Electroacupuncture promotes chondrocyte proliferation via accelerated G_1/S transition in the cell cycle

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Abstract. The aim of the present study was to investigate the effects of electroacupuncture (EA) on the proliferation of chondrocytes and the molecular mechanism(s) involved. Passage 2 chondrocytes were randomly divided into four groups and treated with EA or nocodazole. After treatment, cell proliferation was determined using an MTT assay and DNA staining followed by FACS. The mRNA expression levels of cyclin D1, cyclin-dependent kinase (CDK)4, CDK6, phosphorylated retinoblastoma (pRb) and P16 were detected by RT-PCR, and the protein levels of cyclin D1, CDK4, CDK6, pRb and P16 were detected by western blotting. EA treatment significantly increased cell viability in a time-dependent manner and decreased the number of G_0/G_1 and G_2/M phase chondrocytes and increased the number of S phase cells. The mRNA and protein levels of cyclin D1, CDK4, CDK6, (p)Rb and P16 consistently demonstrated a reverse trend with the levels in the chondrocytes treated with nocodazole. The expression levels of cyclin D1, CDK4, CDK6 and Rb were higher in chondrocytes receiving EA treatment when compared to levels in the untreated cells while expression of P16 was lower. In conclusion, EA treatment promotes chondrocyte proliferation via promotion of G_1/S checkpoint transition in the cell cycle dependent on the activity of the P16-cyclin D1-CDK4/6-pRb pathway and this may, in part, explain its clinical effect in the treatment of osteoarthritis.

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Introduction

Osteoarthritis (OA) is characterized by a basic pathology of cartilage degeneration caused by the mutual influence of mechanical and biological factors. The balance of catabolism and anabolism within chondrocytes helps to maintain the structural and functional integrity of the extracellular cartilage matrix (ECM). In the early stages of OA development, cartilage tissues show self-repair activity, the volume of chondrocytes increases and the synthesis of proteoglycan accelerates. In the late stage of OA, the balance is broken, and the damaging effect of inflammation becomes more dramatic. Chondrocytes can rapidly respond to changes in the microenvironment of the joint and regulate the dynamic equilibrium between the degradation and synthesis of the ECM (1-4). Therefore, the functional changes in chondrocytes play important roles in the degeneration of joint cartilage, and chondrocyte proliferation is one of the important factors contributing to the maintenance of cellular function.

The cell cycle is composed of four different phases, G_1, S, G_2 and M, which regulate cell proliferation, differentiation and apoptosis, essential to cell life. The G_1/S checkpoint that exists at the end of G_1 stage is the key point of intracellular and extracellular signaling which integrates in the nucleus, then stimulates S phase cells to begin a new round of proliferation, differentiation, death or exit the cell cycle into the G_0 phase (5,6). Cell cycle progression involves the activity of cyclin-dependent kinases (CDKs), which are complexes of cyclin and CDK proteins. The G_1/S transition of the cell cycle is dependent on the activity of cyclin D1-CDK4/6. Once activated, these CDK complexes phosphorylate retinoblastoma (Rb) and related family members and release sequestered E2F transcription factors, thereby promoting the transcription of genes required for progression through the cell cycle. Such progression is under the control of CDK inhibitors (CDKIs), such as P16, which cause transient or permanent cell cycle arrest in cells carrying DNA damage. Cell cycle transitions are tightly regulated, and changes in the expression of CDKs or CDKIs may lead to exacerbated cell proliferation (7,8).

Acupuncture, which has been used for the treatment of various types of diseases in Eastern countries for thousands

of years, is currently gaining acceptance as an alternative medicine in Western countries (9,10). Electroacupuncture (EA) is a modified acupuncture technique that utilizes electrical stimulation. Previous studies have demonstrated that EA has therapeutic effects on chondral defects including knee osteoarthritis (11-13) and produces cytokines with multiple biological activities in various types of diseases (14,15). Our previous study showed that EA can be employed as a novel non-drug-inducing method for the differentiation of BMSCs into chondrocytes (16). However, the effects of EA on the proliferation of chondrocytes, have not yet been reported. To further elucidate the precise mechanism of the potential treatment of OA, in the present study we investigated the effect of EA on the proliferation of chondrocytes and investigated the underlying molecular mechanism.

Materials and methods

Materials and reagents. Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), trypsin and toluidine blue stain were purchased from Hyclone Inc. (Carlsbad, CA, USA); MTT, type-II collagenase, and nocodazole were obtained from Sigma. Cell cycle test kit was obtained from Becton-Dickinson (San Jose, CA, USA). A total protein extraction kit and an ECL kit were purchased from Beyotime Biotech (Nanjing, Jiangsu, China). Cyclin D1, CDK4, CDK6, phospho-Rb, $p16^{INK4a}$ and β-actin antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). DNA primers were synthesized by Sangon Biotech (Shanghai, China).

Animals. Healthy and clean, 4-week-old Sprague Dawley rats (90-110 g weight) of either gender (n=42) were purchased from SLAC Laboratory Animal Inc. (Shanghai, China) [Laboratory Animal Use Certificate no. SCXK(SH)2007-0005] and raised in a sterile environment. All experiments involving the animals complied with Guidance Suggestions for the Care and Use of Laboratory Animals 2006 administered by the Ministry of Science and Technology, China.

Isolation and culture of rat chondrocytes. Rat chondrocytes were isolated and cultured as previously described (17). The cells used in these experiments were counted by a hemocytometer and adjusted to 10^4-10^6 cells/ml.

EA stimulation. The EA stimulation method was in accordance with a previously described method (16). The acupuncture stimulation was applied daily for 0, 15, 30, 60 or 120 min.

Cell treatment and grouping. The passage 2 chondrocytes were seeded at $1x10^5$/ml in a T-25 flask, cultured for 48 h and starved in DMEM medium without FBS for 24 h. The cells were then randomly divided into four groups: control group (normal culture without treatment); experimental group 1 (treated with 50 nM nocodazole for 24 h and receiving no EA treatment); experimental group 2 (receiving no nocodazole treatment and treated with EA stimulator for 60 min) and experimental group 3 (treated with 50 nM nocodazole for 24 h and receiving EA stimulator for 60 min). After treatment, cell proliferation was detected using an MTT assay and DNA staining followed by FACS analysis. Cells were also processed to measure the mRNA levels of cyclin D1, CDK4, CDK6, Rb and P16 by RT-PCR and the protein levels of cyclin D1, CDK4, CDK6, pRb and P16 by western blotting.

MTT assay. The passage 2 chondrocytes were seeded in a 6-well plate at $1x10^5$/ml (2 ml/well) and treated with nocodazole and EA stimulator. The chondrocytes were then washed with PBS once, and 1 ml of a 0.5% MTT solution was added to each well. After incubation at 37˚C for 4 h, wells were emptied, supplied with 1 ml of DMSO and shaken for 10 min. The absorbance was measured at 490 nm using an ELx808™ absorbance microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Detection of cell cycle distribution by flow cytometric analysis with propidium iodide (PI) staining. Chondrocytes were digested with 0.25% trypsin and incubated in 25-ml culture flasks at a density of $1x10^5$ cells/ml in 4 ml of medium for 24 h and starved for 24 h in serum-free DMEM medium and were then treated with or without nocodazole and EA stimulator. After treatment, the cell cycle distribution of the chondrocytes was determined by flow cytometric analysis using a fluorescence-activated cell sorting FACSCalibur cytometer and a cell cycle assay kit. PI staining was performed according to the manufacturer's instructions. The percentage of cells in the different phases was calculated by ModFit LT version 3.0 software, and the numbers of cell in the G_0/G_1, S and G_2/M phases were determined.

RNA extraction and RT-PCR analysis. Total RNA from the treated cells was isolated with TRIzol reagent (Invitrogen). Oligo(dT)-primed RNA (2 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 levels of cyclin D1, CDK4, CDK6, Rb and P16 by PCR with *Taq*DNA polymerase (Fermentas). β-actin was used as an internal control. The primers and the annealing temperature (˚C) used for amplification of cyclin D1, CDK4, CDK6, Rb, P16 and β-actin transcripts were as follows: cyclin D1, sense, 5'-GAC ACC AAT CTC CTC AAC GAC-3' and antisense, 5'-AGA CAA GAA ACG GTC CAG GTA G-3' (216 bp, 55˚C); CDK4, sense, 5'-CCT ACG GAC ATA CCT GGA CAA-3' and antisense, 5'-GAG GCA ATC CAA TGA GAT CAA-3' (404 bp, 55˚C); CDK6, sense, 5'-GTT TCA GCT TCT CCG AGG TCT-3' and antisense, 5'-CGT CAA GCA TTT CAG AAG GAG-3' (469 bp, 55˚C); Rb, sense, 5'-CTT TAT TGG CCT GTG CTC TTG-3' and antisense, 5'-ATT CCA TGA TTC GAT GCT CAC-3' (225 bp, 53˚C); P16, sense, 5'-GCT CTC CTG CTC TCC TAT GGT-3', and antisense, 5'-AGA AGT TAT GCC TGT CGG TGA-3' (268 bp, 54˚C); β-actin, sense, 5'-GGG AAG TGC TGG ATA G-3' and antisense, 5'-GTG ATG TTT CGG ATG G-3' (385 bp, 55˚C).

Western blot analysis. After treatment, cells were lysed, and protein concentrations were determined by BCA assay using bovine serum albumin as a standard. Samples were loaded

Table I. Proliferation of chondrocytes as detected by MTT assay.

Group	OD before treatment	OD after treatment
EA treatment for 0 min	0.328±0.006	0.363±0.005[d,e]
EA treatment for 15 min	0.327±0.009	0.369±0.003[b,f]
EA treatment for 30 min	0.333±0.010	0.376±0.003[a,d]
EA treatment for 60 min	0.332±0.004	0.393±0.006[a,c,e]
EA treatment for 120 min	0.335±0.009	0.379±0.005[a,c]

[a]P<0.01, [b]P<0.05 compared with 0-min EA treatment group; [c]P<0.01, [d]P<0.05 compared with 15-min EA treatment group; [e]P<0.01, [f]P<0.05 compared with 30-min EA treatment group. EA, electroacupuncture. OD, optical density. Values are expressed as means ± SD.

Table II. Effect of nocodazole and EA on the proliferation of chondrocytes detected by MTT assay.

Group	Nocodazole (nM)	EA treatment (min)	OD before treatment	OD after treatment
Control	0	0	0.335±0.010	0.364±0.005[b]
Experimental 1	50	0	0.337±0.008	0.348±0.006[a]
Experimental 2	0	60	0.329±0.005	0.391±0.007[a-c]
Experimental 3	50	60	0.336±0.009	0.370±0.006[b]

[a]P<0.01 compared with control group; [b]P<0.01 compared with experimental group 1; [c]P<0.01 compared with experimental group 3. EA, electroacupuncture. OD, optical density. Values are expressed as means ± SD.

with 20 μg of protein and separated by electrophoresis on 12% SDS polyacrylamide gels. After electrophoresis, proteins were transferred to PVDF membranes in 5% w/v nonfat dry milk using a semidry blotting system, and detected with antibodies against cyclin D1, CDK4, CDK6, pRb, P16 and β-actin and developed with ECL. The intensity of each band was quantified utilizing the Image Lab gel analyzing system and normalized to the band intensity of β-actin.

Statistical analysis. Statistical data are expressed as means ± SD. Statistical analysis of the data was performed with the Student's t-test and one-way analysis of variance (ANOVA). Differences were considered statistically significant at P<0.05.

Results

Optimization of EA treatment time. Without treatment, the passage 2 chondrocytes proliferated normally, and there was no significant difference between the four groups. After treatment with EA, the optical densities (ODs) of cells receiving 60- and 120-min treatments were significantly higher than the ODs of cells receiving 0-min (P=0.000) and 15-min treatments (P=0.001). The OD of cells receiving a 60-min treatment was significantly higher than the OD of cells receiving a 30-min treatment (P=0.000), but no significant difference was noted between the OD of cells receiving a 30-min treatment and the OD of cells receiving a 120-min treatment (P=0.242) (Table I).

Effect of the EA treatment on nocodazole-induced proliferation. The effect of the EA treatment on the nocodazole-induced proliferation was assessed by MTT assay. Prior to treatment, there was no difference between the different groups. After treatment, the OD value of experimental group 1 was significantly lower than the OD values of the control group, experimental group 2 and experimental group 3 (P=0.000). Notably, there were significantly more cells in the experimental group 2 compared with the control group (P=0.001) (Table II).

Cell cycle distribution of the treated chondrocytes as determined by FACS. Before treatment, all cells were starved for 24 h to synchronize the cell cycle stage (Fig. 1A-D). The FACS results showed that the cell cycle distribution of the cells in the different groups was similar. The G_0/G_1 ratio of cells in experimental group 1 was significantly higher than the ratio in the cells of experimental group 2 (P=0.000) following treatment, while the G_0/G_1 ratios of cells in experimental group 2 and 3 were significantly lower than that of the control group (P=0.000, P=0.003). The S ratio of cells in experimental group 1 was significantly lower than the ratios of cells of other groups (P=0.000), and the S ratio of cells in experimental group 2 was significantly higher than the ratios of the control group (P=0.000) and experimental group 3 (P=0.001). The G_2/M ratio of cells in experimental group 1 was significantly higher than those of the control group (P=0.000) and experimental group 2 (P=0.002). The G_2/M ratio of cells in the experimental group 2 was higher

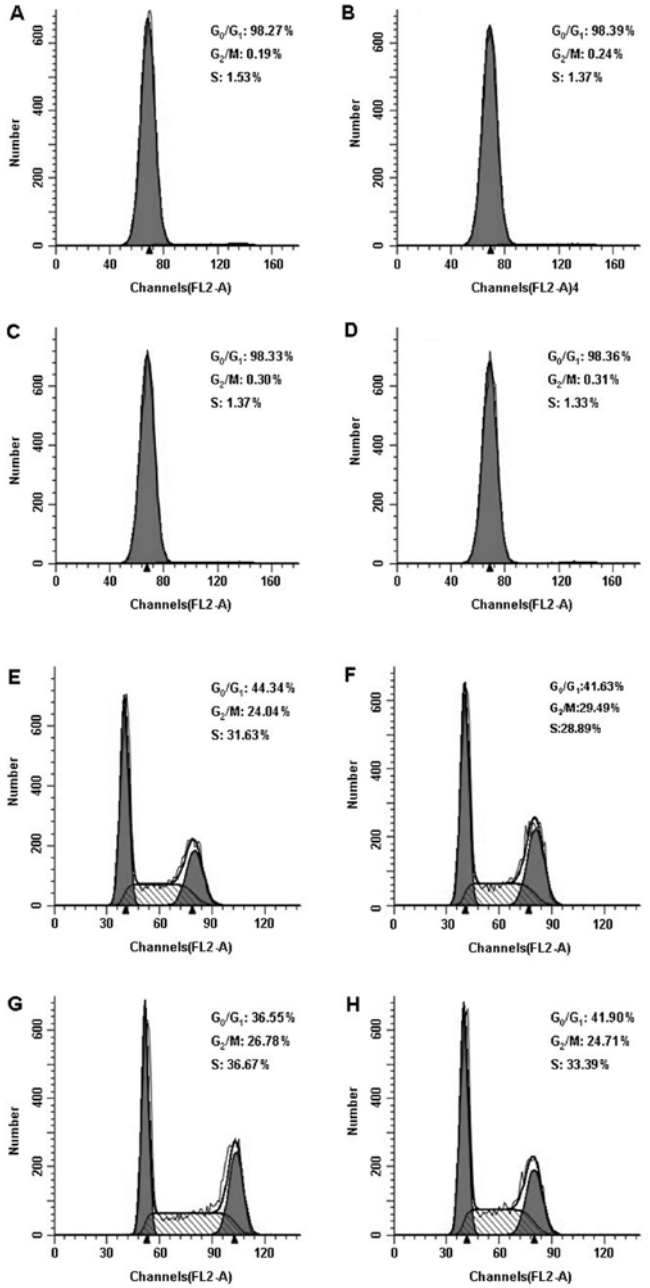

Figure 1. Effect of EA treatment and nocodazole interference on the cell cycle distribution of chondrocytes. After treatment with or without EA and nocodazole, the chondrocytes were collected and stained with PI followed by FACS analysis. Images are representative of three independent experiments. Cells before treatment: (A) control group; (B) experimental group 1; (C) experimental group 2; (D) experimental group 3. Cells after treatment: (E) control group; (F) experimental group 1; (G) experimental group 2; (H) experimental group 3.

than that of the control group (P=0.002) (Fig. 1E-H and Table III).

The mRNA expression of cyclin D1, CDK4, CDK6, Rb and P16 in chondrocytes following EA treatment. To further explore the mechanism of the EA treatment, we analyzed the mRNA expression levels of cyclin D1, CDK4, CDK6, Rb and P16. The amplified products of cyclin D1, CDK4, CDK6, Rb and P16 were clearly visible on the agarose gel (Fig. 2).

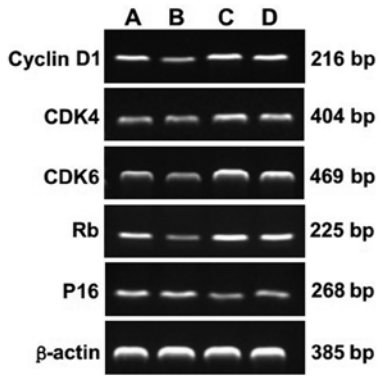

Figure 2. Effect of EA treatment and nocodazole interference on the mRNA expression of cyclin D1, CDK4, CDK6, Rb and P16 in passage 2 chondrocytes. Cells were treated with or without EA and nocodazole for 24 h. The mRNA expression levels of cyclin D1, CDK4, CDK6, Rb and P16 were determined by RT-PCR. β-actin was used as the internal control. Data are representative of three independent experiments. Lane A, control group; lane B, experimental group 1; lane C, experimental group 2; lane D, experimental group 3.

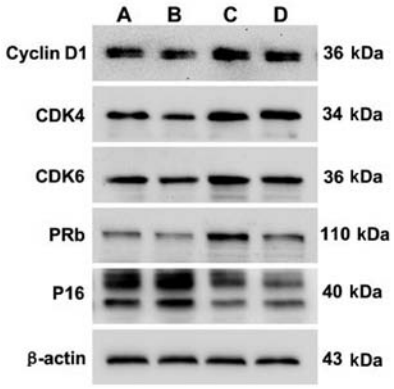

Figure 3. Effect of EA treatment and nocodazole interference on the protein expression of cyclin D1, CDK4, CDK6, pRb and P16 in passage 2 chondrocytes. Cells were treated with or without EA and nocodazole for 24 h. The protein expression levels of cyclin D1, CDK4, CDK6, pRb and P16 were analyzed by western blotting. β-actin was used as the internal control. Data are representative of three independent experiments. Lane A, control group; lane B, experimental group 1; lane C, experimental group 2; lane D, experimental group 3.

Quantification of the PCR products indicated that the levels of cyclin D1, CDK4, CDK6, Rb were significantly higher in control group than in experimental group 2 (P=0.002, P=0.001, P=0.001, P=0.001, respectively). However, the P16 mRNA level in experimental group 2 was significantly lower than that in control group (P=0.005) (Table IV).

Protein expression of cyclin D1, CDK4, CDK6, pRb and P16 in chondrocytes following EA treatment. The protein expression of cyclin D1, CDK4, CDK6, pRb and P16 was also detected using western blotting. Quantification of the western blotting bands showed that levels of cyclin D1, CDK4, CDK6 and pRb proteins in experimental group 2 were significantly higher than levels in the control group (P=0.000, P=0.000, P=0.001, P=0.000, respectively). In contrast, the P16 protein level in experimental group 2 was significantly lower than that in the control group (P=0.000) (Fig. 3 and Table V).

Table III. Cell cycle distribution as detected by FACS (%).

Group	G_0/G_1	S	G_2/M
Before treatment			
Control	98.273±0.129	1.533±0.073	0.193±0.119
Experimental 1	98.390±0.347	1.372±0.378	0.238±0.552
Experimental 2	98.332±0.082	1.370±0.112	0.298±0.097
Experimental 3	98.362±0.381	1.327±0.279	0.313±0.198
After treatment			
Control	44.338±0.038[b]	31.627±0.075[b]	24.035±0.109[b]
Experimental 1	41.627±0.054[a]	28.885±0.038[a]	29.488±0.065[a]
Experimental 2	36.548±0.048[a,b]	36.673±0.056[a,b]	26.778±0.053[a,b]
Experimental 3	41.902±0.072[a]	33.393±0.032[a,b]	24.705±0.091[b]

[a]P<0.01 compared with the control group after treatment; [b]P<0.01 compared with experimental group 1 after treatment. Values are expressed as means ± SD.

Table IV. mRNA expression of cyclin D1, CDK4, CDK6, Rb and P16 in the chondrocytes.

Group	cyclin D1	CDK4	CDK6	Rb	P16
Control	0.750±0.111	0.310±0.036[d]	0.385±0.034[d]	0.335±0.034[c]	0.315±0.038
Experimental 1	0.643±0.064	0.248±0.034[a]	0.327±0.044[b]	0.277±0.028[a]	0.353±0.053
Experimental 2	0.940±0.116[a,c,e]	0.387±0.040[a,c]	0.473±0.039[a,c]	0.412±0.040[a,c,e]	0.238±0.029[b,c]
Experimental 3	0.792±0.076[d]	0.353±0.025[b,c]	0.437±0.031[b,c]	0.367±0.037[b,c]	0.282±0.045[c]

[a]P<0.01, [b]P<0.05 compared with the control group; [c]P<0.01, [d]P<0.05 compared with experimental group 1; [e]P<0.05 compared with experimental group 3. Rb, phosphorylate retinoblastoma; CDK, cyclin-dependent kinase.

Table V. Protein expression of cyclin D1, CDK4, CDK6, pRb and P16 in the chondrocytes.

Group	Cyclin D1	CDK4	CDK6	pRb	P16
Control	0.078±0.012[d]	0.378±0.025[d]	0.425±0.024[c]	0.065±0.005	0.167±0.008[d]
Experimental 1	0.067±0.008[b]	0.305±0.021[b]	0.317±0.029[a]	0.053±0.010	0.177±0.005[b]
Experimental 2	0.107±0.010[a,c]	0.647±0.054[a,c,e]	0.628±0.050[a,c,e]	0.095±0.015[a,e]	0.112±0.008[a,c,e]
Experimental 3	0.097±0.008[a,c]	0.540±0.072[a,c]	0.482±0.022[a,c]	0.071±0.009[d]	0.135±0.010[a,c]

[a]P<0.01, [b]P<0.05 compared with the control group; [c]P<0.01, [d]P<0.05 compared with experimental group 1; [e]P<0.01 compared with experimental group 3. Rb, phosphorylate retinoblastoma; CDK, cyclin-dependent kinase.

Discussion

Osteoarthritis (OA), the most common age-related cartilage and joint disorder (18), is a slowly progressive degenerative disease characterized by degradation of the extracellular matrix and cell death resulting in a gradual loss of articular cartilage integrity (19,20). The only cell type present in mature cartilage is the chondrocyte. This cell type is responsible for repairing damaged cartilage tissue. We previously demonstrated that chondrocytes can be obtained by mechanical and chemical isolation methods and that the purity of isolated chondrocytes is high (17,21). This provided us with the ability to investigate the effect of EA on chondrocyte proliferation.

The working mode of EA is a disant wave and dense wave alternating waveform. The frequency of the distant wave is one fifth of the frequency of the dense wave; the duration time of the distant wave is 5 sec and the duration time of the dense wave is 10 sec. The function uses low-frequency electric current stimulation to change the distribution of hydronium between intracellular and extracellular regions, to interfere with the signal transmission of the organism, thus playing an effective role in cell proliferation. Nocodazole is a drug, like colchicines, that specifically binds to microtubules and interferes with spindle formation, thereby blocking the cell cycle at the G_2/M transition (22-24). In our study, we showed that EA treatment efficiently promoted chondrocyte proliferation,

and the degree of the proliferative effect was dependent on the time of EA treatment in a certain range. After nocodazole treatment, chondrocyte proliferation was obviously inhibited in a dose-dependent manner, but once the cells were treated with EA, the inhibitory effect of nocodazole was weakened, suggesting that EA treatment may function by promoting cartilage cells to pass through the G_1/S transition of the cell cycle.

There are four stages in the cell cycle: G_1, preparation for DNA synthesis; S, DNA synthesis; G_2, preparation for mitosis; and M, mitosis (25). Repetition of this cell cycle carries out cell proliferation. The amount of DNA in a cell changes during the cell cycle, allowing the different stages of the cycle to be identified by analyzing DNA content. The DNA content is 2N in G_0 and G_1 cells. After DNA synthesis in S phase, the DNA content becomes 4N in the G_2 and M phases (26,27). FACS analysis, which measures the DNA content of cells, is more sensitive to the changes during the cell cycle than the MTT method. In the present study, we found that after EA treatment, the G_0/G_1 and G_2/M ratios dropped, and the S ratio increased, indicating that EA treatment promotes cell proliferation by accelerating the G_1/S transition. This conclusion is also supported by our result that after nocodazole treatment, the S phase cell ratio dramatically decreased and the G_2/M cell ratio increased.

Upstream signals are usually transduced by signaling pathways and ultimately function on genes encoding cell cycle regulating factors, leading to cell cycle changes by altering the expression of these factors. The G_1/S checkpoint that exists at the end of G_1 stage is the key point of intracellular and extracellular signaling which integrates in the nucleus, which stimulates cells to begin a new round of proliferation, differentiation, death or exit the cell cycle into the G_0 phase. The G_1/S transition is dependent on the activity of cyclin D1-CDK4/6. Once activated, these CDK complexes phosphorylate retinoblastoma (Rb) and related family members and release sequestered E2F transcription factors, thereby promoting the transcription of genes required for progression through the cell cycle. Such progression is under the control of P16, which causes transient or permanent cell cycle arrest in cells carrying DNA damage. Our results showed that EA treatment effectively enhanced the mRNA and protein levels of cyclin D1, CDK4, CDK6 and (p)Rb and inhibited P16.

In summary, EA treatment effectively promotes chondrocyte proliferation. The molecular mechanism of EA may act by inducing the expression of cyclin D1, CDK4, CDK6 and Rb and inhibiting P16, thereby accelerating G_1/S transition and promoting chondrocyte cell cycle progression. This may, in part, explain its clinical effect in the treatment of osteoarthritis.

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