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Cyclic negative pressure promotes chondrocyte growth: Association of IGF-II with EGR-1

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ABSTRACT

Knee osteoarthritis (KOA) is one of the most common degenerative joint diseases in the elderly worldwide. The primary lesion in patients with KOA is the degeneration of articular cartilage. This study aimed to observe the biological effects of cyclic negative pressure on C28/I2 chondrocytes and to elucidate the underlying molecular mechanisms. We designed a bi-directional intelligent micro-pressure control device for cyclic negative pressure intervention on C28/I2 chondrocytes. Chondrocyte vitality and proliferation were assessed using Cell Counting Kit-8 (CCK-8) and 5-ethynyl-2'-deoxyuridine (EdU) assays. The extracellular matrix was analyzed using real-time fluorescence quantitative polymerase chain reaction (PCR) and western blot, while the molecular mechanism of the chondrocyte response to cyclic negative pressure was explored through mRNA sequencing. Experimental data demonstrated that cyclic negative pressure promoted chondrocyte proliferation and upregulated the expression of chondrocyte-specific protein, namely the collagen type II alpha 1 chain (COL2A1) protein, and the transcription factor SRY-box transcription factor 9 (SOX9). Additionally, RNA sequencing analysis revealed that the gene levels of insulin-like growth factor 2 (*IGF-II*) and early growth response 1 (*EGR-1*) were significantly elevated in the cyclic negative pressure group. This study demonstrates that cyclic negative pressure stimulates the proliferation of C28/I2 chondrocytes by promoting the expression of *EGR-1* and *IGF-II*. This new discovery may provide novel insights into cartilage health and KOA prevention.

Keywords: Osteoarthritis; chondrocytes; biomechanics; cell proliferation; *EGR-1*; *IGF-II*.

INTRODUCTION

Knee osteoarthritis (KOA) is a total knee disorder that typically involves articular cartilage, meniscus, infrapatellar fat pad, synovium, and subchondral bone [1, 2]. In disease progression, the main tissue affected is usually the articular cartilage, which primarily consists of chondrocytes [3]. In the early stages of KOA, the articular cartilage begins to deteriorate, progressing gradually to deeper layers and surrounding tissues, which eventually results in severe damage to the joint structure and function [4]. This process is choronic, progressive and irreversible [5]. Therefore, exploring the mechanisms of articular cartilage degradation is essential for the prevention and early treatment of KOA, in order to slow down the progression of the disease and preserve joint function.

Much attention is focused on the fact that the chondrocytes are in a unique stress environment within the joint. Inside the joint, they are subjected to mechanical loads, primarily from hydrostatic pressure, which is generated by the water pressure within the cartilage space. They also experience tensile forces from neighboring tissues, as well as static and dynamic stresses from changes in stance or posture [6-8]. Some *in vitro* studies showed that different pressures can cause opposite effects in chondrocytes, increased apoptosis and decreased apoptosis [9-12]. These studies used positive pressures higher than atmospheric pressure as the intervention condition. However, the pressure range in the normal human knee joint in a fully relaxed state is about -10-0 mmHg, lower than the atmospheric pressure [13]. This means that physiologically, the human knee cartilage is in a negative pressure environment most of the time. And there has been no research on the effect of negative pressure environment on chondrocytes so far. A clinical trial conducted by our team in 2021 showed that the resting state knee joint cavity pressure in the supine position in healthy volunteers was (-11.32 ± 0.21) cmH2O, while KOA volunteers recorded a much higher pressure of (-3.52 ± 0.34) cm H_2O [14]. It was also observed in the same trial that this negative pressure varied cyclically during

simulated walking in hip flexion and posterior extension maneuvers. Based on the above studies, we hypothesized that the deterioration of chondrocytes and thus the occurrence of KOA may be related to an altered negative pressure environment in the knee joint cavity, and that restoring the negative pressure environment to that of a healthy knee joint cavity may promote chondrocyte growth.

Cell changes in the early environment include the activation of early genes. When cells are stimulated, early growth response factors respond rapidly. *EGR-1* is a member of the early growth-responsive gene family. *EGR-1* plays an important role inside chondrocytes by regulating multiple target genes through DNA-binding activity [15]. Furthermore, it participates in the processes of cell participating in the process of cell proliferation and differentiation [16, 17]. The role of *EGR-1* in cartilage tissue and the mechanisms affecting osteoarthritis are unclear and controversial.

IGF-II is a growth factor with a complex structure and regulatory function [18]. *IGF-II* controls the expression of genes related to cartilage formation in chondrocytes and a decrease in its expression is linked to hampered chondrocyte proliferation. Study demonstrated that *IGF-II* expression is significantly reduced in the cartilage of osteoarthritis patients [19]. In an *in vitro* animal inflammation model assay, increased levels of *IGF-II* enhanced the levels of cartilage matrix, while decreasing the expression of degradation proteins, such as *MMP13*, thereby protecting cartilage integrity [20]. .

The purpose of this study was to observe the effect of cyclic negative pressure within the normal physiological range on the proliferation of C28/I2 chondrocytes and to investigate the related molecular mechanisms. The results of this study would clarify the potential mechanisms behind the effect of cyclic negative pressure on cartilage health.

MATERIALS AND METHODS

Culture of chondrocytes and periodic stress intervention

The C28/I2 cell line was obtained from Qingqi Biotechnology (Shanghai, China). The cells were cultured in DMEM High Sugar Medium (Procell, China) supplemented with 10% FBS (Sigma, USA) and 1% penicillin/streptomycin (Servicebio, China). Cells were cultured at 37°C, 5% CO_2, and 95% humidified air in an incubator for 2-3 generations. Following this, cells displaying logarithmic growth were isolated and selected for subsequent experimental research. Based on experimental requirements, the researchers designed an automatic barometric pressure control system for a biological incubator. The system is based on a Proportional-Integral-Derivative (PID) algorithm and comprises a controller, a motor, a barometric pressure sensor, and a user interface device. The air pressure sensor acquires the air pressure value inside the incubator and inside the air pressure maintenance box in real time and maintains the pressure difference between the two at the a set value. The barometric pressure sensor has a measurement range of -750mmHg to 750mmHg (S2311GN347-1F1, Utility model patent, China) (Figure 1).

Third-generation chondrocytes were subjected to cyclic negative pressure treatment. The cells were seeded at a density of 6×10^3 in 96-well plates and 6×10^5 in 6-well plates before loading pressure. Based on our previous study, the appropriate negative pressure intensity was determined. The cells were then placed in a sealed cassette within a pneumatic pressure maintenance system and exposed to a pressure of -10 mmHg for 1 hour. After that, the pressure was released to maintain a normobaric environment for another 1 hour. The Negative Pressure Group (NP Group) underwent three cyclic interventions in one day, which were repeated for a total of two days. The control group (CON group) was non-negative pressure group placed under the same incubation conditions.

Detection of cell vitality and proliferation

Chondrocyte cell vitality was detected using the CCK-8 method (MeilunBio, China). Cells were inoculated into 96-well plates at 6×10^3 density. Chondrocytes in the NP group were subjected to two days of cyclic negative pressure intervention, while those in the CON group were cultured under normal conditions. Then, CCK-8 reagent was added and incubated at 37°C and 5% CO_2 for 2 hours. The optical density (OD) at 450 nm was detected using a microplate reader (BioTek, USA).

Cell proliferation was detected using the EdU method and immunofluorescence. Cells were inoculated into 6-well plates according to grouping criteria for corresponding treatments, followed by continued incubation of the cells using 1×EdU working solution at 37°C for 2 h. Cells were fixed using 4% paraformaldehyde solution for 15 min. After washing with PBS solution containing 3% BSA, the cells were permeabilized using PBS solution containing 0.3% Triton X-100 for 15 min. Prepare Click Reaction Solution according to the manufacturer's instructions; the mixture contains the compounds required for the bonding of Alexa Fluor® 555 to EdU (Epizyme Biomedical, China). Cells were incubated in Click reaction solution at room temperature and protected from light for 30 min. followed by washing and staining of nuclei using 1× Hoechst33342 (1:1000 in PBS) for 10 min and washing again. Regarding the immunofluorescence imaging system, an inverted microscope (IX73, OLYMPUS, Japan) was used. EdU and nucleus images were acquired using lasers at wavelengths of 555 nm and 346 nm.

RNA-sequencing for the detection of DEGs

RNA sequencing analysis and quantification were utilized to investigate the alterations in cellular mRNA levels after exposure to cyclic negative pressure. Three biological duplicates from each group were sent to the BGI Genomics (Wuhan, China) for transcriptome sequencing, analyzed by the BGISEQ platform, aligned to the reference genome sequence using HISAT,

and analyzed for differential genes (DEGs) using the DEseq2 method. The DESeqDataSetFromMatrix function was used to construct the input matrix, the data were normalized by the DESeq function, and finally the results function was used to perform the difference analysis to obtain the DEGs with significant differential expression between the two groups. Specifically, if the gene's expression level increased in the NP group compared to that in the CON group, the gene was determined to be an up-regulated gene; and conversely, the If the expression level of a gene is decreased in the NP group compared with the CON group, the gene is down-regulated. The screening conditions were set as $|\log2FoldChange| > 0.5$, $P < 0.05$. The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (data number: HRA007672) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa-human/.

Analysis of the differential genes

Differential genes were analyzed for GO and KEGG enrichment by ClusterProfiler package using R4.3.1 software. Protein lnteraction network analysis of differential genes was also performed using the string database (https://cn.string-db.org/), and key gene screening of differential genes was performed using cytoscape software (version 3.7) cytoHubba.

Transient transfection and RNA interference

GenePharmaTM (Suzhou, China) designed siRNA sequences targeting *IGF-II* and *EGR-1* were used to down-regulate their expression. The siRNA sequences are shown in Table 1. The siRNA transfection was performed following the manufacturer's instructions. The siRNA/lipo complex was added to the 6-well plates and incubated at 37°C for at least 24 hours. The knockout efficiency was confirmed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blotting.

The plasmid for *EGR-1* overexpression was purchased from Fenghui Biotechnology™ (China). Cells were seeded into 6-well plates and then transfected with the *h-EGR1* (NM_001964) plasmid using Lipo8000 Transfection Reagent (Beyotime Biotechnology, China). The transfection was carried out at 37°C with 5% CO_2 for 24 to 72 hours. Subsequently, the overexpression of *EGR-1* was confirmed through qRT-PCR and Western blot analysis.

Gene expression analysis

Quantification of mRNA levels for collagen type II (*COL2A1*), *SOX9*, *IGF-II*, and *EGR-1* was performed using qRT-PCR. Total RNA was extracted from cultured cells following the instructions provided by the manufacturer. The RNA from each sample was reverse transcribed into cDNA using a gDNA digester plus kit (Yeasen Biotechnology, China), and the cDNA was stored in aliquots at -20°C until subsequent experiments. Quantitative reverse transcription polymerase chain reaction was then performed using a SYBR Green mix, with the program set to initialize at 95°C for 30 seconds, followed by denaturation at 95°C for 3 seconds and annealing at 55°C for 20 seconds, for a total of 40 cycles. The cDNA was used as a template for qRT-PCR using the CFX Connect™ Real-Time System (Bio-Rad, USA). qRT-PCR was performed using the formula $2^{-\Delta\Delta CT}$ to calculate the amount of target mRNA. The specific primers used are listed in Table 2.

Western blot

For both groups, the cells were rinsed with chilled PBS, then treated with protease inhibitor and phosphatase inhibitor (Beyotime Biotechnology, China) and RIPA lysis buffer (Servicebio, China) for 20 minutes on ice. The appropriate volume of up-sampling buffer was added and the samples were denatured at elevated temperatures, and a total of 15 μg of protein was added to separate the proteins using an SDS-PAGE gel. Subsequently, the proteins were transferred to PVDF membranes with 3% BSA (Beyotime Biotechnology, China) closure. Primary antibodies including *COL2A1* (1:1000) (Proteintech, China), *SOX9* (1:1000) (Proteintech,

China), *IGF-II* (1:1000) (Beyotime Biotechnology, China), *EGR-1* (1:1000) (Proteintech, China), and *β-actin* (1:1500) (Servicebio, China) were incubated. The membranes were subsequently incubated with HRP- conjugated IgG secondary antibodies (Beyotime Biotechnology, China), and protein bands were detected using an enhanced chemiluminescence (ECL) system (Bio-Rad).

Statistical analysis

All data are from independent samples and observations.-The data are presented as the mean ± standard error. One-way ANOVA followed by Dunnett's multiple comparisons test was performed using GraphPad Prism version 9.5.0 for Windows, (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com). Shapiro-Wilk and students' t-tests were performed using GraphPad Prism version 9.5.0 for Windows (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com) to test the normality of the data distribution and to analyze between the two groups. Statistical significance was defined as $P < 0.05$.

RESULTS

Cyclic negative pressure treatment promotes the proliferation of chondrocytes

The results of EdU and CCK-8 experiments showed that chondrocytes under cyclic negative pressure for 3 hours per day for two consecutive days showed an increase in cell proliferation and cell vitality in the negative pressure group (NP group) compared to the normobaric control group (CON group) ($p < 0.001$) (Figure 2A, B).The mRNA and protein expression of the characteristic molecules *COL2A1* and *SOX9* in chondrocytes after cyclic negative pressure intervention were detected by qRT-PCR and protein blotting. The mRNA expression of *COL2A1* and *SOX9* was significantly higher ($p=0.0005$, $p=0.0061$) in NP Group compared to CON Group (Figure 2C, D). Similarly，protein expression of *COL2A1* and *SOX9* were significantly elevated (Figure 2E), and the differences were statistically significant (Figure 2F, G).

mRNA transcriptome sequencing reveals key mechanical stimulus stress response pathways

RNA sequencing data revealed differential gene expression in chondrocytes from the CON and NP groups. A total of 209 DEGs were identified, of which 64 genes were upregulated and 145 genes were downregulated (Figure 3A, B). We performed GO functional annotation analysis of the differentially expressed genes, including molecular functions (Supplementary Figure S1A), biological processes (Supplementary Figure S1B) and cellular components (Supplementary Figure S1C). The top-ranked GO functional annotations were mainly related to cellular receptor signaling pathway and phosphorylated protein modification, suggesting that cell viability and proliferation were promoted in NP Group (Figure 3C). KEGG-enriched pathway was shown to be associated with chemokine signaling pathway and oxidative phosphorylation, suggesting a mechanistic pathway that may be affected by cyclic negative pressure (Figure 3D). Protein Interaction Network (PPI) analysis was performed on DEGs and the top 10 key genes were screened from them, and among these genes, *IGF-II*, which is positively associated with chondrocyte proliferation, was selected (Figure 3E, F).

Cyclic negative pressure significantly promotes *IGF-II* gene expression

Elevated expression of the *IGF-II* gene was confirmed in the NP group, in comparison with the CON group, by both qRT-PCR ($p < 0.01$) and Western blot assay ($p < 0.05$) (Figure 4A, B). To investigate the effect of *IGF-II* on chondrocytes under cyclic negative pressure intervention, we transfected the cells with si-RNA and added si-*IGF-II* mixed with liposomes in the cell culture medium. The results showed that, in the NP group, compared with the si-NC-treated chondrocytes, the *IGF-II* mRNA expression of si-*IGF-II*-treated chondrocytes was significantly decreased ($p < 0.0001$) (Figure 4C).

On the other hand, the protein expression of *IGF-II* in chondrocytes from the CON and NP groups was examined at the protein level, and the results showed a significant decrease in *IGF-*

II protein expression in chondrocytes of both groups after si-*IGF-II* treatment ($p < 0.05$) (Figure 4D). EdU results showed that cell proliferation ability was significantly reduced in si-*IGF-II*-treated CON and NP groups ($p < 0.01$, $p < 0.001$) (Figure 4E). In addition, we also examined the cellular vitality of chondrocytes in CON and NP groups after siRNA treatment, and the results showed that knockdown of *IGF-II* significantly reduced the cell vitality of chondrocytes ($p < 0.05$, $p < 0.001$) (Figure 4F). Meanwhile, the expression of extracellular matrix proteins in chondrocytes of the NP group was examined at the protein level, and the results showed that the expression of *COL2A1* and *SOX9* proteins were significantly decreased in chondrocytes treated with si-*IGF-II* ($p < 0.05$) (Figure 4G). Thus, silencing of *IGF-II* negated the effect of cyclic negative pressure on cell proliferation, suggesting that cyclic negative pressure further promoted chondrocyte proliferation and extracellular matrix synthesis by increasing the expression level of *IGF-II*.

EGR-1 transcriptional regulation of _IGF-II_ activity leads to cyclic negative pressure promoting chondrocyte proliferation

In order to investigate the molecular mechanism of *IGF-II* activation by cyclic negative pressure, we selected *EGR-1*, which has a link with *IGF-II*, through PPI network mapping and querying related literature. The next study will be conducted on *EGR-1* and explore its link with *IGF-II* in cartilage. Our transcriptome sequencing data indicated that *EGR-1* was up-regulated in the NP group. We validated this finding at both mRNA and protein levels, and the results showed that cyclic negative pressure promotes *EGR-1* expression ($p < 0.01$, $p < 0.05$) (Figure 5A, B). To elucidate the effect of *EGR-1* on the increase in *IGF-II* induced by cyclic negative pressure, cells treated with *EGR-1* siRNA were subject to cyclic negative pressure intervention. The data indicated that both groups of *EGR-1* siRNA-treated cells exhibited a significant reduction in mRNA levels compared to negative control (si-NC) cells. Similarly, there was a significant decrease in mRNA expression of *IGF-II* (Figure 5C). At the protein

level, knockdown of *EGR-1* significantly decreased the expression of *EGR-1* and *IGF-II* in chondrocytes of the CON and NP groups ($p < 0.01$) (Figure 5D). Cell proliferation assay of cells transfected with siRNA in CON and NP groups were performed using the EdU assay, and the results showed that cells treated with si-*EGR-1* showed a significant decrease in their cell proliferation ability ($p < 0.0001$) (Figure 5E). On the other hand, CCK-8 data showed that the cell vitality of chondrocytes was significantly reduced when cyclic negative pressure was applied and *EGR-1* was knocked down ($p < 0.001$) (Figure 5F).

In contrast, cells that overexpress *EGR-1* (oe-*EGR-1)* showed a significant increase in mRNA levels, approximately 100-fold higher than cells transfected with the empty plasmid (ve-*EGR-1*) ($p < 0.001$) (Figure 6A), also significantly upregulated at the protein level (Figure 6B). In the NP group, overexpression of *EGR-1* led to a significant upregulation of both mRNA and protein expression of *IGF-II* (Figure 6C, D). Regarding cell vitality, CCK-8 data showed that cells overexpressing *EGR-1* followed by cyclic negative pressure intervention exhibited a significant increase in vitality ($p < 0.01$) (Figure 6E). Meanwhile, the expression of extracellular matrix proteins in chondrocytes from the NP group was examined at the protein level, and the results showed that the expression of both *COL2A1* and *SOX9* proteins in chondrocytes was significantly up-regulated after overexpression of *EGR-1* (Figure 6F). Thus, we confirmed that cyclic negative pressure is involved in the cell proliferation process by inducing the expression of the *EGR-1* gene, which in turn promotes the expression of the target gene *IGF-II*, which is positively correlated with *EGR-1* (Figure 6G).

DISCUSSION

A proper biomechanical environment is essential for maintaining the structure and function of cartilage tissue. It has now been shown that cyclic stress increases the production of cartilage matrix, while excessive static stress significantly reduces collagen synthesis in cartilage [21-23]. In our previous clinical study, we observed a significant difference in the negative pressure

values within the knee joint cavity between healthy volunteers and those with KOA. We found that the pressure within the knee joint cavity exhibited cyclic changes [14]. In this study, we cultured cells at an ambient pressure (approximately equal to 1 atmosphere) as a control group. We then used in vitro assays to confirm that chondrocytes cultured under conditions that simulated the negative pressure found in healthy human joints exhibited a higher proliferative effect. Additionally, the expression of *COL2A1* and *SOX9*, genes associated with cartilage health and the regulation of cartilage growth, was increased. These findings suggest that cyclic negative pressure may be beneficial for cartilage health, indicating that maintaining a certain level of negative pressure in human joints could be advantageous. The chondrocytes of the two groups were further analyzed by transcriptome sequencing. Bioinformatics methods were applied to analyze the data to obtain the differential genes between the two groups, and the *IGF-II* and *EGR-1* genes, which were highly expressed in the NP group, were identified as the focus of the study. Numerous studies have shown that *IGF-II* plays a key role in maintaining cartilage and joint homeostasis as well as promoting bone growth. *IGF-II* activates PI3K/Akt and *TGF-β* signaling pathways in the growth plate to promote endochondral osteogenesis. In skeletal muscle, an amplification cascade occurs with autocrine *IGF-II* synergizing with *MyoD* to promote muscle differentiation [24-27]. Normal chondrocytes secrete *IGF-II*, while in pathological states, the expression level of *IGF-II* is reduced, leading to cartilage matrix damage and subsequent chondrocyte loss [19]. We found that the expression of *IGF-II* in chondrocytes in the cyclic negative pressure group was significantly higher compared with cells cultured in normobaric pressure by RNA-seq analysis, which is consistent with the results of the above studies, suggesting that cyclic negative pressure favored chondrocyte growth. Our inhibition of *IGF-II* reduces cyclic negative pressure-induced chondrocyte proliferation and our inhibition of *IGF-II* reduces cyclic negative pressure-induced chondrocyte proliferation

and extracellular matrix, suggesting that *IGF-II* plays an important role in the promotion of chondrocyte proliferation by cyclic negative pressure.

How cyclic negative pressure induces elevated *IGF-II* expression is our next focus. RNA-seq analysis showed that the expression of *EGR-1* factor was significantly elevated in chondrocytes in the cyclic negative pressure group, which drew our attention. The activation of early genes is first response when the external environment is altered. *EGR-1* is a stress-responsive protein whose expression is up-regulated when the cell is exposed to drugs, inflammation, or growth factors [28, 29]. Studies on the correlation between *IGF-II* and *EGR-1* have not been carried out in the field of cartilage. In the field of oncology, high expression of *EGR-1* was found to drive the expression of growth factors, including *IGF-II* factors. Furthermore, *IGF-II* has been found to act as a physiological target of *EGR-1*, and the promoter region of *IGF-II* contains multiple sites that can bind to *EGR-1* or its ligands, suggesting that the nature of the *IGF-II*-*EGR-1* linkage is the regulation of an autocrine process [30-32]. However, existing studies provide conflicting accounts about the expression and specific role of *EGR-1* in osteoarthritis. On the one hand, some results show that *EGR-1* is abnormally highly expressed in osteoarthritic cartilage, and its ectopic expression exacerbates the degradation of cartilage matrix i*n vivo*; on the other hand, other studies report that the expression of the *EGR-1* gene in normal articular cartilage is higher than that in osteoarthritic cartilage and that it may be involved in the process of chondrogenesis [16, 33, 34]. *IGF-II*, on the contrary, has been shown by many parties to promote chondrocyte synthesis and to promote osteoblast proliferation and differentiation, ameliorating matrix damage in osteoarthritis [20, 35, 36]. In our study, we found that inhibiting or overexpressing *EGR-1* had distinct effects on the expression of *IGF-II* and the proliferation of chondrocytes under cyclic negative pressure stimulation. When *EGR-1* expression was down-regulated under cyclic negative pressure stimulation, the level of *IGF-II* expression decreased, along with reduction in chondrocyte proliferation. Conversely, the overexpression

of *EGR-1* led to increased *IGF-II* expression and further enhanced chondrocyte proliferation. These findings indicate that *IGF-II* expression is positively correlated with the level of *EGR-1* during the stimulation of chondrocytes by cyclic negative pressure.

Although there are important discoveries revealed by these studies, there are also limitations. To ensure the stability and reproducibility of our in vitro experiments, we selected the cell line C28/I2. However, we have now explored a well-established experimental system. To delve deeper into underlying mechanism, an in vivo experimental model can be set up to supplement this study. Moreover, following the methodology presented in this study on healthy human chondrocytes, the biological changes of diseased chondrocytes or tissue progenitor cells can also be explored. In summary, our study demonstrates that cyclic negative pressure using the normal joint pressure range has a pro-proliferative effect on C28/I2 chondrocytes. For the first time, a positive correlation link between *IGF-II* and *EGR-1* in chondrocytes was proposed, suggesting that the combination of *IGF-II* and *EGR-1* is an important link in the response of chondrocytes to changes in the negative pressure environment.

CONCLUSION

In this study, we cultured and observed the proliferation of human C28/I2 chondrocytes in vitro using a device that simulated the negative pressure conditions in healthy human joints. We found that chondrocytes cultured under cyclic negative pressure demonstrated enhanced proliferation and cell vitality. These results suggested that cyclic negative pressure may be beneficial for maintaining cartilage health. Moreover, the positive correlation between cyclic negative pressure and the high expression of *EGR-1* and *IGF-II* suggested that *EGR-1* may be involved in the regulation of *IGF-II* activation in cartilage. This is the first time that a positive correlation between *EGR-1* and *IGF-II* expression has been identified in chondrocytes. The results of this study would provide insights into the pathogenesis of articular cartilage degeneration, KOA prevention and control, and cartilage tissue engineering.

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Data availability

Data are contained within the article and supplementary materials. RNA-seq data will be shared by the corresponding authors upon reasonable request.

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TABLES AND FIGURES WITH LEGENDS

TABLE 1. siRNA sequence

	Sense(5'-3')	Antisense(5'-3')
IGF-II-si#1	UCGAUGCUGGUGCUUCUCACCUUCU	AGAAGGUGAGAAGCACCAGCAUCGA
IGF-II-si#2	CGAUGCGGUGCUUCUCACCUUCUU	AAGAAGGUGAGAAGCACCAGCAUCG
EGR-1-si#1	GCGAUGAACGCAAGAGGCAUACCAA	UUGGUAUGCCUCUUGCGUUCAUCGC
EGR-1-si#2	GGACAAUUGAAAUUUGCUAAA	UAGCAAAUUUCAAUUGUCCUG
NC-siRNA	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

TABLE 2. Gene primer sequences of human used for qRT-PCR

Gene	Forward primers	Reverse primers
COL2A1	TGGACGATCAGGCGAAACC	GCTGCGGATGCTCTCAATCT
SOX9	AGCGAACGCACATCAAGAC	CTGTAGGCGATCTGTTGGGG
IGF-II	CCGTGGCATCGTTGAGGAGTG	CGGGGTATCTGGGGAAGTTGTC
EGR-1	CCACGCCGAACACTGACATT	GAGGGGTTAGCGAAGGCTG
GAPDH	AATTCCATGGCACCGTCAAG	AGCATCGCCCCACTTGATTT

(A)

(B)

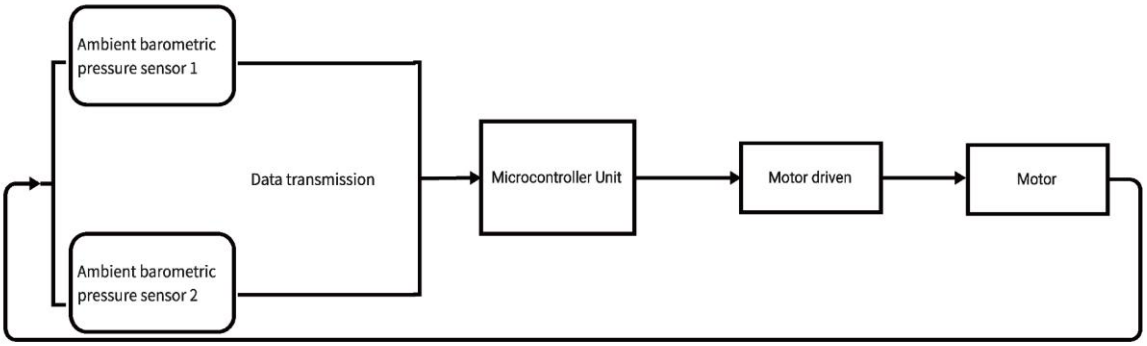

FIGURE 1. Cyclic negative pressure equipment and schematic diagram. (A) Exterior view of cyclic negative pressure equipment; (B) Cyclic negative pressure equipment operating schematic.

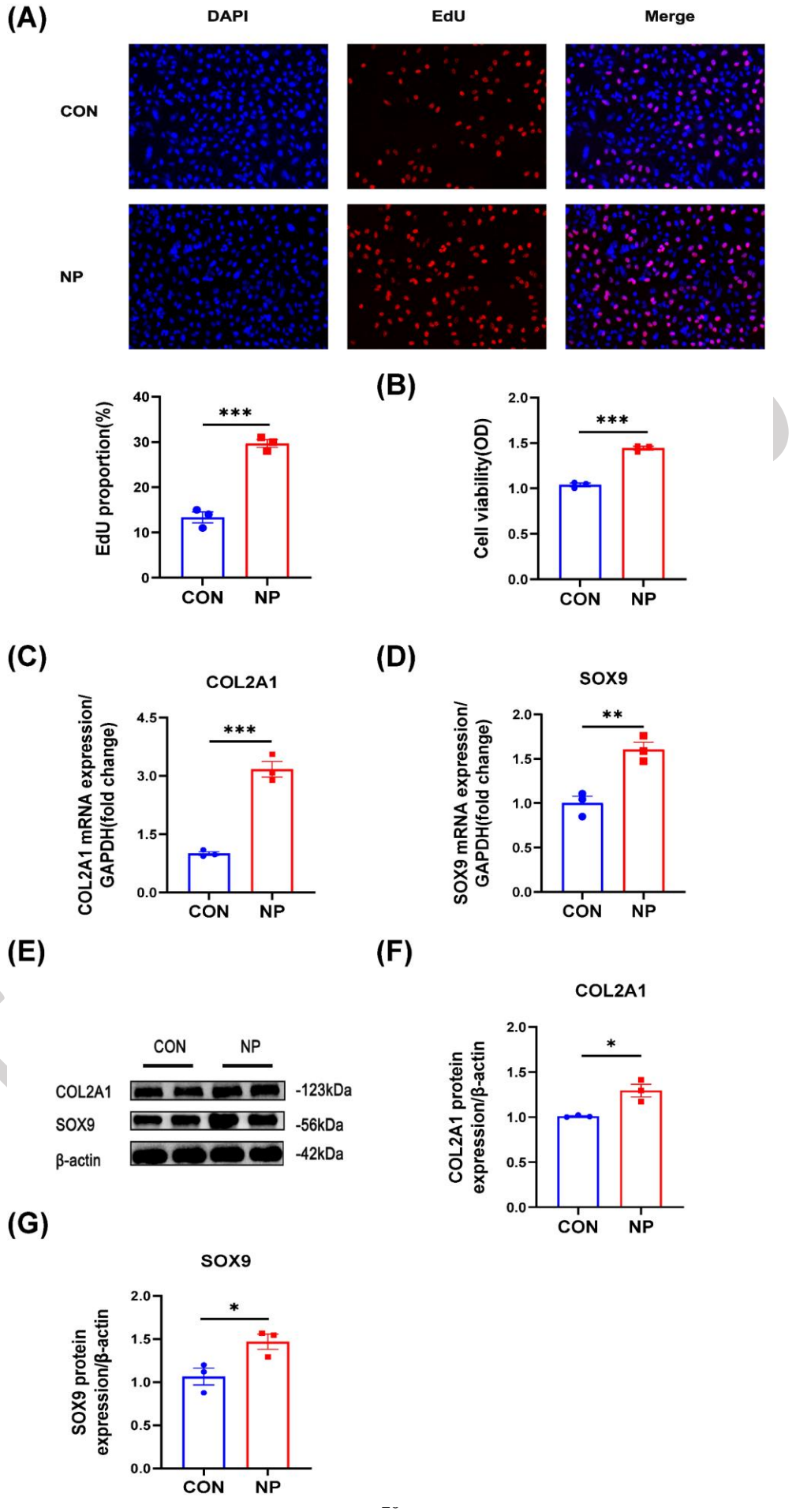
(A)
DAPI
EdU
Merge
CON
NP
(B)
EdU proportion(%)
***
CON
NP
Cell viability(OD)
***
CON
NP
(C)
COL2A1
COL2A1 mRNA expression/ GAPDH(fold change)
***
CON
NP
(D)
SOX9
SOX9 mRNA expression/ GAPDH(fold change)
**
CON
NP
(E)
CON
NP
COL2A1
-123kDa
SOX9
-56kDa
β-actin
-42kDa
(F)
COL2A1
COL2A1 protein expression/β-actin
*
CON
NP
(G)
SOX9
SOX9 protein expression/β-actin
*
CON
NP

FIGURE 2. The effects of cyclic negative pressure on chondrocyte proliferation. (A) C28/I2 chondrocytes exhibited proliferative changes in response to cyclic negative pressure stimulation, as demonstrated by EdU method (n = 3); (B) CCK-8 assay for chondrocyte vitality (n=3); (C) The expression of the *COL2A1* gene was analyzed using qRT-PCR (n = 3); (D) The expression of the *SOX9* gene was analyzed using qRT-PCR (n = 3); (E) The protein expression of *COL2A1* and *SOX9* was analyzed through Western blot; (F) A statistical histogram was created to analyze the expression of the *COL2A1* protein through Western blot (n = 3); (G) A statistical histogram was created to analyze the expression of the *SOX9* protein through Western blot (n = 3). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

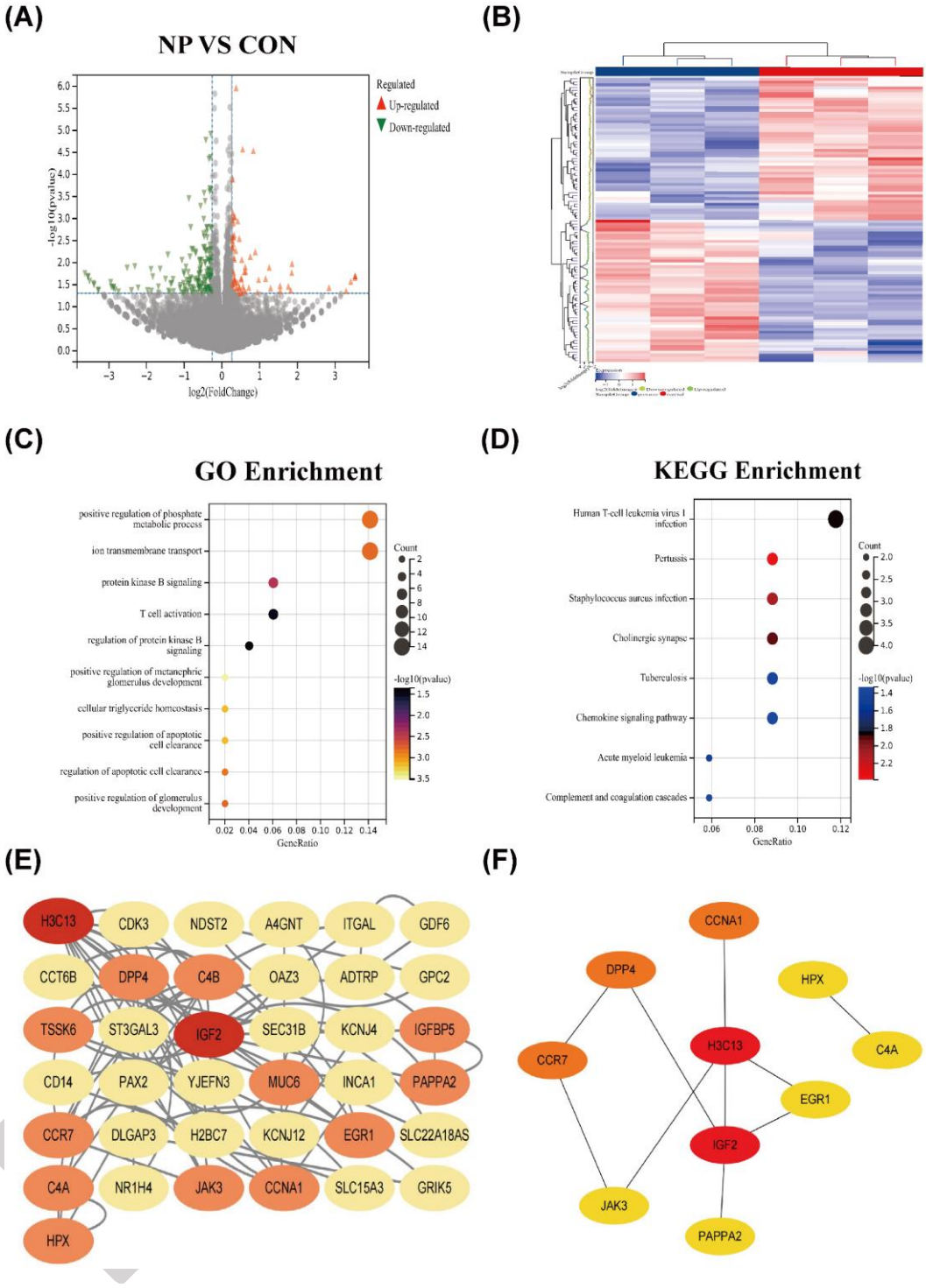

FIGURE 3. Gene expression differences and mechanistic pathways. (A) Volcano plot of expression differences between groups, |log2FoldChange| > 0.5, P < 0.05; (B) A hierarchical clustering heatmap was created to show the related genes between the CON and NP groups. Each group included three samples: Pressure-1, Pressure-2, and Pressure-3 for the NP group, and CON-1, CON-2, and CON-3 for the CON group. The CON group did not receive cyclic negative pressure treatment, while the NP group did (n=3); (C) GO annotation revealed the most enriched terms, P < 0.05; (D) KEGG annotated forward

results, $P < 0.05$; (E) STRING database searches for interactions among differentially expressed genes; (F) Top ten differential genes were retrieved using Cytoscape.

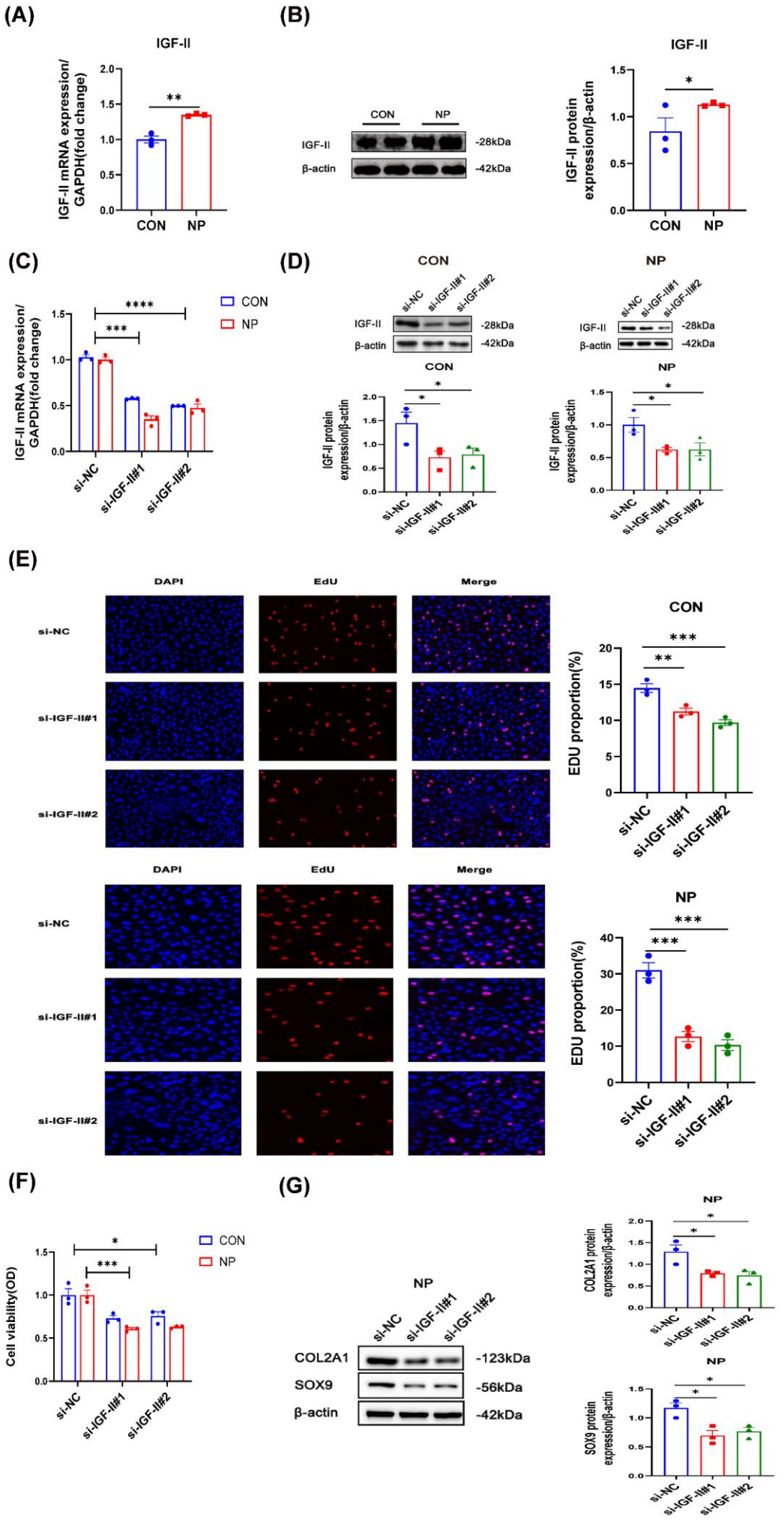
(A)
IGF-II
IGF-II mRNA expression/ GAPDH(fold change)
CON
NP
(B)
CON
NP
IGF-II
β-actin
-28kDa
-42kDa
IGF-II
IGF-II protein expression/β-actin
(C)
CON
NP
IGF-II mRNA expression/ GAPDH(fold change)
si-NC
si-IGF-II#1
si-IGF-II#2
(D)
CON
NP
si-NC
si-IGF-II#1
si-IGF-II#2
IGF-II
β-actin
-28kDa
-42kDa
IGF-II protein expression/β-actin
(E)
DAPI
EdU
Merge
si-NC
si-IGF-II#1
si-IGF-II#2
CON
NP
EDU proportion(%)
(F)
CON
NP
Cell viability(OD)
(G)
NP
si-NC
si-IGF-II#1
si-IGF-II#2
COL2A1
SOX9
β-actin
-123kDa
-56kDa
-42kDa
COL2A1 protein expression/β-actin
SOX9 protein expression/β-actin

FIGURE 4. *IGF-II* molecules as key molecules in cyclic negative pressure affecting cell proliferation. (A) qRT-PCR analysis of *IGF-II* (n=3); (B) Western blot analysis of *IGF-II* molecule expression and statistical histograms (n=3); (C) qRT-PCR was performed to analyze the expression of *IGF-II* mRNA in CON and NP groups after transfection with negative control and *IGF-II* siRNA, respectively (n = 3); (D) Western blot analysis of *IGF-II* molecule expression in cells after transfection of si-NC and si-*IGF-II* in CON and NP groups (n=3); (E) EdU method was used to detect cell proliferation ability after transfection of si-NC and si-*IGF-II* in CON and NP groups (n=3); (F) CCK-8 assay was used to detect the vitality of cells treated with si-NC and si-*IGF-II* (n=3); (G) Western blot analysis of extracellular matrix molecule expression in chondrocytes from the NP group after knockdown of *IGF-II* (n=3). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

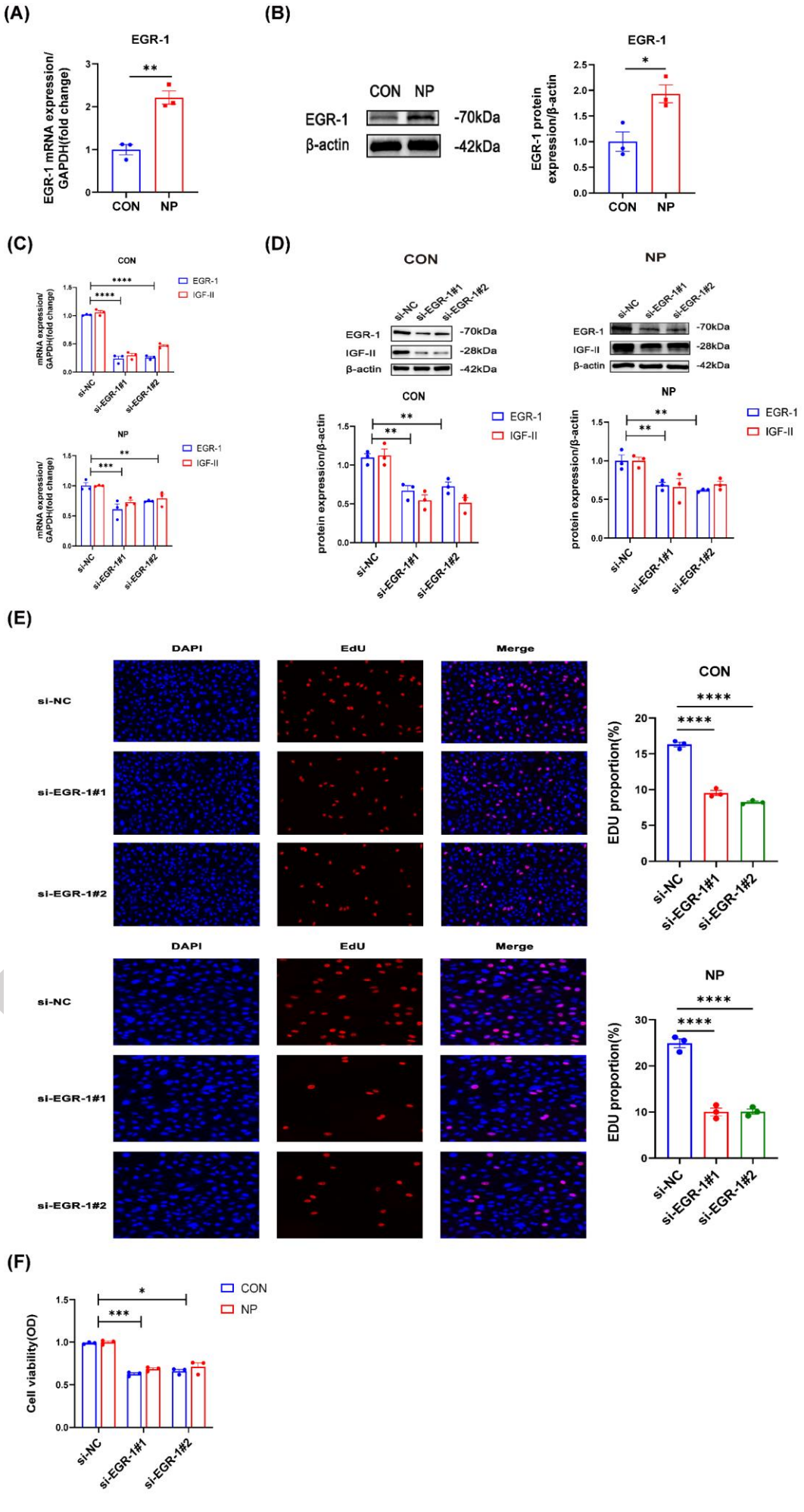
(A)
EGR-1
EGR-1 mRNA expression/ GAPDH(fold change)
**
CON
NP
(B)
CON NP
EGR-1
β-actin
-70kDa
-42kDa
EGR-1
EGR-1 protein expression/β-actin
*
CON
NP
(C)
CON
mRNA expression/ GAPDH(fold change)
EGR-1
IGF-II
****
****
si-NC
si-EGR-1#1
si-EGR-1#2
NP
**
***
(D)
CON
NP
si-NC
si-EGR-1#1
si-EGR-1#2
EGR-1
IGF-II
β-actin
-70kDa
-28kDa
-42kDa
protein expression/β-actin
**
(E)
DAPI
EdU
Merge
si-NC
si-EGR-1#1
si-EGR-1#2
CON
NP
EDU proportion(%)
****
(F)
Cell viability(OD)
CON
NP
*
***

FIGURE 5. Validation of *EGR-1* and effects on *IGF-II* after knockdown of *EGR-1*. (A) qRT-PCR analysis of *EGR-1* (n=3); (B) Western blot analysis of *EGR-1* molecule expression (n=3); (C) The mRNA expression of *EGR-1* and *IGF-II* in chondrocytes transfected with si-NC and si-*EGR-1*, respectively, in CON and NP groups (n=3); (D) Western blot analysis of *EGR-1* and *IGF-II* protein expression in chondrocytes after transfection of si-NC and si-*EGR-1* in CON and NP groups, respectively (n=3); (E) EdU method was used to detect the proliferative capacity of cells in CON and NP groups after treatment with si-NC and si-*EGR-1* (n=3); (F) CCK-8 assay was used to detect the vitality of cells treated with si-NC and si-*EGR-1* (n=3). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

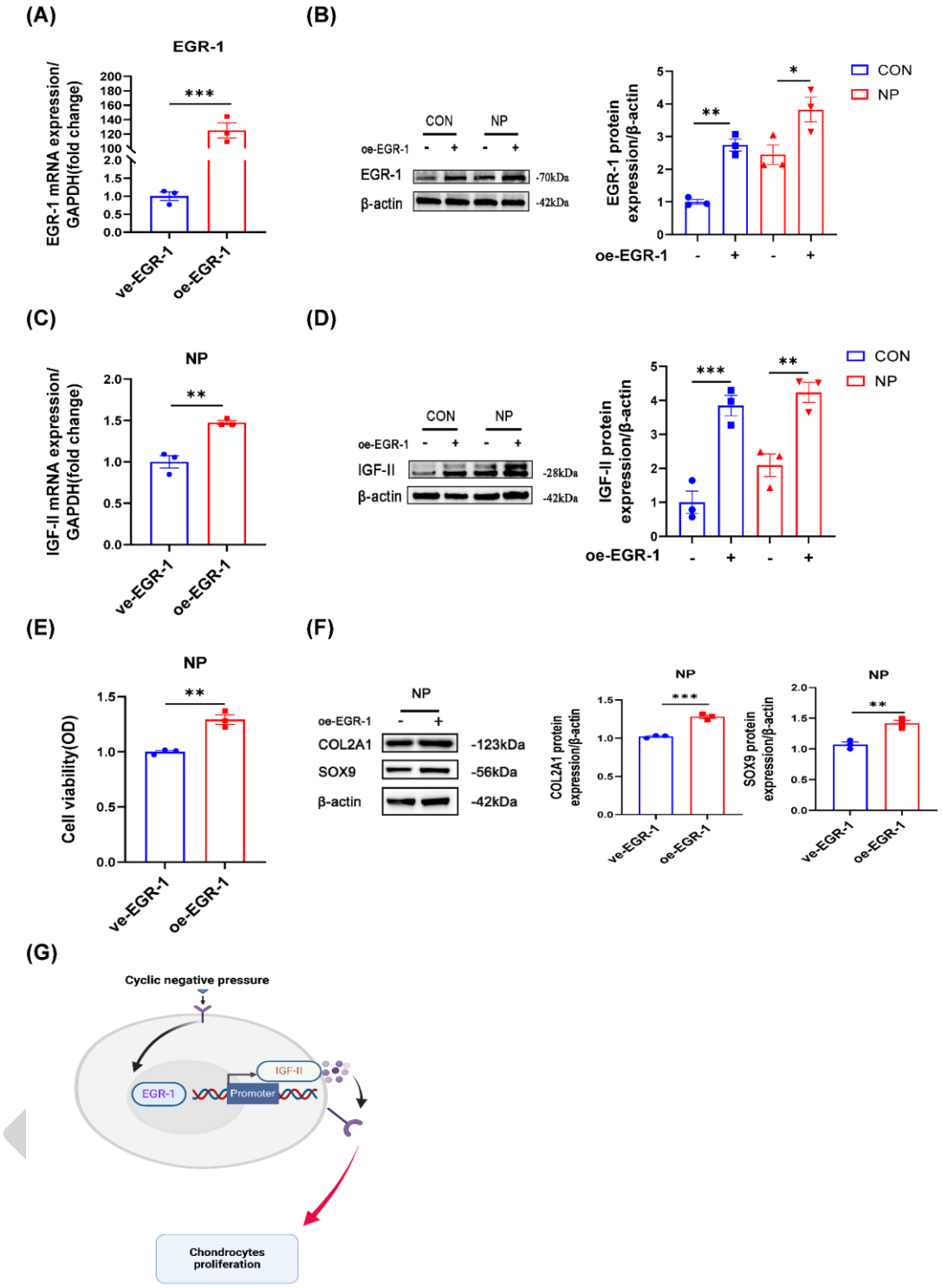

FIGURE 6. Effect of overexpression of *EGR-1* on *IGF-II* and cell proliferation. (A) qRT-PCR analysis of overexpression of *EGR-1* (n=3); (B) Western blot analysis after overexpression of *EGR-1* in CON and NP groups. (n=3); (C) mRNA expression of *IGF-II* after overexpression of *EGR-1* in cells (n=3); (D) *IGF-II* protein expression after overexpression of *EGR-1* in cells of CON and NP groups (n=3); (E) CCK-8 assay was used to detect the vitality of cells treated with overexpressed *EGR-1* (n=3); (F) Western blot analysis of extracellular matrix components of chondrocytes in the NP group after

overexpression of *EGR-1* (n=3); (G) Cyclic negative pressure promotes chondrocyte proliferation by affecting *EGR-1* and *IGF-II* together schematically (https://biorender.com/).*P < 0.05; **P < 0.01; ***P < 0.001.

SUPPLEMENTAL DATA

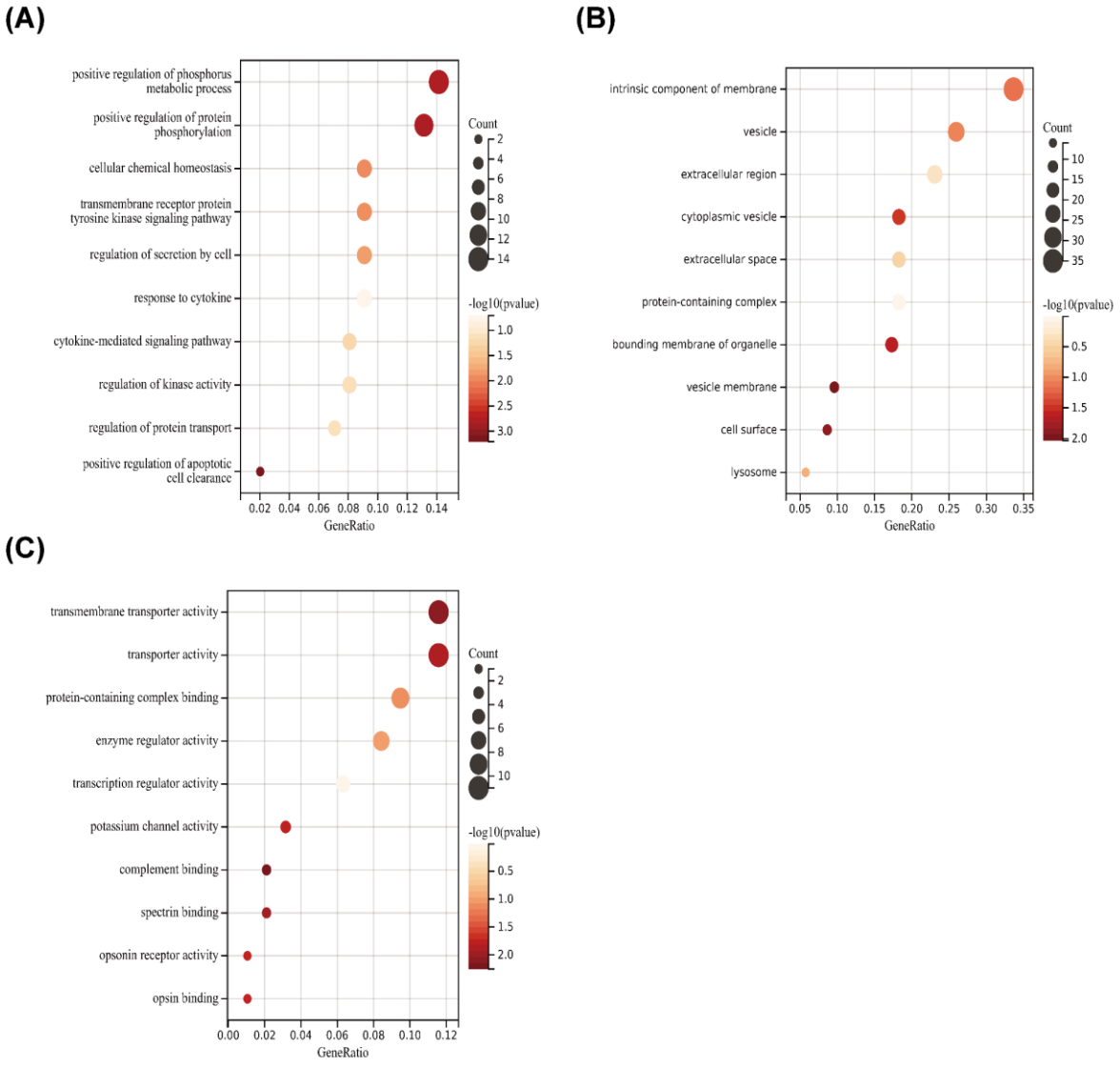

FIGURE S1. GO functional annotation analysis of the differentially expressed genes. (A) GO functional annotation analysis of the molecular function; (B) GO functional annotation analysis of the biological processes; (C) GO functional annotation analysis of the cellular components.