Millimeter wave treatment promotes chondrocyte proliferation via G_1/S cell cycle transition

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Abstract. Millimeter waves, high-frequency electromagnetic waves, can effectively alleviate the clinical symptoms in osteoarthritis patients, as a non-pharmaceutical and non-invasive physical therapy regimen. However, the molecular mechanisms of the therapeutic effects of millimeter wave treatment are not well understood. In the present study, the effect of millimeter waves on the G_1/S cell cycle progression in chondrocytes and the underlying mechanism was investigated. Chondrocytes isolated from the knee of SD rats were cultured and identified using toluidine blue staining. The second generation chondrocytes were collected and stimulated with or without millimeter waves for 48 h. Chondrocyte viability was analyzed using the MTT assay. The cell cycle distribution of chondrocytes was analyzed by flow cytometry. mRNA and protein expression levels of cyclin D1, cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) and p21 were detected using real-time PCR and western blotting, respectively. Millimeter wave stimulation was found to significantly enhance chondrocyte viability. Moreover, the percentage of chondrocytes in the G_0/G_1 phase was significantly decreased, whereas that in the S phase was significantly increased. In addition, following millimeter wave treatment, cyclin D1, CDK4 and CDK6 expression was significantly upregulated, whereas p21 expression was significantly downregulated. The results indicate that millimeter wave treatment promotes chondrocyte proliferation via cell cycle progression.

Introduction

Osteoarthritis (OA), the most frequent arthropathy, is characterized by progressive degradation of hyaline articular cartilage and is associated with aging (1). A complex interplay of mechanical, and functional changes of chondrocytes could precipitate the imbalance between matrix anabolic and catabolic processes in articular cartilage and thus the pathogenesis of OA (2,3). Chondroctyes can rapidly respond to changes in the articular microenvironment and regulate the dynamic equilibrium between the anabolism and catabolism of the extracellular matrix (ECM), which plays a crucial role in the maintenance of the cartilage function (4). Therefore, enhancing chondrocyte function might be an efficient treatment to cure or even delay the progression of OA by promoting chondrocyte proliferation.

The cell cycle plays an important role in the influence on chondrocyte function, which takes place in chondrocytes leading to its division and duplication. Cell cycle control is a highly regulated process that involves a complex cascade of events, in which regulation can be realized through a complicated network by cyclins, cyclin-dependent kinases (CDKs) and cyclin dependent kinase inhibitors (CDKI). G_1/S transition, one of the two main checkpoints, is a rate-limiting step in the cell cycle and regulates cell proliferation (5,6). Cyclin D1 is a key cell cycle regulatory protein, which binds to CDK4 or CDK6 to control cell cycle progression from the G_1 to the S phase (7). Meanwhile, p21 competes with cyclin D1 for binding to CDK4 or CDK6 preventing Rb phosphorylation, leading to the block at the G_1 phase (8). Therefore, the G_1/S progression is highly regulated by cyclin D1, CDK4, CDK6 and p21.

Previously we reported that enhancing chondrocyte proliferation via promoting G_2/M transition might be a mechanism by which millimeter waves treat OA (9). Millimeter wave is a high-frequency electromagnetic wave at a wavelength from 1-10 mm and a frequency of 300 million cycles/sec (300 MHz) to 300 billion cycles/second (300 GHz) (10,11). The millimeter waves within living organisms generate $0.5x10^{10}$-$3x10^{12}$ coherent oscillations during metabolism, which can be absorbed by biological tissues through resonance to produce a non-thermal biological effect (12). Millimeter wave treatment has been used in the treatment of OA, as is convenient, safe and non-invasive. To further research the possible molecular mechanisms of enhancing chondrocyte proliferation, in this study we evaluated the effect of millimeter waves on the cell cycle of cultured primary chondrocytes. We found that

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millimeter waves facilitated chondrocyte proliferation in a dose- and time-dependent manner. In addition, millimeter wave treatment promoted chondrocyte G_1/S transition, which was accompanied by upregulating the expression of cyclin D1, CDK4 and CDK6 as well as downregulating the expression of p21. Our finding suggests the promotion of the chondrocyte cell cycle through the G_1 to S phase transition.

Materials and methods

Reagents. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and trypsin-EDTA were all purchased from HyClone (USA). Type-II collagenase and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased form Sigma-Aldrich (USA). The SYBR Florescence Quantization kit, bis-benzimidazole dye Hoechst 33258 and TRIzol reagent were purchased from Invitrogen (USA). Primer synthesis was performed by Shanghai Sangon Biological Engineering Technology Services Limited (China). Rabbit anti-rat cyclin D1, CDK4, CDK6, p21, β-actin HRP secondary goat anti-rabbit antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Polyvinylidene fluoride (PVDF) membrane was purchased from Millipore (NJ, USA). The cell cycle assay kit was provided by Becton-Dickinson (San Jose, CA, USA). Toluidine blue was obtained from the Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Animals. Thirty-six, 4-week-old male SD rats of specific pathogen-free (SPF) were purchased from the Shanghai Slack Laboratory Animal Co. (Shanghai, China). The qualified number of the rats was SCXK (Shanghai) 2007-0005. Six rats were allocated for primary culture and identification of chondrocytes experiments, and 6 rats for millimeter wave stimulation experiments. All experiments were performed in triplicate. The care and use of the laboratory animals complied with guidance suggestions for the Care and Use of Laboratory animals 2006 by the Ministry of Science and Technology of the People's Republic of China.

In vitro culture of chondrocytes. The SD rats were sacrificed and soaked in 75% alcohol for 5 min. The knee joint was separated, the cartilages removed under sterile conditions. The cartilage was rinsed in PBS three times. Cartilage pieces (1 mm^3) were placed in dishes containing 0.2% Type-II collagenase and transferred to a 37˚C incubator. The supernatant was collected every 60 min, and centrifuged at 800 rpm for 5 min to obtain a cell pellet. This step was repeated four times. The cells were re-suspended in DMEM complete culture medium (containing 10% (v/v) FBS, 50 mg/l vitamin C, 100 U/ml penicillin, and 100 μg/ml streptomycin). The cells were then filtered through 200-mesh stainless steel filters and counted with a blood cell count plate to adjust the concentration of the cell suspension to (2-3)x10^5/ml. The cells were seeded in flasks and cultured in 37˚C, 5% CO_2 incubator. The primary cultured cells were observed under an inverted microscope and passaged when they reached 80% confluency. The second generation of chondrocytes was identified using toluidine blue staining. Chondrocytes used in this study were subjected to no more than the second passage cells.

Millimeter wave exposure. The stability of millimeter waves used for cell exposure is a very important issue. The KFA-100A millimeter wave therapeutic instrument was manufactured by Zhongcheng Kangfu Technology Co., Ltd. (Beijing, China) at wavelengths of 7.5-10.0 mm, a power density of 4 mw/cm^2, and a radiation area of 33.2±3x45.6±4 mm. The millimeter wave was propagated from the top of the Petri dish, and the treatment head was placed at a distance sufficiently near the Petri dish (Fig. 1). Thus, concurrently to each millimeter wave experiment, cells were placed in a 37˚C humidified incubator. Moreover, control cells were kept in the same experimental conditions, but they were not exposed to millimeter waves.

Evaluation of cell viability by MTT assay. The passage 2 chondrocytes were cultured in 96-well plates at a concentration of 1x10^4 cells/well in 100 μl 10% FBS DMEM and cell viability was assessed by the MTT colorimetric assay. The chondrocytes were cultured for 24 h and starved for 24 h in serum-free DMEM medium, and treated with or without millimeter waves for 4 times for 15, 30 and 60 min every 12 h or with millimeter waves for 60 min every 12 h for 12, 24, or 48 h. After treatment, 10 μl MTT (5 mg/ml in phosphate-buffered saline, PBS) was added to each well, and the samples were incubated at 37˚C for 4 h. The purple-blue MTT formazan precipitate was dissolved in 100 μl DMSO and the cells were shaken for 10 min. The absorbance was measured at 490 nm using an ELISA reader (BioTek, Model EXL800, USA).

Observation of chondrocyte morphological changes. Chondrocytes were cultured in 35-mm Petri dishes at a concentration of 5x10^4 cells. The cells were cultured for 24 h and starved for 24 h in serum-free DMEM medium, and treated with millimeter waves for 4 times and for different durations. Chondrocyte morphology was observed using a phase-contrast microscope (Olympus, Japan). The photographs were captured at a magnification of x200.

After treatment with or without millimeter waves, chondrocytes were fixed in 4% neutral formaldehyde for 30 min, washed with PBS for 5 min, than stained with 1% safranin O solution for 5 min and stained with 10 μM Hoechst 33258 at 37˚C for 30 min in the dark, respectively. The cells stained with safranin O were de-colored in 0.1% acetic acid for several seconds, washed with distilled water, dehydrated using 95% ethanol, treated with xylene, dried and sealed for an observation. Cell morphology was observed using a phase-contrast microscope (Olympus). The photographs were captured at a magnification of x200. The bis-benzimidazole dye Hoechst 33258 exhibits high fluorescence upon binding to the double-stranded DNA. Nuclear shape and chromosomal structure can be visualized and counted by staining nuclear DNA with Hoechst 33258 by fluorescent phase-contrast microscopy (Olympus). Photographs of cells were captured at a magnification of x200.

Detection of cell cycle by flow cytometry analysis with PI staining. Chondrocytes were seeded into 35-mm Petri dishes at a density of 5x10^4 cells. The chondrocytes were cultured for 24 h and starved for 24 h in serum-free DMEM medium and were treated for different durations of millimeter waves for 4 times. After millimeter wave treatment, the cell cycle

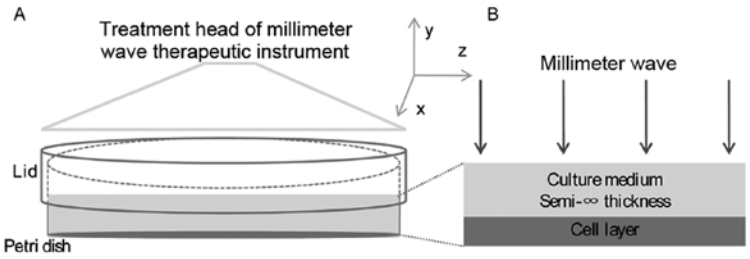

Figure 1. Method of millimeter wave treatment of chondrocytes. (A) Schematic representation of millimeter wave far field exposure set-up used for testing the millimeter wave-promoted effects on chondrocyte proliferation *in vitro* on 35-mm Petri dishes. (B) Enlargement of the Petri dish showing all the dielectric interfaces through which the millimeter wave propagates. The multiple stratified dielectric systems sketched were used to model the reflection properties of chondrocytes cultured in the Petri dish.

of chondrocytes was determined by flow cytometric analysis using a fluorescence-activated cell sorting FACSCalibur cytometer and a cell cycle assay kit (Becton-Dickinson). PI staining was performed according to the manufacturer's instructions. The percentage of cells in the different phases was calculated by the ModFit software, and the cell numbers in the G_0/G_1, S and G_2/M phases were obtained.

RNA extraction and real-time PCR analysis. Chondrocytes were seeded into 35-mm Petri dishes at a concentration of $5x10^4$ cells, and treated for different durations of millimeter waves 4 times. Total-RNA from chondrocytes was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Oligo(dt) -primed RNA (1 μg) was reverse transcribed with SuperScript II reverse transcriptase (Promega) according to the manufacturer's instructions. The obtained cDNA was used to determine the mRNA amount of cyclin D1, CDK4, CDK6 and p21 by real-time PCR with the SYBR Fluorescence Quantization kit (Invitrogen). β-actin was used as an internal control. The primers used for real-time PCR were as follows: cyclin D1 forward, 5'-AAT GCC AGA GGC GGA TGA GA-3' and reverse, 5'-GCT TGT GCG GTA GCA GGA GA-3', 189 bp; CDK4 forward, 5'-GAA GAC GAC TGG CCT CGA GA-3' and reverse, 5'-ACT GCG CTC CAG ATT CCT CC-3', 109 bp; CDK6 forward, 5'-TTG TGA CAG ACA TCG ACG AG-3', and reverse, 5'-GAC AGG TGA GAA TGC AGG TT-3', 151 bp; p21 forward, 5'-ATG TCC GAT CCT GGT GAT GT C-3' and reverse, 5'-CGG CTC AAC TGC TCA CTG TC-3', 92 bp; β-actin forward, 5'-CGT TGA CAT CCG TAA AGA CC-3' and reverse, 5'-GGA GCC AGG GCA GTA ATC T-3', 108 bp. The PCR conditions for all investigated genes had an initial denaturation step at 94˚C, 10 min followed by 45 cycles of denaturation for 30 sec at 94˚C, annealing for 30 sec at 60˚C (for cyclin D1 and CDK4) and at 55˚C (for β-actin, CDK6 and p21), followed by extension of 30 sec at 72˚C. A final extension at 72˚C for 10 min was applied to all the reactions. Fold-changes in the expression of the genes were estimated based on the comparative $2^{-\Delta\Delta Ct}$ method, using the untreated chondrocytes as control.

Western blot analysis. Chondrocytes ($5x10^4$) were seeded into 35-mm Petri dish and treated with or without millimeter waves. The chondrocytes were treated with a lysis buffer for 30 min on ice. Then the lysate was centrifuged for 30 min at 13,000 rpm at 4˚C. Protein concentrations of supernatants were determined with the Bradford protein assay as described. The proteins were separated on 12% SDS-PAGE gels using 110 V for 2 h and transferred to a PVDF membrane (Invitrogen). The membrane was blocked with blocking solution for 2 h at room temperature and was incubated in antibody solution at the concentration of 1:1,000 for cyclin D1 and p21, and 1:500 for CDK4 and CDK6 overnight at 4˚C. β-actin (1:1,000) was also measured as an internal control for protein loading. All antibodies were from Santa Cruz Biotechnology, Inc. Then horseradish peroxidase-linked secondary antibodies were applied for 2 h in blocking solution. Finally, bands were quantified by scanning densitometry (170-8070, Molecular Imager ChemiDoc XRS System, Bio-Rad, USA). Protein concentrations were determined using the Tocan 190 protein assay system and normalized to β-actin in the sample.

Transmission electron microscopy of chondrocytes. After treatment with or without millimeter waves, chondrocytes were digested and collected. The cells were centrifuged and the pellets were fixed with 3% glutaraldheyde and 1.5% paraformaldehyde solution (pH 7.3) at 4˚C for 24 h, rinsed twice with PBS, postfixed with 1% osmic acid and 1.5% potassium hexacyanoferrate (II) solution (pH 7.3) and incubated at 4˚C for 2 h. Subsequently, the cell suspensions were dehydrated in a graded series of alcohol for 5 min each. The dehydrated pellets were embedded three times with propylene oxide for 1 h each and infiltrated with a resin/propylene oxide mixture at a 1:1 ratio for 2 h and then with resin only for 12 h at room temperature. The inclusion was made with epoxide resin 618 and Araldite and the polymerization was performed at 60˚C for 48 h. Ultrathin sections (80 nm) were stained with uranyl acetate and counterstained with lead citrate for 5 min. The stained ultrasections were examined with the H-7650 transmission electron microscope (Hitachi, Japan).

Statistical analysis. All data are expressed as the means of three determinations and were analyzed using the SPSS package for Windows (version 13.0). Statistical analysis of the data was performed with Student's t-test and ANOVA. Differences with $P<0.05$ were considered statistically significant.

Results

Morphological observation and identification of chondrocytes. After isolated from the knee articular cartilage of SD rats, chondrocytes were completely digested with 0.2% type II collagenase. The primary chondrocytes took 8 days to

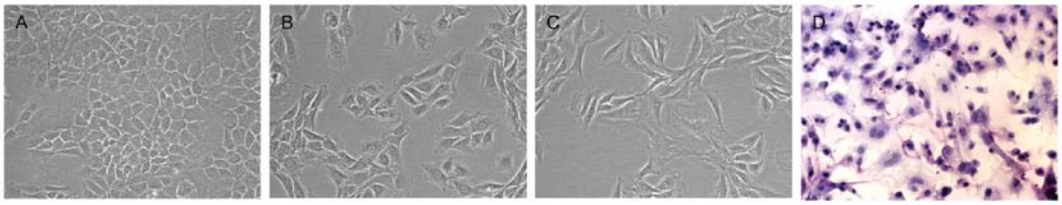

Figure 2. Morphological observation and identification of chrondrocytes (x200). (A) The primary chondrocytes cultured for 8 days were spindle-shapped and polygonal in an irregular 'flagstone' stereo shape. (B) Second generation chondrocytes cultured for 3 days. (C) Third generation chondrocytes cultured for 3 days. (D) Second generation chondrocytes cultured for 3 days, stained with toluidine blue.

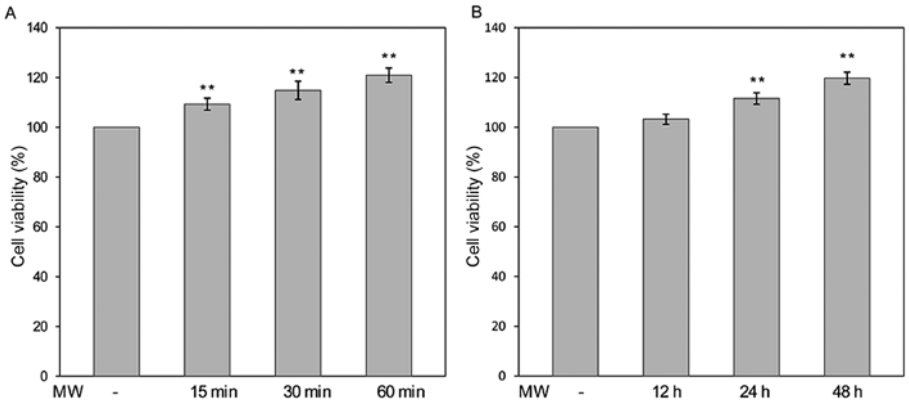

Figure 3. Effect of millimeter wave treatment on chondrocyte viability. (A) Chondrocytes were treated with or without millimeter waves 4 times for 15, 30 or 60 min every 12 h. (B) Chondrocytes were treated with millimeter waves for 60 min every 12 h for 12, 24 or 48 h. Chondrocyte viability was determined by the MTT assay. The data were normalized to the viability of untreated cells (100%). Data are averages ± SD (error bars), **P<0.01, significant vs. untreated cells.

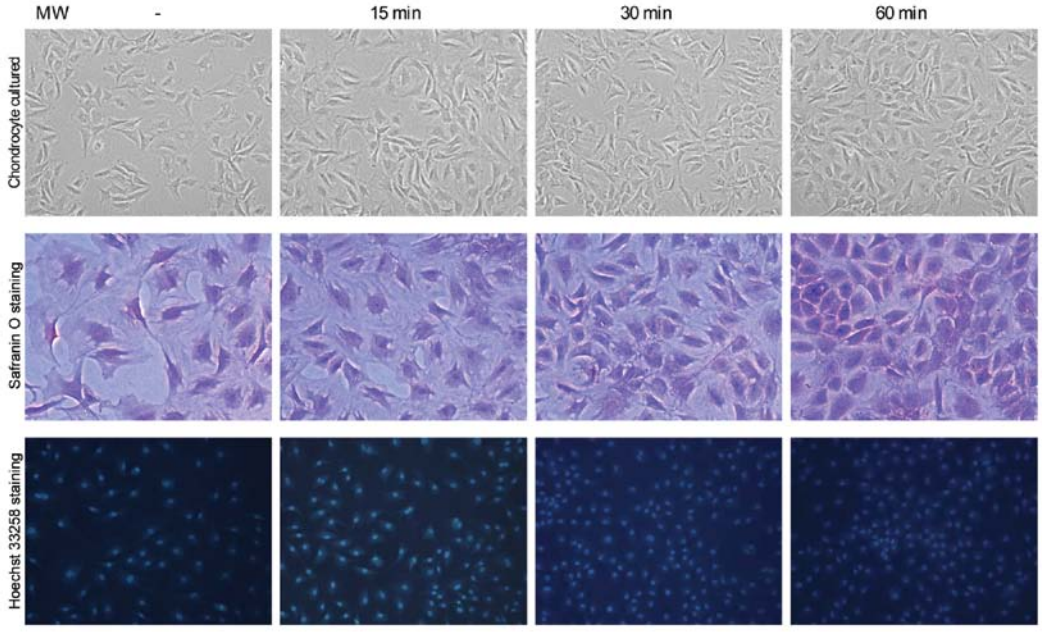

Figure 4. Effect of millimeter wave treatment on the morphological changes of chondrocytes (x200). Chondrocytes were treated with or without millimeter waves 4 times for 15, 30 or 60 min every 12 h. The morphological changes of cultured and safranin O-stained chondrocyte were observed using phase-contrast microscopy. The morphological changes of chondrocytes were also observed with Hoechst 33258 staining by fluorescent phase-contrast microscope.

proliferate into a fusiform overlapping monolayer with typical chondrocytic morphology, i.e., an irregular 'flagstone' stereo shape (Fig. 2A). The second and third generations of chondrocytes grew much faster than the primary cells, which formed fusiform overlapping monolayers within 4-5 days. However, the morphology of the third generation of chondrocytes was changed to a certain extent, whereas the second generation chondrocytes kept the typical morphology (Fig. 2B and C). Toluidine blue can interact with proteoglycan and form a purplish-red multimer, which is a classic method for identification of chondrocytes.

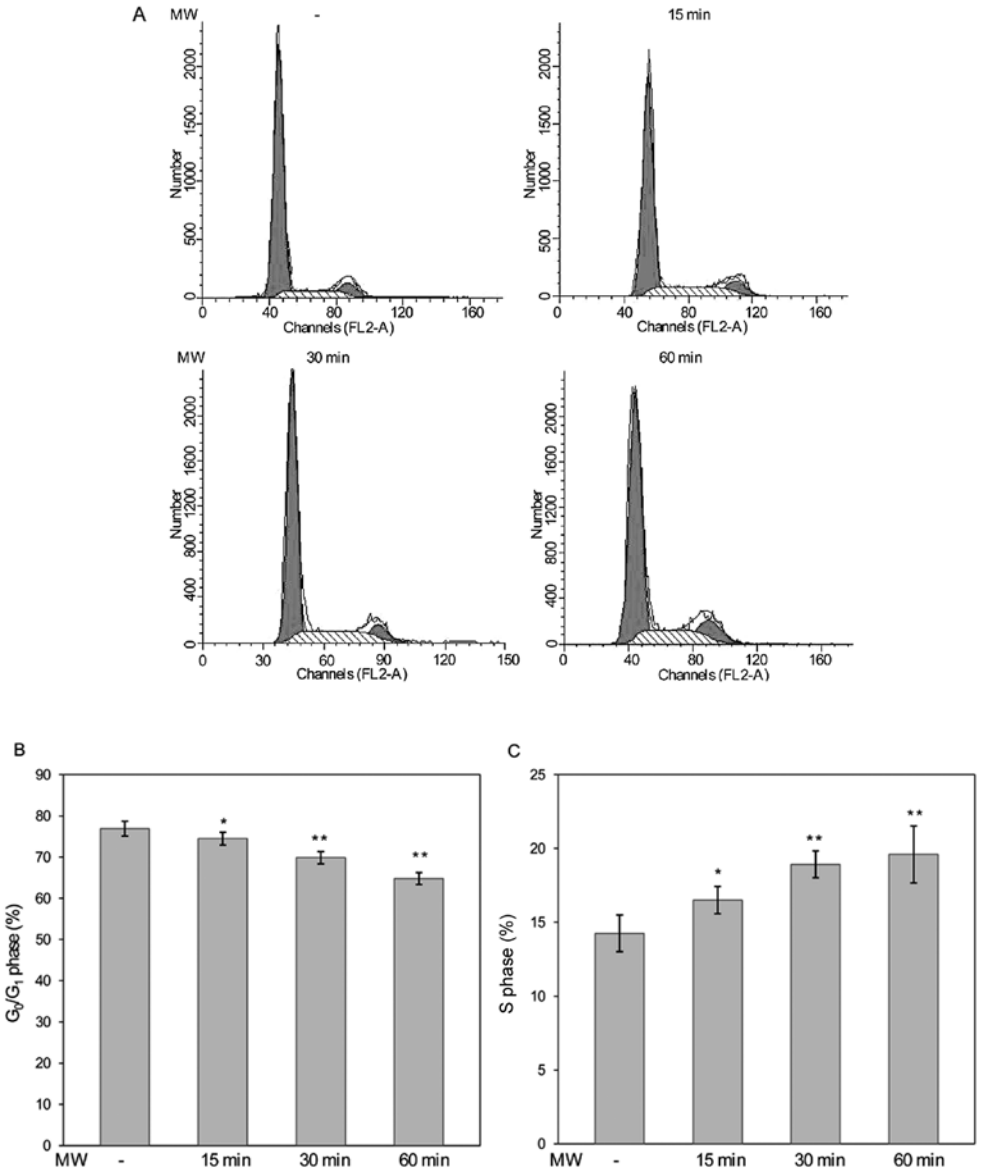

Figure 5. Effect of millimeter wave treatment on the cell cycle of chondrocytes. (A) After treatment with millimeter waves, chondrocytes were collected and stained with PI staining followed by FACS analysis. (B) The percentage of chondrocytes in G_0/G_1 phase cells after treatment with millimeter waves. (C) The percentage of chondrocytes in S phase cells after treatment with millimeter waves. The data shown are averages ± SD (error bars); *P<0.05, **P<0.01, significant vs. untreated cells.

The proteoglycan is a major element of the ECM produced by chondroctyes. The second generation of chondrocytes were cultured 3 days with toluidine blue staining, and the purplish red metachromatic granules were observed both in the cytoplasm and in the extracellular space (Fig. 2D). Therefore, we used the second generation of chondrocytes in the subsequent experiments.

Effect of millimeter waves on chondrocyte viability. The effect of millimeter waves on the viability of chondrocytes was determined by the MTT assay. As shown in Fig. 3, treatment for 4 times with 15, 30 and 60 min/12 h of millimeter waves, respectively increased cell viability by 9.27±2.41, 14.80±3.64 and 20.92±2.87%, and treatment with 60 min/12 h of millimeter waves for 12, 24 and 48 h respectively increased cell viability by 3.24±2.06, 11.57±2.30 and 19.73±2.42%, compared to untreated cells (P<0.01 or P<0.05), suggesting that millimeter wave treatment promotes the growth of chondrocytes in a dose- and time-dependent manner.

Effect of millimeter waves on chondrocytes morphological changes. The effect of millimeter waves on the morphological changes of chondrocytes was evaluated by phase-contrast microscopy, since chondrocyte morphology in culture is indicative of the healthy status. As shown in Fig. 4, untreated chondrocytes were sparsely distributed, whereas treatment with various durations of millimeter wave treatment significantly increased their cell number, suggesting that millimeter wave treatment promotes the growth of chondrocytes. Safranin O staining for proteoglycan was intense around chondrocytes with millimeter wave treatment, indicating high proteoglycan content. Hoechst 33258 staining for the double-stranded DNA displayed a weak fluorescence in normal chondrocytes higher than that of untreated cells, demonstrating that millimeter

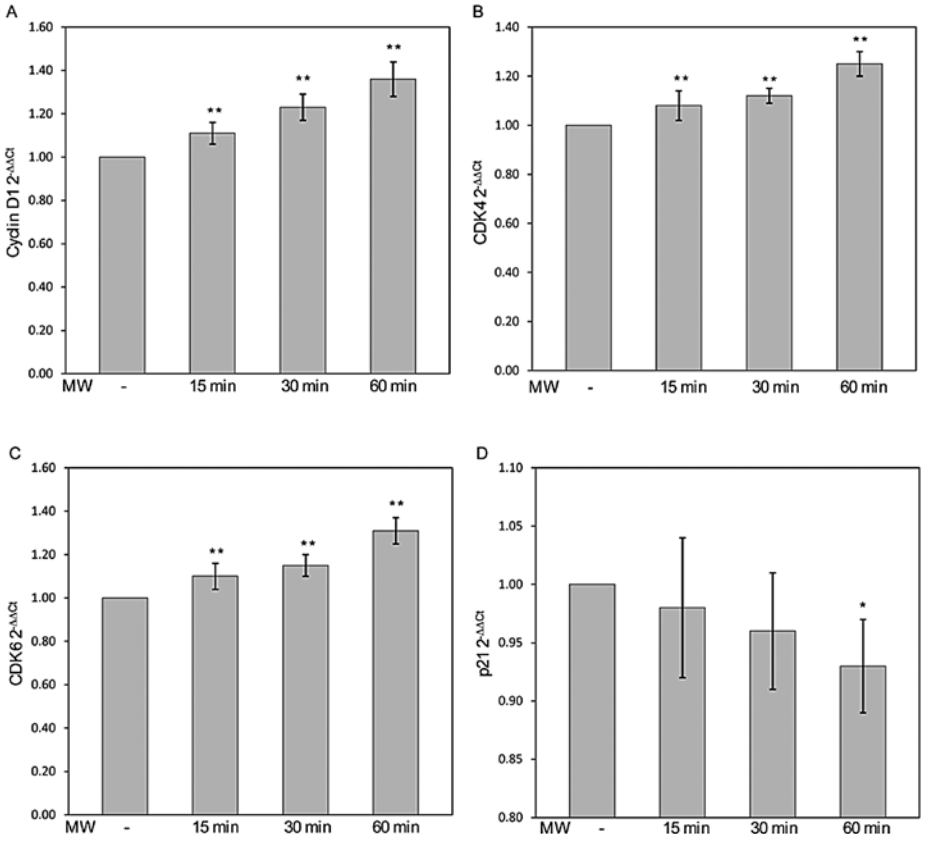

Figure 6. Effect of millimeter wave on the mRNA expression of cyclin D1, CDK4, CDK6 and p21 in chondrocytes. (A) The mRNA expression of cyclin D1 in millimeter wave-treated and untreated cells. (B) The mRNA expression of CDK4 in millimeter wave-treated and untreated cells. (C) The mRNA expression of CDK6 in millimeter wave-treated and untreated cells. (D) The mRNA expression of p21 in millimeter wave-treated and untreated cells. Quantification of real-time PCR analysis, the data shown are averages ± SD (error bars); *P<0.05, **P<0.01, significant vs. untreated cells.

wave treatment enhances the number of chondrocytes and does not induce cell apoptosis.

Effect of millimeter waves on the cell cycle of chondrocytes. Cell cycle plays an important role in chondrocytes leading to its division and duplication. Moreover, the G_1/S transition is one of the two main checkpoints used by the cell to regulate the progression of the cell cycle. Once the checkpoint late in G_1 phase is passed, further progression through the cell cycle occurs with little or no interference from extracellular stimuli followed by the decision to continue cell division. To determine the mechanism of the pro-proliferative activity of millimeter waves, we examined its effect the G_1 to S transition in chondrocytes via PI staining followed by FACS analysis. As shown in Fig. 5, after treatment for the percentage of G_0/G_1 phase cells following treatment for 15, 30 and 60 min of millimeter waves for 4 times, was 74.48±1.54, 69.84±1.46 and 64.80±1.47%, all of which were significantly lower than that of untreated cells (76.90±1.79%; P<0.01 or P<0.05). Consistently, the percentage of S-phase cells showed an opposite trend after millimeter wave treatment. Taken together, it is suggested that millimeter wave treatment can enhance cell cycle of chondrocyte by promoting the cell cycle G_1 to S transition.

Effect of millimeter waves on expression of cyclin D1, CDK4, CDK6 and p21. It is well-known that cyclin D1, CDK4, CDK6 and p21 proteins are key regulators of the G_1 phase progression. The cyclin D1-CDK4/6 complexes regulate mitosis and enhance cell proliferation, whereas p21 inhibits the activity of the cyclin D1-CDK4/6 complexes. To further explore the mechanism by which millimeter waves regulates the cell cycle, we analyzed the mRNA and protein expression levels of cyclin D1, CDK4, CDK6 and p21 after millimeter wave treatment using real-time PCR and western blotting, respectively. The results of the real-time PCR assay showed that millimeter wave treatment significantly increased cyclin D1, CDK4, CDK6 and reduced p21 mRNA expression in chondrocytes (P<0.01 or P<0.05) (Fig. 6); and the protein expression pattern of cyclin D1, CDK4, CDK6 and p21 was similar to that of their respective mRNA level (Fig. 7).

Effect of millimeter waves on the ultrastructure of chondrocytes. The chondrocytes morphology was either round, oval, or polygonal, and the cell nucleus was irregular in shape with lobes, clear nucleoli and evenly distributed chromatin (Fig. 8A). After treatment with millimeter waves, the rough endoplasmic reticulum (ERr) of chondrocytes became more abundant (Fig. 8B), a few chondrocytes demonstrated an increase in condensation of the chromosomes and a disappearance of the nuclear membrane, indicating that these cells were in typical mitosis phenomena (Fig. 8C and D).

Discussion

Millimeter wave treatment has been used as a non-medicinal, non-invasive physical therapeutic regimen for alleviating the

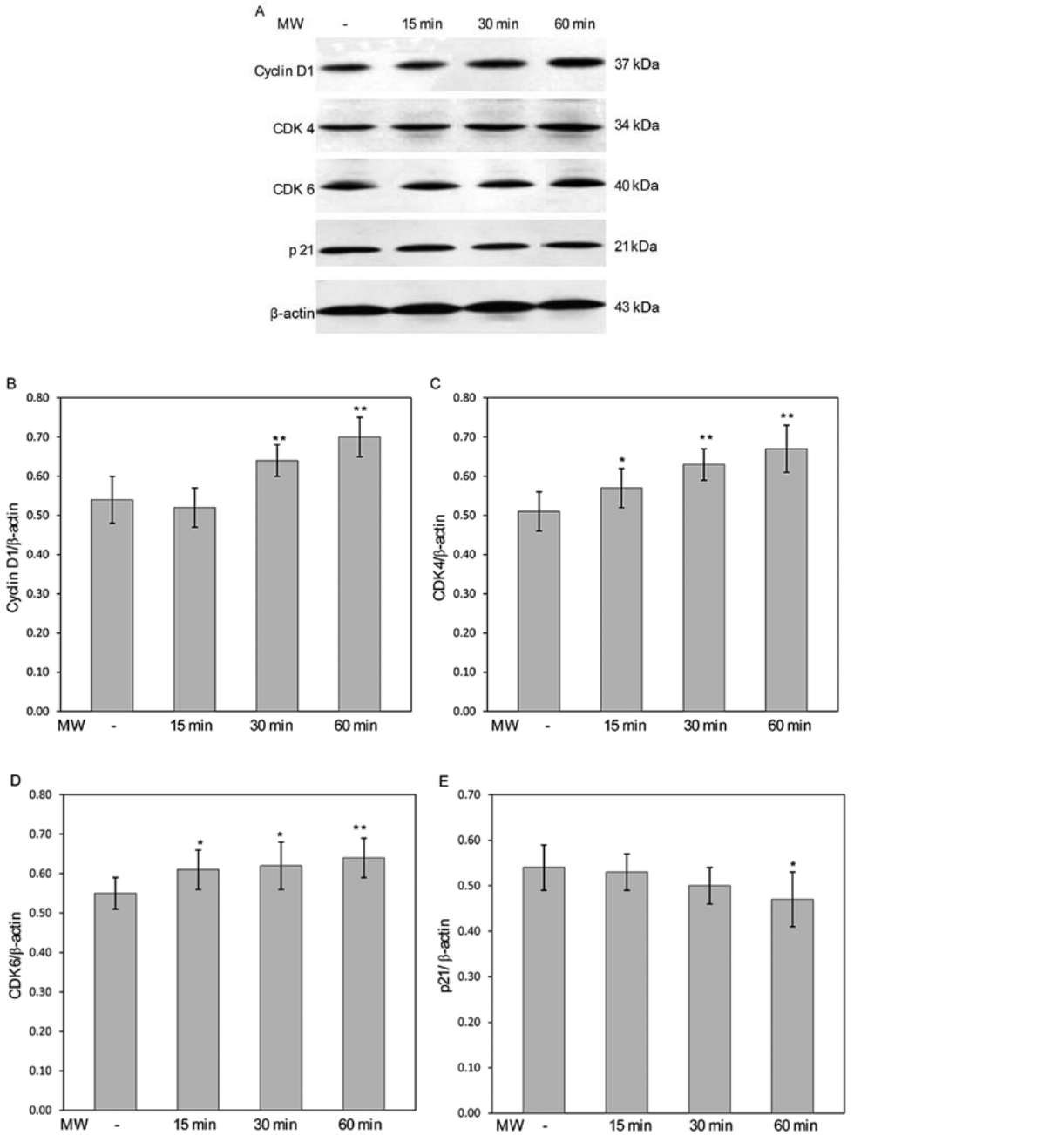

Figure 7. Effect of millimeter wave treatment on the protein expression levels of cyclin D1, CDK4, CDK6 and p21 in chondrocytes. (A) The protein expression levels of cyclin D1, CDK4, CDK6 and p21 were analyzed by western blotting. β-actin was used as the internal controls for the western blotting assays. (B) The protein expression levels of cyclin D1 in millimeter wave-treated and untreated cells. (C) The protein expression levels of CDK4 in millimeter wave-treated and untreated cells. (D) The protein expression levels of CDK6 in millimeter wave-treated and untreated cells. (E) The protein expression levels of p21 in millimeter wave-treated and untreated cells. Quantification of western blotting analysis, the data shown are averages ± SD (error bars); *P<0.05, **P<0.01, significant vs. untreated cells.

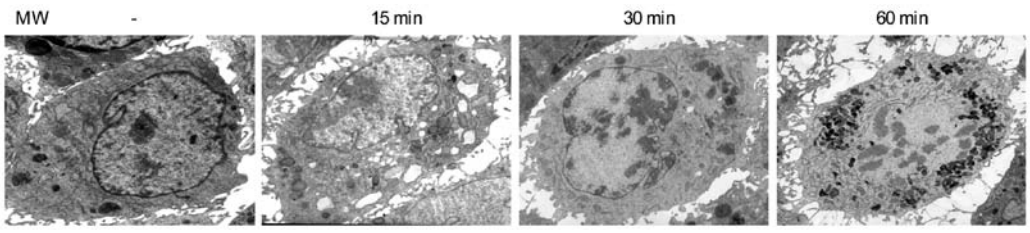

Figure 8. Effect of millimeter wave treatment on the ultrastructural changes of chondrocytes (x6,500). (A) Untreated chondrocytes with a rounded morphology. Some rough endoplasmic reticulum (ERr) and mitochondria are observed within the cytoplasm. The nucleus (N) contains either heterochromatin or euchromatin. (B) The millimeter wave-treated chondrocytes are round with some corrugations and microvilli. The cytoplasm contains a higher number of mitochondria, and the ERr has expanded. (C and D) Cells treated with millimeter wave show typical mitosis phenomena, an increase in condensation of the chromosomes and a disappearance of the nuclear membrane.

clinical symptoms in OA patients. Millimeter waves can be transferred to interfere with signal transduction in the organism and effectively and dynamically regulates cellular metabolism via producing a non-thermal biological effect, since the energy of millimeter waves can be absorbed by biological tissues through resonance (13,14). Our study demonstrated that millimeter wave treatment promotes the cell cycle G_1/S transition in chondrocytes via upregulating the expression of cyclin D1, CDK4 and CDK6, and downregulating the expression of p21, which may be one important molecular mechanisms of the therapeutic effect of millimeter waves.

Chondrocytes are the only cell type present in mature cartilage, which is responsible for extracellular signals and regulates the maintenance of cartilage homeostasis. Therefore, the functional changes of chondrocytes play an important role by contributing to the degradation of the articular cartilage and thus to the pathogenesis of OA (15,16). Several studies have reported that there is a very low proliferative activity in osteoarthritic chondrocytes, which might be an efficient treatment to cure or even delay the progression of OA by promoting chondrocyte proliferation (17,18). Therefore, the present study has focused on chondrocyte proliferation and extends the clinical observations of the potential anti-cartilage degenerative effect of millimeter waves and establishes a scientific foundation for further research.

The cell cycle plays an important role in the influence on division and duplication of chondrocyte, which can be divided into four phases: G_1 (gap 1), S (synthesis), during which DNA is replicated, G_2 and M (mitosis), in which cell division occurs (19,20). The DNA content of cells is a constant parameter that varies over the progression of the cell cycle. The G_1/S transition is one of the two main checkpoints used by a cell to regulate the progression of the cell cycle, and following that a chondrocyte can pass through the S phase and complete cell division (21). Our MTT data showed that millimeter waves promoted chondrocyte viability in a dose- and time-dependent fashion. To investigate the mechanism of how millimeter wave treatment enhances chondrocyte viability, we used FACS analysis with PI staining to examine the change in the cell cycle after millimeter wave treatment, the results showed that the percentage of chondrocytes in the G_0/G_1 phase was significantly reduced and the percentage proportion of chondrocytes in S phases was significantly increased, indicating that millimeter wave treatment causes progression of the cell cycle of chondrocytes *in vitro*, thus promoting chondrocyte proliferation.

Progression through the G_1 phase of the cell cycle is tightly regulated by successive activation of D-type cyclins and CDKs (22). CDKs are expressed and active in a cell cycle phase-specific manner, such that CDK4 and CDK6 activity is necessary for progression through the G_1 phase (23). Cyclin D1 forms complexes with CDK4 or CDK6, which play an important role in the G_1/S transition by phosphorylating Rb (24). As a consequence of Rb phosphorylation, E2F is released from the Rb/E2F complexes and then triggers cell progression from the G_1 to S phase (25). During the progression of the cell cycle, CDK activity can be blocked by binding of CDKIs. The important CDKIs include p21, a potent inhibitor protein of CDK/cyclin complexes, which plays an important role in G_1 and G_2 phase arrest (26). Therefore, differential expression of the cell cycle regulatory factors including cyclin D1, CDK4, CDK6 and p21 may regulate the G_1/S transition in chondrocytes. In this study, we demonstrated that millimeter wave treatment enhances cyclin D1, CDK4 and CDK6 mRNA expression and reduces p21 mRNA expression in chondrocytes. This indicates that millimeter wave treatment results in progression of chondrocytes from the G_1 to the S phase by affecting cyclin D1, CDK4, CDK6 and p21 at the transcriptional level. We further studied the role of millimeter waves on the expression of proteins involved in cyclin D1, CDK4, CDK6 and p21. The results showed that millimeter wave treatment upregulates cyclin D1, CDK4 and CDK6 protein expression and downregulates p21 protein expression, which is in accordance with the pattern of their mRNA expression.

In conclusion, our data demonstrate that millimeter wave treatment promotes chondrocytes from the G_1 to the S phase via upregulating the expression of cyclin D1, CDK4 and CDK6 and downregulating the expression of p21, suggesting that millimeter waves may be a potential novel therapeutic method for the treatment of OA. There remains much to study about millimeter wave treatment, especially given the potential for cross-reactivity and unintended consequences when combined with anti-cartilage degeneration agents.

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