

Article

ESWT Enhances Expression of Pdia-3 which is a Key Factor of $1\alpha,25$ -Dihydroxyvitamin D₃ Rapid Membrane Signaling Pathway in Treatment of Early Osteoarthritis Knee

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Abstract: Dysregulation of cartilage homeostasis and the changes in the density and the architecture of the subchondral bone were postulated as a potent mechanically pathological activity contributing to osteoarthritis (OA) pathogenesis. Extracorporeal shockwave therapy (ESWT) is a new, none invasive and effective method in the treatment of animal OA model. In the current study, we demonstrated that shockwave induced the expression of protein-disulfide isomerase-associated 3 (Pdia-3) which is a multifunctional protein hypothesized to be a significant mediator for $1\alpha,25$ -Dihydroxyvitamin D₃ ($1\alpha,25(\text{OH})_2\text{D}_3$) signaling pathway using two-dimensional electrophoresis. Histological analysis and quantitative polymerase chain reaction (qPCR) were verified and observed that the expression of Pdia-3 at 2 weeks was significantly higher than that of any other group at 4 weeks, 8 weeks, and 12 weeks post-shockwave treatment in early OA knee of rat. The other factors of the $1\alpha,25(\text{OH})_2\text{D}_3$ rapid membrane signaling pathway including extracellular signal-regulated protein kinases 1 (ERK1), osteopontin (OPG), alkaline phosphatase (ALP), and matrix metalloproteinase 13 (MMP13) were measured and significantly increased by qPCR at 2 weeks post-shockwave treatment in early OA knee. Our proteomic data revealed significant Pdia-3 expression in microenvironments of joint tissue that could be actively responded to ESWT, which may potentially regulate biological function of chondrocytes and osteoblasts in the treatment of OA knee.

Keywords: protein-disulfide isomerase-associated 3; osteoarthritis; extracorporeal shockwave therapy; $1\alpha,25$ -Dihydroxyvitamin D₃ signaling pathway; two dimensional electrophoresis

1. Introduction

Osteoarthritis (OA), one of the most common causes of musculoskeletal disorders in the developed countries [1], is characterized by cartilage attrition, reduced subchondral bone remodeling, osteophyte formation and synovial inflammation, and factors inducing cartilage

degeneration such inappropriate mechanical load [2], disturbed biochemical regulation [2] and genetic mutation[3] are potential etiologic causes of OA.

Osteoarthritis had been considered to be primarily cartilage disorder characterized by cartilage degradation. Intensive inflammatory cytokines such as interleukin, tumor necrosis factor and proteases secreted from joint component cells caused by abnormal mechanical force are mainly attributable to accelerate cartilage damage, loss of compensatory synthesis and eventually deteriorate the function of extra-cellular matrix organization[4-6]. Dysregulation of cartilage homeostasis caused by intensive chondrocyte apoptosis has been reported as potent pathological activity in the development of osteoarthritis. Disturbance of oxidative stress [7], proapoptotic/antiapoptotic regulation[8, 9] and mitochondrial dysfunction [10] have been proposed to modulate chondrocyte survival in the progression of osteoarthritis. However the molecular mechanism by which chondrocyte propagates toward to programming cell death is not clearly defined.

1 α ,25-Dihydroxyvitamin D₃ (1 α ,25(OH)₂D₃) is essential in calcium homeostasis to regulation of endochondral ossification [11]. In vitamin D deficiency, bone matrix synthesis and cartilage growth are inhibited. It has been investigated action on osteoblasts and growth plate chondrocytes through classic unclear vitamin D receptor (VDR) and Pdia-3. Pdia-3 was demonstrated to a key factor in 1 α ,25(OH)₂D₃ induced phospholipase A₂ (PLA₂) and protein kinase C (PKC) activation and downstream responses of gene transcription [12, 13].

A growing body of evidence has demonstrated ESWT promoted tissue repair in various tissue and initiated biological responses [14, 15]. Reportedly ESWT ameliorated experimental osteoarthritic cartilage damage and alter angiogenesis pattern [15]. The preliminary proteomic data revealed abundant significant proteins that warrant further characterization [16-18]. These proteins of interest were reported to participate in the cellular response to physical stress, calcium homeostasis, chemotaxis and lipid oxidative stress in several tissue types under pathological contexts. Therefore, we hypothesize that multiple molecules in joint tissue microenvironments may be actively responded to ESWT treatment, which potentially regulates survival and biological function of chondrocytes in OA knee. To test the null hypotheses, we conducted studies to delineating the active responsive molecules in ESWT-regulated biological responses using comparative proteomic technique helps the construct of molecular mechanism of alleviation of OA and whether ESWT changed signaling interfering joint microstructures. Based on these translational experimental data, we could further explore a new regime with good potential for rescuing OA knee joint injury.

2. Results

2.1. The effect of shockwave therapy on articular cartilage

In the macroscopically normal articular cartilage, some changes of cell distribution in the superficial layer were noticed. Furthermore, the differences between OA knee and shockwave treatment were pronounced. After application of ESWT to the subchondral bone of the medial tibia condyle showed regression of osteoarthritis of the knees in rats (Figure

1A). In the OA+ESWT group of knee, the articular cartilage, which Mankin's score ranged from 2 to 5, was better preserved than in the OA group, which Mankin's score ranged from 4 to 8 during 2 weeks to 12 weeks (Figure 1B). In the OA group, significantly greater articular cartilage degeneration was evident and evaluated in wide Mankin's score range.

2.2. Two-dimensional electrophoresis in shockwave knee compared with osteoarthritic knee at 2 weeks after index surgery

Proteome analysis of the left knee was conducted by two-dimensional gel electrophoresis and mass spectrometry. Protein spots in gel were developed by silver stains and scanned by an Amersham ImageScanner. The level of significant spot was 1.5 fold increase or decrease. Twelve spots were found to be differentially abundant, including 9 spots decreased intensity and 3 spots increased signal observed in OA+ESWT group when compared with normal control (NC) group and OA group at 2 weeks after index surgery. (Figure 2 and Supplemental Figure 1)

The 8 identified proteins were significantly different between the shockwave with OA knee and OA knee as well as also changed with disease development (Figure 3). Among those, 2 proteins were up-regulated including Pdia-3 and guanine nucleotide-binding protein subunit beta-2-like. Moreover, six proteins were identified and suppressed in OA+ESWT group, including Beta-enolase, chloride intracellular channel protein 1, malate dehydrogenase, purine nucleoside phosphorylase, creatine kinase M-type and L-lactate dehydrogenase A chain (Table 1). These proteins were involved in regulating various cellular functions, including cytoskeletal structure, ion channel components, energy metabolism, and protein degradation. These findings indicated altered protein expression in the pathogenesis of OA and illuminated a novel therapeutic avenue for treatment in OA disease. Among these proteins, protein Pdia-3 in the knee joint, which was reported to osteoblast and chondrocyte cells integrity and function [19-21].

2.3. The expression of Pdia-3 and extracellular signal-regulated kinases 1 (ERK1) after ESWT on early OA knee

The spot of Pdia-3 observed difference between OA+ESWT group and OA group at 2, 4, 8 and 12 weeks after index surgery were first destaining and enzymatic in gel digestion and then subjected to MALDI/TOF mass spectrometry analysis. The expression of Pdia-3 at each time course was summarized in Figure 4. Significantly, regulation of the mRNA of Pdia-3 at each time course was noted in OA+ESWT group compared with OA group and NC group. It was especially enhanced to 13 fold at 2 weeks than at 4, 8 and 12 weeks after shockwave treatment with OA knee (Figure 4A).

Extracellular signal-regulated kinases (ERKs), acted as an integration point for multiple biochemical signals, and were involved in an osteoblast cellular processes such as proliferation, differentiation, transcription regulation and development [12, 22]. Upon activation by Pdia-3 after ESWT, these kinases translocations to the nucleus of the osteoblast cells, where it phosphorylated nuclear targets. Two alternatively spliced

transcript variants encoding different protein isoforms had been described for this gene. Significant increases of ERK1 in OA+ESWT group as compared with OA and NC groups were found, especially at 2 weeks (Figure 4B).

2.4. The expression of related gene of rapid membrane signaling pathway after ESWT

The addition of shockwave therapy to knee resulted in increase of bone formation markers, including OPG, ALP and MMP13. The ALP increased if there was active bone formation occurring, as ALP was a byproduct of osteoblast activity [23, 24]. OPG in bone was the major determinant of bone mass and strength [25-27]. MMP 13 was established marker gene for bone formation [28, 29]. Significantly less pronounced subchondral bone remodeling with decreases in osteogenesis was noted in OA group compared with the OA+ESWT group. It appeared that application of ESWT to the medial tibia condyle of the knee improved osteogenesis and bone turnover rate of the subchondral bone in OA knees, and the results were comparable to that of the normal control in rats (Figure 4C, 4D and 4E).

2.5. The effect of ESWT on Pdia-3 expression and extracellular matrix in articular cartilage and subchondral bone of OA knee

We further investigated expression of Pdia-3 from our microscopically IHC stain in articular cartilage and subchondral bone of knee at 2 weeks (Figure 5). We observed the expression of Pdia-3 was more 40 % and 15 % concentrated in OA+ESWT group than OA group and NC group (Figure 5A and 5B). The staining signal was particularly enriched on the superficial layer of cartilage and bone marrow of subchondral bone, indicated that Pdia-3 co-responded to ESWT in the chondrocyte and subchondral bone because the up-expression of Pdia-3 significantly increased on the chondrocyte and bone after OA+ESWT compared with OA and NC groups.

The synthesis of extracellular matrix of aggrecan and collagen type II was being investigated for their role in cartilage formation [5, 30, 31]. The OA+ESWT group showed significantly increased amount of the collagen type II (about 3 fold higher) and aggrecan (about 20 fold higher) at 2 and 4 weeks when compared with the NC and OA group ($P < 0.05$). (Supplemental Figure 1A and 1B; Supplemental Table 1)

3. Figures, Tables and Schemes

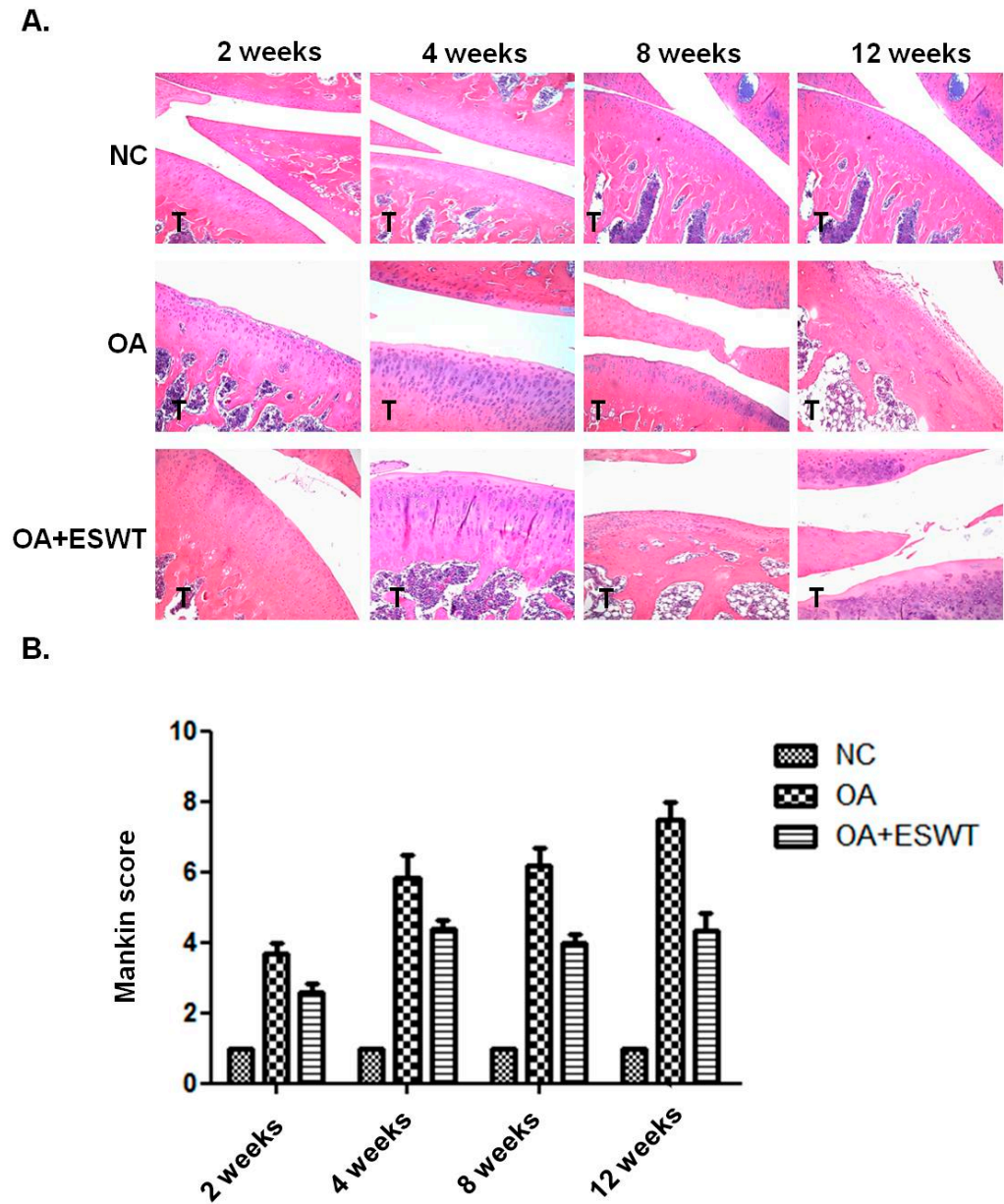


Figure 1. Histologic results of the early OA knee after ESWT. (A) Representative histological photographs of early OA knee after ESWT at 2, 4, 8, 12 weeks. Normal control (NC) showed normal structural integrity, cellularity, and cartilage height and tidemark integrity. (B) Scores across a section of normal cartilage ranged from 0 to 1. OA knee of section with clefts and modest proteoglycan depletion was assigned scores ranging from 4 to 8. The OA+ESWT group showing regression of OA change was given scores from 2 to 5. T was indicated tibia. Specimens were stained by conventional hematoxylin-eosin. Specimens were observed in hundred-fold magnification.

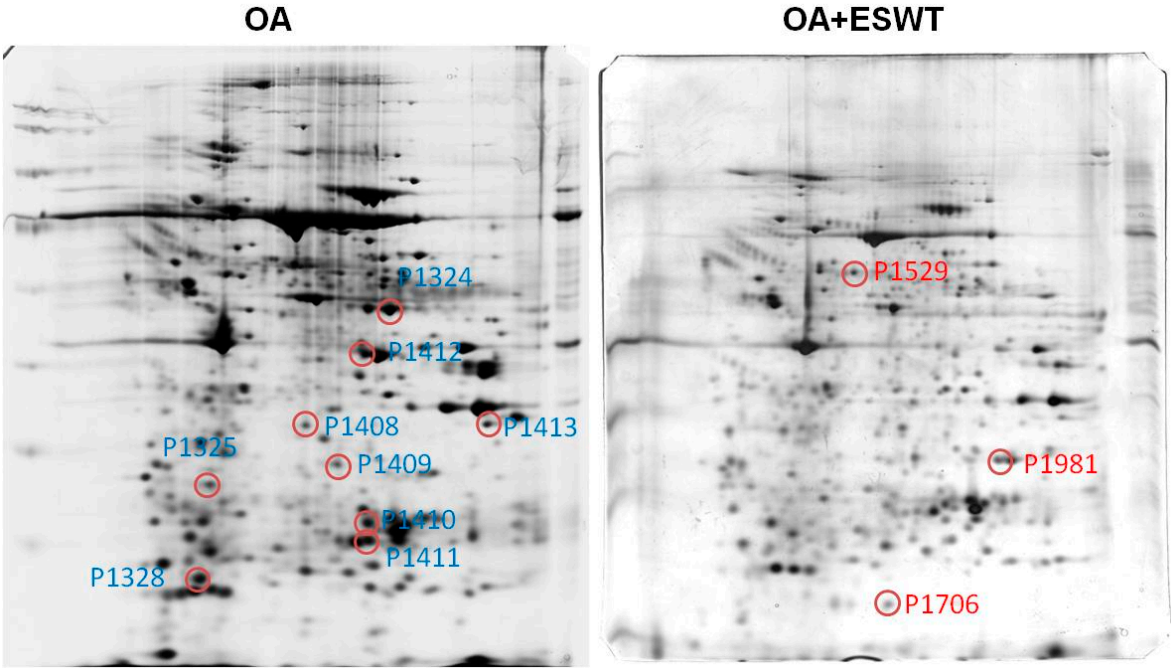
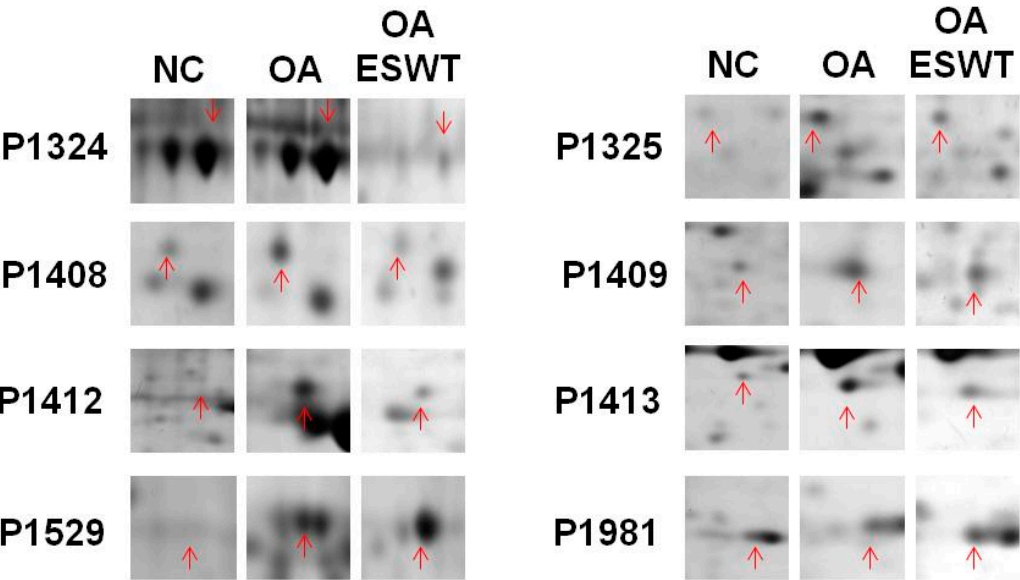


Figure 2. Protein spots of interest in two-dimensional gel electrophoretograms. ESWT induced or suppressed expression of several different proteins at 2 weeks. The level of significant spot was 1.5 fold increase or decrease. Twelve spots were found to be differentially abundant, including 9 spots decreased intensity and 3 spots increased signal observed in OA+ESWT group when compared with



OA group at 2 weeks after index surgery.

Figure 3. Enlarged the regions of the 8 spots of interest in the silver-stained SDS-polyacrylamide gels. The arrows indicated the spot of interest between OA+ESWT and OA groups. ESWT promoted two proteins up-regulation including Pdia-3 (P1529) and guanine nucleotide-binding protein subunit beta-2-like (P1981). Moreover, six proteins were identified that shockwave knee suppressed, including Beta-enolase (P1324), chloride intracellular channel protein 1 (P1325), malate dehydrogenase (P1408), purine nucleoside phosphorylase (P1409), creatine kinase M-type (P1412) and L-lactate dehydrogenase A chain (P1413). Red arrow indicated the position of interest spots.

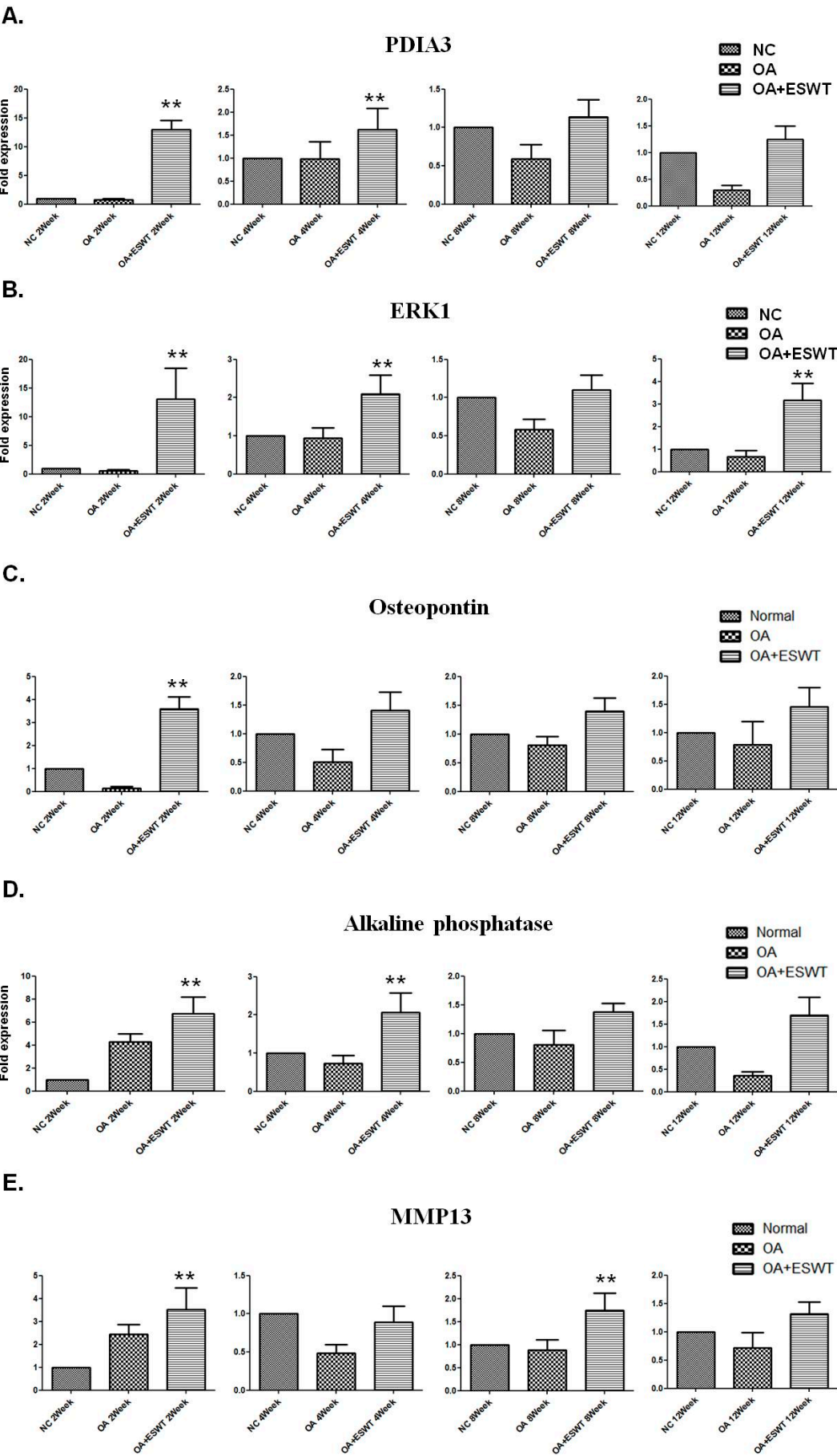


Figure 4. (A) Effect of ESWT on Pdia-3 expression in early OA knee. ESWT rapidly increased Pdia-3 activity at 2 weeks and then decreased at 4, 8 and 12 weeks. When compared with OA knees after index surgery, it had significant difference at 2 and 4 weeks ($P<0.05$). (B) ESWT promoted ERK1 expression at 2 and 4 weeks as compared with OA knee after index surgery ($P<0.05$). (C-E) Effect of ESWT on bone formation markers in early OA knee. Shockwave therapy increased bone formation as implicated by activating OPG, ALP and MMP 13 intensity, especially at 2 weeks after treatment. ($P<0.05$) Real-time PCR was performed against 3 bone related genes: (C) osteoprotegerin; (D) alkaline phosphatase and (E) matrix metalloproteinase 13. The OA+ESWT group showed significant more amount of bone turnover rate than as compared with OA group. ($P<0.05$)

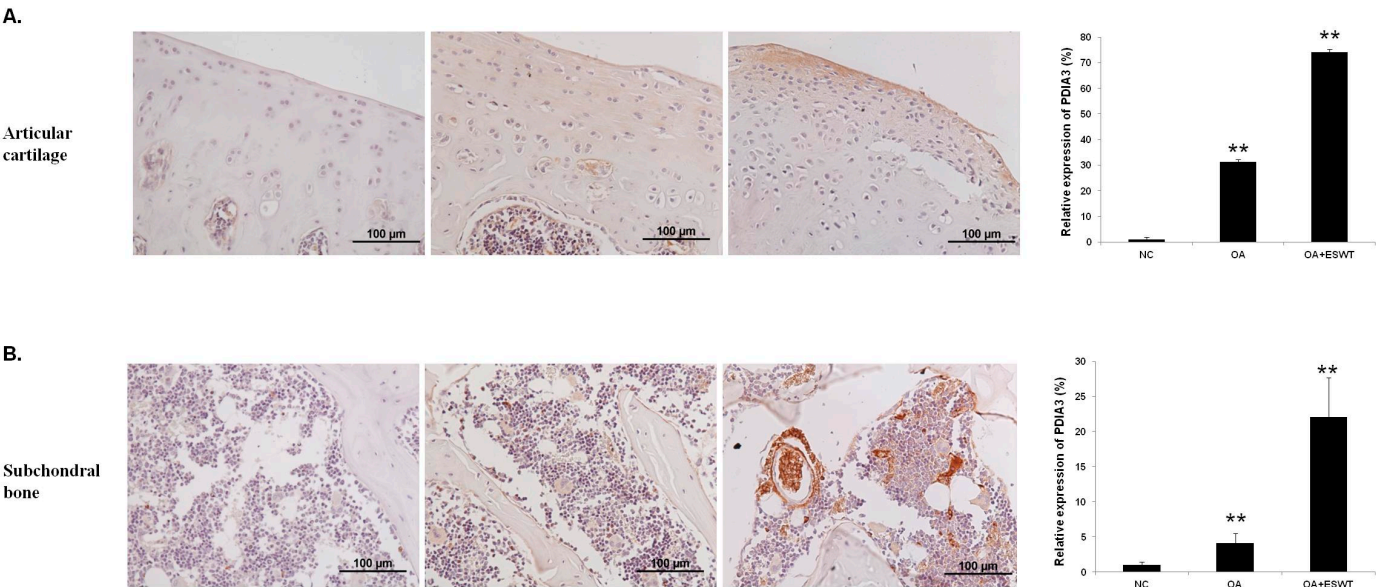


Figure 5. IHC staining of Pdia-3 in chondrocyte and subchondral bone with and without ESWT in early OA knee at 2 weeks. (A) The Pdia3 (brown color) was distributed over the cartilage and was enriched in the articular surface of OA+ESWT group. After quantization of spots by scanning densitometry, the OA+ESWT group showed significantly more chondrocyte expression of Pdia-3 compared with OA knee. ($P<0.05$). (B) NC group showed no staining in cell without primary Pdia-3 bodies. Cells were stained for antibodies against of Pdia-3 (brown spots) and imaged at 100 magnifications. After quantization of spots by scanning densitometry, the OA+ESWT group showed significantly more subchondral bone expression of Pdia-3 compared with OA group ($P<0.05$).

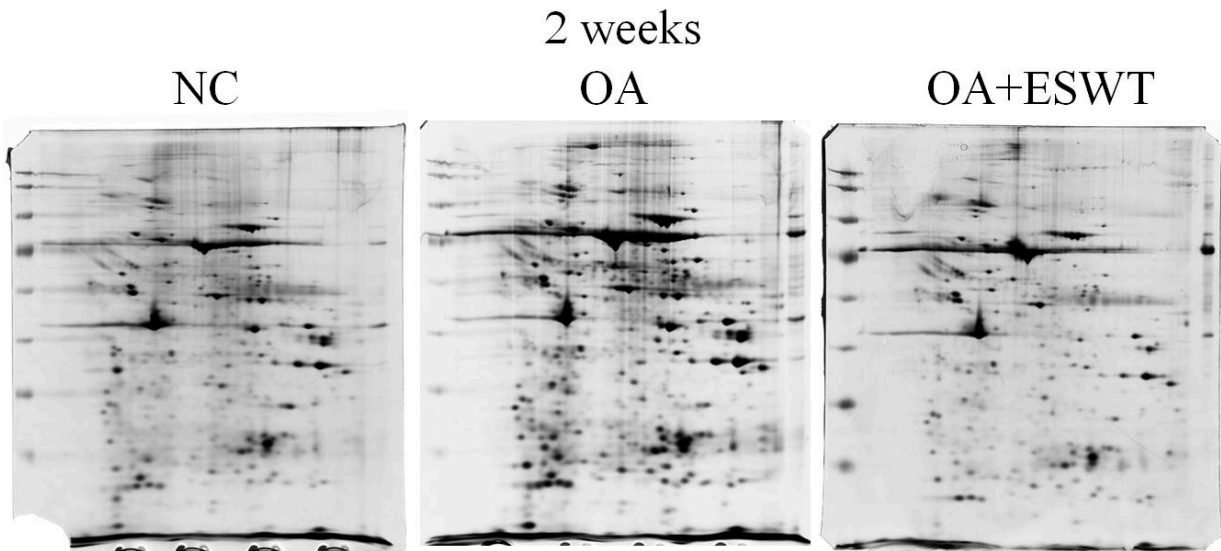
Table 1. Characteristics of positively identified spots by mass spectrometry.

Number	Name of identified protein	Entry name	Score	Molecular weight [kDa]	Theoretical PI value
P1324	Beta-enolase	ENOB_RAT	603.1976361	47.33743	6.886547057
P1325	Chloride intracellular channel protein 1	CLIC1_RAT	260.7394264	27.30589	4.944812479
P1408	Malate dehydrogenase	MDHC_RAT	344.71	36.46005	6.168189325
P1409	Purine nucleoside phosphorylase	PNPH_RAT	376.3744403	32.28104	6.519143097
P1412	Creatine kinase M-type	KCRM_RAT	541.9422014	43.0178	6.632039261
P1413	L-lactate dehydrogenase A chain	LDHA_RAT	589.4044403	36.42734	9.279562822
P1529	Protein disulfide-isomerase A3	PDIA3_RAT	699.4495433	57.04387	5.829417423
P1981	Guanine nucleotide-binding protein subunit beta-2-like	GBLP_RAT	395.3884018	35.51073	8.876677099

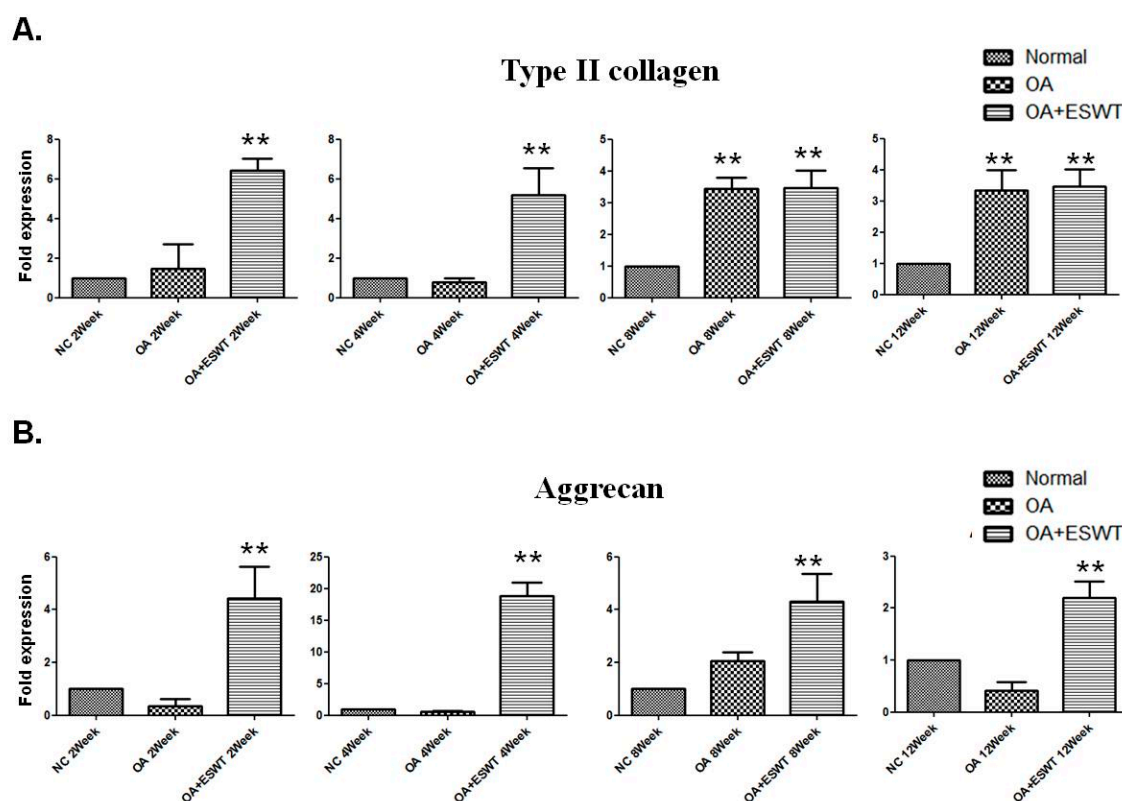
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Table 2. The primers were used for qPCR in this study.

Primer name	Type	Length Sequence (5'–3')
Rat Pdia-3	Forward	20-mer GAGGCTTGCCCCTGAGTATG
Rat Pdia-3	Reverse	19-mer GTTGGCAGTGCAATCCACC
ERK1	Forward	20-mer AGCTGCTAAAGAGCCAGCAG
ERK1	Reverse	20-mer GCAAGGCCAAAATCACAGAT
Osteopontin	Forward	20-mer GTTCTTGACAGCTTCACCA
Osteopontin	Reverse	20-mer AAACAGCCCAGTGACCATTC
Alkaline phosphatase	Forward	20-mer GACAAGAAGCCCTTCACAGC
Alkaline phosphatase	Reverse	20-mer GGGGGATGTAGTTCTGCTCA
MMP13	Forward	20-mer GAGGTGAAAAGGCTCAGTGC
MMP13	Reverse	20-mer TGGGCCCATTGAAAAAGTAG



Supplemental Figure 1. The articular cartilage and subchondral bone of left knee were analysis by two-dimensional gel electrophoresis from NC, OA and OA+ESWT groups.



Supplemental Figure 2. The effect of shockwave therapy induced expression of cartilage related gene in early OA knee. The OA+ESWT group showed significant increases in expression of aggrecan and collagen type II when compared with NC and OA groups at 2, 4 8 and 12 weeks ($P < 0.05$).

4. Discussion

In this study, we expanded the study and confirmed that osteoarthritic knee could be regressed by the application of shockwave therapy. Furthermore, we demonstrated a trend toward increase of Pdia-3 biosynthetic activity in response to pulsed acoustic energy released by shockwave therapy. The mitogenic and anabolic activities of osteoblast and chondrocyte increased relating to elevating ERK phosphorylation in sudchondral bone after ESWT. The growing evidence indicating that Pdia-3 dependent mechanisms are involved in rapid responses to the secosteroid 1, 25-dihydroxyvitamin D₃ ($1\alpha, 25(\text{OH})_2\text{D}_3$) signaling in osteoblast and chondrocyte cells [12, 19, 20]. Pdia-3 has been identified as a potential candidate as an alternate membrane-associated receptor for $1\alpha, 25(\text{OH})_2\text{D}_3$. The $1\alpha, 25(\text{OH})_2\text{D}_3$ directly regulates mineralization from the osteoblasts and matrix formation from the chondrocyte through classic vitamin D receptor (VDR) mediated genomic pathway and membrane receptor-mediated rapid responses via Pdia-3 dependent pathway. Our study provided the first evidence that the effect of ESWT on the OA knee revealed the regulation of protein Pdia-3, linking to osteoblast and chondrocyte cells integrity and function. This finding suggested the proposed biomechanical pathway was likely to be conserved of shockwave therapy in the treatment of OA knee.

It is well established that Pdia-3 mediated the membrane response to $1\alpha, 25(\text{OH})_2\text{D}_3$, including phospholipase A2 (PLA2) stimulation dependent rapid release of prostaglandin E2

(PGE2), activation of protein kinase C (PKC), then regulation of bone related gene transcription and mineralization via phosphorylation of transcription factors such as ERK1/2 in osteoblast-like MC3T3-E1 cells [19]. Jiaxuan et al found that in Pdia-3-silenced (Sh-Pdia-3) cells, $1\alpha,25(\text{OH})_2\text{D}_3$ failed to stimulate PKC and PGE2 reaction and in Pdia-3 overexpression cells (Ov-Pdia-3), the respond to $1\alpha,25(\text{OH})_2\text{D}_3$ were augmented. They suggested the Pdia3 could be a determining factor correlating directly with the magnitude of the membrane response to $1\alpha,25(\text{OH})_2\text{D}_3$ [32]. The principal findings of this study showed that application of ESWT induced Pdia-3 up-regulation leading to subchondral bone remodeling after ACLT OA of knee in rats. The present gene expression showed the ability of the cells to mineralized their extracellular matrix and bone related genes, ALP, OPG and MMP13, significantly increased after shockwave therapy. The results were in agreement with prior studies had demonstrated that ESWT significantly enhances the osteogenic factors reflecting a local stimulation of bone formation during the fracture healing [33-35]. From the results presented, the data clearly showed that a number of genes encoding bone formation and relatively signaling molecules that could potentially transduce osteogenic effect in response to treatment with shockwave changing expression of Pdia3 in the subchondral bone of knee.

Pdia-3 mediated signaling results in gene transcription, which can also modulate bone formation. The key components of ERK1 in this signaling pathway were also activated. ERK had been found to act as an important mediator for mechanical-stimulated proliferation and differentiation of osteogenic cell. Previous studies revealed the ERK was involved in shockwave-augmented bone formation in segmental defects within 14 days after treatment. The phosphorylation of ERK is active throughout the period of ESW-induced bone regeneration and regulated the stimulation of biophysical shockwave therapy, triggering mitogenic and osteogenic responses in the defects [36]. Our present data reveals that the signals of ERK were active and it could play an important role in signaling subchondral bone remodeling after 2 wk local application of shockwave therapy.

Several studies reported positive effects of ESWT in osteoarthritis of different joints in animals [37-41]. The exact mechanism of shockwave therapy was still unknown. Our current study provides the first evidence from immunohistochemistry that shockwave therapy can induce articular cartilages expression of Pdia-3, the critical transcription factor responsible for the matrix formation of chondrocyte. Recent studies reported that $1\alpha,25(\text{OH})_2\text{D}_3$ rapidly stimulated membrane signaling via Pdia-3 dependent activation in growth zone chondrocytes and promotes the production of matrix protein [11, 13, 20, 22, 42, 43]. The present study showed the decrease of cartilage matrix loss and increased aggrecan and collagen II expression in shockwave group. It explained the biomolecular mechanism of shockwave therapy in cartilage development and maintenance of the chondrocyte phenotype. The regression of osteoarthritic knee was supported by the expression of Pdia-3 and biomarkers of the cartilage in the remodeling surface of articular area.

The effect of shockwave therapy in the osteoarthritic rat knee showed time-dependent chondroprotection [44]. We observed that the most beneficial effects of shockwave therapy on the OA knee after 2 wk of shockwave application, and the effects of shockwave seemed to

continue until 12 wks. The novel findings supported the concept that shockwave therapy provided a chondroprotective effect associated with improvement in subchondral bone remodeling, significant decrease in the cartilage degradation and increase in chondrocyte activity in the initiation of ACLT OA. Application of shockwave therapy on the subchondral bone was effective in a time-dependent fashion in OA of the knee.

The exact mechanism of ESWT remains unexplored. The innovative findings in this study may unveil a new concept in the biomolecular pathway and treatment of osteoarthritis of the knee by ESWT. It appeared that local shockwave therapy application to the subchondral bone in medial tibia condyle affected the entire knee joint through stimulating the osteoblast and chondrocyte cells via up-regulation of Pdia-3.

There are some limitations in this study. The data obtained from this study were based on small animals' experiments. The results may differ in larger animals or human subjects. The dose conversion from small animals to larger animals or human subjects must be calculated with additional studies and clinical trial. The optimal ESWT dose and the ideal numbers of ESWT remain unknown. Furthermore, different manufacture companies used different indices of shockwave parameters, and the dose conversion formula among the different devices are not readily available at the present time.

5. Materials and Methods

5.1. Study Design

The Institutional Review Board on animal experiment approved this study. All studies were performed in accordance with the guidelines in the study and the care of animals in experiment. All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital, Taiwan. One hundred forty-four male four-month-old Sprague-Dawley rats with body weight ranging from 250 to 275 mg were used in this study. The rats were randomly divided into three groups with 48 rats in each group and each time course for 12 rats (n=48 per group). The 12 rats were sacrificed at 2, 4, 8 and 12 weeks post-surgery. In 12 rats, six were for proteome analysis and six for histology study. The rats in normal control (NC) group received neither anterior cruciate ligament transection (ACLT) nor ESWT and served as the baseline control. The rats in OA group underwent ACLT, but received no ESWT. The rats in OA+ESWT group underwent ACLT and received ESWT (800 impulse at 0.18 mJ/mm²) to the subchondral bone of the medial tibia condyle.

5.2. *Experimental OA knee model*

Four-month-old male Sprague-Dawley rats were anesthetized using intraperitoneal injection of pentobarbital (50 mg/Kg body weight). The left knees of the animals underwent surgery comprising medial parapatellar arthrotomy and ACLT to induce ACLT-mediated knee OA as previously described [45]. Animals were allowed unrestricted weight-bearing and activity as tolerated. The left knee was excised after animals were sacrificed at each time course.

5.3. *Histology*

Whole knee joints were fixed in 4 % phosphate buffered paraformaldehyde, decalcified in 10 % phosphate buffered EDTA, then embedded in paraffin. Specimens were longitudinally cut into 4- μ m sections for hematoxylin and eosin staining and Alcian blue staining (Sigma-Aldrich). Histomorphometry of articular cartilage was evaluated by a 14-point Mankin scoring system (for structural integrity, from 0 = normal to 6 = complete disorganization; for cells, from 0 = normal to 3 = hypocellularity; for cartilage height, from 0 = normal to 4 = complete loss; for tidemark integrity, from 0 = normal to 1 = disruption) (25). Twelve sections from 6 rats were measured under 100x magnification using a Zeiss Axioskop 2 plus microscope (Carl Zeiss Microimaging) with a cool CCD camera and Image-Pro Plus image analysis software (SNAP-Pro c.f. Digital kit; Media Cybernetics). In some cases, the specimens were fixed in absolute alcohol, embedded in glycolmethylacrylate (Fluka), then cut longitudinally into 8- μ m sections using a rotary tungsten steel-bladed microtome for von Kossa's and tartrate-resistant acid phosphatase histochemical staining (Sigma-Aldrich). All sections were independently assessed by two individuals. Cartilage destruction was assessed using the Mankin scoring system [46]. Each-section was evaluated using Mankin's histological and histochemical grading system, including structural changes in all layers of the uncalcified cartilage, tidemark integrity and Safarine-O staining. Safarine-O staining is the indicator of proteoglycan amount in the extracellular matrix.

5.4. *Two- dimensional electrophoresis*

Samples of 250 μ g protein from 2 comparative subjects were applied on immobilized pH 3-10 nonlinear gradient strips with pretreated CyDye. Isoelectric focusing is performed by an Ettan™ IPGphor II/3. Proteins in the strips were separated in 15 % SDS-PAGE and stained using fluorescence dyes. Protein spots in gels were scanned by an Amersham Image

Scanner. Image, spot match and spot intensity were analyzed and calculated by a Bio-Rad ProteomeWeaver 2-D Analysis Software Version 4.0.

5.5. MALDI-TOF mass spectrometry/LC mass spectrometry

Spots of interest were excised and washed with 10 mM ammonium bicarbonate and 50 % acetonitrile in 10 mM ammonium bicarbonate. After wash and shrinkage with acetonitrile, the dried gels were digested by trypsin at 30°C for 4 hours. The trypsin digest was extracted by trifluoroacetate. Aliquots of the digest were loaded onto AnchorChip for one PMF from MALDI-TOF, and TOF/TOF MS/MS analysis of fragment peptides using the FlexControl™ software. Peptide mass data were submitted to NCBI and Swiss-Port database using MASCOT search engines.

5.6. Quantitative RT-PCR

The primers of bone and cartilage biomarkers including osteoprotegerin (OPG), alkaline phosphatase (ALP), matrix metalloproteinase 13 (MMP13), collagen type II and aggrecan were detected by quantitative RT-PCR (Table 2). Total RNA was extracted and purified from knee joint tissue using QIAzol reagent. Total RNA (1 µg) was reverse transcribed into cDNA. 25 µL of PCR mixture containing cDNA template equivalent to 20 ng total RNA, 2.5 µM each forward, reverse primer and 2X iQ™ SYBR green supermix was amplified using the iCycler iQ® Real time PCR detection system with an initial melt at 95°C for 5 min followed by 40 cycles at 94°C for 15 sec, 52°C for 20 sec and 72°C for 30 sec using following primer oligonucleotide sequences followed by PCR amplification using responsive molecules and rat 18S rRNA primers (forward) (5'-GCAGCTAGGAATAATGGAATAGGA-3'), (reverse) (5'-TAATGAAAACATTCTTG GCAAATG-3'). The number of amplification steps required to reach an arbitrary intensity threshold (Ct) is computed. The relative gene expression level was presented $2^{-\Delta Ct}$, where $\Delta Ct = Ct_{\text{target}} - Ct_{18S \text{ rRNA}}$. Fold change for the treatment was defined as the relative expression, compared with the vehicle and is calculated as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{\text{treatment}} - \Delta Ct_{\text{vehicle}}$.

5.7. In situ immunohistochemistry

Sections were hybridized with relative antibodies against candidate proteins and a nonbiotin HRP detection system (BioGenex). Immunoreactivity in specimens was demonstrated using a horseradish peroxidase (HRP)-3', 3'-diaminobenzidine (DAB) cell and tissue staining kit (R&D systems, Inc. Minneapolis, MN, USA) according to manufacturer's

instructions. Antibodies against discovered molecules were used. Sections were then incubated with biotinylated secondary antibodies with streptavidin conjugated to HRP, followed by chromogen solution and counterstaining with hematoxylin. Sections were finally dehydrated and mounted. Sections without primary antibodies were enrolled as negative controls for the immunostaining. The number of positive immunolabeled cells and total cells in each area were counted and percentages of positive-labeled cells were presented. The osteoblasts and chondrocytes were identified morphologically. A pathologist, blinded to the treatment, performed the measurements of all sections under 100 fold magnifications.

5.8. Statistical analysis

All values were expressed as mean \pm standard error. One-way ANOVA and Tukey tests were used to assess the differences among the groups. The level of statistical significance was set at $P < 0.05$.

6. Conclusions

Our proteomic data revealed abundant significant Pdia-3 expression in joint tissue microenvironments may be actively responded to ESWT treatment, which potentially regulated biological function of chondrocytes and osteoblasts in OA knee. Furthermore, ESWT has the potential ability in the treatment of osteoarthritis of the knee.

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Author Contributions: Shan-Ling Hsu, Jai-Hong Cheng participated in the study with primary responsibility in conception and design drafting, overview the entire study, data collection and analysis, literature review, reference search, draft writing and critically revised the manuscript and read proof of the final manuscript.

Ching-Jen Wang, Jih-Yang Ko, and Chih-Hsiang Hsu participated in the study with primary duty in reference search, literature review and read proof of final manuscript.

Conflicts of Interest: The authors declared that they did not receive any honoraria or consultancy fees in writing this manuscript. No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article. One author (Chin-Jen Wang) serves as a member of the advisory committee of Sanuwave, (Alpharetta, GA) and this study is performed independent of the appointment. The remaining authors declared no conflict of interest.

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