Original Article

Achyranthes bidentata extract protects chondrocytes functions through suppressing glycolysis and apoptosis via MAPK/AKT signaling axis

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Abstract: Osteoarthritis (OA) is considered to be a joint-associated disorder and one of leading reasons for disability, however, potential mechanism has never been clarified. The purpose of this research was to evaluate protective-effects of Achyranthes Bidentata extracts (ABE) on chondrocytes function in osteoarthritis. We performed a systematic investigation of transcriptional and proteomic landscapes to identify the underlying mechanisms behind effects of ABE on chondrocytic functions. OA animal models were generated in the present research. Chondrocytes were isolated and cultured, and then prepared for GeneChip analysis. Two-dimensional gel electrophoresis and LC-MS/MS analysis were conducted to analyze samples. Quantitative real-time PCR (qRT-PCR) and western blotting were used to evaluate expression of protein kinase B (AKT), β-tubulin and β-action. Apoptosis and glycolysis pathway were significantly compromised in chondrocytes with ABE stimulation as revealed by both transcriptional and proteomic data. Consistently, ABE suppressed chondrocytes apoptosis and glycolytic activity in vitro through modulating multiple genes, such as Plk2, Casp1/12 and Cers1 as well as Pkm2, Eno1/3 and Pgk2. Mechanically, ABE activated MAPK signaling pathway and suppressed AKT signaling pathway, therefore, reducing the glycolysis to provide survival benefits. We extended our analysis by verifying insulin-like growth factor 1 (IGF-1) and MAP kinase 1 (MEK1) in chondrocytes function. Depletion of either IGF-1 or MEK1 impaired AKT expression and phosphorylation, leading to the enhanced chondrocyte apoptosis and reduced cell proliferation. In conclusion, our study provided systematic view and molecular basis for ABE to serve as potential intervention of OA via suppressing AKT signaling.

Keywords: Achyranthes bidentata, osteoarthritis, AKT, GLUT1, glycolysis

Introduction

Osteoarthritis (OA) is considered to be a joint-associated disorder and one of leading reasons for disability, influencing appropriate 250 million patients in the whole world [1]. A cohort study [2] shows the prevalence of symptomatic knee osteoarthritis in adults ranges from 7% to 17%, increasing with age, especially post age of 65 years. Multiple risk factors, including age, prior injury, overuse, obesity, and genetic predisposition are particularly relevant with the higher risk of OA [3].

OA is also a degenerative and poor progressive disease, without restoration for OA caused damage. For decades, despite continued efforts have been performed to fully understand OA pathogenesis and treatment, there is currently no cure for OA. Therefore, the present strategies mainly eliminate the stiffness, painful signals and improve the life-quality. Clinically, strategies treating OA mainly includes surgical operation, administrating with drugs or non-drug treatments. Pharmacological therapies in clinical guidelines include capsaicin, topical nephrogenic syndrome of inappropriate antidiuresis (NSIADs), and salicylates [4-7]. Due to the side-effects of drugs and the lower efficacy, it isn’t recommended to administrate medications for long periods. In recent years, several new strategies have also been applied, including mesenchymal stem cells (MSCs) transplantation, injecting intra-articularly with platelet rich plasma (PRP), both of which have been proven to be safe and well tolerated for relieving pain and improving knee functions [8-10]. These are still under investigation and more
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studies and standardization of these therapies would be required.

Therefore, sustained attempts are indispensable for discoveries of novel cellular targets and effective intervention of OA. Nowadays, OA has been proven to be correlated with the metabolic diseases, such as diabetes mellitus type 2 (T2DM), especially for T2DM occurring in older patients. The above description suggests that the metabolic alterations might be a potential link with OA [11-14]. While it is well-characterized that tumor cells prefer to a shift toward increased glycolysis even in presence of oxygen. Meanwhile, an adaptation to stressful and dynamic microenvironment of solid tumor would facilitate the tumor proliferation and invasiveness [15-17]. Strikingly, the microenvironment in swollen joints is considered to be of hypoxia and insufficient nutrients supply [18, 19]. Increased glucose consumption has been observed in swollen joints by fluoro-deoxy-glucose positron emission tomography (FDG-PET) [20-25]. These observations suggested increase of glycolysis might be a critical factor driving the disease progression of OA, therefore, implying targeting glycolysis might provide therapeutic intervention for OA.

Radix achyranthes bidentata (AB), a Traditional Chinese Medicinal Herb, has been extensively used in Chinese Medicinal formulations for treating a variety of diseases, such as OA [26-31]. The underlying molecular basis is poorly clarified, which has limited its potential use of OA therapy. In present study, we have performed Affymetrix GeneChip and proteomic approach for identifying potential effectors or specific signaling events that favor the protecting effects of AB extract (ABE). Interestingly, GeneChip analysis reveals that glycolysis and hypoxia-inducible factor 1 (Hif-1) signaling pathways in chondrocytes were significantly compromised in response to ABE treatment. Of note, mRNA expressions of protein kianse B (AKT2) and glucose transporter 1 (GLUT1) are identified and verified to be remarkably down-regulated upon ABE treatment. Meanwhile, the increased mitogen-activated protein kinase 1 (MAP2K1) and enhanced insulin-like growth factor 1 (IGF-1) are also found. Moreover, proteomic-dissection of altered protein profiles using two-dimensional gel electrophoresis and liquid chromatography-mass spectrometry (LC-MS) also showed that ABE predominately targets the glycolysis. We previously observed that the reduced protein levels of AKT2 are considered as a critical regulator of glucose metabolism. The functional investigations also revealed that depletion of IGF-1 or MAP2K1 dramatically suppresses chondrocytes proliferation and facilitates apoptosis induced by ABE. On the contrary, over-expression of AKT or GLUT1 significantly results in the opposite effects. Furthermore, ablation of IGF-1 or MAP2K1 showed reduced AKT levels and impaired AKT signaling. Collectively, our study for the first time provided experimental evidences that ABE treatment impaired glycolysis in chondrocytes. Therefore, the ABE treatment could facilitate the chondrocytes viability through suppressing AKT signaling, and provide the basis for treating OA by combining ABE treatment with the glycolysis inhibition.

Materials and methods

Animals and OA model establishment

Total of 6 New Zealand white rabbits (male) were employed to generate OA models. Left knee joints were mobilized (1.5 cm below groin to 3 cm above rear-ankle) for 6 weeks with the plaster bandages, keeping knee-flexed at angle of 30°-40°. On each-side, the dorsalis pedis pulses were examined and observed. The plaster tightness was detected for 3 days post the establishment of models. Rabbits were than fed in single cages and were moved or exercise. The other untreated 6 rabbits were employed as the normal control. The successful modeling was evaluated using Pelletier grading and staging system and further confirmed by hematoxylin-eosin (HE) staining and immunohistochemistry (IHC) staining of Collagen II. All of the experiments or tests have been approved by Institutional Animal Care and Use Committee of Shenzhen Traditional Chinese Medicine Hospital.

Isolation and primary culture of chondrocytes

Knee articular cartilages were isolated from OA rabbits models. The isolated chondrocytes were cut into thin-slices. The cartilage was digested using 0.25% Trypsin (Beyotime Bio-tech. Shanghai, China) for 30 min and then cells were obtained. Subsequently, the cells were cultured in F12 medium containing 0.1% collagenase II and 10% fetal bovine serum
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(FBS) and supplementing with penicillin (100 U/ml) and streptomycin (100 mg/mL) in 5% CO₂ for 4 h at 37°C. Chondrocytes were passaged with a ratio of 1:3. Finally, the third passaged chondrocytes were utilized for the following experiments or tests.

**Sample preparation and GeneChip analysis**

Chondrocytes were plated into 6-cm dish. When the cell confluence reaching 80%, chondrocytes were treated with 50 μg/ml and 100 μg/ml ABE contained DMEM:F12 (1:1) solution for low dose and high dose, respectively. Meanwhile, the 10 ng/ml bFGF was also added to the DMEM:F12 (1:1) for 24 h. Cells were then washed with phosphate buffered saline (PBS) for 3 times, followed by RNA extraction. The gene expression profiles were monitored using Agilent SurePrint G3 Rat GE (8*60K, Design ID: 028279) according to protocol provided by manufacture. In order to obtain the raw data, the array-images were analyzed using Feature Extraction software (version 10.7.1.1, Agilent Technologies, Santa Clare, CA, USA). Then, the basic analysis was conducted using Genespring (version 13.1, Agilent Technologies), with raw data as template. Raw data in this study was normalized using quantile algorithm, while probes illustrating more than 100% of values in anyone out of conditions have flags in “Detected” were selected for the following data analysis. The differentially-expressed genes were subsequently identified based on the fold-changes. When a fold change more than 2.0, the up-regulation and down-regulation of genes were assigned. Finally, kyoto encyclopedia of genes and genomes (KEGG) analysis and gene ontology (GO) analysis were conducted to evaluate roles of the above differentially-expressed genes.

**Two-dimensional gel electrophoresis and LC-MS/MS analysis**

Cells and samples were prepared as described above. Briefly, cells with indicated treatment were lysed with radio-immuno-precipitation assay (RIPA). The total protein was extracted and quantified using bicinchoninic acid (BCA) assay. Equal amount of total proteins were loaded by separating with 2D gel electrophoresis. The separation between one or more protein “spots” on the scanned image of a 2-DE gel was analyzed using the software packages, including Delta2D, ImageMaster, Melanie, PDQuest, Progenesis and REDFIN software, as previously described [32].

The excised spots were further analyzed using in-gel digestion and LC-MS/MS as previously described [33]. Briefly, liquid chromatography (LC) was conducted on a NanoAcquity UPLC system (Waters Corporation, Milford, USA) combining with a mass spectrometer (mode: LTQ Orbitrap XL, Thermo Scientific, Bremen, Germany). Peptides were re-suspended with 20 μl solvent A (5% acetonitrile, 0.1% formic acid in water). Total of 18 μl peptide solution was then loaded onto trap-column (100 μm x 2.0 mm Acclaim PepMap C18, Thermo Fisher Scientific, San Jose, CA, USA) at a 20 μl/min flow rate of solvent. Database search and protein identification were performed according to protocol in previous study. Raw files were processed by MaxQuant 1.5.3.8 for peptide/protein identification and quantification with the human International Protein Index database (IPI human 3.45, 71983 entries). The searching parameters were set up as the follows: full trypsin (KR)-cleavage with two-missed cleavage was considered, the oxidation of methionine was specified as the variable modification. Fragment ion tolerance was 0.5 Da and the peptide mass tolerance was 10 ppm. At least two peptides were required for the match of each protein identified.

**Quantitative real-time PCR (RT-PCR) and western blotting**

Total RNAs were extracted and purified using Trizol and the reverse transcription reaction was performed with the First-strand complementary DNA (cDNA) Synthesis Kit. The resulting cDNAs were used as templates for amplifying the indicated genes. qRT-PCR assay was performed using SYBR® Premix Ex Taq™ according to the manual protocol provided by TaKaRa. PCR reactions were done in triplicates with ABI 7500 device (Applied Biosystems Incorporation). Fold enrichment was calculated with the 2^{ΔΔCt} method relative to β-actin.

For western blot assay, the cells were lysed using RIPA lysis buffer (Beyotime Beotech.) supplementing with protease inhibitor (Sigma-Aldrich, St. Louis, Missouri, USA) for 30 min. The supernatants were separated with sodium dodecyl sulphate-polyAcrylamide gel electro-
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phoresis (SDS-PAGE) and subjected for western blotting analysis against indicated antibodies.

Statistical analysis

Data were recorded as mean ± standard deviation (SD) and analyzed using SPSS software (version: 20.0, SPSS Inc., Chicago, Ull, USA). The Tukey’s post-hoc test validated analysis of variance (ANOVA) was used to analyze differences of measurement data among groups. A statistical significance was defined when P<0.05.

Results

Isolation and characterization of chondrocytes in rabbit model of OA

OA modeling was established using knee immobilization, which is reported to achieve a reproducible model to investigate the pathogenesis and therapy of OA [34-36]. The successful modeling was evaluated using Pelletier grading and staging system. The cartilage lesions were obviously discovered in femoral condyles isolating from the knee joints undergoing knee immobilization (Figure 1A). Pelletier grading showed significantly higher scores in modeling group (Figure 1B). HE staining suggested severe fibrillation and erosion of articular cartilage following modeling (Figure 1C). IHC staining of collagen II indicated extensive loss of collagen II, which suggests the cartilage destruction (Figure 1D). These evidences implied the successful modeling of rabbit OA. Chondrocyte was isolated according to the previously described. Chondrocytes displayed fibroblast-like morphology, which is similar to previously described chondrocytes. The identity of isolated chondrocytes was further confirmed using Toluidine Blue O (TBO) staining as well as IHC, which demonstrates the chondrocytes characteristics.

Gene expression profiling of chondrocytes identified the differentially expressed mRNAs upon ABE treatment

To gain insight into the underlying mechanism by which ABE mediated the protecting effect of chondrocytes, we performed GeneChip analysis of RNA expressions in chondrocytes treated with low dose or high dose of ABE. Treatment of chondrocytes with fibroblast growth factor (bFGF) together with insulin-like growth factor-1 (IGF-1) was used in parallel to evaluate the effect of ABE. Differentially expressed mRNAs were filtered through fold changes. Volcano plot filtering analysis (Figure 2A) and the heat map analysis (Figure 2B) demonstrated the differentially-expressed genes, illustrating significant differences among the four groups. Scatter Plot (Figure 2C) and principal component analysis (PCA) (Figure 2D) further supported the good separation and expression signatures among these groups. By the threshold set of fold

Figure 1. Rabbit OA model was successfully established for chondrocytes isolation. A. Macroscopic observations of rabbit articular cartilage in indicated group. Typical changes of the cartilage lesions were illustrated. B. Modeling was evaluated using Pelletier grading. C. Hematoxylin-eosin (HE) staining and immunohistochemistry (IHC) staining verified the proper modeling of OA in rabbit. The IHC-staining was conducted with the anti-Collagen II antibody. D. Isolated chondrocytes was evaluated using Microscopic observation, HE and IHC staining.
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Figure 2. Gene expression profiles revealed ABE predominately targeting glycolysis and apoptosis pathway. A. Volcano plots were used to visualize the differentially-expressed genes among different conditions. Vertical lines correspond to 2.0 folds (log2 scaled) were assigned as up-regulation and down-regulation, respectively. The horizontal line represented a $P$-value of 0.05 (-log10 scaled). The red and blue points in plot represent the differentially expressed genes with statistical significance. B. Heatmap and hierarchical cluster analysis of the most up and down regulated genes. C. Scatter plots were used to evaluate the difference in the expression of genes between experiment group and control group. Values plotted on the X and the Y axes were the mean normalized signal values for each group (log2 scaled).
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change ≥2.0, P<0.05 and FDR <0.05, a total of 498 genes versus 606 genes was found to be differentially regulated. Among which 153 genes and 232 genes were up-regulated, while 374 and 345 down-regulated mRNAs were observed in the low dose and high dose groups respectively, compared with the control group (Figure 2E). Notably, in contrast to low dose treatment, 287 genes were discovered to be changed in chondrocytes with high dose treatment, suggesting a dose-dependent effect of ABE. Strikingly, 2323 genes were significantly altered in bFGF plus IGF-1 including 1232 up-regulated and 1091 down-regulated genes. Venn diagram revealed 654 genes were simultaneously regulated among these groups. These data suggest that ABE exerts transcriptome-wide changes of genes expression in chondrocytes.

ABE treatment connected specific signaling pathways and transcriptional programs for anti-apoptotic response

The gene expression data sets among these groups were integrated and analyzed using multiple bioinformatics methods, including Gene Set Enrichment Analysis (GSEA), GO analysis and KEGG analysis. To this end, the differentially expressed genes were functionally classified into several pathways, including glycolysis, Hif-1 signaling, cell cycle progression, apoptosis and PI3K/AKT signaling pathway and the others. Given that Hif-1 signaling and PI3K/AKT signaling pathway are critical regulators of glycolysis, we assumed that ABE treatment might predominately suppress glucose metabolism. Moreover, cell cycle progression as well as apoptosis downstream of PI3K/AKT signaling is also remarkably enriched. We extended our analysis through integrating key pathway modules and network reconstruction. As showed in Figure 3A, we hypnotized that ABE treatment might mediate the protecting effect through modulating the glycolysis pathway and cell cycle progression through PI3K/AKT signaling pathway.

ABE treatment facilitated chondrocytes cell proliferation and suppressed glycolysis

Next, we performed qRT-PCR analysis to verify the GeneChip data. Cdk14, which positively controlling cell proliferation, was up-regulated in response to ABE stimulation. Plk2, and the canonical targeting genes of p53, including...
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Gadd45a, Gadd45g and Mdm2, were found to be repressed by ABE treatment, suggesting the suppression of negative cell cycle regulators (Figure 3A). Casp1 and Casp12, two potent components of apoptotic cascades, showed reduced levels in chondrocytes following ABE treatment (Figure 3A). Cers1 could encode the CerS1 enzyme implicated in de novo ceramide biosynthesis [37]. Meanwhile, CerS1 mainly generates the C_{18}-Ceramide, which is a critical intracellular inducer of apoptosis [37]. Interestingly, Cers1 was observed to be significantly decreased by ABE stimulation. This evidence indicated that ABE stimulation could promote chondrocytes cell proliferation through suppression of key modulators that negatively govern cell cycle progression. Furthermore, the ABE stimulation could also inhibit the apoptotic process via distinct mechanisms. In agreement with these observations, examination of the proliferation activity and apoptosis showed that the ABE treatment indeed facilitates chondrocytes cell proliferation and represses cell death.

We found the mRNA expressions of certain important metabolic enzymes encoded by Pkm2, Eno1, Eno2, Pdk1, Pgk2, Hk2, were reduced in chondrocytes treated with ABE (Figure 3B, 3C). SLC2A1, as a glucose transporter encoding Glut1, was also decreased. This result suggests the glycolysis pathway is compromised following ABE treatment, possibly providing survival benefits for chondrocytes. Since many genes involved glycolysis were reduced upon ABE treatment in chondrocytes, therefore, ABE treatment indeed affected the glucose metabolism (Figure 3D). Evaluation of the glucose uptake (Figure 3E) and lactate production (Figure 3F) suggested that glycolytic rate decreased by 40% following ABE treatment.

Proteomic-dissection revealed ABE suppresses chondrocytes cell apoptosis and glycolysis

To verify and interpret the transcriptional data, we determined to examine the protein profile of chondrocytes cell following ABE treatment. Two-dimensional gel electrophoresis (2-DE) coupled with LC-MS/MS was a powerful strategy for comparative proteomics research (Figure 4A). Therefore, 2-DE was used to identify the differentiated expressed proteins. A total of 61 proteins were discovered, and were functionally categorized (Figure 4B). The top enriched pathways mainly include cytoskeletal regulation, apoptosis, integrin pathway, glycolysis, FGF sig-
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1. **Signaling, ECM regulation, transcription factors and EGF signaling.** In lines with the transcriptional investigation, these findings here strongly supported that ABE might predominantly modulate apoptosis and glycolysis signaling events. Interestingly, we found the protein level of AKT2 was significantly down-regulated by ABE treatment (**Figure 4C**). This line of evidence might imply that the functions of ABE towards chondrocytes cell apoptosis and glycolysis are probably through activating AKT signaling cascade.

2. **Depletion of IGF-1 and MEK1 in chondrocytes led to increased apoptosis through modulation of AKT**

   Next, we performed in vitro investigation of selected genes for the effects on chondrocytes phenotypes. IGF-1 and MEK1, two newly discovered genes induced by ABE, were selected to verify the following experiments. Ablation of IGF-1 and MEK1 using short hairpin RNA (shRNA) in chondrocytes resulted in 3-5 folds enhanced cell apoptosis, suggesting IGF-1 (**Figure 5A**) or MEK1 (**Figure 5B**) were required for chondrocytes survival. Moreover, knockdown of either IGF-1 (**Figure 5C**) or MEK1 (**Figure 5D**) led to decreased cell proliferation activity. Since IGF-1 acts as an upstream molecule of the MAPK signaling pathway and AKT signaling pathway [38-40], we therefore determined whether IGF-1 and/or MEK1 would play roles through AKT signaling pathway. The protein levels of AKT2, as well as phos-AKT were remarkably increased in chondrocytes undergoing the IGF-1 or MEK1 silencing (**Figure 5E**). These findings suggest that the pro-survival functions of IGF-1 and MEK1 were played through modulating AKT signaling pathway.
Discussion

OA, caused by the irreversible cartilage damage, is considered as a widespread disabling disorder [1, 2]. Although continued efforts have been made to fully understand the OA pathogenesis and therapy, the current management of pharmacotherapies and non-pharmacological therapies has been reported to have limited effectiveness. Considering the side effects, it is not recommended for long-term use. A few new therapeutic approaches and administrations are also undergone the identification, therefore, the effects of these approaches are also needed to be explored [8-10]. Traditional Chinese Medicine might provide alternative intervention for OA. Radix Achyranthes bidentata (AB), a Traditional Chinese Medicinal Herb, has been extensively used in Chinese medical formulations for treating a variety of diseases, such as OA [26-31]. Till now, the protecting effects of ABE on OA seem also ambiguous and the underlying molecular basis of ABE remains poorly understood, therefore, it’s needed to investigate the potential use of ABE on treating OA.

Chondrocytes constitute of the essential component of articular cartilage and are crucial for maintaining the physical structures of cartilage [41, 42]. Here, we performed a systematic study to explore the potential mechanisms hinting in ABE’s effects on the chondrocytic functions. Interestingly, glycolysis pathway was found to be significantly impaired in chondrocytes with ABE stimulation. Moreover, Hif-1 and AKT signaling cascades upstream of glycolysis were spontaneously attenuated. Many key genes, such as Pkm2, Eno1, Eno2, Pdk1, Pgd2, and Hk2, were reduced in chondrocytes treated with ABE. 2-DE coupled with LC-MS/MS also strongly supported that ABE might predominately modulate apoptosis and glycolysis signaling events. Consistently, OA has been proven to be correlated with the metabolic disorders or disturbances [11, 12]. Furthermore, the growing cases of OA and T2DM provide sufficient clinical evidence for correlation between OA and the abnormal metabolism [13, 14]. Enhanced glucose uptake has been seen in swollen joints by FDG-PET [20-25]. These observations increased glycolysis might be a critical factor that drives the disease progression of OA. Our data indicated here that ABE could target glycolysis to protect the functions of chondrocytes. Furthermore, dual treatment of ABE and glycolysis inhibition might provide therapeutic intervention for OA, which requires further investigation in the future work.

In this study, ABE was found to regulate cell proliferation and apoptosis through multiple mechanisms. On one hand, ABE inhibited the expressions of negative cell cycle regulators, such as Plk2, and the canonical target genes of p53, including Gadd45a, Gadd45sg and Mdm2. On the other hand, ABE suppressed the mRNA levels of Casp1 and Casp12, components of apoptotic pathways. Interestingly, Cers1 was observed to be significantly decreased by ABE stimulation. Cers1 was involved in de novo biosynthesis of mainly C18-Ceramide, which potently induces cell apoptosis and autophagy [37]. These findings suggest that ABE could facilitate the cell proliferation via distinct manners.

Eventually, two differentiated genes (IGF-1 and MEK1) were used to verify effects of ABE on chondrocytic functions. Knockdown of IGF-1 or MEK1 significantly promoted cell apoptosis and impaired cell proliferation, suggesting IGF-1 and MEK1 involve in preventing chondrocytes viability. Since ABE primarily modulates glycolysis and apoptosis, the AKT serves as master regulator of glycolysis and apoptosis. Of note, certain genes of upstream of AKT signaling pathway, including Rictor, Pik3ap1 and Inpp5d functions upstream of AKT signaling were found to be simultaneously regulated. Also, many MAPK signaling pathway associated molecules were identified to be regulated by ABE treatment. The previous literatures have demonstrated functions of IGF-1 in MAPK signaling pathway and AKT signaling pathway [38-40]. Therefore, we reasoned whether IGF-1 and/or MEK1 would play functions through activating the AKT signaling pathway. IGF-1 or MEK1 ablation remarkably increased AKT2 protein levels and activated the AKT molecule, suggesting IGF-1 or MEK1 indeed plays roles through activating the AKT signaling pathways to reduce glycolysis.

In conclusion, our study provided systematic view and molecular basis for ABE to serve as potential intervention of OA. Meanwhile, ABE also highlighted that ABE predominately targeting glycolysis via suppressing the AKT signaling pathway.
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Disclosure of conflict of interest

None.

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