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Subchondral bone osteoblasts induce phenotypic changes in human osteoarthritic chondrocytes

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Summary

Objective: To determine the influence of osteoarthritic (OA) phenotype of subchondral osteoblasts on the phenotype of human chondrocytes.

Methods: Human chondrocytes were isolated from OA cartilage and cultured in alginate beads for 4 or 10 days in the absence or in the presence of osteoblasts in monolayer. The osteoblasts were either isolated from non-sclerotic (N) or sclerotic (SC) zones of human subchondral bone. Before co-culture, osteoblasts were incubated for 72 h with or without 1.7 ng/ml interleukin (IL)-1β, 100 ng/ml IL-6 with its soluble receptor (50 ng/ml) or 10 ng/ml oncostatin M. SOX9, type I, II and X collagen (COL1, COL2, COL10), osteoblasts-stimulating factor (OSF)-1, bone alkaline phosphatase (ALP), parathyroid hormone related peptide (PTHrP) and its receptor (PTH-R) messenger RNA (mRNA) levels in chondrocytes were quantified by real-time polymerase chain reaction.

Results: In comparison with chondrocytes cultured alone in alginate beads, chondrocytes after 4 days in co-culture with N or SC osteoblasts expressed significantly less SOX9 and COL2 mRNA. The decrease of SOX9 and COL2 gene expression was significantly more pronounced in the presence of SC than in the presence of N osteoblasts ($P < 0.001$). OSF-1 mRNA level in chondrocyte was increased by both N and SC osteoblasts, but to a larger extent by SC osteoblasts ($P < 0.001$). PTHrP expression in chondrocytes was 21-fold increased by N osteoblasts but four-fold inhibited by SC osteoblasts. PTHrP secretion was also increased by N but reduced by SC osteoblasts. SC, but not N osteoblasts, induced a significant decrease of PTH-R gene expression in chondrocyte. In our experimental conditions, chondrocytes did not express COL1, COL10 or ALP, even after 10 days of co-culture with osteoblasts.

Conclusions: In co-culture, SC subchondral osteoblasts decrease SOX9, COL2, PTHrP and PTH-R gene expression by chondrocytes but increase that of OSF-1. These findings suggest that SC osteoblasts could initiate chondrocyte phenotype shift towards hypertrophic differentiation and subsequently further matrix mineralization.

Key words: Osteoarthritis, Osteoblasts, Cartilage, Chondrocyte, PTH/PTHrP.

In mature adult articular cartilage, chondrocytes have a stable phenotype with a low metabolic activity. Chondrocytic phenotype is characterized by a round shape, a low rate of proliferation and the production of specific extracellular matrix components, including type II collagen and aggrecan. In OA, some phenotypic changes occur and chondrocytes secrete proteins that are normally absent in normal cartilage. They produce type I and III collagens, which could be indicative for a dedifferentiation process of these cells, but also alkaline phosphatase (ALP), osteocalcin (OC) and type X collagen (COL10), which indicate that adult osteoarthritic chondrocyte may differentiate into hypertrophic phenotype[1,2]. Until now, the factors inducing this phenotype shift in OA remain unknown. Herein, we hypothesized that osteoarthritic (OA) subchondral osteoblasts could take part in this pathological mechanism. Since the presence of connections (microcracks, vascular channels, neovascularization) between subchondral bone and cartilage has been demonstrated, it is speculated that mediators produced by subchondral osteoblasts could modulate chondrocyte metabolism and induce chondrocyte phenotype changes[3,4]. Further, it is now well established that OA osteoblasts show an altered phenotype and secrete more ALP, OC, interleukin (IL)-6, transforming growth factor (TGF)-β1, insulin-like growth factor (IGF)-1, urokinase plasminogen activator (uPA) and prostaglandin (PG)E_2 than normal osteoblasts[5–8]. Therefore, we can hypothesize that OA osteoblasts contribute to bone sclerosis and degradation of overlying cartilage.

To verify this hypothesis, we have developed an original co-culture model, in which subchondral osteoblasts in monolayer are cultured together with OA chondrocytes in alginate beads. In a previous work, we have demonstrated that osteoblasts from sclerotic (SC) zones of subchondral OA

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bone, but not non-sclerotic (N) osteoblasts, downregulated aggrecan synthesis and upregulated metalloproteases expression by chondrocytes in this co-culture model[9]. Herein, we propose to investigate the expression of genes in chondrocytes that are commonly considered as markers of normal cartilaginous phenotype [expression of SOX9 and type II collagen (COL2) genes and no expression of type I collagen (COL1)] or as indicators of the hypertrophic differentiation [expression of ALP, COL10, parathyroid hormone related peptide (PTHrP) and its receptor (PTH-R) and osteoblasts-stimulating factor-1 (OSF-1) genes].

SOX9 is a high-mobility-group domain transcription factor that is expressed in chondrocytes and other tissues[10]. During chondrogenesis, SOX9 is expressed in all chondrocyte progenitors and all differentiated chondrocytes, but its expression is completely turned off in hypertrophic chondrocytes[11]. SOX9 expression parallels to that of the gene coding for type II collagen (COL2A1), a specific marker of chondrocyte differentiation[12]. In mouse embryo, SOX9 knock-out embryonic stem cells were excluded from chondrogenic mesenchymal condensations and could not express chondrocyte-specific markers such as COL2A1, COL11A2 and aggrecan[10]. Type I collagen is normally absent of cartilage, and its deposition occurs only in the late stage of OA when fibrocartilage is formed[13,14]. *In vitro*, type I collagen is expressed only when chondrocytes become dedifferentiated, acquiring a fibroblast-like appearance and changing their pattern of gene expression from one that expresses chondrocyte-specific gene to one that resembles a fibroblastic or chondroprogenitor-like pattern[15].

In endochondral growth, PTHrP is expressed principally in the perichondrium and by resting and proliferative chondrocytes, and the PTH/PTHrP receptor by proliferative and prehypertrophic chondrocytes[16,17]. In early endochondral mineralization, PTHrP acts by stimulating proliferation of endochondral chondrocytes and inhibiting apoptosis, partly via induction of bcl-2 (see Ref.[18]). Furthermore, perichondrial PTHrP, acting on prehypertrophic endochondral chondrocytes, acts as an inhibitor of hypertrophic differentiation[19]. In other terms, PTHrP participates in a negative biofeedback loop limiting entry into hypertrophy. Although PTHrP is present in low quantities in normal adult cartilage, it is found in high amount in OA human articular cartilage. It was demonstrated that PTHrP is highly and predominantly expressed by chondrocytes located in degenerative lesions of osteoarthritic tissue[20].

OSF-1, also called pleiotrophin, is a 15.3 kDa heparin-binding peptide that is normally expressed in the epiphyseal growth plate cartilage but not in articular cartilage. It is re-expressed in OA chondrocytes[21]. OSF-1 exerts a chemoattractive effect on osteoblasts and stimulates the osteoblastic maturation[22]. *In vitro*, OSF-1 inhibits mature chondrocyte proliferation and increases glycosaminoglycan synthesis in bovine high-density chondrocytes culture[23]. OA chondrocytes, but not normal cells, also expressed ALP, which is considered as a marker of chondrocytes hypertrophy and mineralization during endochondral ossification[1]. In contrast to mature chondrocytes, hypertrophic cells secrete type X collagen. Type X collagen is found in osteoarthritic cartilage but is absent from normal adult cartilage (including the region of the calcified cartilage)[24,25].

In this work, we demonstrate that OA osteoblasts induce phenotypic changes in chondrocytes, characterized by a decrease of SOX9, COL2 and PTHrP/PTH-R expression and an increase of OSF-1 gene expression in chondrocytes.

Materials and methods

SUBCHONDRAL OSTEOARTHRITIC OSTEOBLASTS IN MONOLAYER CULTURE

Tibial and femoral subchondral bone plates were obtained from the knees of cadavers (three men aged of 48, 57 and 65 years old) with OA immediately after death. After careful elimination of trabecular bone and articular cartilage, OA subchondral bone was dissected under a microscope to separate N from SC zones. N and SC zones were characterized by a marked difference in their thickness. We have considered as SC bone only the subchondral bone zone with a thickness greater than 2 mm and either cartilage denuded or overlaid by fibrillated cartilage. The plates were then cut into small fragments, washed with Dulbecco's modified Eagle's medium (DMEM) and then submitted to enzymatic digestions following a protocol from Hilal *et al.*[6]. Briefly, small pieces of bone (2 mm^3) were sequentially incubated with 0.5 mg/ml hyaluronidase type IV S (Sigma-Aldrich, Bornem, Belgium) for 20 min at 37°C, and 0.6 mg/ml clostridial collagenase IA (Sigma-Aldrich, Bornem, Belgium) for 30 and 240 min successively at 37°C (2 g of bone in 20 ml of enzymatic solution). The digested bone pieces were placed in T-75 flasks and cultured in DMEM supplemented with 15% foetal calf serum (FCS), 10 mM N-(2-hydroxyethyl) piperazin-N′-(2-ethanesulfonic acid HEPES),100 U/ml penicillin and 100 μg/ml streptomycin, until osteoblasts migrated out of bone explants. At this point, the medium was replaced with fresh media containing 10% FCS, 10 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin. At confluence, cells were collected by trypsinization, seeded (50,000 cells/cm^2) in 12-well plates (12-well companion plates, Falcon, BD Biosciences, Erembodegem, Belgium) and grown for 3 days in DMEM containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES. After washings, osteoblasts were maintained for 12 days in a differentiation media, composed of DMEM containing 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 2% Ultroser G, a serum substitute, 10^{-8} M 1,25$(OH)_2$vitaminD_3 (Sigma-Aldrich, Bornem, Belgium), 50 μg/ml ascorbic acid (Sigma-Aldrich, Bornem, Belgium) and 2 mM proline (Invitrogen, Merelbeke, Belgium). At the end of this differentiation period, cells showed an osteoblastic phenotype characterized by the production of OC and ALP. Normal osteoblasts were isolated from normal areas of subchondral bone whereas osteoarthritic osteoblasts were isolated from the SC areas of this tissue. After washings, cells were then cultured for 72 h in the absence or in the presence of an optimal dose of human recombinant IL-1β (1.7 ng/ml or 85 U/ml, Roche, Brussels, Belgium), 100 ng/ml IL-6 in combination with 50 ng/ml IL-6 soluble receptor (IL-6sR) (Biosource Europe, Fleurus, Belgium) or 10 ng/ml oncostatin M (OSM) (Sigma-Aldrich, Bornem, Belgium). These concentrations corresponded to the maximal IL-1β, OSM and IL-6 concentrations found in the synovial fluid of OA patients[26–28]. The nutrient media used in this incubation phase was DMEM supplemented with 1% insulin, transferrin, selenium (ITS+) (ICN Biomedicals, Asse-Relegem, Belgium), 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml ascorbic acid (Sigma-Aldrich, Bornem, Belgium), 2 mM proline (Invitrogen, Merelbeke, Belgium). ITS+ is a premixed cell growth system, which contains in 1 ml: 0.625 mg insulin, 0.625 mg transferrin, 0.625 μg selenious acid, 0.125 g bovine serum albumin and 0.535 mg linoleic acid.

CHONDROCYTES CULTURE IN ALGINATE BEADS

Cartilage was obtained from the knees of cadavers (three men aged 50, 52 and 63 years old) with OA immediately after death, being excised from the superficial and medium layers of cartilage and avoiding the calcified layer. Upon dissection, the femoral, patellar and tibial articular surfaces were evaluated for gross pathological cartilage modifications according to a personal scale[29]. The severity of pitting was recorded for each donor. Four different grades were considered: 0, normal white cartilage in all areas examined; I, the presence of a yellow-grey area with some superficial fibrillations on one or more articular surfaces; II, irregular surface with deep fibrillations on one or more articular surfaces; and III, ulcers penetrating to the subchondral bone on one or more articular surfaces. Experiments were performed with cartilage specimen showing OA cartilage lesions of grade II or III. Cartilage was cut into small fragments and then subjected to enzymatic digestions sequentially with hyaluronidase, pronase and collagenase (3 g of cartilage per 10 ml of enzyme solution) as previously described[30]. The cells were filtered through a nylon mesh with a pore diameter of 70 μm, and then washed three times with sterile saline. Cell viability was estimated by trypan blue exclusion test and in all cases was superior to 95%. Chondrocytes were suspended in 1.2% low viscosity alginate (Sigma-Aldrich, Bornem, Belgium) in sterile saline solution at a density of 4×10^6 cells/ml, which was slowly passed through a 25 gauge needle in a dropwise fashion into 102 mM $CaCl_2$ solution (Sigma-Aldrich, Bornem, Belgium). After instantaneous gelation, the beads were allowed to polymerize further for 10 min in this $CaCl_2$ solution. Ten alginate beads containing OA chondrocytes were placed in porous inserts (with 1 μm pore size; Falcon, BD Biosciences, Erembodegem, Belgium). The chondrocytes in alginate beads were maintained for 2 days in DMEM supplemented with 2% Ultroser G, 10 mM HEPES, penicillin (100 U/ml) and streptomycin (100 μg/ml), as a precaution to avoid contamination with drugs that donors might have taken before death. After this wash out period, cells were washed in phosphate buffered saline (PBS) and the chondrocytes were placed in co-culture with osteoblasts isolated from N or SC subchondral bone or with normal skin fibroblasts in monolayer. Normal fibroblasts were obtained by outgrowth from skin explants as previously described[31].

OA OSTEOBLASTS/CHONDROCYTES CO-CULTURE

The inserts (with a pore size of 1 μm; Falcon, BD Biosciences, Erembodegem, Belgium) containing 10 alginate beads were co-cultured for 4 or 10 days with osteoblasts in monolayer pre-incubated or not with 1.7 ng/ml IL-1β, 100 ng/ml IL-6 or 10 ng/ml OSM. Co-culture medium was DMEM supplemented with 1% ITS+, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml ascorbic acid, 2 mM proline. In the 10 day co-culture experiments, the medium was changed at day 4. The co-culture model is illustrated in Fig. 1. Six wells were pooled to extract the RNA, and the pool was further assayed in triplicate. The experiment was repeated three times, each time using subchondral osteoblasts coming from a different donor. Conversely, the origin of OA chondrocytes selected for each experiment was from a distinct donor than osteoblasts. As controls, OA chondrocytes in alginate beads were cultured alone (mono-culture) or together with normal skin fibroblasts in monolayer. At the end of the culture period, culture medium (S) were carefully collected and kept at −20°C until analysis. The alginate beads were dissolved in 1 ml 0.1 M citrate for 10 min. The resulting suspension was centrifuged at 1200 rpm for 10 min. By this method, two fractions were collected: the supernatant containing macromolecules originated from the further-removed matrix compartment, and a pellet, containing cells with their associated matrix. Osteoblasts in monolayer were collected after 5 min treatment with a solution containing 0.125% trypsin − 0.5 mM ethylenediaminetetraacetic acid (Invitrogen, Merelbeke, Belgium). The cell pellets of chondrocytes or osteoblasts were washed three times with PBS and then either homogenized in 1 ml PBS by ultrasonic dissociation for DNA and AGG quantification or in 175 μl of cell lysis buffer (Promega, Leiden, The Netherlands) for RNA isolation. Cell extracts were kept at −70°C until analysis.

DNA ASSAY

The DNA content of the cultures was measured according to the fluorimetric method of Labarca and Paigen[32].

PTHrP RADIOIMMUNOASSAY

PTHrP was assayed directly in the supernatants according to a specific radioimmunoassay provided by Diagnostic Systems (Oxon, UK). In this assay, the first antibody is coated and the second is ^{125}I labelled. Sample incubation with antibodies was performed overnight at ambient temperature under constant agitation (180 rpm), rinsed with demineralised water and counted. The detection limit of this assay was 3 pg/ml.

QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTION PCR

RNA from 3×10^6 cells was isolated by spin or vacuum total RNA isolation system (Promega, Leiden, The Netherlands). RNA (0.5 μg) was reverse transcribed in 30 μl buffer containing 10 mM dithiothreitol, 1 mM deoxynucleotide triphosphate, 500 ng $(deoxythymine)_{15}$ primer, 140 U mouse moloney leukemia virus reverse transcriptase and 40 U RNaseOUT (Life Technologies, Merelbeke, Belgium) for 60 min at 37°C. The reaction was stopped by incubating the samples at 95°C for 5 min, and 100 μl of H_2O was added. Polymerase chain reaction (PCR) was performed by using the Light Cycler-FastStart DNA Master Sybr Green I (Roche Diagnostics, Brussels, Belgium). The PCR template source was either 3 ng first-strand complimentary DNA (cDNA) or purified DNA standard. Primer sequences used to amplify the desired cDNA are detailed in Table I. Amplification was performed with a spectrofluorometric thermal cycler (LightCycler, Roche Diagnostics, Brussels, Belgium). After an initial denaturation step at 95°C for 10 min, amplification was performed using 40 cycles of denaturation (95°C for 15 s), annealing (temperature

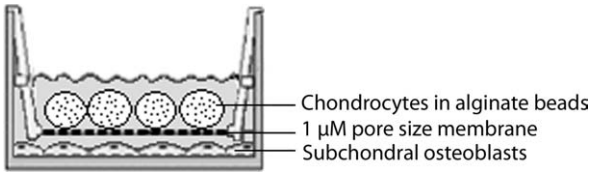

Fig. 1. Schematic representation of the subchondral osteoblasts/chondrocytes co-culture model.

Table I
Primers used for real-time PCR

cDNA		Sequence 5′–3′	Tm	pb
GAPDH	Forward	TTGGTATCGTGGAAGGACTCA	90.9	190
	Reverse	TGTCATCATATTTGGCAGGTTT		
COL1A1	Forward	AGTTCGAGTATGGCGG	90.7	231
	Reverse	CAGTGACGCTGTAGGT		
COL2A1	Forward	TGCTGCCCAGATGGCTGGAGGA	88.7	140
	Reverse	TGCCTTGAAATCCTTGAGGCCC		
COL10A1	Forward	GGGAGTGCCATCATCG	85	328
	Reverse	GAGGCTTCACATACGTTT		
SOX9	Forward	ACAACCCGTCTACACACAGC	85.6	369
	Reverse	ACGATTCTCCATCATCCTCC		
OSF-1	Forward	GTGACCTGAACACAGC	87	265
	Reverse	ACAAAGCCTACGGTACAT		
PAL	Forward	GCTGTAAGGACATCGCC	87.1	311
	Reverse	GTGCTTGTATCTCGGTTT		
PTHrP	Forward	CGGAGACTGGTTCAGC	86.5	200
	Reverse	GTGTGGATTTCTGCGATCA		
PTH-R	Forward	ACCATACACCGAGGTC	85.5	183
	Reverse	CGTGCCTTTCGCTTGA		

gradient: 68–58°C with an increment of 0.5°C per cycle during 20 cycles, continued by 20 cycles at 58°C, for 5 s) and extension (72°C for 15 s). For each run, a standard curve was generated from purified DNA ranging from 10^6 to 10 copies. To standardise messenger RNA (mRNA) levels, we amplified GAPDH, a house keeping gene, as an internal control. Gene expression was normalized by calculating the ratio between the number of cDNA copies of COL1, COL2, COL10, SOX9, OSF-1, PTHrP, PTH-R, ALP and that of GAPDH. After amplification, a final melting curve was recorded by cooling the PCR mixture to 65°C for 30 s and then slowly heating it to 95°C at 0.1°/s. Fluorescence was measured continuously during the slow temperature rise to monitor the dissociation of double stranded DNA. Specificity of the expected Lightcycler products was demonstrated by melting curve analysis. Amplification products performed in the Light Cycler were checked by electrophoresis on 1.5% ethidium bromide stained agarose gel. The estimated size of the amplified fragments matched the calculated size.

CALCULATION AND STATISTICAL ANALYSIS

The results (mean ± S.E.M.) were expressed as GAPDH-normalized gene expression. A non-parametric Mann–Whitney *U* test was performed on all the experiments.

Results

At the beginning of the co-culture period, 3.5 ± 0.5 μg of DNA was measured in alginate beads, osteoblasts and fibroblasts monolayer culture, indicating that a similar number of chondrocytes, osteoblasts or fibroblasts were introduced in the co-culture model. Further, co-culture conditions did not significantly modify DNA content in the different compartments of the co-culture, demonstrating that cell number remained stable over the co-culture period (data not shown).

When they were cultured alone in alginate beads, human OA chondrocytes spontaneously expressed after 4 days of culture 14.25 SOX9 copies, 1.8 COL2 copies, 2.3 OSF-1 copies, 0.6 PTHrP copies and 1.98 PTH-R copies per 1000 GAPDH copies. With the exception of the PTH-R gene, the expression of these genes was strongly decreased after 10 days of culture in alginate beads: 2.9 SOX9 copies, 0.45 COL2 copies, 0.43 OSF-1 copies, 0.15 PTHrP copies and 2.5 PTH-R copies per 1000 GAPDH copies. The expression of GAPDH remained stable whatever the experimental conditions. In our culture conditions, ALP, COL1 and COL10 genes were not expressed by human OA chondrocytes.

INFLUENCE OF OSTEOBLASTS ON HUMAN OA CHONDROCYTE PHENOTYPE

After 4 days in co-culture with osteoblasts isolated from N or SC zones of OA subchondral bone, SOX9 and COL2 gene expression by human chondrocytes was significantly reduced [$P < 0.001$, Fig. 2(A)]. The inhibition of SOX9 and COL2 gene expression was significantly more pronounced in the presence of SC than in the presence of N osteoblasts (SOX9: SC −62% vs N −28%, $P < 0.001$; COL2: SC −78% vs N −52%, $P < 0.001$) [Fig. 2(A)]. When they were co-cultured with chondrocytes, human skin fibroblasts did not modify SOX9 and COL2 gene expression.

After 10 days of co-culture, OSF-1 gene expression by chondrocytes was increased three times ($P < 0.01$) in the presence of SC osteoblasts compared to that in the presence of N osteoblasts or mono-culture [Fig. 2(B)].

When chondrocytes in alginate beads were co-cultured with subchondral osteoblasts for 4 days, OSF-1 mRNA level in chondrocytes was significantly increased. This stimulating effect was significantly more important when chondrocytes were co-cultured with SC osteoblasts than with N osteoblasts [SC +170% vs N +35%, $P < 0.001$, Fig. 2(A)]. As a control, human skin fibroblasts did not modulate OSF-1 gene expression in chondrocytes.

After 10 days of co-culture, OSF-1 gene expression by chondrocytes was three times increased ($P < 0.001$) in the presence of SC osteoblasts compared to N osteoblasts or mono-culture [Fig. 2(B)].

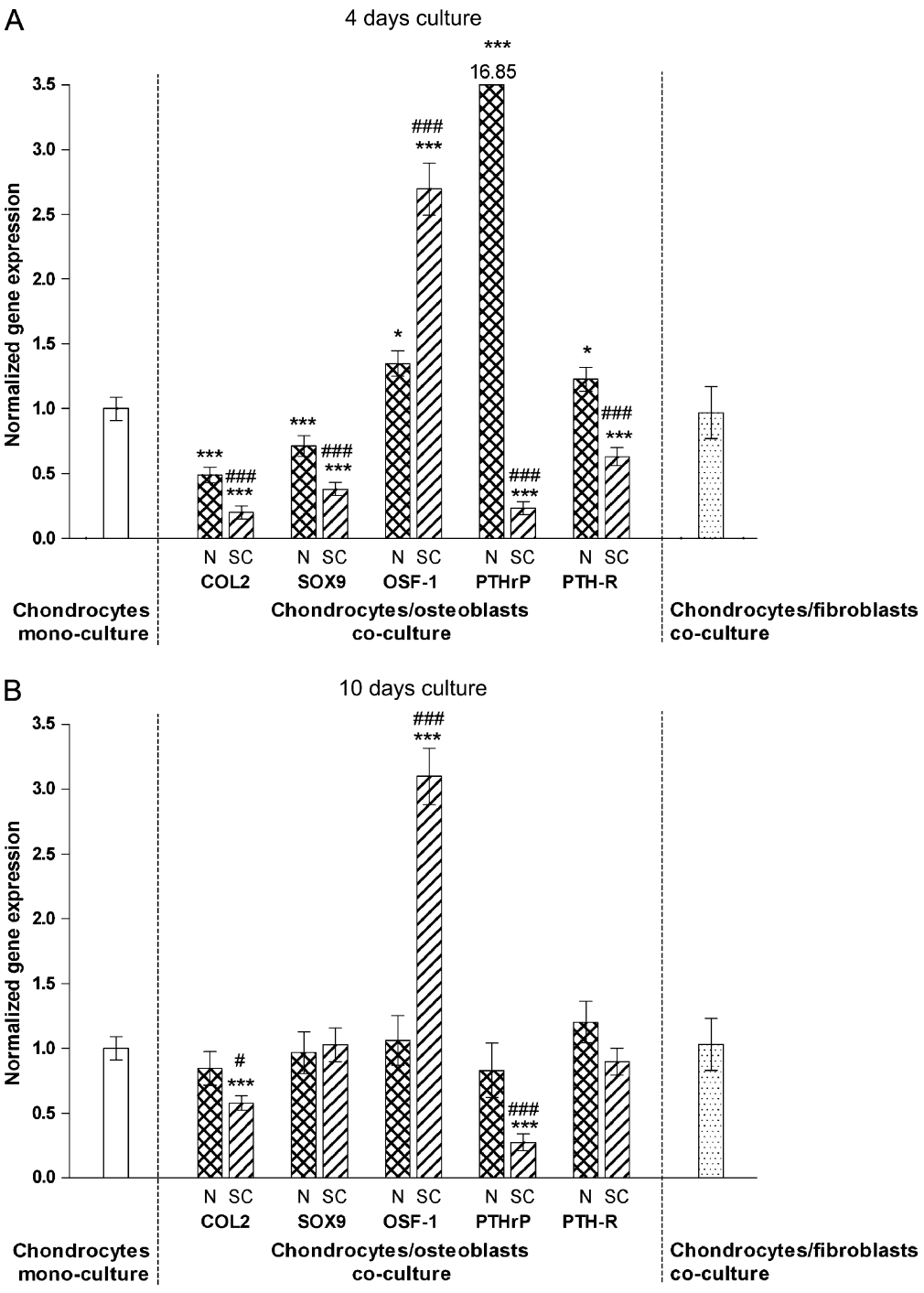

Fig. 2. Gene expressions by human OA chondrocytes in alginate beads after 4 days (A) or 10 days (B) of culture in the absence (mono-culture) or in the presence (co-culture) of osteoblasts isolated from SC or N zones of subchondral bone. As a control, human OA chondrocytes in alginate beads were also co-cultured with normal human skin fibroblasts. The results are expressed as the mean ± S.E.M. of three experiments and performed with cells coming from three different donors. The mRNA copy numbers were normalized against the corresponding copy number of GAPDH mRNA and expressed compared to chondrocytes mono-culture. Comparison of mean values was performed by Mann–Whitney *U* test. Statistical significances: chondrocytes/osteoblasts co-culture compared to mono-culture $^{*}P < 0.05$ and $^{***}P < 0.001$; SC osteoblasts compared to N osteoblasts $\#P < 0.05$ and $\#\#\#P < 0.001$.

After 4 days of culture, a 21-fold increase of PTHrP gene expression in chondrocytes was observed in co-culture with N subchondral osteoblasts [$P < 0.001$, Fig. 2(A)]. In contrast, a four-fold decrease of PTHrP mRNA level in chondrocyte occurred in co-culture with SC subchondral osteoblasts [$P < 0.001$, Fig. 2(A)]. In parallel, PTH-R mRNA level in chondrocytes was enhanced by 23% in co-culture with N osteoblasts ($P < 0.05$) but decreased by 37% in co-culture with SC osteoblasts [$P < 0.001$, Fig. 2(A)].

After 10 days of culture, N osteoblasts didn't modify PTHrP or PTH-R gene expression by chondrocytes [Fig. 2(B)], whereas SC osteoblasts decreased PTHrP expression ($P < 0.001$).

PTHrP protein synthesis was also increased in co-culture with N osteoblasts and decreased in co-culture with SC osteoblasts (Fig. 3).

ALP, COL1 and COL10 genes were not expressed by OA chondrocytes in alginate beads cultured separately or together with osteoblasts, even after 10 days of co-culture.

EFFECT OF PRE-TREATMENT OF OSTEOBLASTS WITH IL-1β ON HUMAN CHONDROCYTES

A 3-day pre-incubation of N or SC subchondral osteoblasts with IL-1β, accentuated the osteoblasts-induced inhibition of COL2 gene expression in chondrocytes ($P < 0.01$), but had no effect on SOX9 expression after 4 days of co-culture (Table II). When N, but not SC osteoblasts, were pre-incubated with IL-1β, OSF-1 gene expression was increased in chondrocytes (IL-1β stimulated vs unstimulated, $P < 0.01$), but the level of expression did not reach to that obtained with SC osteoblasts (Table II).

As described above, after 4 days of co-culture N osteoblasts stimulated PTHrP gene expression and production in chondrocytes, whereas SC osteoblasts inhibited this gene expression. Pre-incubation of osteoblasts with IL-1β did not modify these effects (Table II). Whilst SC osteoblasts decreased PTH-R expression by chondrocytes, IL-1β pre-treated SC osteoblasts had no effect (Table II).

After 10 days of co-culture, N or SC osteoblasts pre-treated with IL-1β did not modify the behaviour of chondrocytes (Table III), and did not induce COL1, COL10 or ALP gene expression in chondrocytes (data not shown).

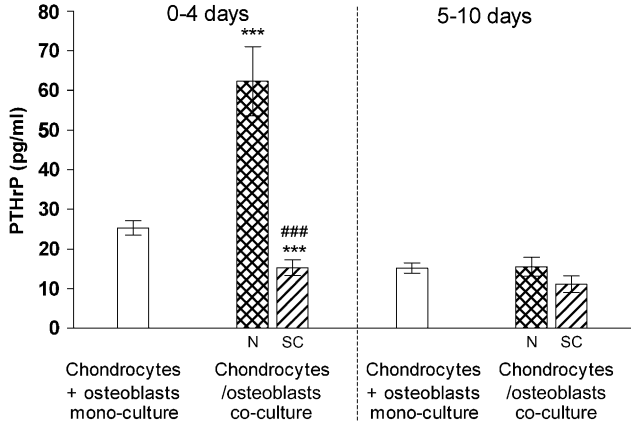

Fig. 3. PTHrP protein measured in the culture supernatant during the first 4 days (0–4 days) and the following 6 days (5–10 days) of culture. The values correspond to the cumulated production of PTHrP by chondrocytes and osteoblasts cultured separately (=chondrocytes + osteoblasts mono-cultures) or the production in co-culture. The results are expressed as the mean ± S.E.M. of three experiments assayed in triplicate and performed with cells coming from three different donors. The results were expressed in pg/ml. Comparison of mean values was performed by Mann–Whitney *U* test. Statistical significances: chondrocytes/osteoblasts co-culture compared to mono-culture ***$P < 0.001$; SC osteoblasts compared to N osteoblasts ###$P < 0.001$.

EFFECT OF PRE-TREATMENT OF OSTEOBLASTS WITH IL-6 OR OSM ON HUMAN CHONDROCYTES

A 3-day pre-incubation of osteoblasts with IL-6 fully reversed the SC osteoblasts-induced inhibition of COL2 gene expression in chondrocytes after 4 days of co-culture ($P < 0.001$), but had no effect on the inhibitory effect of N osteoblasts (Table II). In contrast, the pre-incubation of osteoblasts with OSM increased the inhibitory effect of SC osteoblasts on COL2 gene expression by chondrocytes ($P < 0.05$, Table II), but did not affect the inhibitory effect of N osteoblasts. SOX9 gene expression by chondrocytes in co-culture was not affected by the pre-incubation of osteoblasts with these cytokines.

As described above, N osteoblasts stimulated PTH-R gene expression in chondrocytes, whereas SC osteoblasts inhibited this gene expression. When they were pre-incubated with IL-6, N osteoblasts inhibited PTH-R gene expression in chondrocytes ($P < 0.001$, Table II). In contrast, the pre-incubation of N osteoblasts with OSM stimulated PTH-R gene expression in chondrocytes ($P < 0.01$). The inhibitory effect of SC osteoblasts on PTH-R gene expression in chondrocytes was fully prevented by a 3-day pre-incubation with IL-6 or OSM (Table II).

Finally, the pre-incubation of osteoblasts with IL-6 or OSM did not modify OSF-1 gene expression or PTHrP gene expression and production by chondrocytes in co-culture (Tables II and III).

After 10 days of co-culture, OSM and IL-6 pre-treated osteoblasts did not modify the responses of chondrocytes in co-culture (Table III) and did not induce COL1, COL10 or ALP gene expression in these cells (data not shown).

Discussion

In OA, chondrocytes undergo a phenotypic change in gene expression pattern towards the chondroprogenitor, hypertrophic, and dedifferentiated phenotype[1,2,33,34]. This phenotypic shift is evidenced by the initiation of the synthesis of the interstitial collagens (types I, II and III)[14,34], an increase in the synthesis of fibroblast-type proteoglycans (versican) at the expense of aggrecan[35], the re-expression of type IIA collagen[33] and the expression of a hypertrophic chondrocyte-specific molecule (type X collagen)[36,37]. Although changes in the surrounding matrix, mechanical strain and some growth factors and cytokines have been demonstrated to alter the pattern of genes expression in chondrocyte, the circumstances leading to long-term changes in the phenotype of the cell remain poorly understood. Herein, we assume that factors produced by OA osteoblasts could induce phenotype shift in OA chondrocytes. OA has been associated with a thickening of subchondral bone, and also with abnormally low mineralization pattern. In this respect, it is also important to note that Shimizu *et al.*[38] have shown, using a large number of patients, that progression of joint cartilage degeneration is associated with intensified remodelling of subchondral bone and increased bone stiffness. This is strongly indicative of a cellular bone defect in OA. The hypothesis that abnormal OA osteoblasts directly influence cartilage metabolism was previously put forward by Westacott *et al.*[3]. These authors have shown that conditioned media from primary osteoblasts of OA patients vs subject without arthritis significantly enhanced glycosaminoglycans release from normal cartilage. Furthermore, Hilal *et al.*[6,7] have reported that *in vitro* primary culture of osteoblasts prepared

Table II

*Gene expression by chondrocytes after 4 days of co-culture with osteoblasts isolated from N or SC OA subchondral bone plate. Before co-culture, human osteoblasts were either pre-incubated or not (control) for 72 h with IL-1β (1.7 ng/ml), IL-6 (100 ng/ml in combination with 50 ng/ml of IL-6sR), or OSM (10 ng/ml). Results are expressed as the percentage of the chondrocyte mono-culture (alginate beads only), or as the percentage of the cumulated production of PTHrP protein by chondrocytes and osteoblasts cultivated separately (mono-cultures), and represented by the mean ± S.E.M. of three independent experiments, each assayed in triplicate. Statistical significance: between co-culture and mono-culture *P < 0.05, **P < 0.01 and ***P < 0.001, between N and SC osteoblasts #P < 0.05, ##P < 0.01 and ###P < 0.001, between basal and cytokine pre-incubated osteoblasts ^P < 0.05, ^^P < 0.01 and ^^^P < 0.001*

		4 days of osteoblasts/chondrocytes co-culture			
		Basal	IL-1β	IL-6/IL-6sR	OSM
GAPDH	N	101 ± 4.2	98 ± 7.2	106 ± 7.1	99 ± 5.1
	SC	96 ± 6.2	102 ± 6.3	97 ± 6.5	96 ± 7.8
COL2	N	48.5 ± 3.4***	38.7 ± 2.7***	51.1 ± 3.5***	62.9 ± 4.4***
	SC	19.9 ± 1.4###,***	16.6 ± 1.2###,***	105.1 ± 7.2###,^^^	14.2 ± 1.0###,***,^
SOX9	N	70.9 ± 4.9***	66.7 ± 4.6***	58.9 ± 4.1***	66.7 ± 4.7***
	SC	37.9 ± 2.6###,***	43.2 ± 3.1###,***	36.4 ± 2.5###,***	39.4 ± 2.8###,***
OSF-1	N	134.8 ± 9.1*	195.6 ± 13.7***,^^	126.1 ± 8.8	130.4 ± 9.1
	SC	269.5 ± 18.7###,***	282.6 ± 19.6###,***	304.3 ± 21.3###,***	347.8 ± 24.3###,***
PTHrP mRNA	N	1683 ± 116***	1583 ± 110***	1402 ± 98***	1583 ± 111***
	SC	23.2 ± 1.5###,***	40.5 ± 2.8###,***	38.8 ± 2.7###,***	24.9 ± 1.7###,***
PTHrP protein	N	246.0 ± 34.5***	253.0 ± 41.2***	261.4 ± 53.1***	234.5 ± 43.8***
	SC	60.2 ± 7.9###,***	67.2 ± 8.9###,***	64.5 ± 9.4###,***	68.1 ± 11.2###,***
PTH-R	N	122.5 ± 10.1*	121.4 ± 6.2*	75.8 ± 3.8**,^^^	138.2 ± 12.0**
	SC	63.1 ± 3.2###,***	102.3 ± 5.1#,^^^	100.2 ± 5.0##,^^^	126.0 ± 6.3*,^^^

from human OA subchondral bone plates showed an altered phenotype, and that uPA/plasmin system activity and IGF-1 levels are altered in these cells. In a previous work, we have shown that SC osteoblasts overproduced cytokines and growth factor, such as IL-6 and TGF-β1 (see Ref.[9]), that are involved in local remodelling of bone tissues, and these could also contribute to remodelling the overlying cartilage after seeping through microcracks in the calcified layer of articular cartilage. Whether these changes in subchondral bone cells are responsible for OA or contribute to its progression is not definitely known. To better explore this pathophysiological axis, we have developed a co-culture system in which osteoblasts isolated from the SC area of the OA subchondral bone are cultured simultaneously with OA chondrocytes. In a previous work, we have clearly demonstrated that osteoblasts isolated from the SC OA subchondral bone induced a significant inhibition of aggrecan production and a significant increase of MMP-3 and MMP-13 synthesis[9]. These metabolic changes are related to the SC phenotype of osteoblasts as suggested by

Table III

*Gene expression by chondrocytes after 10 days of co-culture with osteoblasts isolated from N or SC OA subchondral bone plate. Before co-culture, human osteoblasts were either pre-incubated or not (control) for 72 h with IL-1β (1.7 ng/ml), IL-6 (100 ng/ml in combination with 50 ng/ml of IL-6sR), or OSM (10 ng/ml). Results are expressed as the percentage of the chondrocyte mono-culture (alginate beads only) or as the percentage of the cumulated production of PTHrP protein by chondrocytes and osteoblasts cultivated separately (mono-cultures), and represented by the mean ± S.E.M. of three independent experiments, each assayed in triplicate. Statistical significance: between co-culture and mono-culture ***P < 0.001, between N and SC osteoblasts #P < 0.05, ##P < 0.01 and ###P < 0.001*

		10 days of osteoblasts/chondrocytes co-culture			
		Basal	IL-1β	IL-6/IL-6sR	OSM
GAPDH	N	105 ± 6.5	96 ± 7.6	103 ± 5.3	94 ± 8.4
	SC	101 ± 3.3	105 ± 4.4	96 ± 5.4	98 ± 5.3
COL2	N	84.4 ± 13.1	91.1 ± 10.9	93.3 ± 14.4	80.1 ± 8.8
	SC	57.8 ± 5.6#,***	66.7 ± 4.7##,***	64.4 ± 7.3#,***	56.9 ± 3.9##,***
SOX9	N	96.5 ± 16	93.1 ± 15.8	95.5 ± 12.3	86.3 ± 12.6
	SC	102.7 ± 13	88.6 ± 14.8	95.2 ± 12.5	104.8 ± 13.5
OSF-1	N	106 ± 19	116.3 ± 16.6	120.2 ± 18.4	118.6 ± 19.2
	SC	309.7 ± 21.9###,***	249.8 ± 29.8###,***	260.5 ± 22.1###,***	279.1 ± 19.9###,***
PTHrP mRNA	N	82.9 ± 21.3	116.4 ± 25.1	96.6 ± 17.7	84.9 ± 25.1
	SC	27.4 ± 6.6###,***	34.2 ± 11.6###,***	39.7 ± 10.9###,***	37.6 ± 9.6###,***
PTHrP protein	N	102.0 ± 15.8	104.4 ± 5.9	94.6 ± 10.3	89.8 ± 12.4
	SC	79.0 ± 13.8	85.4 ± 11.1	91.2 ± 18.7	87.7 ± 8.6
PTH-R	N	120.2 ± 16.2	110.8 ± 12.8	120.4 ± 10.8	135.5 ± 21.5
	SC	89.6 ± 10.4	95.2 ± 4.0	119.3 ± 11.9	104.0 ± 16.9

the absence of effect of osteoblasts isolated from N subchondral bone and skin fibroblasts. In our culture condition, SC osteoblasts secrete more ALP, OC, IL-6 and TGF-β1 than N cells corroborating the results of some previous work comparing normal and OA osteoblasts[5,6]. These findings indicate that SC subchondral osteoblasts used in this study showed an altered phenotype compared to osteoblasts isolated from N subchondral bone, and that within the subchondral bone, local changes in osteoblasts can occur. These local metabolic changes in subchondral bone could result from abnormal mechanical stress[39]. We also hypothesized that OA chondrocytes contribute or modulate these changes in osteoblasts through the localized microfractures. Nevertheless, this pathological pathway needs to be demonstrated. In this work, we have demonstrated that osteoblasts, essentially SC osteoblasts, decreased COL2, SOX9 and PTHrP/PTH-R gene expression but increased OSF-1 mRNA level. For the first time, these data indicate that SC osteoblasts may induce alteration in the gene expression pattern of OA chondrocytes and initiate phenotypic changes in these cells. Indeed, OSF-1, which is not expressed by normal chondrocytes (personal communication), is expressed by OA chondrocytes. These data corroborate those of Pufe *et al.*[21] demonstrating that OSF-1 is re-expressed in OA cartilage. In our co-culture model, OSF-1 mRNA level is highly (+170%) increased by SC, but very slightly by N osteoblasts (+35%), indicating that this effect is related to the SC phenotype of OA subchondral osteoblasts. Our study proposes a new regulatory pathway involving osteoblasts stimulation of OSF-1 expression in OA cartilage. In parallel, SC osteoblasts downregulate SOX9 expression. During endochondral ossification, the expression of SOX9 gene and of COL2A1 is high in resting and proliferating cells, and rapidly turned off when chondrocytes undergo hypertrophy in the end of their differentiation pathway[40]. These findings indicate that SOX9 expression is essential to maintain the chondrocyte-specific phenotype. Interestingly, SOX9 mRNA level was decreased in OA cartilage[41], suggesting that SOX9 expression decrease could be a marker of the onset of chondrocyte phenotype shift in OA. One of the most interesting results of our study is that SC osteoblasts decrease PTHrP and PTH-R expression in OA chondrocytes, whereas osteoblasts isolated from N subchondral bone increase both PTHrP and PTH-R expression. In endochondral ossification, PTHrP participates in a negative feedback loop regulating the onset of hypertrophic differentiation[42]. Again, our data suggest that SC osteoblasts could contribute to hypertrophic differentiation of chondrocyte by suppressing the inhibitory retrocontrol exerted by PTHrP. Thus, a decrease of SOX9 and PTHrP/PTH-R and an increase of OSF-1 gene expression could be interpreted as a signal of chondrocyte phenotype shift towards hypertrophic phenotype. Nevertheless, we fail to demonstrate the expression by chondrocyte of type X, which is considered as a molecule specifically expressed by hypertrophic cells. Our study has some limitations. The most important is the duration of the observation that is limited to the first 10 days of culture. We cannot exclude that this culture period is too short to allow a complete differentiation process. Indeed, other authors have demonstrated that *in vitro* type X collagen is expressed by chondrocytes after 11 days in a 5-azacytidine treated monolayer model[43]. Further research works are needed to investigate the long-term effect of OA osteoblasts on the hypertrophic differentiation of OA chondrocytes. Moreover, we cannot exclude that chondrocyte phenotypical changes were limited by our culture conditions. In alginate beads, cartilage matrix is formed during the first 6 days and thereafter remains in homeostasis[30,44]. Further, we have observed that some genes involved in chondrogenesis were highly expressed after the first 4 days of culture, but expressed in very low level after 10 days, indicating a stabilization of the chondrocyte phenotype. This observation is in agreement with those of Binette *et al.*[45] who have reported that type X collagen and osteopontin were not expressed by articular chondrocytes cultivated in alginate beads, even after 5 months.

We have also co-cultured human OA chondrocytes with N or SC osteoblasts pre-incubated with IL-1β, IL-6 or OSM. We have tested these cytokines because they play an important role in bone remodelling and they could be involved in bone sclerosis process that occurs in OA. IL-6, in the presence of IL-6sR, increases collagenase-3 and IGF-1 expression in osteoblasts from foetal rat calvariae[46,47]. IL-6 also stimulates bone resorption by increasing osteoclasts recruitment and differentiation. The effects of IL-6 on osteoclasts recruitment require the presence of osteoblasts and depend on IL-6 receptors and receptor activator of nuclear factor κB (RANK)/RANK ligand (RANKL)[48]. In primary neonatal murine or foetal rat calvariae osteoblasts culture, OSM induces proliferation, collagen synthesis, and IL-6 secretion, whereas it inhibits ALP[49]. In primary culture of rat osteoblast cells, IL-1β decreases OC synthesis but has no effect on ALP activity[50]. IL-1β also stimulates PTHrP, PGE_2, IL-6 and uPA secretion and decreases TGF-β1 production by human osteoblast-like cells[51–53]. In a previous study, we have demonstrated that IL-1β, IL-6 and OSM increased ALP, but decreased OC synthesis by normal osteoblasts. Further, IL-6 and OSM increased TGF-β synthesis but had no effect on IL-1β production by normal osteoblasts[9]. Curiously, IL-1β, IL-6, or OSM pre-incubated N osteoblasts did not modify SOX9, COL2, or PTHrP/PTH-R mRNA levels in OA chondrocytes. These data suggest that IL-1β, IL-6 or OSM-treated N osteoblasts fail to reproduce SC osteoblasts-induced phenotype shift in chondrocytes, and that probably TGF-β1, which is increased by IL-6 and OSM, is not the mediator of the OA-osteoblasts effects. We are aware that further investigations are needed to identify the factor(s) involved in the SC osteoblasts effects. Some potential candidate could be responsible for these effects. For example, IL-1β is known to decrease COL2 and SOX9 expression by chondrocytes[54]. However, we have previously shown that N and SC osteoblasts produced similar amount of IL-1β (see Ref.[9]), so it's probably not this factor that is involved in the present results. IL-6 is overproduced and therefore could be the mediators of osteoblasts effects. In fact, IL-6/IL-6sR has been shown to decrease COL2 and SOX9 gene expression by chondrocytes[55]. To verify these hypotheses, we have programmed some experiments using neutralizing antibodies against numerous cytokines and growth factors. The results will be published in a separate paper.

In summary, our work demonstrates that SC osteoblasts induced a phenotypic shift in OA chondrocytes that could lead to hypertrophic phenotype and subsequently to matrix mineralization. Interestingly, IL-1β, IL-6 or OSM-treated osteoblasts isolated from N subchondral bone fail to reproduce the SC osteoblasts effects, suggesting that the factor(s) involved in this process is (are) not produced or produced in an inactive form by IL-1β, IL-6 or OSM stimulated normal osteoblasts. These findings provide further insights into the pathogenic role of osteoblasts in OA.

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