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Extracorporeal shock wave promotes activation of anterior cruciate ligament remnant cells and their paracrine regulation of bone marrow stromal cells' proliferation, migration, collagen synthesis, and differentiation

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Aims

Proliferation, migration, and differentiation of anterior cruciate ligament (ACL) remnant and surrounding cells are fundamental processes for ACL reconstruction; however, the interaction between ACL remnant and surrounding cells is unclear. We hypothesized that ACL remnant cells preserve the capability to regulate the surrounding cells' activity, collagen gene expression, and tenogenic differentiation. Moreover, extracorporeal shock wave (ESW) would not only promote activity of ACL remnant cells, but also enhance their paracrine regulation of surrounding cells.

Methods

Cell viability, proliferation, migration, and expression levels of Collagen-I (COL-I) A1, transforming growth factor beta (TGF-β), and vascular endothelial growth factor (VEGF) were compared between ACL remnant cells untreated and treated with ESW (0.15 mJ/mm^2, 1,000 impulses, 4 Hz). To evaluate the subsequent effects on the surrounding cells, bone marrow stromal cells (BMSCs)' viability, proliferation, migration, and levels of Type I Collagen, Type III Collagen, and tenogenic gene (*Scx, TNC*) expression were investigated using coculture system.

Results

ESW-treated ACL remnant cells presented higher cell viability, proliferation, migration, and increased expression of COL-I A1, TGF-β, and VEGF. BMSC proliferation and migration rate significantly increased after coculture with ACL remnant cells with and without ESW stimulation compared to the BMSCs alone group. Furthermore, ESW significantly enhanced ACL remnant cells' capability to upregulate the collagen gene expression and tenogenic differentiation of BMSCs, without affecting cell viability, TGF-β, and VEGF expression.

Conclusion

ACL remnant cells modulated activity and differentiation of surrounding cells. The results indicated that ESW enhanced ACL remnant cells viability, proliferation, migration, and expression of collagen, TGF-β, VEGF, and paracrine regulation of BMSC proliferation, migration, collagen expression, and tenogenesis.

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Keywords: Anterior cruciate ligament, Extracorporeal shock wave, ACL reconstruction, Graft maturation

Article focus

- The interaction between anterior cruciate ligament (ACL) remnant and its surrounding cells after ACL reconstruction with remnant preservation.

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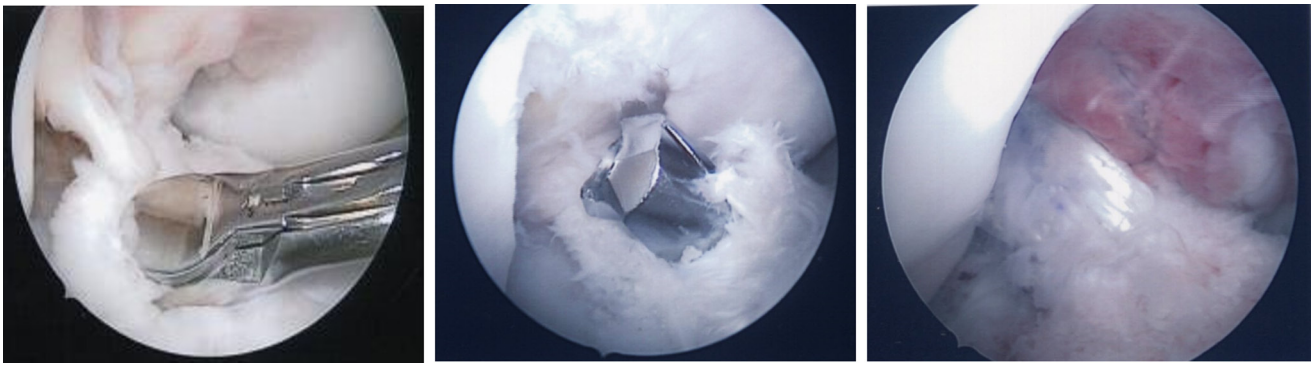

Fig. 1

Arthroscopic pictures of anterior cruciate ligament (ACL) remnant harvest and reconstruction. ACL remnant was harvested by arthroscopic punch (Left image). The ACL remnant was partially preserved and reamed to create bone tunnel (Middle image). The reconstructed graft was passed through the bone tunnel and surrounded by ACL remnant (Right image).

- The effects of extracorporeal shock wave (ESW) on activity of the ACL remnant cells and paracrine regulation of surrounding cells.

Key messages

- After ACL reconstruction with remnant preservation, the ACL remnant regulated bone marrow stromal cells' (BMSCs) proliferation, migration, collagen gene expression, and tenogenesis potential to help graft maturation.
- ESW stimulated the viability, proliferation, migration, and expression of collagen synthesis, transforming growth factor beta (TGF-β), and vascular endothelial growth factor (VEGF) of ACL remnant cells.
- Overall, ESW treatment not only stimulated the activity of ACL remnant cells, but also significantly enhanced its regulatory capability to the surrounding cells, which would help in graft maturation.

Strengths and limitations

- The first study to confirm the interaction between ACL remnant and its surrounding cells and the effects of ESW on ACL remnant cells and their regulatory capability to the surrounding cells.
- We investigated the effects of ESW on ACL remnant cells in vitro but did not validate these findings using animal studies.
- The optimal dose and frequency of ESW to treat ACL remnant cells and promote tissue regeneration was not investigated.

Introduction

Knee injuries, especially those involving the anterior cruciate ligament (ACL), are common sports injuries.[1-3] Due to its intra-articular location, limited blood supply, and intrinsic cell loss, the injured ACL heals poorly.[4] Surgical ACL reconstruction recovers joint function and athletic ability in clinical practice.[3,5,6] The major concerns of implanted graft after ACL reconstruction are osseous integration at the graft-tunnel interface and remodelling of the intra-articular graft (ligamentization).[7,8] After implantation, the graft necrosis leads to secretion of growth factors and cytokines that direct cell migration from the graft periphery (bone marrow, synovium, and remnant of the native ACL) to the injury site. These migrated cells further proliferate and produce extracellular matrix, and proceed tenogenic differentiation to promote ligament healing under growth factor stimulation.[9,10]Hence, insights into the regulation of ACL remnant tissue and cells and surrounding cells in terms of proliferation, migration, collagen synthesis, secretion of growth factors, and differentiation is important to accelerate early graft healing and prevent graft rupture after ACL reconstruction.[11,12]

The remnant preservation technique for ACL reconstruction has been proposed to improve functional result and augment graft incorporation by enhancing cell proliferation, revascularization, and regeneration of proprioceptive organs.[13-15] However, the clinical value and result after remnant preservation in ACL reconstruction is still debated.[14-16] The ACL remnant was demonstrated to contain stem cells and present the capability to augment the healing of tendon to bone tunnel in in vitro studies.[13,17,18] During ACL reconstruction, ACL remnant surrounded the implanted graft and contacted the surrounding tissue and cells (i.e. bone marrow stromal cells (BMSCs) released from the drilled bone tunnel that covered ACL remnant and implanted graft; Figure 1 middle and right images). However, the actual role of ACL remnant cells in graft regeneration and their interaction with surrounding cells is not clear.

Extracorporeal shock wave (ESW), a sound wave characterized with fast pressure rise and high peak-pressure in a short lifecycle,[19] has been applied in treating multiple

orthopaedic disorders.[20-22] The possible mechanisms of ESW treatment include mechanical force stimulation, signalling pathway transduction, biomolecule secretion, ion channel alteration, and cytokine and growth factor release.[23,24] ESW treatment potentiates soft tissue regeneration.[25-27] Studies have shown that ESW treatment activates proliferation, migration, and gene expression in different cells.[28-31] ESW has been proposed as a non-invasive treatment that potentiates graft healing after ACL reconstruction surgery.[32-34] Wang et al[34] treated rabbits with 500 impulses of shock waves at 14 kV after ACL reconstruction using long digital extensor tendons, and demonstrated a significant improvement in healing at the grafted tendon to bone tunnel interface. While these results are promising, the effects of ESW on the biochemical processes of ACL remnant and surrounding cells must be elucidated in vitro to better inform subsequent clinical studies evaluating ESW to improve the graft maturation after ACL reconstruction.

In this study, we hypothesized that ACL remnant cells would modulate the behaviour of surrounding cells including their exhibited proliferation, migration, and collagen formation. We also hypothesized that ESW treatment would not only activate the ACL remnant cells, but also strengthen their paracrine and downstream capability to modulate BMSC activity and differentiation.

Methods

Cell isolation. ACL remnant tissues were obtained from eight patients (five males and three females; mean age 23.5 years (SD 3.46); mean injury time 3.3 months (SD 1.6)) who underwent ACL reconstruction surgery. These ACL remnant tissues were harvested by arthroscopic punch during operation (Figure 1 left image). This study procedure was approved by the Institutional Review Board (KMUHIRB-F(I)-20160112). After removing the epitendon and peritendon sheaths, the remnant tissues were minced into pieces 1 mm to 2 mm thick and washed with phosphate-buffered saline (PBS). Tissues were spun down at 1,000 rpm, 25 °C for five minutes, collected, and cultured in low-glucose Dulbecco's modified Eagle's medium (Gibco DMEM; Thermo Fisher Scientific, Waltham, Massachussetts, USA) supplemented with 10% foetal bovine serum (Gibco FBS; Thermo Fisher Scientific) and 1% antibiotics (penicillin/streptomycin; Gibco; Thermo Fisher Scientific) at 37°C in a 5% CO_2 incubator. Cells that migrated from the remnant tissue without collagenase digestion after seven to ten days were designated the P0 passage and subcultured with 0.25% trypsin (Gibco; Thermo Fisher Scientific) until 80% confluence was reached. The third passage ACL remnant cells were used for this study.

Rabbit BMSCs were isolated from 8 ml of bone marrow, previously aspirated from iliac crest, and cultured. The animal experiment protocol was approved by the Institutional Animal Care and Use Committees (IACUC) (KMU-105263). The third passage BMSCs were used for this study.

ESW treatment protocol. ESW was administered in focused mode with an electromagnetic shock wave generator (Duolith SD1; Storz Medical AG, Trägerwilen, Switzerland). Third-passage remnant cells at a density of 1×10^6 cells/ml were placed in a cryogenic vial and subjected to 1,000 shock wave impulses at 0.15 mJ/mm^2.[30,31] The control group did not receive ESW treatment.

Coculture of ACL remnant cells with BMSCs. To investigate the regulatory capability of ACL remnant cells on other cells, we cocultured the human ACL remnant cells with rabbit BMSCs using a non-contact method with the transwell system (4 µm pores).[35] BMSCs (1×10^5) were plated on the floor of the culture plate (lower well) with MEM α (Gibco; Thermo Fisher Scientific) supplemented with 10% FBS and 1% antibiotics (penicillin/streptomycin) incubated at 37°C and 5% CO_2. After 24 hours the culture medium was discarded, and the non ESW-treated ACL remnant cells (ACL-ESW coculture group) or the ACL remnant cells that were treated with ESW (1,000 shock wave impulses at 0.15 mJ/mm^2; ACL+ESW coculture group) were seeded on the transwell insert (upper well) with medium (low-glucose DMEM, serum-free, 1% antibiotics (penicillin/streptomycin); all from Gibco; Thermo Fisher Scientific). As control group, 1×10^5 BMSCs were cultured in monolayers without coculture with ACL remnant cells. After seven days, the BMSCs in each group were detached with 0.25% trypsin and subcultured to be used in other experiments.

Cell viability. Cell viability was determined for ESW-treated and untreated ACL remnant cells and for all BMSCs in the coculture experiments (control group, ACL-ESW coculture group, and ACL+ ESW coculture group) by the MTT assay following the manufacturer's instructions. The third passages of ACL remnant cells and BMSCs were used in this study. These cells were cultured in 96-well plates at a density of 5×10^3 cells/well containing 200 µl medium (MEM α supplemented with 10% FBS and 1% penicillin/streptomycin) at 37°C for 48 hours. Absorbance was measured at 570 nm using a Bio-Rad Microplate Manager Benchmark Plus Reader (Bio-Rad Laboratories, Hercules, California, USA). The results were compared between the ESW-treated and untreated ACL remnant cells, and between the BMSCs in the control group, ACL-ESW coculture group, and ACL+ESW coculture group.

Cell proliferation- EdU assay. Cell proliferative ratio was analyzed with a Click-iT EdU assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The third passages of ESW-treated and untreated ACL remnant cells and BMSCs in different coculture groups were cultured in 12-well plates at a density of 2×10^4 cells/well containing 2 ml medium (MEM α supplemented with 10% FBS and 1% penicillin/streptomycin) at 37°C for 48 hours. Cell slides were prepared with mounting medium and counterstained with Hoechst 33342 (Sigma-Aldrich, St. Louis, Missouri, USA) for ten minutes.

Table I. The primers for complementary DNA (cDNA) sequences.

Gene name	Amplicon size, bp	Primer sequence		Accession no.
Hu-Ki67	78	ForwardReverse	5′-GAGGTGTGCAGAAAATCCAAA-3′5′- CTGTCCCTATGACTTCTGGTTGT-3′	NM_001145966.2
Hu-GAPDH	576	ForwardReverse	5′-CCATCACCATCTTCCAGGAG-3′5′-CCTGCTTCACCACCTTCTTG-3′	NM_001256799.3
Rb-COL-1	73	ForwardReverse	5′- TTCTGCAGGGCTCCAATGA-3′5′-TCGACAAGAACAGTGTAAGTGAACCT-3′	NM_001195668.1
Rb-COL-3	92	ForwardReverse	5′-CCTGAAGCCCCAGCAGAA -3′5′-AACAGAAATTTAGTTGGTCACTTGTACTG-3′	XM_002712333.3
Rb-TGFβ	140	ForwardReverse	5′-CAGTGGAAAGACCCCACATCTC-3′5′- GACGCAGGCAGCAATTATCC-3′	XM_008268050
Rb-VEGF	122	ForwardReverse	5′- ATCATGCGGATCAAACCTCA-3′5′- CAAGGCCCACAGGGATTTTC-3′	XM_020912728.1
Rb-Ki67	232	ForwardReverse	5′-GTCACCGAGAGGCAGAGAAC-3′5′- TTTGCCCTTCTTCCACATTC-3′	XM_008251084.2
Rb-SCX	165	ForwardReverse	5′- CAGCGGCACACGGCGAAC-3′5′- CGTTGCCCAGGTGCGAGATG-3′	BK000280
Rb-TNC	78	ForwardReverse	5′- CAGAAGCCTTGGCCATGTG-3′5′- GCACTCTCTCCCCTGTGTAGGA-3′	XM_017350093
Rb-GAPDH	103	ForwardReverse	5′-AGTGACACCCACTCCTCCAC-3′5′- TGCTGTAGCCAAATTCGTTG-3′	NM_001082253

Hu: human; Rb: rabbit; COL-1: Collagen-I; COL-3: Collagen-III; TGFβ: transforming growth factor beta 1; VEGF: vascular endothelial growth factor; Ki67: marker of proliferation Ki-67; SCX: scleraxis; TNC: Tenascin D; GAPDH: glyceraldehyde 3-phosphate dehydrogenase

Slides were viewed under fluorescence microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany). The cell proliferation rate was calculated by ImageJ (64-bit Java v. 1.6.0_24; National Institutes of Health (NIH), Bethesda, Maryland, USA) using five randomized areas per sample that was captured by computerized stage set and analyzed by scientists who were blinded to the study groups.

RNA isolation and real-time polymerase chain reaction. The third passages of ESW-treated and untreated ACL remnant cells and BMSCs in different coculture groups were cultured in six-well plates at a density of 2 × 10^5 cells/well containing 3 ml medium (MEM α supplemented with 10% FBS and 1% penicillin/streptomycin) at 37°C for 48 hours. Total RNA was extracted with RNAzol reagent (MRC Inc, Cincinnati, Ohio, USA). Then, 2 µg of purified total RNA was reverse-transcribed using the Thermo Scientific Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Real-time PCR was carried out using the SYBR Green PCR Master Mix (Thermo Fisher Scientific) and was processed on ABI 7900 real time PCR instrument. Each reaction (20 µl) was run in triplicate and contained 1 µl of complementary DNA (cDNA) template along with the relevant primers (Table I). Threshold cycle (Ct) for all tested genes were normalized to that of GAPDH (ΔCt). Each experimental sample was referred to its control (ΔΔCt). Fold change values were expressed as $2^{-\Delta\Delta Ct}$.

Scratch migration assay. This test was conducted to determine the migration rates of ACL remnant cells with or without ESW treatment and BMSCs in different coculture groups. Third-passage cells from each group were seeded into a six-well plate at a density of 3 × 10^5 cells/plate and cultured at 37°C in a 5% CO_2 incubator. When the cells reached > 90% confluence, the plates were scratched with a sterile 200 µl pipette tip. Real-time cell migration status was investigated by closing the scratch gap at regular intervals (8, 16, 24, 32 hours in the ESW treated and untreated ACL remnant cells; 12, 24, 36, 48 hours in the BMSCs in different coculture groups) under microscope. ImageJ was used to calculate the relative changes in cell migration rate.

Transwell migration assay. An in vitro cell migration assay was performed in a 6.5 mm Transwell chamber with an 8 µm pore diameter (EMD Millipore, Billerica, Massachusetts, USA). The third passages of ACL remnant cells with or without ESW treatment and BMSCs in different coculture groups (3 × 10^4 cells/per well) in serum-free medium were seeded on the upper chamber compartment for the migration assay. MEM α supplemented with 10% FBS and 1% penicillin/streptomycin was added to the lower chamber compartment as a chemoattractant. After incubation for 20 hours at 37°C, cells that migrated to the lower membrane surface were fixed with 4% paraformaldehyde (PFA) for ten minutes, followed by staining with 0.5% crystal violet for 20 minutes. Migrated cells were counted in each chamber under a microscope and the numbers were normalized to those of the control cells. The relative cell migration ability was calculated as the ratio of migrating treated cells to the control cells and designated as the migration rate (% of the control). Each reaction was performed in triplicate and the mean was obtained from those results.

Immunofluorescence staining. ACL remnant cells with or without ESW treatment and BMSCs in different coculture groups were seeded in 16 mm cover glasses at a density of 2 × 10^4 cells/well containing 2 ml medium (MEM α supplemented with 10% FBS and 1% penicillin/streptomycin) and incubated at 37°C for 48 hours. The cells were then fixed with 4% PFA for 15 minutes and 1% Triton-X-100 for one minute, and then preincubated with 100 µl blocking solution (1% FBS in PBS) for 20 minutes. Primary antibodies against Type I Collagen (Sigma-Aldrich), Type III Collagen (Arigo Biolaboratories, Hsinchu, Taiwan), transforming growth factor beta (TGF-β; Sigma-Aldrich), and VEGF proteins (Arigo Biolaboratories) were used to stain the cells overnight at 4°C. The cells were then stained with fluorescence secondary antibodies; Donkey anti-Goat immunoglobulin G (IgG) (H + L)-FAM (1:250; Leadgene Biomedical, Tainan, Taiwan) for Type

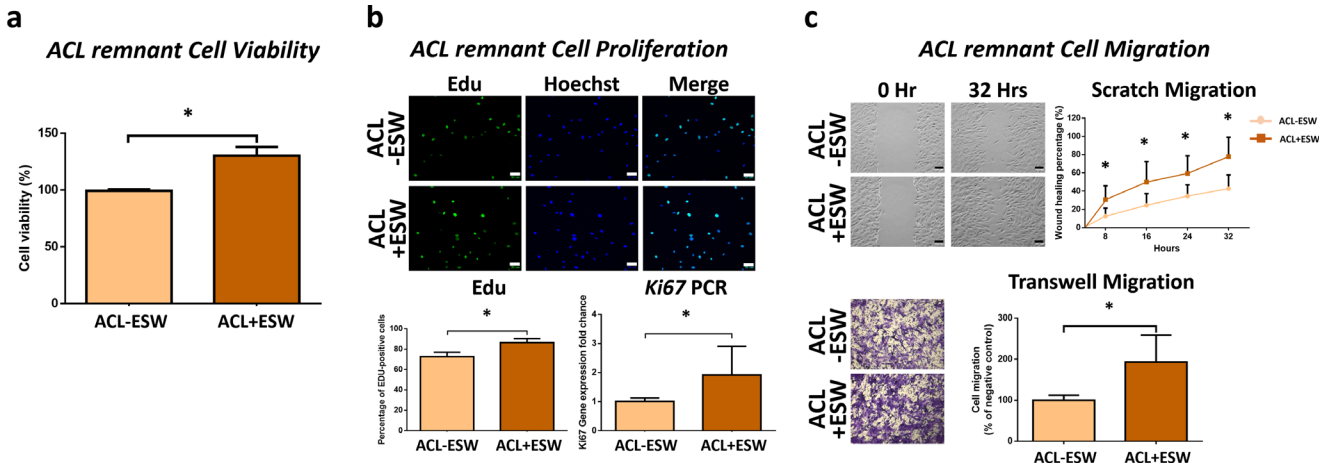

Fig. 2

a) Anterior cruciate ligament (ACL) remnant cell viability significantly increased at 72 hours post extracorporeal shock wave (ESW) treatment, according to MTT assay (n = 8). b) ACL remnant cells showed a significant increase in cell number and EdU content at 24 hours post ESW treatment compared to untreated cells (Alexa Fluro 488 stained in green; Hoechst 33342 in blue; magnification 200×). The ESW-treated ACL remnant cells showed significantly higher Ki67 messenger RNA (mRNA) expression levels compared to the control groups (n = 7). Scale bar = 50 μm. c) ESW-treated ACL remnant cells actively migrated compared to untreated cells. The ESW-treated ACL remnant cells revealed significantly higher cell migration rate in both the scratch (upper panel) and transwell assays (lower panel) (n = 8). Scale bar = 100 μm. Data are indicated as means (SD). *p < 0.01.

I Collagen and TGF-β in ACL remnant cells and Type I and Type III Collagen in BMSCs; Goat anti-Mouse IgG (H + L)-TAMRA (1:250; Leadgene Biomedical) for TGF-β in BMSCs and VEGF in both ACL remnant cells and BMSCs for one hour and rinsed thrice with PBS. The slides were counterstained with 1 μg/ml Hoechst solution (Sigma-Aldrich) for ACL remnant cells and DAPI for BMSCs for ten minutes, rinsed thrice with PBS, and mounted before observation under a confocal microscope. ImageJ was used to quantify fluorescence intensity in five random fields of six samples that was captured by computerized stage set and analyzed by scientists who were blinded to the study groups. The results of fluorescence intensity in ACL remnant cells after ESW treatment were shown in fold change compared with those of ACL remnant cells without ESW treatment.

Statistical analysis. The differences between ESW-treated and untreated ACL remnant cells were analyzed by the paired *t*-test. In the coculture experiment, we compared the results among the control, ACL-ESW coculture, and ACL+ESW coculture groups using one-way analysis of variance (ANOVA) with Tukey's post hoc test. All data are presented as mean (SD). $p < 0.05$ was considered statistically significant. All statistical analysis was performed using SPSS software version 20 (IBM, Armonk, New York, USA).

Results

ESW treatment increased ACL remnant cell viability, proliferation, and migration. The ACL remnant cell viability significantly increased within 72 hours of ESW treatment (Figure 2a). The proliferation rates markedly increased in ACL remnant cells after ESW treatment in both EdU assay and *Ki67* gene expression compared with that of untreated cells (Figure 2b). Furthermore, the ACL remnant cells treated with ESW more actively migrated into the scratched area (upper panel) or lower chamber compartment (lower panel) than the untreated cells (Figure 2c).

ESW treatment upregulated COL-I A1, TGF-β, and VEGF expression in ACL remnant cells. We conducted immunofluorescence staining to detect Collagen-I (COL-I) A1, TGF-β, and VEGF expression after ESW treatment. COL-I A1, TGF-β, and VEGF protein levels were all significantly upregulated in ESW-treated ACL remnant cells relative to those in the untreated cells (Figure 3).

BMSC proliferation and migration rate increased after coculture with ACL remnant cells with and without ESW stimulation. The cell viability of BMSCs did not reveal significant change between control, ACL-ESW coculture, and ACL+ESW coculture group (Figure 4a). BMSCs showed higher cell proliferation rate than control group after coculture with ACL remnant cells (in both ESW-treated and non-treated groups), according to EdU assay and *Ki67* gene expression levels (Figure 4b). The scratch migration test revealed significantly higher BMSC migration rate after 12 or more hours of coculture with ACL remnant cells, and the BMSCs in the ACL+ESW coculture group showed highest migration rate among the three groups at all timepoints (Figure 4c upper panel). These results were consistent with the transwell migration study results (Figure 4c lower panel). In both the proliferation and migration studies, the ESW-treated ACL remnant cells presented a more profound effect on BMSC activity compared to non ESW-treated ACL remnant cells.

ESW enhanced ACL remnant cells' capability to upregulate BMSC collagen gene expression and tenogenic differentiation, without affecting TGF-β and VEGF expression. The BMSCs cocultured with ACL remnant cells showed significantly increased levels of Type I and Type III collagens (Figure 5) and tenogenic gene (*Scx* and *TNC*) expression

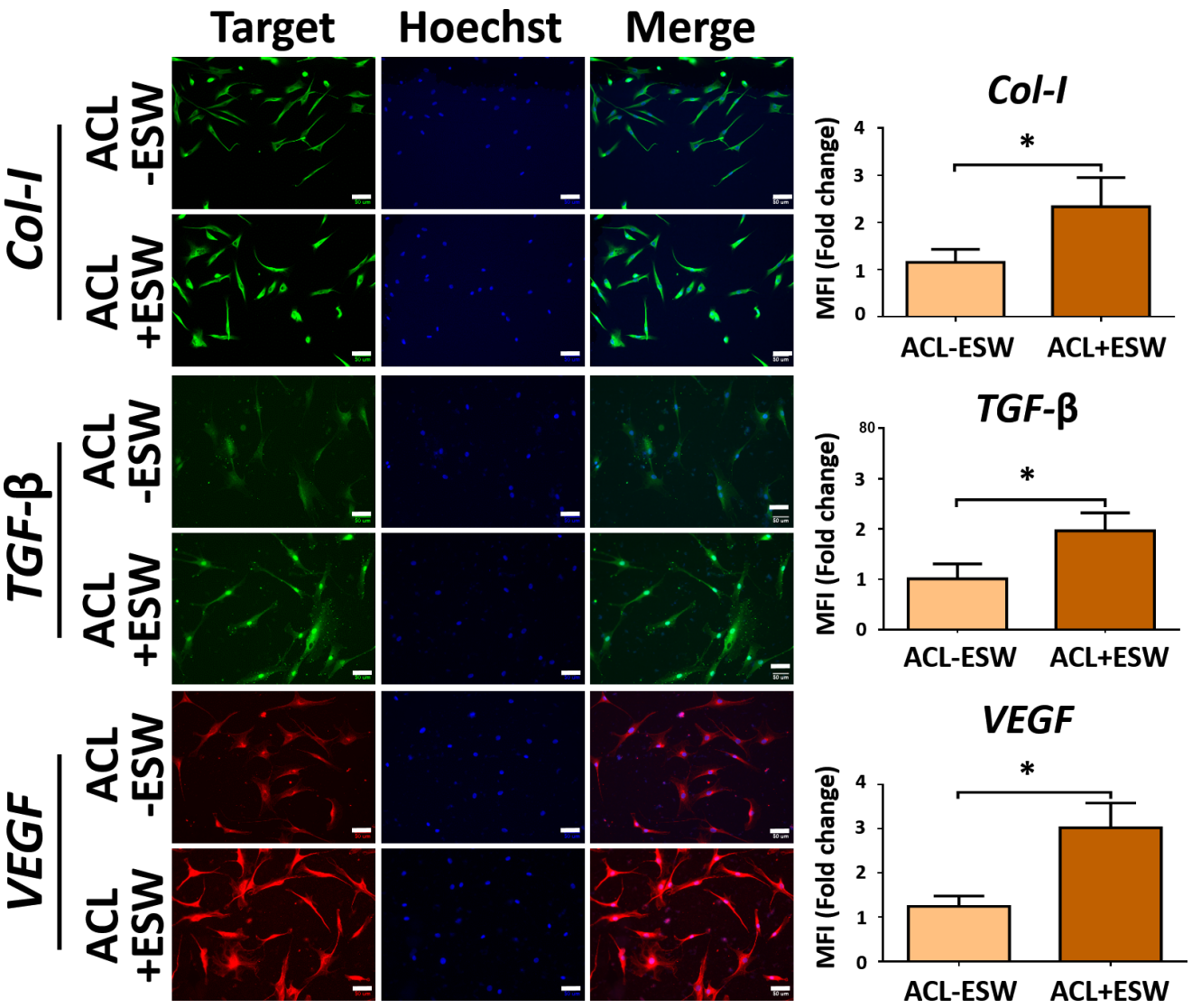

Fig. 3

Effects of extracorporeal shock wave (ESW) treatment on Collagen-I (COL-I) A1, transforming growth factor beta (TGF-β), and vascular endothelial growth factor (VEGF) expression in anterior cruciate ligament (ACL) remnant cells. Immunofluorescence imaging and results of mean fluorescence intensities (MFI) (n = 6) showed that COL-I A1 (upper panel; stained with FAM in green), TGF-β (middle panel; stained with FAM in green), and VEGF (lower panel; stained with TAMRA in red) protein expression levels in the ESW-treated ACL remnant cells were significantly higher than those in untreated cells. Cell nuclei were counterstained with Hoechst in blue. All images are shown under 200× magnification. Data are indicated as means (SD). Scale bar = 50 µm. *p < 0.01.

(Figure 6) compared with those in the control group. The BMSCs in the ACL+ESW coculture group revealed the highest gene expression of collagen and tenogenic markers among the three groups. No significant difference was observed in *TGF-β* and *VEGF* gene expression between the control and ACL remnant coculture groups.

Discussion

Enhancing the proliferation, migration, and differentiation of ACL remnant and peripheral cells is important to improve graft maturation after ACL reconstruction with remnant preservation. This study demonstrated that ACL remnant cells exerted a paracrine effect to regulate proliferation, migration, collagen synthesis, and tenogenic differentiation of BMSCs. ESW treatment activated cell viability, proliferation, migration, and increased COL-I A1, TGF-β, and VEGF expression in ACL remnant cells compared to those of untreated cells, and their paracrine effect on BMSCs was further increased after ESW stimulation, compared to untreated ACL remnant cells. Therefore, ESW treatment could improve graft maturation after ACL reconstruction by both activating remnant cells and enhancing their paracrine effect to regulate the surrounding cells to proliferate, migrate, and synthesize collagen and differentiate.

Studies have reported that ACL remnant tissue contains both mesenchymal and vascular stem cells, which play important roles in injury repair.[13,17,18] However, another study showed poor structure and low graft healing capacity in the presence of ACL remnant cells.[36] Similarly,

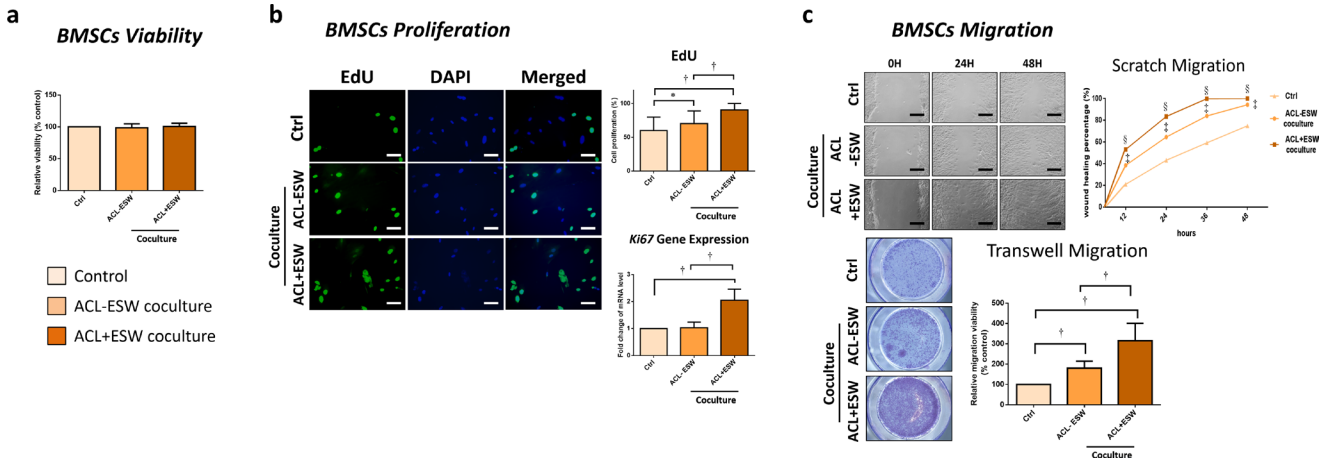

Fig. 4

Effects of anterior cruciate ligament (ACL) remnant cells on bone marrow stromal cells (BMSCs)' viability, proliferation, and migration. a) No significant difference of BMSCs viability was found between the control group, ACL-extracorporeal shock wave (ESW) coculture group, and ACL+ESW coculture group (n = 6). b) BMSCs proliferation rate in ACL remnant cells coculture group was significantly higher than that in the control group. The ESW-treated ACL remnant cells coculture group showed a more pronounced effect than non-treated ACL remnant cells coculture group (n = 6). Scale bar = 50 μm. c) Significantly higher BMSCs migration rate was noted in the ACL coculture group compared to the control group. The cell migration rate of BMSCs in the ESW-treated ACL remnant cells coculture group was the highest among the three groups. (Scratch migration, n = 6; Insert migration, n = 4). Scale bar = 100 μm. All data are means (SD). *p < 0.05; †p < 0.01 between two comparison groups; ‡p < 0.05, ACL-ESW coculture group versus control group; §p < 0.05, ACL+ESW coculture group versus ACL-ESW coculture and control group. Ctrl, control; mRNA, messenger RNA.

Lu et al[37] noted that tendon stump preserved the stemness and presented lower proliferation rate compared to normal tendon. Hence, improvement of graft regeneration only by ACL remnant cells without stimulation is limited. ESW modulation of cell proliferation and migration was demonstrated in different cells through different pathways.[30,31,38-41] Shock waves improved neural stem cell proliferation via the PI3K/AKT pathway, and regulated adipose-derived mesenchymal stem cell proliferation by activating the mammalian target of rapamycin complex 1/focal adhesion kinase (mTORC1/FAK) signalling pathway.[38,39]ESW treatment also induced the proliferation and migration of human tenocytes from ruptured Achilles tendons.[31] In the present study, the ESW treatment enhanced the proliferation, migration, and collagen synthesis of the ACL remnant cells, which promoted cell migration to and proliferation in the graft after ACL reconstruction. However, the actual mechanism through which ESW enhances ACL remnant cells still needs further clarification.

Growth factor secretion and induction are important processes in tissue regeneration as they promote graft healing.[10,42,43] Shock wave mechanotransduction induced TGF-β expression in BMSCs, mesenchymal stem cells, and ligament fibroblasts.[44-46] During ACL reconstruction, TGF-β in the TGF-β/MAPK signalling pathway strengthened the tendon-bone junction, reduced scar tissue entrance to the tunnel, promoted collagen fibre formation, and increased fibroblast cell number with a superior prognosis in a rabbit model.[47] Furthermore, TGF-β mediated the ACL remodelling by enhancing the proliferation and matrix synthesis of ACL fibroblast.[48-50] Several studies reported that shock waves improved angiogenesis by upregulating VEGF or its receptor VEGFR2 levels and promoting heart, tendon, and tendon-bone junction regeneration.[51-54] In immunodeficient rats, ACL remnant-derived CD34+ (endothelial stem cell marker) cells enhanced tendon-bone healing by activating angiogenesis and VEGF secretion.[18] In this study, we observed elevated TGF-β and VEGF secreted levels in ACL remnant cells following ESW treatment. Therefore, ESW-based mechanotransduction may activate secretion of TGF-β and VEGF from ACL remnant cells, which may further augment the regeneration of graft after ACL reconstruction.

In the situation of remnant preservation during ACL reconstruction, the actual relationship between ACL remnant and surrounding tissue and cells is unknown. This study simulated the situation presented during ACL reconstruction, in which the implanted graft passes the ACL remnant and is covered by the released BMSCs from the drilled bone tunnel. Our results showed the coculture with ACL remnant cells enhanced BMSC proliferation, migration, and differentiation, as well as collagen synthesis and tenogenic gene expression. However, we also observed that BMSC viability and growth factor secretion (TGF-β and VEGF) was not altered after coculture with ACL remnant cells, which indicated that ACL remnant cells preserved the capability to secrete paracrine factors to regulate the surrounding cells' activity and differentiation without affecting the cell viability and secretion of growth factors. These results provide a new concept that ACL remnant cells could regulate the surrounding cells' activity and differentiation, enhancing graft healing during ACL reconstruction.

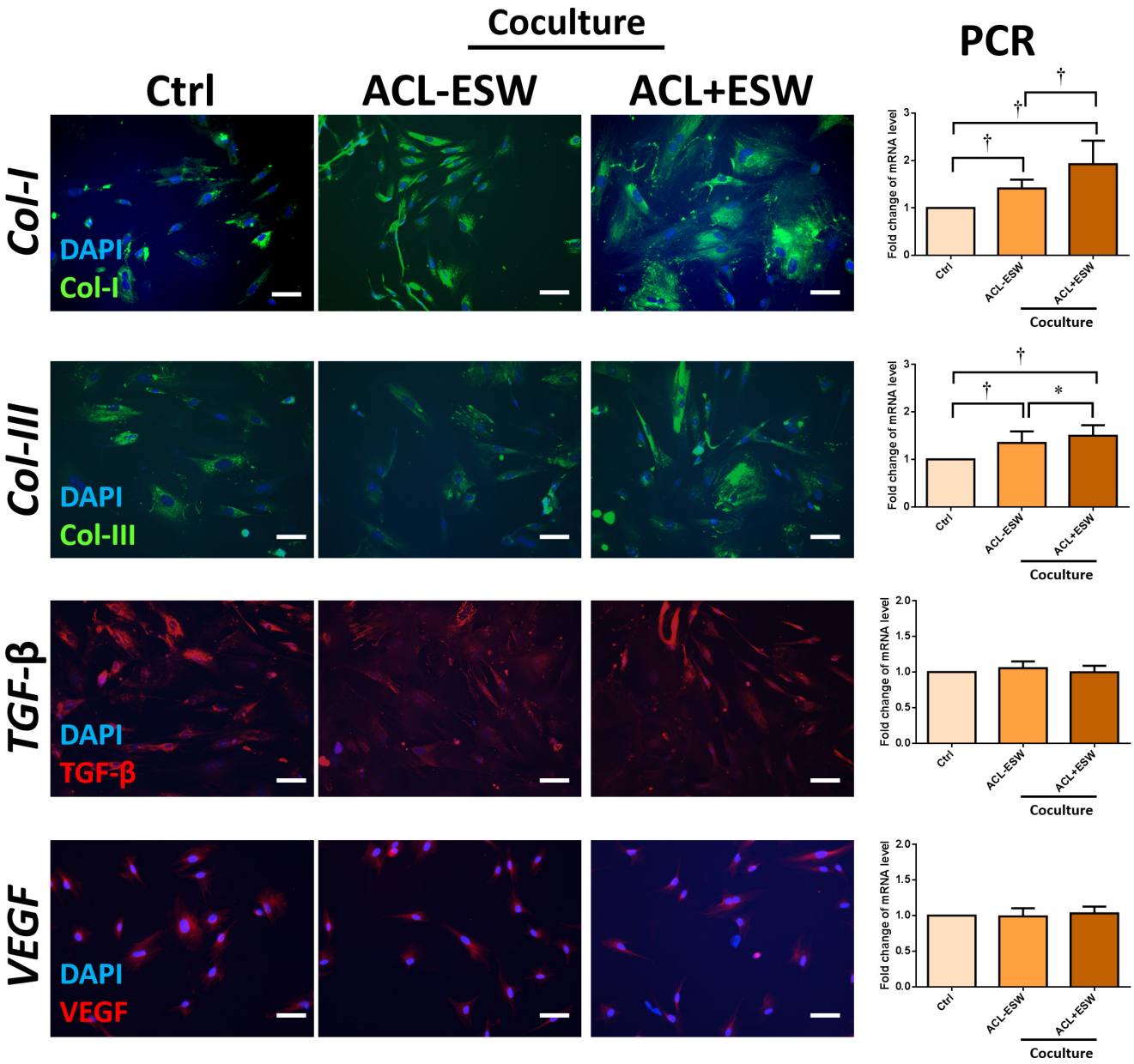

Fig. 5

Effects of anterior cruciate ligament (ACL) remnant cells on Type I Collagen (Col-I), Type III Collagen (Col-III), transforming growth factor beta (TGF-β), and vascular endothelial growth factor (VEGF) expression in bone marrow stromal cells (BMSCs). After coculture with ACL remnant cells, BMSCs evidenced higher Type I and Type III Collagen gene expression in both immunofluorescence staining (both were stained with FAM in green) and reverse transcription polymerase chain reaction (RT-PCR) results. BMSCs presented the highest gene expression of Type I Collagen and Type III Collagen in the ACL+ESW coculture group. No significant difference in the expression of TGF-β and VEGF (both were stained with TAMRA in red) between the control and ACL coculture groups with and without ESW treatment was noted (n = 6). Cell nuclei were counterstained with DAPI in blue. All images are shown with 200× magnification. Scale bar = 50 μm. Data are indicated as means (SD). *p < 0.05; †p < 0.01. Ctrl, control; mRNA, messenger RNA.

ESW treatment was shown to stimulate target cell activity. However, the interaction between ESW-treated cells and other cells has not been well investigated. In this study, the ESW treatment activated the ACL remnant cell viability, migration, collagen synthesis, and TGF-β and VEGF expression. Moreover, coculture with ESW-treated ACL remnant cells more profoundly regulated BMSC proliferation, migration, expression of collagen, and tenogenic differentiation compared to non ESW-treated ACL remnant cells and control group. The results indicated that ESW may not only activate the target cell directly, but also surrounding cells by promoting secretion of factors that regulate the activity and differentiation of the surrounding cells in a paracrine fashion. However, BMSC viability and secretion of growth factors did not improve after coculture with ESW-treated ACL remnant cells; consequently no significant difference to the results was obtained with non ESW-treated ACL remnant cells

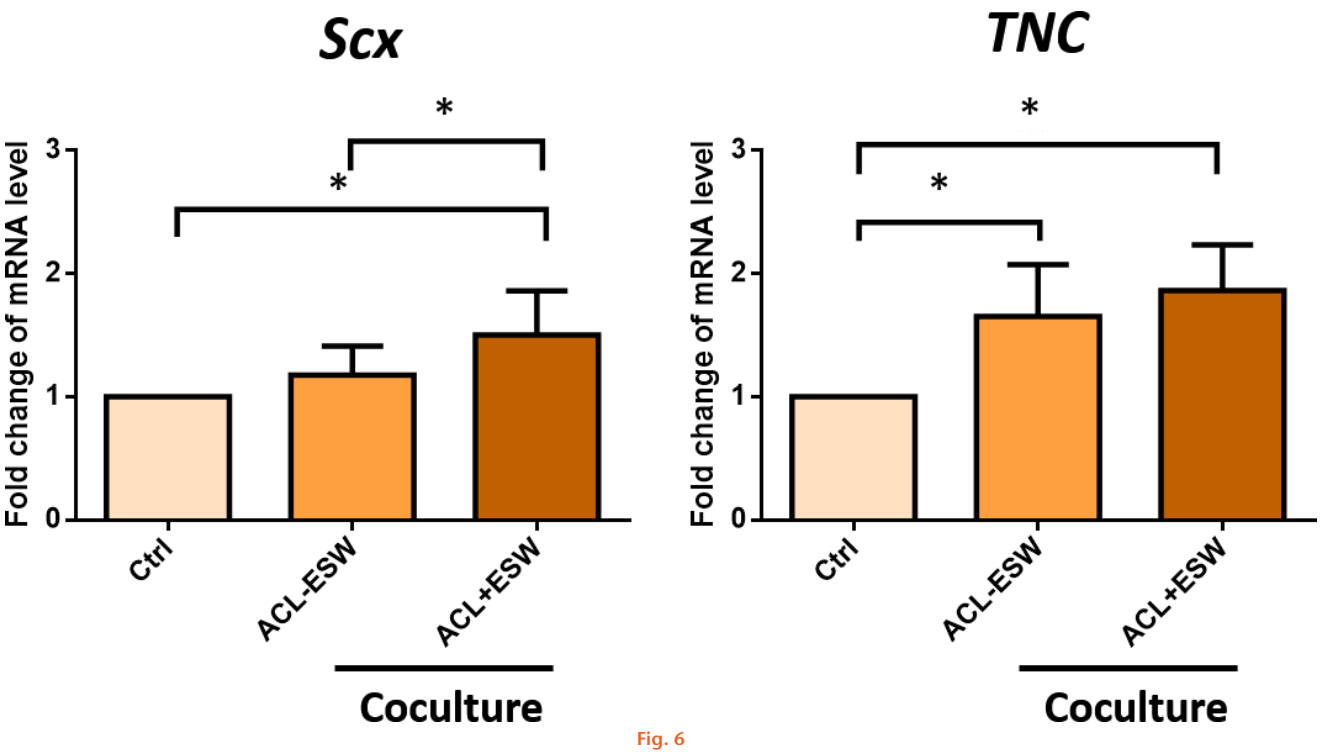

Fig. 6

Effects of anterior cruciate ligament (ACL) remnant cells on bone marrow stromal cells (BMSCs) tenogenic differentiation (Scx, TNC). After coculture with ACL remnant cells, BMSCs revealed higher Scx and TNC gene expression in reverse transcription polymerase chain reaction (RT-PCR) results. BMSCs cocultured with extracorporeal shock wave (ESW)-treated ACL remnant cells presented the highest Scx and TNC gene expression (n = 6). Data are indicated as means (SD). *p < 0.01. Ctrl, control.

coculture and control coculture groups. These findings showed that ESW treatment could enhance native capability with inability to create new function of the target cell to regulate surrounding cells.

In clinical practice, ESW would not only be delivered to ACL remnant, but also to other tissues in the reconstruction area (e.g. tendon graft, bone marrow, synovium). Vetrano et al[31] investigated the effect of ESW on tenocyte in semitendinosus tendon harvested during graft preparation in ACL reconstruction. Their result showed that ESW promotes the proliferation and collagen synthesis in tenocytes of the semitendinosus tendon. Suhr et al's study[55] reported that ESW application increases human BMSC growth rate, proliferation, and migration and reduces the apoptosis rate. Their experimental result also showed that ESW could induce the remodelling of actin cytoskeleton and maintain the differentiation potentials in BMSCs after ESW treatment. Wang et al[34] first investigated the effect of ESW in rabbit ACL reconstruction model; their result showed that ESW treatment significantly enhanced the interfacial healing between graft tendon and bone tunnel from the histomorphological and tensile strength test. Further, Wang et al[33] applied the ESW treatment (1,500 impulses at 20 kV) delivery to tibial tunnel in patients who received remnant preservation ACL reconstruction surgery immediately during the same anaesthesia. At two years follow-up, the Lysholm score was significantly improved and tibial tunnel enlargement was decreased in the ACL reconstruction patients who received ESW compared to that in the no-ESW treatment patients. The current study is the first to hypothesize and demonstrate that ESW could enhance the activity of ACL remnant cells and its regulatory effect on BMSCs that might benefit in graft maturation. Nevertheless, the interaction between ACL remnant, graft tenocytes, BMSCs, and other tissue under ESW stimulation requires further investigation to clarify the effect of ESW on graft maturation in ACL reconstruction.

This study had some limitations. First, we investigated the effects of ESW on ACL remnant cells in vitro, but did not validate these findings with in vivo animal studies. Second, we acknowledge that the indirect coculture of human ACL remnant cells and rabbit BMSCs to simply observe the regulatory effect of ACL remnant cells on surrounding cells is a limitation. To understand the clinical situation in ACL reconstruction, a study using direct contact coculture of ACL remnant cells and surrounding cells from the same species will be needed. Third, we did not elucidate the clinical effects and precise mechanism of ESW treatment on ACL remnant tissue. Prior to the clinical application of ESW, its optimal dose and frequency to treat ACL remnant cells and promote tissue regeneration remain to be determined. Several studies reported dose-dependent influence of ESW on various cell activities and injured tissue healing.[56-58] To the best of our knowledge, no study to date has validated the optimal ESW dose for

ACL remnant cells. Future experiments should be directed toward performing in vitro cell studies and ACL reconstruction with remnant preservation in animal models to confirm the efficacy and safety of ESW application before proceeding with clinical trials in human volunteers.

In conclusion, our study revealed a few novel findings. We demonstrated that ACL remnant cells regulated the activities of surrounding cells by increasing their proliferation, migration, collagen synthesis, and differentiation. The ESW treatment not only activated the ACL remnant cells' viability, migration, collagen synthesis, and secretion of TGF-β and VEGF, but also significantly enhanced their paracrine capabilities to regulate the surrounding cells, thereby enhancing graft healing. These results support the evidence of application of ESW in ACL reconstruction with remnant preservation to enhance graft maturation.

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Ethical review statement

- This study was approved by the Kaohsing Medical University Hospital Institutional Review Board (IRB) (KMUHIRB-F(I)-20160112) and Institutional Animal Care and Use Committees (IACUC) (KMU-105263).