Demethylation of an NF-κB enhancer element orchestrates *iNOS* induction in osteoarthritis via cell cycle regulation

Demethylation of an NF-κB enhancer element orchestrates *iNOS* induction in osteoarthritis and is associated with altered chondrocyte cell cycle

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Abstract

Objective: To examine the methylation profile of the NF-κB enhancer region at −5.8 kb of *iNOS* and the subsequent role in the induction of osteoarthritis (OA) via cell cycle regulation.

Methods: Percentage methylation was determined by pyrosequencing, gene expression by qPCR and cell proliferation was determined using the MTT assay. Transient transfections were induced to determine the effect of the NF-κB enhancer region on cell proliferation and the influence of DNA methylation.

Results: *In vitro* de-methylation with 5-aza-dC showed decreased levels of DNA methylation at CpG sites localised at −5.8 kb, which correlated with higher levels of *iNOS* expression. *In vitro* methylation of the NF-κB enhancer region at −5.8 kb increased the percentage of cells at G0/G1 cell cycle phase. Loss of methylation within this region correlated with, enhanced proliferation and increased number of cells at G2/M phase. OA chondrocytes demonstrated up-regulation of the G0/G1 cell cycle progression markers Cyclin D1 and CDK6 in contrast to control cells. We demonstrate the loss of methylation that occurs at specific CpG sites localised at the −5.8 kb NF-κB enhancer region of the *iNOS* gene in OA chondrocytes permits the binding of this transcription factor activating the expression of *iNOS*. This results in subsequent altered cell cycle regulation, altered proliferative phenotype and transmission of the pathogenic phenotype to daughter cells.

Conclusions: This study indicates that inhibition of cell cycle progression by *iNOS* enhancer hypermethylation is capable of reducing pro-inflammatory responses via down-regulation of NF-κB with important therapeutic implications in OA.

Keywords: Epigenetics; DNA methylation; iNOS; chondrocytes; osteoarthritis, cell cycle

Introduction

Osteoarthritis (OA), the most common form of arthritis world-wide, is characterized by progressive failure of the extracellular cartilage matrix, along with changes in the synovium and subchondral bone. In normal adult cartilage, chondrocytes present, in the non-stressed steady state, as quiescent cells demonstrating negligible turnover of the cartilage matrix. In contrast, in OA, the chondrocytes become "activated", characterized by cell proliferation, cluster formation, and the increased production of matrix proteins and matrix-degrading enzymes [1]. Degradative chondrocytes, cells that express degradative enzymes, undergo cell division, a phenomenon that is rarely seen in normal, mature, articular chondrocytes. We have previously shown that abnormal synthesis of metalloproteinases (MMPs) does not occur in all OA chondrocytes, but is notably present in chondrocyte clusters, indicating acquisition of altered phenotype by daughter cells from a single abnormal chondrocyte [2].

Typically, hyaline cartilage does not undergo terminal differentiation under normal conditions, however OA chondrocytes may be the result of articular chondrocytes differentiating to give a hypertrophic and thus an altered phenotype [3]. In support of such a mechanism, genetic modifications that stimulate chondrocyte hypertrophy-like changes are frequently associated with a higher incidence of OA or accelerated OA development [3].

Nitric oxide (NO) and its redox derivatives display a number of different regulatory functions in both normal and pathophysiological joint conditions [4] and, has been extensively demonstrated to play a role in the regulation of bone cell metabolism, bone remodelling and in the modulation of chondrocyte physiology in OA [4-9]. This catabolic factor is the product of inducible nitric oxide synthase (*iNOS*), which not only suppresses the synthesis of cartilage matrix, but also increases expression of proteases in OA [10].

There is growing evidence to support a role for epigenetics in the pathogenesis of OA. Epigenetic mechanisms explain changes in gene function that are not a consequence of modifications in the DNA sequence and of these, DNA methylation has been implicated in the induction of *iNOS* [11]. We have recently described that demethylation of the NF-κB enhancer region at −5.8 kb of the *iNOS* gene leads to increased transcription through elevated binding of this transcription factor [12]. Indeed, it has previously been shown that there are multiple functional NF-κB binding sites in iNOS so far upstream, a unique property of the human *iNOS* promoter [13-15]. However, it is not known if all these binding sites are functional, regulated by DNA methylation and if regulated by DNA methylation, which site is critical for *iNOS* induction in chondrocytes

Nuclear factor (NF)-κB represent a family of proteins, many of which are ubiquitously expressed and inducible by a variety of extracellular growth stimuli [16]. Key gene members within the family include RelA, RelB, Nfκb1, Nfκb2, and Iκ Bα which regulate the transcription of genes that mediate a variety of cellular functions, including cell proliferation, differentiation, apoptosis and inflammation [16]. Within the regulation of cell proliferation, cyclins, cyclin dependent kinases (CDKs) and other relative proteins are expressed according to space and time and tightly regulate cell cycle progression [17]. Interestingly, down-regulation of NF-κB has been implicated in the inhibition of cell-cycle progression [18]. Critically, differences in the molecular mechanisms underlying cell cycle regulation between *iNOS* and p65 NF-kB subunit in human chondrocytes remain far from clear. In the current study, we hypothesised that loss of methylation of the crucial enhancer element within OA chondrocytes permits p65 binding and subsequent induction of expression of *iNOS*. This results in regulation of key factors in G1/S, decreasing p16, increasing CDK6 and cyclin D, inducing cell cycle progression and proliferation. We hypothesise this leads, ultimately, to transmission of this altered phenotype to daughter cells and propagation of OA.

Material and Methods

Cartilage dissection and articular chondrocytes isolation

Human articular cartilage samples were obtained from 12 patients following hemiarthroplasty as a consequence of femoral neck fracture (#NOF) (3 men and 9 women; mean±SD age of 83.2±8.0 years) and from 16 OA patients (OA Research Society International– modified Mankin score [19] of 3–5) who underwent total hip arthroplasty (8 men and 8 women; mean±SD age of 70.6±12.3 years). Informed consent was obtained from all patients, and the study was approved by the Southampton and West Hampshire local ethics committee. Cartilage obtained from patients with femoral neck fracture is widely used as a suitable non-OA control. Only chondrocytes from the superficial layer of OA cartilage or the deep zone of cartilage from patients with femoral neck fracture were isolated in order to differentiate both phenotypes. Cartilage samples were obtained from individual subjects and cartilage fragments digested as previously described [20].

Chondrocyte culture

Isolated chondrocyte samples were divided into 4 groups: (i) non-cultured, (ii) cultured without treatment (control culture), (iii) cultured in 10 ng/ml IL-1β plus 10 ng/ml oncostatin M (OSM), and (iv) cultured using 2 μM 5-azadeoxycytidine (5-aza-dC). Prior to treatment, chondrocytes were cultured as previously described [12].

For samples cultured in 5-aza-dC, the histone deacetylase inhibitor trichostatin A (TSA; 300 nM) was added to facilitate access to 5-aza-dC (cytidine analogue that inhibits DNA

methyltransferase 1) [21]. Sample media was changed twice weekly and primary cultures maintained for 5 weeks until the samples reached confluence.

DNA and RNA extraction, quantitative reverse transcription–polymerase chain reaction (qRT-PCR) and pyrosequencing

Total RNA and genomic DNA were extracted simultaneously from harvested chondrocytes using AllPrep DNA/RNA Mini kit (Qiagen), according to the manufacturer's instructions. *GAPDH*, *iNOS*, *CCND1*, *CDK6* and *CKN2A* relative gene expression and the percentage DNA methylation in the −5.8 kb NF-kB *iNOS* enhancer element were quantified using qRT-PCR and pyrosequencing and were performed as previously described [12, 20, 22]. See supplementary table for primer information.

Plasmid construction

The *iNOS* promoter (piNOS) and three enhancer elements plus promoter (e1piNOS, e2piNOS and e3piNOS) constructs were generated by PCR amplification using Platinum PCR Supermix High Fidelity (Invitrogen) with genomic DNA from human articular chondrocytes as a template (see supplementary table for primer information). The resulting PCR products were cloned into the CpG-free-luc vector [23]. The piNOS-luc construct was generated by using the FastDigest restriction enzymes Bgl II and Nco I (Thermo) to cut the DNA from −1144 to +25 bp. Constructs containing the different enhancers were generated using FastDigest restriction enzymes Sbf I and Bgl II (Thermo) to cut the DNA from −5996 to −4906 bp (e1iNOS), from −7257 to −5927 bp (e2iNOS), and from −17000 to −11127 (e3iNOS) and subsequently, the epiNOS constructs were generated by ligation of promoter

plus enhancer constructs. The authenticity of the DNA insert was confirmed by sequence analysis. E coli GT115 competent cells (InvivoGen) were used for the transformation.

In vitro DNA methylation and transient transfection

Methylated plasmids (Met-e1piNOSluc, Met-e2piNOSluc and Met-e3piNOSluc) were generated following incubation of plasmid DNA with CpG methyltransferase (M.SssI; NEB) according to the manufacturer's instructions. Complete methylation was verified by plasmid DNA bisulfite modification and pyrosequencing. The methylated plasmids DNA were purified using a QIAquick PCR purification kit (Qiagen) and transfected (0.5 µg) into the immortalized chondrocytic cell line C28/I2 (provided by Dr Mary Goldring, Hospital for Special Surgery, NYC), in parallel with unmethylated plasmids and the control Renilla vector using Fugene® HD reagent (Promega). In co-transfections, the expression vectors for NF-κB (p50 [Addgene plasmid 21965], p65 [Addgene plasmid 21966], or p50/p65) were used (0.06 µg). Blank expression vector pCMV4 (generously provided by Dr. David Russell, University of Texas) was used as a control. Total DNA was normalized with empty vectors in the transfection mixture. In all transient transfections, the internal control reporter (pRL-TK Renilla luciferase) was present at 1 ng/well. The luciferase assay was conducted using a 96-well luminometer with the dual luciferase substrate system (Promega) 48 hours after transfection. Relative luciferase activity was normalized to the internal control Renilla luciferase activity. Each experiment was repeated at least 3 times, and each data point was calculated as the mean±SD of 3 wells per experiment.

Chromatin immunoprecipitation (ChIP) assay.

A ChIP-IT Express Enzymatic kit (Active-Motif) was used for ChIP assays according to the manufacturer's instructions, as described previously [24]. Briefly, C28/I2 cells were co-transfected with unmethylated or methylated pCpG-free-Luc-epiNOS vector and the expression vector encoding p65 using FuGene HD (Promega). After 48hours, precleared chromatin was stored as assay input or incubated overnight at 4°C with 4 µg of mouse monoclonal anti-NFκB p65 (RelA) antibody or normal mouse IgG (Millipore). After reverse cross-linking and purification, the final DNA preparations were subjected to qPCR analysis using 2 µl of the eluted DNA. For analysis, the Ct of each sample was normalized to the Ct of the input sample. Specific primers flanking the NF-κB response elements in the human *iNOS* promoter were designed (see supplementary table for primer information).

MTT assay

Cell viability assay was determined using the MTT protocol. Briefly, C28/I2 cells (3 x 10^4 cells/well) in DMEM/F12 media at a final volume of 400 µl were seeded into 24-wells culture plate and incubated overnight at 37°C in 5% CO_2. The cells were transfected with e1piNOS-luc and Met-e1piNOS-luc; in co-transfections, expression vectors for NF-κB (p50, p65, or p50/p65) were incubated for 48 hours. Finallythe cells were treated with 40 µl of MTT (5 mg/ml) alone and incubated for 1.5 hours at 37°C in 5% CO_2. The blue formazan products formed in the cells were dissolved in DMSO (400 µl) and measured at 540 nm using a spectrophotometer.

Cell cycle analysis

C28/I2 cells were transfected with e1piNOS-luc and Met-e1piNOS-luc; in co-transfections, the expression vectors for NF-κB (p50, p65, or p50/p65) were used. Transfected empty plasmids served as a negative control group and after 48hours were analysed by fluorescence-activated cell sorting (FACS). In brief, cells were harvested and fixed in 70% ice-cold ethanol at 4°C for at least 12 hours. Subsequently, cells were incubated in 100 µg/ml of propidium iodide (PI) and 50 µg/ml of RNase for 30 min. Cell cycle distribution was analysed by GUAVA flow cytometer (Millipore), and results processed using FLOWJO version 10 software (TreeStar, San Carlos, CA).

Statistics analysis

Statistical analysis was performed using SPSS software version 21.0 Unless otherwise indicated, data are presented as the mean±SD of at least 3 multiple independent experiments. Significance was determined by Mann-Whitney U test to compare gene expression and analysis of variance with post hoc t-test was used to analyse transfection assays. *P* values less than 0.05 were considered significant.

Results

The NF-κB element at −5.8 kb is crucial for induction of the human *iNOS* promoter in articular chondrocytes

Previous analysis of the 5'-flanking region of the human *iNOS* gene revealed three regions containing cytokine-responsive elements and functional NF-κB enhancer elements localised at −3.8 to −5.8, −5.8 to −7.0, and −7.0 to −16.0 kb [13-15]. In order to determine

whether any of these putative elements conferred enhanced *iNOS* gene activity in human chondrocytes, transient co-transfections in C28/I2 cells were performed with three different constructs each containing the enhancer element cloned into the CpG-free vector (Figure 1A). Reporter vectors were subsequently transfected in combination with or without co-transfection of expression vectors coding p50 (pCMV4-p50) and p65 (pCMV4-p65), alone and in combination, to analyse the effect of NF-κB on *iNOS* activity.

Reporter gene assays demonstrated a 47-fold induction of luciferase activity with e1piNOS-luc in combination with p65 subunit as published [12] (Figure 1B). Analysis of e2piNOS-luc and e3piNOS-luc indicated a 5-fold and 19-fold increase in luciferase expression, respectively (Figures 1C-D). Interestingly, with e3piNOS-luc the luciferase activity was higher (31-fold) following co-transfection with the classical heterodimer p50/p65 (Figure 1D).

Plasmids were methylated *in vitro* using CpG methyltransferase (M.SssI) to analyse the effect of CpG methylation on *iNOS* activity. Using the e1piNOS-luc construct, enhanced *iNOS* activity induced by p65 was significantly reduced following methylation treatment (47.6±23.8% for e1piNOS versus 11.7± .2% for Met-e1piNOS) (Figure 1B). Following application of e3piNOS-luc, the enhanced *iNOS* activity promoted by p50/p65 was significantly reduced following methylation treatment (31.4±7.0% for e3piNOS versus 16.9±12.5% for Met-e3piNOS) (Figure 1D).

We investigated whether the CpG methylation status directly affected p65 binding to the -5.8 kB NF-κB enhancer element using ChIP assays performed using C28/I2 chondrocytes cotransfected with unmethylated or methylated e1piNOS constructs and expression vectors encoding p65. *iNOS* enhancer binding was analysed with specific PCR primers that recognised only the transiently transfected construct. ChIP assays revealed that methylation

197 treatment significantly reduced p65 binding to the NF-κB enhancer element of the *iNOS* gene
198 (Figure 2).
199
200 **CpG demethylation *in vitro* with 5-aza-dC correlates with enhanced levels of *iNOS* in**
201 **both healthy and OA human chondrocytes**
202
203 Long-term culture of NOF human articular chondrocytes in 5-aza-dC resulted in a 4-fold
204 increase in *iNOS* expression compared to control cultures (mean±SD 4.2±4.4 *versus* 1.4±0.8
205 fold increase). In contrast, in cultured OA chondrocytes while *iNOS* expression was observed
206 to increase, this proved variable and sample (patient) dependent (mean±SD 10.4±21.5 *versus*
207 1.4±1.7 fold decrease) (Figure 3A).
208 To determine if the changes in *iNOS* expression correlated with loss or gain of DNA
209 methylation in the CpG sites localised at the −5.8 kb NF-κB enhancer element, the
210 percentage methylation was quantified using pyrosequencing. Both CpG sites (−5853 and
211 −5843) were significantly demethylated in both NOF and OA samples following 5-aza-dC
212 treatment, with loss of methylation more pronounced in the pathologic samples. The loss of
213 methylation in the NOF samples was ~ 10%: 93.9±0.4 *versus* 84.9±2.5 at −5853 CpG and,
214 87.0±2.9 *versus* 79.3±3.1 at −5843 CpG (Figure 3B). In contrast, within the OA samples; the
215 loss of methylation was considerably higher reaching 30-40%: 93.4±0.6 *versus* 65.2±21.7 at
216 −5853 CpG and, 90.0±1.0 *versus* 51.8±16.1 at −5843 CpG (Figure 3C).
217
218 **Loss of methylation at the CpGs localised at −5.8 kb NF-κB enhancer element enhances**
219 **C28/12 chondrocyte cell line proliferation.**
220

An increase in cell proliferation after 48 hours, determined using the MTT proliferation assay, was observed in C28/12 chondrocytes transfected with the unmethylated plasmid (e1piNOS-luc) compared to cells transfected with methylated plasmid (Met-e1piNOS-luc): 73.1±8.6% *versus* 81.6±7.2% (Figure 4). However, no significant influence on MTT assay results was observed following co-transfections with NF-κB subunits in C28/12 cells (Suppl. Figure 1).

Alteration of cell cycle distribution is induced by loss of methylation of the crucial *iNOS* enhancer element and correlates with lower G0/G1 cell cycle arrest

To evaluate the effect of DNA methylation status on the −5.8 kb NF-κB enhancer element on cell cycle activity of chondrocytes, C28/I2 cells were transiently co-transfected with methylated (e1piNOS-Met) and unmethylated vector *iNOS* vectors (e1piNOS) together with the p65 NF-κB subunit. In these studies, the empty transfected plasmid served as the negative control group and the non-transfected group as the control group.

Flow cytometry analysis demonstrated that in cells transfected with the unmethylated plasmid (e1piNOS-luc), a decreased proportion of cells (59%) could be detected in the G0/G1 phase compared to cells transfected with the methylated plasmid (64%) (Figure 5A). Furthermore, FACS analysis using propidium iodine-stained DNA demonstrated an alteration in cell cycle distribution in cells transfected with methylated or unmethylated −5.8 kb *iNOS* enhancer. These results indicate chondrocytes with enhanced *iNOS* expression were active proliferating cells (Figure 5B).

Differential cell-cycle-specific gene expression profile in osteoarthritic chondrocytes

To determine if the loss of methylation of the NF-κB crucial enhancer element is an OA-related phenomenon, resulting in altered OA cell cycle phase distribution, the expression profile of cell-cycle genes was also analysed in NOF chondrocytes in direct comparison to OA cells. Thus differential expression of *CCDN1*, *CDK6*, and *CDKN2A* was examined in articular chondrocytes obtained from control subjects (NOF) and OA patients using qRT-PCR. A significant increase in relative *CCDN1* and *CDK6* expressions was observed in OA chondrocytes in comparison to control (1.6±1.1 in control *versus* 3.7±2.2 in OA) for *CCDN1* (Figure 6A), and similarly for *CDK6* (1.3±0.6 in controls *versus* 7.6±4.5 in OA) (Figure 6B). In marked contrast, *CDKN2A* was significantly decreased in OA samples compared to controls (1.1±1.1 *versus* 3.3±2.5) (Figure 6C). Finally, long-term culture and stimulation with the cytokines IL-1β and OSM produced a significant increase in *CCDN1*expression, a significant decrease in *CDK6* expression although no differences were observed in *CDKN2A* expression (Suppl. Figure 2).

Discussion

The current study demonstrates that in human OA articular chondrocytes, the enhancer element localised at −5.8 kb is crucial for *iNOS* induction following DNA demethylation with subsequent enhancement of NF-κB binding. Critically, the results demonstrate that the subsequent loss of methylation in this region correlated with enhanced chondrocyte proliferation evidenced by chondrocytic cells observed in the G2/M cell cycle phase. We have recently published that demethylation of the NF-κB enhancer region at −5.8 kb of the *iNOS* gene leads to increased transcription through elevated binding of this transcription factor [12]. Indeed the presence of multiple functional NF-κB binding sites this far upstream has been demonstrated, although not studied in chondrocytes and is unique to the human

iNOS promoter [13-15]. Furthermore, we show that in chondrocytes, CpG methylation of this enhancer element specifically impairs p65-driven *iNOS* promoter activation by altering p65 binding to the DNA. Studies using the luciferase reporter assays together with a CpG-free vector containing distinct *iNOS* enhancer element indicate the NF-κB enhancer region at −5.8 kb of *iNOS* is critical for the induction of *iNOS* expression in articular chondrocytes. Thus epigenetic regulation of an enhancer element can be pivotal if a sparse CpG promoter also contains a strong enhancer element allowing gene transcription even when CpG sites are methylated [25].

iNOS expression in both normal and OA chondrocytes was induced with the demethylating agent 5-aza-dC and expression was significantly enhanced in OA chondrocytes. Interestingly, we had anticipated less *iNOS* expression in OA chondrocytes (*iNOS* is highly expressed in OA chondrocytes) given culture of cells is sufficient to induce hypermethylation and loss of expression in some genes [24]. In addition, in the current study, the loss of methylation following treatment with 5-aza-dC at the CpG sites of the −5.8 kb NF-kB enhancer elements was higher in OA cells.

Aged articular chondrocytes from normal individuals do not undergo cell division; in contrast, in OA cartilage, the chondrocytes that survive form clones typical of severe OA [26], consistent with cell proliferation [27]. Indeed, cartilage neighbouring the damaged tissue location appear to contain a population of proliferative chondrocytes, which may be involved in an attempt to repair the damage tissue [3]. In support of this possible approach, recent investigations applying different treatments to promote chondrocyte proliferation suggest the potential to delay or enhance OA progression [28, 29]. The current study indicates a proliferative role of aberrant chondrocytes in the aetiology of OA.

Cell replication and the process of cell cycling are central in complex multicellular processes, such as injury repair or mounting an immune response [30]. Microarray

296 experiments with synchronized cells indicate that the majority of IL-1-triggered genes, like
297 *iNOS,* are preferentially up-regulated in cells released from the G1 phase [31]. The current
298 studies indicate chondrocyte progression from G1 phase to S and G2/M phases in cells
299 transfected with the unmethylated −5.8 kb NF-kB enhancer correlated with higher *iNOS*
300 expression levels.

301 While the regulation of cell-cycle genes in endochondral bone growth has been
302 extensively studied [32, 33], to date, there is little is known regarding the role of cell-cycle
303 genes in adult human articular cartilage. Cell-cycle genes are targeted by a number of
304 mitogenic and anti-mitogenic pathways in chondrocytes [32]. Cyclin D1 (*CCDN1*) is a
305 positive regulator of cell G1/S transition and is a key restriction point in the cell cycle
306 binding to CDK4 and CDK6 to control cell cycle progression from G1 to S phase [34]. The
307 *CDKN2A* gene (p16) belongs to the Ink4 family of proteins that act to inhibit CDK4 and
308 CDK6 upon binding, resulting in the arrest of the cell cycle in G1 or apoptosis [35].
309 Handschick and collaborators have identified CDK6 as a molecular link between the
310 inflammatory microenvironment and permanent cell cycle progression. Thus, in the G1
311 phase, D1 cyclins activate cell cycle progression through phosphorylation of negative-growth
312 regulators [31]. The current study demonstrates an increase in the proliferation rate of OA
313 chondrocytes together with increased gene expression of cyclin D1 (*CCDN1*), *CDK6*, and
314 decreased expression of p16 (*CDKN2A*). Recently, it has been published that p21, a cell
315 cycle-related protein, may function as a regulator of transcriptional factors other than the
316 inhibitor of cell cycle progression in the cartilage tissue [36]. Another study showed that
317 interleukin (IL)-1β increased proliferation and caused a G_1-to-S phase shift in chondrocytes,
318 accompanied by a reduction of *p21*, and reduction of *p21* caused delayed cell differentiation
319 [37].

Interestingly, Wang and colleagues have published a model in which chondrocyte proliferation is promoted through *iNOS*-NO-mediated induction of cyclin D1 expression. Furthermore, reduced chondrocyte proliferation was observed in *iNOS*-deficient mice [38]. Jhou et al reported inhibition of the cell-cycle progression is capable of reducing pro-inflammatory responses *via* down-regulation of NF-kB [18], whilst increased NF-kB activity during growth of mitogen-stimulated and transformed cells has been linked to cell cycle progression through transcriptional activation of the cyclin D1 gene, leading to increased abundance of cyclin D1 and increased activity of cyclin D1 kinase [39].

The current observations that loss of methylation in the crucial enhancer element and subsequent induction of *iNOS* expression is complimentary to reports from Van der Kraan and van der Berg postulating OA has an age and senescence dependent component as a consequence of changes in gene methylation [3]. Furthermore, Yan and colleagues have shown in mice that a deficiency of the neuronal (nNOS) and endothelial (eNOS) members of the nitric oxide synthase family results in reduced chondrocyte proliferation and earlier cell cycle exit [40, 41], presenting an area of further future investigation in human articular chondrocytes.

Epigenetics changes have been shown to be key intermediates in signalling pathways that control proliferation. Thus chromatin compaction levels are directly correlated with DNA methylation levels resulting in altered gene expression and proliferation [42]. In support of this observation, methylation of H4K20 has been implicated in cell cycle progression, chromosome condensation and transcriptional regulation [30] with cross-talk between histone phosphorylation and acetylation at distinct amino acids with H3S28phK27ac and H3S10phK14ac responsible for epigenetic regulation of transcription and cell cycle regulation [43].

The results of the current study of NF-κB-*iNOS*, cell cycle and DNA methylation signalling in OA chondrocytes demonstrate that the loss of methylation that happens at specific CpG sites localised at the −5.8 kb NF-κB of the *iNOS* gene in OA chondrocytes facilitate the binding of NF-κB and subsequent activation of *iNOS*. We hypothesise this has important implications in the pro-inflammatory response in OA, cell cycle regulation and propagation of the aberrant OA phenotype to daughter cells (Figure 7). In conclusion, this study suggests that inhibition of cell cycle progression by *iNOS* enhancer hypermethylation is capable of reducing pro-inflammatory responses via down-regulation of NF-κB with important therapeutic implications in OA.

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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. In detail: MCDA— conception, design, acquisition of data, analysis and interpretation of data, drafting the manuscript; AT—acquisition of data, analysis and interpretation of data, critical revision

of the manuscript; ROCO—conception, design, analysis and interpretation of data, critical revision of the manuscript.

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Competing interests

The authors have no conflict of interest to declare.

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Introduction

Osteoarthritis (OA), the most common form of arthritis world-wide, is characterized by progressive failure of the extracellular cartilage matrix, along with changes in the synovium and subchondral bone. In normal adult cartilage, chondrocytes present, in the non-stressed steady state, as quiescent cells demonstrating negligible turnover of the cartilage matrix. In contrast, in OA, the chondrocytes become "activated", characterized by cell proliferation, cluster formation, and the increased production of matrix proteins and matrix-degrading enzymes [1]. Degradative chondrocytes, cells that express degradative enzymes, undergo cell division, a phenomenon that is rarely seen in normal, mature, articular chondrocytes. We have previously shown that abnormal synthesis of metalloproteinases (MMPs) does not occur in all OA chondrocytes, but is notably present in chondrocyte clusters, indicating acquisition of altered phenotype by daughter cells from a single abnormal chondrocyte [2].

Typically, hyaline cartilage does not undergo terminal differentiation under normal conditions, however OA chondrocytes may be the result of articular chondrocytes differentiating to give a hypertrophic and thus an altered phenotype [3]. In support of such a mechanism, genetic modifications that stimulate chondrocyte hypertrophy-like changes are frequently associated with a higher incidence of OA or accelerated OA development [3].

Nitric oxide (NO) and its redox derivatives display a number of different regulatory functions in both normal and pathophysiological joint conditions [4] and, has been extensively demonstrated to play a role in the regulation of bone cell metabolism, bone remodelling and in the modulation of chondrocyte physiology in OA [4-7]. This catabolic factor is the product of inducible nitric oxide synthase (*iNOS*), which not only suppresses the synthesis of cartilage matrix, but also increases expression of proteases in OA [8].

There is growing evidence to support a role for epigenetics in the pathogenesis of OA. Epigenetic mechanisms explain changes in gene function that are not a consequence of modifications in the DNA sequence and of these, DNA methylation has been implicated in the induction of *iNOS* [9]. We have recently described that demethylation of the NF-κB enhancer region at −5.8 kb of the *iNOS* gene leads to increased transcription through elevated binding of this transcription factor [10]. Indeed, it has previously been shown that there are multiple functional NF-κB binding sites in iNOS so far upstream, a unique property of the human *iNOS* promoter [11-13]. However, it is not known if all these binding sites are functional, regulated by DNA methylation and if regulated by DNA methylation, which site is critical for *iNOS* induction in chondrocytes

Nuclear factor (NF)-κB represent a family of proteins, many of which are ubiquitously expressed and inducible by a variety of extracellular growth stimuli [14]. Key gene members within the family include RelA, RelB, Nfκb1, Nfκb2, and Iκ Bα. Protein products of the RelA, Nfκb1 and Nfκb2 genes are processed to p65, p50 and p52, respectively, to form p65/p50 and p52/RelB dimmers which regulate the transcription of genes that mediate a variety of cellular functions, including cell proliferation, differentiation, apoptosis and inflammation [14]. Within the regulation of cell proliferation, cyclins, cyclin dependent kinases (CDKs) and other relative proteins are expressed according to space and time and tightly regulate cell cycle progression [15]. Interestingly, down-regulation of NF-κB has been implicated in the inhibition of cell-cycle progression [16]. Critically, differences in the molecular mechanisms underlying cell cycle regulation between *iNOS* and p65 NF-kB subunit in human chondrocytes remain far from clear. In the current study, we hypothesised that loss of methylation of the crucial enhancer element within OA chondrocytes permits p65 binding and subsequent induction of expression of *iNOS*. This results in regulation of key factors in G1/S, decreasing p16, increasing CDK6 and cyclin D, inducing cell cycle

progression and proliferation. Significantly we suggest this leads, ultimately, to transmission of this altered phenotype to daughter cells and propagation of OA.

Material and Methods

Cartilage dissection and articular chondrocytes isolation

Human articular cartilage samples were obtained from 12 patients following hemiarthroplasty as a consequence of femoral neck fracture (#NOF) (3 men and 9 women; mean ± SD age of 83.2 ± 8.0 years) and from 16 OA patients (OA Research Society International– modified Mankin score [17] of 3–5) who underwent total hip arthroplasty (8 men and 8 women; mean ± SD age of 70.6 ± 12.3 years). Informed consent was obtained from all patients, and the study was approved by the Southampton and West Hampshire local ethics committee. Cartilage obtained from patients with femoral neck fracture is widely used as a suitable non-OA control. Only chondrocytes from the superficial layer of OA cartilage or the deep zone of cartilage from patients with femoral neck fracture were isolated. Cartilage fragments were digested as previously described [18].

Chondrocyte culture

Isolated chondrocyte samples were divided into 4 groups: (i) non-cultured, (ii) cultured without treatment (control culture), (iii) cultured in 10 ng/ml IL-1β plus 10 ng/ml oncostatin M (OSM), and (iv) cultured using 2 μM 5-azadeoxycytidine (5-aza-dC). Prior to treatment, chondrocytes were cultured for a minimum of 48 hours at 37°C at a density of 2-4 x 10^5 cells in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 5% fetal

calf serum, 1% insulin-transferrin-selenium, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 100 µg/ml ascorbic acid in an atmosphere of 5% CO_2.

For samples cultured in 5-aza-dC, the histone deacetylase inhibitor trichostatin A (TSA; 300 nM) was added to facilitate access to 5-aza-dC (cytidine analogue that inhibits DNA methyltransferase 1) [19]. Sample media was changed twice weekly and primary cultures maintained for 5 weeks until the samples reached confluence.

DNA and RNA extraction, quantitative reverse transcription–polymerase chain reaction (qRT-PCR) and pyrosequencing

Total RNA and genomic DNA were extracted simultaneously from harvested chondrocytes using AllPrep DNA/RNA Mini kit (Qiagen), according to the manufacturer's instructions. *GAPDH*, *iNOS*, *CCND1*, *CDK6* and *CKN2A* relative gene expression and the percentage DNA methylation in the −5.8 kb NF-kB *iNOS* enhancer element were quantified using qRT-PCR and pyrosequencing and were performed as previously described [10, 18, 20]. Primer information used in these studies for qRT-PCR and pyrosequencing is available upon request.

Plasmid construction

The *iNOS* promoter (piNOS) and three enhancer elements plus promoter (e1piNOS, e2piNOS and e3piNOS) constructs were generated by PCR amplification using Platinum PCR Supermix High Fidelity (Invitrogen) with genomic DNA from human articular chondrocytes as a template. Primer information for cloning is available upon request from the corresponding author. The resulting PCR products were cloned into the CpG-free-luc vector

[21]. The piNOS-luc construct was generated by using the FastDigest restriction enzymes Bgl II and Nco I (Thermo) to cut the DNA from −1144 to +25 bp. Constructs containing the different enhancers were generated using FastDigest restriction enzymes Sbf I and Bgl II (Thermo) to cut the DNA from −5996 to −4906 bp (e1iNOS), from −7257 to −5927 bp (e2iNOS), and from −17000 to −11127 (e3iNOS) and subsequently, the epiNOS constructs were generated by ligation of promoter plus enhancer constructs. The authenticity of the DNA insert was confirmed by sequence analysis. E coli GT115 competent cells (InvivoGen) were used for the transformation.

In vitro DNA methylation and transient transfection

Methylated plasmids (Met-e1piNOSluc, Met-e2piNOSluc and Met-e3piNOSluc) were generated following incubation of 1 µg of plasmid DNA with 4 units/µl of CpG methyltransferase (M.SssI; NEB) according to the manufacturer's instructions. Complete methylation was verified by plasmid DNA bisulfite modification and pyrosequencing. The methylated plasmids DNA were purified using a QIAquick PCR purification kit (Qiagen) and transfected (0.5 µg) into the immortalized chondrocytic cell line C28/I2 (provided by Dr Mary Goldring, Hospital for Special Surgery, NYC), in parallel with unmethylated plasmids and the control Renilla vector using Fugene® HD reagent (Promega). In co-transfections, the expression vectors for NF-κB (p50 [Addgene plasmid 21965], p65 [Addgene plasmid 21966], or p50/p65) were used (0.06 µg). Blank expression vector pCMV4 (generously provided by Dr. David Russell, University of Texas) was used as a control. Total DNA was normalized with empty vectors in the transfection mixture. In all transient transfections, the internal control reporter (pRL-TK Renilla luciferase) was present at 1 ng/well. The luciferase assay was conducted using a 96-well luminometer with the dual luciferase substrate system

(Promega) 48 hours after transfection. Relative luciferase activity was normalized to the internal control Renilla luciferase activity. Each experiment was repeated at least 3 times, and each data point was calculated as the mean ± SD of 3 wells per experiment.

MTT assay

Cell viability assay was determined using the MTT protocol. Briefly, C28/I2 cells (3 x 10^4 cells/well) in DMEM/F12 media at a final volume of 400 µl were seeded into 24-wells culture plate and incubated overnight at 37°C in 5% CO_2. The cells were transfected with e1piNOS-luc and Met-e1piNOS-luc; in co-transfections, expression vectors for NF-κB (p50, p65, or p50/p65) were incubated for 48 hours. Finally, following a wash with PBS, the cells were treated with 40 µl of MTT (5 mg/ml) alone and incubated for 1.5 hours at 37°C in 5% CO_2. The blue formazan products formed in the cells were dissolved in DMSO (400 µl) and measured at 540 nm using a spectrophotometer.

Cell cycle analysis

C28/I2 cells were transfected with e1piNOS-luc and Met-e1piNOS-luc; in co-transfections, the expression vectors for NF-κB (p50, p65, or p50/p65) were used. Transfected empty plasmids served as a negative control group and after 48hours were analysed by fluorescence-activated cell sorting (FACS). In brief, cells were harvested by trypsinization, centrifuged and cell pellets resuspended and fixed in 70% ice-cold ethanol at 4°C for at least 12 hours. Subsequently, cells were incubated in 100 µg/ml of propidium iodide (PI) (Sigma) and 50 µg/ml of RNase (Sigma) for 30 min. Cell cycle distribution was

analysed by GUAVA flow cytometer (Millipore), and results processed using FLOWJO version 10 software (TreeStar, San Carlos, CA).

Statistics analysis

Statistical analysis was performed using SPSS software version 17.0. Unless otherwise indicated, data are presented as the mean ± SD of at least 3 multiple independent experiments. Significance was determined by analysis of variance with post hoc t-test and by Mann-Whitney U test. P values less than 0.05 were considered significant.

Results

The NF-κB element at −5.8 kb is crucial for induction of the human *iNOS* promoter in articular chondrocytes

Previous analysis of the 5'-flanking region of the human *iNOS* gene revealed three regions containing cytokine-responsive elements and functional NF-κB enhancer elements localised at −3.8 to −5.8, −5.8 to −7.0, and −7.0 to −16.0 kb [11-13]. In order to determine whether any of these putative elements conferred enhanced *iNOS* gene activity in human chondrocytes, transient co-transfections in C28/I2 cells were performed with three different constructs each containing the enhancer element cloned into the CpG-free vector (Figure 1A). Reporter vectors were subsequently transfected in combination with or without co-transfection of expression vectors coding p50 (pCMV4-p50) and p65 (pCMV4-p65), alone and in combination, to analyse the effect of NF-κB on *iNOS* activity.

Reporter gene assays demonstrated a 47-fold induction of luciferase activity with e1piNOS-luc in combination with p65 subunit as published [10] (Figure 1B). Analysis of e2piNOS-luc and e3piNOS-luc indicated a 5-fold and 19-fold increase in luciferase expression, respectively (Figures 1C-D). Interestingly, with e3piNOS-luc the luciferase activity was higher (31-fold) following co-transfection with the classical heterodimer p50/p65 (Figure 1D).

Plasmids were methylated *in vitro* using CpG methyltransferase (M.SssI) to analyse the effect of CpG methylation on *iNOS* activity. Using the e1piNOS-luc construct, enhanced *iNOS* activity induced by p65 was significantly reduced following methylation treatment (47.6 ± 23.8% for e1piNOS versus 11.7 ± 7.2% for Met-e1piNOS) (Figure 1B). Following application of e3piNOS-luc, the enhanced *iNOS* activity promoted by p50/p65 was significantly reduced following methylation treatment (31.4 ± 7.0% for e3piNOS versus 16.9 ± 12.5% for Met-e3piNOS) (Figure 1D).

CpG demethylation *in vitro* with 5-aza-dC correlates with enhanced levels of *iNOS* in both healthy and OA human chondrocytes

Long-term culture of NOF human articular chondrocytes in 5-aza-dC resulted in a 4-fold increase in *iNOS* expression compared to control cultures (mean ± SD 4.2 ± 4.4 *versus* 1.4 ± 0.8 fold increase). In contrast, in cultured OA chondrocytes while *iNOS* expression was observed to increase, this proved variable and sample (patient) dependent (mean ± SD 10.4 ± 21.5 *versus* 1.4 ± 1.7 fold decrease) (Figure 2A).

To determine if the changes in *iNOS* expression correlated with loss or gain of DNA methylation in the CpG sites localised at the −5.8 kb NF-κB enhancer element, the percentage methylation was quantified using pyrosequencing. Both CpG sites (−5853 and

198 −5843) were significantly demethylated in both NOF and OA samples following 5-aza-dC
199 treatment, with loss of methylation more pronounced in the pathologic samples. The loss of
200 methylation in the NOF samples was ~ 10%: 93.9 ± 0.4 *versus* 84.9 ± 2.5 at −5853 CpG and,
201 87.0 ± 2.9 *versus* 79.3 ± 3.1 at −5843 CpG (Figure 2B). In contrast, within the OA samples;
202 the loss of methylation was considerably higher reaching 30-40%: 93.4 ± 0.6 *versus* 65.2 ±
203 21.7 at −5853 CpG and, 90.0 ± 1.0 *versus* 51.8 ± 16.1 at −5843 CpG (Figure 2C).
204
205 **Loss of methylation at the CpGs localised at −5.8 kb NF-κB enhancer element enhances**
206 **C28/12 chondrocyte cell line proliferation.**
207
208 An increase in cell proliferation after 48 hours, determined using the MTT proliferation
209 assay, was observed in C28/12 chondrocytes transfected with the unmethylated plasmid
210 (e1piNOS-luc) compared to cells transfected with methylated plasmid (Met-e1piNOS-luc):
211 73.1 ± 8.6% *versus* 81.6 ± 7.2% (Figure 3). However, no significant influence on MTT assay
212 results was observed following co-transfections with NF-κB subunits in C28/12 cells (Suppl.
213 Figure 1).
214
215 **Alteration of cell cycle distribution is induced by loss of methylation of the crucial *iNOS***
216 **enhancer element and correlates with lower G0/G1 cell cycle arrest**
217
218 To evaluate the effect of DNA methylation status on the −5.8 kb NF-κB enhancer
219 element on cell cycle activity of chondrocytes, C28/I2 cells were transiently co-transfected
220 with methylated (e1piNOS-Met) and unmethylated vector *iNOS* vectors (e1piNOS) together
221 with the p65 NF-κB subunit. In these studies, the empty transfected plasmid served as the
222 negative control group and the non-transfected group as the control group.

Flow cytometry analysis demonstrated that in cells transfected with the unmethylated plasmid (e1piNOS-luc), a decreased proportion of cells (59%) could be detected in the G0/G1 phase compared to cells transfected with the methylated plasmid (64%) (Figure 4A). Furthermore, almost 100% of these cells were observed in the G2/M phase afterwards, indicating a proliferative stage. Furthermore, FACS analysis using propidium iodine-stained DNA demonstrated an alteration in cell cycle distribution in cells transfected with methylated or unmethylated −5.8 kb *iNOS* enhancer. These results indicate chondrocytes with enhanced *iNOS* expression were active proliferating cells with reduced levels of apoptotic cells (Figure 4B).

Differential cell-cycle-specific gene expression profile in osteoarthritic chondrocytes

To determine if the loss of methylation of the NF-κB crucial enhancer element is an OA-related phenomenon, resulting in altered OA cell cycle phase distribution, the expression profile of cell-cycle genes was also analysed in NOF chondrocytes in direct comparison to OA cells. Thus differential expression of *CCDN1*, *CDK6*, and *CDKN2A* was examined in articular chondrocytes obtained from control subjects (NOF) and OA patients using qRT-PCR. A significant increase in relative *CCDN1* and *CDK6* expressions was observed in OA chondrocytes in comparison to control (1.6 ± 1.1 in control *versus* 3.7 ± 2.2 in OA) for *CCDN1* (Figure 5A), and similarly for *CDK6* (1.3 ± 0.6 in controls *versus* 7.6 ± 4.5 in OA) (Figure 5B). In marked contrast, *CDKN2A* was significantly decreased in OA samples compared to controls (1.1 ± 1.1 *versus* 3.3 ± 2.5) (Figure 5C). Finally, long-term culture and stimulation with the cytokines IL-1β and OSM produced a significant increase in *CCDN1*expression, a significant decrease in *CDK6* expression although no differences were observed in *CDKN2A* expression (Suppl. Figure 2).

Discussion

The current study demonstrates that in human OA articular chondrocytes, the enhancer element localised at −5.8 kb is crucial for *iNOS* induction following DNA demethylation with subsequent enhancement of NF-κB binding. Critically, the results demonstrate that the subsequent loss of methylation in this region correlated with enhanced chondrocyte proliferation evidenced by chondrocytic cells observed in the G2/M cell cycle phase. We have recently published that demethylation of the NF-κB enhancer region at −5.8 kb of the *iNOS* gene leads to increased transcription through elevated binding of this transcription factor [10]. Indeed the presence of multiple functional NF-κB binding sites this far upstream has been demonstrated, although not studied in chondrocytes and is unique to the human *iNOS* promoter [11-13]. Studies using the luciferase reporter assays together with a CpG-free vector containing distinct *iNOS* enhancer element indicate the NF-κB enhancer region at −5.8 kb of *iNOS* is critical for the induction of *iNOS* expression in articular chondrocytes. Thus epigenetic regulation of an enhancer element can be pivotal if a sparse CpG promoter also contains a strong enhancer element allowing gene transcription even when CpG sites are methylated [22].

iNOS expression in both normal and OA chondrocytes was induced with the demethylating agent 5-aza-dC and expression was significantly enhanced in OA chondrocytes. Interestingly, we had anticipated less *iNOS* expression in OA chondrocytes (*iNOS* is highly expressed in OA chondrocytes) given culture of cells is sufficient to induce hypermethylation and loss of expression in some genes [23]. In addition, in the current study, the loss of methylation following treatment with 5-aza-dC at the CpG sites of the −5.8 kb NF-kB enhancer elements was higher in OA cells.

Aged articular chondrocytes from normal individuals do not undergo cell division; in contrast, in OA cartilage, the chondrocytes that survive form clones typical of severe OA [24], consistent with cell proliferation [25]. Indeed, cartilage neighbouring the damaged tissue location appear to contain a population of proliferative chondrocytes, which may be involved in an attempt to repair the damage tissue [3]. In support of this possible approach, recent investigations applying different treatments to promote chondrocyte proliferation suggest the potential to delay or enhance OA progression [26, 27]. The current study adds further support to a proliferative role of aberrant chondrocytes in the aetiology of OA. Thus, transient transfection of the −5.8 kb NF-kB enhancer element in the chondrocytic cell line C28/I2 induced higher proliferation in contrast to the methylated enhancer element.

Cell replication and the process of cell cycling are central in complex multicellular processes, such as injury repair or mounting an immune response [28]. Microarray experiments with synchronized cells indicate that the majority of IL-1-triggered genes, like *iNOS*, are preferentially up-regulated in cells released from the G1 phase [29]. The current studies indicate chondrocyte progression from G1 phase to S and G2/M phases in cells transfected with the unmethylated −5.8 kb NF-kB enhancer correlated with higher *iNOS* expression levels.

While the regulation of cell-cycle genes in endochondral bone growth has been extensively studied [30, 31], to date, there is little is known regarding the role of cell-cycle genes in adult human articular cartilage. Cell-cycle genes are targeted by a number of mitogenic and anti-mitogenic pathways in chondrocytes [30]. Cyclin D1 (*CCDN1*) is a positive regulator of cell G1/S transition and is a key restriction point in the cell cycle binding to CDK4 and CDK6 to control cell cycle progression from G1 to S phase [32]. The *CDKN2A* gene (p16) belongs to the Ink4 family of proteins that act to inhibit CDK4 and CDK6 upon binding, resulting in the arrest of the cell cycle in G1 or apoptosis [33].

Handschick and collaborators have identified CDK6 as a molecular link between the inflammatory microenvironment and permanent cell cycle progression. Thus, in the G1 phase, D1 cyclins activate cell cycle progression through phosphorylation of negative-growth regulators [29]. The current study demonstrates an increase in the proliferation rate of OA chondrocytes together with increased gene expression of cyclin D1 (*CCDN1*), *CDK6*, and decreased expression of p16 (*CDKN2A*). Interestingly, Wang and colleagues have published a model in which chondrocyte proliferation is promoted through *iNOS*-NO-mediated induction of cyclin D1 expression. Furthermore, reduced chondrocyte proliferation was observed in *iNOS*-deficient mice [34]. Jhou et al reported inhibition of the cell-cycle progression is capable of reducing pro-inflammatory responses *via* down-regulation of NF-kB [16], whilst increased NF-kB activity during growth of mitogen-stimulated and transformed cells has been linked to cell cycle progression through transcriptional activation of the cyclin D1 gene, leading to increased abundance of cyclin D1 and increased activity of cyclin D1 kinase [35].

The current observations that loss of methylation in the crucial enhancer element and subsequent induction of *iNOS* expression is complimentary to reports from Van der Kraan and van der Berg postulating OA has an age and senescence dependent component as a consequence of changes in gene methylation [3]. Furthermore, Yan and colleagues have shown in mice that a deficiency of the neuronal (nNOS) and endothelial (eNOS) members of the nitric oxide synthase family results in reduced chondrocyte proliferation and earlier cell cycle exit [36, 37], presenting an area of further future investigation in human articular chondrocytes.

Epigenetics changes have been shown to be key intermediates in signalling pathways that control proliferation. Thus chromatin compaction levels are directly correlated with DNA methylation levels resulting in altered gene expression and proliferation. In support of this

observation, methylation of H4K20 has been implicated in cell cycle progression, chromosome condensation and transcriptional regulation [28]. There is now emerging evidence of the cross-talk between histone phosphorylation and acetylation at distinct amino acids with H3S28phK27ac and H3S10phK14ac shown to be responsible for epigenetic regulation of transcription and cell cycle regulation [38].

The results of the current study of NF-κB-*iNOS*, cell cycle and DNA methylation signalling in OA chondrocytes demonstrate that the loss of methylation that happens at specific CpG sites localised at the −5.8 kb NF-κB of the *iNOS* gene in OA chondrocytes facilitate the binding of NF-κB and subsequent activation of *iNOS*. We suggest this has important implications in the pro-inflammatory response in OA as well as cell cycle regulation of OA chondrocytes culminating in the propagation of the aberrant OA phenotype to daughter cells (Figure 6). In conclusion, this study suggests that inhibition of cell cycle progression by *iNOS* enhancer hypermethylation is capable of reducing pro-inflammatory responses via down-regulation of NF-κB with important therapeutic implications in OA.

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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. In detail: MCDA— conception, design, acquisition of data, analysis and interpretation of data, drafting the manuscript; AT—acquisition of data, analysis and interpretation of data, critical revision of the manuscript; ROCO—conception, design, analysis and interpretation of data, critical revision of the manuscript.

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Competing interests

The authors have no conflict of interest to declare.

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Figure legends

Figure 1. NF-κB-mediated iNOS transactivation is affected by the CpG methylation of specific enhancer regions in human chondrocytes. (A) Different NF- κB enhancer elements were inserted in the CpG-free vector containing the iNOS promoter and transfected into C28/I2 cells. (B-D) Luciferase activity was measured before and after in vitro methylation as described in material and methods. Values are expressed as mean ± SD (n = 3 independent experiments, each performed in duplicate). * = P < 0.05 by analysis of variance with post hoc t-test.

Figure 2. Attenuated p65 binding to the NF-κB enhancer region at -5.8 kB in the presence of CpG methylation. ChIP assays were performed using cell lysates from C28/I2 cells that had been stably transfected with unmethylated vector (Meth-) and with methylated vector (Meth+) e1piNOS construct (Input) and the expression vector encoding p65. Binding of transcription factor to the human *iNOS* enhancer element was analysed by qPCR reaction using primers specifically bracketing NF-κB-binding sites. The results were quantified and are shown as the percentage input. Values are the mean ± SEM of 3 experiments and represent the fold-change versus IgG. * = P < 0.05.

Figure 3. DNA demethylation of the −5.8 kb NF-κB enhancer element and aberrant *iNOS* expression following long-term culture in 5-azadeoxycytidine (5-aza-dC) in healthy and OA human chondrocytes. (A) *iNOS* expression in primary human chondrocytes was analysed by quantitative reverse transcription-polymerase chain reaction. (B-C) Pyrosequencing analysis of the DNA methylation status of CpG sites in the NF-κB binding element at −5.8 kb. NOF = 7 and OA = 5; * = *P* < 0.05 by Wilcoxon's signed rank test.

Figure 4. Loss of methylation at the CpGs localised at −5.8 kb NF-κB enhancer element increase chondrocytic cell line proliferation. MTT assay was performed in C28/I2 cells after transfection with Met-e1piNOS and e1piNOS plasmids for 48 hours. Control bar represents cells transfected with empty vectors. Values are expressed as mean ± SD (n = 3 independent experiments, each performed in triplicate). * = $P < 0.05$ by analysis of variance with post hoc *t*-test.

Figure 5. NF-κB-mediated *iNOS* transactivation is affected by the loss of CpG methylation of specific CpG sites in human chondrocytes and results in an alteration in cell cycle distribution. Results shown are from cell cycle analysis of C28/I2 cells stably transfected with methylated vector (e1piNOS-Met) and with unmethylated vector (e1piNOS). Values are expressed as mean ± SD. * = $P < 0.05$ by analysis of variance with post hoc *t*-test.

Figure 6. Cell cycle specific gene expression in primary human chondrocytes obtained from control subjects with femoral neck fracture and patients with osteoarthritis (OA). Differential expression of A: *CCDN1*; B: *CDK6* C: *CDKN2A* in primary human chondrocytes obtained from control subjects with femoral neck fracture (#; n = 11) and from OA patients (n = 11), analysed by quantitative reverse transcription–polymerase chain reaction. Values are the mean ± SD of triplicate determinations per sample.

Figure 7. Proposed model of NF-κB-*iNOS*, cell cycle and DNA hypomethylation signalling in chondrocytes. Loss of methylation at specific CpG sites localised at the −5.8 kb NF-κB of the *iNOS* gene in OA chondrocytes permits binding of NF-κB transcription factor activating

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Supplementary Figures

Supplementary Figure 1. Metabolic activity of transfected C28/I2 is affected by DNA methylation. MTT assay was performed in C28/I2 cells following co-transfections of Met-e1piNOS and e1piNOS plasmids with NF-κB subunits (p50, p65 and p50/p65) for 48 hours. Values are expressed as mean ± SD (n = 3 independent experiments, each performed in triplicate).

Supplementary Figure 2. Cell cycle specific gene expression in primary human chondrocytes obtained from control subjects with femoral neck fracture before and after long-term culture with IL-1β plus OSM. Differential expression of A: *CCDN1*; B: *CDK6* C: *CDKN2A* (#; n = 7) analysed by quantitative reverse transcription–polymerase chain reaction. Values are the mean ± SD of triplicate determinations per sample.

Supplementary table. Primer information.

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Figure 1

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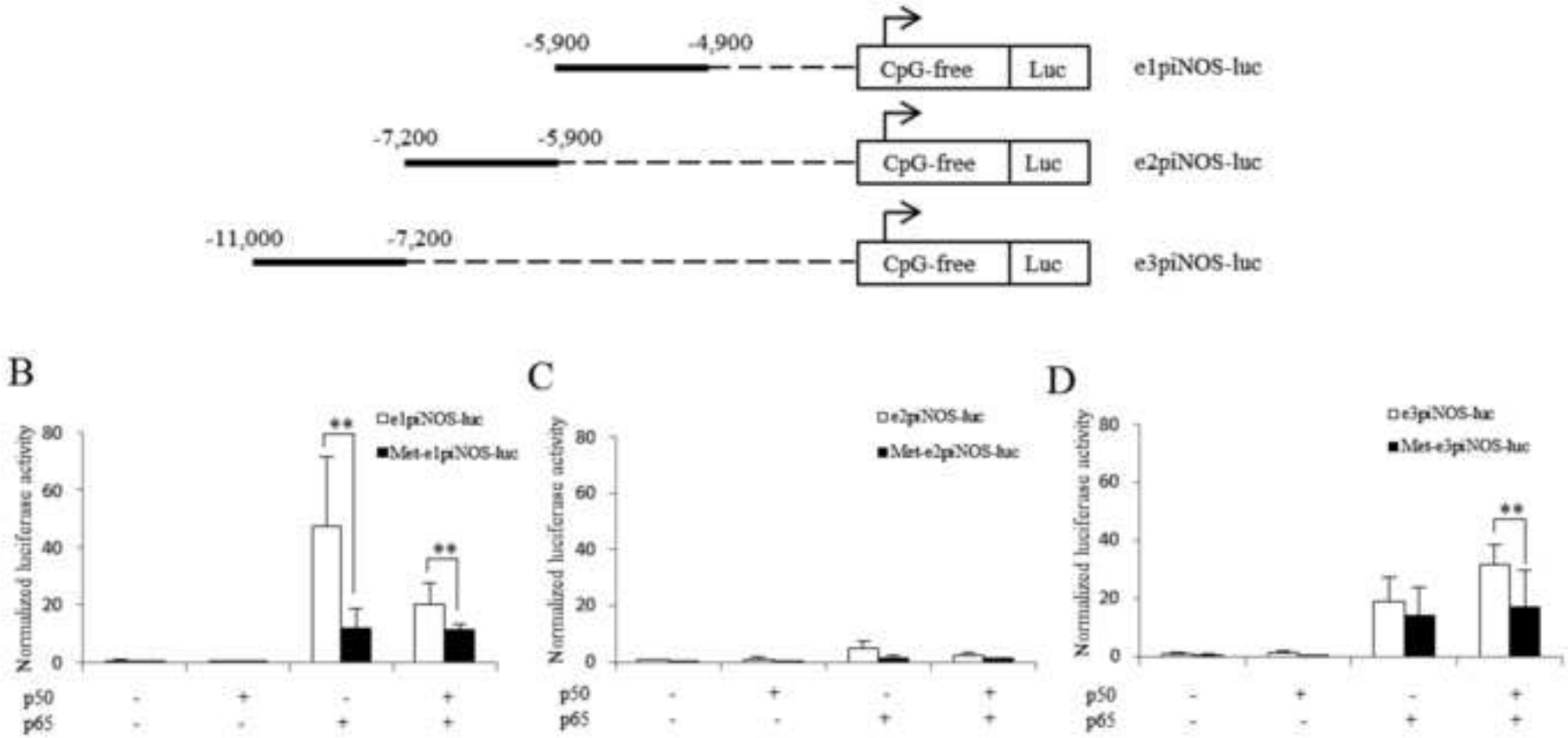

Figure2

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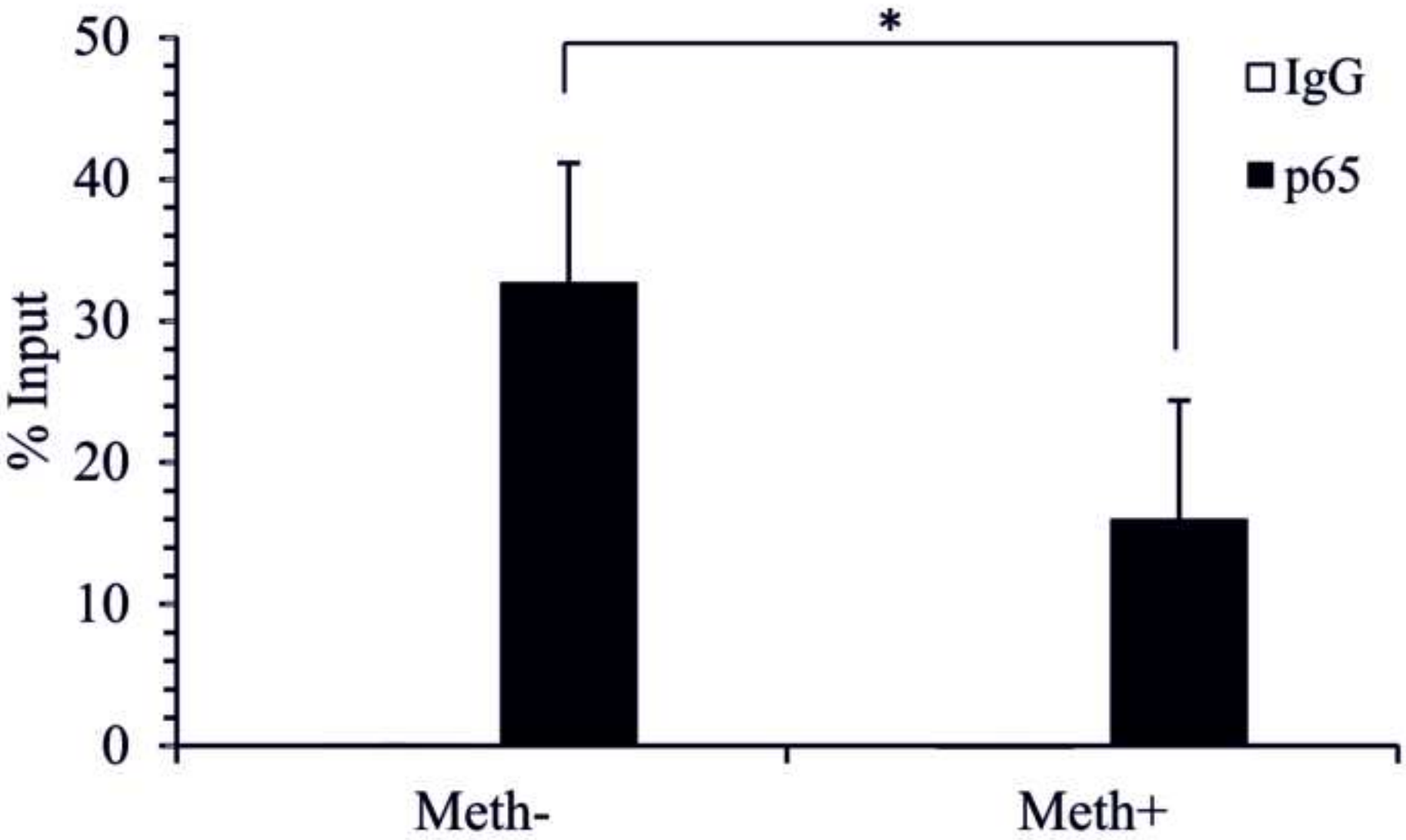

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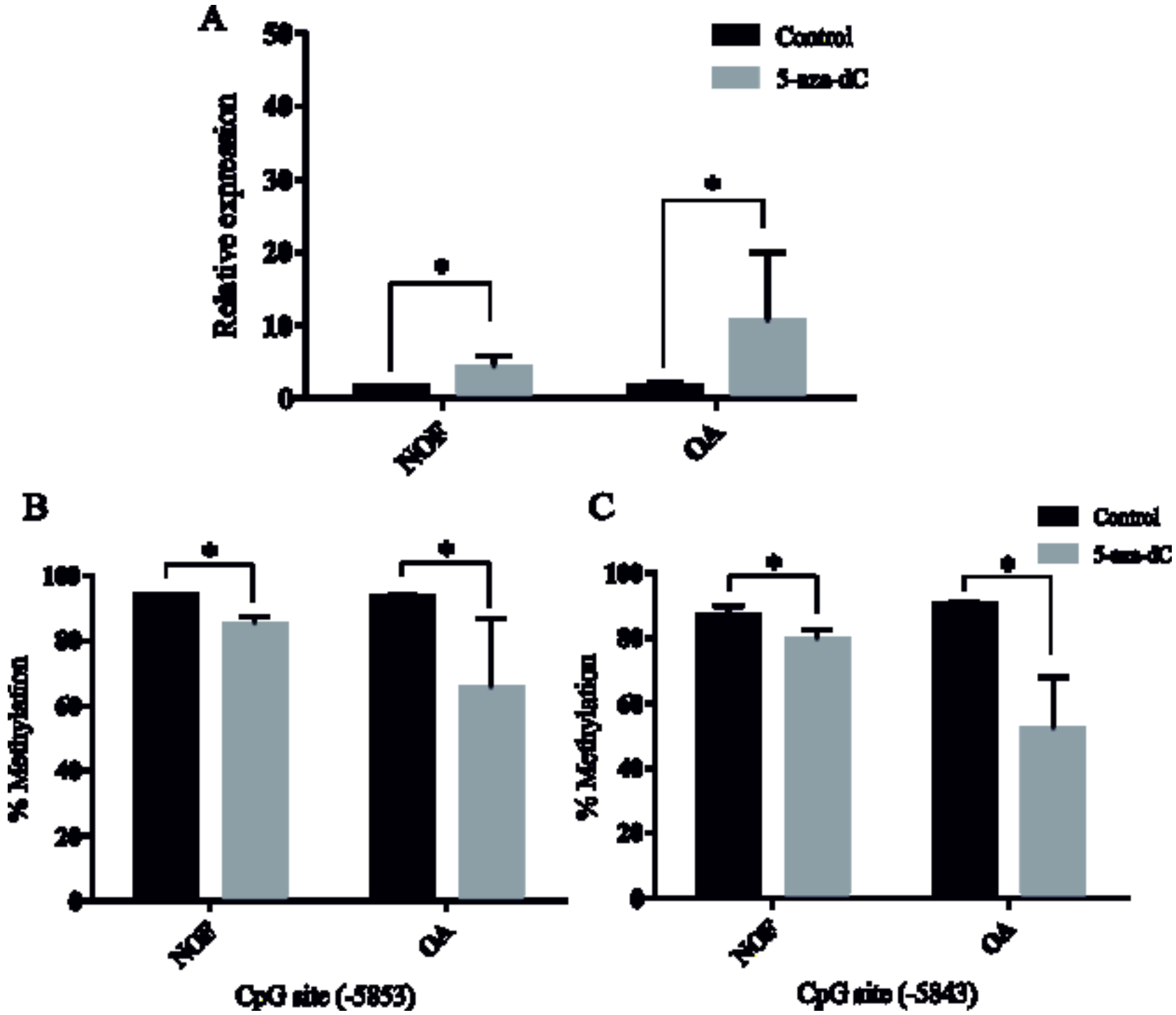

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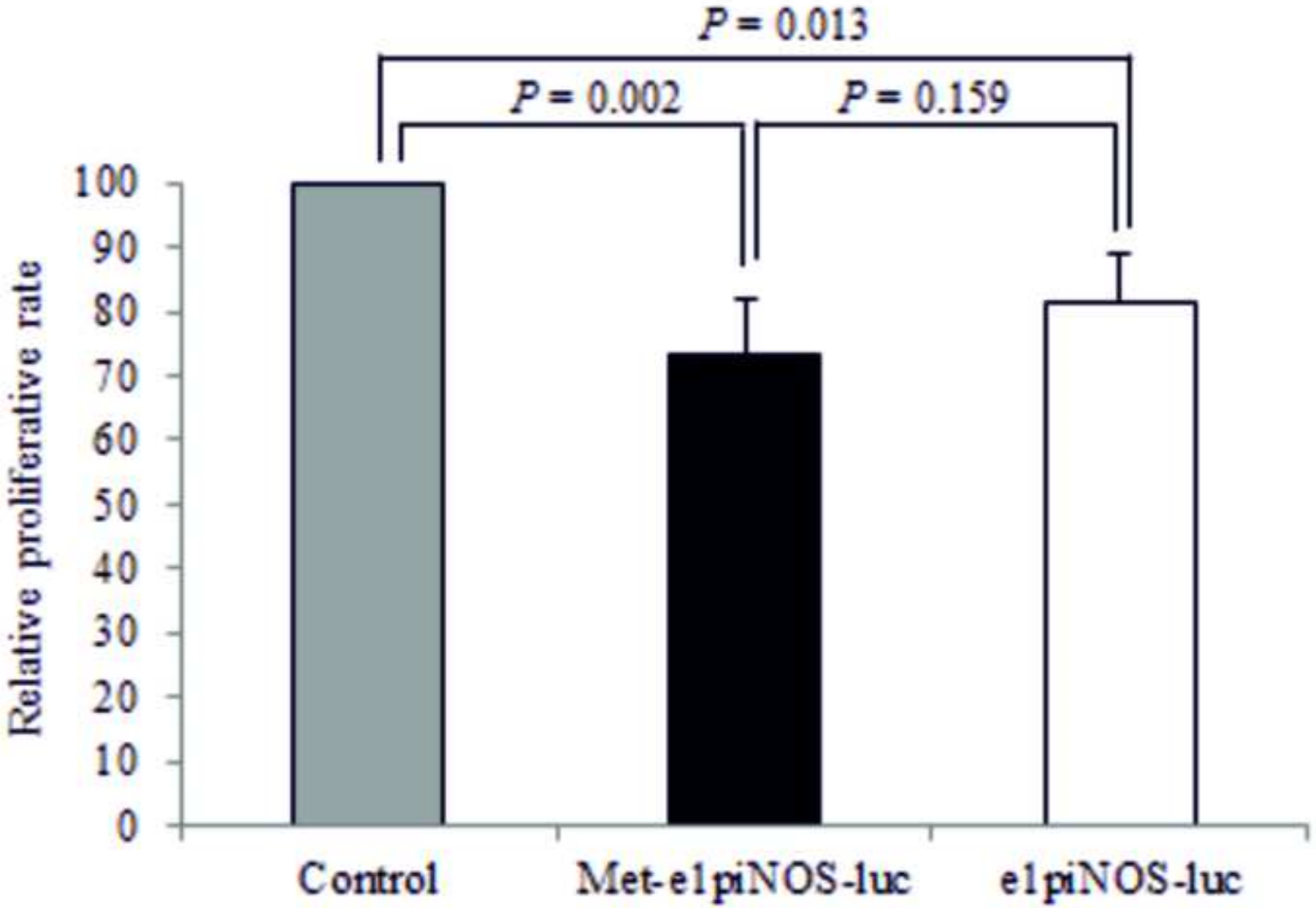

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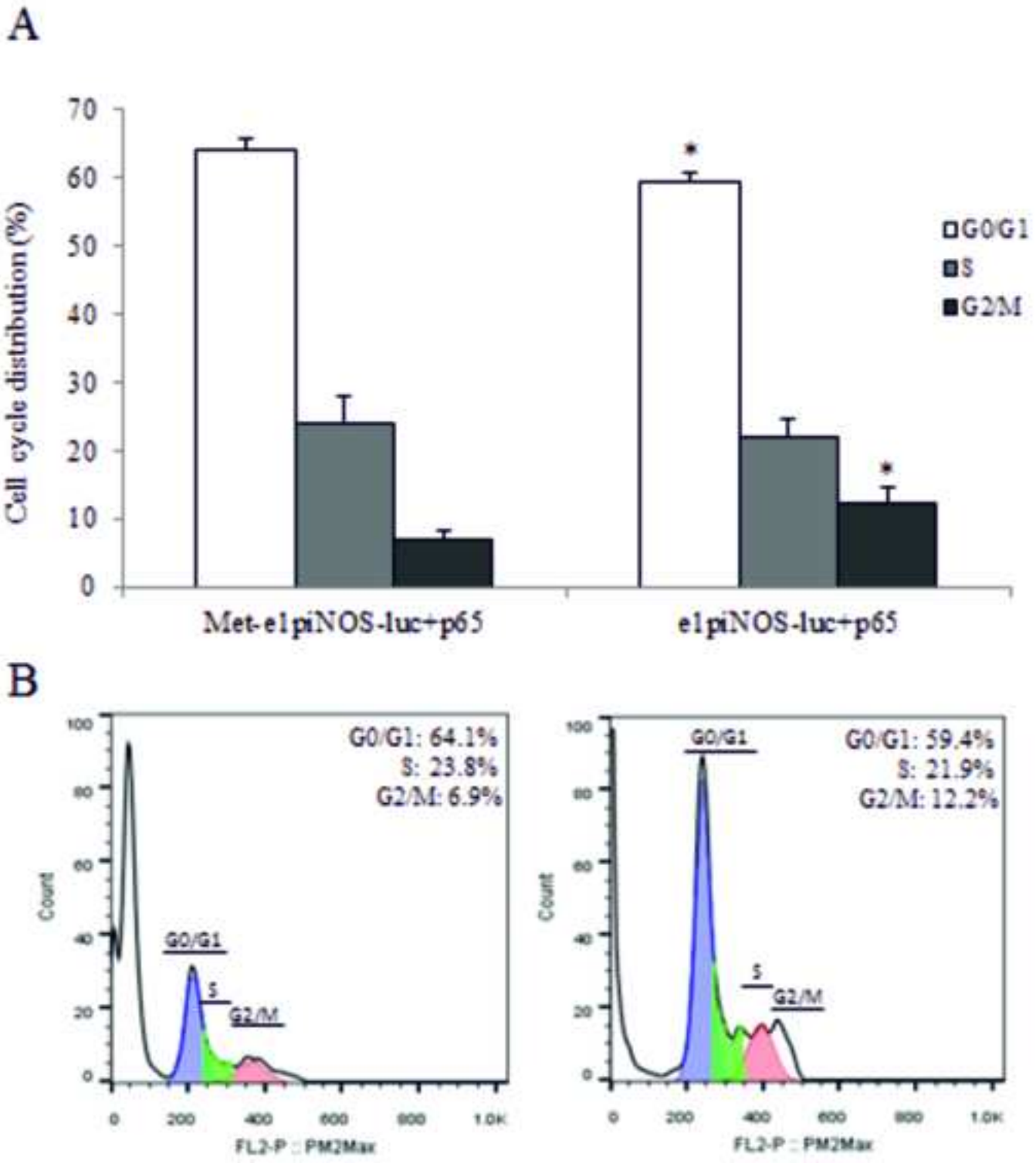

Figure6

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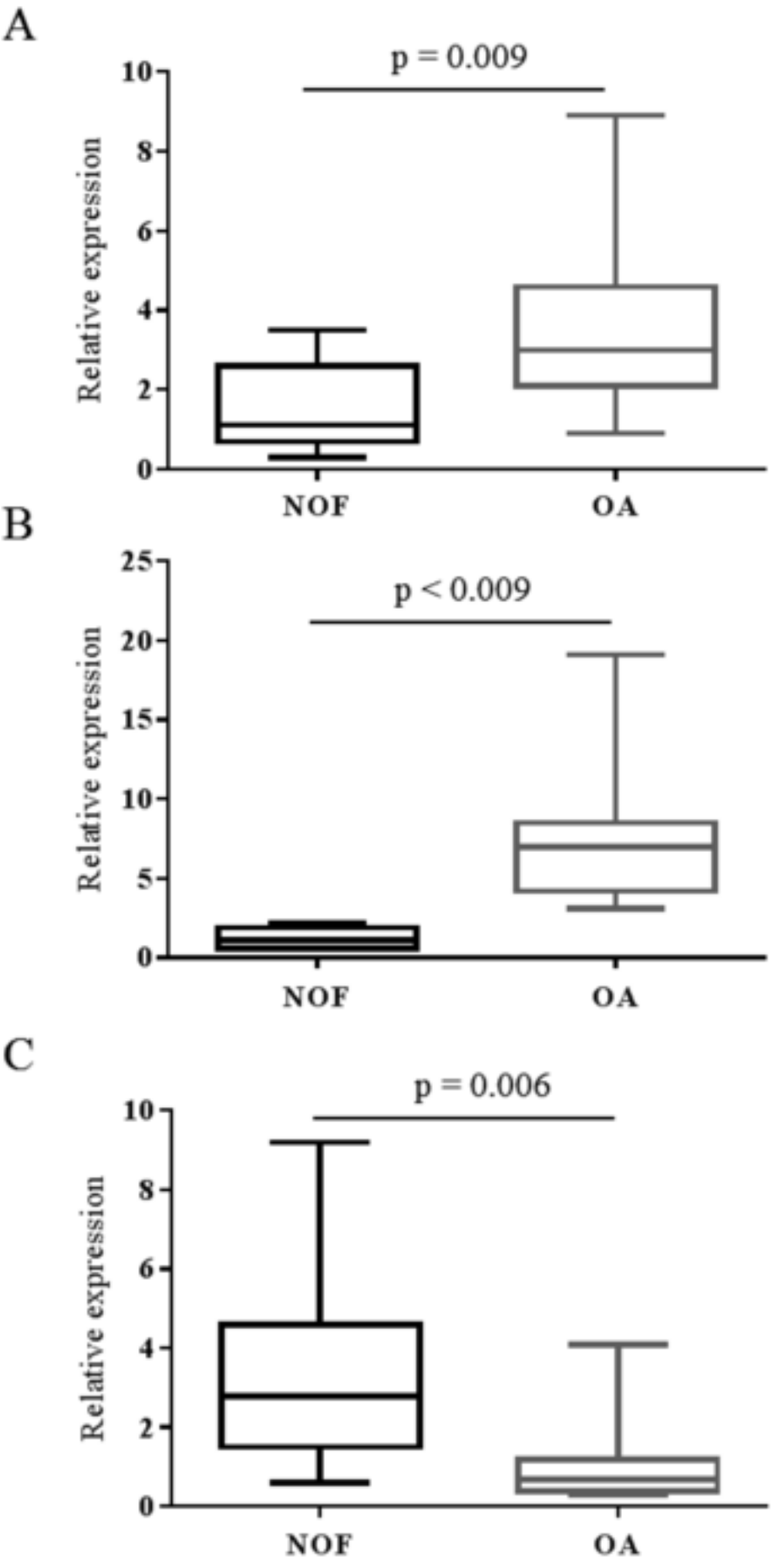

Figure7

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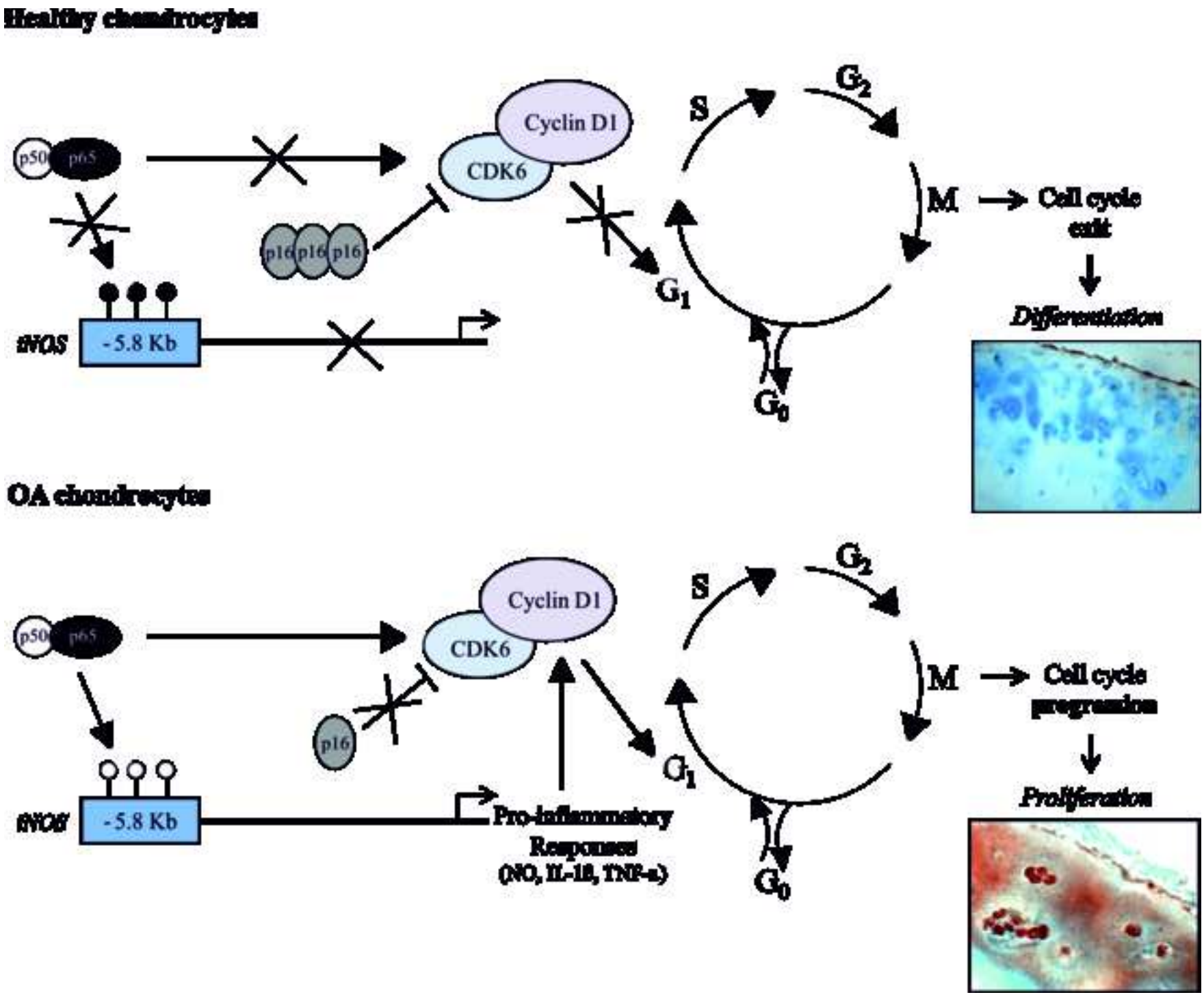

Supplementary Figure 1

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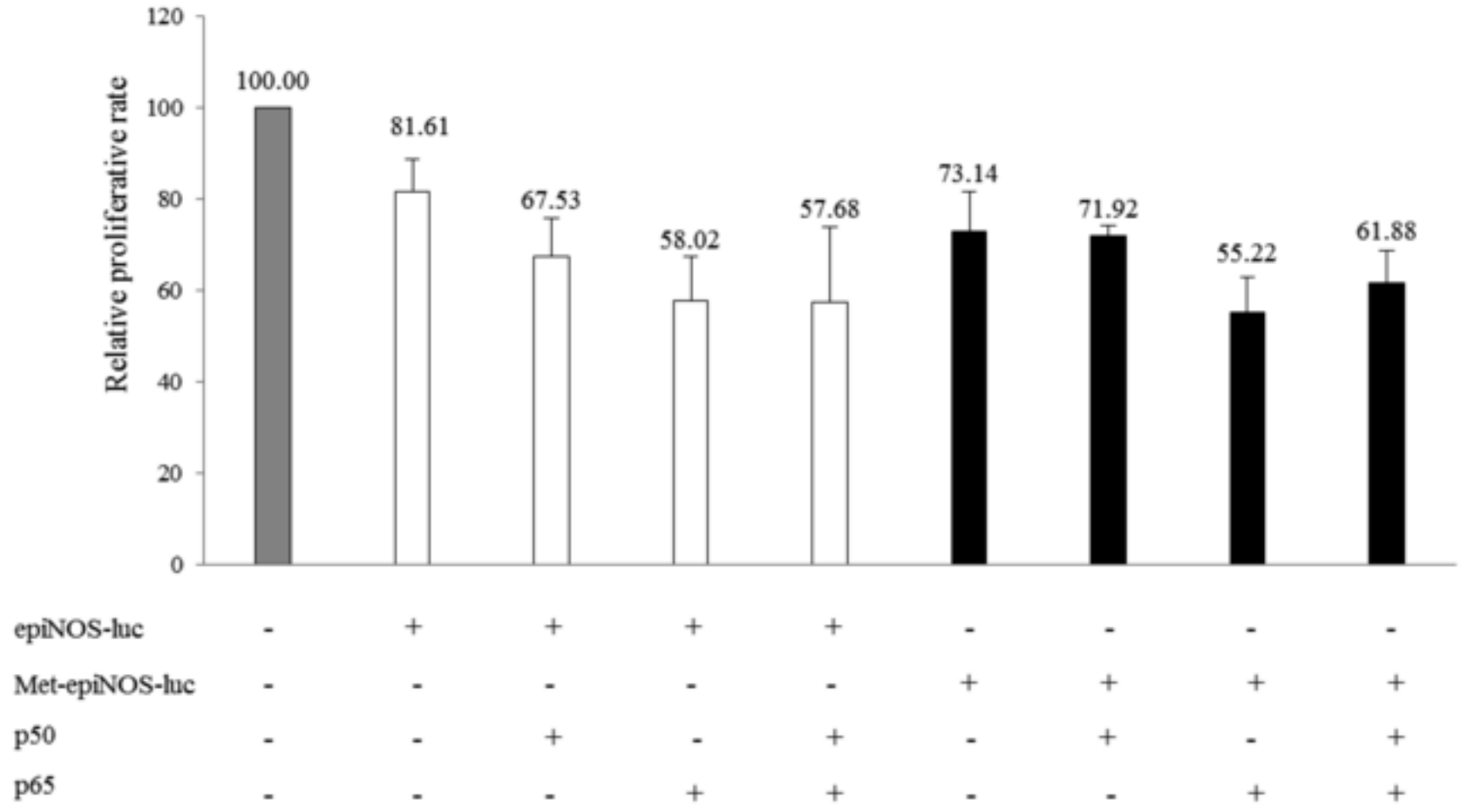

Supplementary Figure 2

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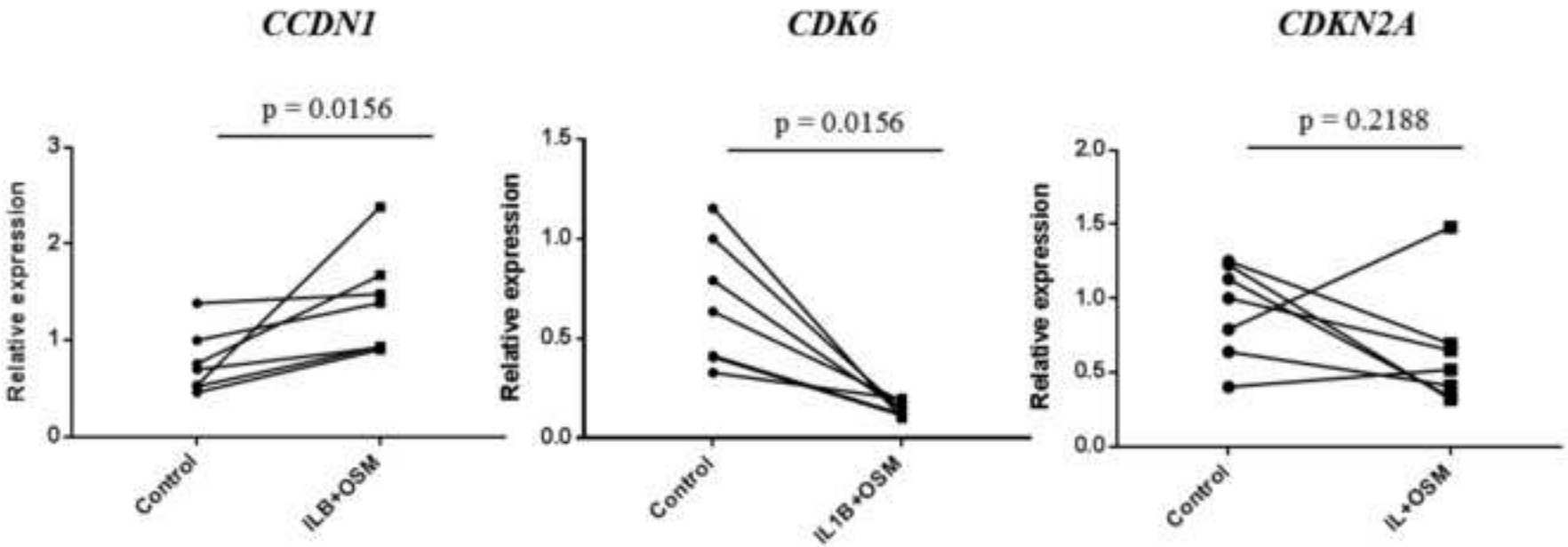

Supplementary Table

Gene	Sequence (5'-3')
qPCR	
GAPDH	F CCAGGTGGTCTCCTCTGACTTC
	R TCATACCAGGAAATGAGCTTGACA
iNOS	F GAGGAGCAGGTCGAGGACTAT
	R TCTTCGCCTCGTAAGGAAATAC
CCND1	F CTACCGCCTCACACGCTT
	R CTTGGGGTCCATGTTCTGC
CDK6	F TTTCGTGGAAGTTCAGATGTTG
	R CATCTCTAGGCCAGTCTTCTTCT
CKN2A	F GTGGACCTGGCTGAGGAG
	R CTTTCAATCGGGGATGTCTG
Pyrosequencing	
Enhancer -5.8 kB	F GTTTTTTTTGGTTTTGGGAAAGTT
	R TTAACCCAATTCTAAACCCCCTAT
	S TTATAAAGTGTATTGGAATGAG
Cloning	
Promoter	F ATTAGATCTTTTGCTTCTCAACTTCTCCCTAAT
	R ATACCATGGAGTTTTCGACTCGCTACAAAGTTA
Enhancer 1	F ATTCCTGCAGGGGATACAAGAGGGTGGGCTTAG
	R ATTAGATCTGGGCGTGGCTCTTACTCTCTA
Enhancer 2	F ATTCCTGCAGGTTGTCTGCCATCA
	R ATTAGATCTGATGCTGATCCAAGAAGT
Enhancer 3	F ATTCCTGCAGGTAATGAGGAGTTCTT
	R ATTAGATCTCAATAGAGATGGCAAGATG
ChIP	
RelA	F GGGCTTATGTGGCCTAACCAA
	R CCACCAGGGAACTTGAAAAA

Introduction

Osteoarthritis (OA), the most common form of arthritis world-wide, is characterized by progressive failure of the extracellular cartilage matrix, along with changes in the synovium and subchondral bone. In normal adult cartilage, chondrocytes present, in the non-stressed steady state, as quiescent cells demonstrating negligible turnover of the cartilage matrix. In contrast, in OA, the chondrocytes become "activated", characterized by cell proliferation, cluster formation, and the increased production of matrix proteins and matrix-degrading enzymes [1]. Degradative chondrocytes, cells that express degradative enzymes, undergo cell division, a phenomenon that is rarely seen in normal, mature, articular chondrocytes. We have previously shown that abnormal synthesis of metalloproteinases (MMPs) does not occur in all OA chondrocytes, but is notably present in chondrocyte clusters, indicating acquisition of altered phenotype by daughter cells from a single abnormal chondrocyte [2].

Typically, hyaline cartilage does not undergo terminal differentiation under normal conditions, however OA chondrocytes may be the result of articular chondrocytes differentiating to give a hypertrophic and thus an altered phenotype [3]. In support of such a mechanism, genetic modifications that stimulate chondrocyte hypertrophy-like changes are frequently associated with a higher incidence of OA or accelerated OA development [3].

Nitric oxide (NO) and its redox derivatives display a number of different regulatory functions in both normal and pathophysiological joint conditions [4] and, has been extensively demonstrated to play a role in the regulation of bone cell metabolism, bone remodelling and in the modulation of chondrocyte physiology in OA [4-9]. This catabolic factor is the product of inducible nitric oxide synthase (*iNOS*), which not only suppresses the synthesis of cartilage matrix, but also increases expression of proteases in OA [10].

There is growing evidence to support a role for epigenetics in the pathogenesis of OA. Epigenetic mechanisms explain changes in gene function that are not a consequence of modifications in the DNA sequence and of these, DNA methylation has been implicated in the induction of *iNOS* [11]. We have recently described that demethylation of the NF-κB enhancer region at −5.8 kb of the *iNOS* gene leads to increased transcription through elevated binding of this transcription factor [12]. Indeed, it has previously been shown that there are multiple functional NF-κB binding sites in iNOS so far upstream, a unique property of the human *iNOS* promoter [13-15]. However, it is not known if all these binding sites are functional, regulated by DNA methylation and if regulated by DNA methylation, which site is critical for *iNOS* induction in chondrocytes

Nuclear factor (NF)-κB represent a family of proteins, many of which are ubiquitously expressed and inducible by a variety of extracellular growth stimuli [16]. Key gene members within the family include RelA, RelB, Nfκb1, Nfκb2, and Iκ Bα~~. Protein products of the RelA, Nfκb1 and Nfκb2 genes are processed to p65, p50 and p52, respectively, to form p65/p50 and p52/RelB dimmers~~ which regulate the transcription of genes that mediate a variety of cellular functions, including cell proliferation, differentiation, apoptosis and inflammation [16]. Within the regulation of cell proliferation, cyclins, cyclin dependent kinases (CDKs) and other relative proteins are expressed according to space and time and tightly regulate cell cycle progression [17]. Interestingly, down-regulation of NF-κB has been implicated in the inhibition of cell-cycle progression [18]. Critically, differences in the molecular mechanisms underlying cell cycle regulation between *iNOS* and p65 NF-kB subunit in human chondrocytes remain far from clear. In the current study, we hypothesised that loss of methylation of the crucial enhancer element within OA chondrocytes permits p65 binding and subsequent induction of expression of *iNOS*. This results in regulation of key factors in G1/S, decreasing p16, increasing CDK6 and cyclin D, inducing cell cycle

progression and proliferation. We hypothesise this leads, ultimately, to transmission of this altered phenotype to daughter cells and propagation of OA.

Material and Methods

Cartilage dissection and articular chondrocytes isolation

Human articular cartilage samples were obtained from 12 patients following hemiarthroplasty as a consequence of femoral neck fracture (#NOF) (3 men and 9 women; mean–±–SD age of 83.2–±–8.0 years) and from 16 OA patients (OA Research Society International– modified Mankin score [19] of 3–5) who underwent total hip arthroplasty (8 men and 8 women; mean–±–SD age of 70.6–±–12.3 years). Informed consent was obtained from all patients, and the study was approved by the Southampton and West Hampshire local ethics committee. Cartilage obtained from patients with femoral neck fracture is widely used as a suitable non-OA control. Only chondrocytes from the superficial layer of OA cartilage or the deep zone of cartilage from patients with femoral neck fracture were isolated in order to differentiate both phenotypes. Cartilage samples were obtained from individual subjects and cartilage fragments digested as previously described [20].

Chondrocyte culture

Isolated chondrocyte samples were divided into 4 groups: (i) non-cultured, (ii) cultured without treatment (control culture), (iii) cultured in 10 ng/ml IL-1β plus 10 ng/ml oncostatin M (OSM), and (iv) cultured using 2 µM 5-azadeoxycytidine (5-aza-dC). Prior to treatment, chondrocytes were cultured as previously described [12]~~.for a minimum of 48 hours at 37°C~~

~~at a density of 2-4 x 10^5 cells in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 5% fetal calf serum, 1% insulin-transferrin-selenium, 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 100 µg/ml ascorbic acid in an atmosphere of 5% CO_2.~~

For samples cultured in 5-aza-dC, the histone deacetylase inhibitor trichostatin A (TSA; 300 nM) was added to facilitate access to 5-aza-dC (cytidine analogue that inhibits DNA methyltransferase 1) [21]. Sample media was changed twice weekly and primary cultures maintained for 5 weeks until the samples reached confluence.

DNA and RNA extraction, quantitative reverse transcription–polymerase chain reaction (qRT-PCR) and pyrosequencing

Total RNA and genomic DNA were extracted simultaneously from harvested chondrocytes using AllPrep DNA/RNA Mini kit (Qiagen), according to the manufacturer's instructions. *GAPDH*, *iNOS*, *CCND1*, *CDK6* and *CKN2A* relative gene expression and the percentage DNA methylation in the −5.8 kb NF-kB *iNOS* enhancer element were quantified using qRT-PCR and pyrosequencing and were performed as previously described [12, 20, 22]. See supplementary table for p~~P~~rimer information. ~~used in these studies for qRT-PCR and pyrosequencing is available upon request.~~

Plasmid construction

The *iNOS* promoter (piNOS) and three enhancer elements plus promoter (e1piNOS, e2piNOS and e3piNOS) constructs were generated by PCR amplification using Platinum PCR Supermix High Fidelity (Invitrogen) with genomic DNA from human articular

chondrocytes as a template. (see supplementary table for primer information) ~~Primer information for cloning is available upon request from the corresponding author~~. The resulting PCR products were cloned into the CpG-free-luc vector [23]. The piNOS-luc construct was generated by using the FastDigest restriction enzymes Bgl II and Nco I (Thermo) to cut the DNA from −1144 to +25 bp. Constructs containing the different enhancers were generated using FastDigest restriction enzymes Sbf I and Bgl II (Thermo) to cut the DNA from −5996 to −4906 bp (e1iNOS), from −7257 to −5927 bp (e2iNOS), and from −17000 to −11127 (e3iNOS) and subsequently, the epiNOS constructs were generated by ligation of promoter plus enhancer constructs. The authenticity of the DNA insert was confirmed by sequence analysis. E coli GT115 competent cells (InvivoGen) were used for the transformation.

In vitro DNA methylation and transient transfection

Methylated plasmids (Met-e1piNOSluc, Met-e2piNOSluc and Met-e3piNOSluc) were generated following incubation ~~of 1 µg~~ of plasmid DNA with ~~4 units/µl of~~ CpG methyltransferase (M.SssI; NEB) according to the manufacturer's instructions. Complete methylation was verified by plasmid DNA bisulfite modification and pyrosequencing. The methylated plasmids DNA were purified using a QIAquick PCR purification kit (Qiagen) and transfected (0.5 µg) into the immortalized chondrocytic cell line C28/I2 (provided by Dr Mary Goldring, Hospital for Special Surgery, NYC), in parallel with unmethylated plasmids and the control Renilla vector using Fugene® HD reagent (Promega). In co-transfections, the expression vectors for NF-κB (p50 [Addgene plasmid 21965], p65 [Addgene plasmid 21966], or p50/p65) were used (0.06 µg). Blank expression vector pCMV4 (generously provided by Dr. David Russell, University of Texas) was used as a control. Total DNA was normalized

with empty vectors in the transfection mixture. In all transient transfections, the internal control reporter (pRL-TK Renilla luciferase) was present at 1 ng/well. The luciferase assay was conducted using a 96-well luminometer with the dual luciferase substrate system (Promega) 48 hours after transfection. Relative luciferase activity was normalized to the internal control Renilla luciferase activity. Each experiment was repeated at least 3 times, and each data point was calculated as the mean ± SD of 3 wells per experiment.

Chromatin immunoprecipitation (ChIP) assay.

A ChIP-IT Express Enzymatic kit (Active-Motif) was used for ChIP assays according to the manufacturer's instructions, as described previously [24]. Briefly, C28/I2 cells were co-transfected with unmethylated or methylated pCpG-free-Luc-epiNOS vector and the expression vector encoding p65 using FuGene HD (Promega). After 48hours, precleared chromatin was stored as assay input or incubated overnight at 4°C with 4 µg of mouse monoclonal anti-NFκB p65 (RelA) antibody or normal mouse IgG (Millipore). After reverse cross-linking and purification, the final DNA preparations were subjected to qPCR analysis using 2 µl of the eluted DNA. For analysis, the Ct of each sample was normalized to the Ct of the input sample. Specific primers flanking the NF-κB response elements in the human *iNOS* promoter were designed ~~(F GGGCTTATGTGGCCTAACCAA and R CGACCAGGGAACTTGAAAAA).~~(see supplementary table for primer information).

MTT assay

Cell viability assay was determined using the MTT protocol. Briefly, C28/I2 cells (3 x 10^4 cells/well) in DMEM/F12 media at a final volume of 400 µl were seeded into 24-wells

culture plate and incubated overnight at 37°C in 5% CO_2. The cells were transfected with e1piNOS-luc and Met-e1piNOS-luc; in co-transfections, expression vectors for NF-κB (p50, p65, or p50/p65) were incubated for 48 hours. Finally~~, following a wash with PBS,~~ the cells were treated with 40 µl of MTT (5 mg/ml) alone and incubated for 1.5 hours at 37°C in 5% CO_2. The blue formazan products formed in the cells were dissolved in DMSO (400 µl) and measured at 540 nm using a spectrophotometer.

Cell cycle analysis

C28/I2 cells were transfected with e1piNOS-luc and Met-e1piNOS-luc; in co-transfections, the expression vectors for NF-κB (p50, p65, or p50/p65) were used. Transfected empty plasmids served as a negative control group and after 48hours were analysed by fluorescence-activated cell sorting (FACS). In brief, cells were harvested ~~by trypsinization, centrifuged and cell pellets resuspended~~ and fixed in 70% ice-cold ethanol at 4°C for at least 12 hours. Subsequently, cells were incubated in 100 µg/ml of propidium iodide (PI) ~~(Sigma)~~ and 50 µg/ml of RNase ~~(Sigma)~~ for 30 min. Cell cycle distribution was analysed by GUAVA flow cytometer (Millipore), and results processed using FLOWJO version 10 software (TreeStar, San Carlos, CA).

Statistics analysis

Statistical analysis was performed using SPSS software version ~~17~~21.0 Unless otherwise indicated, data are presented as the mean ± SD of at least 3 multiple independent experiments. Significance was determined by Mann-Whitney U test to compare gene

expression and analysis of variance with post hoc t-test was used to analyse transfection assays~~and by Mann-Whitney U test~~. *P* values less than 0.05 were considered significant.

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Results

The NF-κB element at −5.8 kb is crucial for induction of the human *iNOS* promoter in articular chondrocytes

Previous analysis of the 5'-flanking region of the human *iNOS* gene revealed three regions containing cytokine-responsive elements and functional NF-κB enhancer elements localised at −3.8 to −5.8, −5.8 to −7.0, and −7.0 to −16.0 kb [13-15]. In order to determine whether any of these putative elements conferred enhanced *iNOS* gene activity in human chondrocytes, transient co-transfections in C28/I2 cells were performed with three different constructs each containing the enhancer element cloned into the CpG-free vector (Figure 1A). Reporter vectors were subsequently transfected in combination with or without co-transfection of expression vectors coding p50 (pCMV4-p50) and p65 (pCMV4-p65), alone and in combination, to analyse the effect of NF-κB on *iNOS* activity.

Reporter gene assays demonstrated a 47-fold induction of luciferase activity with e1piNOS-luc in combination with p65 subunit as published [12] (Figure 1B). Analysis of e2piNOS-luc and e3piNOS-luc indicated a 5-fold and 19-fold increase in luciferase expression, respectively (Figures 1C-D). Interestingly, with e3piNOS-luc the luciferase activity was higher (31-fold) following co-transfection with the classical heterodimer p50/p65 (Figure 1D).

Plasmids were methylated *in vitro* using CpG methyltransferase (M.SssI) to analyse the effect of CpG methylation on *iNOS* activity. Using the e1piNOS-luc construct, enhanced

iNOS activity induced by p65 was significantly reduced following methylation treatment (47.6 ± 23.8% for e1piNOS versus 11.7 ± 7.2% for Met-e1piNOS) (Figure 1B). Following application of e3piNOS-luc, the enhanced *iNOS* activity promoted by p50/p65 was significantly reduced following methylation treatment (31.4 ± 7.0% for e3piNOS versus 16.9 ± 12.5% for Met-e3piNOS) (Figure 1D).

We investigated whether the CpG methylation status directly affected p65 binding to the -5.8 kB NF-κB enhancer element using ChIP assays performed using C28/I2 chondrocytes cotransfected with unmethylated or methylated e1piNOS constructs and expression vectors encoding p65. *iNOS* enhancer binding was analysed with specific PCR primers that recognised only the transiently transfected construct. ChIP assays revealed that methylation treatment significantly reduced p65 binding to the NF-κB enhancer element of the *iNOS* gene (Figure 2).

CpG demethylation *in vitro* with 5-aza-dC correlates with enhanced levels of *iNOS* in both healthy and OA human chondrocytes

Long-term culture of NOF human articular chondrocytes in 5-aza-dC resulted in a 4-fold increase in *iNOS* expression compared to control cultures (mean ± SD 4.2 ± 4.4 *versus* 1.4 ± 0.8 fold increase). In contrast, in cultured OA chondrocytes while *iNOS* expression was observed to increase, this proved variable and sample (patient) dependent (mean ± SD 10.4 ± 21.5 *versus* 1.4 ± 1.7 fold decrease) (Figure 3A).

To determine if the changes in *iNOS* expression correlated with loss or gain of DNA methylation in the CpG sites localised at the −5.8 kb NF-κB enhancer element, the percentage methylation was quantified using pyrosequencing. Both CpG sites (−5853 and −5843) were significantly demethylated in both NOF and OA samples following 5-aza-dC

treatment, with loss of methylation more pronounced in the pathologic samples. The loss of methylation in the NOF samples was ~ 10%: 93.9 ± 0.4 *versus* 84.9 ± 2.5 at −5853 CpG and, 87.0 ± 2.9 *versus* 79.3 ± 3.1 at −5843 CpG (Figure ~~2B~~3B). In contrast, within the OA samples; the loss of methylation was considerably higher reaching 30-40%: 93.4 ± 0.6 *versus* 65.2 ± 21.7 at −5853 CpG and, 90.0 ± 1.0 *versus* 51.8 ± 16.1 at −5843 CpG (Figure ~~2C~~3C).

Loss of methylation at the CpGs localised at −5.8 kb NF-κB enhancer element enhances C28/12 chondrocyte cell line proliferation.

An increase in cell proliferation after 48 hours, determined using the MTT proliferation assay, was observed in C28/12 chondrocytes transfected with the unmethylated plasmid (e1piNOS-luc) compared to cells transfected with methylated plasmid (Met-e1piNOS-luc): 73.1 ± 8.6% *versus* 81.6 ± 7.2% (Figure ~~3~~4). However, no significant influence on MTT assay results was observed following co-transfections with NF-κB subunits in C28/12 cells (Suppl. Figure 1).

Alteration of cell cycle distribution is induced by loss of methylation of the crucial *iNOS* enhancer element and correlates with lower G0/G1 cell cycle arrest

To evaluate the effect of DNA methylation status on the −5.8 kb NF-κB enhancer element on cell cycle activity of chondrocytes, C28/I2 cells were transiently co-transfected with methylated (e1piNOS-Met) and unmethylated vector *iNOS* vectors (e1piNOS) together with the p65 NF-κB subunit. In these studies, the empty transfected plasmid served as the negative control group and the non-transfected group as the control group.

Flow cytometry analysis demonstrated that in cells transfected with the unmethylated plasmid (e1piNOS-luc), a decreased proportion of cells (59%) could be detected in the G0/G1 phase compared to cells transfected with the methylated plasmid (64%) (Figure ~~4A~~5A). ~~Furthermore, almost 100% of these cells were observed in the G2/M phase afterwards, indicating a proliferative stage.~~ Furthermore, FACS analysis using propidium iodine-stained DNA demonstrated an alteration in cell cycle distribution in cells transfected with methylated or unmethylated −5.8 kb *iNOS* enhancer. These results indicate chondrocytes with enhanced *iNOS* expression were active proliferating cells ~~with reduced levels of apoptotic cells~~ (Figure ~~4B~~5B).

Differential cell-cycle-specific gene expression profile in osteoarthritic chondrocytes

To determine if the loss of methylation of the NF-κB crucial enhancer element is an OA-related phenomenon, resulting in altered OA cell cycle phase distribution, the expression profile of cell-cycle genes was also analysed in NOF chondrocytes in direct comparison to OA cells. Thus differential expression of *CCDN1*, *CDK6*, and *CDKN2A* was examined in articular chondrocytes obtained from control subjects (NOF) and OA patients using qRT-PCR. A significant increase in relative *CCDN1* and *CDK6* expressions was observed in OA chondrocytes in comparison to control (1.6~~–~~±~~–~~1.1 in control *versus* 3.7~~–~~±~~–~~2.2 in OA) for *CCDN1* (Figure ~~5A~~6A), and similarly for *CDK6* (1.3~~–~~±~~–~~0.6 in controls *versus* 7.6~~–~~±~~–~~4.5 in OA) (Figure ~~5B~~6B). In marked contrast, *CDKN2A* was significantly decreased in OA samples compared to controls (1.1~~-~~±~~-~~1.1 *versus* 3.3~~-~~±~~-~~2.5) (Figure ~~5C~~6C). Finally, long-term culture and stimulation with the cytokines IL-1β and OSM produced a significant increase in *CCDN1*expression, a significant decrease in *CDK6* expression although no differences were observed in *CDKN2A* expression (Suppl. Figure 2).

Discussion

The current study demonstrates that in human OA articular chondrocytes, the enhancer element localised at −5.8 kb is crucial for *iNOS* induction following DNA demethylation with subsequent enhancement of NF-κB binding. Critically, the results demonstrate that the subsequent loss of methylation in this region correlated with enhanced chondrocyte proliferation evidenced by chondrocytic cells observed in the G2/M cell cycle phase. We have recently published that demethylation of the NF-κB enhancer region at −5.8 kb of the *iNOS* gene leads to increased transcription through elevated binding of this transcription factor [12]. Indeed the presence of multiple functional NF-κB binding sites this far upstream has been demonstrated, although not studied in chondrocytes and is unique to the human *iNOS* promoter [13-15]. Furthermore, we show that in chondrocytes, CpG methylation of this enhancer element specifically impairs p65-driven *iNOS* promoter activation by altering p65 binding to the DNA. Studies using the luciferase reporter assays together with a CpG-free vector containing distinct *iNOS* enhancer element indicate the NF-κB enhancer region at −5.8 kb of *iNOS* is critical for the induction of *iNOS* expression in articular chondrocytes. Thus epigenetic regulation of an enhancer element can be pivotal if a sparse CpG promoter also contains a strong enhancer element allowing gene transcription even when CpG sites are methylated [25].

iNOS expression in both normal and OA chondrocytes was induced with the demethylating agent 5-aza-dC and expression was significantly enhanced in OA chondrocytes. Interestingly, we had anticipated less *iNOS* expression in OA chondrocytes (*iNOS* is highly expressed in OA chondrocytes) given culture of cells is sufficient to induce hypermethylation and loss of expression in some genes [24]. In addition, in the current study,

298 the loss of methylation following treatment with 5-aza-dC at the CpG sites of the −5.8 kb NF-
299 kB enhancer elements was higher in OA cells.

300 Aged articular chondrocytes from normal individuals do not undergo cell division; in
301 contrast, in OA cartilage, the chondrocytes that survive form clones typical of severe OA
302 [26], consistent with cell proliferation [27]. Indeed, cartilage neighbouring the damaged
303 tissue location appear to contain a population of proliferative chondrocytes, which may be
304 involved in an attempt to repair the damage tissue [3]. In support of this possible approach,
305 recent investigations applying different treatments to promote chondrocyte proliferation
306 suggest the potential to delay or enhance OA progression [28, 29]. The current study
307 indicates a proliferative role of aberrant chondrocytes in the aetiology of OA. ~~Thus, transient~~
308 ~~transfection of the −5.8 kb NF-kB enhancer element in the chondrocytic cell line C28/I2~~
309 ~~induced higher proliferation in contrast to the methylated enhancer element.~~

310 Cell replication and the process of cell cycling are central in complex multicellular
311 processes, such as injury repair or mounting an immune response [30]. Microarray
312 experiments with synchronized cells indicate that the majority of IL-1-triggered genes, like
313 *iNOS*, are preferentially up-regulated in cells released from the G1 phase [31]. The current
314 studies indicate chondrocyte progression from G1 phase to S and G2/M phases in cells
315 transfected with the unmethylated −5.8 kb NF-kB enhancer correlated with higher *iNOS*
316 expression levels.

317 While the regulation of cell-cycle genes in endochondral bone growth has been
318 extensively studied [32, 33], to date, there is little is known regarding the role of cell-cycle
319 genes in adult human articular cartilage. Cell-cycle genes are targeted by a number of
320 mitogenic and anti-mitogenic pathways in chondrocytes [32]. Cyclin D1 (*CCDN1*) is a
321 positive regulator of cell G1/S transition and is a key restriction point in the cell cycle
322 binding to CDK4 and CDK6 to control cell cycle progression from G1 to S phase [34]. The

CDKN2A gene (p16) belongs to the Ink4 family of proteins that act to inhibit CDK4 and CDK6 upon binding, resulting in the arrest of the cell cycle in G1 or apoptosis [35]. Handschick and collaborators have identified CDK6 as a molecular link between the inflammatory microenvironment and permanent cell cycle progression. Thus, in the G1 phase, D1 cyclins activate cell cycle progression through phosphorylation of negative-growth regulators [31]. The current study demonstrates an increase in the proliferation rate of OA chondrocytes together with increased gene expression of cyclin D1 (*CCDN1*), *CDK6*, and decreased expression of p16 (*CDKN2A*). Recently, it has been published that p21, a cell cycle-related protein, may function as a regulator of transcriptional factors other than the inhibitor of cell cycle progression in the cartilage tissue [36]. Another study showed that interleukin (IL)-1β increased proliferation and caused a G_1-to-S phase shift in chondrocytes, accompanied by a reduction of *p21*, and reduction of *p21* caused delayed cell differentiation [37].

Interestingly, Wang and colleagues have published a model in which chondrocyte proliferation is promoted through *iNOS*-NO-mediated induction of cyclin D1 expression. Furthermore, reduced chondrocyte proliferation was observed in *iNOS*-deficient mice [38]. Jhou et al reported inhibition of the cell-cycle progression is capable of reducing pro-inflammatory responses *via* down-regulation of NF-kB [18], whilst increased NF-kB activity during growth of mitogen-stimulated and transformed cells has been linked to cell cycle progression through transcriptional activation of the cyclin D1 gene, leading to increased abundance of cyclin D1 and increased activity of cyclin D1 kinase [39].

The current observations that loss of methylation in the crucial enhancer element and subsequent induction of *iNOS* expression is complimentary to reports from Van der Kraan and van der Berg postulating OA has an age and senescence dependent component as a consequence of changes in gene methylation [3]. Furthermore, Yan and colleagues have

shown in mice that a deficiency of the neuronal (nNOS) and endothelial (eNOS) members of the nitric oxide synthase family results in reduced chondrocyte proliferation and earlier cell cycle exit [40, 41], presenting an area of further future investigation in human articular chondrocytes.

Epigenetics changes have been shown to be key intermediates in signalling pathways that control proliferation. Thus chromatin compaction levels are directly correlated with DNA methylation levels resulting in altered gene expression and proliferation [42]. In support of this observation, methylation of H4K20 has been implicated in cell cycle progression, chromosome condensation and transcriptional regulation [30] with . There is now emerging evidence of the cross-talk between histone phosphorylation and acetylation at distinct amino acids with H3S28phK27ac and H3S10phK14ac shown to be responsible for epigenetic regulation of transcription and cell cycle regulation [43].

The results of the current study of NF-κB-*iNOS*, cell cycle and DNA methylation signalling in OA chondrocytes demonstrate that the loss of methylation that happens at specific CpG sites localised at the −5.8 kb NF-κB of the *iNOS* gene in OA chondrocytes facilitate the binding of NF-κB and subsequent activation of *iNOS*. We suggest hypothesise this has important implications in the pro-inflammatory response in OA, as well as cell cycle regulation andof OA chondrocytes culminating in the propagation of the aberrant OA phenotype to daughter cells (Figure 67). In conclusion, this study suggests that inhibition of cell cycle progression by *iNOS* enhancer hypermethylation is capable of reducing pro-inflammatory responses via down-regulation of NF-κB with important therapeutic implications in OA.

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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. In detail: MCDA— conception, design, acquisition of data, analysis and interpretation of data, drafting the manuscript; AT—acquisition of data, analysis and interpretation of data, critical revision of the manuscript; ROCO—conception, design, analysis and interpretation of data, critical revision of the manuscript.

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Competing interests

The authors have no conflict of interest to declare.

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Prof. Stefan Lohmander
Editor-in-Chief; Osteoarthritis and Cartilage

Dear Professor Lohmander,

Please find enclosed our manuscript for consideration for publication in Osteoarthritis & Cartilage.

Manuscript: **Demethylation of an NF-κB enhancer element orchestrates *iNOS* induction in osteoarthritis via cell cycle regulation**

Authors: María C. de Andrés, Atsushi Takahashi and Richard O.C. Oreffo

Osteoarthritis (OA), the most common form of arthritis world-wide, is characterized by progressive failure of the extracellular cartilage matrix, along with changes in the synovium and subchondral bone. In normal adult cartilage, the articular chondrocytes are quiescent cells demonstrating negligible turnover of the cartilage matrix. In contrast, in OA, the chondrocytes become "activated", characterized by cell proliferation, cluster formation, and the increased production of matrix proteins and matrix-degrading enzymes. We have recently described that demethylation of the NF-κB enhancer region at −5.8 kb of the *iNOS* gene leads to increased transcription through elevated binding of this transcription factor.

Here we show that the loss of methylation that occurs at specific CpG sites localised at the −5.8 kb NF-κB enhancer region of the *iNOS* gene in OA chondrocytes permits the binding of this transcription factor activating the expression of *iNOS*. This results in subsequent altered cell cycle regulation, altered proliferative phenotype and transmission of the pathogenic phenotype to daughter cells. This study indicates that inhibition of cell cycle progression by *iNOS* enhancer hypermethylation is capable of reducing pro-inflammatory responses via down-regulation of NF-κB with important therapeutic implications in OA.

We anticipate that the study will be of considerable interest to the readership of Osteoarthritis & Cartilage including epigeneticists and musculoskeletal scientists.

We can confirm this manuscript has neither been published nor is currently under consideration for publication either in whole or in part, by any other journal. Submission has been approved by each co-author and the authors have not received anything of value from a commercial or other party related directly or indirectly to the work. The authors have not previously discussed this manuscript with an Osteoarthritis & Cartilage one editor. Laboratory costs were funded by grants awarded from Leverhulme Trust and Biotechnology and Biological Sciences Research Council, UK.

I look forward to hearing from Osteoarthritis & Cartilage.

Yours sincerely,

Richard OC Oreffo DPhil CBiol FSB

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AUTHORS' DISCLOSURE

Manuscript title **Demethylation of an NF-κB enhancer element orchestrates iNOS induction in osteoarthritis via cell cycle regulation**

Corresponding author **Richard O.C. Oreffo**
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Authorship
All authors should have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted. By signing below each author also verifies that he (she) confirms that neither this manuscript, nor one with substantially similar content, has been submitted, accepted or published elsewhere (except as an abstract).

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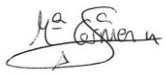	18/01/2016		
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