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Brief report
DNA methylation is not responsible for p21WAF1/CIP1 down-regulation in osteoarthritic chondrocytes

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Summary

Objective: In this study, we were interested in the overall methylation level in aged and degenerated cartilage. Also, we looked at one gene which might be involved in the re-initiation of replicative activity in osteoarthritis (OA) chondrocytes, $p21^{WAF1/CIP1}$. $p21^{WAF1/CIP1}$ was previously suggested to be down-regulated in OA chondrocytes and is known to be regulated by epigenetic modulation.

Methods: Total methylation levels were analyzed by high pressure liquid chromatography (HPLC), mRNA expression of $p21^{WAF1/CIP1}$ and DNMT enzymes by real-time polymerase chain reaction. The methylation status of the $p21^{WAF1/CIP1}$- promotor using bisulfite genomic sequencing was evaluated.

Results: General methylation analysis of genomic DNA showed no difference in between normal and aged/OA chondrocytes. Also no difference in methylation of the promotor of the $p21^{WAF1/CIP1}$ gene was detectable, which was significantly down-regulated in OA chondrocytes. DNMT1 and DNMT3a were expressed with no significant changes of expression levels found in OA chondrocytes.

Conclusion: Cell cycle progression inhibitor $p21^{WAF1/CIP1}$ is expressed in normal and significantly down-regulated in OA articular chondrocytes, which may mediate the re-initiation of cell proliferation in OA cartilage. However, the suppression of $p21^{WAF1/CIP1}$ mRNA expression is not due to hypermethylation of its promotor. No overall changes in genome methylation levels were found in aged or OA cartilage. Interestingly, significant expression of DNA methyltransferases was found in articular chondrocytes, which supports that DNA methylation could still be a relevant mechanism of gene regulation in (osteoarthritic) chondrocytes, though not on an overall genomic level nor specifically for the regulation of the $p21^{WAF1/CIP1}$ gene.

Key words: Cartilage, Osteoarthritis, Methylation, p21, Methylases.

Introduction

Osteoarthritis (OA) is one of the most common disabling diseases worldwide. OA is caused by mechanical factors, but also a severe (dis)regulation of numerous genes important for tissue homeostasis. Additionally, other more general cell biological phenomena such as cell apoptosis and proliferation play an important role in maintaining tissue integrity. The latter is related to a re-initiation of the proliferative machinery within the chondrocytes in OA cartilage[1], whereas normal articular chondrocytes are considered to be largely post-mitotic after puberty. One important factor involved in this particular phenotypic trait of articular chondrocytes may be mediated by $p21^{WAF1/CIP1}$, which is known to block proliferation in many cell types.

Clearly, one major issue during disease progression is a severe alteration of the gene expression phenotype of the chondrocytes. Besides gene regulation by ordinary transcription factors, basic genetic and epigenetic alterations (for review see Ikegawa[2] and Roach and Aigner[3]) may play an important role in determining gene expression levels. In fact, recent experimental data indicate that differences in the methylation status within disease-relevant promotors are likely to induce/repress respective gene expressions[4]. This is, however, not true for all genes since we were not able to identify changes in the methylation levels of the aggrecan gene in aged and diseased chondrocytes[5]. At present, nothing is known about the general methylation level in diseased chondrocytes.

Therefore, in this paper we were interested to determine whether there is a difference in the overall methylation level in degenerated cartilage compared to normal chondrocytes. As we could not find any evidence for a general hyper- or hypomethylation we looked at one gene that might be involved in the re-initiation of replicative activity in OA chondrocytes, $p21^{WAF1/CIP1}$. $p21^{WAF1/CIP1}$ was suggested to be down-regulated in OA compared to normal chondrocytes by previous cDNA-array analysis[6]. Also, $p21^{WAF1/CIP1}$ expression has been previously shown in neoplastic cells to be, at least in part, regulated by epigenetic modulation[7].

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Materials and methods

CARTILAGE SAMPLES

Cartilage from normal human femoral condyles was obtained from autopsies within 48 h of death and processed as described previously[6]. Osteoarthritic cartilage was obtained during total knee replacement surgery. Cartilage was considered to be normal if it showed no significant fibrillation/erosion. Only primary degenerated and not regenerative cartilage (osteophytic tissue) was used. For quantitative real-time polymerase chain reaction (PCR) studies 10 samples of normal articular cartilage (48–83 years, mean 64.5 years) and 10 samples of osteoarthritic cartilage (60–83 years, mean 70.0 years) were analyzed. For bisulfite methodology three samples of normal articular cartilage (52–72 years, mean 62 years) and six samples of osteoarthritic cartilage (57–78 years, mean 67 years) were used. For HPLC five samples of normal articular cartilage (43–72 years, mean 53 years) and six samples of osteoarthritic cartilage (67–82 years, mean 71 years) were analyzed.

ISOLATION OF TOTAL RNA FROM CARTILAGE TISSUE – cDNA SYNTHESIS – QUANTITATIVE REAL-TIME PCR

Total RNA was isolated from cartilage tissue and transcribed into cDNA for real-time PCR analysis as described previously[8]. mRNA expression levels were measured by real-time quantitative PCR (qPCR) using an ABI Prism® 7700 Sequence Detection System (Applied Biosystems). Primers (MWG Biotech, Germany) and the TaqMan probe (Eurogentec, Seraing, Belgium) were designed using the PRIMER EXPRESS® software (Applied Biosystems) (Table I). All experiments were performed in triplicates using titrated standard curves as described elsewhere[9]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression levels were used for normalization of gene expression values. Statistical evaluation of expression levels was calculated using the non-parametric Wilcoxon–Mann–Whitney test.

ISOLATION OF GENOMIC DNA

For isolation of genomic DNA from cartilage, tissue samples were ground under liquid nitrogen using a freezer mill (Spex certiprep 6800) as described previously[8]. Genomic DNA was isolated using the Blood and Cell culture DNA Midi Kit in combination with the genomic DNA buffer set (Qiagen, Hilden, Germany).

HPLC SCREENING FOR GENOMIC METHYLATION OF CYTOSINES

The 5mdC content in genomic DNA was determined by HPLC. Residual RNA was removed by alkaline hydrolysis with 0.5 M NaOH at 37°C for 1 h. After neutralization with HCl and DNA precipitation 10 μg DNA of each sample were digested with two units micrococcal nuclease (United States Biochemical, Cleveland, USA) and 1 μg phosphodiesterase II (Roche, Basel, Switzerland) at 37°C for 3 h in 10 mM $CaCl_2$ and 20 mM succinate at pH 6.0 according to the protocol of Young[10]. For complete digestion to nucleosides, phosphate groups were removed by digestion with 20 units alkaline phosphatase (Roche) after adding $MgCl_2$ and $ZnCl_2$ to final concentrations of 1 mM each and Tris (pH 8.0) to 20 mM for 16 h at 37°C. Samples were lyophylized and stored at −20°C.

5mdC content was measured with a Beckmann Ultrasphere ODS column, 4.6 mm × 250 mm with 5 μm pore size (Beckmann Coulter, Krefeld, Germany) on a Merck–Hitachi (Merck–Hitachi, Amsterdam, The Netherlands) HPLC System (L-6200 gradient pump, L-7250 autosampler and L-7450 DA-Detection System) at room temperature. After injection of the samples, a programmed linear gradient was applied from 100% solution A (5% acetonitrile with 0.04% acidic acid in H_2O) to 100% solution B (100% methanol) over 10 min at 1 ml/min. The ratio between 5mdC and dC was determined by the ratio between the elution peak areas. All samples were measured in triplicates.

METHYLATION ANALYSIS – BISULFITE METHOD

For bisulfite genomic sequencing 2 μg genomic DNA was bisulfite treated with the EpiTec 96 Bisulfite Kit (Qiagen, Hilden, Germany). A 576 bp region spanning a CpG island which includes the core promotor of the $p21^{WAF1/CIP1}$ gene was amplified by the primers p21-fw (5′-AGGGAAGTGTTTTTTTGTAGT-3′) and p21-rev (5′-TAACCAAAAATTCCTATACTTA-3′)[11] [Fig. 4(a,b)]. The amplification conditions were: 0.8 pmol/μl primers, 2.5 units AmpliTaq Gold (Perkin Elmer, Waltham, USA), 2.5 mM $MgCl_2$, 0.2 mM dNTPs and buffer according to the supplier. PCR products were subcloned using the pGEM-T Easy Vector System (Promega, Mannheim, Germany). XL1-Blue competent cells (Stratagene, La Jolla, USA) were transformed and at least 10 clones picked (from each donor) and sequenced. The methylation status was analyzed by BIQ_Analyzer software according to Bock *et al.*[12].

Results

GENERAL METHYLATION ANALYSIS OF THE CHONDROCYTIC GENOME

First, we were interested to determine whether there was a difference in the general methylation level of genomic DNA in OA and normal articular chondrocytes. These analyses showed no statistically significant differences [Fig. 1(a,b)] between OA and normal articular chondrocytes. Also, no correlation was found with regard to the age of the donors investigated [Fig. 1(c)].

EXPRESSION ANALYSIS OF DNMT ENZYMES

Next, we were interested in the expression of *DN*A *m*ethyl*t*ransferase (DNMT) enzymes within the articular chondrocytes as these enzymes are the only means by which the methylation level could increase within the cells.

Conventional PCR demonstrated the expression of all three DNMT enzymes in normal and osteoarthritic articular cartilage with no obvious changes in between both. Real-time quantitative PCR confirmed these results showing that DNMT1 and DNMT3a were strongly expressed DNMTs (both about 0.006 times GAPDH). DNMT3b was considerably less expressed and hardly above background detection levels (below 0.0001/GAPDH). In OA chondrocytes no significant change of expression levels compared to normal was found for any of the three methylases (Fig. 2); again DNMT3b was hardly detectable at all.

EXPRESSION OF $p21^{WAF1/CIP1}$ IN NORMAL AND OA CARTILAGE

We then investigated the expression levels of $p21^{WAF1/CIP1}$ of articular chondrocytes *in vivo* by conventional PCR analysis of RNA isolated directly from normal articular cartilage. This showed a strong amplification product for $p21^{WAF1/CIP1}$ in normal articular chondrocytes [Fig. 3(a)]. The expression levels were clearly reduced in OA cartilage cells compared to the normal cells [Fig. 3(a)]. This was confirmed by real-time PCR, which showed a highly significant down-regulation in OA chondrocytes (about fourfold; $P < 0.001$) [Fig. 3(b)].

METHYLATION ANALYSIS OF THE $p21^{WAF1/CIP1}$-PROMOTOR REGION

Next we were interested to investigate whether the observed down-regulation of $p21^{WAF1/CIP1}$ expression may be due to an increased methylation grade of the $p21^{WAF1/CIP1}$-promotor region, namely the CpG island present (for a representation of the $p21^{WAF1/CIP1}$-promotor region including the CpG island see Fig. 4). Sequencing analysis of bisulfite treated DNA from three normal and six osteoarthritic donor samples showed no significant methylation in normal or in osteoarthritic samples.

Discussion

Methylation of cytosines is an important epigenetic mechanism for gene regulation, essential for normal cellular functions as well as genomic imprinting of specific genes such as X chromosome inactivation in the female. A change of the overall methylation level was suggested to be one

Table I
Sequences of primers and probes used for quantitative PCR experiments

Gene	Account number	Primer/probe	Sequence	nM*	Mg†
GAPDH	NM_002046	Forward	5′-GAAGGTGAAGGTCGGAGTC-3′	50	5.5
		Reverse	5′-GAAGATGGTGATGGGATTTC-3′	900	
		Probe	5′-CAAGCTTCCCGTTCTCAGCC-3′	100	
p21$^{WAF1/CIP1}$	NM_000389	Forward	5′-GCAGACCAGCATGACAGATTTC-3′	300	7
		Reverse	5′-GCGGATTAGGGCTTCCTCTT-3′	900	
		Probe	5′-CACTCCAAACGCCGGCTGATCTTC-3′	100	
DNMT1	NM_001379	Forward	5′-GAGGAGGGCTACCTGGCTAAA-3′	300	5
		Reverse	5′-CGTTTTCTAGACGTCCATTCACTTC-3′	300	
		Probe	5′-CTTGGAGAACGGTGCTCATGCTTACAACC-3′	100	
DNMT3a	NM_175629	Forward	5′-CAGAAGTGCCGGAACATTGA-3′	300	3
		Reverse	5′-TTCTTGCAGTTTTGGCACATTC-3′	900	
		Probe	5′-CCTGGAACACCCCCTCTTCGTTGG-3′	100	
DNMT3b	NM_006892	Forward	5′-GGAGTGTAATCCAGTGATGATTGATG-3′	900	6
		Reverse	5′-ATGCTATCACGGGCCTGTTC-3′	300	
		Probe	5′-TTCTGCTGCTCACAGGGCCCGATAC-3′	100	

*Concentration of primer/probe in TaqMan reaction.
†$MgCl_2$ concentration in TaqMan reaction in mM.

possible core mechanism of aging of cells and tissues[13]. Thus, the first important result of our study is that we were not able to detect any significant change in general methylation levels in osteoarthritic and aging articular chondrocytes. Obviously, this does not exclude differences in methylation levels in selected promotor regions[14] as reported for some genes previously[4,15,16]. A caveat might also be that measuring total DNA methylation might be too crude to detect relevant changes in methylation levels of relevant sites. Millions of CpG sites are located throughout the genome, many in introns, transposons and repetitive sequences are largely methylated. Compared to this, CpG

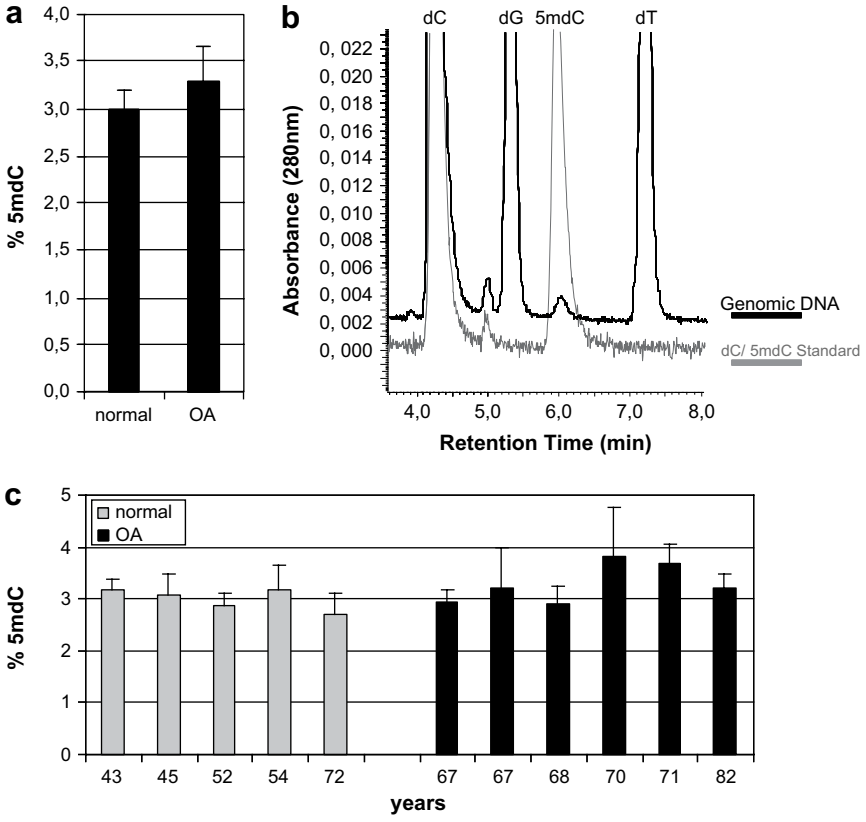

Fig. 1. General methylation level of genomic DNA derived from normal and OA cartilage measured by HPLC (a). Representative absorption curve of a single sample of digested DNA as compared to a standard curve of 10 mM of dC and 5mdC (b). Methylation of the individual samples shown relative to the age of the donors (c).

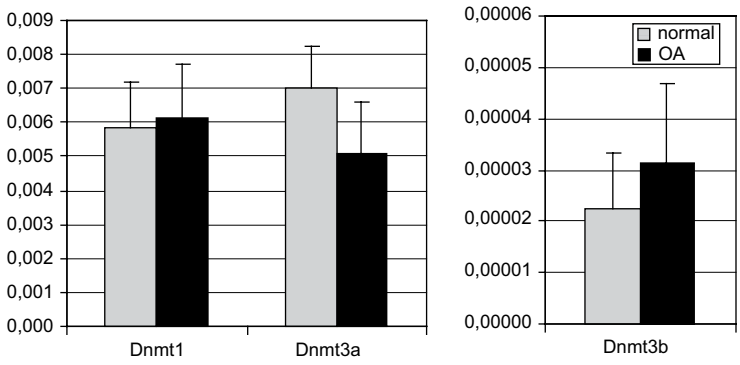

Fig. 2. qPCR-analysis of the expression of the DNMTs, DNMT1, DNMT3a and DNMT3b using real-time PCR (n(normal) = 8, n(late-osteoarthritic) = 9). Shown are values normalized to GAPDH.

sites in the promotors of genes represent a relatively small percentage of total CpG sites. Thus, a more focussed investigation of specific promotor CpG sites is needed. In addition, our results do not exclude major changes taking place during childhood and adolescence, in particular as during these life periods the organism grows. Specifically chondrocytes also divide[17] and methylation levels can in particular be strongly modified during DNA-replication. Thus, one potential reason for the stability in overall methylation levels in adult articular chondrocytes could be largely related to the post-mitotic status of these cells in the adult[1].

$p21^{WAF1/CIP1}$ is one classical inhibitor of cell proliferation and in fact normal non-proliferating adult chondrocytes show a significant expression of $p21^{WAF1/CIP1}$. In contrast, OA chondrocytes, which re-initiate cell cycle activity[1], do show a highly significant down-regulation of $p21^{WAF1/CIP1}$ expression. Thus, down-regulation of $p21^{WAF1/CIP1}$ expression might well be one reason or at least one mediator of the "de-blocking" of cell cycle progression in OA cartilage cells. The reasons of this down-regulation of $p21^{WAF1/CIP1}$ expression are, however, largely unclear. This current study provides experimental evidence that no *de novo* methylation of the $p21^{WAF1/CIP1}$-promotor-CpG island is involved in this process, though $p21^{WAF1/CIP1}$ is known to be regulated by methylation, for example, in some cases of neoplastic transformation[7,18,19]. Other reports, however, could not find such changes of methylation pattern in the *p21* promotor region[20]. Potentially, this down-regulation of $p21^{WAF1/CIP1}$ mRNA expression might be due to a reduction in p53 activity, of which $p21^{WAF1/CIP1}$ is one direct major target gene, but no experimental data are so far available about p53 activity in articular chondrocytes.

Methylation of CpG sites within the genome is mediated by enzymes known as DNMTs, namely DNMT1, DNMT3a and DNMT3b (for review see Pradhen and Esteve[21]). Of the three DNMTs, DNMT1 is responsible for maintaining established methylation patterns during cell division. DNMT3a and DNMT3b are known to play key roles in the *de novo* methylation of primarily unmethylated DNA. In our study, we found low, but still significant expression levels of DNMT1 and DNMT3a, whereas DNMT3b was hardly expressed at all. This is similar to other adult tissues[22] and in line with the assumption that DNMT3b is mostly involved in embryonic differentiation processes. Most interestingly, however, and different to other tissues is that cartilage is largely post-mitotic, i.e., DNMT-expression is not needed for maintenance of methylation pattern during replication; thus, the expressed DNMTs might well be involved in *de novo* methylation in articular chondrocytes. However, so far no evidence of *de novo* methylation has been reported in adult or OA cartilage: $p21^{WAF1/CIP1}$ and *aggrecan* promotors appear not to have any changes in methylation levels at all[5], other genes such as *matrix metalloproteinases (MMPs)* and *leptin* appear to lose methylation during OA progression[4,15,16].

Epigenetic disregulation in OA chondrocytes is clearly one potentially important new topic for understanding the cellular (dis)behavior during the disease process[3,23]. The implications of epigenetic alterations, namely changes in methylation pattern, for OA are only in part investigated so far. Important chondrocytic genes, for example collagen type II and aggrecan, contain CpG islands in their promotors, but no evidence to date exists that epigenetic alterations are involved in the regulation of, for instance the aggrecan gene in OA chondrocytes[5]. More relevant, Roach

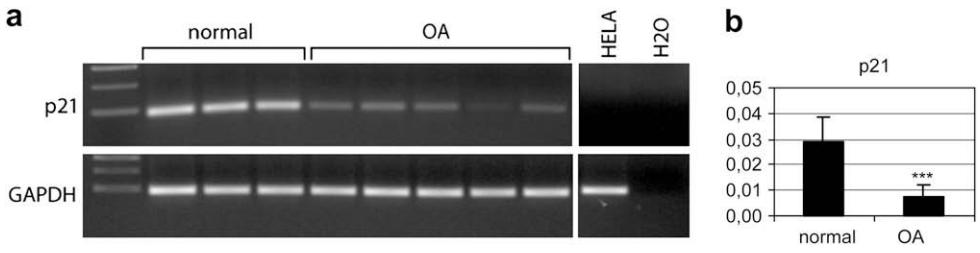

Fig. 3. Analysis of the expression of $p21^{WAF1/CIP1}$ mRNA by conventional (a: lane 1: standard; lanes 2–4: normal samples; lanes 5–9: late OA samples; lane 10: Hela cell line; lane 11: water (negative control)) and real-time PCR (b: n(normal) = 10, n(late-osteoarthritic) = 10; shown are values normalized to GAPDH).

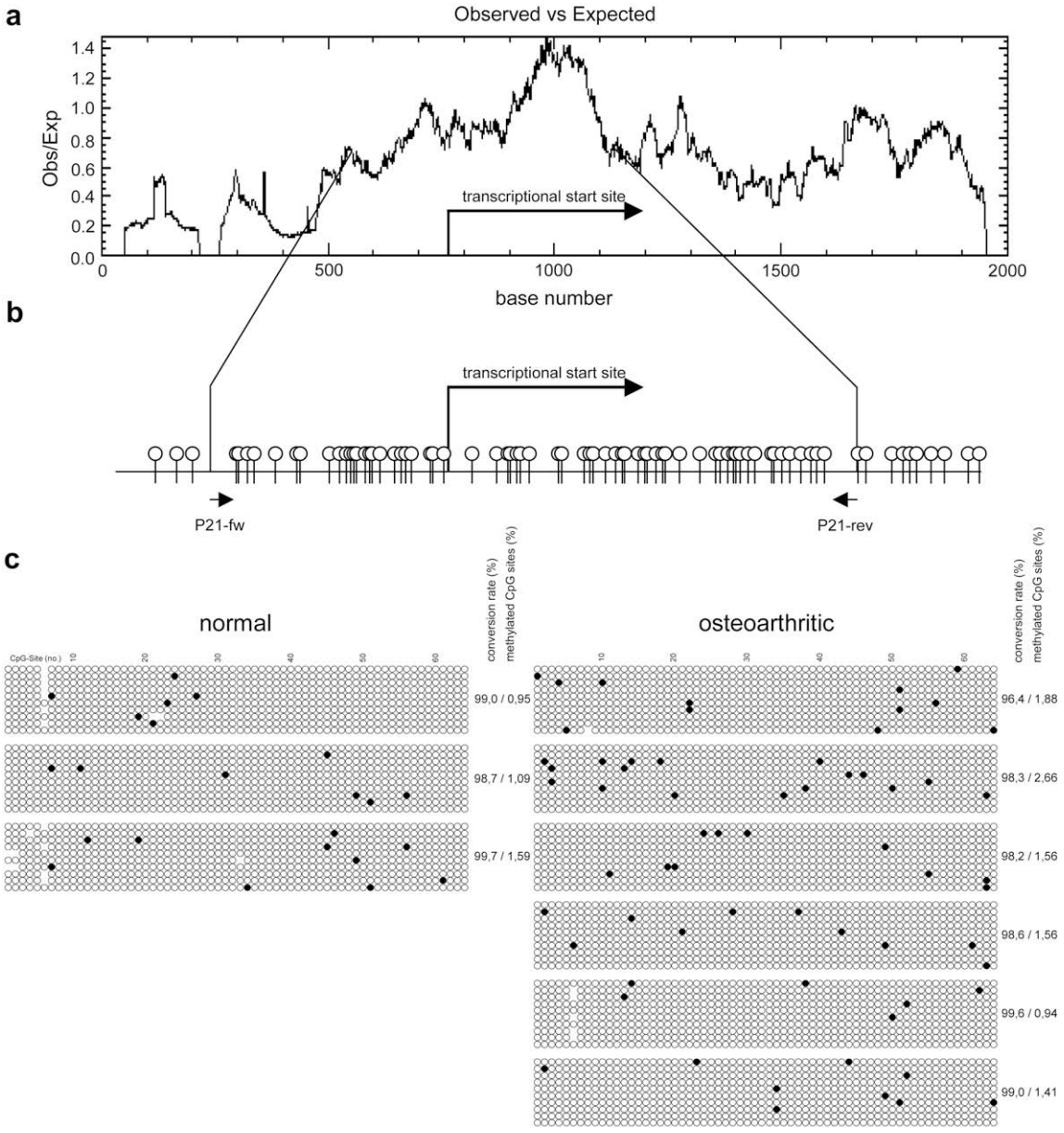

Fig. 4. Analysis of p21 promoter methylation by bisulfite genomic sequencing. (a) Analysis of the CpG island in the *p21* promoter region. (b) A region containing 64 CpG sites was chosen for analysis. Arrows delineate the PCR product used for sequencing, individual CpG sites are indicated by circles. (c) Ten individual alleles were analyzed for each cartilage sample (three normal, six OA). Individual alleles are arranged in rows. Filled circles depict methylated CpG sites, open circles represent unmethylated sites. The percentage of methylated CpG sites is shown next to each sample. "Conversion rate" shows the percentage of non-CpG site cytosine residues converted after bisulfite treatment.

and colleagues showed a hypomethylation of *MMP*-promotors in OA[4,16], which could be an alternative explanation for the switch on of these matrix degrading genes than the pure induction through inflammatory cytokines such as interleukin-1 (IL-1). However, these changes in gene expression appear not to be due to a general loss of methylation in OA chondrocytes. As the human epigenome project has shown, 88% of promotors with CpG islands are always hypomethylated and show no correlation between DNA methylation and gene expression at least for three chromosomes investigated[24,25]. On the other hand, methylation is not the only epigenetic mechanism and DNA methylation frequently synergizes with histone modifications, as there are several examples of cancer-related genes that are silenced by the combinatory effects of DNA methylation and histone modifications.

In summary, in this paper we show that the cell cycle progression inhibitor $p21^{WAF1/CIP1}$ is expressed in normal and significantly down-regulated in OA articular chondrocytes, which may mediate the re-initiation of cell proliferation in OA cartilage. However, the suppression of $p21^{WAF1/CIP1}$ mRNA expression is not due to hypermethylation of its promotor. Also, no overall changes in genome methylation levels are found in aged or OA cartilage. Still, more focussed studies on promotor specific methylation and other epigenetic events in other genes could provide

a clue for the regulation of protein expression in the onset and development of OA.

Conflict of interest

The authors declare that no potential conflicts of interest exist.

Acknowledgments

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