Intermittent PTHrP(1–34) Exposure Augments Chondrogenesis and Reduces Hypertrophy of Mesenchymal Stromal Cells

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Phenotype instability and premature hypertrophy prevent the use of human mesenchymal stromal cells (MSCs) for cartilage regeneration. Aim of this study was to investigate whether intermittent supplementation of parathyroid hormone-related protein (PTHrP), as opposed to constant treatment, can beneficially influence MSC chondrogenesis and to explore molecular mechanisms below catabolic and anabolic responses. Human MSCs subjected to chondrogenic induction in high-density culture received PTHrP(1–34), forskolin, dbcAMP, or PTHrP(7–34) either constantly or via 6-h pulses (three times weekly), before proteoglycan, collagen type II, and X deposition; gene expression; and alkaline phosphatase (ALP) activity were assessed. While constant application of PTHrP(1–34) suppressed chondrogenesis of MSCs, pulsed application significantly increased collagen type 2 (*COL2A1*) gene expression and the collagen type II, proteoglycan, and DNA content of pellets after 6 weeks. Collagen type 10 (*COL10A1*) gene expression was little affected but Indian hedgehog (IHH) expression and ALP activity were significantly downregulated by pulsed PTHrP. A faster response to PTHrP exposure was recorded for ALP activity over *COL2A1* regulation, suggesting that signal duration is critical for catabolic versus anabolic reactions. Stimulation of cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling by forskolin reproduced major effects of both treatment modes, whereas application of PTHrP(7–34) capable of protein kinase C (PKC) signaling was ineffective. Pulsed PTHrP exposure of MSCs stimulated chondrogenesis and reduced endochondral differentiation apparently uncoupling chondrogenic matrix deposition from hypertrophic marker expression. cAMP/PKA was the major signaling pathway triggering the opposing effects of both treatment modes. Intermittent application of PTHrP represents an important novel means to improve chondrogenesis of MSCs and may be considered as a supporting clinical-treatment mode for MSC-based cartilage defect regeneration.

Introduction

MUCH EFFORT HAS BEEN INVESTED in the use of mesenchymal stromal cells (MSCs) as a possible cellular source for tissue engineering of cartilage constructs [1,2]. The advantage in using MSCs over articular chondrocytes lies in an easier retrieval without irreversible damage, their rapid expansion in culture, and documented in vitro differentiation capacity toward the chondrogenic lineage [3,4]. Yet, there are still major obstacles for the routine application of MSCs for cell-based cartilage repair, one of the most urgent of which is a phenotype instability of the generated chondrocytes in vitro [5–7] and in vivo [7–9].

Although common protocols for chondrogenic in vitro differentiation of MSCs evoke the expression of key molecules characteristic for articular cartilage, the natural course

toward a stable articular chondrocyte phenotype is not recapitulated, as a process reminiscent of endochondral bone formation is triggered [7]. The hypertrophic phenotype of chondrocytes is mainly characterized by a strong increase in cell volume and the onset of expression of Indian hedgehog (IHH), collagen type X, and alkaline phosphatase (ALP), which is closely associated with matrix mineralization.

In the growth plate, the extent and velocity of hypertrophic differentiation are tightly controlled, most prominently by the parathyroid hormone-related protein (PTHrP)/IHH feedback loop [10,11]. The N-terminal region of PTHrP shares a high sequential and structural homology with parathyroid hormone (PTH) and signals via a common receptor, the G protein-coupled PTH/PTHrP receptor (PTH1R). Activation of the receptor triggers several different intracellular signaling pathways, including $G\alpha_s$ /adenylate cyclase/cyclic

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adenosine monophosphate (cAMP)/protein kinase A (PKA) and $G\alpha_{q/11}$ /phospholipase C β /protein kinase C (PKC) [12]. More recently, signal transduction via ERK1/2 and β -arrestin has been reported as well [13]. The principal receptor-binding domain of PTHrP lies within amino acids (aa) 15–34, but the first 2 amino acids of PTH/PTHrP are essential for the activation of the cAMP/PKA-dependent pathway. The C-terminus of PTH/PTHrP(1–34), where PTH and PTHrP share structural rather than sequential homology, contains a domain necessary for PKC activation within aa 28–34. N-terminally truncated peptide analogs such as PTHrP(7–34) can bind to the receptor but do not elicit a cAMP/PKA response [14]. As PTHrP(7–34) can cell-type-dependently activate PKC [15,16], it may represent a useful tool to antagonize PTHrP(1–34)-related cAMP/PKA signaling while simultaneously maintaining PKC signaling.

There is evidence that PTHrP is operative also in post-natal articular cartilage [17] and might therefore be involved in the maintenance of the stable articular phenotype. Capable to modulate chondrocyte maturation and hypertrophy, PTHrP is, thus, a highly interesting candidate molecule to improve chondrogenesis of MSCs. However, constant application of PTHrP in chondrogenic medium suppressed chondrogenesis instead of selectively inhibiting hypertrophic features only [6,18]. Interestingly, PTH can exert opposing effects on the osteogenic differentiation of osteoblasts [19,20], rat MSCs [21], and on bone mass dependent on a constant versus intermittent application regime. Whereas constant application acted catabolically on bone, an intermittent application (eg, once daily) provided anabolic effects, resulting in a net increase in bone mass [22,23]. Similar effects were reported for PTHrP [24–26] and first clinical trials are conducted to evaluate the benefit of intermittent PTHrP administration for the treatment of osteoporosis [27].

Although chondrocytes and osteoblasts share common progenitors, comparably little is known about a potential effect of intermittent application of PTH/PTHrP on chondrocytes in vitro and on cartilage tissue in vivo. Few studies reported on an improved osteochondral repair [28,29], stimulation of chondrogenesis in bone healing during callus formation [30], and inhibition of osteoarthritis progression in an in vitro and in vivo azacytidine-induced osteoarthritis model [31]. In contrast, an inhibition of cartilage formation was observed in a microfracture study after intermittent treatment with PTH [32].

How intermittent application of PTHrP affects in vitro chondrogenesis of MSCs remained so far unexplored. We here hypothesized that, opposite to constant application, intermittent application of PTHrP can exert beneficial effects on MSC chondrogenesis and investigated whether this may include stimulation of extracellular matrix deposition and suppression of hypertrophic chondrocyte development. Beyond, we intended to identify the signaling pathways relevant for constant versus intermittent PTHrP effects on human MSCs. Bone-marrow-derived MSCs were subjected to 6 weeks of chondrogenesis, receiving either constant or intermittent PTHrP(1–34) or chondrogenic medium alone. Markers for chondrogenesis and hypertrophy were quantitatively evaluated on the mRNA and protein levels and the contribution of different signaling pathways was explored by treatment with forskolin, dbcAMP, and PTHrP(7–34).

Materials and Methods

Isolation and expansion of MSCs

MSCs were isolated from fresh bone marrow aspirates obtained from 24 patients undergoing total-hip replacement by fractionation on a Ficoll-Paque Plus density gradient (GE Healthcare Europe), and the low-density mononuclear cell fraction was washed and seeded in expansion medium [33] consisting of high-glucose Dulbecco's modified Eagle's medium (DMEM), 12.5% fetal calf serum (FCS), 2 mM L-glutamine, 1% nonessential amino acids, 0.1% β -mercaptoethanol (all Life Technologies), 1% penicillin/streptomycin (Biochrom), and 4 ng/mL of recombinant basic fibroblast growth factor (R&D Systems). After 24–48 h, cells were washed to remove nonadherent material. For expansion, cells were replated at a density of 5×10^3 cells/cm² in gelatine-coated culture flasks and used at passage 3.

Written consent was obtained from all tissue donors and the study received approval from the local ethics committee.

Chondrogenic induction of MSCs

Pellets consisting of 5×10^5 cells were exposed for 6 weeks to chondrogenic induction medium (high-glucose DMEM with 0.1 μ M dexamethasone, 0.17 mM ascorbic acid-2 phosphate, 5 μ g/mL insulin, 5 μ g/mL transferrin, 5 ng/mL selenous acid, 1 mM sodium pyruvate, 0.35 mM proline, 1.25 mg/mL bovine serum albumin, and 1% penicillin/streptomycin) supplemented with 10 ng/mL recombinant human TGF- β_1 (Pepro-Tech). Medium was changed three times a week. Where indicated, 2.5 nM of PTHrP(1–34), 2.5 nM or 1 μ M of [Asn¹⁰, Leu¹¹, D-Trp¹²] PTHrP(7–34) NH₂ [PTHrP(7–34); all from Bachem], 10 μ M of forskolin (Calbiochem/Merck Millipore), or 1 mM of N⁶,2'-O-dibutyryl adenosine-3':5'-cyclic monophosphate sodium salt (dbcAMP; Sigma-Aldrich Chemie) was supplemented.

For intermittent application, PTHrP(1–34), PTHrP(7–34), or forskolin was added to the culture medium to yield a final concentration of 2.5 nM or 10 μ M, respectively, 6 h prior to every medium change starting on day 7 of the pellet culture. Culture supernatants were then replaced with fresh medium containing no PTHrP or forskolin. Since forskolin was dissolved in dimethyl sulfoxide (DMSO), respective DMSO controls were prepared for the pulsed application of forskolin. DMSO treatment alone yielded no significant effect in all experiments allowing to omit these controls in subsequent experiments.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from two to six pooled, homogenized pellets per guanidinium isothiocyanate/phenol extraction (peqGOLD Trifast; Peqlab). Polyadenylated mRNA was purified from total RNA with oligo(dT)-coupled magnetic beads (Dynabeads; Invitrogen/Life Technologies). The mRNA was then subjected to first-strand cDNA synthesis using reverse transcriptase (Omniscript1; Qiagen) and oligo(dT) primers. The expression level of individual genes was analyzed by RT-PCR using the MX3000P QPCR System (Stratagene/Agilent Technologies). The following forward (F) and reverse (R) primer pairs were used for amplification: β -actin-F, 5'-CTCTTCCAGCCTTCCTT CCT-3'; β -actin-R, 5'-GATCCACACGGAGTACTTG-3';

Col2A1-F, 5'-TGGCCTGAGACAGCATGAC-3'; Col2A1-R, 5'-AGTGTGGGAGCCAGATTGT-3'; Col10A1-F, 5'-CCC TTTTGTCTGCTAGTATCC-3'; Col10A1-R, 5'-CTGTTGT CCAGGTTTTCCTGGCAC-3'; IHH-F, 5'-CGACCGCAAT AAGTATGGAC-3'; IHH-R, 5'-GGTGAGCGGGTGTGA GTG-3'; PTH1R-F, 5'-GGTGAGGTGGTGGCTGT-3'; PTH 1R-R, 5'-AGCATGAAGGACAGGAAC-3'; and MMP13-F, 5'-CTGGAGATATGATGATACTAAC-3'; MMP13-R, 5'-CACGCATAGTCATATAGATACT-3'. Gene expression levels were calculated as percentage of β -actin expression using the Δ Ct method.

Measurement of ALP activity

Supernatants conditioned for 2 days were incubated with substrate solution (10 mg/mL of *p*-nitrophenyl phosphate in 0.1 M glycine, 1 mM $MgCl_2$, and 1 mM $ZnCl_2$, pH 9.6). After 150 min, the ALP activity was measured spectrophotometrically at 405/490 nm and was referred to a standard curve made from *p*-nitrophenol (Sigma-Aldrich Chemie).

Collagen extraction, quantification of collagen type II, and western blotting

Collagens were extracted as described elsewhere [5]. Briefly, pellets were homogenized, digested in pepsin buffer (0.5 M acetic acid, 0.2 M NaCl, and 2.5 mg/mL of pepsin), and precipitated with 4.5 M NaCl. Extracted collagens were resuspended in 400 μ L of precipitation buffer (0.4 M NaCl and 0.1 M Tris Base, pH 7.4) and 1.2 mL of 100% ethanol and incubated for 4 h at -20°C . Precipitated collagens were resolved in lysis buffer (1% Triton X-100, 150 mM NaCl, and 50 mM Tris, pH 8.0). The collagen type II content of each lysate was determined by a native type II collagen detection ELISA (Chondrex).

For western blotting, amounts of lysate corresponding to 30 ng collagen type II were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane (Amersham Biosciences/GE Healthcare Europe). The membrane was cut horizontally and the lower part was probed with mouse anti-type X collagen antibody (1:500; Quartett) whereas the upper part was probed with mouse anti-type II collagen antibody (1:1,000; MP Biomedicals). Peroxidase-coupled goat anti-mouse antibody (1:5,000; Jackson ImmunoResearch Laboratories) was utilized as secondary antibody. Bands were visualized by enhanced chemiluminescence (Roche Diagnostics) and signals were quantified by densitometric integrity measurements using Bio1D v. 15.01.

Measurement of cAMP activity

Pellets at day 25 of chondrogenic differentiation were treated with indicated concentrations of forskolin, PTHrP(1–34), and PTHrP(7–34) in chondrogenic medium for 1 h. Culture supernatants were collected and the cAMP activity was determined by ELISA (R&D Systems).

Quantification of proteoglycan content

Pellets were lysed in guanidine-hydrochloride (GuHCl) buffer (50 mM Tris and 4 M GuHCl, pH 8.5) and insoluble components were removed by centrifugation. One hundred

microliters of each supernatant was mixed with 50 μ L of SAT buffer (0.3% H_2SO_4 and 0.75% Triton X-100) and then supplemented with 750 μ L of Alcian blue solution (0.3% Alcian blue, 0.16% H_2SO_4 , 0.04 M GuHCl, and 0.35% Triton X-100). Precipitates were pelleted by centrifugation, resuspended in DMSO buffer (40% DMSO and 0.05 M $MgCl_2$), and centrifuged at 16,000 g for 15 min at room temperature. The pellets were then solubilized in Gu-Prop buffer (4 M GuHCl, 33% 2-propanol, and 0.25% Triton X-100) and the proteoglycan content was determined by measurement of the absorbance at 570 nm.

After initial results from four donors, it became desirable to additionally determine the DNA content from the same sample. For this purpose we switched to an alternative proteoglycan quantification protocol (10/14 donors), in the course of which the pellets were digested with 100 μ g/mL proteinase K (Roche Diagnostics), dissolved in Tris-HCl buffer (0.05 M Tris and 1 mM $CaCl_2$, pH 8) overnight at 60°C . After digestion, 30 μ L of each sample was mixed with 200 μ L of a 1,9-dimethyl-methylene blue (DMMB) dye solution (40 mM glycine, 40 mM NaCl, and 48 μ M DMMB) and absorbance was measured immediately at 530 nm.

Quantification of DNA content

The DNA content of pellets was determined using the Quanti-iT Pico-Green dsDNA kit (Invitrogen). For this assay, 20 μ L of the Proteinase K-digested pellet samples prepared for the proteoglycan quantification was mixed with 80 μ L TE buffer (200 mM Tris HCl and 20 mM EDTA) and PicoGreen solution. DNA content was determined by fluorescence measurement at 485/535 nm.

Histology

Pellets were fixed in 4% paraformaldehyde as described previously [34]. Sections (5 μ m) were stained with Safranin O (Fluka) and counterstained with Fast Green (Chroma) to analyze the proteoglycan content. Immunohistologic staining of collagen type II was performed as described elsewhere [35]. Briefly, sections were incubated with a monoclonal mouse anti-human type II collagen antibody (1:1,000; MP Biomedicals) and reactivity was detected using biotinylated goat anti-mouse secondary antibody (1:500; Dianova), streptavidin-ALP (Dako), and fast red (Sigma-Aldrich Chemie).

Statistics

The mean and SD values were calculated for all variables. Differences between two groups were analyzed with the nonparametric Mann–Whitney *U* test or with Wilcoxon test for paired samples. Correlations were analyzed by determination of the Pearson's coefficient (r^2). $P \leq 0.05$ was considered significant. Data analysis was performed using SPSS 20.0.

Results

Intermittent PTHrP supplementation stimulates in vitro chondrogenesis

In confirmation of previous results [6], constant PTHrP treatment of MSCs throughout in vitro chondrogenesis resulted in cell pellets with virtually no proteoglycan and collagen type II deposition at day 42 (Fig. 1). When MSCs

FIG. 1. Histology of cartilaginous matrix deposition. Mesenchymal stromal cell (MSC) pellets were treated with chondrogenic medium alone (**A**) or chondrogenic medium additionally supplemented with 2.5 nM PTHrP(1–34) either constantly (**B**) or intermittently for 6 h prior to every medium change (three times per week) (**C**). Paraffin sections of day-42 pellets were stained for proteoglycans by Safranin O or collagen type II by immunohistochemistry (*insets*). Representative results from one of four donors are shown. Scale bars represent 500 μ m. Color images available online at www.liebertpub.com/scd

received intermittent PTHrP(1–34) treatment for 6 h three times a week, however, a collagen type II- and proteoglycan-rich matrix was deposited like in the non-PTHrP-treated control group according to histology (Fig. 1A, C). Quantitative analysis of the proteoglycan and collagen type II content of pellets (Fig. 2) revealed a significantly higher proteoglycan (mean 1.54-fold, $P < 0.01$) and collagen type II (mean 2.94-fold, $P < 0.05$) deposition in the PTHrP-pulsed group compared with controls. DNA levels were slightly, but significantly, elevated ($P < 0.05$; Fig. 2C), suggesting enhanced survival or proliferation of cells during chondrogenesis under intermittent PTHrP treatment. Since the absolute proteoglycan/DNA content ($n = 10$) was significantly higher (1.2-fold, $P < 0.01$) after pulsed PTHrP treatment, not only higher cell numbers but also more proteoglycan deposition per cell contributed to superior matrix deposition (data not shown). Thus, as opposed to constant application, intermittent PTHrP treatment three times a week stimulated *in vitro* chondrogenesis of MSCs compared with the standard protocol.

Intermittent PTHrP supplementation reduces hypertrophy

To investigate whether the anabolic effect of intermittently supplemented PTHrP affected expression of chondrogenic and hypertrophic differentiation markers, gene expression of collagen type 2 (*COL2A1*), collagen type 10 (*COL10A1*), *IHH*, *PTHRI*, and matrix metalloproteinase (*MMP13*) was quantified by quantitative real-time PCR. In

FIG. 2. Biochemical analysis of MSC pellets after chondrogenesis. Pellets received chondrogenic medium alone (ctrl) or were additionally stimulated for 6 h with 2.5 nM PTHrP(1–34) before each medium change [parathyroid hormone-related protein (PTHrP) pulse; three times a week]. On day 42, pellets were harvested and the proteoglycan (**A**) and collagen type II content (**B**) and DNA content (**C**) were determined. Values are shown as mean $\pm$ SD of 14 (**A**, **B**) or 10 (**C**) independent experiments and normalized to ctrl set to 1. * $P \leq 0.05$, ** $P \leq 0.01$, Wilcoxon test.

line with collagen type II protein deposition (Fig. 2B), *COL2A1* mRNA levels at day 42 were significantly higher in the PTHrP-pulsed group (2.1-fold; $P < 0.05$, Fig. 3A). The *COL10A1* mRNA levels were, however, rather similar (Fig. 3A) and no significant difference was obtained for the *COL10A1/COL2A1* ratio (Fig. 3B). Time course analysis (Fig. 3C) demonstrated that *COL2A1* expression was stimulated especially in the late phase of MSC chondrogenesis.

Samples with better chondrogenesis show higher collagen type II content and usually contain also more collagen type X. To normalize collagen type X deposition to the degree of chondrogenesis of pellets, collagen type II was used as reference protein in western blot analysis. Densitometric analysis of protein bands from the same pellet reflected successful adjustment to collagen type II content and revealed no coherent tendency with respect to an increase or decrease of collagen type X deposition at termination of culture (Fig. 4A, B). As a result, neither the *COL10A1/COL2A1* mRNA ratio (Fig. 3B) nor the collagen type X/collagen type II protein ratio (Fig. 4C) was different between the PTHrP pulse and the control groups, indicating no specific effect of pulsed PTHrP on the hypertrophic marker collagen type X.

Pulsed application of PTHrP did not alter *PTHIR* mRNA expression levels versus the positive control, indicating no differential ligand sensitivity in response to intermittent

FIG. 3. Gene expression analysis of collagen type 2 (*COL2A1*) and collagen type 10 (*COL10A1*). MSC pellets were treated with chondrogenic medium alone (ctrl) or chondrogenic medium additionally supplemented with 2.5 nM PTHrP(1–34) either constantly (PTHrP const.) or intermittently (PTHrP pulse). RNA was extracted from MSC pellets at the indicated time points and mRNA levels of *COL2A1* and *COL10A1* were determined using quantitative real-time PCR and standardized to β -actin levels. (A) *COL2A1* and *COL10A1* expression on day 42 ($n=10$). (B) *COL10A1* mRNA levels were related to the *COL2A1* expression level in the same sample to standardize for the degree of chondrogenic induction ($n=10$). (C) Time course analysis of mRNA expression levels of *COL10A1* and *COL2A1* ($n=4$). Values are shown as the mean $\pm$ SD. ** $P \leq 0.01$ versus control, Wilcoxon test.

PTHrP treatment. However, samples with higher *PTH1R* expression levels showed a higher stimulation of *COL2A1* expression compared with samples with lower *PTH1R* expression levels. Pearson's coefficient of *PTH1R* and Δ *COL2A1* (*COL2A1* expression pulse group – *COL2A1* expression control group) demonstrated a significant correlation ($r^2=0.902$, $P<0.01$, $n=16$ samples from four experiments), suggesting that a correlation between ligand sensitivity and the extent of *COL2A1* stimulation may exist (Fig. 5B). At day 42, *IHH* mRNA levels were, however, significantly reduced under pulsed PTHrP treatment (Fig. 5C) (decrease to 70%, $P<0.05$) in line with an inhibitory effect of PTHrP on *IHH* expression leading to reduced hypertrophy. The mRNA levels of the terminal differentiation marker *MMP13* were not altered (Fig. 5D). Importantly, the enzyme activity of ALP in culture supernatants was significantly reduced in the pulsed PTHrP group from day 14 on, reaching 67% of control ALP levels at day 42 ($P<0.01$, Fig. 5E). Altogether this demonstrated that intermittent PTHrP supplementation of MSCs during chondrogenesis enhanced *COL2A1* expression and concomitantly reduced hypertrophy according to *IHH* levels and ALP enzyme activity.

Constant PTHrP(1–34) inhibits chondrogenesis via cAMP/PKA signaling

Since constant and intermittent application of PTHrP exerted opposing effects on MSC chondrogenesis, we sought to clarify whether these might be mediated via divergent signaling pathways. Forskolin is an activator of adenylate cyclase, whereas the N-terminally truncated PTHrP(7–34) fails to elicit a cAMP response. Detection of cAMP levels confirmed a stimulation of cAMP levels by 10 μ M forskolin in

FIG. 4. Western blot analysis of the collagen type X/collagen type II expression ratio of pellets from the untreated positive control group receiving transforming growth factor-beta (TGF- β) but no PTHrP (C) compared with pellets that additionally received intermittent PTHrP(1–34) (P) during chondrogenesis. (A) Collagens were extracted from individual pellets on day 42 and collagen type II content was determined by ELISA. ELISA values were used to load approximately equal amounts of type II collagen per lane for each collagen extract of the control group and the pulsed group. This was done to standardize the samples on the same degree of chondrogenesis. By cutting the blot, collagen type II and collagen type X from the same pellet were stained. Representative results from nine sample pairs from four donors are shown. (B) Densitometric analysis of the western blots. (C) The densitometric volume of the collagen type X bands was related to the corresponding collagen type II band of the same sample, shown as the mean $\pm$ SD. No significant differences were detected between the two groups, Wilcoxon test.

FIG. 5. Expression of PTH/PTHrP receptor (*PTH1R*), Indian hedgehog (*IHH*), *MMP13*, and alkaline phosphatase (ALP) enzyme activity. Pellets received chondrogenic medium alone (ctrl) or were additionally stimulated with 2.5 nM PTHrP(1–34) either constantly (PTHrP const.) or intermittently (PTHrP pulse). (A–D) RNA was extracted from MSC pellets and mRNA levels were determined using quantitative real-time PCR as described for Figure 3. (A) Time course analysis of *PTH1R* expression. (B) Correlation of *PTH1R* mRNA expression levels of samples of the PTHrP pulse group with the stimulation of *COL2A1* mRNA levels of the pulse group relative to *COL2A1* mRNA levels of the control group (*COL2A1* pulse group – *COL2A1* control group, Δ COL2A1). Shown are the single values of four independent experiments grouped by the indicated time points ($n = 16$). $r^2 = 0.902$, $P < 0.01$, Pearson's correlation. (C, D) *IHH* (B) and *MMP13* (C) expression at day 42. (E) ALP enzyme activity was measured in culture supernatants conditioned for 2 days each. (A, C–E) Values are given as the mean $\pm$ SD of 10 (*IHH* and ALP) or 4 (*MMP13* and *PTH1R*) independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$ versus control, Wilcoxon test. *MMP13*, matrix metalloproteinase.

MSCs at day 25 of chondrogenesis and a lack of cAMP signaling by PTHrP(7–34) but not PTHrP(1–34) treatment (Fig. 6A). In line, constant application of 2.5 nM PTHrP(1–34) or 10 μ M forskolin blocked chondrogenic differentiation, according to strongly decreased proteoglycan and collagen type II staining in histology, while 2.5 nM PTHrP(7–34) displayed no effect (Fig. 6B). To investigate how long it takes until constant PTHrP exposure displays its negative effect on *COL2A1* and *COL10A1* expression and on ALP enzyme activity and to decide whether small-molecule-induced activation of the cAMP/PKA pathway parallels timing of PTHrP(1–34) effects, chondrogenic pellets were treated for 2 or 4 days with PTHrP(1–34), forskolin, and the cAMP-analogue dbcAMP, respectively. Stimulation of the cAMP/PKA pathway with forskolin and dbcAMP from day 21 on reached a significant decline of *COL2A1* and *COL10A1* mRNA levels at 96 h but not 48 h (Fig. 6C) and paralleled the effects of PTHrP(1–34). This indicated that not hours but rather several days of PTHrP exposure are needed before negative effects on *COL2A1* expression become significant. Inhibition of ALP activity, however, was already significant after 48 h in all three treatment groups. This demonstrated that constant presence of PTHrP(1–34) during chondrogenesis influenced MSCs predominantly via activation of the cAMP/PKA pathway and may affect ALP enzyme activity earlier than *COL2A1* expression.

Intermittent PTHrP(1–34) affects proteoglycan and DNA via cAMP/PKA signaling

To test whether the effects of the intermittent PTHrP treatment may be explained by activation of the cAMP/PKA or the PKC pathway, MSCs were intermittently treated with either 2.5 nM PTHrP(1–34), 2.5 nM PTHrP(7–34), or 10 μ M forskolin. Intermittent forskolin, but not PTHrP(7–34), mimicked the positive effects of pulsed PTHrP(1–34) on proteoglycan (Fig. 7A) and DNA content of day-42 pellets (Fig. 7B). The significant suppression of ALP activity by pulsed PTHrP(1–34) to 61% at day 42 ($P < 0.05$, Fig. 7C) was, however, not fully imitated by intermittent forskolin treatment, which induced only a trend to lower ALP values (74%, $P = 0.063$). Pulsed PTHrP(7–34) could neither enhance proteoglycan and DNA content nor influence ALP activity (Fig. 7). In conclusion, positive effects of intermittent PTHrP(1–34) on MSC chondrogenesis were imitated by cAMP/PKA (forskolin) but not PKC [PTHrP(7–34)] signaling while ALP downregulation was not fully reproduced by either pathway.

Discussion

Under common in vitro differentiation protocols, chondrogenesis of MSCs leads to undesired phenotype instability

FIG. 6. Effects of agonists of PTHrP signaling on MSC chondrogenesis. **(A)** After 25 days of chondrogenic induction, pellets were treated for 1 h with dimethyl sulfoxide (DMSO), 10 μM Forskolin, 2.5 nM PTHrP(1–34), or 2.5 nM PTHrP(7–34) or were left untreated (control). Then, the cyclic adenosine monophosphate (cAMP) content of supernatants was determined by ELISA ($n=4$). Data show the mean $\pm$ SD, * $P \leq 0.05$ versus control. **(B)** Collagen type II-stained paraffin sections from day-42 pellets, treated with chondrogenic medium alone (control) or supplemented with 2.5 nM PTHrP(7–34), 2.5 nM PTHrP(1–34), or 10 μM forskolin. Representative results of three donors are shown. Scale bars represent 200 μm. **(C)** Pellets were cultivated under standard chondrogenic conditions up to day 21. Then, pellets received either chondrogenic induction medium supplemented with 2.5 nM PTHrP(1–34), 10 μM forskolin, or 1 mM dbcAMP for 48 h (corresponding to day 23) or for 96 h (corresponding to day 25) or remained under standard conditions (control). One group received constant PTHrP(1–34) (2.5 nM) from day 0 to 25 ($n=4$). *COL2A1* and *COL10A1* mRNA levels were determined as described in Figure 3. ALP activity was determined from supernatants conditioned for 2 days each (lower panel). Data show the mean $\pm$ SD normalized to control on day 21 set as 100%. * $P \leq 0.05$ versus all other groups, # $P \leq 0.05$ versus forskolin and dbcAMP, Mann–Whitney *U* test. Color images available online at www.liebertpub.com/scd

and premature hypertrophy of the developing chondrocytes. Due to its documented antihypertrophic properties, PTHrP is an interesting candidate molecule to improve chondrogenesis of MSCs; however, constant treatment of MSCs with PTHrP during chondrogenesis, unfortunately, resulted in a suppression of differentiation rather than a selective inhibition of hypertrophic features of chondrocyte development [6]. In view of a well-documented switch from a catabolic to an anabolic response in bone when PTH/PTHrP is administered intermittently instead of constantly [22], we here exposed MSCs to a pulsed instead of constant PTHrP regimen during chondrogenesis, in order to evoke beneficial effects on MSC chondrogenesis. This indeed allowed us to guide MSCs to chondrogenic differentiation under significant elevation of the DNA, proteoglycan, and collagen type II content of pellets and reduced signs of hypertrophy.

A significantly increased proteoglycan/DNA ratio argued in favor of the envisaged anabolic stimulation of the cells. Further, higher cell numbers per pellet suggested either a reduced loss of cells by apoptosis, which is abundant in MSC pellets during early chondrogenesis [36], and indeed PTHrP action has been linked to the prevention of apoptosis before [37]. Alternatively, pulsed PTHrP may have increased proliferation during pellet culture [33] in line with a documented influence on cell proliferation via cyclin D1 [38] and the cyclin kinase inhibitor p57 [39]. In the context of bone formation, the anabolic action of PTH was attributed to enhanced osteoblast or preosteoblast survival or differentiation and not to cell proliferation [40,41], but to unravel the exact mechanism operational in our anabolic chondrogenic setting, highly sensitive quantitative assays applicable to 3D culture are required.

Higher *COL2A1* mRNA levels versus controls were observed especially during the last 2 weeks of chondrogenesis

FIG. 7. Biochemical analysis after intermittent forskolin or PTHrP(7-34) treatment. Pellets received chondrogenic medium alone (ctrl) or were additionally stimulated for 6 h with either 2.5 nM PTHrP(1-34), 2.5 nM PTHrP(7-34), or 10 μ M forskolin (forsk) before each medium change, respectively. (A, B) On day 42, pellets were harvested and proteoglycan (A) and DNA content (B) were determined. The values are given as the mean $\pm$ SD of 5 [PTHrP(7-34)] or 10 (all other groups) independent experiments. (C) ALP enzyme activity was measured in culture supernatants conditioned for 2 days each. Values are given as the mean $\pm$ SD of five to six independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$, Wilcoxon test.

when PTHrP receptor levels are high, indicating an anabolic effect mainly on the more mature cells. This rules out that PTHrP just delayed the progression of chondrocyte development due to its presence for about 10% of the total time interval. Thus, enhanced proteoglycan deposition and *COL2A1* expression per cell is not just a result of keeping the cells longer in a matrix-producing stage, but it is a true anabolic action based on stimulation of gene expression via PTHrP. Several reports suggested that the anabolic action of PTH involves, at least in part, the action of insulin-like growth factor 1 (IGF-1) [19] and studies are ongoing to evaluate whether regulation of IGF-1 may also be important for stimulation of chondrogenesis.

Interestingly, we see no uniform tendency for *COL10A1* mRNA levels and collagen type X deposition in pellets. Yoshida et al. monitored the time course of *IHH*, patched 1 (*PTCH1*), osteopontin (*OPN*), and *COL10A1* mRNA inhibition by PTH(1-84) in chondrocytes in vitro [42] and found that *IHH*, *PTCH1*, and *OPN* mRNA expression was reduced rapidly within 4–6 h after PTH treatment, whereas *COL10A1* was downregulated only after 24 h. Others similarly reported a peak influence of PTHrP on collagen type X expression in growth plate chondrocytes after 24 h [43]. Pulsed PTHrP treatment for 6 h was obviously sufficient to effectively stimulate *COL2A1* expression but too short to exert a profound effect on *COL10A1* expression. After

longer PTHrP exposure at 48 h (day 23), mean mRNA levels for *COL2A1*, *COL10A1* (Fig. 6C), and *IHH* (not shown) were reduced and a significant downregulation was reached after 96 h. We here selected a PTHrP pulse duration of 6 h in accordance with a previous study, where 6 h—but not 1, 3, or 24 h—of pulsed PTH treatment exerted anabolic effects on osteoblastic monolayer cultures [19]. Further, 6 h but not constant treatment of rat MSCs with PTH in monolayer cultures reduced ALP activity [21]. In view of the clearly time-dependent effects of PTHrP on MSCs during chondrogenesis, it is next important to clarify whether a time window of PTHrP exposure may exist, in which not only ALP and *IHH* but also *COL10A1* can be downregulated while *COL2A1* is still stimulated. However, as *MMP13*, *PTH1R*, and *COL10A1* expression was not significantly affected by intermittent treatment with PTHrP compared with the positive control, this clearly indicates that hypertrophic maturation is further linked to other signaling pathways apart from PTHrP signaling, such as the transforming growth factor-beta (TGF- β) superfamily pathways [44].

Interestingly, ALP activity in pellets is often more intense around cells located in the periphery of the pellet in areas of less proteoglycan deposition possibly due to higher oxygen and nutrient exposure favoring endochondral differentiation [9]. These cells often do not look like chondrocytes but have a more fibroblastic or osteoblast-like appearance and may contribute a large part of the ALP secreted into culture supernatants. The distance of PTHrP diffusion is an important parameter in growth plate development and the rate of diffusion of PTHrP may strongly depend on the presence and composition of the cartilaginous matrix. Thus, it is tempting to speculate that in our model peripheral cells experience higher concentration of PTHrP or longer PTHrP exposure than chondrocytes in the pellet center. If the antihypertrophic action of PTHrP may guide more cells in the periphery toward a chondrogenic instead of endochondral development, then this could contribute to more matrix deposition per pellet and reduced ALP activity in supernatants. According to Figure 1C, some pellets exposed to pulsed PTHrP indeed seemed to be devoid of a noncartilaginous peripheral cell layer; however, this was not a consistent observation throughout. A spatially restricted or stronger inhibition of the peripheral cells by pulsed PTHrP could also allow more nutrients and oxygen to pass into the pellet center, stimulating improved cartilage matrix deposition by an indirect effect even not requiring PTHrP signaling in the chondrocytes themselves. However, the importance of *PTH1R* signaling duration is also seen in osteoblast monolayers, where 1 h and continuous PTH stimulation decreased ALP activity of rat osteoblasts, while 3- and 24-h stimulation had no effect and 6 h stimulated ALP activity [19]. Further, we reproduced most of the effects of pulsed PTHrP by the small-molecule inhibitor forskolin having different diffusion kinetics, suggesting that 3D effects are only of minor importance for an explanation of anabolic versus catabolic PTHrP effects in MSC chondrogenesis.

Overall, we encountered high donor variabilities leading to high standard deviations and the need to provide a high sample number to achieve statistical significance throughout all experiments. A high donor variability regarding chondrogenic progression of MSCs in pellet culture and in parameters of chondrogenic matrix deposition

is well established in the field. This striking interdonor variability and even intradonor variation between individual pellet samples was a main topic of one of our recent studies [45]. We demonstrated that the proliferation capacity of MSCs at initiation of chondrogenic culture strongly correlated with speed of chondrogenic progression during culture and with outcome. Obviously high anabolic conditions favor reduced apoptosis and faster start of chondrogenesis since less cells may die and more cells can enter matrix deposition under such conditions. Further, cells underwent a transient proliferation stage essential for a successful chondrogenesis starting earlier in “fitter” cultures compared with others [33]. We here examined all pellets at fixed time points after switch to induction conditions, not taking into account the individual timing of the sample population. Therefore the cells may be in different maturation stages at the time of investigation, yielding different expression levels of chondrogenic and hypertrophic markers. In line with this, the standard deviation usually increases with time in culture and typically is highest at the late time points.

PTHrP signals via numerous divergent pathways [46,47], but most of its impact on chondrocyte hypertrophy [48,49] as well as the PTH/PTHrP-mediated anabolic effects on bone mass or osteoblast differentiation [50,51] has been linked to the cAMP/PKA-dependent pathway. Little was so far known about the molecular mechanisms of PTHrP action on MSCs during in vitro chondrogenesis. In line with the previous results we show here for the first time that stimulation of cAMP/PKA signaling by forskolin was able to fully reproduce both the negative effects of constant PTHrP application and the positive anabolic effects of intermittent PTHrP exposure on chondrogenic extracellular matrix deposition. PTHrP(7–34) capable of PKC but not of PKA activation [15,16,52] instead was ineffective under both application regimes. This demonstrated that PTHrP exerted its catabolic and anabolic influence on MSC chondrogenesis mainly via the cAMP/PKA pathway under both modes of administration, underlining that signal duration is the decisive variable. Differential duration of PKA signals may feed back on PTH1R expression levels, ligand sensitivity of cells, and crosstalk with other downstream signaling pathways, leading to receptor desensitization or altered availability of costimulatory molecules and repressors to regulate gene transcription. In line with the first two explanations, *PTH1R* is not upregulated during chondrogenesis of MSCs under constant PTHrP exposure [6] and samples with high *PTH1R* levels responded with a higher stimulation of *COL2A1* expression compared with samples with lower levels as evident from the strong correlation of receptor levels with this anabolic response (Fig. 5B).

Besides its ability to mimic PTHrP effects on MSC anabolism, forskolin only showed a nonsignificant tendency to reduce ALP activity. It is possible that higher concentrations of forskolin would have induced more pronounced effects, but cAMP generation of forskolin at the chosen concentration of 10 μ M nearly equaled that of 2.5 nM PTHrP(1–34) and therefore represented a good approximation to the used PTHrP concentration. Ishizuya et al. proposed a cooperative contribution of both PKA and PKC activation on the anabolic stimulation of osteoblasts, where only a simultaneous activation of both pathways could mi-

mic anabolic intermittent PTH effects [19]. We therefore also tested a combined administration of pulsed PTHrP(7–34) and forskolin, which had, however, no additional effect to forskolin in our model (data not shown). Thus, the cAMP/PKA pathway seems to be dominant for the prochondrogenic and antihypertrophic effects of intermittent PTHrP in MSC chondrogenesis, whereas involvement of the PKC-dependent pathway seems less important. Additional studies on the noncanonical pathways of PTH1R signaling are, however, needed to fully clarify this issue.

In conclusion, intermittent treatment of MSCs during chondrogenesis represents a novel and simple means to augment cartilaginous matrix deposition together with a simultaneous suppression of undesired hypertrophic features. In spite of opposing effects of constant versus intermittent PTHrP application on chondrogenic differentiation markers, both treatment modes influenced the MSCs mainly via the cAMP/PKA-dependent pathway, suggesting that PKC signaling is less important. Overall our data are encouraging to further optimize signal duration and pulse frequency, in relation to agonist concentration, to fully deploy the potential of pulsed PTHrP application on MSC chondrogenesis, and in order to try to group *COL10A1* regulation together with the other hypertrophic markers of reduced expression. In this context, ectopic transplantation of treated pellets in mice will be important to verify that the beneficial results of pulsed PTHrP treatment are not just a transient effect.

In view of this prochondrogenic and antihypertrophic effect seen in vitro and first promising in vivo results [28], it is now attractive to consider PTHrP injections, as used for osteoporosis prevention, as a means to support MSC-based cartilage regeneration strategies in a clinical setting.

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Author Disclosure Statement

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