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Superoxide dismutase downregulation in osteoarthritis progression and end-stage disease

Jenny L Scott,[1,2] Christos Gabrielides,[1] Rose K Davidson,[3] Tracey E Swingler,[3] Ian M Clark,[3] Gillian A Wallis,[4] Raymond P Boot-Handford,[4] Tom B L Kirkwood,[5] Robert W Talyor,[5] David A Young[1]

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[1]Musculoskeletal Research Group, Institute of Cellular Medicine, Newcastle University, Newcastle-upon-Tyne, UK
[2]Autoimmunity and Inflammation Program, Hospital for Special Surgery, New York, USA
[3]School of Biological Sciences, University of East Anglia, Norwich, UK
[4]Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, Manchester, UK
[5]Institute for Ageing and Health, Newcastle University, Newcastle-upon-Tyne, UK

Correspondence to
Dr David Young, Musculoskeletal Research Group, Institute of Cellular Medicine, Newcastle University, Newcastle-upon-Tyne, UK;
d.a.young@ncl.ac.uk

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ABSTRACT

Background Oxidative stress is proposed as an important factor in osteoarthritis (OA).
Objective To investigate the expression of the three superoxide dismutase (SOD) antioxidant enzymes in OA.
Methods SOD expression was determined by real-time PCR and immunohistochemistry using human femoral head cartilage. SOD2 expression in Dunkin–Hartley guinea pig knee articular cartilage was determined by immunohistochemistry. The DNA methylation status of the SOD2 promoter was determined using bisulphite sequencing. RNA interference was used to determine the consequence of SOD2 depletion on the levels of reactive oxygen species (ROS) using MitoSOX and collagenases, matrix metalloproteinase 1 (MMP-1) and MMP-13, gene expression.
Results All three SOD were abundantly expressed in human cartilage but were markedly downregulated in end-stage OA cartilage, especially SOD2. In the Dunkin–Hartley guinea pig spontaneous OA model, SOD2 expression was decreased in the medial tibial condyle cartilage before, and after, the development of OA-like lesions. The SOD2 promoter had significant DNA methylation alterations in OA cartilage. Depletion of SOD2 in chondrocytes increased ROS but decreased collagenase expression.
Conclusion This is the first comprehensive expression profile of all SOD genes in cartilage and, importantly, using an animal model, it has been shown that a reduction in SOD2 is associated with the earliest stages of OA. A decrease in SOD2 was found to be associated with an increase in ROS but a reduction of collagenase gene expression, demonstrating the complexities of ROS function.

INTRODUCTION

Osteoarthritis (OA) is a common degenerative joint disease characterised by focal degradation of articular cartilage, centred on loadbearing areas. The primary risk factor for OA development is age, but the mechanisms by which ageing contributes to OA susceptibility and progression remain poorly understood. Articular cartilage extracellular matrix (ECM), which is maintained by chondrocytes, undergoes significant changes in structure and composition with age, closely linked to a decline in chondrocyte function.[1 2] Many studies have identified molecular characteristics of ageing in OA cartilage or chondrocytes which may contribute to the onset of OA, including telomere genomic instability, formation of advanced glycation end products, increased apoptosis and senescence.[3] Many such changes could be associated with the elevated levels of oxidative stress that occur in OA and aged cartilage,[4] leading to an altered chondrocyte phenotype that renders cells unable to respond effectively to normal loading regimens[5] and potentially contributes to disease onset.

Although generally considered a hypoxic environment, oxygen diffuses through articular cartilage and is used by chondrocytes to derive approximately 25% of their ATP. Oxidative phosphorylation is a major source of reactive oxygen species (ROS); however, chondrocytes also express NADPH oxidase and nitric oxide (NO) synthase family members together with various oxygenases which principally generate the ROS NO and the superoxide anion ($O_2^{\bullet-}$).[4] These ROS generate derivatives including hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and hydroxyl radicals ($OH^\bullet$). ROS are highly reactive and therefore transient so their production in cartilage has been determined indirectly; an accumulation of lipid peroxidation products[6] and nitrotyrosine residues[7] have been observed in aged and OA cartilage. ROS can cause cartilage degradation directly by cleaving collagen and aggrecan and activating matrix metalloproteinases (MMP),[8–10] a family of enzymes that have a key role in cartilage destruction in OA.[11] ROS also act indirectly by modulating redox-sensitive signalling pathways that control MMP expression, and in cultured chondrocytes may lead to decreased collagen and aggrecan synthesis.[12 13]

To prevent an accumulation of ROS-mediated damage chondrocytes produce a number of antioxidant enzymes, including the superoxide dismutases (SOD), catalase and glutathione peroxidase.[4] There are three SOD family members; SOD1 (Cu/Zn-SOD) found principally in the cytosol, SOD2 (Mn-SOD) found in the mitochondrial matrix and SOD3 (EC-SOD) found in the ECM. The SOD family catalyses the dismutation of $O_2^{\bullet-}$ to O_2 and H_2O_2, thereby limiting the formation of highly aggressive compounds such as $ONOO^-$ and $OH^\bullet$.

SOD2 has been shown to be downregulated in OA cartilage.[14 15] SOD3 is also decreased in human OA cartilage and in a mouse model of OA.[16] SOD3-deficient mice show an increased severity of collagen-induced arthritis.[17] Together, these and other data have led to the proposal that supplementary antioxidants may represent a potential treatment for OA.[18]

We considered that the SOD family may have a key role in controlling ROS levels in cartilage and that any deficiency may account for the elevated oxidative stress observed. Here, we report a significant decrease in the expression of all SOD family members in OA cartilage compared with macroscopically normal cartilage (from patients with a neck of femur (NOF) fracture) at both the RNA and protein levels. We also show that the decline in SOD2 expression correlates with the onset of, and importantly precedes, OA-like lesions in an animal model. We also demonstrate that the SOD2 promoter is significantly differentially methylated in OA compared with NOF cartilage, representing a potential mechanism for the reduction in expression seen in OA. Functionally, SOD2 depletion in chondrocytes resulted in a significant increase in ROS but a decrease in the interleukin (IL)1-induced levels of MMP-1 and MMP-13. Together, our data suggest that a decrease in SOD2 expression may be presymptomatic of disease and potentially an attempt to reduce the expression of collagenases, enzymes which contribute to cartilage degradation, but concomitantly also contributes to increased oxidative stress.

MATERIALS AND METHODS

Materials

Antibodies against SOD1 and SOD2 were from Stressgen Bioscience (Cambridge, UK) and SOD3 from Abcam (Cambridge, UK). Rabbit immunoglobulin G (IgG) isotype control antibody, oligonucleotides (table 1), PCR Mastermix and 5-aza-2′-deoxycytidine (AZA) were from Sigma-Aldrich (Poole, UK). Anti-rabbit Vectastain ABC Elite kit and Vectashield were from Vector Laboratories (Peterborough, UK). Recombinant human IL1α was a gift from GlaxoSmithKline (Stevenage, UK). Taqman Low Density Arrays (TLDA) were from Applied Biosystems (ABI, Foster City, California, USA). Sybr-Green Mastermix was from Invitrogen (Paisley, UK). SmartPool small interfering RNA (siRNA) against SOD2 (table 1) and siControl2 were from Dharmacon (Cramlington, UK).

RNA extraction from cartilage samples, chondrocyte isolation and cell culture

Articular cartilage was obtained from patients undergoing joint replacement surgery. Hip cartilage samples from patients with OA were compared with lesion-free cartilage from patients with fracture to the NOF with no known history of joint disease. Femoral heads were processed and RNA extracted using established methodology.[19] Human articular chondrocytes (HACs) were isolated from OA cartilage and cultured as previously described.[20] This study was performed with ethical committee approval from Norfolk and Norwich University Hospital Trust, Oxford Radcliffe Hospitals NHS Trust and Newcastle and North Tyneside Health Authority and all patients provided informed consent.

Real-time RT-PCR and siRNA-mediated gene silencing

RNA interference (RNAi) in HACs was as previously described[19] with the siRNA used at 100 nmol/l. Briefly, $2.5 \times 10^4/cm^2$ (HAC) cells were seeded overnight and the following day transfected with Dharmafect 1 (Dharmacon) and siRNA (SOD2 (table 1) or non-targeting control, siControl2). Cells were washed 24 h after transfection with phosphate-buffered saline (PBS) and cultured in serum-free medium for a further 24 h before 24 h stimulation±0.05 ng/ml IL1, or as described. Total RNA was isolated using the Cells-to-cDNA II Kit (ABI) and MMP-1 and MMP-13 expression determined by real-time RT-PCR, as previously described.[19] Gene depletion was confirmed by real-time RT-PCR and immunoblotting of total cell lysates prepared as described.[19] SOD family gene expression in cartilage cDNA was assessed using a TLDA according to the manufacturer's (ABI) instructions and as previously described.[19] Throughout, mRNA levels were normalised to a housekeeping gene (ABI) using the calculation $2^{-\Delta Ct}$.

Immunohistochemistry

Human patient samples

Serial sections (12 μm) were prepared and processed from full-depth cartilage taken from intact cartilage of OA and NOF femoral heads, as previously described.[21] Serial sections were incubated for 60 min at room temperature (RT) with either a polyclonal primary (rabbit) antibody to a SOD or a normal rabbit IgG isotype-matched control. All antibodies were used at a concentration of 1 μg/ml except for SOD3 which was used at a 1:2000 dilution. All antibodies were prepared in PBS containing 1.5% (v/v) normal goat serum.

Dunkin Hartley guinea pig knee samples

Male Dunkin–Hartley guinea pigs (Harlan Olac, UK) aged from 1 to 8 months (five per group) were used for histology. Animals were humanely killed according to UK Home Office regulations. Tibial heads were fixed in 4% (v/v) formaldehyde for 2 days and decalcified in 0.1 mol/l EDTA (pH 7.0) in PBS for 3 weeks. Blocks that included articular cartilage and bone (lateral and medial tibial plateaus) were dehydrated and embedded in paraffin. Serial, coronal sections (5 μm) were deparaffinised with xylene and rehydrated through a graded ethanol series and, finally, incubated in 10 mmol/l sodium citrate buffer, pH 6.0, for 2 h at RT, followed by 3% (v/v) H_2O_2 in Tris-buffered saline (TBS) for 15 min. Serial sections were blocked with 1.5% (v/v) normal goat serum in TBS for 30 min and then incubated

Table 1 Oligonucleotides: real-time RT-PCR, bisulphite PCR primers and superoxide dismutase 2 (SOD2) siGENOME SMART pool siRNA sequences

Gene	Method	Primer	Sequence
SOD2	Sybr Green RT-PCR	Forward	5′-CTGGACAAACCTCAGCCCTA
		Reverse	5′-TGATGGCTTCCAGCAACTC
SOD2	DNA methylation analysis	Forward	5′-GTAATTAAAATTTAGGGGTAGG
		Reverse	5′-AAAAAAAACTACAAACTAACCTC
SMARTpool Duplex	Target strand		Sequence
1	Sense		5′-GGACAAACCUCAGCCCUAAUU
	Antisense		5′-PUUAGGGCUGAGGUUUGUCCUU
2	Sense		5′-GGAGCACGCUUACUACCUUUU
	Antisense		5′-PAAGGUAGUAAGCGUGCUCCUU
3	Sense		5′-AAAGAUACAUGGCUUGCAAUU
	Antisense		5′-PUUGCAAGCCAUGUAUCUUUUU
4	Sense		5′-GUAAUCAACUGGGAGAAUGUU
	Antisense		5′-PCAUUCUCCCAGUUGAUUACUU

for 90 min at RT with an antibody to SOD2 or normal rabbit IgG (1 μg/ml in TBS/1.5% (v/v) normal goat serum). The remainder of the staining procedure and mounting was performed as previously described.[21] Representative sections from each animal were stained with safranin 'O'/fast green (0.02% (w/v) fast green for 3 min, 1% (v/v) acetic acid for 30 s, 0.1% (w/v) safranin 'O' for 5 min).

MitoSOX Red staining

HACs were transfected with siRNA against SOD2 or a control (see above) and 24 h later serum starved overnight. Cells were then stained with 2 μmol/l MitoSOX Red (Invitrogen) in serum-free media for 15 min at 37°C, washed twice in PBS for 5 min and fixed in 4% (w/v) cold paraformaldehyde in PBS for 10 min at RT or, for SOD2 staining, permeabilised and blocked with 0.5% (v/v) Saponin/PBG (0.5% (w/v) BSA solution/0.2% (v/v) fish skin gelatin) in PBS for 10 min at RT, incubated with 1 μg/ml rabbit anti-human SOD2 antibody in 0.5% Saponin/PBG for 45 min at RT, washed twice with 0.5% Saponin/PBG and then incubated with secondary antibody (goat anti-rabbit Alexa 488) (1:750 in 0.5% Saponin/PBG) for 45 min at RT, before fixing as above. All slides were washed twice with PBS and mounted in Vectashield and visualised using confocal microscopy.

Image analysis

Fluorescent and immunohistochemical images were analysed using ImageJ analysis software (Wayne Rasband, NIH, USA). For fluorescent images, individual cells from random fields were selected and the average red (MitoSOX Red) and/or green (SOD2) pixels present in the cell (per area) calculated after average background subtraction. Immunological staining from cartilage sections was quantified for each section area for SOD3 or on a per cell basis for SOD1 and SOD2.

DNA methylation analysis

HACs were cultured±10 μmol/l AZA for 35 days before RNA isolation, with fresh AZA added daily. SOD2 expression was determined by real-time RT-PCR. Genomic DNA (gDNA) was purified from cartilage samples during RNA extraction[22] by ethanol precipitation of the first wash of Qiagen RNeasy columns (Qiagen, Crawley, UK). gDNA (200–300 ng) was treated with bisulphite using the Epitect Bisulfite kit (Qiagen). PCR was performed to amplify a SOD2 promoter region from 10–15 ng bisulphite-treated gDNA using Titanium Taq (TaKaRa Biomedicals, Wokingham, UK). PCR conditions were 95°C 1 min, (95°C 15 s, 64.2°C 30 s, 68°C 1 min) × 40 cycles, 68°C for 7 min. PCR products were cloned and DNA sequenced until 10 unique clones from each patient sample were obtained. DNA methylation analysis was performed using the BiQ analyzer software.[23]

Statistical analysis

Differences between NOF and OA groups and cultured cell treatments were defined using a two-tailed Mann–Whitney U test. Fisher's exact test was used for statistical analysis of DNA methylation. Student t-tests were performed when quantifying the level of immunological or MitoSOX staining.

RESULTS

The SOD gene family are highly but differentially expressed between NOF and OA human cartilage

A screen for SOD expression found that all three genes have significantly reduced expression in OA compared with NOF cartilage. The median expression levels of SOD1, SOD2 and SOD3 were reduced 3.4- (p=0.0014), 41.5- (p≤0.0001) and 3.9- (p=0.0009) fold, respectively, in OA cartilage. This reduction in SOD2 and SOD3 expression compares with previous reports.[14 16] The quantitative nature of this screen disclosed the very high abundance of all the SOD family, particularly SOD2, with the relative abundance of each in NOF samples being: SOD2>>SOD3>actin B (ACTB)>SOD1 (figure 1).

To validate our RNA expression data we performed immunohistochemistry using OA and NOF cartilage (figure 2A). As expected SOD1 expression was cellular with staining obvious throughout the sections (ie, from articular surface to subchondral bone), though staining was more intense at the articular surface. Staining within the full-depth cartilage was significantly reduced in OA sections (figure 2B). SOD2 staining was cellular and strongest near the articular surface. There was a clear, significant, decrease in the expression of SOD2 in the surface zones of the diseased tissue though the protein was still detected. A SOD3 antibody gave the expected diffuse staining pattern representative of ECM staining. This was visible throughout the tissue, though staining was more intense at the articular surface and again significantly reduced in OA compared with NOF cartilage.

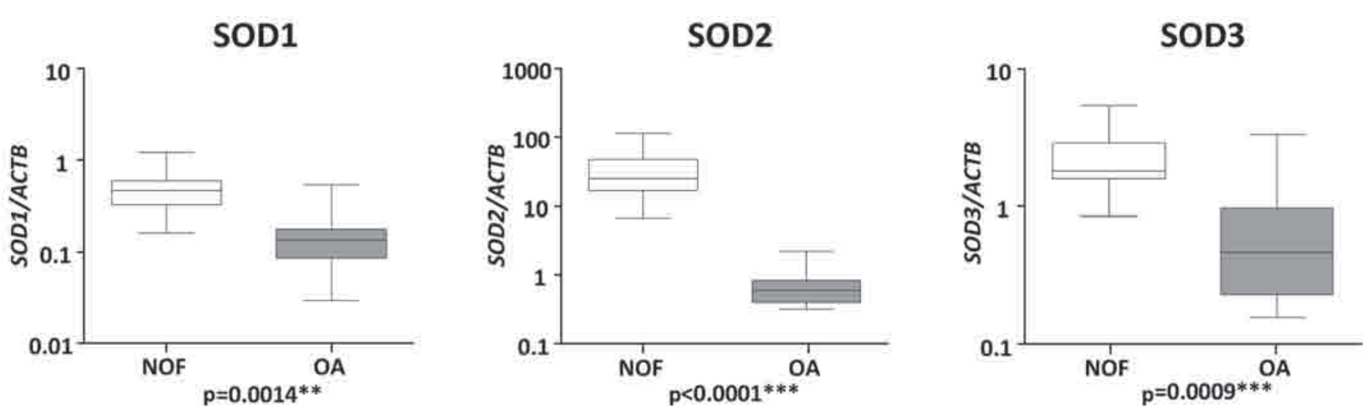

Figure 1 Comparison of superoxide dismutase (SOD) gene expression between cartilage from neck of femur (NOF) and patients with osteoarthritis (OA). SOD family gene expression was determined by real-time reverse transcriptase PCR from cartilage RNA isolated from 12 NOF (open) and 12 patients with OA (shaded). Gene expression data are presented as a ratio of SOD gene levels to that of the housekeeping gene, actin B (ACTB), using the calculation $2^{-\Delta Ct}$. Lines within the boxes represent the median, the boxes represent the 25th and 75th centiles, and the lines outside the boxes correspond to the minimum and maximum values. The p values were calculated using the Mann–Whitney U test: **p<0.01; ***p<0.001.

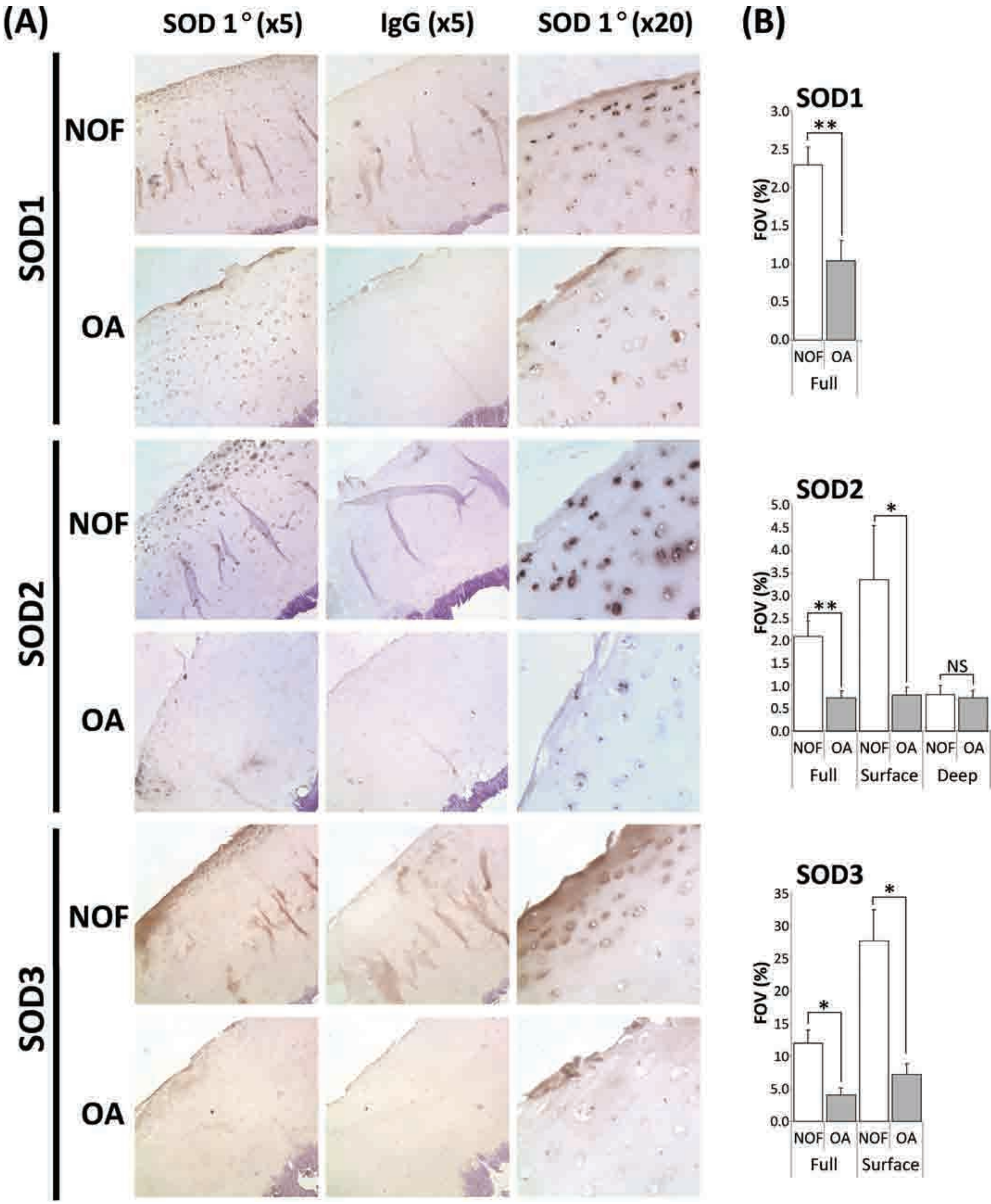

Figure 2 Immunohistochemical analysis of superoxide dismutase (SOD) expression in representative specimens of human articular cartilage from neck of femur (NOF) and patients with osteoarthritis (OA). (A) Sections were subjected to immunohistochemistry using the labelled anti-SOD or normal rabbit IgG (negative control) and counterstained with haematoxylin. Full-depth sections are shown at ×5 magnification and for clarity the articular surface is shown at ×20. Images are representative of sections from five NOF and five patients with OA. (B) Immunohistochemical images, of fixed exposure, from five NOF and five patients with OA were quantified using the ImageJ software. For each image the percentage staining in each field of view (FOV) was calculated and the mean with standard error of the mean (SEM) NOF and OA FOV presented. Percentage FOV was calculated for full-depth sections or after dividing the section into two quadrants 'deep' and 'surface'. p Values were calculated using the Student t test,: *$p<0.05$; **$p<0.01$.

SOD2 expression declines before the appearance of OA erosions

Since the changes in SOD2 expression were most significant, we next ascertained its expression during the onset of OA using joint sections from Dunkin–Hartley guinea pigs of different ages; an established model of spontaneous OA.[24] In this model the medial tibial plateau develops OA-like lesions as the animal ages, while the lateral tibial plateau develops significantly fewer lesions as highlighted by cellular and surface changes together with a decrease in safranin 'O' staining, representative of proteoglycan loss (figure 3A). In young animals (1 month) SOD2 staining was strongest near the articular surface of both medial and lateral plateaus (figure 3A), as observed in human NOF cartilage (figure 2). At 4 months of age OA-like lesions could be seen on the medial but not lateral plateau. Concomitantly SOD2 expression was significantly reduced in the diseased medial, but not the normal lateral plateau (figure 3A,B). Interestingly, SOD2 expression within the medial plateau significantly declined before observation of lesions (ie, in 2-month-old animals), suggesting that this change in expression may be symptomatic of early changes in OA (figure 3A,B).

The SOD2 promoter is differentially methylated between NOF and OA cartilage

As SOD2 promoter hypermethylation correlates with decreased gene expression (eg, Hurt *et al*[25]), we investigated the part that DNA promoter methylation may play in the SOD2 downregulation seen in OA chondrocytes. First, we found that SOD2 expression increased twofold (p≤0.001) in OA HAC treated with

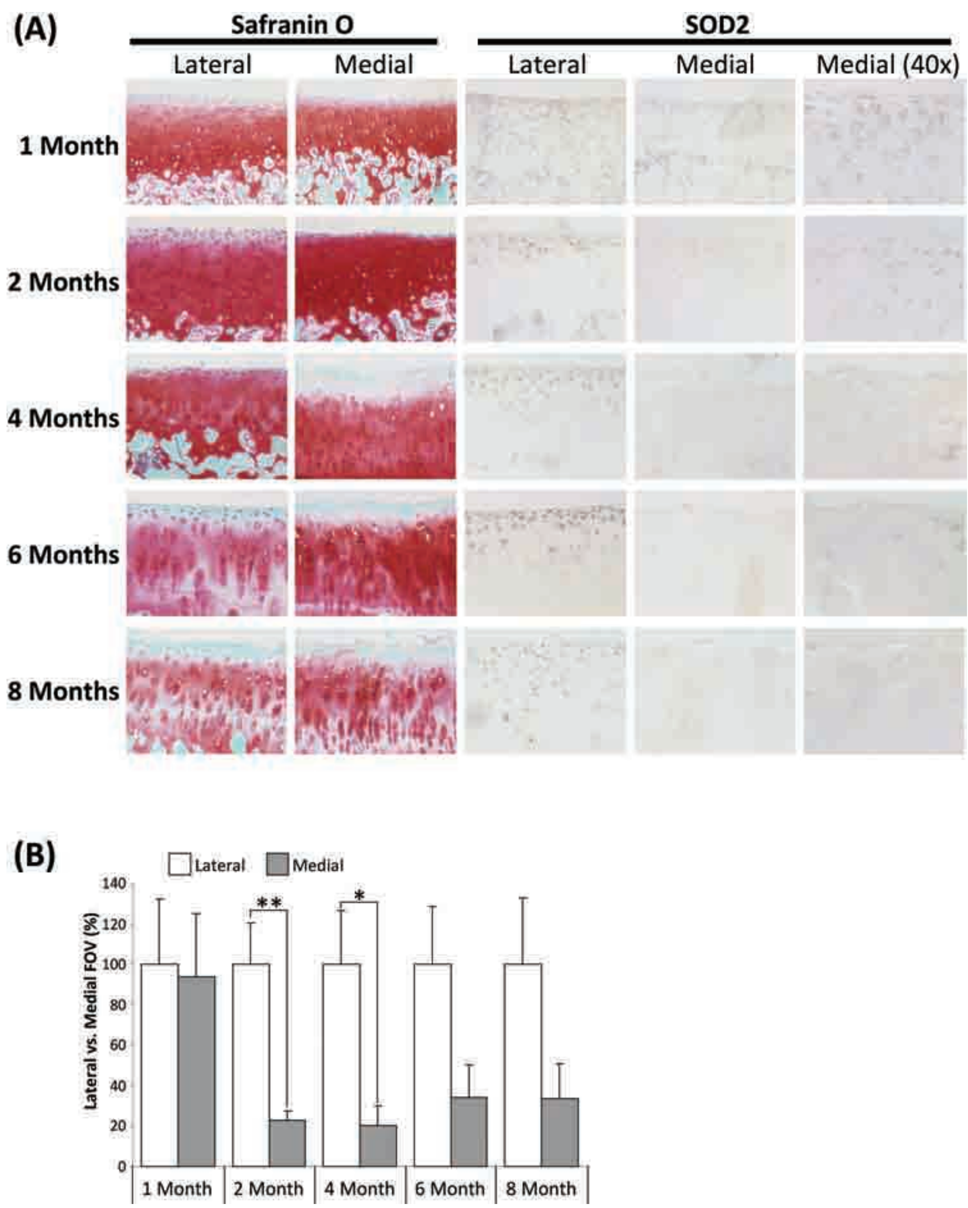

Figure 3 Immunohistochemical analysis of superoxide dismutase 2 (SOD2) expression in representative specimens of Dunkin–Hartley guinea pig articular cartilage. (A) Cartilage sections are representative of five animals at each time point (1–8 months of age). Sections were either stained with safranin 'O' as a marker of proteoglycan or subjected to immunohistochemistry using the labelled anti-SOD2 or normal rabbit IgG (negative control—data not shown). Lateral and medial tibial plateaus are as indicated. Images shown are at ×10 magnification, unless indicated otherwise. (B) Immunohistochemical images of lateral and medial tibial plateaus, of fixed exposure, from five animals were quantified using the ImageJ software. For each image the percentage staining in each field of view (FOV) was calculated. For each age group the staining in the lateral plateau is arbitrarily 100% + SEM. p Values were calculated using the Student t test,: *p<0.05; **p<0.01.

the DNA demethylating agent, AZA (figure 4A), indicating a role for DNA methylation in SOD2 regulation. Next, we analysed a region of the SOD2 promoter upstream of the transcription start site at −297 to −87 (online supplementary figure 1) known to be subject to differential methylation.[25] Bisulphite sequencing of, on average, 9.6 clones from each of 12 OA and NOF patient cartilage samples identified three CpG sites, CpG6, 10 and 14, corresponding to nucleotides −222, −183 and −154, respectively, with significant differences in methylation status between NOF and OA cartilage (figure 4B). CpG14 and CpG10 showed significantly ($p \leq 0.001$ and $p \leq 0.05$, respectively) lower methylation in OA cartilage, whereas CpG6 was more methylated ($p \leq 0.001$) in OA cartilage. CpG14 is encompassed within a putative AP-2 binding site while CpG10 is located at a single

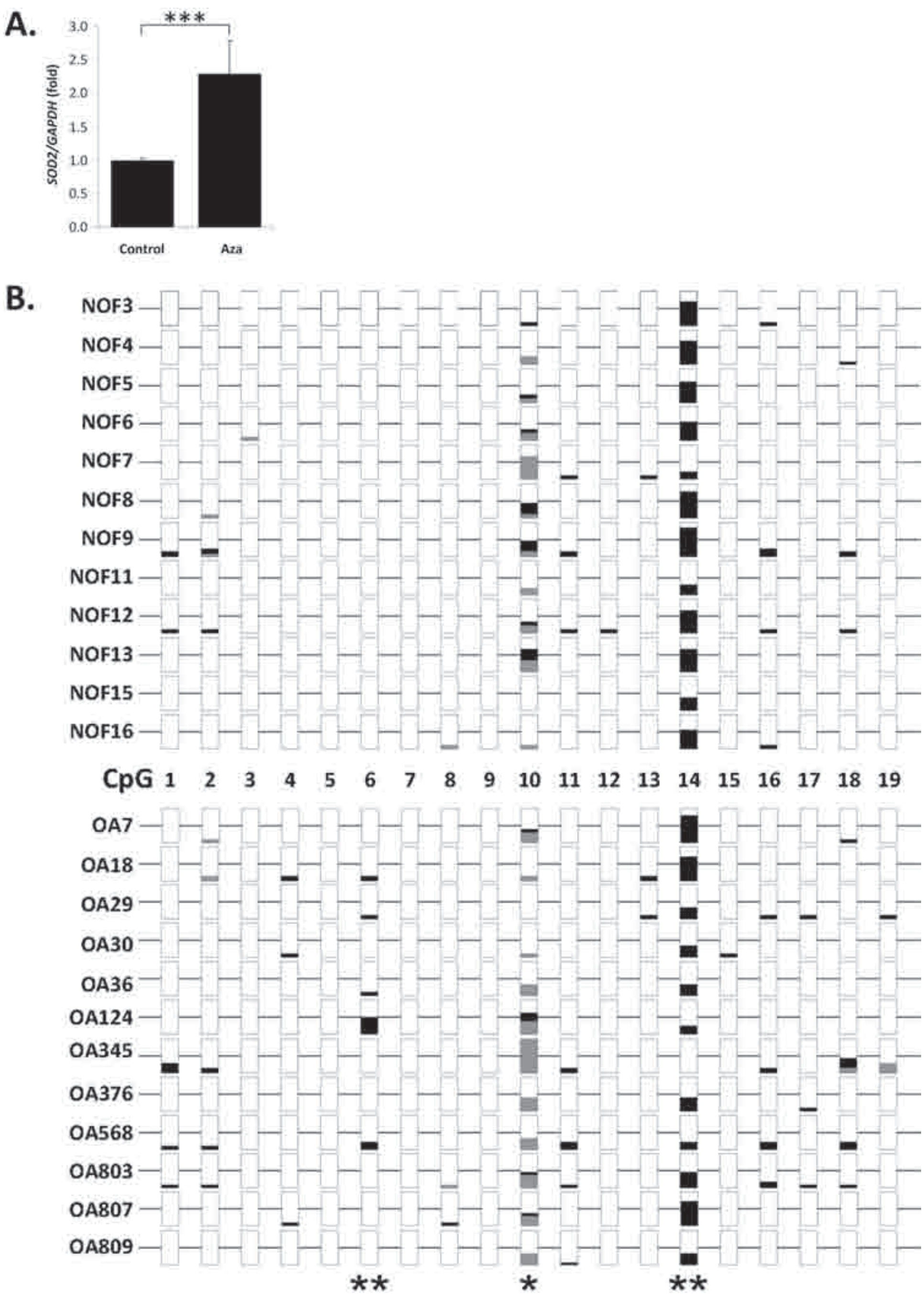

Figure 4 Analysis of the regulation of superoxide dismutase 2 (SOD2) in human articular chondrocytes (HAC) by DNA methylation. (A) Primary HAC were treated with the DNA demethylating agent 5-aza-2′deoxycytidine (AZA) for 35 days (two passages) as described and SOD2 expression measured by real-time RT-PCR and normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression levels. Data are presented fold over control and error bars represent SEM. Data are combined from four HAC populations treated in duplicate. Statistical significance was calculated using the Mann–Whitney U test: ***$p<0.001$. (B) DNA methylation analysis of the SOD2 promoter in cartilage genomic DNA from neck of femur (NOF) and patients with osteoarthritis (OA). Genomic DNA from cartilage of 12 NOF and 12 patients with OA was analysed to determine the methylation status of the SOD2 promoter region spanning 19 potential CpG dinucleotides. An average of 9.6 non-clonal DNA sequences per patient sample were analysed and the methylation percentage for each is plotted. Methylation CpG sequences are represented by black bars, open are non-methylated and the bar is grey when the CpG sequence was not detected, generally owing to a single nucleotide polymorphism at that locus (eg, CpG10). Fisher's exact test was used for statistical analysis: *$p<0.05$; **$p<0.01$.

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nucleotide polymorphism (NCBI reference rs2758343). Overall, OA cartilage had, as hypothesised, a significant increase ($p\leq0.05$) in methylated CpG.

SOD2 depletion modulates MMP-1 and MMP-13 expression in HAC

A reduction in SOD levels is likely to lead to an increase in ROS. Since SOD2 is localised to the mitochondria, the major source of ROS, we used the mitochondria-targeting MitoSOX Red, which is oxidised by $O_2^{\bullet-}$, but not other ROS or reactive nitrogen species, to confirm that SOD2 depletion increased $O_2^{\bullet-}$ levels in chondrocytes (figure 5A, B). A further consequence of a reduction in SOD2 is a decrease in H_2O_2 which acts as an intracellular signalling moiety.[26] Hence, we tested whether a reduction in SOD2 in chondrocytes would effect basal and proinflammatory cytokine (IL1)-induced collagenase (MMP-1 and MMP-13) expression. RNAi depletion of SOD2 in chondrocytes (figure 5C) resulted in a significant reduction of basal MMP-1 but not MMP-13 expression (figure 5D). OA has an inflammatory component[27] and IL1, a potent inducer of SOD2 and the collagenases, can be detected within the OA joint. In line with this, IL1 stimulation led to a robust increase in SOD2 and collagenase expression. However, depletion of SOD2 significantly decreased the IL1-induced expression of both collagenases (figure 5E).

DISCUSSION

Here, we found that all of the SOD family of antioxidant enzymes are highly expressed in NOF human cartilage in relation to an established housekeeping gene, which suggests that cartilage must generate significant levels of ROS and that ROS scavenging is necessary for tissue maintenance and homoeostasis.[28] Our findings show that expression of all SOD enzymes is significantly reduced in diseased cartilage, confirming and expanding on previous data for SOD2 and SOD3.[14–16] Regan *et al*, found that SOD3 was reduced in OA compared with normal (again, hip fracture) human tissue and in the cartilage of the STR/ort mouse spontaneous OA model, interestingly also before histological evidence of disease.[16] SOD3 levels in synovial fluid are also reduced in patients with OA compared with patients with injured/painful joints but no cartilage damage.[29] We have now shown that SOD2 expression is significantly downregulated in OA chondrocytes in vivo, and that this precedes the development of OA lesions in the Hartley guinea pig, raising the possibility that alterations in SOD2 expression are associated with the earliest stages of OA pathogenesis.

SOD2 expression is subject to epigenetic control via promoter DNA methylation in a number of carcinoma cell lines.[25] Furthermore, aberrant DNA methylation occurs with disease and ageing.[30] In chondrocytes, SOD2 expression increased after inhibition of DNA methylation (figure 4A) and analysis of the SOD2 promoter identified a significant overall increase in the number of CpG sites partially methylated in OA compared with NOF cartilage (figure 4B). Promoter DNA methylation is generally repressive to transcription, thus even the modest increase in CpG methylation seen here may significantly impact on the SOD2 expression level. Reinstigation of SOD2 expression using DNA methyltransferase inhibitors is one way in which they may benefit patients with OA, as proposed.[31]

SOD are required for the conversion of reactive $O_2^{\bullet-}$ to O_2 and H_2O_2 and thus a downregulation of SOD would increase oxidative stress, which is implicated in a number of disease, including arthritis.[32] Thus, research has investigated the consequences of modulating SOD levels in both cells and animal models. SOD1 and SOD3 knockout mice display relatively mild phenotypes[33] but exhibit an increase in oxidative stress markers.[34] SOD2 deletion in mice results in lethality by neonatal day 10.[35] SOD2 is localised to the mitochondria and dismutates $O_2^{\bullet}$ produced as a by-product of oxidative phosphorylation. It has been proposed that depletion of SOD2 leads to increased mitochondrial DNA mutation and subsequent dysfunction. Interestingly, mitochondrial DNA mutations increase in OA,[36] and mitochondrial DNA haplogroups themselves contribute to the pathogenesis of OA.[37]

The combined effect of a decrease in all three SOD enzymes could significantly contribute to the increased oxidative stress found in OA cartilage.[38] Using RNAi we confirmed that depletion of SOD2 from chondrocytes leads to an increase in $O_2^{\bullet-}$, which can result in telomere instability and a downregulation of chondrocyte function[38] as well as damage to cartilage macromolecules such as hyaluronan and collagen.[9 39] An increase in ROS could also potentially contribute to the onset of senescence, which may have a role in OA.[1] Together, these and other data suggest that SOD may have a protective role in the joint and cartilage. However, the results here demonstrate that downregulation of SOD2 is, in part, chondroprotective, reducing the activation of redox-sensitive signalling pathways and thus collagenase expression. Similarly, in fibroblasts, SOD2 overexpression increases MMP-1 expression via increased H_2O_2-dependent activation of ERK1/2[40] and AP-1,[41] while SOD2-deficient fibroblasts cannot induce MMP-1 and MMP-13 after cytokine stimulation.[40] It is therefore perhaps unsurprising that the depletion of SOD2 in HACs resulted in a decrease in basal MMP-1, an observation seen in end-stage OA,[22] as well as a decrease in production of both collagenases after IL1 stimulation (figure 5), presumably by reduced H_2O_2 levels. H_2O_2 levels are also controlled by catalase, whose expression we found to be significantly increased in OA cartilage (data not shown); indeed, increased catalase expression can reverse the H_2O_2-mediated induction of MMP-1 in fibroblasts.[40] Therefore one potential benefit of a downregulation in SOD2 would be to reduce the expression of matrix-degrading enzymes by modulation of redox-controlled signalling pathways. However, the role of ROS is complex and increased levels can actually activate latent MMP,[8] while more aggressive ROS such as $ONOO^-$, generated from $O_2^{\bullet-}$ and NO, can also modulate redox-sensitive signalling pathways.[42] As a result, elucidation of the overall consequences of SOD2 downregulation requires more detailed analysis of both the levels of $O_2^{\bullet-}$ present in NOF and OA chondrocytes and the activity of the associated redox-sensitive signalling pathways.

Several studies have used antioxidants as treatments for arthritis (reviewed by Afonso *et al*[18]). Ex vivo SOD3 gene transfer or SOD mimetics (eg, M40403) can reduce the severity of collagen-induced arthritis in models,[43 44] and recombinant SOD1 can inhibit cartilage damage in hens. A trial of intra-articular injections of bovine SOD1 (Orgotein) has also been carried out in patients with OA with some success; however, the drug was withdrawn owing to adverse side effects (reviewed by Afonso *et al*[18]). As dietary supplements, vitamins have been shown to decrease OA development and increase the expression of antioxidant enzymes in an OA model.[18] However, epidemiological studies examining the benefits of antioxidants in human OA, especially vitamin E (α-tocopherol), are contradictory (reviewed by Henrotin *et al*[4]).

In conclusion, this is the first detailed study to examine the expression of all three SOD antioxidant enzymes in cartilage and OA. Our data clearly show a decrease in the expression of all three enzymes in OA cartilage, with SOD2 and SOD3

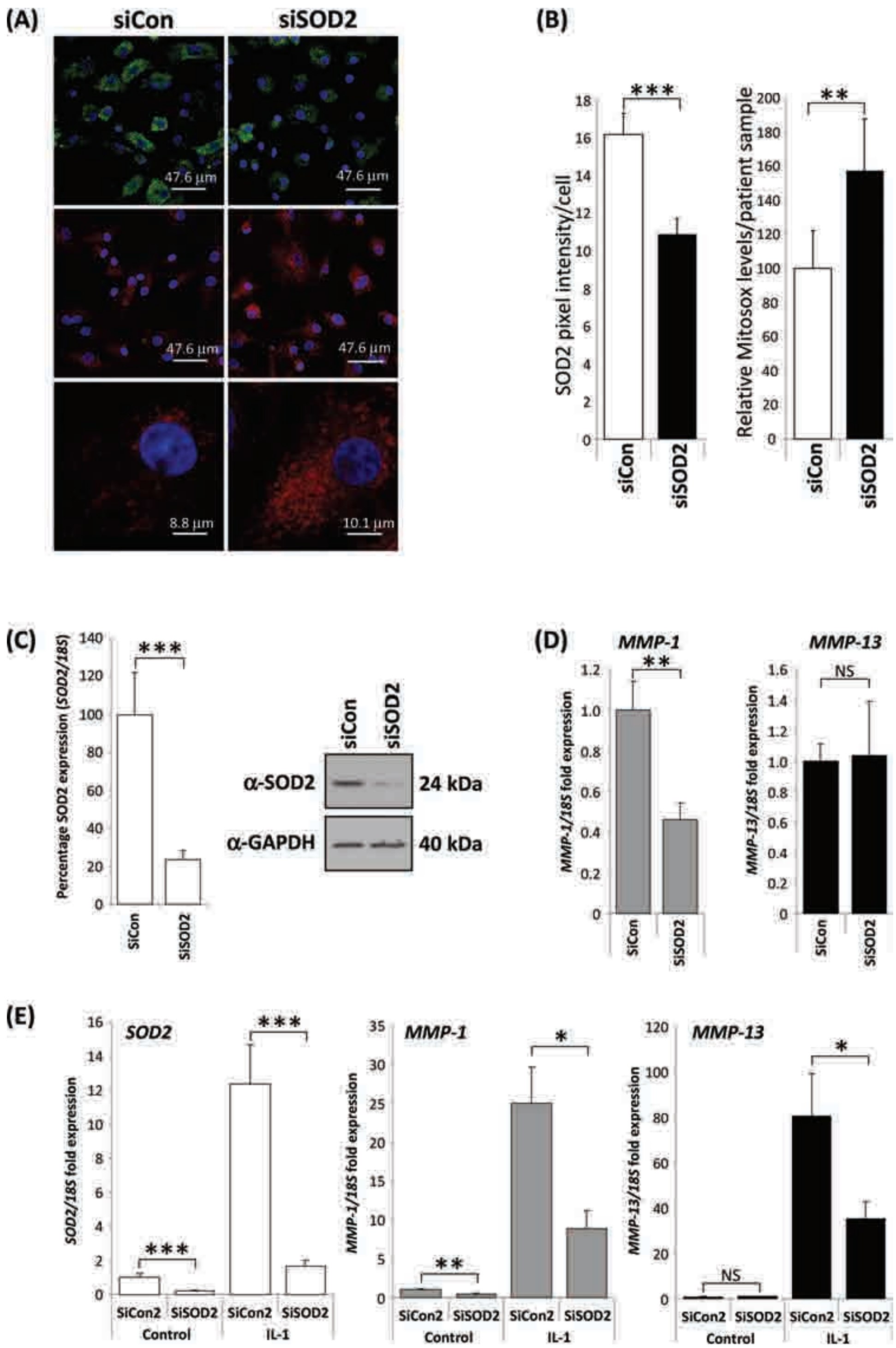

Figure 5 Functional consequences of superoxide dismutase 2 (SOD2) depletion. (A) and (B) SOD2 depletion (green) by RNA interference (RNAi) led to a significant increase in MitoSOX Red mitochondrial staining as determined by confocal microscopy. Data are taken from four patient chondrocyte preparations. Images were quantified using ImageJ software on a per cell basis from random field images. p Values were calculated using a Student t test: **p<0.01; ***p<0.001. (C–E) Depletion of SOD2 leads to reduction in collagenase gene expression. (C) Transfection of primary human articular chondrocytes (HAC) from patients with OA with siRNA to SOD2 (siSOD2) for 48 h under basal conditions (24 h serum free) compared with transfection with an equivalent amount (100 nmol/l) of a non-targeting control siRNA (siCon) resulted in a highly significant ~80% reduction in SOD2 RNA level as determined by real-time RT-PCR. Concomitantly, a substantial reduction in SOD2 protein expression was measured by immunoblotting (an anti-glyceraldehyde 3-phosphate dehydrogenase antibody was used as a protein loading control). (D) Matrix metalloproteinase (MMP)-1 and MMP-13 basal expression levels were measured as described above. SOD2 RNAi resulted in a significant reduction in MMP-1 but no change in the levels of MMP-13. (E) Interleukin 1 (IL1, 0.05 ng/ml) stimulation for 24 h induced the expression of SOD2, MMP-1 and MMP-13; however, the induced-expression of all three genes was significantly reduced when SOD2 was depleted by RNAi (siSOD2). Throughout, RNA expression levels were normalised to the 18S gene and data plotted as fold over control levels. Data presented are from a single experiment (n=8) using cells from one donor. The experiment was performed three times using different patient cell populations. p Values were calculated using the Mann–Whitney U test: *p<0.05; **p<0.01; ***p<0.001. Error bars shown are SEM.

expression decreasing before the appearance of histologically detectable lesions. ROS are normal and useful metabolites, serving important roles in a number of processes, including their action as a signalling molecule. Depletion of SOD2 reduced the IL1-induced expression of MMP-1 and MMP-13, indicating that the decrease of SOD2 expression in OA cartilage may represent a chondroprotective mechanism. However, SOD2 depletion also leads to an increase in ROS and such an overproduction, as occurs in injury, disease or ageing, can lead to cell dysfunction and death.[32] Clearly, the balance of ROS levels and the consequences of altered levels on cartilage are complex. Further work is required to determine whether the decrease in antioxidant enzymes reported here is an attempt by the joint to reduce cartilage destruction, mediated by modulating the levels of the collagenases but which ultimately leads to an increase in ROS and loss of cartilage homoeostasis. Such information is pertinent if antioxidant treatments hold the key to potential OA treatments.

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