Regulated Transcription of Human Matrix Metalloproteinase 13 (*MMP13*) and Interleukin-1β (*IL1B*) Genes in Chondrocytes Depends on Methylation of Specific Proximal Promoter CpG Sites*

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Background: The role of DNA methylation in musculoskeletal diseases is poorly understood.
Results: Methylation of specific CpG sites in the *MMP13* and *IL1B* promoters correlates strongly with gene activity in human cartilage.
Conclusion: Specific CpG site methylation inhibits *MMP13* transactivation by HIF-2α.
Significance: Our findings offer new insight on the impact of epigenetic control of a joint disease that can affect adults throughout life.

The role of DNA methylation in the regulation of catabolic genes such as *MMP13* and *IL1B*, which have sparse CpG islands, is poorly understood in the context of musculoskeletal diseases. We report that demethylation of specific CpG sites at −110 bp and −299 bp of the proximal *MMP13* and *IL1B* promoters, respectively, detected by *in situ* methylation analysis of chondrocytes obtained directly from human cartilage, strongly correlated with higher levels of gene expression. The methylation status of these sites had a significant impact on promoter activities in chondrocytes, as revealed in transfection experiments with site-directed CpG mutants in a CpG-free luciferase reporter. Methylation of the −110 and −299 CpG sites, which reside within a hypoxia-inducible factor (HIF) consensus motif in the respective *MMP13* and *IL1B* promoters, produced the most marked suppression of their transcriptional activities. Methylation of the −110 bp CpG site in the *MMP13* promoter inhibited its HIF-2α-driven transactivation and decreased HIF-2α binding to the *MMP13* proximal promoter in chromatin immunoprecipitation assays. In contrast to HIF-2α, *MMP13* transcriptional regulation by other positive (RUNX2, AP-1, ELF3) and negative (Sp1, GATA1, and USF1) factors was not affected by methylation status. However, unlike the *MMP13* promoter, *IL1B* was not susceptible to HIF-2α transactivation, indicating that the −299 CpG site in the *IL1B* promoter must interact with other transcription factors to modulate *IL1B* transcriptional activity. Taken together, our data reveal that the methylation of different CpG sites in the proximal promoters of the human *MMP13* and *IL1B* genes modulates their transcription by distinct mechanisms.

Somatic cell phenotypes can be stably regulated by epigenetic mechanisms, including DNA methylation (1, 2), which may regulate gene transcription by recruiting chromatin remodeling proteins and by modulating the binding affinities of specific transcription factors (3–7). In contrast to the more dynamic and reversible histone modifications (1), CpG methylation is generally stable in somatic cells throughout adult life (8, 9). Investigations have mainly focused on the so-called CpG islands but have ignored the contributions of sparse CpG sites. Moreover, only a few reports have addressed how methylation of individual CpG sites influences the promoter activity of a specific gene (6, 10, 11), and the relative contributions of the methylation status of each individual CpG site to the transcriptional regulation of a given gene *in vivo* remain largely unexplored. In addition, although epigenetic gene regulation has been broadly investigated in other contexts, little is known about the specific impact of this mechanism of gene control in musculoskeletal diseases (12–29).

Osteoarthritis (OA)[3] is characterized by the progressive loss of cartilage matrix, accompanied by deleterious changes in the

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3 The abbreviations used are: OA, osteoarthritis, MMP, matrix metalloproteinase; HIF-2α, hypoxia-inducible factor-2α; EPAS1, endothelial PAS domain protein 1; 5-aza-dC, 5-aza-deoxycytidine; bHLH-PAS, basic helix-loop-helix Per-ARNT-Sim; mt, mutant.

TABLE 1
Primer sequences for pyrosequencing

Name (range)/direction/purpose (covering CpG sites)	Sequence (5′ to 3′)
MMP13-Pyro-1 (−402/−188)	
Forward	AATTAGTATTAAGTTTTTTTTTATGGAAGT
Reverse	TTCAACAAAATCTCAAAACCCATCTAA
Sequencing 1	AAATTTTTTTTTTTTTTTACCTTCTAT
Sequencing 2	CTCAAAACCCATCTAAC
MMP13-Pyro-2 (−210/+36)	
Forward	ATGGGTTTTGAGATTTTG
Reverse	ACCCCTAAATACATCTTAAATA
Sequencing 1	CAATCACTTAAAAATAAACATACTT
Sequencing 2	AATAATACCTAAAAACTATTATC
Forward	ATGGAAGGGTAAGGAGTAGTAA
IL1B-Pyro-1 (−331/−175)	
Reverse	CCCACATATACTAAATTTAAACATTCTT
Sequencing	ATACTAAATTTAAACATTCTTCTA
IL1B-Pyro-2 (−64/+65)	
Forward	ATGAAGATTGGTTGAAGAGAATTTTAGA
Reverse	ATTTCTCAACCTCCTACTTCTACTTTTAA
Sequencing	ATTTTAGAGTAGTTTGTTGTG

synovium and subchondral bone (30, 31). In OA disease, chondrocytes undergo phenotypic changes with altered patterns of gene expression that are stably transmitted to subsequent cell generations (25, 32), suggesting the involvement of epigenetic mechanisms in disease onset and progression. We previously described a novel relationship between CpG demethylation within the CpG-sparse promoters of the *MMP3, -9, -13*, and *ADAMTS4* genes and their aberrant, enhanced expression in OA chondrocytes, although a direct relationship between the methylation status of a specific CpG site and the respective promoter activity was not investigated (25). We also found that long term, repetitive stimulation of primary human chondrocytes with inflammatory cytokines induced demethylation of specific CpG sites in the *IL1B* proximal promoter, leading to increased and sustained *IL1B* expression (22). More recently, Reynard *et al.* (24) reported CpG methylation-dependent regulation of growth differentiation factor 5 expression in OA, along with direct evidence of suppressed gene activity in response to methylation of its CpG islands *in vitro*.

Here, we report that the methylation of specific CpG sites in the proximal promoters of both the human *MMP13* and *IL1B* genes correlates with their expression in cartilage and alters their transcriptional activities in chondrocytes. In addition, we provide the first evidence that binding to and transactivation of the *MMP13* promoter by hypoxia-inducible factor-2α (HIF-2α), also known as EPAS1 (endothelial PAS domain protein 1), are controlled by these epigenetic events. Importantly, our *in vitro* results correlate strongly with the *in vivo* methylation status of the same individual CpG sites in the *MMP13* and *IL1B* promoters and with their relative levels of gene activity in chondrocytes isolated directly from healthy and OA human cartilage.

EXPERIMENTAL PROCEDURES

Human Primary Chondrocyte Isolation—Human articular cartilage was obtained after hemiarthroplasty following femoral neck fracture or total hip arthroplasty for OA with patient consent and prior approval of the local Institutional Review Board. Cartilage was dissected within 6 h of surgery, and non-OA fractured neck of femur and OA human primary chondrocytes were isolated as described previously (22). Briefly, samples were obtained from the deep zone of cartilage from patients with femoral neck fracture for isolation of non-OA/healthy chondrocytes, whereas cartilage pieces adjacent to weight-bearing areas of OA femoral heads (lacking surface zones) were harvested to obtain OA chondrocytes. Cartilage samples were then cut in small fragments and digested with 10% trypsin (Lonza) in PBS for 30 min, followed by sequential digestions in 1 mg/ml of hyaluronidase (Sigma-Aldrich) in PBS for 15 min, and in 10 mg/ml of collagenase B (Roche Applied Science) in α-modified Eagle's medium (Sigma-Aldrich) for 12–15 h at 37 °C. Following isolation, the chondrocytes were either subjected to bisulfite pyrosequencing or placed in primary culture.

Pyrosequencing—Genomic DNA extracted directly from neck of femur and OA chondrocytes or from cultured chondrocytes was treated with sodium bisulfite to convert unmethylated cytosine in CpG sites to uracil using the EZ DNA methylation Gold kit (Zymo Research). PCR and sequencing primers for pyrosequencing were designed using the Biotage Assay Design Software (version 2.0; Table 1). PCR was performed with Premium® PCR Supermix High Fidelity (Invitrogen). Pyrosequencing reactions were run in the PyroMark Q96 MD system (Qiagen), using 20 μl and 5 μl of the PCR products for CpG methylation analysis of *MMP13* and *IL1B* promoters, respectively. Data are represented as percentage of methylation of each CpG site in OA or 5-aza-deoxycytidine (5-aza-dC)-treated samples set against 100% in the non-OA or untreated controls.

Human Primary Chondrocyte Culture and 5-Aza-dC Treatment—For culture and *in vitro* analysis, only deep-zone primary non-OA chondrocytes were utilized, as described (21). Before treatment, chondrocytes were cultured for 48 h at an average density of 16×10^3 cells/cm^2 in a 25-cm^2 flask in 5 ml of α-modified Eagle's medium supplemented with 10% FBS (Invitrogen), 1% insulin-transferrin-selenium (Sigma-Aldrich), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (Lonza) and 100 μg/ml of ascorbic acid (Sigma-Aldrich). The chondrocytes were then left untreated (control) or incubated with 2 μM 5-aza-dC and added freshly at twice weekly medium changes for 4 to 5 weeks until confluence.

DNA and RNA Extraction—Genomic DNA and total RNA were extracted simultaneously from the harvested chondrocytes using AllPrep DNA/RNA mini kit (Qiagen). RNA was reverse-transcribed with avian myeloblastosis virus reverse transcriptase and combination of oligo(dT)$_{15}$ and random primers (Promega).

Quantitative RT-PCR—Gene expression was analyzed with the ABI Prism 7500 gene detection system (Applied Biosystems) using the following primers, designed using Primer Express software (version 3.0; Applied Biosystems) to bracket exon-exon boundaries: *GAPDH*, 5′-CCAGGTGGTCTCCTCTGACTTC-3′ (forward) and 5′-TCATACCAGGAAATGAGCTTGACA-3′ (reverse); *MMP13*, 5′-TTAAGGAGCATGGCGACTTCT-3′ (forward) and 5′-CCCAGGAGGAAAAGCATGAG-3′ (reverse). The *IL1B* primers were designed by and purchased from PrimerDesign, Ltd. (Southampton, UK): 5′-TGGCAATGA-GGATGACTTGTTC-3′ (forward) and 5′-CTGTAGTGGT-GGTCGGAGATT-3′ (reverse). Reactions were performed in triplicate, with GAPDH as the normalizing control gene. The $2^{-\Delta\Delta Ct}$ method was employed for relative quantification.

Plasmid Constructions—A CpG-free reporter vector, pCpG-free-Luc vector, was generated according to the literature (33). The *MMP13* promoter constructs spanning −372/+14, −214/+14, and −86/+14 bp of the human *MMP13* promoter sequence and containing seven, four, and one CpG sites, respectively, were generated by PCR amplification, utilizing genomic DNA from the human chondrocyte cell line C28/I2 (34) as a template and the following PCR primers: MMP13(−372/+14), 5′-CCG<u>ACTAGT</u>GGAAGTAAACATGCCATCTTGATA-3′ (forward); MMP13(−214/+14), 5′-CCG<u>ACTAGT</u>ATTTTG-CCAGATGGGTTTTG-3′ (forward); MMP13(−86/+14), forward: 5′-CCG<u>ACTAGT</u>CAAGTGACTAGGAAGTGGAA-ACC-3′; and reverse (common to all three MMP13 promoter constructs): 5′-CCG<u>AAGCTT</u>CCTGGGGACTGTTGTC-TTT-3′. Underlined letters indicate SpeI and HindIII recognition sequences in the forward and reverse primers, respectively. The resultant PCR products were digested with SpeI and HindIII and transferred into the multiple cloning site of a pCpG-free-Luc vector treated with the same enzymes using TaKaRa DNA ligation kit (version 2.1; TaKaRa).

The human *IL1B* promoter constructs spanning −521/+56, −276/+56, and −183/+56 bp and containing four, three, and two CpG sites, respectively, were generated by PCR amplification utilizing the following PCR primers: IL1B(−521/+56), 5′-CCG<u>ACTAGT</u>AGAGAGCTCCTGAGGCAGAG-3′ (forward); IL1B(−276/+56), 5′-CCG<u>ACTAGT</u>TGTGGACATCAACTGC-ACAA-3′ (forward); IL1B(−183/+56), 5′-CCG<u>ACTAGT</u>CCCT-TCCATGAACCAGAGA-3′ (forward); and common to all three *IL1B* promoter constructs, 5′-CCG<u>AGATCT</u>GGCTGAAGAG-AATCCCAGAG-3′ (reverse). *Underlined letters* indicate SpeI and BglII recognition sequences on the forward and reverse primers, respectively. The resultant PCR products were digested with SpeI and BglII and transferred into the multiple cloning site of the pCpGfree-Luc vector.

Point mutations of CpG sites were generated by converting CG to TG by a two-step PCR mutagenesis approach as described previously (35) using the primers shown in Table 2 and the wild type (WT) −214/+14 bp *MMP13* (MMP13–214)

or −521/+56 bp *IL1B* (IL1B-521) promoter constructs, linearized by double enzyme digestion by SacI and KpnI as templates. After two-step PCR, the resulting products were digested with SpeI/HindIII and SpeI/BglII for MMP13–214 and IL1B-521, respectively, and cloned into the pCpGfree-Luc vector backbone treated with the same enzymes using TaKaRa DNA ligation kit (version 2.1, TaKaRa). Promoter constructs with multiple CpG mutations were generated by a repeated two-step PCR mutagenesis, utilizing different PCR mutagenesis primers and linearized CpG-mutated MMP13–214 or IL1B-521 construct as template. Expression vectors encoding ELF3, HIF1α, HIF-2α, Sp1, RUNX2, NF-κB, and AP-1 (cFos/cJun) were purchased from Addgene and/or described elsewhere (35–37). The sequences of all constructs were confirmed by DNA sequencing at the Cornell University Life Sciences Core Laboratories Center.

In Vitro Methylation, Transfections, and Luciferase Assay—The methylated plasmids were generated by incubating 1 μg of plasmid DNA with 4 units/μl of the CpG methyltransferase, M.SssI (Zymo Research) in reaction buffer supplemented with 1600 μM *S*-adenosylmethionine according to the manufacturer's instructions. Complete methylation was verified by plasmid DNA bisulfite modification and pyrosequencing using specific primers. The methylated plasmid DNA was purified by the PureLink® Quick Plasmid Miniprep kit (Invitrogen). The methylated and non-methylated vectors were then transfected in immortalized human chondrocytes (C28/I2) seeded 24 h prior to transfection in 24-well tissue culture plates at 3.0×10^4 cells/well, using Lipofectamine PLUS^{TM} reagents as described (35). Briefly, transfections were carried out in triplicate in serum-free antibiotic-free conditions, with a total of no more than 475 ng of plasmid DNA per well, including 300 ng of non-methylated or methylated *MMP13* or *IL1B* luciferase reporter construct and the expression vectors. The ratios of luciferase reporter construct to expression vector were established by titrations to ensure that concentrations of expressed transcription factors were not limiting to the promoter transactivations under the conditions of the co-transfections. Reporter constructs were treated with DNA methyltransferase to methylate all available CpG sites in the promoters. Luciferase assays were performed using the luciferase assay system (Promega) and detected using a LMaxII^{384} Luminometer system (Molecular Devices). Unless otherwise specified, the firefly luciferase activities were normalized to the *Renilla* activity of pGL4.74-hRluc-TK (Promega).

ChIP Assay—The ChIP-IT Express Enzymatic Kit (Active-Motif) was used to perform ChIP assays according to the manufacturer's instructions with minor modifications, as described (35), using C28/I2 chondrocytic cells co-transfected with non-methylated or methylated pCpGfree-Luc-MMP13 vector (−214/+14bp) and expression vectors encoding HA-tagged HIF-2α or FLAG-tagged ELF3. At 24 h after transfection, precleared chromatin was stored as assay input or incubated at 4 °C overnight with 1.25 μg of rabbit anti-HA antibody (Invitrogen), rabbit anti-FLAG antibody (Sigma), or normal rabbit IgG (Cell Signaling). After reverse cross-linking and purification on DNA mini-columns (Qiagen), the final DNA preparations were subjected to quantitative PCR analysis using 5 μl of the eluted DNA and the following primers specific for the transiently trans-

TABLE 2
Primer sequences for two-step promoter mutagenesis

Name (location of mutated CpG)/part of amplicon direction	Sequence (5′ to 3′)[a]
MMP13–214-Mut1 (-14)	
1st half (forward)	TATGTGAGCAAACAGCAGATTAAAA
1st half (reverse)	TTGTCTTTCCaCAGAGATTACCTTT
2nd half (forward)	AAAGGTAATCTCTGtGGAAAGACAA
2nd half (reverse)	AGACATCTCAAAGTATTCAGCATAGGT
MMP13–214-Mut2 (−110)	
1st half (forward)[b]	AATAAATCTCTTTGTTCAGCTCTCTGTTTC
1st half (reverse)	CTTACaTGGCGACTTTTTCTTTTCCCT
2nd half (forward)	AGGGAAAAGAAAAAGTCGCCAtGTAAG
2nd half (reverse)[c]	AGACATCTCAAAGTATTCAGCATAGGTGAT
MMP13–214-Mut3 (−115)	
1st half (forward)[b]	AATAAATCTCTTTGTTCAGCTCTCTGTTTC
1st half (reverse)	CTTACGTGGCaACTTTTTCTTTTCCCT
2nd half (forward)	AGGGAAAAGAAAAAGTtGCCACGTAAG
2nd half (reverse)[c]	AGACATCTCAAAGTATTCAGCATAGGTGAT
MMP13–214-Mut4 (−135)	
1st half (forward)	ACCCAATAAATAATAAATCTCTTTGTTCAGCTCT
1st half (reverse)	TTCTTTTCCCTCCCaAGTGTGGTTT
2nd half (forward)	AAACCACACTtGGGAGGGAAAAGAA
2nd half (reverse)	GACATCTCAAAGTATTCAGCATAGGTGATG
IL1B-521-Mut1 (+13)	
1st half (forward)[d]	TTGTTTATGTGAGCAAACAGCA
1st half (reverse)	CCTTGTGCCTCaAAGAGGTT
2nd half (forward)	AACCTCTTtGAGGCACAAGG
2nd half (reverse)[e]	CCAGCCTCACAGACATCTCA
IL1B-521-Mut2 (−20)	
1st half (forward)[d]	TTGTTTATGTGAGCAAACAGCA
1st half (reverse)	CCCTCaCTGTTTTTATGGCTTT
2nd half (forward)	AAAGCCATAAAAACAGtGAGGG
2nd half (reverse)[e]	CCAGCCTCACAGACATCTCA
IL1B-521-Mut3 (−256)	
1st half (forward)	TTTGTTTATGTGAGCAAACAGCAG
1st half (reverse)	TTTTCCTGACAATCaTTGTGCAGT
2nd half (forward)	ACTGCACAAtGATTGTCAGGAAAA
2nd half (reverse)	TGCCATCTTCCAGAGGGTAG
IL1B-521-Mut4 (−299)	
1st half (forward)	TATGTGAGCAAACAGCAGATTAAAAG
1st half (reverse)	AATACTGGATTTTCCCACaTTAGAAG
2nd half (forward)	CTTCTAAtGTGGGAAAATCCAGTATT
2nd half (reverse)	ACTAAGCTTAAGATCTGGCTGAAGAG

[a] Lowercase letters indicate a mutated base.
[b] Common primers.
[c] Common primers.
[d] Common primers.
[e] Common primers.

fected *MMP13* reporter construct: 5′-TCAAATTCTACCAC-AAACCACA-3′ (forward) and 5′-TAATATTCTTGGCAT-CCTCCAT-3′ (reverse). Primer efficiency was calculated utilizing serial dilutions of the pooled input DNA samples. For real time PCR analysis, the C_T of each sample was normalized to the C_T of the input sample (10%).

Statistical Analysis—Each data point is reported as the means $\pm$ S.E. of at least three independent experiments. Statistical significance was evaluated using Student's *t* test, with *p* values of < 0.05 considered significant.

RESULTS

Specific MMP13 and IL1B Proximal Promoter CpG Sites Are Demethylated in OA Chondrocytes and Correlate with Higher Levels of Gene Expression in OA Disease—We initially quantified the CpG methylation status of the −343/−14 bp *MMP13* and −299/+13 bp *IL1B* proximal promoter sequences in human primary chondrocytes isolated from articular cartilage obtained from non-OA donors (*n* = 12) and patients with OA (*n* = 17). Pyrosequencing analysis of the *MMP13* promoter

revealed that five of the seven CpG sites contained in the proximal promoter region were significantly demethylated in OA compared with non-OA chondrocytes. The highest degree of demethylation was observed at the −110-bp CpG site (Fig. 1*A*). The *IL1B* promoter was also significantly demethylated at the −299-bp CpG and −256-bp CpG sites in OA compared with non-OA chondrocytes, whereas there was no significant difference in methylation status at the −20-bp CpG and +13-bp CpG sites (Fig. 1*B*). Finally, the decreased methylation of the *MMP13* (Fig. 1*C*) and *IL1B* (Fig. 1*D*) proximal promoters correlated with higher levels of their mRNAs in OA chondrocytes (Fig. 1*C*).
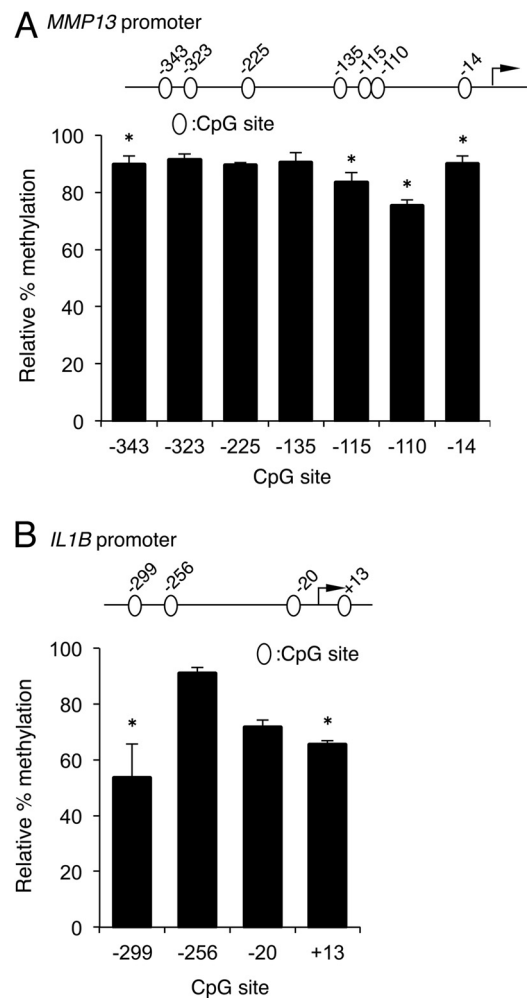
CpG Demethylation in Vitro Correlates with Enhanced Levels of MMP13 and IL1B Gene Expression in Cultured Human Primary Chondrocytes—To determine the functional effects of demethylating the CpG sites in the *MMP13* and *IL1B* promoters, we cultured human primary chondrocytes in the presence or absence of 5-aza-dC, a CpG demethylating agent, as described (22). Treatment with 5-aza-dC resulted in significant

A *MMP13 promoter*

B *IL1B promoter*

C

D
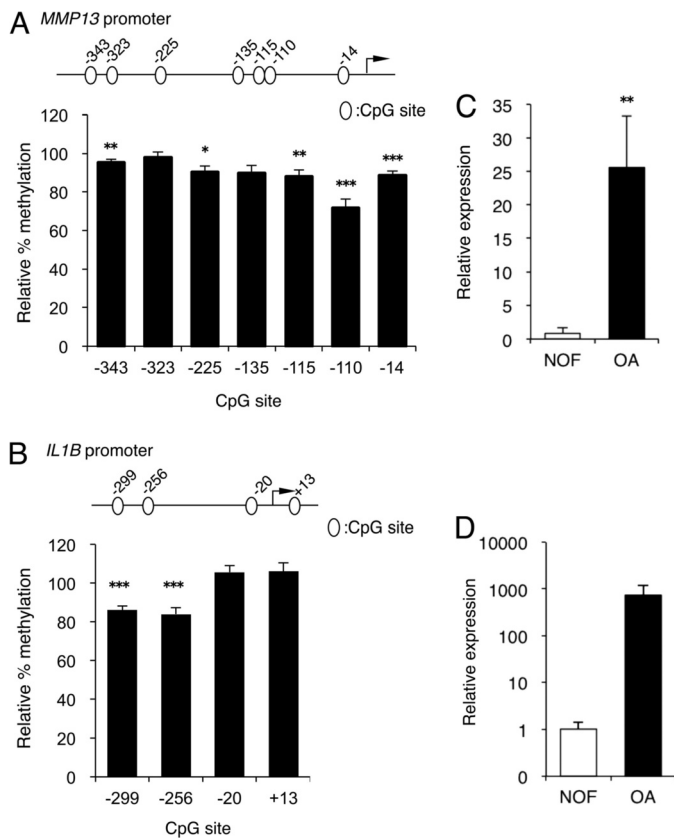
FIGURE 1. **Demethylation of specific *MMP13* and *IL1B* proximal promoter CpG sites in OA chondrocytes correlates with higher levels of gene expression in OA disease.** Bisulfite pyrosequencing analysis was performed to determine the relative methylation status of the indicated CpG sites on the (*A*) *MMP13* or (*B*) *IL1B* proximal promoter sequences in healthy (fractured neck of femur (*NOF; n* = 12) and OA (*n* = 17) human chondrocytes isolated directly from cartilage. Data are represented as percentage of methylation in OA samples relative to that in non-OA specimens set at 100% for each CpG site. *MMP13* (*C*) or *IL1B* (*D*) mRNA was analyzed by quantitative RT-PCR and normalized against GAPDH. All of the data are shown as means ± S.E. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

A *MMP13 promoter*

B *IL1B promoter*

FIGURE 2. **CpG demethylation *in vitro* correlates with enhanced levels of *MMP13* and *IL1B* gene expression in cultured human primary chondrocytes.** Primary human chondrocytes were cultured without or with 5-aza-dC and the CpG methylation status of the *MMP13* proximal promoter (*A*) or the *IL1B* proximal promoter (*B*) was analyzed. All data are shown as means ± S.E. and represented as percentage of methylation in the 5-aza-dC-treated cells relative to that in the non-treated control cells set at 100% for each CpG site (*, $p < 0.05$).

demethylation of the CpG sites in the *MMP13* promoter at −343 bp, −115 bp, −110 bp, and −14 bp (Fig. 2*A*). In the *IL1B* promoter, significant loss of methylation occurred at the −299 bp CpG after 5-aza-dC treatment (Fig. 2*B*). In accordance with these results, we had reported previously that *MMP13* and *IL1B* mRNA levels increased by 2- and 5-fold, respectively, in response to treatment with 5-aza-dC (22, 38).

Methylation of Unique CpG Sites in the Proximal Human MMP13 and IL1B Gene Promoters Directly Affects Transcriptional Activities—We next investigated the contribution of each of the methylation-sensitive CpG sites to *MMP13* and IL1B promoter activities using a CpG-free reporter system. The activities of deletion constructs of the *MMP13* promoter, spanning −372/+14, −214/+14, and −86/+14 bp, were significantly reduced in their methylated state (Fig. 3*A*). Next, we generated mutant constructs of the −214/+14-bp *MMP13* promoter harboring site-specific CpG mutation(s), replacing unique cytosine (C) with thymine (T) residues, as indicated in Fig. 3*B*. A single CpG mutation at −110 bp (−110 mt) significantly decreased promoter activity compared with the WT promoter; in contrast, no other individual CpG mutant caused a significant change in *MMP13* promoter activity. In addition,

methylation significantly suppressed the activities of all single CpG mutants except for the −110 mt and −14 mt (Fig. 3*B*). Moreover, a triple CpG mutant construct (−135/−115/−14 mt), which preserves the −110 bp CpG site, retained promoter activity comparable with WT; all other triple mutants with a mutated −110 bp CpG site showed significantly decreased promoter activity (Fig. 3*C*). Importantly, CpG methylation treatment of the −135/−115/−14-mt construct significantly decreased reporter activity. These results indicate that the methylation status of the −110-bp CpG is crucial for *MMP13* promoter activity. In contrast, activity of the methylated −135/−110/−14-mt construct, which only preserves the −115-bp CpG site, increased by 3.7-fold (Fig. 3*C*).

We also generated three *IL1B* promoter constructs. Methylation treatment significantly decreased the activities of the WT −521/+56 and −276/+56 reporter constructs, with no significant effect on the −183/+56-bp promoter (Fig. 3*D*). Single and triple CpG mutant constructs of the −521/+56 WT *IL1B* promoter revealed that a single CpG mutation at −299 bp (−299
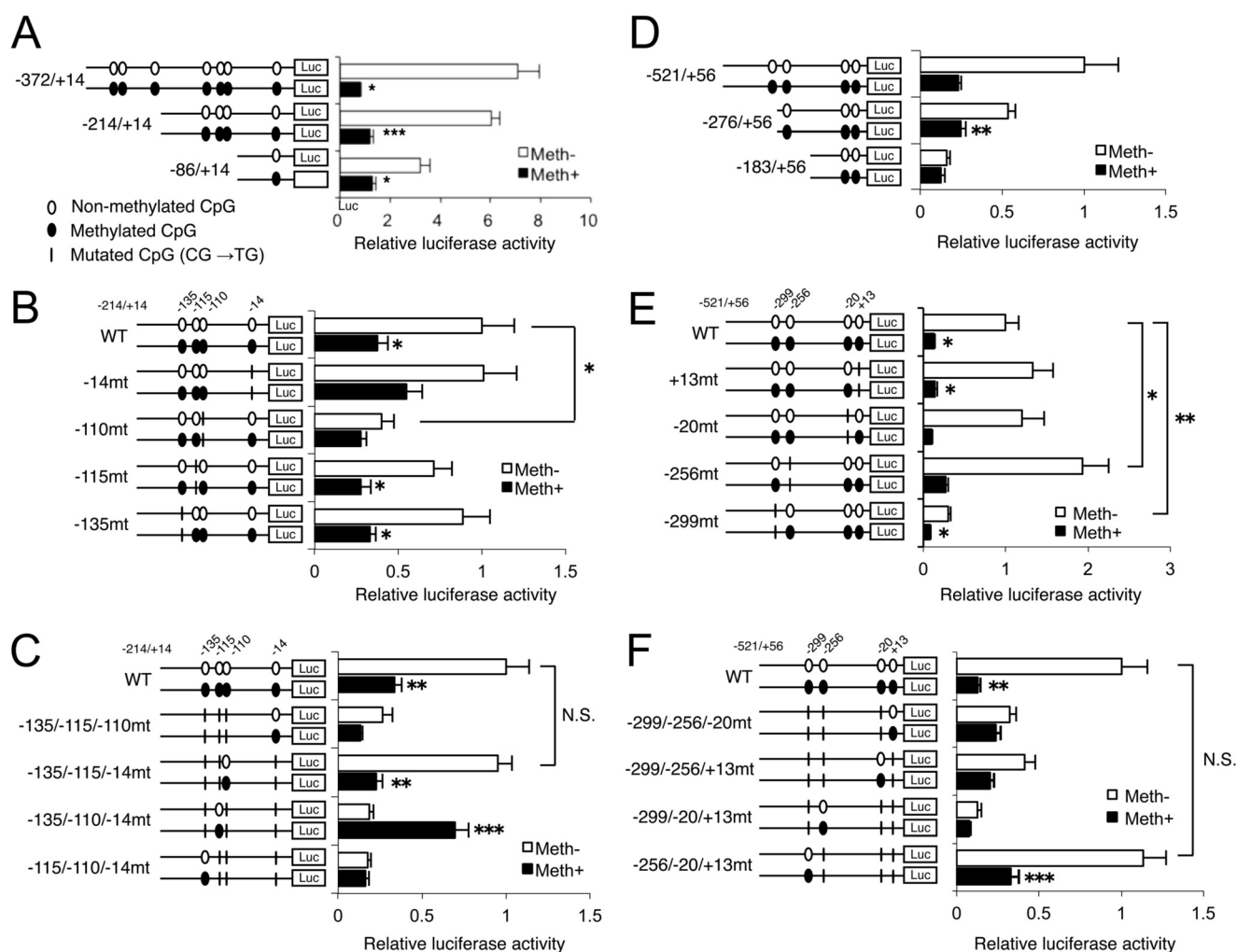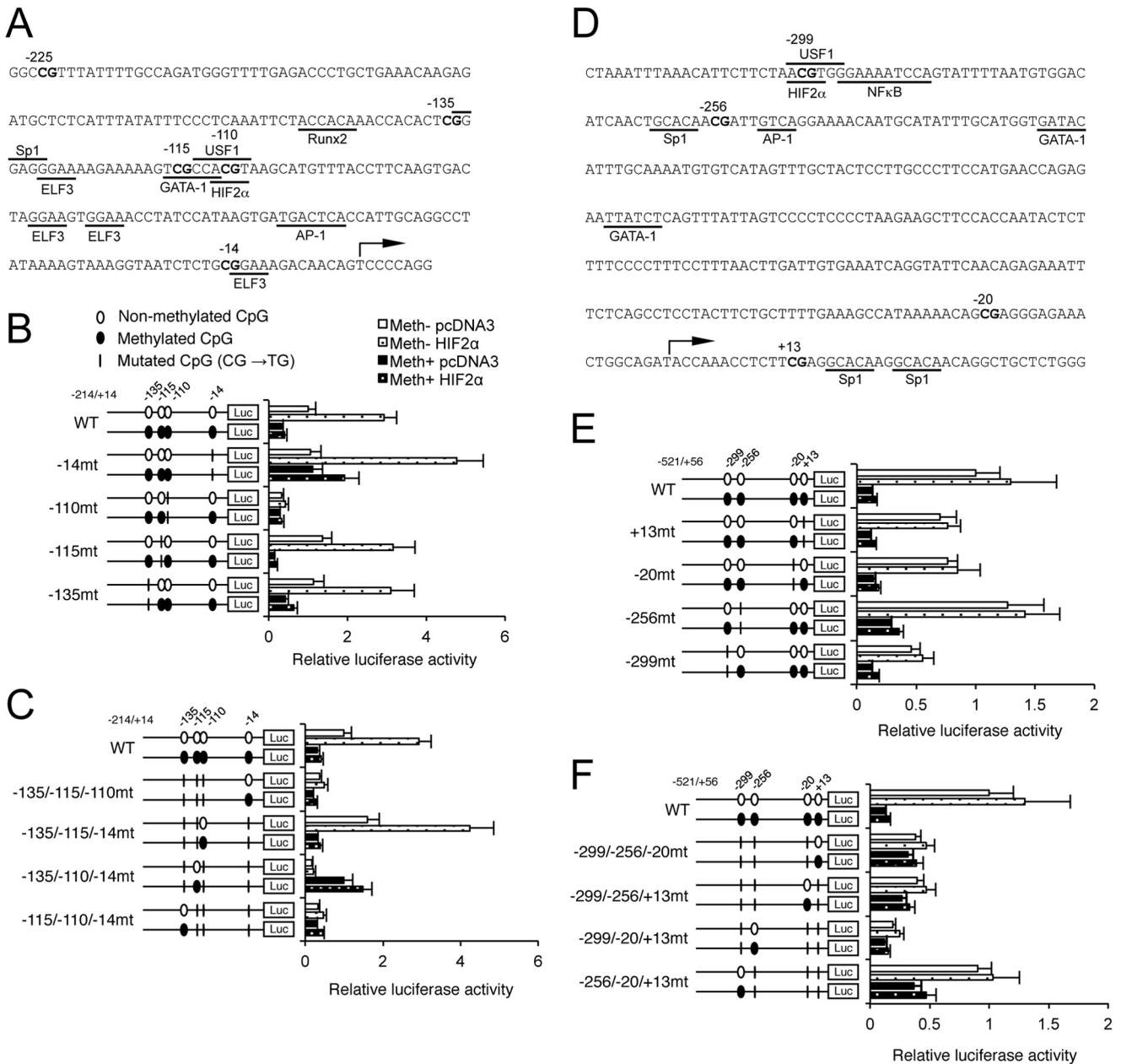
FIGURE 3. **Methylation of unique CpG sites in the proximal human *MMP13* and *IL1B* gene promoters directly affects transcriptional activity.** Basal activities of *MMP13* and *IL1B* promoter constructs with deletions from 5′ ends or specific CpG mutation(s) were analyzed with (*Meth+*, *closed bars*) or without (*Meth−*, *open bars*) CpG methylation treatment in luciferase assays performed in C28/I2 chondrocytes. *MMP13* promoter activity was analyzed in cells transfected with WT −372/+14, −214/+14, and −86/+14-bp constructs (*A*), −214/+14-bp constructs containing single CpG mutations (*B*), and −214/+14-bp promoter constructs containing triple CpG mutations (*C*), leaving only one intact CpG site. *IL1B* promoter activity was analyzed in cells transfected with −521/+56, −276/+56, and −183/+56-bp constructs (*D*), −521/+56-bp constructs with single CpG mutations (*E*), and −521/+56-bp constructs with triple-CpG mutations (*F*), leaving only one intact CpG site. All data are shown as means ± S.E. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). *Luc*, luciferase. *N.S.,* not significant.

mt) significantly decreased promoter activity, whereas the −256-bp CpG mutant was significantly more active than WT; mutations of the other two CpG sites had no effect on promoter activity (Fig. 3*E*). Among the triple CpG mutants with only one intact CpG site, the basal activity of the −256/−20/+13-mt promoter did not change, whereas the basal activities of all constructs containing a mutated −299-bp CpG decreased significantly (Fig. 3*F*). Furthermore, methylation treatment significantly decreased the activity of the −256/−20/+13 mutant (Fig. 3*F*). These results indicate that, similar to the −110-bp CpG site in the *MMP13* promoter, the methylation status of one CpG at −299 bp in the *IL1B* promoter uniquely impacts on its transcriptional activity.

CpG Methylation Impairs HIF-2α-driven MMP13 Promoter Transactivation but Not IL1B Promoter Activation—CpG methylation may regulate gene transcriptional activities by differentially and directly modulating the binding affinities of trans-acting factors (6). Hence, we next investigated the effects

of CpG methylation status on the actions of *MMP13* trans-acting factors selected based on previous reports (35, 39–44) and on bioinformatic analyses using the Transcription Element Search System (Fig. 4*A*). In agreement with prior work (43, 44), HIF-2α overexpression increased the activity of the nonmethylated WT −214/+14-bp promoter construct; interestingly, CpG methylation significantly attenuated this HIF-2α-mediated promoter activation (Fig. 4*B*). In addition, HIF-2α overexpression did not significantly activate the nonmethylated −110-mt construct (Fig. 4*B*), whereas HIF-2α transactivated the non methylated triple mutant with an intact −110-bp CpG to an extent comparable with the WT construct. All other triple mutant constructs showed decreased HIF-2α-driven transactivation, compared with the WT reporter construct (Fig. 4*C*). Methylation treatment of the triple mutant with an intact −110-bp CpG significantly decreased *MMP13* promoter activity in response to HIF-2α overexpression (Fig. 4*C*). Together, these results indicate that targeted −110-bp CpG methylation

A

-225
GGC**CG**TTTATTTTGCCAGATGGGTTTTGAGACCCTGCTGAAACAAGAG

-135
ATGCTCTCATTTATATTTCCCTCAAATTCT<u>ACCACA</u>AACCACACT**CG**G
      <u>Runx2</u>

<u>Sp1</u>   -115 <u>USF1</u>   -110
<u>GAGGGA</u>AAAGAAAAAGT**CG**CCA**CG**TAAGCATGTTTACCTTCAAGTGAC
<u>ELF3</u>   <u>GATA-1</u> <u>HIF2α</u>

TA<u>GGAAGT</u><u>GGAA</u>ACCTATCCATAAGTG<u>ATGACTCA</u>CCATTGCAGGCCT
  <u>ELF3</u> <u>ELF3</u>   <u>AP-1</u>

                          -14
ATAAAAGTAAAGGTAATCTCTGC**CG**GAAAGACAACAGTCCCCAGG
                        <u>ELF3</u>

B

0   Non-methylated CpG   ☐ Meth− pcDNA3
●   Methylated CpG   ▨ Meth− HIF2α
| Mutated CpG (CG →TG)   ■ Meth+ pcDNA3
                                ▩ Meth+ HIF2α

D

                -299
                USF1
CTAAATTTAAACATTCTTCTAA<u>A**CG**T</u><u>GGG</u>AAAATCC<u>AGT</u>ATTTTTAATGTGGAC
                      <u>HIF2α</u>   <u>NFκB</u>

          -256
ATCAACT<u>GCACA</u>**CG**ATT<u>GTC</u>AGGAAAACAATGCATATTTGCATGGT<u>GATAC</u>
      <u>Sp1</u>   <u>AP-1</u>   <u>GATA-1</u>

ATTTGCAAAATGTGTCATAGTTTGCTACTCCTTGCCCTTCCATGAACCAGAG

<u>AATTATCT</u>CAGTTTATTAGTCCCCTCCCCTAAGAAGCTTCCACCAATACTCT
<u>GATA-1</u>

TTTCCCCTTTCCTTTAACTTGATTGTGAAATCAGGTATTCAACAGAGAAATT

                                              -20
TCTCAGCCTCCTACTTCTGCTTTTGAAAGCCATAAAAACAG**CG**AGGGAGAAA

  →   +13
CTGGCAGATACCAAACCTCTT**CG**AGG<u>CACAA</u>GG<u>CACAA</u>CAGGCTGCTCTGGG
                          <u>Sp1</u>   <u>Sp1</u>

FIGURE 4. **CpG methylation impairs the HIF-2α-driven *MMP13* promoter transactivation.** Shown is the schematic representation of the proximal human *MMP13* (42) (*A*) and *IL1B* (*D*) promoter sequences. Putative binding sites for trans-acting factors are *underlined*. Methylated (*Meth+*; *closed bars*) or non-methylated (*Meth−*; *open bars*) *MMP13* promoter constructs with single CpG mutations (*B*) or triple CpG mutations (*C*) were co-transfected with the empty control vector (pcDNA3) or with the HIF-2α expression vector. Methylated (*Meth+*; *closed bars*) or non-methylated (*Meth−*; *open bars*) *IL1B* promoter constructs with single CpG mutations (*E*) or triple CpG mutations (*F*) were co-transfected with the empty control vector (pcDNA3) or with the HIF-2α expression vector. All data are shown as means ± S.E. *Luc,* luciferase.

impairs HIF-2α transactivation of the *MMP13* promoter *in vitro.*

To assess the specificity of the above effects of HIF-2α, we interrogated the dependence of activities of multiple transcription factors on CpG site-specific methylation of the *MMP13* promoter. Overexpression of RUNX2, ELF3, or AP-1 (c-Fos/c-Jun) activated the *MMP13* promoter constructs as shown previously (35, 42); but in contrast to our results with HIF-2α, these transactivations were unaffected by any CpG site mutation or by alteration of methylation status (data not shown). Moreover, in contrast to HIF-2α, HIF-1α had no significant effect when co-transfected with any *MMP13* promoter construct in the

absence or presence of methylation (data not shown). Similarly, overexpressing Sp1, USF-1, or GATA-1 had no effect on *MMP13* promoter activity and did not correlate with mutation or methylation of any specific CpG site (data not shown).

We also investigated the effects of CpG methylation on the actions of candidate *IL1B* trans-acting factors (Fig. 4*D*). HIF-2α overexpression had no significant effect on *IL1B* promoter activity (Fig. 4, *E* and *F*). In contrast, HIF-1α or NF-κB (p65/p50) overexpression significantly increased *IL1B* promoter activity, but this enhancement was independent of mutation of any CpG site or alteration of CpG methylation status (data not shown). Furthermore, overexpression of USF-1 or the combi-

nation of Single-minded (Sim) 2 and aryl hydrocarbon receptor nuclear translocator (ARNT) significantly repressed *IL1B* promoter activity, but the latter responses were not significantly changed by the methylation status of the promoter or by targeted mutation of any CpG site (data not shown).

CpG Methylation Attenuates HIF-2α Binding to the MMP13 Promoter—Given the results above, we investigated whether CpG methylation status directly affected HIF-2α binding to the proximal *MMP13* promoter in ChIP assays. In the context of endogenous chromatin, methylating/demethylating agents would impact transcription affecting not only in the proximal promoter region analyzed in our study but also distal sequences, therefore introducing further complexity to the analysis. Hence, to avoid confounding effects of CpG sites in distal *MMP13* promoter regions and their contributions to overall endogenous chromatin structure, ChIP assays were performed in chondrocytes transfected with methylated and non-methylated WT −214/+14-bp MMP13 constructs in combination with expression vectors encoding HA-tagged HIF-2α or FLAG-tagged ELF3; and *MMP13* promoter binding was analyzed with specific PCR primers that only recognize the transiently transfected promoter constructs, as described in the "Experimental Procedures." ChIP assays revealed that methylation treatment significantly reduced HIF-2α binding to the *MMP13* proximal promoter (Fig. 5A), in agreement with our results showing decreased HIF-2α-driven *MMP13* transactivation after methylation treatment of the WT and/or mutant *MMP13* reporter constructs (Fig. 4, *B* and *C*). In contrast, as a control, ELF3 binding to the proximal *MMP13* promoter was not affected by promoter methylation status (Fig. 5B), in accordance with our results showing that ELF3-driven *MMP13* transactivation is methylation-insensitive, as indicated above. Our results show that CpG methylation of the *MMP13* proximal promoter specifically impairs HIF-2α-driven promoter activation by altering its binding to DNA.

DISCUSSION

MMP-13, or collagenase 3, is the major type II collagen-degrading enzyme that drives erosion of the cartilage collagen network in OA disease (45–48). This enzyme plays essential roles in extracellular matrix remodeling in the late hypertrophic zone of the growth plate prior to endochondral ossification, but articular chondrocytes express low basal MMP-13 levels under normal, physiological conditions *in vivo* (49). High levels and activity of MMP-13 and other matrix-degrading enzymes in OA chondrocytes are due in part to their activated state in response to enhanced stress and inflammatory signaling (reviewed in Ref. 39). *MMP13* transcription is subject to regulation by a number of transcription factors, including ELF3, RUNX2, AP-1 (cFos/cJun), p130^cas nuclear matrix transcription factor 4 (NMP-4), the canonical NF-κB subunits p65/p50, and HIF-2α (35, 40–44, 50). NF-κB also plays a major role in increased *IL1B* expression in various cell types under stress/proinflammatory conditions by directly activating the *IL1B* promoter (51, 52). Moreover, as shown previously, NF-κB modulates gene expression in some contexts via epigenetic mechanisms, affecting the level of CpG demethylation (53).
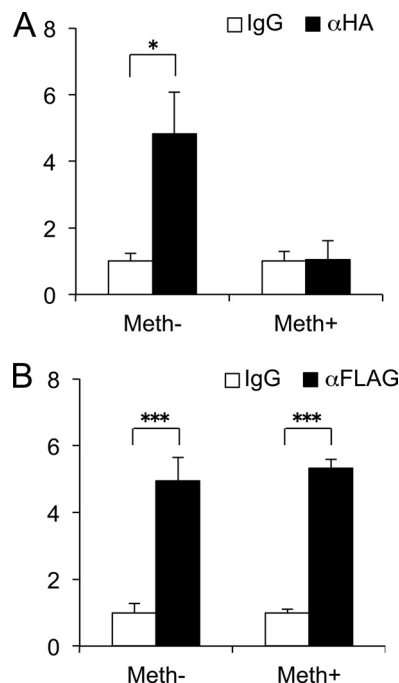
FIGURE 5. **CpG methylation attenuates HIF-2α binding to the *MMP13* promoter.** ChIP assays were performed using cell lysates of C28/I2 cells transfected with methylated (*Meth−*) or non-methylated (*Meth+*) WT −214/+14-bp *MMP13* promoter constructs and expression vectors encoding HA-tagged HIF-2α (*A*) or FLAG-tagged ELF3 (*B*). At 24 h after transfection, chromatin was cross-linked and enzymatically sheared, and after reverse cross-linking of the DNA-protein complexes, the precleared lysates were incubated with antibodies against HA, FLAG, or normal IgG overnight at 4 °C. The HA-HIF-2α and FLAG-ELF3 binding to the human *MMP13* promoter region was analyzed by real time PCR, utilizing PCR primers bracketing the transfected reporter vector boundaries. All data are shown as means ± S.E. and represented as fold-change *versus* IgG (*, $p < 0.05$; ***, $p < 0.001$).

Our previous studies showed epigenetic changes associated with abnormal *MMP13* and *IL1B* gene expression in chondrocytes of OA cartilage (25) and in long term cytokine-treated human primary chondrocyte cultures (22). Although other studies have also described associations between CpG methylation status and deregulation of OA-associated genes (23, 24, 54), this does not appear to be the case for all genes in human chondrocytes (26, 55). However, most previous studies of OA-associated genes have investigated their CpG islands, ignoring the fact that 5′-UTR with non-island CpG sites may also impact functionally on gene regulation by alterations in CpG methylation status (56). Indeed, there is a paucity of studies that have functionally analyzed alterations in promoter activity induced by the methylation of a single CpG site (6, 10, 11). In our study, the use of a CpG-free luciferase reporter gene system (33) facilitated the investigation of the direct cause-and-effect relationships between CpG methylation and altered promoter activities of specific genes, and the dissection of the relative contribution of individual CpG sites to the transcriptional control of a given gene.

Here, we have shown that methylation of unique CpG sites in the proximal *MMP13* and *IL1B* promoters regulates their transcriptional activities in human chondrocytes via different mechanisms (Fig. 6). We initially quantified the methylation status of the promoters of the human genes in chondrocytes isolated from normal and OA articular cartilage and found that
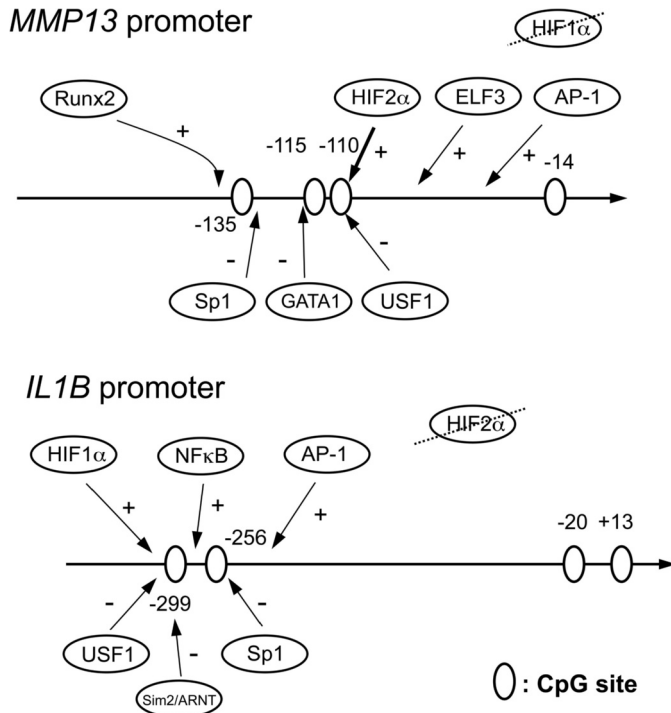
MMP13 promoter

IL1B promoter

FIGURE 6. **Scheme of CpG sites on the proximal *MMP13* and *IL1B* promoters.** The CpG sites are indicated by *open ovals*. The positive (+) and negative (−) transcription factors that bind to these promoters are indicated with *arrows above* and *below*, respectively. In co-transfection experiments, only HIF-2α-driven *MMP13* promoter activity was modulated by CpG methylation status and methylation attenuated HIF-2α binding to the −110 CpG in ChIP assays. On the *IL1B* promoter, HIF-1α and NF-κB, but not HIF-2α, enhanced transcriptional activity, but there was no relationship with CpG methylation status for activities of the transcription factors studied.

the −110-bp CpG and −299-bp CpG in the proximal *MMP13* and *IL1B* promoters, respectively, were most subject to demethylation. Interestingly, 5-aza-dC treatment of human primary chondrocyte cultures induced demethylation of the same CpG sites in *MMP13* and *IL1B* promoters, accompanied with increased expression of both genes. Although the possible involvement of other pathways in promoter-activating effects of 5-aza-dC cannot be excluded, these findings indicate that each CpG site has a different sensitivity to demethylation and that demethylation of specific CpG sites has the potential to activate or derepress *MMP13* and *IL1B* gene expression.

We directly investigated the cause-and-effect relationship between the methylation of these specific CpG sites and the transcriptional activities of *MMP13* and *IL1B* promoters by comparative analysis of their non-methylated and methylated forms. The activities of WT *MMP13* and *IL1B* promoters with methylated CpG sites were reduced compared with their non-methylated counterparts; this was true even for promoter constructs containing longer 5′-flanking promoter sequences, indicating that the methylation of sparse CpG sites in the proximal promoters of these two genes can have a direct suppressive effect on transcriptional activity. In addition, analyses of single and triple CpG mutants within the proximal *MMP13* and *IL1B* promoters further clarified that their respective −110-bp CpG and −299-bp CpG sites have key roles in the methylation-dependent regulation of these two genes in human chondrocytes. Importantly, the latter specific CpG sites correspond to the

most demethylated CpG sites in OA chondrocytes *ex vivo* and in 5-aza-dC-treated primary chondrocytes *in vitro*.

The −110-bp CpG and −299-bp CpG sites in the proximal promoters of the human *MMP13* and *IL1B* genes contain putative ACGT hypoxia-responsive element consensus core sequences, which in some contexts respond to both HIF-1α and HIF-2α (57–61). HIF-2α, but not HIF-1α, controls MMP-13 expression in chondrocytes *in vitro* and *in vivo* and regulates both endochondral ossification processes and cartilage destruction in OA disease (43, 44). HIF-2α, the gene product of *EPAS1*, is an IL-1β-inducible member of the basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) family of transcription factors that directly targets the hypoxia-responsive elements within the *COL10A1*, *MMP13*, and *VEGFA* promoters in chondrocytic ATDC5 cells and in HeLa cells (43). HIF-2α binding to the endogenous *MMP13* promoter containing the proximal hypoxia-responsive element has been demonstrated previously by ChIP *in vivo* and by EMSA *in vitro* (43). In addition, HIF-2α-mediated *MMP13* transcriptional control has been established both *in vitro* and *in vivo* (43, 44). Moreover, enforced expression of HIF-2α *in vivo* has been shown to result in enhanced cartilage damage in association with enhanced MMP-13 expression. Furthermore, $Epas1^{+/-}$ mice, which are haploinsufficient in HIF-2α, have reduced levels of MMP13 mRNA and are resistant to surgically or chemically induced knee OA disease (44).

Here, we have shown that enforced HIF-2α expression transactivates a nonmethylated WT *MMP13* promoter construct and that its transactivating effect is reduced by CpG methylation. Moreover, HIF-2α-driven *MMP13* activation is solely dependent on the presence of the −110-bp CpG site in the proximal promoter of the gene and targeted methylation of the −110-bp CpG also diminishes *MMP13* promoter transactivation by HIF-2α, suggesting that demethylation of this specific CpG site is essential for HIF-2α-mediated *MMP13* promoter activation and contributes to increased *MMP13* expression in OA chondrocytes. Consistent with our finding that CpG methylation treatment suppresses HIF-2α-driven activation of the *MMP13* promoter, ChIP assays performed with cells co-transfected with HIF-2α and a *MMP13* promoter construct with a methylation-resistant reporter gene revealed that targeted methylation of the *MMP13* proximal promoter significantly decreased HIF-2α binding affinity.

In addition to our observations, Bui *et al.* (10) recently reported that the same −110-bp CpG site (positioned at −104 in their report, based on the selected TSS) affects *MMP13* transactivation via basal and IL1-β-induced CREB recruitment *in vitro*. The latter observations, taken together with our results demonstrating that HIF-2α-driven *MMP13* control depends upon the methylation status of the −110-bp CpG site, highlight the multifaceted importance of the methylation status of this specific CpG site in the control of *MMP13* transcription by different trans-acting factors responding to diverse disease-associated environmental cues and thereby revealing its potentially important role in the dysregulation of *MMP13* expression observed in OA cartilage.

In contrast to the *MMP13* promoter, HIF-2α had no activating effect on the *IL1B* promoter, even though the critical

−299-bp CpG for *IL1B* promoter activity is located within a putative hypoxia-responsive element core sequence (Fig. 4*D*), a non-canonical "E-box-like" sequence (CANNTG), to which several bHLH-PAS family transcription factors can bind. A heterodimer of two bHLH-PAS family members, Sim2 and ARNT, binds to non-canonical E-boxes, repressing (62, 63) or activating (64) transcription depending upon the promoter and cellular contexts. However, overexpression of Sim2 and ARNT repressed *IL1B* promoter activity independent of CpG methylation status. Although we found that the −299-bp CpG is essential for *IL1B* promoter activity and that its demethylation correlates with enhanced *IL1B* gene expression in human primary chondrocytes *ex vivo* and *in vitro*, mutation of this site does not attenuate *IL1B* promoter transactivation by NF-κB. Early studies in myeloid cell lines demonstrated that two other functional NF-κB sites, which exist upstream in the *IL1B* promoter, were influenced by CREB, whereas mutation of the −299-bp NF-κB site attenuated transactivation by C/EBPβ but not by CREB (51, 52). Whether methylation at −299-bp CpG directly affects the binding and activities of other bHLH family members or it modulates *IL1B* transcription via other indirect mechanisms merits future investigation.

Our finding that methylation of the −115-bp CpG site enhances *MMP13* promoter activity (Fig. 3*C*), as opposed to the inhibitory effect of −110-bp CpG methylation, suggests alternative mechanisms, as reported for half-Cre sequences, where CpG methylation creates C/EBPα binding sites and activates gene expression (6). The −115-bp CpG overlaps a GATA-1 binding sequence, although the inhibitory effect of GATA-1 overexpression on *MMP13* promoter activity was not altered by methylation status (data not shown). Further analysis will be required to elucidate the role of the −115-bp CpG site in methylation-dependent *MMP13* promoter activation.

Interestingly, treatment with 5-aza-dC *in vitro* produced a more marked decrease in the methylation of CpG sites in the proximal *MMP13* and *IL1B* promoters than that observed *ex vivo* comparing OA and non-OA articular chondrocytes. However, whereas OA chondrocytes showed 25-fold and 800-fold *MMP13* and *IL1B* gene expression compared with non-OA chondrocytes, the *MMP13* and *IL-1β* expression levels were only modestly elevated by the more pronounced 5-aza-dC-induced demethylation *in vitro*. Moreover, in reporter assays, differences in promoter activities with or without methylation were also modest even when the methylation status of a specific CpG was all or none in the reporter constructs. Taken together, our observations suggest that even a small change in CpG methylation status may have a significant impact on gene expression *in vivo*, where CpG methylation is likely to be a contributing factor not only by altering the binding of trans-acting factors, but also by acting together with histone-modifying, chromatin-remodeling activities and other transcriptional regulatory elements. Indeed, 5-aza-dC treatment of primary cultures does not necessarily reflect the more complex *in vivo* physiological or pathological condition, where more prolonged exposure to different stimuli may induce not only differential methylation patterns but also other epigenetic changes that may be absent in our chondrocyte culture systems and reporter assays. Furthermore, analysis of the cell populations *in vivo*/*ex vivo* is limited in that cell-to-cell variability is not taken into account, whereas *in vitro* studies are performed with synchronized cells. Hence, limitations in current methylation and epigenomic mechanistic studies stem from the lack of technology and models that more precisely reflect the *in vivo* scenario. Techniques that allow for specific modulation of CpG methylation patterns *in vivo* are required, therefore, for a better understanding of the precise contributions of this means of transcriptional control (65, 66).

In summary, we have investigated the relative contributions of the methylation status of individual CpG sites in the *MMP13* and *IL1B* proximal promoters to modulation of their transcriptional activities and found that methylation of unique CpG sites has promoter-specific effects. Moreover, we show that the DNA binding and transactivation activity of HIF-2α, a key transcription factor in *MMP13* gene regulation, is prevented by methylating only one unique CpG site in the proximal *MMP13* promoter. Although the use of epigenetic inhibitors as cancer therapies is closer to reality than their use in musculoskeletal diseases (67), specific targeting of DNA methylation may eventually represent a potential therapeutic strategy to prevent or delay OA disease progression by suppressing the activities of catabolic genes causing cartilage matrix destruction. Our findings may also suggest the utility of methylation marks as advantageous early diagnostic markers of OA disease.

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