Mitochondrial genome sequencing of chondrocytes in osteoarthritis by Human Mitochondria RT^2 Profiler™ PCR Array

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Abstract. Mitochondria are not only the main energy generators of the cell, but also mediate several critical biochemical processes such as apoptosis, proliferation and redox homeostasis. As such, mitochondrial dysfunctions can lead to a wide variety of human diseases, including cancer and osteoarthritis (OA). In OA, mitochondrial-associated signaling has been implicated in the molecular events leading to cartilage degradation, including oxidative stress, defective chondrocyte biosynthesis and growth responses, increased cytokine-induced chondrocyte inflammation and matrix catabolism, cartilage matrix calcification and increased chondrocyte apoptosis. Thus, the mitochondrial genome represents an attractive target for molecular therapy and OA research has focused on determining its role in chondrocyte metabolism and subsequent cartilage degradation. In this study, we analyzed the mitochondrial gene expression changes that characterize chondrocytes in OA using the Human Mitochondria RT^2 Profiler™ PCR Array. Twenty-six differentially expressed genes were identified that discriminated chondrocytes in OA from those in normal cartilage, including 17 upregulated and 9 downregulated genes. These genes represent diverse functional categories, including mitochondrial membrane polarization and potential, mitochondrial transport, small molecule transport, targeting proteins to the mitochondria, mitochondrial protein import, outer and inner membrane translocation, mitochondrial fission and fusion, mitochondrial localization and apoptosis. Western blot analysis confirmed that the p53 upregulated modulator of apoptosis (PUMA; encoded by the BB3 gene) was significantly upregulated in OA cartilage. In conclusion, our study generates a differential mitochondrial gene expression profile for chondrocytes in OA and demonstrates that mitochondrial genome dysregulation occurs in cartilage cells during OA. Finally, our results indicate that PUMA may be a new diagnostic and therapeutic target for OA.

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Introduction

Osteoarthritis (OA) is one of the most common causes of musculoskeletal disorders related to aging. This irreversible degenerative disease is characterized by slowly progressive matrix destruction and chondrocyte cell death, which eventually results in the loss of articular cartilage integrity and joint space (1,2). The articular cartilage is necessary for protecting the bones from friction damage caused by normal movement at the joints, and is composed of collagen fibrils, extracellular matrix (ECM) components, and chondrocytes - the only resident cells in human mature articular cartilage. The chondrocytes produce and secrete collagen, proteoglycans and enzymes that are critical for the repair of damaged cartilage tissue and the maintenance of cartilage integrity (3). In addition, chondrocytes have finely balanced anabolic and catabolic properties that contribute to the maintenance of normal homeostasis in articular cartilage (4,5). As such, chondrocytes are generally considered to be key players in the cartilage degeneration events associated with OA.

OA not only destroys joint function, thereby disabling the sufferer, but is associated with significant pain. These symptoms of the disease can severely impact a sufferer's quality of life by hindering functional and social activities, which in turn can erode interpersonal relationships, socioeconomic status, body image and emotional well-being (6). A number of non-pharmacological treatments are currently being applied routine clinical practice which include patient education, self-management programs, weight loss, physical and occupational therapy and external devices that assist function. Pharmacological therapies are available if the non-pharmacological interventions fail; generally, these are traditional non-steroidal anti-inflammatory drugs (NSAIDs), chondroitin sulfate, glucosamine and intra-articular glucocorticoids. In a number of OA cases, surgical therapy may be required, and this usually involves arthroscopy and joint replacement.

With recent advances in molecular technology, much human disease research has focused on identifying the underlying genetic, proteomic and metabolic processes that contribute to disease onset and progression, and may be effectively and safely manipulated by molecular targeted therapies (7,8). As a result, many studies have uncovered a pathogenic role for mitochondrial-related dysfunctions (9,10). The mitochondrion is a complex membrane-enclosed organelle in eukaryotic cells that harbors a DNA genome distinct from the nuclear genome and mediates many life-sustaining biochemical processes. A typical eukaryotic cell contains approximately 2,000 mitochondria, which occupy approximately 20% of the total cell volume. Each mitochondrion is comprised of an outer membrane, an intermembrane space, an inner membrane and a matrix, all of which interact with non-mitochondrially expressed and translated proteins.

Mitochondrial dysfunctions have been implicated in aberrant apoptosis and proliferation events, natural aging, and various human pathologies, including Parkinson's, Alzheimer's diseases and OA. Mitochondrial dysfunctions are believed to affect a number of pathways implicated in OA-related cartilage degradation, including oxidative stress, defective chondrocyte biosynthesis and growth responses, increased cytokine-induced chondrocyte inflammation and matrix catabolism, cartilage matrix calcification and increased chondrocyte apoptosis (11). Thus, it is believed that the study of mitochondrial gene expression may provide insights into the metabolism of chondrocytes and their role in cartilage degradation (12).

The Human Mitochondria RT^2 Profiler™ PCR Array was developed to detect the expression of 84 genes involved in mitochondrial-related biogenesis processes and functions (Fig. 1). The genes monitored by this array include regulators and mediators of mitochondrial molecular transport not only of the metabolites needed for the electron transport chain and oxidative phosphorylation, but also of the ions required for maintaining the mitochondrial membrane polarization and those involved in ATP synthesis. In addition, the intrinsic apoptotic pathway genes, which are known to be activated by intracellular damage signaling, are also represented on this array. Despite the mitochondrion's non-eukaryotic origins and presence of its own chromosome, the majority of proteins essential for mitochondrial replication and function are encoded by nuclear genomic DNA. Therefore, this array also targets non-mitochondrially expressed genes of proteins that translocate into the outer and/or inner mitochondrial membranes and/or into the mitochondrial matrix.

This mitochondrial PCR array technology was used to perform niche targeting analysis of changes in the mitochondrial gene expression profile of chondrocytes in OA articular cartilage, with the aim of gaining insights into the underlying mechanisms of OA pathogenesis. In addition, this mitochondrial gene profiling study was expected to identify potential biomarkers or targets of molecular therapeutics for OA diagnosis and treatment, respectively.

Materials and methods

Reagents, chemicals, human mitochondria PCR array and antibodies. Culture medium (Dulbecco's modified Eagle's medium; DMEM), fetal calf serum (FCS), and culture flasks were purchased from Invitrogen. The Human Mitochondria RT^2 Profiler PCR Array was from SABiosciences. Hyaluronidase, pronase, collagenase and other chemicals were from Sigma-Aldrich. Monoclonal antibodies (rabbit anti-human) against human p53 upregulated modulator of apoptosis (PUMA) and GAPDH were from Cell Signaling Technology, Inc.

Cartilage procurement and processing. Healthy human knee cartilage was obtained from 6 cadaver donors (2 males and 4 females, 42-70 years of age) with no history of joint disease. All donors had died from traumatic injuries or brief illnesses unrelated to knee joints, and none had been taking corticosteroids or cytostatic drugs. All specimens were obtained within 24 h after autopsy. OA-affected cartilage was obtained from 8 patients (3 males and 5 females, 48-78 years of age) undergoing total knee arthroplasty. This study was approved by the Institutional Ethics Committee of Sun Yat-sen University, Guangzhou, China. After excision, the knee cartilage sample was rinsed with saline and diced into small fragments, which were immediately incubated in trypsin at 37˚C for 10 min. The cartilage fragments were then treated with type IV clostridial collagenase in DMEM with 5% FCS for 12-16 h in order to release the chondrocytes from the cartilage.

Primary culture of chondrocytes from the cartilage. The isolated chondrocytes from the cartilage were seeded into 75 cm^2 flasks at high density in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1% glutamine and 10% FCS. The cells were incubated in a 5% CO_2 humidified atmosphere, and were used after 2-3 weeks in primary culture (8).

RNA isolation. Total RNA was extracted from the chondrocytes using standard procedures. Briefly, cells were lysed directly in the culture flask with the TRIzol reagent, and the resultant cell lysate was passed several times through a pipette. The homogenized samples were then incubated for 5 min at 15-30˚C to permit the complete dissociation of nucleoprotein complexes. Chloroform (0.2/1 ml of TRIzol reagent) was then added. The tubes were shaken vigorously by hand for 15 sec, incubated at 15-30˚C for 2-3 min, and centrifuged at 12,000 x g for 15 min at 4˚C. The aqueous phase was transferred to a fresh tube and isopropyl alcohol (0.5 ml/1 ml of TRIzol reagent) was added to precipitate the RNA from the aqueous phase. Samples were incubated at 15-30˚C for 10 min and then centrifuged at 12,000 x g for 10 min at 4˚C. The RNA pellet was washed once with 75% ethanol and allowed to air-dry for 5-10 min before dissolving in RNase-free water by passing the solution through a pipette tip a few times and incubating at 55-60˚C for 10 min. The concentration and purity of the RNA was determined by spectrophotometric measurement on the NanoDrop® ND-1000 and by electrophoretic resolution through a denaturing agarose gel.

mRNA isolation. In a sterile RNase-free 1.5-ml tube, 0.1-1.0 mg of total RNA was brought to a final volume of 500 μl in RNase-free water. The samples were heated at 65˚C in a heating block for 10 min. Then, 3 μl of biotinylated-oligo(dT) probe and 13 μl of 20X sodium chloride-sodium citrate (SSC) buffer were added. For each isolation, one tube of Promega's

Streptavidin MagneSphere Paramagnetic Particles (SA-PMPs) was resuspended by gently flicking the bottom of the tube until the particles were completely dispersed. The SA-PMPs were captured by placing the tube on a magnetic stand. The supernatant was removed using the magnetic stand and the SA-PMPs were washed three times with 0.5X SSC (300 μlwash). The washed SA-PMPs were resuspended in 100 μl of 0.5X SSC and then the entire contents of the annealing reaction were added to the tube. The samples were incubated at room temperature for 10 min. The SA-PMPs were captured using the magnetic stand and the supernatant was removed carefully. The particles were washed 4 times with 0.1X SSC (300 μl/wash) by gently flicking the bottom of the tube until all particles were resuspended. After the final wash, as much supernatant as possible was removed. The final SA-PMP/mRNA-bound pellet was resuspended in 100 μl of RNase-free water, and the particles were resuspended by flicking the tube. The unbound SA-PMPs were captured magnetically, and the eluted mRNA in the supernatant was transferred to a sterile RNase-free tube. The elution step was repeated once by resuspending the SA-PMP pellet in 150 μl of RNase-free water. The entire capture process was repeated, and the eluted mRNAs were pooled (for a total volume of 250 μl).

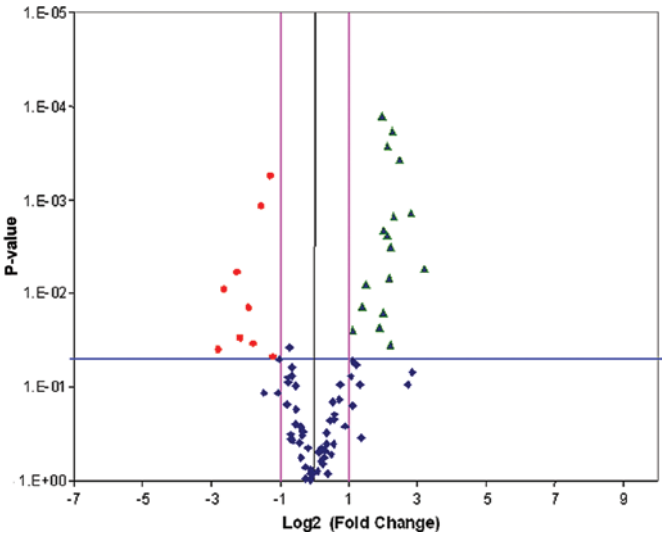

Figure 1. Volcano plot of the mitochondria real-time PCR array results. The black line indicates a 1.0-fold change in gene expression. The pink lines indicate the desired threshold of a 2.0-fold change in gene expression. The blue line indicates the desired 0.05 threshold for the P-value of the t-test.

First-strand cDNA synthesis. cDNA was synthesized from 0.5 μg total RNA in a reaction mix containing 1 μl of 500 ng oligo(dT)18, 1 μl of 10 mM dNTP mix, and sterile distilled water to bring the volume to 13 μl. The mixture was heated to 65˚C for 5 min, and incubated on ice for at least 1 min. The contents of the tube were collected by brief centrifugation and the following reagents were added: 4 μl 5X First-Strand buffer, 1 μl 0.1 M DTT, 1 μl RNase inhibitor, 1 μl SuperScript III reverse transcriptase. The sample was mixed by pipetting gently and incubated at 50˚C for 60 min. The reaction was inactivated by heating at 70˚C for 15 min. A total amount of 91 μl of double-distilled water (ddH_2O) was added to each 20 μl of cDNA synthesis reaction.

Mitochondria PCR arrays and data analysis. The following components were mixed in a 5-ml tube or a multi-channel reservoir: 550 μl of 2X SuperArray PCR master mix, 102 μl of the diluted first-strand cDNA synthesis reaction and 448 μl of ddH_2O. The cocktails were then added to the PCR array. Real-time PCR detection was performed under the following thermal cycling conditions: 95˚C for 10 min; 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The results were analyzed by the ΔΔCt method.

Western blot analysis. Total cellular proteins were extracted according to standard procedures and the protein concentration was determined using the Bradford reagent (Bio-Rad). The extracted proteins were separated by SDS-PAGE and electrotransferred to polyvinyl difluoride membranes (Millipore). Non-specific binding was blocked by incubating the membrane in PBSA (5% of dried milk and 0.1% of Tween-20 in phosphate buffered saline (PBS) for 1 h at room temperature. First, antibodies against PUMA or Bcl-2 were added and incubation was carried out overnight at 4˚C. The appropriate peroxidase-conjugated secondary antibody was then added and immunoreactive bands were detected by chemiluminescence. Band intensities were quantified by the use of ImageQuant software (GE Healthcare). The relative abundance of PUMA and Bcl-2 was calculated as the ratio of the normalized densitometric values between OA and normal samples. Intergroup differences (of the densitometry data) were calculated by the Mann-Whitney U test using SPSS version 15.0 software. P-value <0.05 was considered to indicate a statistically significant difference.

Results

Comparison of the mitochondrial gene expression between chondrocytes in OA and normal cartilage. The cartilage of 8 OA donors and 6 normal controls (NC) was used to isolate total RNA and proteins. Donors had been clinically diagnosed with OA and were of similar ages.

In this study, we sought to compare the mitochondrial gene expression profile of chondrocytes in OA cartilage with those in normal cartilage by using mitochondrial real-time PCR microarray technology. A total of 84 genes related to mitochondrial function were compared between the 2 cell types (diseased vs. healthy). Plotting of the detected transcripts on a volcano plot (Fig. 1) indicated that 26 genes were differentially expressed in the chondrocytes from the OA and normal cartilage by 2-fold or greater. A total amount of 17 genes were upregulated and 9 were downregulated. When the functions of these genes were investigated by searching the GenBank database, it was found that they represented diverse functional categories related to mitochondria, including membrane polarization and potential, mitochondrial transport, small molecule transport, targeting proteins to the mitochondria, mitochondrial protein import, outer and inner membrane translocation, mitochondrial fission and fusion, mitochondrial localization and apoptosis (Fig. 1 and Table I).

Of the genes involved in apoptosis, the expressions of BBC3, BCL2, and BCL2L1 were increased, whereas the

Table I. Genes identified by mitochondrial real-time PCR arrays as differentially expressed in osteoarthritis cartilage.

Gene ID	Gene	Fold change	P-value	Mitochondrial function
BBC3	BCL2 binding component 3	4.48	0.006	Apoptosis
BCL2	B-cell CLL/lymphoma 2	5.51	0.001	Membrane polarization and potential; mitochondrial transport; apoptosis
BCL2L1	BCL2-like 1	4.71	0.003	Membrane polarization and potential; mitochondrial transport; apoptosis
COX18	COX18 cytochrome c oxidase assembly homolog	2.20	0.024	Mitochondrion protein import; mitochondrial fission and fusion
FXC1	Fracture callus 1 homolog	4.02	0.002	Mitochondrial transport
MFN2	Mitofusin 2	4.88	0.001	Mitochondrial transport; mitochondrial fission and fusion
NEFL	Neurofilament, light polypeptide	4.57	0.035	Mitochondrial localization
SLC25A2	Solute carrier family 25, member 2	7.04	0.001	Small molecule transport
SLC25A22	Solute carrier family 25, member 22	3.72	0.023	Small molecule transport
SLC25A25	Solute carrier family 25, member 25	2.80	0.008	Small molecule transport
SLC25A31	Solute carrier family 25, member 31	4.95	0.002	Small molecule transport
SLC25A37	Solute carrier family 25, member 37	9.01	0.005	Small molecule transport
STARD3	StAR-related lipid transfer (START) domain containing 3	4.02	0.016	Mitochondrial transport
TIMM17B	Translocase of inner mitochondrial membrane 17 homolog B	4.35	0.002	Inner membrane translocation
TP53	Tumor protein p53	2.66	0.014	Membrane polarization and potential
UCP1	Uncoupling protein 1	4.43	0.001	Membrane polarization and potential; mitochondrial transport
UCP3	Uncoupling protein 3	3.89	0.001	Membrane polarization and potential; mitochondrial transport
CPT1B	Carnitine palmitoyltransferase 1B	-2.87	0.001	Mitochondrial transport
DNAJC19	DnaJ (Hsp40) homolog, subfamily C, member 19	-2.32	0.048	Mitochondrial transport; targeting proteins to mitochondria; mitochondrion protein import
LRPPRC	Leucine-rich PPR-motif containing	-2.38	0.001	Mitochondrial localization
MFN1	Mitofusin 1	-3.40	0.034	Mitochondrion protein import; mitochondrial fission and fusion
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1	-6.87	0.040	Apoptosis
SLC25A10	Solute carrier family 25, member 10	-4.44	0.029	Small molecule transport
SLC25A12	Solute carrier family 25, member 12	-6.19	0.009	Small molecule transport
SLC25A16	Solute carrier family 25, member 16	-4.77	0.006	Small molecule transport
SLC25A24	Solute carrier family 25, member 24	-3.73	0.014	Small molecule transport

Gene identity according to the GenBank databases. P-values show the comparison results between the patients and the normal controls.

expression of PMAIP1 was decreased. The other genes were mostly involved in small molecule transport, mitochondrial transport, and membrane polarization and potential.

Increased presence of PUMA in chondrocytes from OA cartilage. PUMA gene expression was found to be significantly increased in the chondrocytes in OA by our mitochondrial real-time PCR array analysis. The increase in PUMA protein expression was confirmed by western blot analysis of 8 protein extracts from chondrocytes from NC and OA (Fig. 2). Two independent samples t-tests were used to compare the different protein levels between the 2 groups. The results indicated that

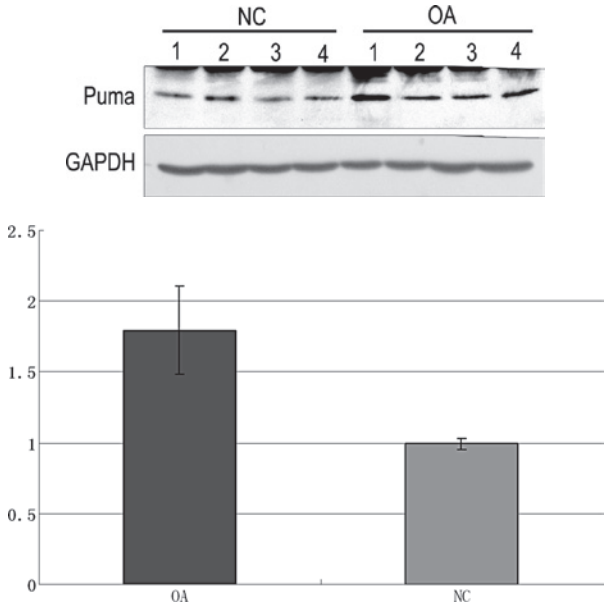

Figure 2. Mitochondrial p53 upregulated modulator of apoptosis (PUMA) is increased in chondrocytes in osteoarthritis (OA). Western blot analysis of PUMA protein levels in the chondrocytes from normal and OA cartilage showed a significant increase in the diseased cells in the OA cartilage ($P<0.05$). A representative blot is shown along with the numeric data obtained by densitometry analysis of the blots (n=8). Data are presented as the mean values and error bars indicate standard error of the mean.

the average level of PUMA in OA was significantly higher than that in NC ($P<0.05$).

Discussion

OA has emerged as the most common arthropathy related to aging. This slowly progressive degenerative disease is characterized by matrix destruction and chondrocyte death, which ultimately results in the loss of articular cartilage integrity and joint space and has a severe impact on the quality of life (2). Based on previous research reporting on specific mitochondrial alterations in chondrocytes in OA, we designed a microarray-based comparative analysis to evaluate the mitochondrial gene expression profile of cartilage cells extracted from age-matched healthy and OA donors. The Human Mitochondria RT^2 Profiler PCR Array was used to comprehensively assess mitochondrial pathway alterations in OA and possibly identify novel pathogenic mechanisms. This array was designed to profile the expressions of 84 genes involved in the biogenesis and function of the mitochondria, including genes from both the mitochondrial and the nuclear genomes. Using this technology, we identified 26 differentially expressed genes that clearly discriminate between chondrocytes in OA and normal cartilage, including 17 upregulated and 9 downregulated genes. All these differentially expressed genes should be studied in more depth in future studies since they may be useful for the development of new diagnosis tools, or may represent useful and novel therapeutic targets for OA.

The collective body of research on OA has generated a molecular image of the disease process, which includes cell death, matrix degradation and subchondral bone remodeling (13). The loss of articular cartilage, however, is considered the central event in OA. Studies have shown that cell apoptosis is the primary pathogenic mechanism underlying OA-related cartilage destruction (14). Mitochondrial dysregulation has been related to aberrant apoptotic processes and itself has been implicated in OA development and progression (15,16). Adams and Horton demonstrated that chondrocyte apoptosis increases with age in the articular cartilage of adult mice and rats (17). Likewise, apoptosis in human OA cartilage has been shown to be greater than that in normal cartilage (18,19). Furthermore, apoptotic chondrocyte death occurs more frequently in OA than in normal cartilage, and in OA lesional than in OA non-lesional cartilage. The differential expression patterns of the apoptosis-related genes, Bcl-2 and Fas, in OA lesional and non-lesional cartilage further confirms the contribution of apoptotic events, and suggests that these genes may be critical in the pathogenesis of OA (18).

In this study, we focused on the gene, BB3, that encodes the apoptosis-related PUMA protein, but which has yet to be extensively studied in OA. PUMA is a Bcl-2 homology 3 (BH3)-only Bcl-2 family member, and is characterized as an essential mediator of p53-dependent and -independent apoptotic pathways (19,20). Structural analyses by others have shown that the BH3 domain of PUMA forms an amphipathic α-helical structure that directly binds to anti-apoptotic Bcl-2 protein family members with a remarkably high affinity (21). PUMA exerts pro-apoptotic activity by this interaction with Bcl-2 family members and by mediating their mitochondrial localization. Previous studies using the human embryonic kidney cell line, 911, have revealed that PUMA co-precipitates with a large fraction of Bcl-2 or Bcl-xL (22). Under most conditions and in most cell types, PUMA accounts for a majority of the p53-dependent apoptosis. Many types of PUMA-knockout cells exhibit high resistance to apoptosis induced by p53 overexpression or DNA-damaging agents (23,24). In addition, PUMA is known to activate Bax/Bak directly or indirectly by relieving the inhibition of all anti-apoptotic Bcl-2 family proteins to promote mitochondrial dysfunction, and release of the mitochondrial apoptogenic proteins, cytochrome c, SMAC and apoptosis-inducing factor (AIF), leading to caspase activation and cell death. Under certain conditions, PUMA may directly activate Bax/Bak, or cytoplasmic p53 (by displacing it from Bcl-xL), to promote cell death through the mitochondrial pathway. Our study indicates that p53-dependent PUMA induction may be an important mechanism leading to chondrocyte apoptosis, and may contribute to the resulting OA. Thus, this study suggests that PUMA may be a potential target for anti-apoptotic treatments in OA.

On the other hand, the perturbation of oxidative stress balance caused by mitochondrial dysregulation has also been reported to play an essential role in OA development and progression (25,26). A previous proteomic-based study of OA-related mitochondrial proteins supports the hypothesis that inadequate control of reactive oxygen species (ROS), known to affect chondrocyte intracellular metabolism, is critically involved in OA pathophysiology (27). In addition, OA chondrocytes have been shown to have higher ROS generation than normal chondrocytes. It has been hypothesized that a global alteration of the redox balance in the bone, cartilage and synovial fluid of the OA joint could be due to changes in the expression of antioxidant proteins (12).

PUMA is known to be induced by ROS, anoxia, or oxidative stress in some types of cells (28,29). ROS is generated during PUMA-mediated apoptosis, as a result of mitochondrial damage, and the induction of PUMA by ROS may provide a feed-forward mechanism for signal amplification in the execution of apoptosis (30). A number of studies have described how PUMA regulates ROS production, thereby defending the cells from oxidative stress-mediated apoptosis (29). Although no evidence has yet indicated that an additional antioxidant supply or inhibition of ROS-related gene expression could effectively resolve OA symptoms, and/or prevent structural damage in OA cartilage (31), this study suggests the potential utility of inhibiting PUMA expression as a treatment of OA.

In conclusion, this study provides some novel insights into the apoptotic pathways that are altered in the mitochondria in OA and may aid in the future development of molecular-based diagnostic or therapeutic strategies. The increased mitochondrial PUMA is a relevant finding relative to apoptosis and/or maintenance of a normal intracellular redox balance in chondrocytes. Further studies to elucidate the precise mechanism by which PUMA participates in OA pathogenesis are required.

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