



The modulation of the oxidative stress response in chondrocytes by Wip1 and its effect on senescence and dedifferentiation during *in vitro* expansion

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ABSTRACT

Obtaining a sufficient number of cells *ex vivo* for tissue regeneration, which are appropriate for cartilage repair, requires improved techniques for the continuous expansion of chondrocytes in a manner that does not change their innate characteristics. Rapid senescence or dedifferentiation during *in vitro* expansion results in loss of chondrocyte phenotype and the formation of fibrous cartilage replacement tissue, rather than hyaluronic cartilage, after transplantation. As demonstrated in the current study, wild-type p53-inducible phosphatase (Wip1), a well-established stress modulator, was highly expressed in early-passage chondrocytes, but declined rapidly during *in vitro* expansion. Stable Wip1-expressing chondrocytes generated by microporation were less susceptible to the onset of senescence and dedifferentiation, and were more resistant to oxidative stress. The increased resistance of Wip1 chondrocytes to oxidative stress was due to modulation of p38 mitogen-activated protein kinase (MAPK) activity. Importantly, chondrocytes expressing Wip1 maintained their innate chondrogenic properties for a longer period of time, resulting in improvements in cartilage regeneration after transplantation. Chondrocytes from Wip1 knockout (Wip1^{-/-}) mice were defective in cartilage regeneration compared with those from wild-type mice. Thus, Wip1 expression represents a potentially useful mechanism by which a chondrocyte phenotype can be retained during *in vitro* expansion through modulation of cellular stress responses.

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1. Introduction

Cartilage is an important tissue that forms a flexible cushion-like layer at active joints to alleviate compression and frictional stress. The primary cellular component of cartilage is the chondrocyte, and the flexibility of the tissue is entirely dependent on the level of complexity of the extracellular matrix (ECM), which includes chondrocyte-specific glycoproteins such as collagen II (Col II). Once cartilage is damaged, spontaneous regeneration is limited due to the lack of blood vessels surrounding and infiltrating the cartilage tissue. Therefore, chondrocyte implantation is proposed as

one approach to tissue recovery after cartilage damage [1,2]. As with nearly all cell-based therapies, successful cartilage regeneration requires techniques for the expansion of clinical-grade chondrocytes to obtain a sufficient number of cells for transplantation [3]. To this end, *ex vivo* monolayer cultures of isolated chondrocytes have been widely adopted due, in large part, to their simplicity. However, chondrocytes cultured under monolayer conditions undergo senescence and dedifferentiation, resulting in the loss of chondrocyte properties [4]. This results in the formation of undesirable fibrous cartilage at the transplantation site [5].

Senescence and dedifferentiation of chondrocytes in monolayer cultures is accompanied by fibroblastic morphological changes and decreased expression of chondrocyte-specific glycoproteins such as Col II, proteoglycans, and glycoproteins [5]. As is observed in other cell types, this loss of chondrocyte-specific properties is thought to be due to stress responses triggered in response to the *ex vivo* culture conditions [6,7]. Oxidative stress due to the increased production of reactive oxygen species (ROS) upon abnormal mechanical loading [8], damage, or aging [9] results in the onset of senescence or

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decreased survival. Similarly, exposure to ionizing radiation and the subsequent generation of ROS induces chondrocyte senescence [10]. Antioxidants such as N-acetyl cysteine (NAC) and ascorbic acid delay chondrocyte senescence and degeneration by attenuating ROS insult [10,11]. Alternatively, a reduction in oxygen tension in monolayer cultures induces the re-differentiation of dedifferentiated chondrocytes [12], consistent with the idea that low oxygen tension protects cells from the onset of senescence [13,14]. Oxidative stress in chondrocytes induces telomere genomic instability [15], expression of matrix metalloproteinases (MMPs) [16], and downregulation of Sirt1, a mammalian Sir2 ortholog, through p38 MAPK activation [10]. The latter two phenomena are responsible for the onset of senescence in chondrocytes and other cartilage diseases, respectively.

A variety of environmental stresses (including ROS) and stress responses can lead to permanent senescence through a number of different stress signaling pathways (e.g. the p38MAPK-p16Ink4a or p53-p21CIP pathways) [6,17]. Modulation of stress signaling, therefore, is one way to delay or attenuate the stress response; in the case of chondrocytes, this is the onset of senescence. In fact, this relatively simple approach has been demonstrated in a number of different cell types [16–18]. We previously reported that the “stemness” of human mesenchymal stem cells is prolonged under conditions of oxidative stress by modulating stress signaling thorough the stable expression of wild-type p53-inducible phosphatase-1 (Wip1) gene [7].

Wip1 is encoded by *PPM1D* (for protein phosphatase 1D Mg²⁺-dependent, delta isoform) and is a type 2C protein phosphatase (PP2C). A number of stress mediators such as p53 [19], p38 [20], Chk1/2 [21], ATM [22] and γ -H2AX [23,24] are dephosphorylated and inactivated by Wip1. Through inactivation of stress mediators, Wip1 can modulate stress responses such as growth arrest or cellular senescence under conditions of stress [24].

In this study, we hypothesized that senescence and dedifferentiation of chondrocytes is closely associated to ROS induced stress response. To aim this, we examined whether the modulation of stress modulator Wip1 in chondrocytes was able to successfully retard onset of senescence and dedifferentiation, and subsequently sustain the chondrogenic properties for extended periods of time both *in vitro* and *in vivo*. In addition, we investigated the phenotype of cartilage tissue from Wip1 knockout mice for the understanding of molecular mechanisms underlying the aging process.

2. Materials and methods

2.1. Human chondrocytes isolation and cell culture

Human cartilage tissue was obtained from nasal septum by surgical operations from informed and consenting patients with the approval of the institutional review board of Seoul St. Mary's Hospital. Chondrocytes were isolated from cartilage tissue using enzymatic digestion with phosphate-buffered saline (PBS) containing 0.2% (w/v) bovine serum albumin and 2 mg/ml collagenase type II (Sigma, St. Louis, MO). Undigested tissue was separated from cells using a 40 mm filter; cells were centrifuged, washed at least three times, and re-suspended in culture media. Freshly isolated chondrocytes were either cultured in cell culture plates for expansion or cryopreserved in liquid nitrogen. Cultures were incubated in Dulbecco's Modified Eagle Medium (DMEM, Gibco BRL, Gaithersburg, MD) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco BRL) and 100 units/ml penicillin (Gibco BRL) in humidified air with 5% (v/v) CO₂ at 37 °C. At 80% confluency, cells were harvested by 0.5% Trypsin-EDTA (Invitrogen, Grand Islands, NY) and seeded on culture plates (2 × 10⁴ cells/cm²), which was considered a two-dimensional (2D) culture condition. For three-dimensional (3D) culture, cell suspension was centrifuged at 1200 rpm for 5 min, and the cell pellet (2 × 10⁵ cells/pellets) was cultured in humidified air with 5% (v/v) CO₂ at 37 °C.

2.2. Construction of expression vectors

Full-length human Wip1 (accession number: U78305) was ligated into pcDNA3.1 (Invitrogen) to generate the pcDNA3.1-Wip1/HA expression vector. The PCR amplified products were digested with Xho I. Meanwhile, the sense and antisense full-length coding sequence of Wip1, which was amplified by PCR using sense primer (5'-GGG ATC CCG GCC AGC CGG CC -3') and antisense primer (5'-GCA AAC

ACA AAC AGT TTT CC -3'), was ligated into pcDNA3.1/V5-His-TOPO vector (Invitrogen) following the manufacturer's protocol to construct the mammalian expression vector. The plasmid pEGFP-N3 vector (Clontech, Mountain View, CA) was used for expression of green fluorescent protein (GFP). Constructs were subcloned, and the plasmid cDNAs were purified by Mini plasmid preparation kit (QIAprepSpin, Qiagen, Valencia, CA). All of the newly generated expression vectors in this work were confirmed by nucleotide sequencing analysis.

2.3. Gene delivery using microporation transfection

Chondrocytes were transfected with Mock (empty) and Wip1 plasmid vector using a Microporator (Neon™, Invitrogen). First, sub-confluent human chondrocytes were harvested and washed with PBS. Cells were re-suspended in re-suspension buffer R with a density of 1 × 10⁷ cells/ml, followed by incubation of 100 μ l of cell suspension solution with 5 μ g of plasmid. Subsequently, microporation was performed at a voltage of 1400 for 20 ms with 2 pulses. Following microporation, cells were re-plated into cell culture dishes and placed at 37 °C in a 5% CO₂ humidified atmosphere. Delivery efficiency of the microporator transfection was evaluated by GFP expression and FACS analysis. Twenty-four hours after GFP gene transfection, fluorescence was visualized using a Nikon microscope Eclipse 55i (Nikon, Kanagawa, Japan) and quantified by a BD FACS Calibur system (BD bioscience, San Jose, CA) (Supplementary Fig. S2) [25].

2.4. Reverse transcription-polymerase chain reaction and quantitative real-time PCR analysis

Total RNA was extracted from transfected cells using TRIzol (Invitrogen) and 2 μ g of total RNA was used for cDNA synthesis with RT-PreMix (Bioneer, Daejeon, Korea). PCR was performed with PCR-PreMix (Bioneer) under standard PCR conditions: collagen type II (Col II), collagen type X (Col X) aggrecan (AGG), and GAPDH primers are shown in Table 1. PCR cycles consisted of an initial denaturation step at 94 °C for 5 min, followed by 32 amplification cycles consisting of 30 s of denaturation at 94 °C, 30 s of annealing at 62 °C, and 1 min of extension at 72 °C. Last, a final extension was performed at 72 °C for 10 min. PCR products were analyzed by UV irradiation on a 1.2% agarose gel stained with ethidium bromide. For quantitative real-time PCR analysis, gene-specific primers were designed to amplify Col II, Col X, and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primer pairs are as follows: Col II (5'-CAC GTA CAC TGC CCT GAA GGA-3, 5'-CGA TAA CAG TCT TGC CCC ACT T-3), Col X (5'-ACG CTG AAC GAT ACC AAA TG-3, 5'-TGC TAT ACC TTT ACT CTT TAT GGT GTA-3), and GAPDH (5'-ACA TCG CTC AGA CAC CAT G-3, 5'-TGT AGT TGA GGT CAA TGA AGG G-3). All amplifications were performed in a final reaction mixture (20 μ l) containing 1 final concentration of SYBR supermix, 500 nmol/l of gene-specific primers, and 1 μ l of template, using the following conditions: an initial denaturation at 95 °C for 1 min, followed by 45 cycles of 95 °C for 15 s, 56 °C for 15 s, and 72 °C for 15 s, with a final extension at 72 °C for 5 min. After amplification, the baseline and threshold levels for each reaction were determined using the company's software package (Exicycler™96; Bioneer). For validation of polymerase chain reaction (PCR), amplified products were separated on 1% agarose gels and visualized by ethidium bromide staining.

2.5. Antibodies

The anti-Wip1 antibody was obtained from Bethyl Laboratories (Montgomery, TX). The anti-phosphorylated Rb (p-Rb) antibody was obtained from Cell Signaling Technology (Danvers, MA). The anti-type II collagen (Col II) antibody was obtained from Millipore (Billerica, MA). Alexa 594-conjugated goat anti-mouse and anti-rabbit IgGs antibody was purchased from Molecular Probes (Eugene, OR). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgGs antibody was purchased from Applied Biological Materials Inc. (Cambridge, MA).

Table 1

Nucleotide sequences of primer sets used for type II collagen (Col II), type X collagen (Col X) aggrecan (AGG), and GAPDH in RT-PCR analysis.

Gene	Human primer sequence	Product size (bp)
Type II Collagen (Col II)		
Sense	5'- TTC AGC TAT GGA GAT GAC AAT C -3'	472 bp
Antisense	5'- AGA GTC CTA GAG TGA CTG AG -3'	
Type X Collagen (Col X)		
Sense	5'- AGG GTT ACC AGG TCC AAA AG -3'	553 bp
Antisense	5'- TTC CAG TCC TTG GGT CAT AA -3'	
Aggrecan (AGG)		
Sense	5'- GAA TCT AGC AGT GAG ACG TC -3'	540 bp
Antisense	5'- CTG CAG CAG TTG ATT CTG AT -3'	
GAPDH		
Sense	5'- AGG CCG GTG CTG AGT ATG TCT -3'	529 bp
Antisense	5'- TGC CTG CTT CAC CAC CTT CT -3'	

bp: base pair.

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