

## DNA Demethylation at Specific CpG Sites in the *IL1B* Promoter in Response to Inflammatory Cytokines in Human Articular Chondrocytes

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**Objective.** To determine whether changes in the DNA methylation status in the promoter region of the gene encoding interleukin-1 $\beta$  (IL-1 $\beta$ ) account for expression of *IL1B* messenger RNA (mRNA) after long-term treatment of human articular chondrocytes with inflammatory cytokines.

**Methods.** IL-1 $\beta$ , tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) plus oncostatin M (OSM), or 5-azadeoxycytidine (5-aza-dC) was added twice weekly for 4–5 weeks to primary cultures of normal human articular chondrocytes derived from the femoral head cartilage of patients with a fracture of the femoral neck. Expression of *MMP13*, *IL1B*, *TNFA*, and *DNMT1* was determined by SYBR Green-based quantitative reverse transcription–polymerase chain reaction (RT-PCR) analysis of genomic DNA and total RNA extracted from the same sample before and after culture. Bisulfite modification was used to identify which CpG sites in the *IL1B* promoter showed differential methylation between *IL1B*-expressing and *IL1B*-nonexpressing cells. The percentages of cells that were methylated at that critical CpG site (–299 bp) were quantified by a method that depended on methylation-sensitive restriction enzymes and real-time RT-PCR.

Secretion of IL-1 $\beta$  into the culture media was assessed by enzyme-linked immunosorbent assay.

**Results.** Healthy chondrocytes did not express *IL1B* mRNA, but the levels were increased 5-fold by treatment with 5-aza-dC and were increased 100–1,000-fold by treatment with TNF $\alpha$ /OSM. The percentage CpG methylation was decreased by 5-aza-dC treatment but was reduced considerably more by IL-1 $\beta$  and was almost abolished by TNF $\alpha$ /OSM. The mRNA was translated into protein in cytokine-treated chondrocytes.

**Conclusion.** These novel findings indicate that inflammatory cytokines can change the DNA methylation status at key CpG sites, resulting in long-term induction of *IL1B* in human articular chondrocytes.

Idiopathic osteoarthritis (OA) is a late-onset, complex disease of the joint. It is characterized by progressive failure of the extracellular cartilage matrix as well as changes in the synovium and subchondral bone (1,2). Susceptibility genes for OA, which represent potential risks, have been indentified (3). However, the disease probably develops following interactions with the environment. Such interactions may be mediated by epigenetically induced changes in gene expression that are then transmitted to generations of daughter cells (4).

As in all adult somatic cells, the phenotype of normal adult chondrocytes is stabilized by epigenetic mechanisms, such as DNA methylation of CpG sites, modifications of histone tails, and changes in chromatin structure. DNA methylation is generally stable in somatic cells throughout adult life (5,6), whereas histone modifications are readily reversible by specific enzymes (7). During DNA replication, the methylation pattern is rapidly reproduced on the nascent strand by DNA methyltransferase 1 (DNMT-1), the maintenance DNA methyltransferase (8,9). The histone code can be re-

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established after cell division by interactions of methyl binding domains and DNMTs with histone methyltransferases and histone deacetylases (10,11). As a result, both the DNA methylation pattern and the histone code are reproduced during mitosis.

In normal adult articular cartilage, chondrocytes synthesize cartilage matrix proteins, such as types II, IX, and XI collagen as well as aggrecan. These molecules impart tensile strength and compressive resistance to the joint. Normally, chondrocytes maintain a low turnover of cartilage matrix proteins, but in OA, many chondrocytes undergo a phenotypic change and acquire a gene expression repertoire that is characterized by the aberrant expression of numerous catabolic genes, including matrix metalloproteinases (MMPs), aggrecanases (ADAMTS-4 and ADAMTS-5), inducible nitric oxide synthetase and prostaglandins (12), interleukin-1 $\beta$  (IL-1 $\beta$ ) and other cytokines (13,14), and many more genes (15,16). This change in gene expression does not take place in all OA chondrocytes, but predominantly in chondrocytes of the surface zone and near weight-bearing regions (17). To distinguish these chondrocytes from OA chondrocytes with a normal gene expression repertoire, they will be referred to as “degradative” chondrocytes. Disease progression correlates with an increase in the number of these degradative chondrocytes.

Gene expression is regulated by both epigenetic and nonepigenetic mechanisms. Genes that are part of the repertoire of a particular cell type show DNA hypomethylation, and chromatin has an open structure. This permits the binding of specific transcription factors in combination with cofactors (e.g., histone acetylases and methylases) that mediate rapid responses (minutes to hours) to inductive or repressive factors. Genes that are not part of a given repertoire tend to be permanently silenced by DNA hypermethylation; this prevents access of the relevant transcription factors to their promoter. Epigenetic silencing is essential to insure genomic stability throughout life.

Epigenetic disruption may activate normally silent genes (18), or it may silence normally expressed genes. This is precisely the situation in degradative OA chondrocytes, where many nonchondrocytic genes are permanently activated. So far, only a few studies have dealt with the question of whether the aberrant gene expression in OA is linked to loss of DNA methylation. Our group (19,20) has previously demonstrated hypomethylation at specific CpG sites in the promoters of MMP-3, MMP-9, MMP-13, and ADAMTS-4 in degradative OA chondrocytes. Iliopoulos et al (21) have shown that the aberrant induction of leptin in OA is

associated with loss of DNA methylation. On the other hand, loss of osteogenic protein 1 (OP-1) expression in aged chondrocytes is correlated with hypermethylation of the OP-1 promoter (22).

Because of the association of aberrant expression of proteases with DNA demethylation, it is of interest to identify factors that cause a loss of DNA methylation. The aberrant expression of nonchondrocytic genes that occurs in OA can, to some extent, be reproduced *in vitro* by treating healthy chondrocytes with either IL-1 $\beta$  (23) or tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), especially in combination with oncostatin M (OSM) (24). However, it is not known whether this aberrant induction depends solely on nonepigenetic regulation by transcription factors or whether epigenetic changes are also involved. In the first case, one would expect expression to be induced within hours and be readily reversible by cytokine withdrawal. In the second case, induction might take days or weeks, but expression would probably persist after cytokine withdrawal.

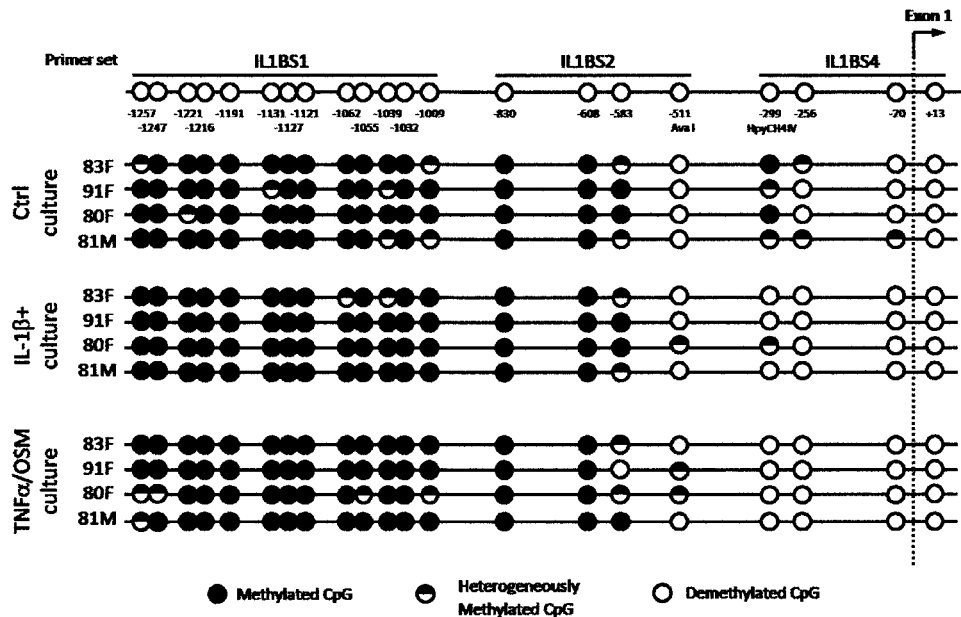
We hypothesized that the latter situation is linked to demethylation of specific CpG sites in the relevant promoter regions, whereas this is not the case for short-term induction. To test our hypothesis, messenger RNA (mRNA) expression of *IL1B*, an aberrantly induced gene, was compared with the DNA methylation status in primary human chondrocytes cultured in the presence and absence of inflammatory cytokines.

## PATIENTS AND METHODS

**Chondrocyte isolation.** Human articular cartilage was obtained after hemiarthroplasty following femoral neck fracture or following total hip arthroplasty for OA. Cartilage was dissected from femoral heads within 6 hours of surgery. In total, tissues from 21 patients with femoral neck fracture and 12 patients with OA were used in this study. Permission of the Local Ethics Committee and the consent of the patients were obtained prior to the study.

A previous study (25) showed that the deep zones of cartilage from patients with femoral neck fracture contained healthy, albeit aged, chondrocytes that did not aberrantly express proteases and cytokines. However, the superficial zone contained a few cells with aberrant expression. This zone typically displayed a pinkish coloring, so that it was relatively easy to separate this zone from the deep zone with a scalpel. In the OA patients, a significant amount of cartilage had already been degraded, especially at the weight-bearing regions. To obtain enough cartilage, we collected the “surface zone” cartilage irrespective of whether this was actually the superficial, intermediate, or deep zone. It was possible to obtain proper deep-zone OA cartilage only if thick cartilage had remained at non-weight-bearing regions, which was not always the case.

To liberate the cells, cartilage pieces were cut into small fragments and digested with 10% trypsin (Lonza, Wok-



**Figure 1.** CpG methylation map for the proximal *IL1B* promoter. Normal chondrocytes were isolated from 4 patients with femoral neck fracture and cultured for 4–5 weeks with or without interleukin-1 $\beta$  (IL-1 $\beta$ ) or tumor necrosis factor  $\alpha$  (TNF $\alpha$ )/oncostatin M (OSM). Methylation status was determined by bisulfite modification. Each circle represents the average result of 6 sequenced clones. The CpG site at  $-299$  bp was selected for quantification.

ingham, UK) in phosphate buffered saline (PBS) for 30 minutes, in 1 mg/ml of hyaluronidase (Sigma-Aldrich, Gillingham, UK) in PBS for 15 minutes, and in 10 mg/ml of collagenase B (Roche, Lewes, UK) in  $\alpha$ -modified Eagle's medium ( $\alpha$ -MEM; Sigma-Aldrich, Gillingham, UK) for 12–15 hours at 37°C.

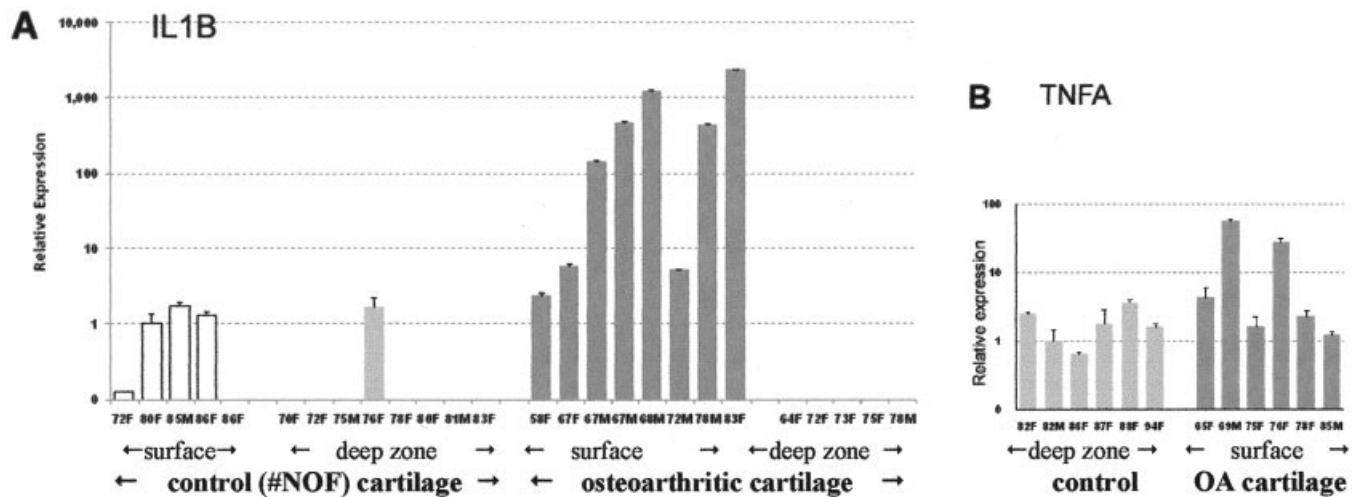
**Chondrocyte culture.** For culture, we used only non-OA chondrocytes from the deep zone of tissues from patients with femoral neck fracture (25). Before treatment, chondrocytes were cultured for 48 hours at a density of  $2\text{--}4 \times 10^5$  cells/25-cm<sup>2</sup> flask in 5 ml of  $\alpha$ -MEM supplemented with 10% fetal calf serum (FCS; Invitrogen, Paisley, UK), 1% insulin–transferrin–selenium (Sigma-Aldrich), 100 units/ml of penicillin and 100  $\mu$ g/ml of streptomycin (Lonza), and 100  $\mu$ g/ml of ascorbic acid (Sigma-Aldrich) in an atmosphere of 5% CO<sub>2</sub> at a temperature of 37°C. In the first experiment, chondrocytes were cultured until confluence, passaged once, incubated with a single addition of 10 ng/ml of IL-1 $\beta$  plus 10 ng/ml of OSM, and harvested after 24 or 72 hours. Other passage 1 cultures were treated with cytokines at each change of medium for a total of 3 weeks. Half of the cultures were then harvested by trypsinization, whereas the other half was passaged again and cultured without cytokine treatment until confluence at  $\sim$ 2 weeks.

In another experiment, chondrocytes were divided into 5 groups immediately after isolation: noncultured, cultured without treatment (control culture), cultured with 2  $\mu$ M 5-azadeoxycytidine (5-aza-dC), cultured with 10 ng/ml of IL-1 $\beta$ , and cultured with a mixture of 10 ng/ml of TNF $\alpha$  plus 10 ng/ml of OSM. For the group cultured with 5-aza-dC, the

histone deacetylase inhibitor trichostatin A (300 nM) was added just once, at the first treatment, to facilitate access of 5-aza-dC, a cytidine analog that inhibits the activity of DNMT-1 (26). This results in the nonspecific loss of DNA methylation during cell division. The media were changed twice each week, when reagents were also added. These primary cultures were maintained for 4–5 weeks until they reached confluence.

**DNA and RNA extraction.** Genomic DNA and total RNA were extracted simultaneously from the harvested chondrocytes with the use of an AllPrep DNA/RNA Mini kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. RNA was immediately reverse-transcribed with avian myeloblastosis virus reverse transcriptase and both oligo(dT)<sub>15</sub> and random primers (27).

**Quantitative reverse transcription–polymerase chain reaction (RT-PCR).** Relative quantification of gene expression was performed with an ABI Prism 7500 detection system (Applied Biosystems, Warrington, UK). Reactions were performed in triplicate, with GAPDH as the internal control. Primer Express 3.0 software (Applied Biosystems) was used to design primers across exon–exon boundaries. The primers for *IL1B* were commercially designed by PrimerDesign (Southampton, UK), and the sequence for *COL2A1* was taken from reference 28. Messenger RNA expression was quantified according to the  $2^{-\Delta\Delta C_t}$  method. The 25- $\mu$ l reaction mixture we used contained 1  $\mu$ l of complementary DNA, 12.5  $\mu$ l of 2 $\times$  Power SYBR Green PCR Master Mix, and 500  $\mu$ M of each primer. Thermocycler conditions consisted of an initial activation step at 95°C for 10 minutes, followed by a 2-step PCR



**Figure 2.** Differential expression of **A**, *IL1B* and **B**, *TNFA*, as determined by quantitative reverse transcription–polymerase chain reaction analysis. Articular cartilage was obtained from patients with femoral neck fracture (#NOF) or from patients with osteoarthritis (OA). Surface zones of the cartilage were removed and analyzed separately from the deep zones. Results are shown for individual patients, arranged in order of age. For each gene, femoral neck fracture and OA samples were analyzed in the same assay. The low levels of *IL1B* expression in the surface zone of cartilage from femoral neck fracture patients contrast with the 100–1,000-fold increased expression in the surface zone of cartilage from OA patients. With 1 exception (a 72-year-old man), expression increased with age in the OA samples. There was no expression of *IL1B* in the deep zones of cartilage from the femoral neck fracture patients (1 exception, a 76-year-old woman) or the OA patients. In contrast, *TNFA* was expressed in the control chondrocytes located in the deep zone of cartilage from femoral neck fracture patients and expression did not increase significantly in chondrocytes from the surface layer of cartilage from OA patients. Values are the mean and SD.

program of 95°C for 15 seconds and 60°C for 60 seconds for 40 cycles. A dissociation curve was obtained for each quantitative PCR run.

**Bisulfite modification.** Genomic DNA was modified with MethylDetector (Active Motif, Rixensart, Belgium), according to the manufacturer's instructions. The MethPrimer website (<http://www.urogene.org/methprimer>) was used to design primers that contained no CpG sites. Twenty-one CpG sites located between –1,300 and +15 bp were investigated with 3 pairs of nested PCR primers (Figure 1). The sequences of all primers we used can be found at <http://www.som.soton.ac.uk/research/dohad/groups/bone/Supplementary.asp>.

Thermocycler conditions comprised an activation step at 94°C for 2 minutes, followed by a 3-step PCR program, which consisted of 94°C for 30 seconds, 55°C for 60 seconds, 72°C for 60 seconds for 35 cycles, and a final extension at 72°C for 3 minutes. The PCR products were diluted 50×, and inner reactions were run. The PCR products were cloned with the TOPO TA Cloning kit using One Shot TOP 10 Chemically Competent *Escherichia coli* (Invitrogen, Paisley, UK), followed by purification of the plasmids with a PureLink Quick Plasmid MiniPrep kit (Invitrogen). Six plasmids were sequenced for each sample by Eurofins MWG Operon (Ebersberg, Germany).

**Determining the percentage methylation at –299 bp in the *IL1B* promoter.** The CpG site at –299 bp in the *IL1B* promoter was selected for quantification because it was differentially methylated in control versus cytokine-treated cultured cells (Figure 1) and because it is recognized by a methylation-sensitive restriction enzyme, namely, *Hpy* CH4IV (New England BioLabs, Frankfurt, Germany). Fully methylated DNA and fully nonmethylated DNA were used to estimate nonspecific digestion and incomplete digestion, respectively, as well as

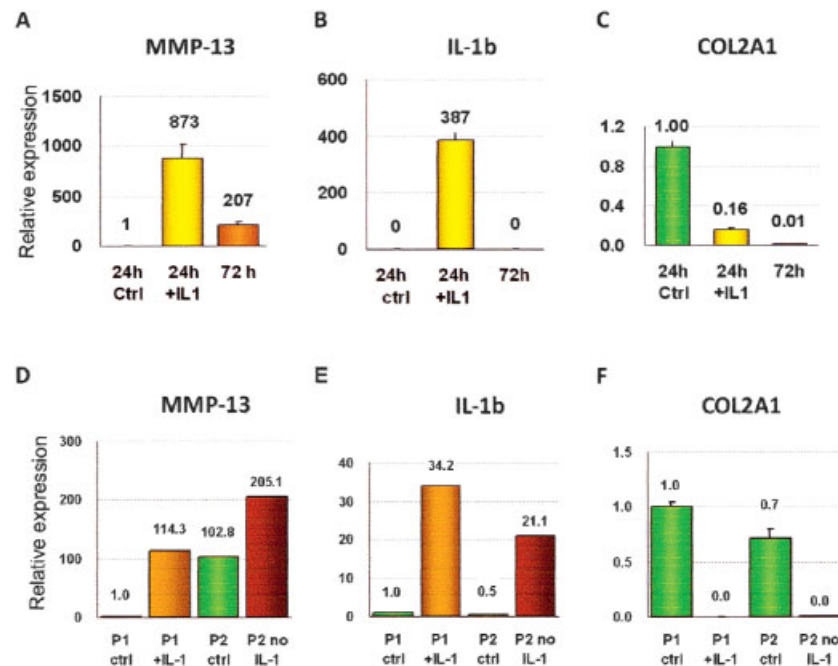
to generate a standard curve. CpGenome Universal Methylated DNA was obtained from Millipore (Eastleigh, UK), and fully nonmethylated DNA was generated from the fully methylated DNA by whole-genome amplification with an Illustra GenomiPhi V2 DNA Amplification kit (GE Healthcare Life Sciences, Little Chalfont, UK).

DNA concentrations were measured using a Nano-Drop instrument (Thermo Scientific, Welwyn Garden City, UK) and were adjusted to 4 ng/μl (20 ng of DNA in 5 μl). *Hpy* CH4IV was diluted to contain 2 units/2.5 μl of enzyme solution. These concentrations had previously been determined as optimal (27). Each digestion sample consisted of a final volume of 8.3 μl, containing 5 μl of diluted DNA, 2.5 μl of diluted *Hpy* CH4IV, and 0.8 μl of 10× NE Buffer I. All prepared samples, together with controls, were incubated at 37°C for 12 hours, followed by heat inactivation at 65°C for 20 minutes.

PCR for the percentage methylation analysis was performed as for the RT-PCR analysis. Each run included no-enzyme controls, fully methylated and nonmethylated DNA controls, the standard curve, and the enzyme-treated DNA samples. The DNA content of each enzyme-digested sample was normalized to its corresponding no-enzyme control, and the percentage methylation was calculated from the standard curve (27).

When articular cartilage from the femoral neck fracture samples were compared with articular cartilage from the OA samples for mRNA expression or for the percentage of DNA methylation, all samples were analyzed in the same assay.

**Enzyme-linked immunosorbent assay (ELISA) for IL-1β in culture media.** At the end of culture, the media containing the cytokines were removed, and after thorough wash-



**Figure 3.** Effects of cytokine withdrawal following short-term (A–C) or long-term (D–F) treatment with interleukin-1 $\beta$  (IL-1 $\beta$ )/oncostatin M (OSM) on expression of the indicated genes. A–C, The catabolic genes *MMP13* and *IL1B* were up-regulated several hundredfold at 24 hours following the addition of a single dose of IL-1 $\beta$ /OSM, whereas *COL2A1* was down-regulated. After 72 hours with no further cytokine treatment, expression of the catabolic genes was down-regulated again to near-normal levels, but expression of *COL2A1* was not regained. D–F, When IL-1 $\beta$ /OSM was added twice a week for 3 weeks to passage 1 cells (P1+ IL-1), the catabolic genes *MMP13* and *IL1B* were again considerably up-regulated. Cells were then passaged and cultured for a further 3 weeks during which no cytokines were added (P2 no IL-1). This time, expression was maintained even in the absence of cytokines, a finding consistent with the permanent induction of gene expression. *COL2A1* expression was abolished by the cytokines, and the levels remained low throughout the experiment. Results are representative of 4 experiments. Values are the mean and SD. Numbers at the top of the bars are the values that are represented by the bars.

ings to remove any remaining exogenous IL-1 $\beta$ , 5 ml of fresh medium with 1% FCS was added. After 48 hours, the media were harvested and stored at  $-80^{\circ}\text{C}$  until analysis with the human IL-1 $\beta$ /IL-1F2 Quantikine ELISA kit (R&D Systems, Abingdon, UK).

**Statistical analysis.** The data for IL-1 $\beta$  expression and percentage methylation were analyzed in Microsoft Excel (Microsoft, Redmond, WA) using Wilcoxon's signed rank test. *P* values less than 0.05 were considered significant. Other data are expressed as the mean  $\pm$  SD.

## RESULTS

### Identifying differentially methylated CpG sites.

Of the 20 CpG sites in the 1,300-bp sequence upstream of exon 1 (Figure 1), 16 were methylated in all cultures. This suggests that epigenetic regulation does not involve the region between  $-1,300$  and  $-583$  bp. The *Ava* I site at  $-511$  bp and the 2 CpG sites that encompass the transcription start site ( $-20$  and  $+13$ ) were essentially

nonmethylated in all groups. In contrast, the 2 CpG sites at  $-299$  and  $-256$  bp were methylated in control samples, but had become demethylated in cytokine-treated chondrocytes. Because only the site at  $-299$  bp was cleavable by a methylation-sensitive restriction enzyme, this site was selected for quantitative analysis in subsequent experiments.

### Differential expression of *IL1B*, but not *TNFA*, in control and OA cartilage.

To investigate the zonal distribution of *IL1B*-expressing chondrocytes in vivo, mRNA expression in the superficial and deep zones of OA and control cartilage was assessed separately. Expression was 10–1,000-fold higher in the surface layer of OA cartilage as compared with femoral neck fracture cartilage (Figure 2A). The variability among patients bore some relationship to age, in that, with 1 exception, expression increased markedly with age. In contrast to

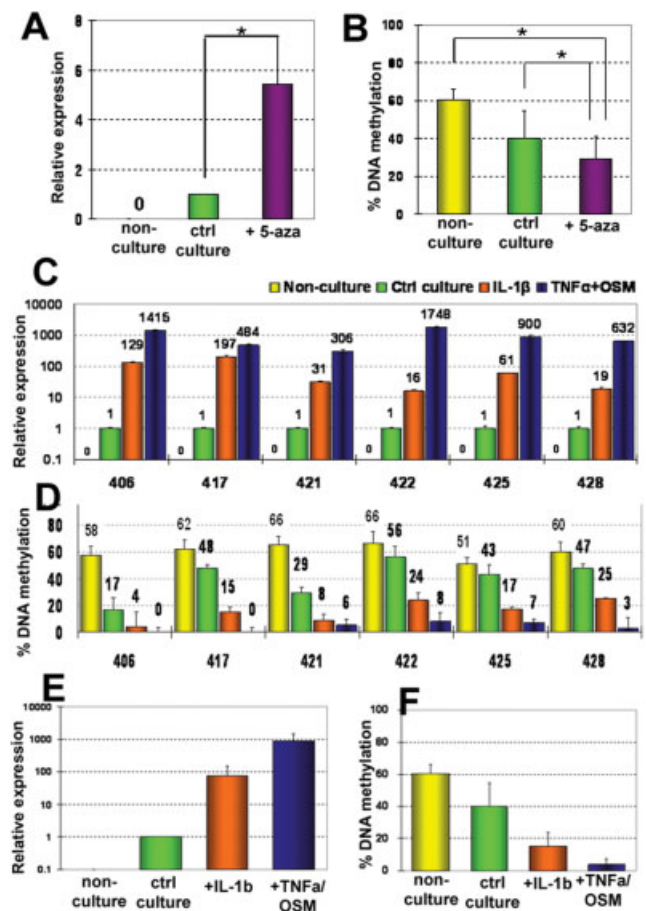
the findings in the superficial zone, no expression of *IL1B* was apparent in the deep zone of OA cartilage. In the femoral neck fracture samples, very low expression was found in the surface layer, but not in the deep zone.

These findings confirm that the deep zone of cartilage from patients with femoral neck fracture contains chondrocytes that do not express *IL1B* and are therefore suitable for culture experiments. A previous study of ADAMTS-4 expression showed similar differential expression (20). In contrast to *IL1B*, *TNFA* expression was not significantly different in the surface and deep zones (Figure 2B); this supports the notion that IL-1 $\beta$ , but not TNF $\alpha$ , is the major cytokine involved in the pathology of OA.

**Persistent aberrant expression of *MMP3*, *MMP13*, and *IL1B* with long-term cytokine treatment.** A single treatment with IL-1 $\beta$ /OSM induced the aberrant expression of *MMP3* (data not shown), *MMP13* (Figure 3A), and *IL1B* (Figure 3B) within 24 hours (Figure 3A). By 72 hours, enzyme expression had declined or disappeared. In contrast, when expression was induced by the repeated addition of IL-1 $\beta$ /OSM during 3 weeks of incubation, the expression persisted for 3 more weeks without further cytokine treatment (Figures 3D and E). *COL2A1* expression was reduced after a single addition of cytokine and remained low throughout the experiments (Figures 2C and F)

**Increased expression of *IL1B* following experimental demethylation.** If DNA demethylation underpins aberrant *IL1B* expression in chondrocytes, then experimentally induced demethylation should result in increased expression. When normal chondrocytes were cultured with 5-aza-dC, the expression of *IL1B* increased 3.6–8.6-fold (mean  $\pm$  SD  $5.5 \pm 2.2$ -fold increase) compared with control cultures (Figure 4A). To check that 5-aza-dC treatment had actually resulted in loss of DNA methylation in the *IL1B* promoter, we quantified the percentage of methylation. As shown in Figure 4B, a mean  $\pm$  SD of  $61.0 \pm 6.1\%$  of the chondrocytes were methylated in uncultured chondrocytes. In cultured samples, this value was reduced to  $44.8 \pm 9.9\%$ , indicating that culture per se could cause a limited loss of DNA methylation. However, 5-aza-dC further reduced the percentage of DNA methylation to  $33.6 \pm 5.7\%$ . The results showed that the experimentally induced loss of DNA methylation results in increased gene transcription, thus confirming a cause-and-effect relationship between DNA demethylation and transcription.

**Increased expression of *IL1B* (100–1,000-fold) with long-term exposure to cytokines.** Healthy chondrocytes were cultured for 4–5 weeks with twice-weekly



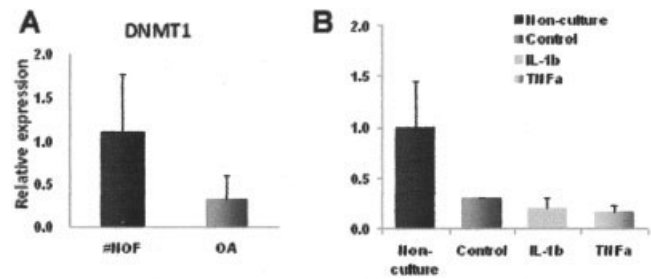
**Figure 4.** DNA demethylation and aberrant gene expression following long-term treatment with cytokines. Chondrocytes were cultured with 5-azadeoxycytidine (5-aza-dC) (A and B) or with interleukin-1 $\beta$  (IL-1 $\beta$ ) or tumor necrosis factor  $\alpha$  (TNF $\alpha$ )/oncostatin M (OSM) (C–F), and the relative expression of mRNA for *IL1B* was compared with the percentage of DNA methylation at  $-299$  bp in the *IL1B* promoter. A and B, Culture itself induced some *IL1B* expression (A) and a 20% loss in DNA methylation (B). Addition of 5-aza-dC increased the expression 5-fold as compared with control culture and caused a further loss of DNA methylation. Values are the mean and SD of 6 samples. \* =  $P < 0.05$ . C and D, Relative expression of *IL1B* (C) and percentage of DNA methylation (D) in chondrocytes from 6 individual patients (patient numbers shown across the x-axis). Chondrocytes were not cultured, were cultured alone, or were cultured with IL-1 $\beta$  or TNF $\alpha$ /OSM. No *IL1B* was detected in noncultured chondrocytes. Culture itself induced low expression (set at 1), but culture with IL-1 $\beta$  or TNF $\alpha$ /OSM considerably increased *IL1B* expression. Before culture,  $\sim 60\%$  of cells were methylated. Culture alone reduced DNA methylation, which was significant in patients 406 and 421. However, cytokine treatment caused a greater loss of DNA methylation, particularly the combined treatment with TNF $\alpha$ /OSM. Values are the mean and SD of 6 individual samples. Numbers at the top of the bars are the values that are represented by the bars. E and F, Overall mean and SD values for the relative expression of *IL1B* (E) and the percentage of DNA methylation (F) in the 6 individual samples shown in C and D, respectively. The differences between all groups were significant at  $P < 0.05$ .

additions of either IL-1 $\beta$  or TNF $\alpha$ /OSM (Figure 4C). The absence of expression in noncultured chondrocytes was confirmed, and the low expression induced by culture alone was set at 1. Treatment with IL-1 $\beta$  increased the expression of *IL1B* 16–197-fold, while the combination of TNF $\alpha$ /OSM increased expression of *IL1B* by 306–1,748-fold compared with control cultures ( $P < 0.01$ ). This showed that the capacity of the cytokines to increase expression was several orders of magnitude greater than that of 5-aza-dC.

**Loss of DNA methylation with long-term exposure to cytokines.** Quantitative analysis showed that culture alone reduced DNA methylation in cartilage samples from 2 patients (patients 406 and 421), but this decrease was not significant as compared with the other patients. In contrast, the effects of the cytokines were very prominent. Only 4–25% (mean  $\pm$  SD 17.9  $\pm$  6.9%) of the IL-1 $\beta$ -treated chondrocytes were still methylated at –299 bp, and with the combined TNF $\alpha$ /OSM treatment, only 0–8% (mean  $\pm$  SD 4.5  $\pm$  3.7%) of the cells remained methylated. Although there was no direct linear correlation between the fold increases in gene expression and the loss of DNA methylation, the trends are very clear: samples with the greatest loss of DNA methylation showed, on the whole, the greatest induction of gene transcription.

**Association of increased *IL1B* expression with the release of IL-1 $\beta$  protein into the medium.** Significant amounts of IL-1 $\beta$  protein were produced by chondrocytes that had been cultured with either IL-1 $\beta$  or TNF $\alpha$ /OSM, but no IL-1 $\beta$  was detected in control cultures or in cultures treated with 5-aza-dC. This indicates that the small increases in transcription induced by 5-aza-dC did not produce measurable amounts of IL-1 $\beta$  protein. Treatment with exogenous IL-1 $\beta$  resulted in the release of 0.011–0.225 pg of IL-1 $\beta$  per  $\mu$ g of DNA per hour, while TNF $\alpha$ /OSM treatment caused the release of 0.066–0.169 pg of IL-1 $\beta$  per  $\mu$ g of DNA per hour. These findings indicate that the increased expression of *IL1B*, a likely result of transcriptional promoter activation, resulted in increased synthesis of IL-1 $\beta$  protein.

**No increase in *TNFA* expression by chondrocytes following treatment with cytokine.** Because IL-1 $\beta$  induced its own expression in vitro, we explored whether the same was true for TNF $\alpha$ . Noncultured chondrocytes expressed *TNFA* (Figure 2B), and culture of chondrocytes had no further effect. The expression of *TNFA* in chondrocyte cultures treated with IL-1 $\beta$  or TNF $\alpha$ /OSM was not significantly different from that in the cultured controls, with a mean  $\pm$  SD increase in expression of 0.59  $\pm$  0.44-fold and 0.91  $\pm$  0.49-fold, respectively.



**Figure 5.** Expression of *DNMT1* in articular chondrocytes in vivo (A) and in vitro (B). *DNMT1* expression in articular cartilage chondrocytes from patients with osteoarthritis (OA;  $n = 5$ ) was less than half that in control chondrocytes from patients with femoral neck fracture (#NOF;  $n = 5$ ) ( $P < 0.05$ ). In vitro, culture alone had the greatest effect ( $P < 0.01$ ), and the decreased expression in control chondrocytes from patients with femoral neck fracture was of similar magnitude to that in chondrocytes from patients with OA. In addition, treatment with tumor necrosis factor (TNF $\alpha$ )/oncostatin M (OSM) further decreased expression compared with noncultured cells ( $P < 0.01$ ). Values are the mean and SD of chondrocyte cultures from 6 patients.

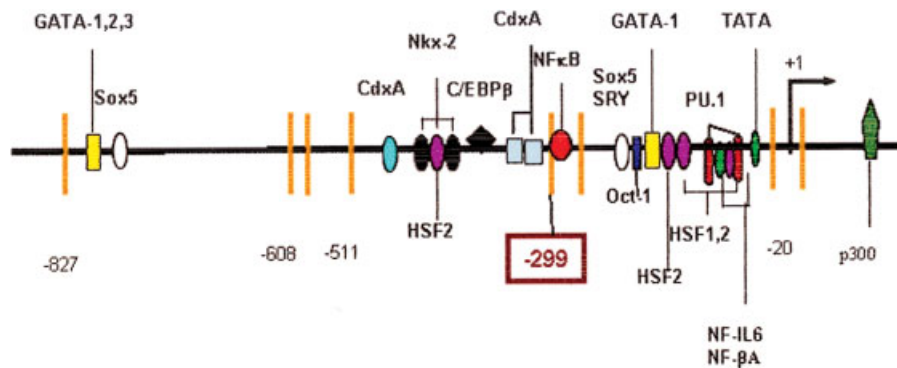
Thus, in contrast to the expression of *IL1B*, treatment with inflammatory cytokines did not increase *TNFA* expression in vitro.

**Decreased *DNMT1* expression following culture and cytokine treatment.** To determine whether decreased expression of *DNMT1* contributed to the observed loss of DNA methylation, we used quantitative RT-PCR to investigate its expression. Indeed, *DNMT1* expression in OA chondrocytes from the surface zone was half that in control chondrocytes from patients with femoral neck fracture (Figure 5A). Culture alone also reduced *DNMT1* expression to around half that in noncultured chondrocytes, which may partly explain the loss of DNA methylation in control cultures. Addition of IL-1 $\beta$  or TNF $\alpha$ /OSM caused only a small further decrease compared with control (Figure 5B).

## DISCUSSION

The novel findings of the present study are that inflammatory cytokines have the capacity not only to induce changes in gene expression, but also to demethylate specific CpG sites in the proximal *IL1B* promoter. IL-1 $\beta$  is at the apex of several inflammatory cascades and is one of the most important cytokines in OA, although the debate is ongoing.

Because IL-1 $\beta$  has a very short half-life and the mRNA transcript levels correspond to the protein levels, it is important to understand transcriptional regulation of this protein. Nonepigenetic regulation of the *IL1B*



**Figure 6.** Proximal promoter of *IL1B*. Vertical bars indicate the positions of CpG sites. The CpG sites at  $-299$  and  $-256$  bp bracket an NF- $\kappa$ B binding site.

promoter has been studied extensively in monocyte/macrophages. In these cells, *IL1B* is readily induced by a variety of stimuli, including IL-1 $\beta$  itself (29,30). The CpG region between  $-131$  and  $+12$  bp is sufficient to direct the expression of a reporter gene (31), but an inducible enhancer located between  $-3,134$  and  $-2,729$  bp (31,32) is also involved in signal-dependent expression. In monocytes, this promoter is packaged into a nontranscribed, but “poised,” promoter structure that is characterized by a histone-free transcription start site and constitutive association with the transcription factors PU.1 and CCAAT/enhancer binding protein  $\beta$  (c/EBP $\beta$ ) (30). Monocytes are thus primed for rapid activation in response to pathologic or other stimuli.

Articular chondrocytes do not normally express *IL1B*, as confirmed here by quantitative RT-PCR. The low level of expression observed in the superficial zone of articular cartilage from patients with femoral neck fracture probably indicates an age-related change to “degradative” chondrocytes in a few cells. In contrast, the chondrocytes located near the surface of OA cartilage showed very high levels of *IL1B* expression, whereas this was undetectable in deep-zone OA cartilage. We hypothesized that *IL1B* was silenced by DNA methylation in normal chondrocytes and that demethylation had occurred in OA, as previously reported for MMPs and ADAMTS-4 (19).

To investigate the epigenetic regulation of *IL1B*, we studied the proximal promoter because its activity regulates cell-type-specific expression (33). When the CpG locations were compared with the transcription factor binding sites (Figure 6), the latter were concentrated in 2 CpG-free regions, each bracketed by CpG sites. Interestingly, the site at  $-511$  bp and the 2 CpG sites around the transcription start site ( $-20$  and  $+13$

bp) were unmethylated, whether *IL1B* was expressed or not. The 2 CpG sites that were differentially methylated and where the methylation status was inversely correlated with expression ( $-256$  and  $-299$  bp) were located between the 2 regions that contain multiple transcription factor binding sites and bracketed an NF- $\kappa$ B binding site. Both IL-1 $\beta$  and TNF $\alpha$  activate NF- $\kappa$ B (34–36), which in turn, activates many genes (37), including *IL1B* and other catabolic genes induced in OA. Perhaps more relevant in the present context is the fact that NF- $\kappa$ B may be involved in demethylation (38), although the details are not clear. The observation (39) that in intestinal inflammation, TNF $\alpha$ -induced NF- $\kappa$ B expression was associated with demethylation of CpG islands supports the inference that NF- $\kappa$ B may play a role in demethylation in the experiments described herein.

Having identified the differentially methylated CpG sites, we determined the percentage of cells that were methylated at  $-299$  bp (27). Even in control chondrocytes, which do not express *IL1B*, the percentage methylation was only  $\sim 60\%$ , rather than the expected 100%. This raises the question of why 40% of the nonmethylated cells did not express *IL1B*. Hypomethylation is a necessary, but not sufficient, condition for gene activation, inasmuch as histone modifications are also required. Deacetylated histones or methylated histone H3 lysine 9 (H3K9) and H3K27 can, in theory, silence a gene even in the absence of DNA methylation. An alternative possibility is that normal articular chondrocytes do not contain the transcription factors required for *IL1B* expression.

Thus, the rapid induction after 24 hours could depend on cytokine-induced histone modifications and/or activation of transcription factors in cells with preexisting demethylation. In support of this notion,



IL-1 $\beta$  has been shown to translocate to the nucleus and to interact with histone acetyltransferase in yeast strains (40). Whether this also applies to IL-1 $\beta$  is not known. Up-regulation of *IL1B* after short-term treatment did not persist, yet after long-term treatment, the expression of *IL1B* persisted for at least 2 weeks after the inducer had been withdrawn. This is consistent with permanent epigenetic changes. Because the pathology of OA involves long-term aberrant induction of IL-1 $\beta$  and proteases, short-term in vitro experiments are unlikely to model the situation in vivo.

Further evidence of a cause-and-effect relationship between DNA methylation and gene expression was obtained in 5-aza-dC-treated cultures, in which the experimentally induced loss of DNA methylation increased the expression of *IL1B* by 5-fold. Treatment with 5-aza-dC reduced, yet did not abolish, the percentage of methylation. One reason may be that 5-aza-dC, whose half-life is ~4 hours (26), can act only on those cells that undergo cell division during that time. Because 5-aza-dC was added 3 times per week, it is likely that active 5-aza-dC was not present at all times when cell division took place.

Treatment with cytokines, rather than with 5-aza-dC, was much more effective at inducing gene expression. This was especially true for the combination of the 2 cytokines (TNF $\alpha$ /OSM). TNF $\alpha$  with or without OSM has frequently been used to induce the expression of degradative proteases in cultured chondrocytes (34,41). The findings presented here show that the TNF $\alpha$ /OSM combination is also highly effective in stimulating *IL1B* in chondrocytes, increasing its expression up to 1,000-fold. OSM belongs to the IL-6 family, acts via JAK/STAT signaling, and can, on its own, induce cartilage degradation in vitro (42), but it is usually used together with TNF $\alpha$  (43) or IL-1 $\beta$  (24,41) to enhance the cytokine effect. The percentage of DNA methylation correlated negatively with increases in *IL1B* expression, although this varied considerably among patients. Samples treated with TNF $\alpha$ /OSM had the highest expression level and the greatest loss of DNA methylation. Indeed, DNA methylation was completely abolished in some samples.

Based on these findings, it is evident that long-term aberrant expression of *IL1B* was a consequence of DNA demethylation, presumably caused by inflammatory cytokines. The question then arises of how cytokines can achieve greater DNA demethylation than 5-aza-dC. Because 5-aza-dC inhibits *DNMT1*, we sought to determine whether cytokines also alter *DNMT1* expression. This was indeed reduced in vitro by the cyto-

kines and in OA chondrocytes as compared with femoral neck fracture control chondrocytes in vivo. However, since culture per se significantly reduced expression, lower expression of *DNMT1* alone could not explain the results. We hypothesize that activation of NF- $\kappa$ B plays a major role in cytokine-induced DNA demethylation (38), but further studies are required.

There are several caveats that should be borne in mind. When chondrocytes are grown in monolayer for several weeks, their phenotype is lost. However, explants of articular cartilage in organ culture, in which the chondrocyte phenotype is maintained, exhibit little or no loss of DNA methylation or gene induction. This suggests that the extracellular matrix protects the cells from loss of phenotype and possibly from loss of DNA methylation. To maintain genomic stability in vivo, one would expect resistance to changes in DNA methylation status in healthy, fully differentiated cells, and the extracellular matrix might provide increased resistance in explant cultures. Monolayer cultures probably sensitize chondrocytes to epigenetic changes, so that one can obtain within weeks the phenotype changes that probably take years to occur in OA. The extent to which the protease-producing “degradative” cells are still chondrocytes is a matter for debate.

One should also be careful about extending these in vitro findings to the in vivo situation. In vitro, decreased expression of *COL2A1* was observed after a single addition of cytokine, whereas in vivo, increased *COL2A1* expression was observed in surface-zone OA chondrocytes as compared with femoral neck fracture chondrocytes (data not shown), suggesting that the cultures do not model the vivo situation as far as *COL2A1* is concerned. There is also some disagreement about the importance of IL-1 $\beta$  in OA. We have observed significant expression of *IL1B* in chondrocytes of the superficial zone of OA cartilage, where cytokines have also been immunolocalized (44,45). In contrast, Fan et al (46) found no significant up-regulation of *IL1B* in OA patients, but those investigators had used early degenerated cartilage from the knee joint, with the deep zone included. In IL-1 $\beta$ -knockout mice, experimentally induced OA was either enhanced or reduced (47,48), depending on genetic background, whereas overexpression of human TNF $\alpha$  induced severe inflammatory arthritis (49). However, the mouse models may not be representative of human OA.

In summary, our novel findings support the conclusion that the long-term cytokine-stimulated induction of *IL1B* in human articular chondrocytes in vitro involves the loss of DNA methylation. If applicable in vivo,

then the mechanisms for OA progression may involve the following. An initial episode of inflammation in the synovium, perhaps as a consequence of mechanical stress, activates synovial macrophages to produce IL-1 $\beta$  and TNF $\alpha$ . These cytokines diffuse into the articular cartilage. There, the cytokines induce aberrant expression of proteases and *IL1B* in the chondrocytes (50). If this induction leads to the loss of DNA methylation, *IL1B* will now be included in the expression repertoire of the OA chondrocytes, even after the synovial inflammation has abated. This scenario may explain why protease inhibitors, such as tissue inhibitors of metalloproteinases, have little effect when injected into the joint and why, once degradative processes have been operative, OA progression cannot be halted.

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#### 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. Roach 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.** Gibson, Goldring, Roach.

**Acquisition of data.** Hashimoto.

**Analysis and interpretation of data.** Hashimoto, Oreffo, Roach.

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