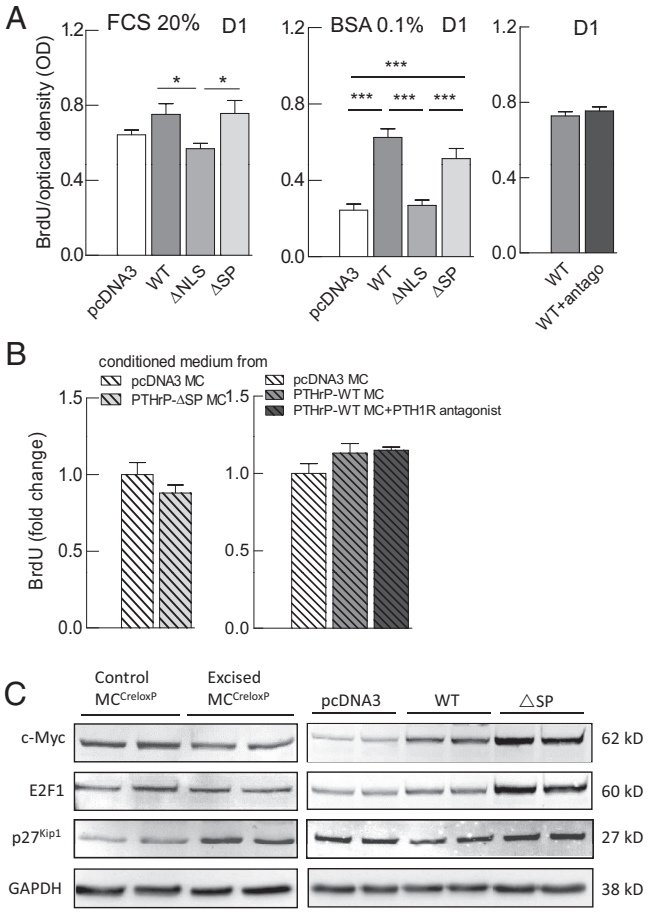


**Figure 5.** MC proliferation after transfection with various constructs of PTHrP. A, Control $MC^{CreloxP}$ were transfected with constructs of PTHrP devoid of its intracrine (ΔNLS) or paracrine (ΔSP) effects, as well as with the WT form or the empty vector (pcDNA3). MC proliferation was assayed by BrdU incorporation over 24 hours in the presence or absence of serum. Intracrine (PTHrP-ΔSP) and PTHrP-WT but not autocrine/paracrine PTHrP (PTHrP-ΔNLS) were mitogenic for MC. The PTHrP-WT–induced response was not modified by the PTH1R antagonist [($Asn^{10}$, $Leu^{11}$, D-$Trp^{12}$)-PTHrP(7–34)amide, 1 μM]. B, MC proliferation was also assessed by BrdU incorporation over a 24-hour period, during which the cells were exposed to conditioned medium, without FBS, obtained from MC transfected with PTHrP-ΔSP, the empty vector, or PTHrP-WT (with or without addition of the PTH1R antagonist). The conditioned medium from MC overexpressing PTHrP-WT or PTHrP-ΔSP did not modify MC proliferation. Results are given as means ± SEM, n = 5. Tukey test, * $P < .05$ and *** $P < .001$. C, Representative immunoblots for c-Myc, E2F1, and $p27^{Kip1}$ of control $MC^{CreloxP}$ and PTHrP-excised $MC^{CreloxP}$, as well as of MC overexpressing PTHrP-WT, PTHrP-ΔSP, or the empty vector (pcDNA3). GAPDH is used as a housekeeping protein control on the same membrane.

## cAMP/PKA and PI3-K/Akt pathways are involved in PTHrP-induced MC survival

PTH1R activation by PTHrP was classically associated with the cAMP/PKA pathway. This is also the case in MC. PTHrP induced a dose-dependent accumulation of cAMP (Figure 7A), a response shared with PTH(1–34), forskolin, and isoproterenol. PTHrP(1–36)-induced accumulation of cAMP was selectively decreased by the PTH1R antagonist, ($Asn^{10}$, $Leu^{11}$, D-$Trp^{12}$)PTHrP(7–34)amide.

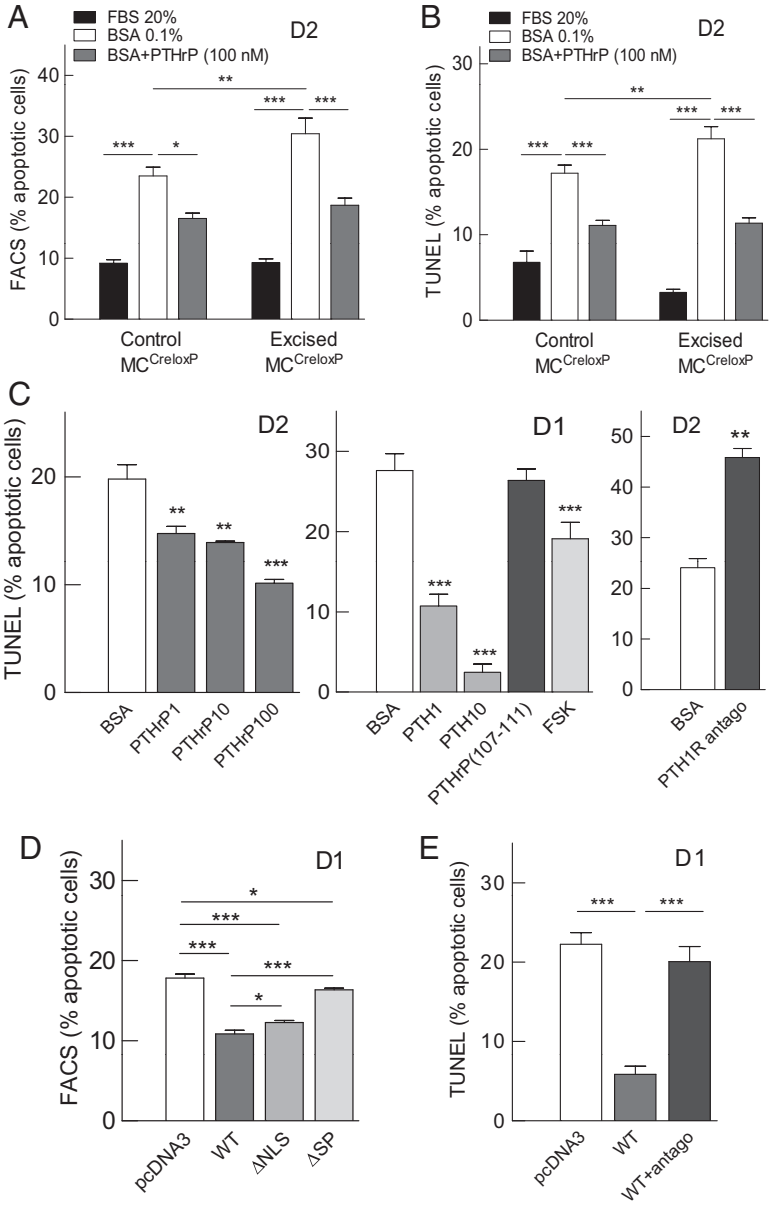


**Figure 6.** Antiapoptotic effects of PTHrP on MC. PTHrP gene excision was induced as shown previously. MC were synchronized for 24 hours and apoptosis was induced by serum-deprivation for an additional 24-hour (day 1) or 48-hour period (day 2). Apoptosis, analyzed by both (A) FACS and (B) TUNEL, was higher in PTHrP-excised $MC^{CreloxP}$ and was partially reversed by treatment of the cells with PTHrP(1–36) (100 nM). C, Incrementing PTHrP(1–36) concentrations from 1 to 100 nM (n = 8) showed dose dependence. PTH(1–34) (1 and 10 nM) and forskolin (FSK, 1 $\mu$M) also reduced MC apoptosis, whereas PTHrP(107–111) was inactive and the PTH1R antagonist ($Asn^{10}$, $Leu^{11}$, D-$Trp^{12}$)-PTHrP(7–34)amide, 1 $\mu$M) increased MC apoptosis. D, Transfection of control $MC^{CreloxP}$ with various constructs of PTHrP (WT, ΔNLS, ΔSP or empty vector) decreased MC apoptosis assessed by FACS, particularly with the WT and ΔNLS constructs, whereas the ΔSP one had minimal effects. E, The increased survival of MC overexpressing PTHrP-WT was reversed by the PTH1R antagonist. PTHrP active by the autocrine/paracrine pathway appears as a survival factor for MC. Results are given as means ± SEM, n = 9 (FACS) and n = 3 (TUNEL). Student *t* test or Tukey test, * $P < .05$, ** $P < .01$ and *** $P < .001$.

PTHrP has also been shown to promote cell survival through PI3-K/Akt activation in renal carcinoma cells, renal fibroblasts, and epithelial cells (17, 22). On MC, PTHrP(1–36) induced time-dependent phosphorylation of Akt at both Thr308 and Ser473, as shown by Western blot (Figure 7B), and confirmed for Ser473 by immunostaining (Supplemental Figure 5).

To see if the PTHrP(1–36) effect on MC survival is associated with the activation of cAMP/PKA or Akt phosphorylation, we used serum-deprived PTHrP-excised $MC^{CreloxP}$, a situation in which MC apoptosis is maximal. A 24-hour cell exposure to PTHrP(1–36) reduced apoptosis analyzed by TUNEL, a response that was reversed by inhibitors of PKA (H89, 1 $\mu$M) or PI3-K (LY249002, 20 $\mu$M) (Figure 7C). Otherwise, an inhibitor of PKC (Gö6983, 5 $\mu$M) decreased basal apoptosis of MC by itself and was unable to block PTHrP-induced response. These data highlight the critical role of the PI3-K/Akt and cAMP/PKA pathways in PTHrP-induced MC survival.

## Discussion

PTHrP is a locally active cytokine with well-known effects on proliferation and apoptosis in different cell types through distinct intracrine or paracrine pathways. PTHrP was found to be up-regulated in MC after protein overload in rat, as well as in human and experimental diabetic nephropathies (19, 23, 32). Here we explored the effects of intracrine and paracrine PTHrP on the two aforementioned processes on mouse MC in primary culture. Our results show that intracrine PTHrP enhances MC proliferation, whereas paracrine PTHrP exerts a major antiapoptotic effect.

We first analyzed the effect of PTHrP(1–36) on the proliferation of MC prepared from C57BL/6 mice kidneys. Treatment with PTHrP(1–36) did not elicit any mitogenic effect on MC, whatever the dose used (1–100 nM, once or daily treatment) or the time point considered (1–3 days). PTH(1–34), another ligand of the PTH1R, was similarly inactive. In contrast, MC re-

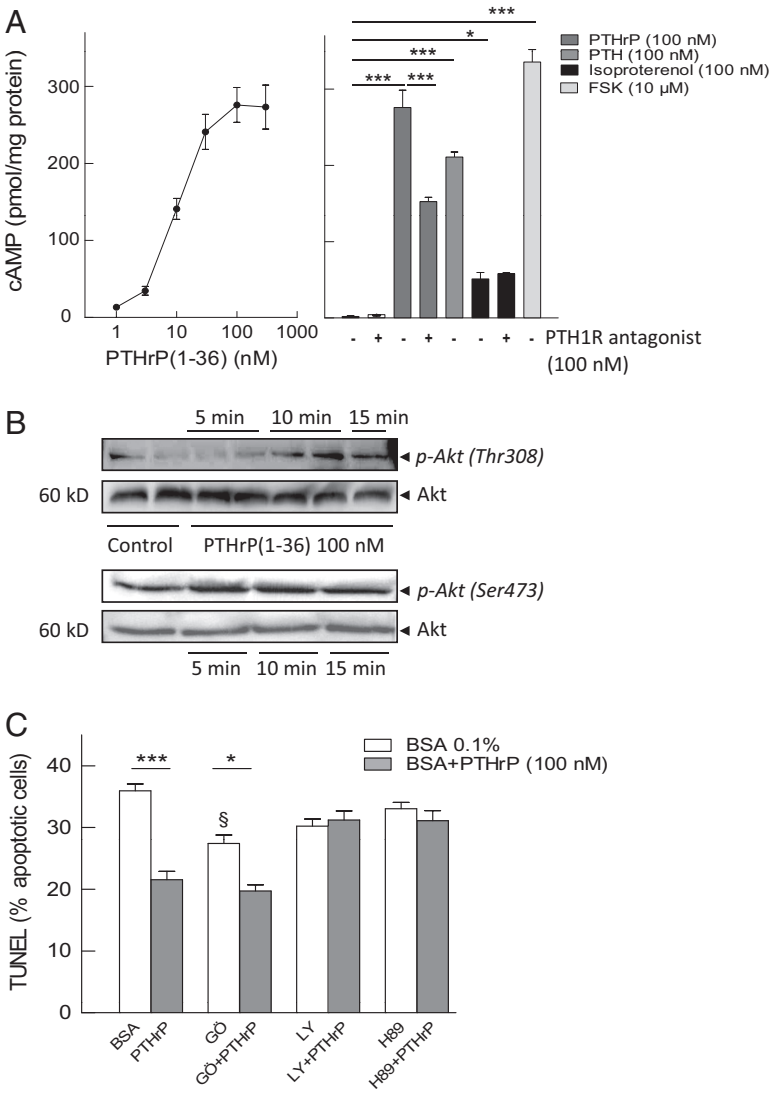


**Figure 7.** Signaling pathways activated by PTHrP and involvement in PTHrP-induced MC survival. A, Control $MC^{CreloxP}$ were synchronized for 24 hours after 15 min pretreatment with 200 $\mu$M 3-isobutyl-1-methyl-xanthine. MC were incubated for 15 min with increasing concentrations of PTHrP(1–36) (1–300 nM), PTH(1–34) (100 nM), isoproterenol (100 nM), or forskolin (FSK 10 $\mu$M), with or without the PTH1R antagonist (100 nM). Cyclic AMP was measured by enzyme immunoassay in cell lysates and culture medium. Data are expressed as picomole per milligram protein and given as means ± SEM, n = 3. Tukey test, * $P < .05$ and *** $P < .001$. B, Quiescent control $MC^{CreloxP}$ were exposed or not to 100 nM PTHrP(1–36) for 5 to 15 min. Representative immunoblots for Akt phosphorylated at Thr308 or Ser473, and total Akt are shown. Experiments were repeated at least three times with similar results. C, PTHrP-excised $MC^{CreloxP}$ were synchronized for 24 hours. Apoptosis was induced by serum deprivation for an additional 24-hour period, during which the cells were exposed to PTHrP(1–36) at 100 nM, with or without a PKC inhibitor (Gö6983, 5 $\mu$M), a PI3-K inhibitor (LY294002, 20 $\mu$M), or a PKA inhibitor (H89, 1 $\mu$M). Apoptosis was analyzed by TUNEL. Data show that the PKA and PI3-K pathways contribute to PTHrP-induced MC survival. Results are given as means ± SEM of three independent experiments. Tukey test, * $P < .05$ and *** $P < .001$ vs the corresponding control group; § $P < .05$ vs the BSA group.

sponded to PDGF-BB by an increase in proliferation. Our results with PTHrP(1–36) differ from those reported previously by Soifer *et al.* (20) and Bosch *et al.* (25) on rat and human MC, respectively, in which PTH1R activation by PTHrP(1–36) increased MC proliferation. In a recent study, Ortega *et al.* (32) also found that PTHrP(1–36) exerted a short-lasting proliferative effect of human MC (24 hours), which ended with MC hypertrophy. As in mouse MC, PTHrP(1–36) stimulated cAMP accumulation in human MC (25), while its signaling pathway has not been identified in rat MC. However, cAMP accumulation was described to be antimitogenic in these cells (33–35). We therefore searched for any antiproliferative effect of PTHrP on MC and again found none. We think that the contrast between our results and those of the studies on human and rat MC may be related to species discrepancies or differences in the population doubling times.

Next, we wanted to see if endogenous PTHrP exerts any effect on MC proliferation. For this purpose, we used MC obtained from CreloxP transgenic mice that allowed conditional PTHrP gene excision by 4-OH-Tam treatment in cells expressing $\alpha$SMA (26, 27). This was feasible because MC in culture express this protein. One important observation is that the deletion of endogenous PTHrP in MC reduced their proliferation even in serum-supplemented media. Attempts to restore MC proliferation by the addition of exogenous PTHrP(1–36) were again unsuccessful. It is worth mentioning that PTHrP gene excision slightly decreased PTH1R protein, but a functional PTH1R was still present, as shown by the effects of paracrine PTHrP on survival. On the other hand, the overexpression of PTHrP-WT elicited a mitogenic effect on MC. These results indicate that another PTHrP fragment or pathway accounted for the proliferative effect. In fact, post-translational modifications of PTHrP through proteolytic cleavage may generate different secreted fragments, such as PTHrP(67–86), PTHrP(38–64), PTHrP(38–94) in the midregion, or PTHrP(107–139) in the C-terminal tail (11, 36). Among these, PTHrP(67–86) and PTHrP(107–139) have been shown to inhibit breast cancer cell proliferation

(37), whereas PTHrP(107–139) through its active pentapeptide PTHrP(107–111), but also PTHrP(38–64), increased proliferation of osteoblasts and epithelial pulmonary cells, respectively (38, 39). In addition, PTHrP(38–94) was shown to inhibit breast cancer cell proliferation through nuclear translocation (40). We therefore tested the conditioned medium from MC overexpressing PTHrP-WT, but found no effect on cell proliferation. On the other hand, PTHrP(1–108) has been shown to reach cell nucleus in a PTH1R-dependent manner (41). However, PTH1R blockade by a selective antagonist did not change MC proliferation. Altogether, these results exclude any effect on mitogenesis of secreted PTHrP fragments, whether linked to the activation of PTH1R or yet unknown receptor(s), or to the PTH1R-mediated internalization of some fragments.

Otherwise, PTHrP acting through intracrine pathway has been shown to increase proliferation in VSMC (12, 13) or prostate and colon cancer cells (42, 43). We observed similar induction of proliferation in cells overexpressing PTHrP-WT or PTHrP-ΔSP, with PTHrP-ΔNLS lacking any mitogenic effect. These results indicate that only intracrine PTHrP was responsible for the MC mitogenesis. In addition, the absence of mitogenic effect on MC exposed to conditioned media from MC transfected with PTHrP-ΔSP suggests that PTHrP-induced proliferation is related to a direct intracellular effect of PTHrP but not to the release of other mitogenic factors in the medium. We next showed that PTHrP deletion increased $p27^{Kip1}$ protein level, and PTHrP-WT overexpression decreased it. $p27^{Kip1}$ is a well-known inhibitor of the cell cycle, and MC mitogens such as PDGF-BB and basic fibroblast growth factor diminish $p27^{Kip1}$ expression in MC (44). Nuclear PTHrP has also been shown to target $p27^{Kip1}$ to proteasomal degradation in VSMC (13, 31). In addition, transfection of MC with PTHrP-WT or PTHrP-ΔSP increased c-Myc and E2F1 protein levels, whereas PTHrP deletion reduced them. E2F1 is required for cell cycle progression and was found, as well as the proto-oncogen c-Myc, to be upregulated in proliferating MC in vitro and in vivo (45, 46). Intracrine PTHrP-induced expression of c-Myc was also associated with VSMC proliferation (31). The $p27^{Kip1}$ level seemed normal in MC transfected with PTHrP-ΔSP. These cells showed particularly high levels of c-Myc and E2F1, which may exert opposite regulations on $p27^{Kip1}$ expression (47–49).

MC apoptosis characterizes the initial event of experimental mesangial proliferative glomerulonephritis. However, apoptosis is also seen in advanced stages when excessive MC proliferation occurs (50). In this study, we report an antiapoptotic function of PTHrP in MC, and to the best of our knowledge, this has not been described previously. Our results show that PTHrP gene deletion or PTH1R antagonism increased the number of apoptotic cells subsequent to serum withdrawal, which indicates that under basal conditions, PTHrP is sufficiently produced and secreted by cultured MC to enhance their survival. Otherwise the treatment with PTHrP(1–36) or the overexpression of PTHrP (WT,-ΔNLS,-ΔSP) reduced MC apoptosis. Survival response was particularly marked in cells transfected with PTHrP-WT or PTHrP-ΔNLS, suggesting that PTHrP mainly acted through the autocrine/paracrine pathway. This was confirmed by the rescue of apoptotic PTHrP-deleted MC through exogenous PTHrP(1–36) treatment. The activation of the PTH1R seems to be particularly important because 1) PTH1R antagonism increased MC apoptosis induced by serum deprivation, 2) the enhanced survival of cells transfected with PTHrP-WT was completely reversed by a selective antagonist, 3) PTH(1–34) also decreased MC apoptosis and 4) PTHrP(107–111) was devoid of any effect in our study, although PTHr(107–139) increased osteoblastic cell survival (51). These results are in accordance with results seen in other cell types. PTHrP was shown to protect renal fibroblasts, tubuloepithelial cells, pancreatic β-cells, and renal carcinoma cells against apoptosis principally through the autocrine/paracrine pathway (52–54). However, the nuclear translocation of PTHrP also was associated with enhanced survival in chondrocytes (16).

We next examined the signaling pathways that mediate the antiapoptotic effects of PTHrP in MC. The stimulation of the PTH1R has been classically associated with adenylyl cyclase and PKA activation (55). The PTH1R can also bind to NHERF1 and NHERF2 and recruit PLCβ, leading to PKC activation (56). Our results show that the activation of the PTH1R by PTHrP or PTH elicits a large increase in cAMP. Both agonists, as well as the direct activation of the cAMP/PKA pathway by forskolin, enhanced MC survival. Interestingly, we also found that PTHrP activates Akt in MC through the phosphorylation of both Thr308 and Ser473. The PI3-K/Akt signaling is a key pathway involved in cell survival (57, 58). Our group reported previously that Akt activation was responsible for PTHrP-induced cell survival in renal carcinoma cells (17). Here, we show that inhibition of both the PI3-K/Akt and cAMP/PKA pathways, but not PKC, reversed the antiapoptotic effects of PTHrP in MC. These results are in agreement with those of other studies (52, 59). The complete reversion of PTHrP-induced survival in MC through the inhibition of either PKA or Akt suggests that these two factors may work in concert to activate or inhibit the same downstream signaling pathway. Possible targets are, for example, NFκB, Bad, and GSK-3β, which may be differentially phosphorylated by each PKA and Akt. They contribute to MC survival in response to PDGF and insulin (60, 61), but many other candidates also exist. Additional studies are needed to clarify this point.

We noticed that PKC inhibition solely diminished MC apoptosis but did not reverse the response to PTHrP. In fact, interaction between PTH1R and NHERF1/2 has been shown to be necessary for PKC activation (56). In this respect, we found that NHERF1 and NHERF2 were expressed in our cells, but surprisingly NHERF1 was localized to the nucleus (Supplemental Figure 6). Multiple coimmunoprecipitation analysis did not show any interaction between PTH1R and NHERF1 or NHERF2 (M. Hochane, personal data), and colocalization was not found by immunostaining (Supplemental Figure 6).

In conclusion, this study reveals the complexity of PTHrP signaling in MC. PTHrP is a mitogenic and a survival factor through intracrine and paracrine pathways, respectively. PTHrP shares many effects with PDGF, increasing proliferation and survival, and stimulating Akt phosphorylation. Given the prominent role of these processes in the progression of glomerulonephritis, PTHrP may be a potential actor in this pathology. Additional studies are needed to evaluate the role of PTHrP in this context in vivo. The clinical relevance of PTHrP also should be considered through analysis of PTHrP expression in human samples of glomerulonephritis.

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