ARTHRITIS & RHEUMATISM
Vol. 62, No. 9, September 2010, pp 2696–2706
DOI 10.1002/art.27565
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Human Articular Chondrocytes Secrete Parathyroid Hormone–Related Protein and Inhibit Hypertrophy of Mesenchymal Stem Cells in Coculture During Chondrogenesis

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Objective. **The use of bone marrow–derived mesenchymal stem cells (MSCs) has shown promise in cell-based cartilage regeneration. A yet-unsolved problem, however, is the unwanted up-regulation of markers of hypertrophy, such as alkaline phosphatase (AP) and type X collagen, during in vitro chondrogenesis and the formation of unstable calcifying cartilage at heterotopic sites. In contrast, articular chondrocytes produce stable, nonmineralizing cartilage. The aim of this study was to address whether coculture of MSCs with human articular chondrocytes (HACs) can suppress the undesired hypertrophy in differentiating MSCs.**

Methods. **MSCs were differentiated in chondrogenic medium that had or had not been conditioned by parallel culture with HAC pellets, or MSCs were mixed in the same pellet with the HACs (1:1 or 1:2 ratio) and cultured for 6 weeks. Following in vitro differentiation, the pellets were transplanted into SCID mice.**

Results. **The gene expression ratio of COL10A1 to COL2A1 and of Indian hedgehog (IHH) to COL2A1 was significantly reduced by differentiation in HAC-conditioned medium, and less type X collagen protein was deposited relative to type II collagen. AP activity was significantly lower ($P < 0.05$) in the cells that had been differentiated in conditioned medium, and transplants showed significantly reduced calcification in vivo. In mixed HAC/MSC pellets, suppression of AP was dose-dependent, and in vivo calcification was fully inhibited. Chondrocytes secreted parathyroid hormone–related protein (PTHrP) throughout the culture period, whereas PTHrP was down-regulated in favor of IHH up-regulation in control MSCs after 2–3 weeks of chondrogenesis. The main inhibitory effects seen with HAC-conditioned medium were reproducible by PTHrP supplementation of unconditioned medium.**

Conclusion. **HAC-derived soluble factors and direct coculture are potent means of improving chondrogenesis and suppressing the hypertrophic development of MSCs. PTHrP is an important candidate soluble factor involved in this effect.**

Due to the limited self-repair capacity of articular cartilage and the lack of efficient pharmacologic treatments for chondral defects, cell-based approaches for articular cartilage regeneration have been developed. One of these approaches, autologous chondrocyte transplantation (ACT), has been used with encouraging clinical results (1–5). For ACT, chondrocytes are harvested by biopsy of a non–weight-bearing region of the damaged joint, expanded ex vivo, and then reinjected into the site of the defect. Among the limitations of ACT are a paucity of the cell source and tissue damage at the donor site, with the risk of emerging osteoarthritis. To overcome these problems, adult mesenchymal stem cells (MSCs) have been proposed as an alternative cell source. MSCs can be easily obtained from different sources, such as bone marrow (6,7) and adipose tissue (8), and possess good proliferation and differentiation potential, including differentiation into a chondrogenic phenotype (8,9).

Supported in part by the German Research Foundation (DFG grant Ri707/7-1), the German Ministry of Education and Research (grants 0313.755 and 0315.579), and Orthopaedic University Hospital Heidelberg.

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Submitted for publication January 18, 2010; accepted in revised form May 11, 2010.

Chondrogenesis is a complex and tightly regulated process, the underlying molecular mechanisms of which are not yet fully understood. During early chondrogenesis, progenitor cells condense and differentiate into resting chondrocytes, producing aggregating proteoglycans and types II, IX, and XI collagen. This phenotype is stably maintained in the hyaline cartilage of joints, whereas further differentiation occurs during endochondral ossification in the development and growth of bones. These maturing chondrocytes proliferate and subsequently become hypertrophic, characterized by a marked increase in metabolic activity and cell volume. Hypertrophic cells deposit large quantities of extracellular matrix, including type X collagen, which is a marker of this stage of differentiation (10). Hypertrophic chondrocytes also begin to produce alkaline phosphatase (AP), an enzyme involved in matrix mineralization, marking the late stage of terminal differentiation. Mineralized cartilage is then invaded and replaced by bone cells and bone marrow cells.

To better understand the chondrogenesis of MSCs as well as to produce MSC-derived tissue-engineered cartilage for use in cartilage repair, various protocols for the in vitro chondrogenesis of MSCs have been developed. These include pellet culture of cells in serum-free chondrogenic medium supplemented with dexamethasone and transforming growth factor β (TGFβ) (11–13). This pellet culture results in the up-regulation of type II collagen and proteoglycans, but it also up-regulates markers of hypertrophy, such as type X collagen, AP, and matrix metalloproteinase 13 (MMP-13) (11,14,15). Predifferentiated chondrogenic MSC constructs transplanted subcutaneously into immunodeficient mice have been shown to undergo calcification, vascular invasion, and micro-ossicle formation (15), suggesting the formation of undesirable, transient cartilage reminiscent of endochondral ossification, rather than stable articular cartilage. In contrast, articular chondrocytes have been shown to maintain their nonhypertrophic phenotype and to be capable of stable ectopic cartilage formation, unless they are subjected to too extensive dedifferentiation by monolayer culture (15,16).

Improvement in the differentiation protocols is therefore needed in order to generate MSC-derived chondrocytes that have a stable, nonhypertrophic phenotype. This is particularly challenging because the differentiation of MSCs reflects the natural pathway of endochondral ossification, and the molecular events that trigger terminal differentiation and how this can be suppressed remain largely unclear.

Interestingly, coculture experiments have demonstrated that immature or articular chondrocytes produce soluble factors that are able to suppress the terminal differentiation of maturing growth plate chondrocytes in vitro (17–19). This indicates that resting chondrocytes maintain their stable phenotype by actively inhibiting terminal differentiation and could potentially teach differentiating MSCs to become stable chondrocytes in coculture. It has been suggested that coculture-induced mechanisms may rely on the soluble factors TGFβ2 and fibroblast growth factor 2 (FGF-2) acting in synergy (17), whereas other postulated mechanisms are TGFβ-independent (18).

Three published studies have addressed the coculture of MSCs and articular chondrocytes; however, those studies focused on the promotion of chondrogenesis of MSCs (20–22). Two of the studies found positive effects on the induction of type II collagen messenger RNA (mRNA) and/or protein. However, the animal MSCs that were chosen for study (20,21) also exhibited chondrocyte marker induction, including type II collagen, in the absence of exogenous TGFβ and thus displayed some degree of autoinduction. While this has not been observed with primary human MSCs, it may apply to the immortalized human MSC line that was used in coculture with immortalized human chondrocytes in the third study (22).

It thus remains unknown whether primary human articular chondrocytes (HACs) can suppress the hypertrophic development of primary human MSCs in coculture and prevent ectopic matrix calcification. Such knowledge is not only important for unraveling the molecular mechanisms and mediators of articular chondrocyte phenotype stability that are relevant to osteoarthritis (23,24), but may also help to further improve clinical stem cell–based cartilage repair strategies.

In the present study, we therefore sought to determine whether direct or indirect coculture with HACs is able to suppress hypertrophy during TGFβ-driven chondrogenesis of MSCs and whether either technique will reduce the calcification of ectopic transplants in vivo. We further searched for candidate soluble mediators of this action in a novel indirect coculture system in which conditioned medium from 3-dimensional (3-D) chondrocyte pellet cultures was transferred to parallel 3-D MSC-pellet cultures during a followup period of 6 weeks.

MATERIALS AND METHODS

Isolation and expansion of MSCs and chondrocytes. MSCs were isolated from fresh bone marrow samples obtained

from patients undergoing total hip replacement or iliac bone graft harvest, as described elsewhere (25). Briefly, cells were fractionated on a Ficoll-Paque Plus density-gradient (GE Healthcare), and the low-density cell fraction was washed and seeded in expansion medium (7,14) consisting of high-glucose Dulbecco's modified Eagle's medium (DMEM), 40% MCDB 201 medium, 2% fetal calf serum (FCS), 2×10^{-8}*M* dexamethasone, 10^{-4}*M* ascorbic acid 2-phosphate, 10 μg/ml of insulin, 10 μg/ml of transferrin, 10 ng/ml of sodium selenite, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 10 ng/ml of recombinant epidermal growth factor (Miltenyi Biotec), and platelet-derived growth factor BB (Active Bioscience). Nonadherent material was removed after 24–48 hours. For expansion, cells were replated at a density of 5×10^3 cells/cm^2 and used at passage 3.

Samples of human articular cartilage were obtained from patients undergoing total knee replacement surgery. Cartilage from regions with no evident degeneration was harvested, minced, and digested overnight with 1.5 mg/ml of collagenase B and 0.1 mg/ml of hyaluronidase. Washed chondrocytes were plated at 5×10^3 cells/cm^2 and expanded for 1 passage in low-glucose DMEM supplemented with 10% FCS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin.

Chondrogenic differentiation of MSCs. Pellets consisting of 5×10^5 expanded MSCs were exposed to chondrogenic medium (high-glucose DMEM with 0.1 μ*M* dexamethasone, 0.17 m*M* ascorbic acid-2 phosphate, 5 μg/ml of insulin, 5 μg/ml of transferrin, 5 ng/ml of selenous acid, 1 m*M* sodium pyruvate, 0.35 m*M* proline, 1.25 mg/ml of bovine serum albumin, 100 units/ml of penicillin, and 100 μg/ml of streptomycin) supplemented with 10 ng/ml of recombinant human TGFβ1 (PeproTech) and cultivated for 6 weeks. Where indicated, 10 ng/ml or 1.2 ng/ml of parathyroid hormone–related protein (1–34) (PTHrP[1–34]) (Bachem) was added from day 21 or day 14, respectively.

Chondrogenic differentiation of MSCs in conditioned medium. Pellets consisting of 5×10^5 expanded HACs were cultivated in parallel with MSC pellets (2.5×10^5 cells) in chondrogenic medium containing 10 ng/ml of TGFβ1. HAC pellets and MSC control pellets received 250 μl or 200 μl of fresh medium, respectively, 3 times a week. The MSC-conditioned medium group received 66.6% of centrifugation-cleared (10 minutes at 660*g*) conditioned medium from the HAC pellets. This was further supplemented with 33.3% fresh chondrogenic medium. Fresh TGFβ1 was kept constant at 2 ng per feeding for all MSC cultures.

Chondrogenic differentiation of MSCs in mixed pellets. For direct coculture experiments, 2.5×10^5 MSCs were mixed with 2.5×10^5 or with 5×10^5 chondrocytes prior to pellet formation. Pellets consisting of 2.5×10^5 MSCs or 7.5×10^5 HACs were used as a control. All cells were cultured in chondrogenic medium supplemented with 10 ng/ml of TGFβ1.

RNA isolation and quantitative real-time polymerase chain reaction (PCR). A total of 3–4 pellets per donor and per group were pooled and then minced, and total RNA was isolated with a phenol/guanidine isothiocyanate extraction reagent (peqGOLD TriFast; PeqLab). Polyadenylated mRNA was purified from total RNA with oligo(dT)-coupled magnetic beads (Dynabeads; Invitrogen) according to the manufacturer's instructions. The mRNA was then subjected to first-strand complementary DNA (cDNA) synthesis using Sensiscript reverse transcriptase (Qiagen) and oligo(dT) primers. The expression levels of individual genes were analyzed by quantitative PCR using a LightCycler (Roche) and the following forward and reverse primer pairs: for β-actin, 5′-CTCTTCCAGCCTTCCTTCCT-3′ and 5′-CGATCCACACGGAGTACTTG-3′; for COL2A1, 5′-TGGCCTGAGACAGCATGA-3′ and 5′-AGTGTTGGGAGCCAGATTGTC-3′; for COL10A1, 5′-CCCTTTTTGCTGCTAGTATCC-3′ and 5′-CTGTTGTCCAGGTTTTCCTGGCAC-3′; for COL1A1, 5′-GATGCCAATGTGGTTCGTGA-3′ and 5′-TCAGCTGGATGGCCACATC-3′; for AP, 5′-CACCAACGTGGCTAAGAATG-3′ and 5′-ATCTCCAGCCTGGTCTCCTC-3′; for MMP-13, 5′-CTGGAGATATGATGATACTAAC-3′ and 5′-CACGCATAGTCATATAGATACT-3′; for IHH, 5′-CGACCGCAATAAGTATGGAC-3′ and 5′-GGTGAGCGGGTGTGAGTG-3′; and for PTHrP (26), 5′-CGGTGTTCCTGCTGAGCTA-3′ and 5′-TGCGATCAGATGGTGAAGGA-3′.

The number of cDNA copies was correlated with the apparent threshold cycle (C_t). Building the difference between C_t of the gene of interest and the C_t of β-actin (housekeeping gene) from each sample gave ΔC_t values that were expressed as a percentage of β-actin.

Measurement of AP activity. Two-day culture supernatants (6 pellets per group) were collected, pooled, and incubated with substrate solution (10 mg/ml of *p*-nitrophenyl phosphate in 0.1*M* glycine, 1 m*M* $MgCl_2$, and 1 m*M* $ZnCl_2$, pH 9.6). AP activity was measured spectrophotometrically at 405/490 nm.

Histologic assessment. Pellets were fixed in 4% paraformaldehyde as described previously (27). Sections (5 μm) were stained with 1% Alcian blue (Waldeck Division of Chroma) and counterstained with fast red (Sigma-Aldrich). Immunohistologic staining for type II and type X collagen was performed as described elsewhere (14). Briefly, sections were incubated with a monoclonal mouse anti-human type II collagen antibody (clone II-4C11; MP Biomedicals). Reactivity was detected using biotinylated goat anti-mouse antibody (Dianova), streptavidin–alkaline phosphatase (Dako), and fast red. To detect calcification, sections were stained with alizarin red (0.5%; Chroma) and counterstained with Certistain fast green FCF (0.04% in 0.1% acetic acid; Merck). The percentage of alizarin red–stained area compared with the total area of 2 pellets per donor and per group was rated by 2 observers who were blinded to the study group. A semiquantitative histologic scoring system was used, with a scale of 0–4, where 0 = no alizarin red staining, 1 = >0% but ≤25% alizarin red–stained area, 2 = >25% but ≤50% alizarin red–stained area, 3 = >50% but ≤75% alizarin red–stained area, and 4 = >75% but ≤100% alizarin red–stained area. Bone formation was evaluated after toluidine blue (28) and osteocalcin (29) staining.

Combined staining for AP and proteoglycan. Pellets were fixed in phosphate buffered saline (PBS) containing 4% paraformaldehyde, washed with PBS, and embedded in Tissue-Tek (Sakura Finetek). Cryosections (10 μm) were prepared, washed with Tris buffered saline, pH 7.6, and incubated with 20 μl of nitroblue tetrazolium/BCIP stock solution (Roche Diagnostics) per milliliter of 0.1*M* Tris, pH 9.5. The reaction was stopped by washing with PBS. Safranin O was used to visualize proteoglycans on AP-stained sections.

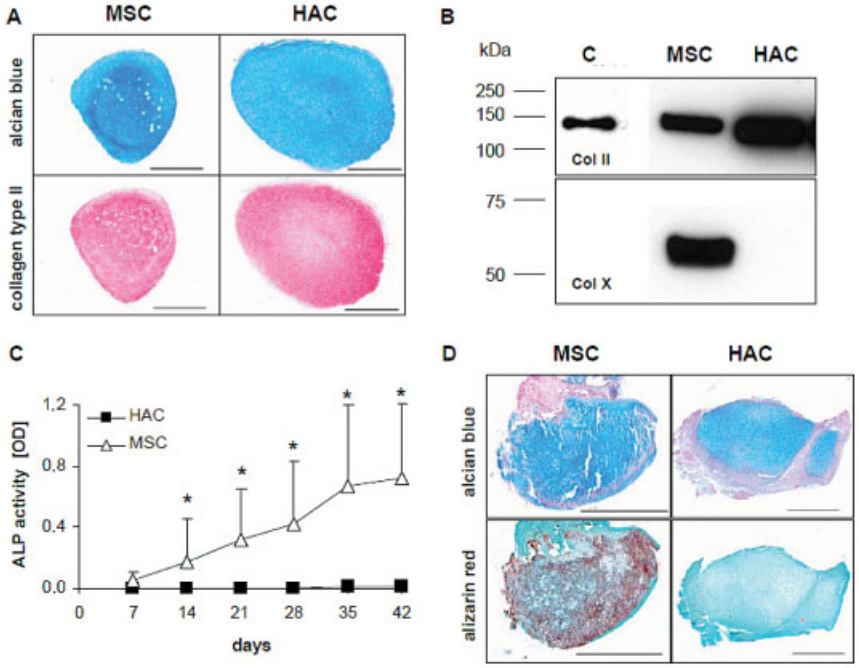

Figure 1. Characterization of the differences between chondrogenic mesenchymal stem cells (MSCs) and human articular chondrocytes (HACs). HAC and MSC pellets were cultured for 6 weeks in chondrogenic medium containing 10 ng/ml of transforming growth factor β. **A,** Paraffin sections of MSC and HAC pellets stained for proteoglycans and for type II collagen show cartilaginous matrix deposition. **B,** Collagens were extracted from MSC and HAC pellets and subjected to Western blotting. A type II collagen standard sample (c) was included. The gel was cut at ~85 kd and the upper part was stained for type II collagen (Col II), the lower part for type X collagen (Col X). Molecular weight markers are shown on the left. **C,** Culture supernatants of MSC and HAC pellets were collected during chondrogenesis, and the alkaline phosphatase (ALP) activity per 250,000 cells and after 48 hours of secretion was determined. In contrast to MSCs, HACs secreted no alkaline phosphatase. Values are the mean and SD optical density (OD) obtained from 9 independent experiments. $* = P < 0.05$. **D,** After 6 weeks of in vitro chondrogenic preinduction, pellets were transplanted subcutaneously into SCID mice for 4 weeks. Sections of explants were stained for proteoglycan deposition by Alcian blue or for mineral deposition with alizarin red/fast green. Only MSC-derived pellets underwent calcification. Bars = 500 μm.

Subcutaneous transplantation into SCID mice. After 6 weeks of culture under chondrogenic conditions in vitro, the pellets were transplanted into subcutaneous pockets that had been prepared in the upper dorsal area of anesthetized SCID mice (15). After 4 weeks, the transplants were excised and analyzed histologically. All animal experiments were approved by the local animal experimentation committee in Karlsruhe.

Collagen extraction and Western blotting. Pellets were homogenized and subjected to pepsin digestion overnight at 4°C (0.5*M* acetic acid, 0.2*M* NaCl, and 2.5 mg/ml of pepsin). The pH was then adjusted to neutral pH 7 with 1*M* Tris Base prior to extraction of the collagens with 4.5*M* NaCl (overnight at 4°C). The following day, the extracted collagens were pelleted by centrifugation at 16,000*g* at 4°C for 30 minutes and subsequently precipitated with 400 μl of precipitation buffer (0.4*M* NaCl and 0.1*M* Tris Base, pH 7.4) and 1,200 μl of ethanol per sample for 4 hours at –20°C. The precipitated collagens were pelleted by centrifugation at 16,000*g* for 30 minutes at 4°C and resolved in 50 μl of lysis buffer (1% Triton X-100, 150 m*M* NaCl, and 50 m*M* Tris, pH 8.0).

The type II collagen content of each lysate was determined by enzyme-linked immunosorbent assay (ELISA) (Chondrex). For Western blotting, either equal amounts of each lysate or, where indicated, amounts normalized to the type II collagen content of the corresponding control sample of the same donor, were separated by denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were blotted onto a nitrocellulose membrane (Amersham Biosciences). The lower part of the membrane was probed with mouse anti–type X collagen antibody (clone X-53; Quartett), and the upper part was probed with mouse anti–type II collagen antibody (MP Biomedicals). Bands were visualized with peroxidase-coupled goat anti-mouse antibody (Jackson ImmunoResearch) using an ECL detection system from Roche Diagnostics.

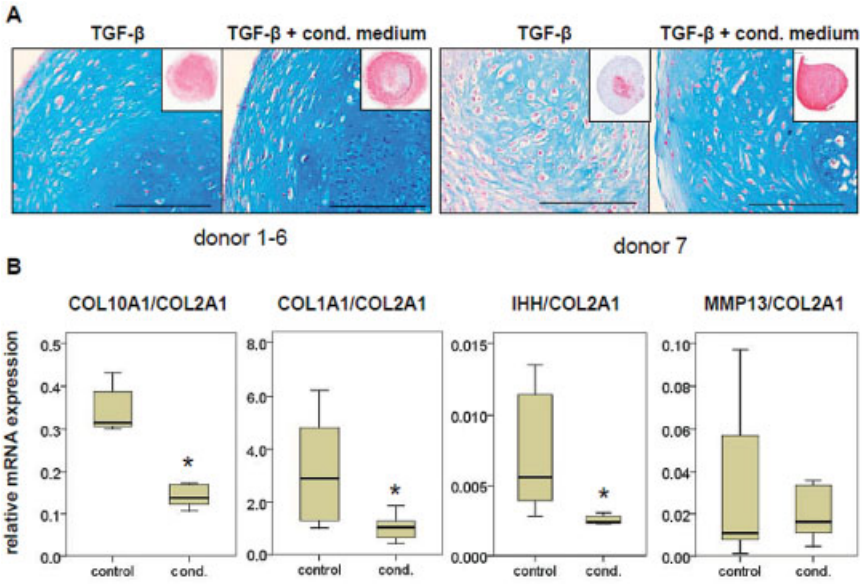

Figure 2. Effect of conditioned medium on MSC chondrogenesis. MSC pellets were analyzed after 6 weeks of chondrogenic induction in the presence or absence of HAC-conditioned medium. **A,** Paraffin sections of MSC control (TGFβ) or conditioned (TGFβ + cond. medium) pellets were stained for proteoglycan (main image) and type II collagen (**inset**) deposition. Similar staining for both groups was observed with samples from donors 1–6, whereas improved chondrogenesis was evident in conditioned medium from donor 7. Bars = 200 μm. **B,** The expression of COL10A1, COL1A1, and Indian hedgehog (IHH), but not matrix metalloproteinase 13 (MMP-13), was down-regulated relative to COL2A1 expression by treatment with conditioned medium. On day 42, RNA was extracted from MSC pellets, and mRNA levels were determined by quantitative real-time polymerase chain reaction analysis. The expression of COL10A1, COL1A1, IHH, and MMP-13 was standardized to the β-actin signals and was subsequently related to the COL2A1 mRNA level in the same sample to standardize for the degree of chondrogenic induction. Data are shown as box plots. Each box represents the interquartile range (IQR) extending between the 25th and 75th percentiles. Lines inside the boxes represent the median of 7 independent experiments. Whiskers extend to a maximum of 1.5 IQR. $* = P < 0.05$. See Figure 1 for other definitions.

Determination of PTHrP and FGF-2 protein in culture supernatants. The PTHrP content of culture supernatants was determined by a peptide enzyme immunoassay from Bachem. The FGF-2 content was measured by ELISA (R&D Systems).

Statistical analysis. The mean and SD values were calculated for all variables. The nonparametric Wilcoxon test (for paired analyses) or Mann-Whitney test (unpaired analyses) were applied to analyze differences between time points and between groups. P values less than or equal to 0.05 (2-tailed exact test) were considered significant. Data analysis was performed with SPSS for Windows version 16.0.

RESULTS

Comparison of HACs and MSC-derived chondrocytes. When HACs or MSCs were subjected to 6 weeks of culture under chondrogenic conditions, both groups of cells showed deposition of a proteoglycan-rich and type II collagen–rich extracellular matrix, as determined histologically (n = 8 donors) (Figure 1A). Western blot analysis demonstrated that only MSCs, and not HAC pellets, contained type X collagen (n = 10 donors) (Figure 1B). Only MSC cultures showed AP induction, while HACs remained negative throughout (n = 9 donors) (Figure 1C). When the pretreated pellets were transplanted into the subcutaneous pouches of SCID mice, all MSC-derived pellets underwent mineralization and formed calcified cartilage but no osteocalcin-positive bone tissue. In contrast, no mineral deposition was evident in any of the HAC-derived pellets (Figure 1D). Thus, HACs from passage 1 maintained a stable, nonhypertrophic phenotype even after 6 weeks of chondrogenic culture, whereas under the same conditions, MSCs were prone to hypertrophy and matrix calcification.

Chondrogenesis altered in chondrocyte-conditioned medium. Among the MSC cultures derived from 7 different donors that were continuously exposed to HAC-conditioned medium, 6 cultures revealed histologic features similar to those of the corresponding unconditioned control pellets. One of the control group cultures was not fully differentiated (possibly due to donor-dependent features), but showed strong type II collagen and proteoglycan deposition in conditioned medium (Figure 2A). This indicated a positive effect of conditioned medium on chondrogenic differentiation.

Consistent with this finding, the expression of COL2A1 revealed a trend toward increased mRNA levels (mean 180%; *P* not significant) in the conditioned medium group as compared with the unconditioned control pellets, whereas the mean COL10A1 (86%; *P* not significant) and the mean IHH (86%; *P* not significant) mRNA levels were lower. (Data showing the effect of indirect coculture on the gene expression are available upon request from the author.) In order to standardize gene expression to the degree of chondrogenic differentiation of each donor culture, the gene expression values were divided by the COL2A1 mRNA values from the same samples. A significant decrease in the mRNA expression ratio of COL10A1 to COL2A1 (to 32% of the mean levels; $P = 0.031$) (Figure 2B), COL1A1 to COL2A1 (14%; $P = 0.016$), and IHH to COL2A1 (18%; $P = 0.031$) was evident in the conditioned medium group as compared with the control group (set at 100%). In contrast, the mRNA expression ratio of MMP-13 to COL2A1 showed no significant alteration between the 2 groups (Figure 2B). This indicated a specific suppression of the relative COL10A1, COL1A1, and IHH levels by soluble factors released from chondrocytes.

Lower levels of type X collagen deposition in chondrocyte-conditioned medium. Consistent with the levels of the mRNA transcripts, a trend toward increased deposition of type II collagen in pellets cultured in conditioned medium did not reach statistical significance (250%; $P = 0.156$) (Figure 3A). The type X collagen content of conditioned MSC pellets was, however, always considerably lower than that of the corresponding control pellets when lysates of similar type II collagen content were compared by Western blot analysis (Figure 3B). Thus, HAC-derived soluble factors induced a trend toward improved chondrogenic differentiation and lowered the specific amount of type X collagen deposition relative to that of type II collagen, suggesting the potential to suppress hypertrophy during chondrogenesis.

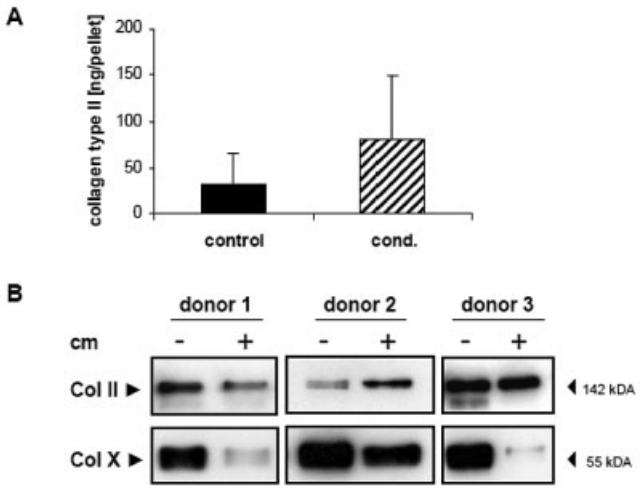

Figure 3. Effect of conditioned medium on types II and X collagen protein deposition. **A,** The type II collagen content per MSC pellet was determined by enzyme-linked immunosorbent assay (ELISA). A trend toward enhanced levels of type II collagen was evident for the conditioned medium group. Values are the mean and SD of MSC samples from 7 donors. **B,** ELISA values were used to load approximately equal amounts of type II collagen per lane for the control extract (–) and the corresponding extract from the conditioned medium (cm) group (+). This was done to standardize the samples on the same degree of chondrogenesis. By cutting the blot so that the upper half was stained for type II collagen and the lower half for type X collagen, both types of collagen could be compared in the same sample. Deposition of type X collagen was reduced in relation to type II collagen in the conditioned medium group, as determined by Western blot analysis. Representative samples from 3 of 9 donors are shown. See Figure 1 for other definitions.

Reduction of AP activity by treatment with chondrocyte-conditioned medium. There were significantly lower AP mRNA levels in conditioned MSC pellets as compared with controls (Figure 4A). Consistent with that finding, the AP enzyme activity was much weaker in cryosections of pellets cultured in conditioned medium as compared with the control group (Figure 4B). AP enzyme activity in culture supernatants of pellets treated with conditioned medium rose later during chondrogenesis and remained significantly lower as compared with the control values (Figure 4C), whereas HAC pellets remained AP-negative throughout culture (Figure 1). Taken together, these findings demonstrated that HAC-derived soluble factors reduced the production and/or activation of AP, an enzyme that is crucial for matrix mineralization.

Reduced calcification of MSC pellets treated with chondrocyte-conditioned medium. To test whether the reduced AP activation may have long-term effects on the mineralization of MSC pellets in vivo, corresponding

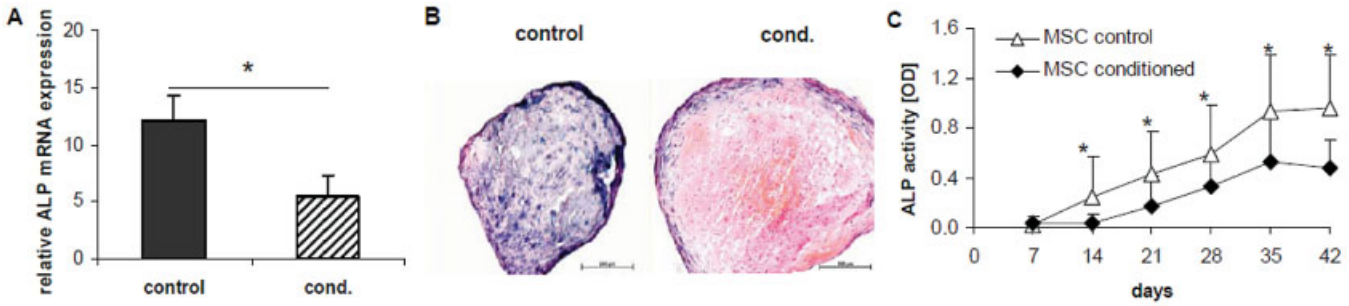

Figure 4. Effect of conditioned medium on alkaline phosphatase activity. MSC pellets were cultured for 6 weeks with or without conditioned medium. **A,** After 6 weeks of chondrogenic induction, mRNA was extracted from MSC pellets. Lower relative levels of mRNA for alkaline phosphatase (standardized to the β-actin signals) were obtained by quantitative real-time polymerase chain reaction analysis of pellets cultured in conditioned medium. Values are the mean and SD of 7 independent experiments. $* = P = 0.031$. **B,** Visualization of alkaline phosphatase activity in cryosections of MSC pellets by nitroblue tetrazolium/BCIP reveals less activity in conditioned MSC pellets. Proteoglycans were stained with Safranin O. Bars = 200 μm. **C,** Culture supernatants were collected during chondrogenesis and analyzed for alkaline phosphatase activity per 250,000 cells and after 48 hours. Values are the mean and SD OD obtained from 6 independent experiments. $* = P < 0.05$. See Figure 1 for definitions.

pellets were ectopically transplanted into SCID mice. At 4 weeks, the explants still contained proteoglycan (Figure 5). Semiquantitative histomorphometric rating of calcification after alizarin red staining revealed significantly reduced mineralization of MSC pellets treated with conditioned medium (median score 2; n = 5 donors) as compared with the control pellets (median score 4; $P = 0.001$). This analysis demonstrated that MSC pellets acquired differences during in vitro culture in HAC-conditioned medium that mediated a reduced mineralization of their extracellular matrix after in vivo transplantation.

Reduced hypertrophy and calcification by direct coculture with chondrocytes. Since we were unable to fully suppress AP activity and in vivo calcification by indirect coculture, we wanted to determine whether direct coculture could intensify the effects of HACs on the differentiation of MSCs. This was tested in mixed pellets containing an equal or double amount of chondrocytes as compared with MSCs. Culture supernatants from the last 2 days of a 6-week in vitro culture period showed significantly decreased AP activity for the 1:1 mixed-pellet group (to 43%; $P = 0.031$ versus control) (Figure 5I). This effect was significantly enhanced in the

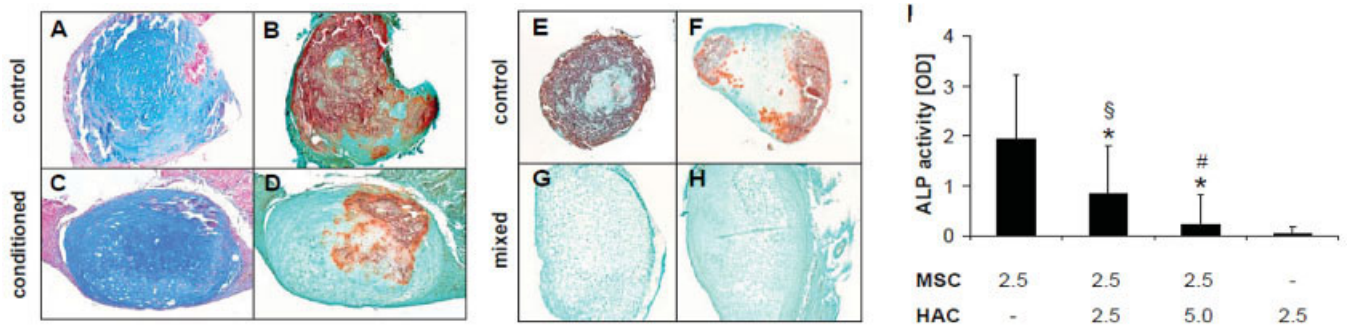

Figure 5. Effect of HAC-conditioned medium or direct coculture (mixed) on in vitro alkaline phosphatase activity and in vivo calcification. **A–D,** MSC pellets (2.5×10^5 cells/pellet) were cultured for 6 weeks in chondrogenic medium with (**C** and **D**) or without (**A** and **B**) addition of conditioned medium. **E–H,** Alternatively, 2.5×10^5 MSCs were mixed with either 2.5×10^5 (**G**) or 5×10^5 (**H**) HACs and cultivated as mixed pellets for 6 weeks in chondrogenic medium. Pellets of 2.5×10^5 (**E**) or 7.5×10^5 (**F**) MSCs served as controls. The pellets were then transplanted subcutaneously into SCID mice, harvested after 4 weeks, embedded in paraffin, sectioned, and then stained with Alcian blue (**A** and **C**) or alizarin red/fast green (**B** and **D–H**). Representative samples from 1 of 5 donors are shown. **I,** Corresponding culture supernatants obtained during the last 2 days of the in vitro culture period were analyzed for alkaline phosphatase activity. Values are the mean and SD OD obtained from 6 independent experiments. $* = P < 0.05$ versus the MSC control group; $\# = P < 0.05$ versus the 2.5×10^5 MSCs + 2.5×10^5 HACs group; $\S = P < 0.05$ versus the 2.5×10^5 HACs group. See Figure 1 for definitions.

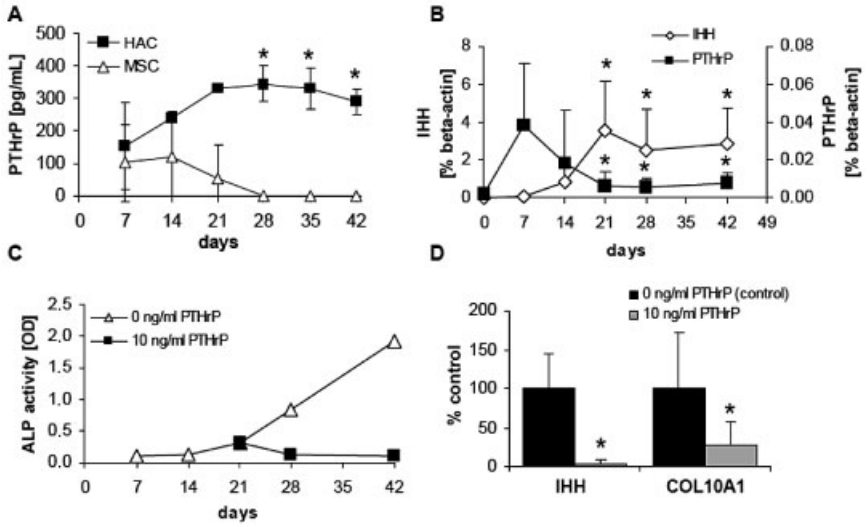

Figure 6. Regulation of parathyroid hormone–related protein (PTHrP) in HACs and MSCs and its effect on the expression of alkaline phosphatase, Indian hedgehog (IHH), and COL10A1. **A,** The release of PTHrP by MSC pellets into culture supernatants decreased within the first 3 weeks of chondrogenesis, whereas PTHrP secretion by HAC pellets persisted, as determined by enzyme immunoassay. Values are the mean ± SD of 3 donors for the HAC pellets and 4 donors for the MSC pellets. $* = P < 0.05$. **B,** Inverse regulation of PTHrP and IHH mRNA levels in MSC pellets during chondrogenesis. Values are the mean ± SD of 8 independent experiments. $* = P < 0.05$ versus day 7. **C,** MSC pellets were subjected to in vitro chondrogenesis for 6 weeks with or without the addition of 10 ng/ml of PTHrP from day 21 onward. Alkaline phosphatase activity in culture supernatants remained low in the presence of PTHrP. **D,** Supplementation of PTHrP from day 21 to day 42 of in vitro chondrogenesis resulted in significant suppression of IHH and COL10A1 mRNA. The mRNA levels were standardized to the β-actin signals. Values are the mean and SD of 4 independent experiments. $* = P < 0.05$. See Figure 1 for other definitions.

1:2 mixed-pellet group (11%; $P = 0.031$ versus control), demonstrating a dose-dependent suppressive effect of HACs on the AP activity in cultures.

The corresponding 4-week ectopic explants were almost unmineralized, based on staining with alizarin red/fast green (Figures 5G and H), and had significantly lower histologic scores for both of the mixed-pellet groups (median score 0.5 in the 1:1 group and 0 in the 1:2 group) as compared with the MSC control groups (median score 4 in the 2.5×10^5 MSC group and 3.5 in the 7.5×10^5 MSC group; $P = 0.001$).

In conclusion, direct coculture of HACs and MSCs showed a dose-dependent reduction in the AP activity in culture supernatants and almost complete suppression of mineralization in vivo, demonstrating stronger effects in direct coculture versus conditioned medium ($P < 0.05$).

Soluble factors derived from HACs include PTHrP. In our search for soluble factors that mediate the reduced expression of AP, IHH, and COL10A1, we determined the levels of mRNA for PTHrP and FGF-2 in HAC (n = 3 donors) and MSC (n = 2) pellets that had undergone standard chondrogenic induction for 6 weeks. HACs expressed PTHrP mRNA throughout the followup period (data not shown), and consistent with this, PTHrP was detected in the culture supernatants by enzyme-linked immunoassay (Figure 6A). In contrast, MSCs secreted PTHrP only during the early phase of differentiation, until days 14–21 (Figure 6A), and mRNA levels for PTHrP declined, whereas those for IHH were up-regulated, for the remaining weeks of culture (Figure 6B). FGF-2 protein expression was detected in HAC medium on day 7, but later decreased below the limits of detection, whereas MSC culture supernatants were negative throughout the culture period (data not shown).

Based on these results, we tested whether supplementation of unconditioned chondrogenic medium with PTHrP from day 14 or from day 21 of culture could mimic the inhibitory effects of chondrocyte-conditioned

medium. First, we determined in the PTHrP quantification assay, what amount of a synthetic PTHrP(1–34) peptide would be needed to produce the same signal intensity as HAC-conditioned medium on days 14–42. This concentration of PTHrP(1–34) (1.2 ng/ml) plus 10 ng/ml of PTHrP in chondrogenic medium was then used to treat MSC pellet cultures. While 1.2 ng/ml of PTHrP from day 14 of culture had no evident effect on AP activity, 10 ng/ml of PTHrP from day 21 of culture strongly reduced AP activity (Figure 6C) and significantly decreased the COL10A1 and IHH mRNA levels (Figure 6D). These results demonstrated that PTHrP supplementation of unconditioned medium could reproduce the main inhibitory effects of HAC-conditioned medium.

DISCUSSION

Resting chondrocytes in the growth plate and articular chondrocytes maintain their nonhypertrophic phenotype by active inhibition of terminal differentiation, and soluble factors have been discussed as the main mediators of this action (17–19,30). This encouraged us to evaluate whether articular chondrocytes can teach MSCs to become stable chondrocytes, either by direct or indirect coculture, during TGFβ-driven in vitro chondrogenesis.

Direct coculture was chosen for these experiments since we expected the strongest effects when MSCs and HACs were mixed in a single pellet. Furthermore, we established a novel indirect coculture system in which conditioned medium was generated by 3-D HAC pellets cultivated in parallel with MSC pellets under standard chondrogenic conditions. TGFβ was added to the medium to be conditioned, since a pilot study showed that HACs deposited very little proteoglycan and type II collagen in chondrogenic medium without TGFβ. Serum-free conditions were used, since this is standard for chondrogenic induction and because the inhibitory effects of serum components on chondrogenesis were seen in a previous study (31).

HAC-conditioned medium stimulated COL2A1 mRNA levels—and thus, in vitro chondrogenesis—in most MSC donor cultures, except those which reached very high COL2A1 mRNA levels (>900% β-actin) already under control conditions (data not shown). Most strikingly, however, the HAC-conditioned medium induced significant inhibition of hypertrophy, as evidenced by the in vitro down-regulation of the COL10A1 to COL2A1 mRNA ratio, the relative amount of type X collagen deposition, and the AP enzyme activity. As a consequence, matrix calcification was reduced after ectopic transplantation of pellets in vivo, an effect that was even more pronounced in direct coculture experiments. Our finding of repressed type X collagen production while maintaining type II collagen production is consistent with the results of studies using a rat model, in which MSCs were cocultured with cartilage chips in a Transwell system (21). In that study, vascular endothelial growth factor, MMP-13, and tissue inhibitor of metalloproteinases types 1 and 2 were identified in the conditioned medium and were suggested to be involved in the regulation of type X collagen.

Our objective was to identify the candidate inhibitory factors released by HACs, which may modulate hypertrophy. We therefore performed assays for FGF-2 and PTHrP, both of which are negative regulators of chondrocyte differentiation (32) and MSC chondrogenesis (33). FGF-2 and PTHrP have been shown to severely reduce type X collagen expression, AP activity, and cell enlargement of lower sternal chondrocytes from immature chicken (34,35), and both molecules are soluble factors produced by articular chondrocytes (36–38).

While we detected FGF-2 secretion by HACs on day 7 only, PTHrP mRNA and protein were produced throughout the culture period, making PTHrP a likely candidate for this action. Indeed, exposure of MSCs to 10 ng/ml of PTHrP in chondrogenic medium from day 21 of culture until week 6 significantly reduced COL10A1 and IHH expression and AP activity. However, the complete suppression seen with HACs was not reached by MSCs exposed to PTHrP. We, therefore, propose that PTHrP is one of the main candidate mediators of coculture-induced reduction of hypertrophy in our model. This is consistent with the established direct suppression of COL10A1 by PTHrP via a PTH/PTHrP-responsive region in the human COL10A1 enhancer (39) and parallels the suggestion that PTHrP may mediate the suppression of AP activity and matrix mineralization in a coculture model of deep-zone with surface-zone bovine cartilage (30).

Most importantly, our study established a causal relationship between the presence of PTHrP during late MSC chondrogenesis and a reduced, but not fully suppressed, hypertrophic development of cells. The final proof that PTHrP is the sole relevant inhibitor of HAC-conditioned medium is not provided by this study, however. This would require the prevention of PTHrP actions in HAC culture supernatants by a specific inhibitor that would block PTHrP. Known inhibitors, such as PTHrP(7–34), target the PTHrP receptor PTHR-1, and our previous results (33) suggest that PTHrP exerts

receptor-independent effects during early chondrogenesis, when PTHR-1 mRNA is not yet detected (33,40).

In light of the improved chondrogenesis in HAC-conditioned medium, as opposed to the down-regulation of COL2A1 in the presence of 10 ng/ml of PTHrP observed in our previous study (33), we believe that PTHrP is certainly not the only factor involved in the attenuation of hypertrophy by HAC-conditioned medium. Most likely, factors that support anabolic differentiation act in synergy with more than one negative regulator; however, PTHrP is a strong candidate negative regulator. Candidate stimulatory factors could further be examined by use of limiting concentrations of TGFβ in our model, an appealing approach for upcoming studies.

One remarkable finding was that MSCs produced PTHrP only during the first 2–3 weeks of chondrogenesis, which was then down-regulated in favor of a strong induction of IHH (Figure 6B). Thus, MSCs differed from phenotypically stable HAC cultures by a loss of endogenous PTHrP expression during culture, while no such autocrine PTHrP/IHH regulation was observed in HACs. Only MSC differentiation was reminiscent of growth plate development, during which PTHrP is expressed by immature chondrocytes, whereas IHH is secreted later on, allowing for further maturation of the cells toward hypertrophy. This implies that we should try to understand why PTHrP is down-regulated during MSC chondrogenesis and that we should try to prevent this step with new protocols that induce a stable chondrogenic phenotype, as is desirable for cartilage repair studies.

In conclusion, HAC-derived soluble factors and direct coculture were potent means of improving TGFβ-driven chondrogenesis by suppressing the development of hypertrophy and ectopic matrix mineralization of MSCs. PTHrP was identified as an important candidate soluble factor involved in this action. Our study suggests that second-generation differentiation protocols for MSCs be developed in which the PTHrP/IHH autoregulation discovered in this study is modulated in favor of the generation of chondrocytes, which display no hypertrophic phenotype and thus seem more suitable as a substitute for articular chondrocytes for use in cartilage repair. The knowledge of how to stop MSC differentiation before and after hypertrophy is important, since calcified hypertrophic cartilage is highly relevant for the intimate anchoring of articular cartilage to the subchondral bone. A functional cartilage repair tissue should therefore always consist of both a stable middle and upper cartilage layer and a hypertrophic mineralized lower region (which only together can form a functional unit) as a prerequisite for a long-lasting success of MSC-based cartilage repair strategies.

ACKNOWLEDGMENTS

Special thanks to Julia Glockenmeier, Michaela Burkhardt, Katrin Goetzke, and Birgit Frey for excellent technical assistance, to Kathrin Brohm for help with the heterotopic transplantations into the SCID mice, to Simone Gantz for advice concerning the statistical evaluations, and to Dr. Eric Steck for extensive help in interpreting the experimental data.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Richter had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Fischer, Dickhut, Rickert, Richter.

Acquisition of data. Fischer, Dickhut.

Analysis and interpretation of data. Fischer, Dickhut, Rickert, Richter.

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