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Anterior cruciate ligament remnant preservation attenuates apoptosis and enhances the regeneration of hamstring tendon graft

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Aims

The effects of remnant preservation on the anterior cruciate ligament (ACL) and its relationship with the tendon graft remain unclear. We hypothesized that the co-culture of remnant cells and bone marrow stromal cells (BMSCs) decreases apoptosis and enhances the activity of the hamstring tendons and tenocytes, thus aiding ACL reconstruction.

Methods

The ACL remnant, bone marrow, and hamstring tendons were surgically harvested from rabbits. The apoptosis rate, cell proliferation, and expression of types I and III collagen, transforming growth factor-β (*TGF-β*), vascular endothelial growth factor (*VEGF*), and tenogenic genes (scleraxis (*SCX*), tenascin C (*TNC*), and tenomodulin (*TNMD*)) of the hamstring tendons were compared between the co-culture medium (ACL remnant cells (ACLRCs) and BMSCs co-culture) and control medium (BMSCs-only culture). We also evaluated the apoptosis, cell proliferation, migration, and gene expression of hamstring tenocytes with exposure to co-culture and control media.

Results

Compared to BMSCs-only culture medium, the co-culture medium showed substantially decreased early and late apoptosis rates, attenuation of intrinsic and extrinsic apoptotic pathways, and enhanced proliferation of the hamstring tendons and tenocytes. In addition, the expression of collagen synthesis, *TGF-β*, *VEGF*, and tenogenic genes in the hamstring tendons and tenocytes significantly increased in the co-culture medium compared to that in the control medium.

Conclusion

In the presence of ACLRCs and BMSCs, the hamstring tendons and tenocytes significantly attenuated apoptosis and enhanced the expression of collagen synthesis, *TGF-β*, *VEGF*, and tenogenic genes. This in vitro study suggests that the ACLRCs mixed with BMSCs could aid regeneration of the hamstring tendon graft during ACL reconstruction.

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Keywords: Anterior cruciate ligament reconstruction, Hamstring tendon, Remnant preservation, Apoptosis, Ligamentization

Article focus

- The effects of hamstring tendons and tenocytes with remnant preservation on anterior cruciate ligament (ACL) reconstruction were investigated.
- To simulate clinical conditions, we applied the ACL remnant cells (ACLRCs)/bone marrow stromal cells (BMSCs) co-culture medium to investigate its effects on the activity of hamstring tendons and tenocytes in vitro.

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Key messages

- The ACLRCs/BMSCs co-culture medium attenuated the apoptosis rate of the hamstring tendons and tenocytes by reducing the expression of genes involved in both the intrinsic and extrinsic apoptosis pathways.
- In this in vitro study, the coexistence of ACLRCs and BMSCs enhanced the proliferation capability, expression of collagen synthesis genes, growth factor secretion, and tenogenesis of the hamstring tendons and tenocytes.
- The ACLRCs not only provided stem cells but also regulated and cooperated with the surrounding tissue, which enhanced graft maturation during ACL reconstruction.

Strengths and limitations

- This study provides strong supportive evidence for the importance of remnant preservation to enhance hamstring graft maturation in ACL reconstruction.
- The findings of this study were drawn from the in vitro experiments, which need to be corroborated by an in vivo animal study.
- The exact mechanism and content of the ACLRCs/BMSCs co-culture medium need to be further investigated.

Introduction

Anterior cruciate ligament (ACL) injuries are common sports injuries of the knee joint.[1-3] Surgical ACL reconstruction aids recovery of joint function and restores athletic ability in patients who fail conservative treatment and those with associated knee injuries.[3-5] Osseous integration at the graft-tunnel interface and remodelling of the intra-articular graft (ligamentization) are the major concerns of implanted tendon grafts after ACL reconstruction.[6-8] After implantation, the hamstring tendon graft undergoes three stages: hypovascularization with inflammation and graft cell apoptosis; repair; and remodelling.[6] In the early stage of ACL reconstruction, the implanted tendon graft undergoes cell apoptosis and death, which decreases the number of live cells, destroys the graft structure, weakens the mechanical strength of the graft, and delays graft maturation.[9-11] Therefore, reducing apoptosis of implanted tendon grafts, and enhancing tendon and tenocyte proliferation and regeneration, is critical to prevent graft tear and accelerate graft maturation after ACL reconstruction.[12,13]

Preservation of the ACL remnant during ACL reconstruction potentially enhances the implanted graft incorporation and improves clinical function.[14-16] Furthermore, ACL remnant contains stem cells that contribute to the tendon-bone healing after ACL reconstruction.[14,17-19] During ACL reconstruction, the ACL remnant remains in contact with the bone marrow stromal cells (BMSCs) released from the drilled bone tunnel surrounding the implanted tendon grafts, and has been shown to improve the functional results.[20] However, the clinical value of ACL remnant has not been fully realized due to its distorted structure and limited cells. Lu et al[20] demonstrated that ACL remnant cells (ACLRCs) increase the proliferation, migration, collagen synthesis, and differentiation of the surrounding cells in a paracrine manner in a Transwell co-culture model. However, the effects of the implanted hamstring tendon with ACL remnants and released BMSCs during ACL reconstruction remain unclear.

This study aimed to investigate changes in the hamstring tendon graft with remnant preservation for ACL reconstruction. We hypothesized that the coexistence of ACL remnant (ACL preservation) and BMSCs released from the drilled bone tunnel reduces apoptosis and regulates the activity/differentiation of the grafted tendon, which enhances graft maturation after ACL reconstruction. In this study, we harvested the conditioned medium from direct co-culture of ACLRCs and BMSCs, and observed its effects on the hamstring tendons and tenocytes compared to the BMSCs-only culture medium.

Methods

Tissue harvest and cell isolation of ACL remnant, bone marrow, and hamstring tendon. A total of six skeletally mature New Zealand male rabbits weighing 2.5 to 3.0 kg were used in this study. The animal experiment protocol was conducted and approved by the Institutional Animal Care and Use Committees (Kaohsiung Medical University) with the approval number (KMU-107193). We have included an ARRIVE checklist to show that we have conformed to the ARRIVE guidelines. After anaesthetization with intramuscular injections of ketamine (40 mg/kg) and xylazine (10 mg/kg), the rabbit was maintained with gaseous anaesthesia of isoflurane (2%)/O_2.[21,22] A medial parapatellar approach with the lateral patella subluxation was employed. The normal ACL was detached from the femoral insertion following the procedure described in a previous study (Figure 1, left panel).[17] After surgery, these rabbits were kept in individual cages without activity restriction. To prevent infection and alleviate pain after surgery, these rabbits received intramuscular administration of Cefa (cefazoline, 15 mg/kg; Taiwan Biotech, Taiwan) twice, Keto (ketorolac, 0.7 to 1 mg/kg; Yungshin Pharm, Taiwan) once, and Temgesic (Buprenorphine, 0.01 to 0.05 mg/kg; Reckitt Benckiser, UK) twice per day for three days. As per clinical convention,[23] the ACL remnant (Figure 1, right panel, blue circle) was harvested at four weeks after the initial surgery. Before tissue harvest, these rabbits were sedated by isoflurane (2%)/O_2 first and then euthanized via carbon dioxide inhalation. At the same time, we also harvested the bone marrow from the proximal tibia (Figure 1, right panel, yellow frame) and hamstring tendons from both knees.

The hamstring tendons harvested from six rabbits were cut transversely into two pieces for further experiments. The ACL remnant and six pieces (from six rabbits) of the hamstring tendons were digested overnight using 5 mg/ml type I collagenase (MilliporeSigma, USA) in low

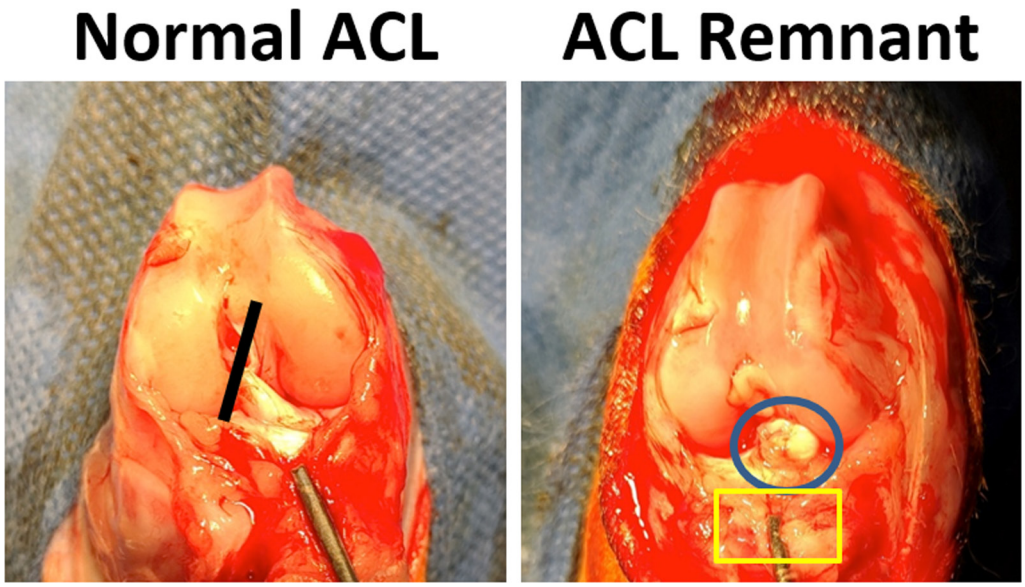

Fig. 1

Representative pictures of anterior cruciate ligament (ACL) remnant harvest. The ACL was detached from the femoral insertion (black line) to create the ACL complete tear model (left panel). Four weeks later, the rabbit's ACL remnant (blue circle) and proximal tibia (yellow frame) were harvested for further ACL remnant cell (ACLRC) and bone marrow cell culture (right panel).

glucose Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Thermo Fisher Scientific, USA) without agitation at 37°C in a 5% CO_2 incubator, and subcultured to obtain ACLRCs and hamstring tenocytes.[20,24] The remaining fresh hamstring tendons were washed twice with phosphate-buffered saline (PBS) for tissue culture to investigate the expression of the hamstring tendons in different conditioned media.

The bone marrow was subcultured to isolate BMSCs, as described previously.[20,25] The ACLRCs, hamstring tenocytes, and BMSCs at passage three were used for further experimentation.

The sample size was determined based on the findings of a pilot study using G*Power version 3.1.9.7 (developed by Franz Faul, Edgar Erdfelder, Axel Buchner, and Albert-Georg Lang, Germany).[26,27] Briefly, the ACLRCs, BMSCs, and hamstring tenocytes were harvested from three rabbits for pilot study to determine the sample size. The hamstring tenocytes were treated with BMSCs-only or ACLRCs/BMSCs co-culture media to investigate the cell proliferation (Click-iT EdU Cell Proliferation Kit (Cat. No. 1906238; Thermo Fisher Scientific)) and early and late apoptosis (flow cytometry) with the experimental methods described below. The power test was performed with a significance level (α) of 0.05, power level (1-β) of 0.8 and effect size which was calculated from the difference of mean and standard deviations (SDs) between the groups of hamstring tenocytes treated with BMSC-only medium or coculture medium, using both the two-sided Wilcoxon signed-rank test and two-sided paired *t*-test. The analysis revealed that the minimal sample size was six from cell proliferation study (power 0.832), four from early apoptosis study (power 0.832), or five from late apoptosis study (power 0.903), respectively. Therefore, we performed the experiments with the ACL remnant, bone marrow, and hamstring tendon from six rabbits.

Culture media preparation. The culture media comprised low-glucose DMEM, 10% exosome-depleted fetal bovine serum (FBS; Gibco), and 1% antibiotics (penicillin/streptomycin). ACLRCs (1.5×10^5 cells) and BMSCs (1.5×10^5 cells) at passage three were co-cultured (co-culture medium) in 10 ml medium in a 10 cm culture dish incubated at 37°C in humidified conditions with 5% CO_2. In control medium, only BMSCs (3×10^5 cells) were cultured using the same procedure. The ACLRCs/BMSCs co-culture and BMSCs-only culture (control) media were collected every two days for six days.

LIVE/DEAD cell staining assay. To observe the cell viability of hamstring tendon explants in different media, hamstring tendons were cultured separately in co-culture (n = 5) or control (n = 5) media. After seven days of culture, these tendons were prepared to evaluate cell viability using LIVE/DEAD Cell Viability Assay (Cat. No.L3224; Invitrogen, Thermo Fisher Scientific).[28] Briefly, these tendon tissues were washed with PBS and immersed in low-glucose DMEM in 12-well culture plate. The LIVE/DEAD working solution (2 μM calcein-AM and 4 μM ethidium homodimer-1 in PBS) was added and incubated at 37°C for 1.5 hours in a dark condition. Afterwards, the tendons were washed with PBS and fixed in 4% formaldehyde for 15 minutes. Subsequently, they were embedded in optimal cutting temperature compound (Tissue-Tek; Sakura Finetek, Japan), cut into 3 μm-thick sections, and observed under a confocal microscope (Olympus IX-81-FV100; Olympus, Japan). The live cells stained green with calcein-AM, and the dead cells stained red

Table I. The primers of complementary DNA sequences.

Gene name	Amplicon size, bp		Primer sequence	Accession No.
Rb-COL-1	73	Forward	5′-TTCTGCAGGGCTCCAATGA-3′	NM_001195668.1
		Reverse	5′-TCGACAAGAACAGTGTAAGTGAACCT-3′	
Rb-COL-3	92	Forward	5′-CCTGAAGCCCCAGCAGAA-3′	XM_002712333.3
		Reverse	5′-AACAGAAATTTAGTTGGTCACTTGTACTG-3′	
Rb-VEGF	122	Forward	5′-ATCATGCGGATCAAACCTCA-3′	XM_020912728.1
		Reverse	5′-CAAGGCCCACAGGGATTTTC-3′	
Rb- TGF-β	140	Forward	5′-CAGTGGAAAGACCCCACATCTC-3′	XM_008268050
		Reverse	5′-GACGCAGGCAGCAATTATCC-3′	
Rb-Ki-67	232	Forward	5′-GTCACCGAGAGGCAGAGAAC-3′	XM_008251084.2
		Reverse	5′-TTTGCCCTTCTTCCACATTC-3′	
Rb-SCX	165	Forward	5′-CAGCGGCACACGGCGAAC-3′	BK000280
		Reverse	5′-CGTTGCCCAGGTGCGAGATG-3′	
Rb-TNC	78	Forward	5′-CAGAAGCCTTGGCCATGTG-3′	XM_017350093
		Reverse	5′-GCACTCTCTCCCCTGTGTAGGA-3′	
Rb-TNMD	122	Forward	5′-CCATGCTGGATGAGAGAGGTT-3′	NM_001109818.1
		Reverse	5′-CCGTCCTCCTTGGTAGCAGT-3′	
Rb-Caspase 3	129	Forward	5′-GCTGGACAGTGGCATCGAGA-3′	NM_001082117.1
		Reverse	5′-TCCGAATTTCGCCAGGAATAGTAA-3′	
Rb-Caspase 8	179	Forward	5′-ATGCAGAGGCTTTGAGCAAT3′	XM_017343029.1
		Reverse	5′-GCCATAGATGATGCCCTTGT-3′	
Rb-Caspase 9	133	Forward	5′-CTGTTTCCGAGCGAGGGATT-3′	XM_008249762.2
		Reverse	5′-CGCAGGAAGGTTTTGGGGTA-3′	
Rb-Bax	250	Forward	5′-GTCGCCCTGTTTTACTTTGC-3′	XM_002723696.3
		Reverse	5′-CTCAGCCCATCTTCTTCCAG-3′	
Rb-Bcl-2	221	Forward	5′-GATTGTGGCCTTCTTTGAGTTC-3′	XM_008261439.2
		Reverse	5′-AAGTCTTCAGAGACACCCAGGA-3′	
Rb-Fas	442	Forward	5′-CAAACCAGCAACACCAAATGC-3′	XM_008269902.2
		Reverse	5′-CCGCAAGAGCACAAAGATTAG-3′	
Rb-GAPDH	103	Forward	5′-AGTGACACCCACTCCTCCAC-3′	NM_001082253
		Reverse	5′-TGCTGTAGCCAAATTCGTTG-3′	

Bax, Bcl2-associated X protein; Bcl2, B-cell lymphoma 2; COL-1, type I collagen; COL-3, type III collagen; Fas, Fas cell surface death receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Ki-67, marker of proliferation Ki-67; Rb, rabbit; SCX, scleraxis; TGF-β, transforming growth factor-β; TNC, tenascin C; TNMD, tenomodulin; VEGF, vascular endothelial growth factor.

with ethidium homodimer-1. The number of live or dead cells was counted using ImageJ software (64-bit Java v. 1.6.0_24; National Institutes of Health, USA). The ratio of live cells within the hamstring tendon was calculated as the number of live cells divided by the count of live cells plus dead cells from three randomly selected areas (400 × 250 μm) in each of the five samples per study group, and expressed as a percentage. Following the same procedure described above, the ratio of dead cells was also calculated. The image of one fresh hamstring tendon was used as the control.

Terminal deoxynucleotidyl transferase dUTP nick end labelling staining. To detect apoptotic cells within the hamstring tendon treated with co-culture and control media, we performed terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining (TUNEL Assay Kit, Cat. No ab206386; Abcam, UK) according to the manufacturer's protocol. Briefly, the cryosections were fixed in 4% formaldehyde for 15 minutes at room temperature, and the slides were immersed in Tris-buffered saline (TBS) for 15 minutes. After hydration, these tissues were permeabilized with proteinase K for five minutes and then incubated with 3% hydrogen peroxide (H_2O_2) to inactivate endogenous peroxidases. Afterwards, the terminal deoxynucleotidyl transferase (TdT) enzyme and TdT labelling reaction mix was added and incubated for 90 minutes at room temperature (TdT binds to exposed 3'-OH ends of DNA fragments generated in response to apoptotic signals, and catalyzes the addition of biotin-labelled deoxynucleotides). Then, the reaction was stopped by adding the stop buffer at 37°C for five minutes. To determine the biotinylated DNA, the sections were incubated with a streptavidin-HRP conjugate for 30 minutes at room temperature. Subsequently, they were washed with TBS, diaminobenzidine (DAB) substrate was added, and the reaction was stopped. The slides were then counterstained with Methyl Green, dehydrated, cover-slipped, and observed under a light microscope (Axio Imager Z2; Carl Zeiss Microscopy GmbH, Germany). TUNEL expression in the hamstring tendon with co-culture and control media was calculated from three randomized areas per sample (n = 6 in both groups) and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, USA).

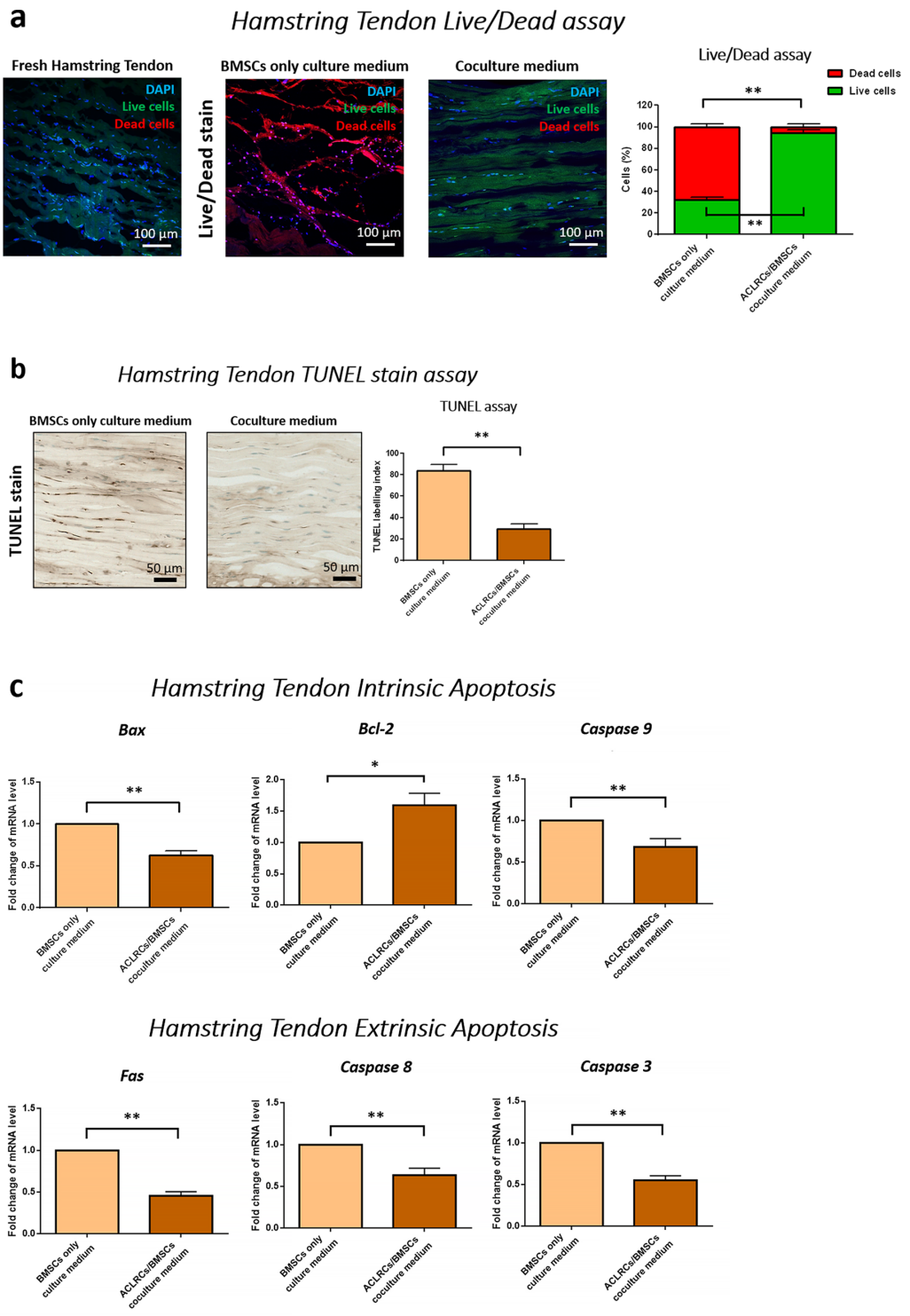

Fig. 2

Effects of co-culture medium of anterior cruciate ligament remnant cells (ACLRCs) and bone marrow stromal cells (BMSCs) on hamstring tendon explant viability. Hamstring tendon explants treated with BMSCs-only culture medium or ACLRCs/BMSCs co-culture medium were detected using LIVE/DEAD staining. a) The representative immunofluorescence pictures show the LIVE/DEAD staining in fresh hamstring tendons and hamstring tendons after BMSCs-only culture (n = 5) or co-culture medium (n = 5) treatment. Live cells, dead cells, and nuclei are stained green, red, and blue, respectively. Data represent the mean (standard deviation (SD)) of triplicate measurement. b) The representative immunohistochemistry pictures of terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining in hamstring tendon after BMSCs-only culture or co-culture media treatment. The TUNEL assay was performed to analyze the level of apoptosis in hamstring tendons in BMSCs-only culture (n = 6) or co-culture medium (n = 6). The expression was calculated from three randomized areas per sample; scale bar: 50 μm. c) The expression of Bcl2-associated X protein (Bax), B-cell lymphoma 2 (Bcl-2), Caspase 9, Fas cell surface death receptor (Fas), Caspase 8, and Caspase 3 detected by real-time polymerase chain reaction (PCR) (n = 6). Data were analyzed with paired *t*-test. *p < 0.05; **p < 0.01. DAPI, 4′,6-diamidino-2-phenylindole; mRNA, messenger RNA.

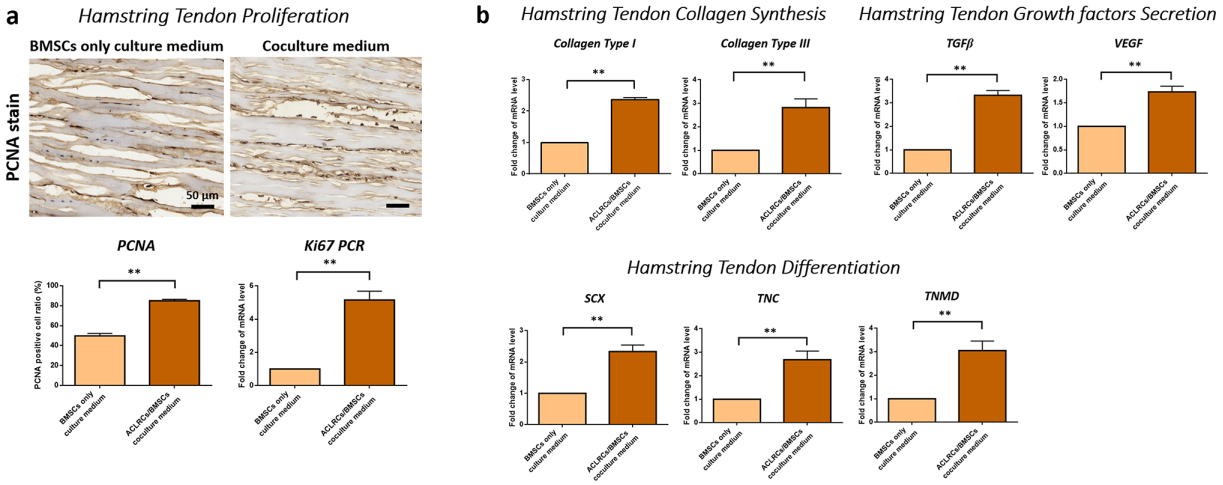

Fig. 3

Effects of anterior cruciate ligament remnant cells (ACLRCs)/bone marrow stromal cells (BMSCs) co-culture medium on hamstring tendon proliferation, collagen synthesis, growth factor secretion, and tenogenesis. a) The representative immunohistochemistry pictures show the proliferating cell nuclear antigen (PCNA) staining in hamstring tendons after BMSCs-only culture or ACLRCs/BMSCs co-culture media treatment. The bar chart shows the difference in PCNA positive cells ratio and gene expression of Ki-67 in the hamstring tendon treated with BMSCs-only culture (n = 6) or ACLRCs/BMSCs co-culture (n = 6) media; scale bar: 50 μm. b) Expression of collagen types I and III, transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), and tenogenesis-related genes detected by real-time polymerase chain reaction in hamstring tendon treated with BMSCs-only culture (n = 6) or ACLRCs/BMSCs co-culture (n = 6) media. Data are indicated as mean (standard deviation) of triplicate measurements and analyzed with paired *t*-test. **p < 0.01 compared with BMSCs-only culture medium-treated group. mRNA, messenger RNA; SCX, scleraxis; TNC, tenascin C; TNMD, tenomodulin.

Annexin V-fluorescein isothiocyanate/propidium iodide assay for early and late apoptotic cells. The apoptosis of hamstring tenocytes treated with co-culture (n = 6) and control (n = 6) media was detected using flow cytometry (FC500; Beckman Coulter, USA) after staining with Annexin V-fluorescein isothiocyanate/propidium iodide (PI) (Cat. No. 32113; Leadgene Biomedical, Taiwan) following the manufacturer's instructions.[29] In brief, 1 to 5 × 10^5 tenocytes were detached by 1× trypsin, washed with PBS, and collected by centrifugation at 300× g for five minutes. The cell pellet was resuspended with 500 μl of 1× Binding Buffer (Leadgene Biomedical, Taiwan). Then, 5 μl Annexin V-FAM (carboxyfluorescein) and 5 μl PI were added to the mixture and incubated for 15 to 20 minutes at room temperature in the dark. After incubation, the cells would be kept on ice and performed by flow cytometry using filters appropriated for fluorescein isothiocyanate (FITC, corresponding to Annexin V-FAM). The percentage of cells in early apoptosis was calculated by Annexin V-positivity and PI-negativity, and the cells in late apoptosis were calculated by Annexin V-positivity and PI-positivity.

Cell viability. The cell viability of hamstring tenocytes treated with co-culture (n = 6) and control (n = 6) media was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Cat. No. M2003; MilliporeSigma).[20] Cells were cultured in 96-well plates at a density of 5 × 10^3 cells/well containing 200 μl medium (low-glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin) at 37°C for 72 hours. Then, 50 μl MTT stock solution (2 mg/ml) was added to each well and incubated at 37°C with 5% CO_2 for four hours in the dark. After incubation, the medium was removed, and 150 μl dimethyl sulfoxide was added to each well to dissolve the crystal. Absorbance was measured at 570 nm using a Bio-Rad Microplate Manager Benchmark Plus Reader (Bio-Rad Laboratories, USA).

Cell proliferation-EdU assay. The cell proliferation ratio of the hamstring tenocytes in response to co-culture (n = 6) and control (n = 6) media was measured by Click-iT EdU Cell Proliferation Kit (Cat. No. 1906238) according to the manufacturer's instructions.[20] In brief, the hamstring tenocytes were cultured in 12-well plates at a density of 2 × 10^4 cells/well in 2 ml medium (low-glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin) at 37°C for 48 hours. Next, 10 μl of 10 mM EdU stock solution was diluted in 5 ml of prewarmed tissue culture medium to obtain a 20 μM EdU labelling solution. Half of the medium was removed and replaced with an equal volume of EdU labelling solution (final concentration 10 μM), and the cells were incubated at 37°C with 5% CO_2 overnight. After that, 1 ml of 4% formaldehyde in PBS was added to each well and allowed to stand at room temperature for 15 minutes. Subsequently, the formaldehyde was removed and washed twice with 1 ml of 3% BSA in PBS. After removing the wash solution, 1 ml of 0.5% Triton X-100 in PBS was added to each well and incubated at room temperature for 20 minutes. The wells were washed twice with 1 ml of 3% BSA in PBS. Then, 0.5 ml of Click-iT reaction cocktail was added to each well and the plates were incubated at room temperature for 30 minutes, protected from light. Following repeated washing with 1 ml of 3% BSA in PBS, 1 ml of 1× Hoechst 33342 solution (Thermo Fisher Scientific) was added to each well and incubated at room temperature for 30 minutes, protected from light. Subsequently, each well was

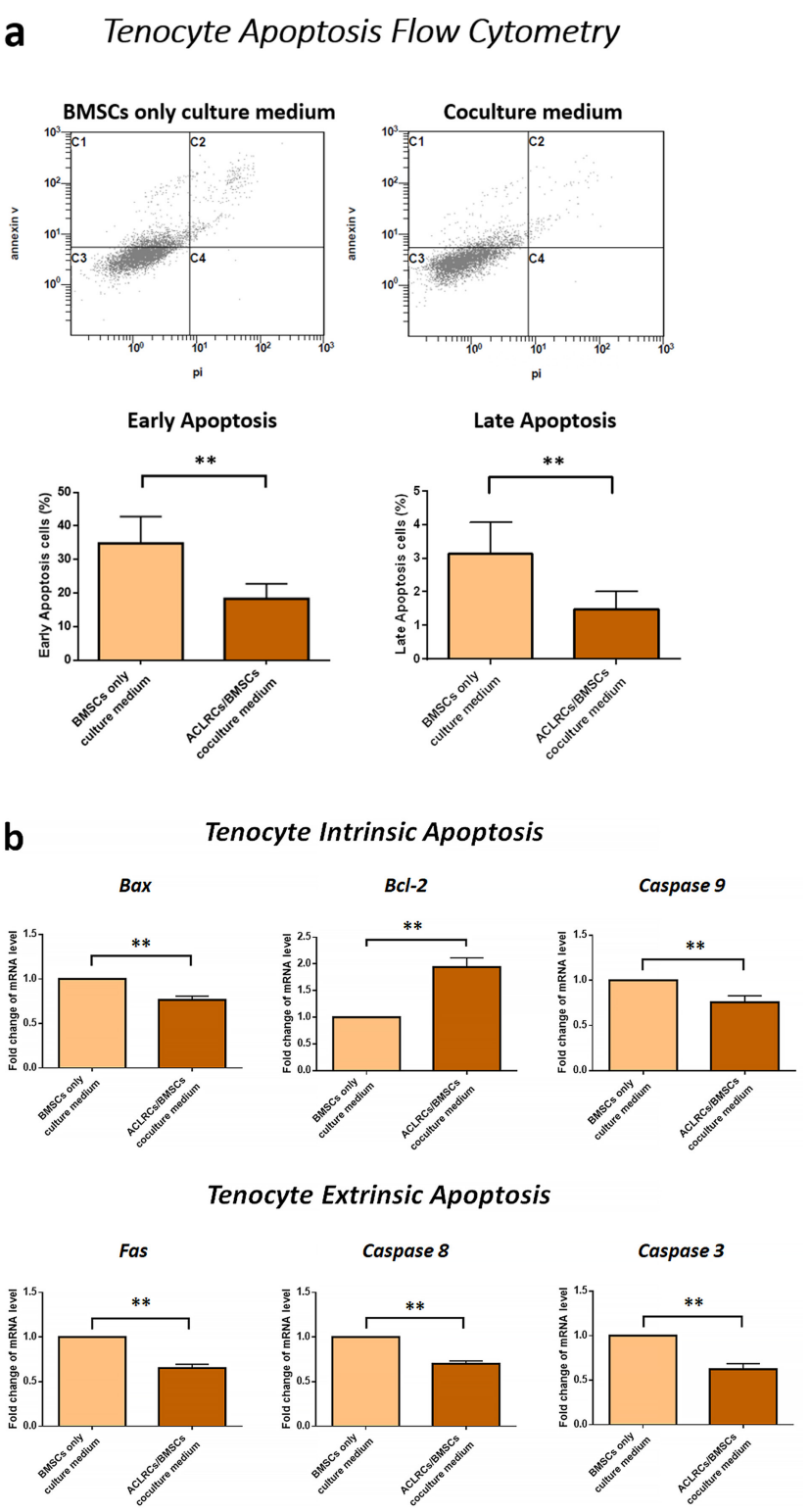

Fig. 4

Effects of anterior cruciate ligament remnant cells (ACLRCs)/bone marrow stromal cells (BMSCs) co-culture medium on hamstring tenocyte apoptotic regulation. a) Hamstring tenocytes cultured in the BMSCs-only culture (control) medium (n = 6) or ACLRCs/BMSCs co-culture medium (n = 6). The dot plot diagrams represent cell populations undergoing apoptosis detected using fluorescein isothiocyanate-conjugated Annexin V/propidium iodide (PI) staining followed by flow cytometry analysis. The lower left quadrants of the panels represent live cells (Annexin V–/PI–), the upper left quadrants represent early apoptotic cells (Annexin V+/PI–), the lower right quadrants represent necrotic cells (Annexin V–/PI+), and the upper right quadrants represent late apoptotic cells (Annexin V+/PI+). b) Expression of proapoptotic and antiapoptotic genes. Data show the mean (standard deviation) of triplicate measurements and analyzed with paired *t*-test. **p < 0.01 compared with BMSCs-only culture medium-treated group. Bax, Bcl2-associated X protein; Bcl2, B-cell lymphoma 2; Fas, Fas cell surface death receptor; mRNA, messenger RNA.

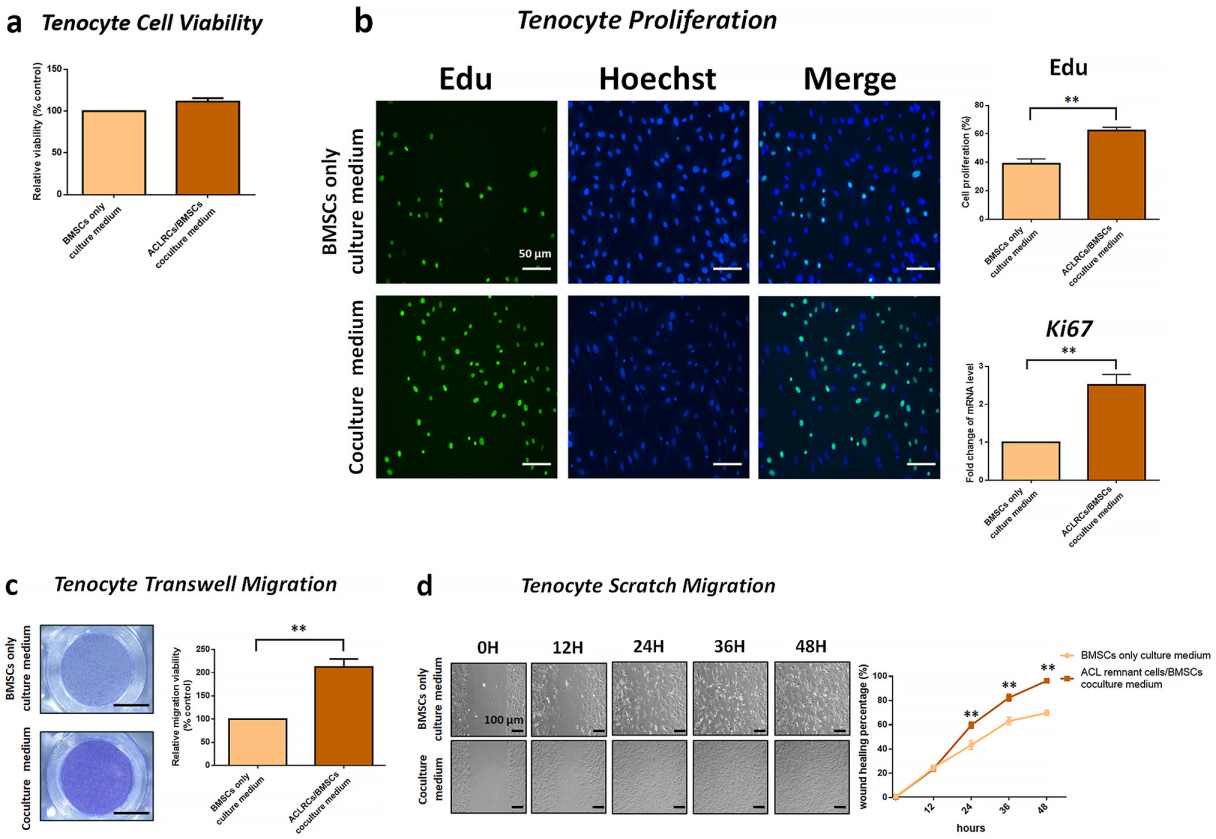

Fig. 5

Effects of anterior cruciate ligament remnant cells (ACLRCs)/bone marrow stromal cells (BMSCs) co-culture medium on hamstring tenocyte cell viability, proliferation rate, and migration ability. a) Cell viability determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay of the tenocytes treated with BMSCs-only culture (n = 6) or ACLRCs/BMSCs co-culture (n = 6) media. Data are expressed as the mean (standard deviation (SD)) of triplicate measurements. b) The representative immunofluorescence pictures show the result of EdU assay in BMSCs-only culture or ACLRCs/BMSCs co-culture media-treated hamstring tenocytes. The EdU incorporation assay and Ki-67 mRNA expression were assessed to detect the proliferation of BMSCs-only culture (n = 6) or ACLRCs/BMSCs co-culture (n = 6) media-treated hamstring tenocytes; scale bar: 50 μm. c) Migration activity of hamstring tenocytes treated with the BMSCs-only culture or ACLRCs/BMSCs co-culture media plated on the upper chamber of Transwell inserts. d) Cell migration ability of hamstring tenocytes treated with BMSCs-only culture (n = 6) or ACLRCs/BMSCs co-culture (n = 6) media estimated using scratch wound healing assay. Wounds were evaluated at 12, 24, 36, and 48 hours after medium administration. The wound healing area was measured by calculating the wound area in each period and expressed as a percentage of the initial wound area at time zero. Data show the mean (SD) of triplicate measurements and analyzed with paired *t*-test. **p < 0.01 compared with BMSCs only culture medium-treated group. mRNA, messenger RNA.

washed twice with 1 ml of PBS and observed under confocal microscopy (Olympus IX-81-FV100; Olympus, Japan). The cell proliferation rate was calculated using ImageJ with five randomized areas per sample.

Transwell migration assay. To investigate the change in the cell migration rate, the hamstring tenocytes were tested after administration of co-culture (n = 6) and control (n = 6) media using a cell migration assay in a 6.5 mm Transwell chamber with a pore size of 8 μm (MilliporeSigma, USA), as described previously.[20] The counted tenocytes were resuspended in serum-free medium and seeded in the upper compartment of the chamber for the migration assay. The culture medium supplemented with 10% FBS was added to the bottom chamber to act as a chemoattractant. After incubation at 37°C for 20 hours in the migration assay, cells that were translocated to the bottom surface of the membrane were fixed with 100% methanol for ten minutes, followed by staining with 0.1% crystal violet for 30 minutes. The migrated cells were counted under a microscope, and the relative cell migration rate was calculated as the ratio of migrating co-culture medium-treated tenocytes to the control medium-treated cells (% of the control).

Scratch migration assay. The scratch migration test was conducted to examine the change in migration rate between the co-culture- and control medium-treated tenocytes (n = 6 in both groups), as described previously.[20] The cell migration rate was investigated in real time by closing the scratch gap at regular intervals (12, 24, 36, and 48 hours) under a microscope (Leica DMI6000B; Leica Microsystems, Germany). The relative changes were calculated using ImageJ and presented as: (1–[residual area/initial scratch area]) × 100%.

RNA isolation and real-time polymerase chain reaction. Total RNA from the hamstring tendons and tenocytes treated with co-culture (n = 6) and control (n = 6) media was extracted using RNAzol reagent (Cat. No. RN-190; Molecular Research Center, USA), and 2 μg

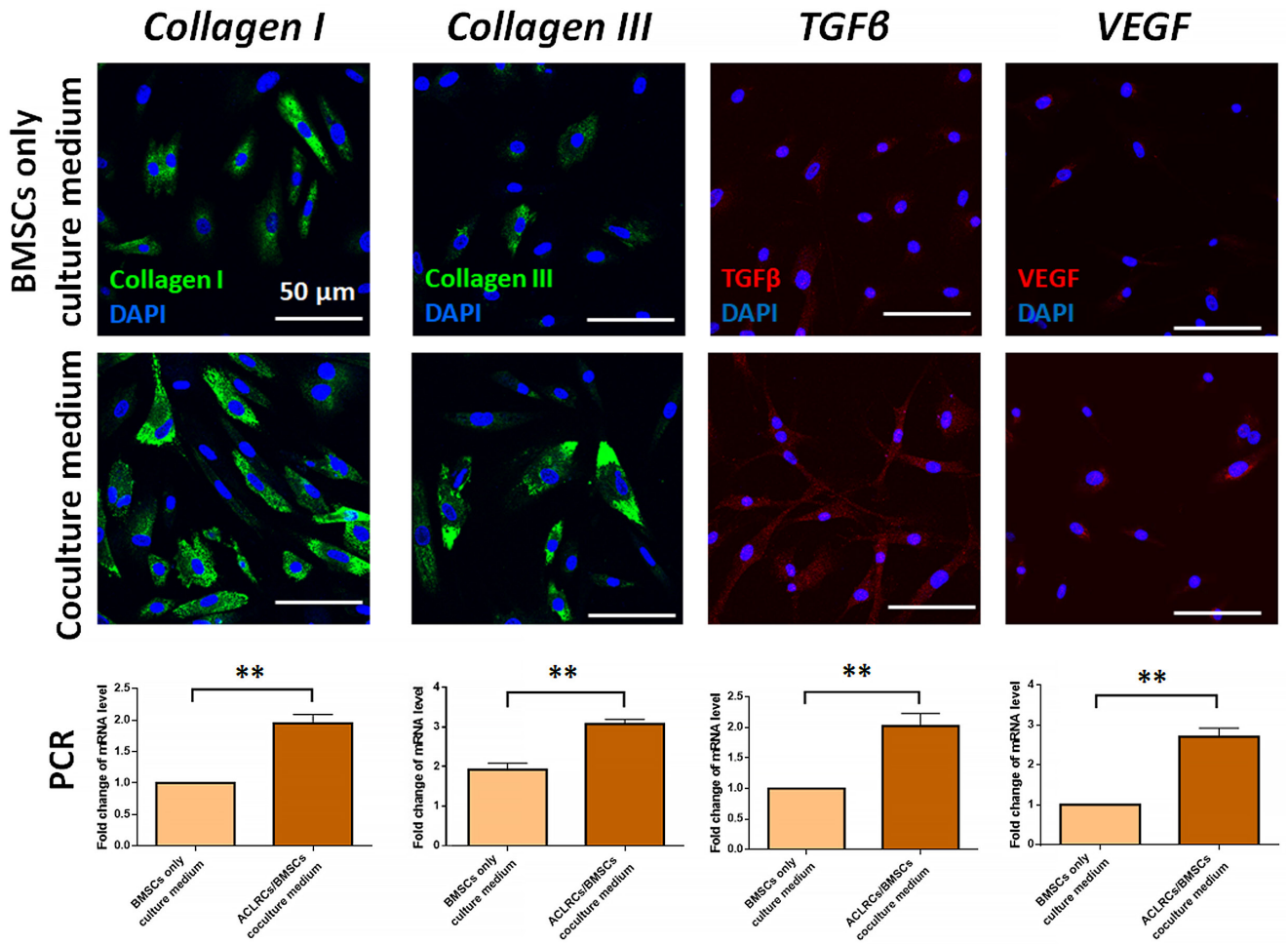

Fig. 6

Effects of anterior cruciate ligament remnant cells (ACLRCs)/bone marrow stromal cells (BMSCs) co-culture medium on hamstring tenocytes on the expression of collagen types I and III, transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF). The representative immunofluorescence pictures show the expression of types I and III collagen, TGF-β, and VEGF in BMSCs-only culture (n = 6) or ACLRCs/BMSCs co-culture (n = 6) media-treated hamstring tenocytes. Scale bar: 50 μm. Data are indicated as mean (standard deviation) of triplicate measurements and analyzed with paired *t*-test. **p < 0.01. mRNA, messenger RNA; PCR, polymerase chain reaction.

total RNA was reverse-transcribed using the Maxima First Strand cDNA Synthesis Kit (Cat. No. K1642, Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, the solution was incubated at 65°C for five minutes, and was then mixed with first-strand buffer, Dithiothreitol (DTT), and RNaseOUT in a final volume of 20 μl. Then, the solution was incubated at 42°C for 60 minutes, followed by at 70°C for 15 minutes to inactivate the reverse transcriptase activity. Real-time polymerase chain reaction (PCR) was carried out using the SYBR Green PCR Master Mix (Cat. A25780, Thermo Fisher Scientific) and was processed on a LightCycler PCR and detection 7500 system (Bio-Rad, USA). The complementary DNA (cDNA) samples (2 μl samples in a total volume of 20 μl per reaction) were amplified using the primers listed in Table I and the following the cycling condition: 95°C for 15 minutes (to activate DNA polymerase), followed by 40 cycles of 95°C for 15 seconds, 60°C for 20 seconds, and 72°C for 30 seconds.[20,30] Threshold cycles (*Ct*) for each gene tested were normalized to the housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene value (Δ*Ct*), and every experimental sample was referred to its control (ΔΔ*Ct*). Fold-change values were expressed as $2^{-\Delta\Delta Ct}$ values. The experimental data of the co-culture medium-treated group were expressed as fold-change values compared to that of the control medium set as 1.

Immunofluorescence staining. Hamstring tenocytes treated with co-culture (n = 6) or control (n = 6) medium were assessed for collagen types I and III, transforming growth factor (*TGF-β*), and vascular endothelial growth factor (*VEGF*), as described previously.[20] Primary antibodies against collagen types I and III, *TGF-β*, and *VEGF* (all from Arigo Biolaboratories, Taiwan) were used to stain the cells overnight at 4°C. The cells were then stained with fluorescent secondary antibodies (donkey anti-goat IgG (H+L)-FAM (Leadgene Biomedical; 1:250) for collagen types I and III, and goat anti-mouse

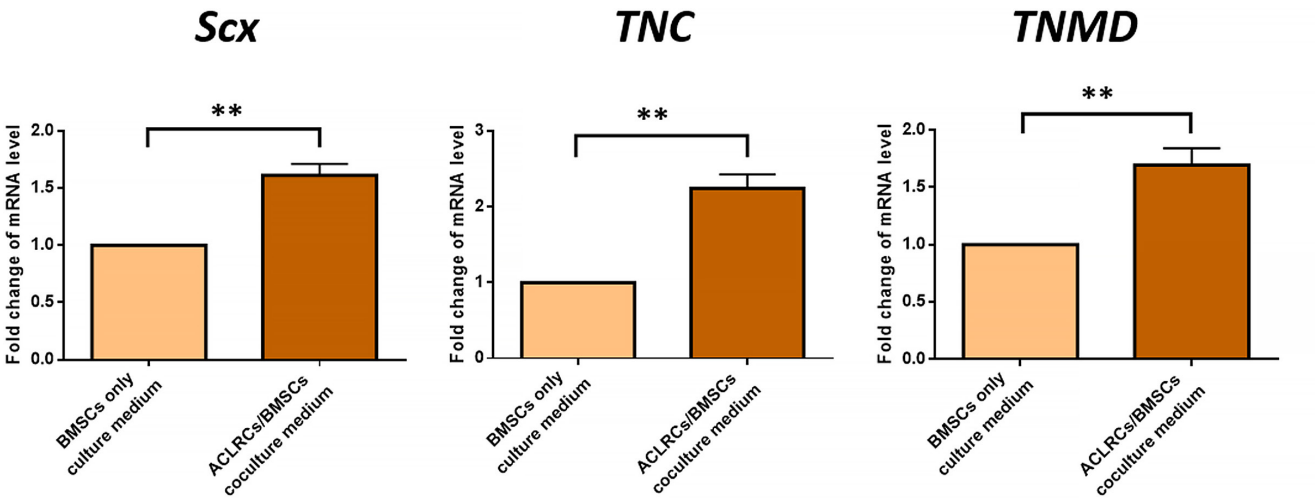

Fig. 7

Effects of anterior cruciate ligament remnant cells (ACLRCs)/bone marrow stromal cells (BMSCs) co-culture medium on hamstring tenocytes on the expression of tenogenesis genes (scleraxis (SCX), tenascin C (TNC), and tenomodulin (TNMD)). The hamstring tenocytes show significantly higher gene expression of tenogenic markers (SCX, TNC, TNMD) after co-culture medium (n = 6) treatment compared to that of BMSCs-only culture medium (n = 6). Data are indicated as mean (standard deviation) of triplicate measurements and analyzed with paired *t*-test. **p < 0.01. mRNA, messenger RNA.

IgG (H+L)-TAMRA (Tetramethylrhodamine; Leadgene Biomedical; 1:250) for *TGF-β* and *VEGF*) for one hour and rinsed twice with PBS. The slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific), mounted, and observed under a confocal microscope (Olympus IX-81-FV100; Olympus, Japan).

Statistical analysis. The sample size calculation was performed using G*Power version 3.1.9.7. The differences between co-culture and control media in hamstring tendons and tenocytes were analyzed using the paired *t*-test. All data are presented as mean (SD) with triple measurements. All statistical analyses were performed using SPSS software version 20 (IBM, USA). Statistical significance was set at $p < 0.05$.

Results

ACLRCs/BMSCs co-culture medium attenuated apoptosis in hamstring tendons. Under co-culture (ACLRCs/BMSCs) medium treatment, the hamstring tendon presented statistically significantly fewer dead cells (those stained red) compared to those in control (BMSC-only culture) medium in the LIVE/DEAD assay (Figure 2a). TUNEL staining also showed a substantial decrease in the number of apoptotic cells in the hamstring tendon treated with co-culture medium than that in the control medium (Figure 2b). Furthermore, in co-culture medium, the expression of apoptotic genes in the intrinsic pathway including *Bax* ($p < 0.001$) and Caspase 9 ($p = 0.005$) was reduced, and that of *Bcl-2* ($p = 0.018$; all paired *t*-test) was elevated significantly compared with that in control medium. Similarly, significant reductions in the expression of extrinsic apoptosis pathway genes, including *Fas* ($p < 0.001$), Caspase 8 ($p = 0.002$), and Caspase 3 ($p < 0.001$; all paired *t*-test), were observed in co-culture medium compared with those in the control medium (Figure 2c).

ACLRCs/BMSCs co-culture medium activated proliferation and expression of collagen synthesis, TGF-β, VEGF, and tenogenic genes in the hamstring tendon. The substantially higher expression of proliferating cell nuclear antigen (*PCNA*) revealed by immunohistochemistry (IHC) and higher gene expression of *Ki-67* ($p < 0.001$, paired *t*-test) indicated active proliferation in the hamstring tendon after treatment with co-culture medium compared to that with control medium (Figure 3a). The expression of collagen synthesis genes (collagen types I ($p < 0.001$) and III ($p < 0.001$, both paired *t*-test)), growth factor secretion (*TGF-β* ($p < 0.001$) and *VEGF* ($p < 0.001$, both paired *t*-test)), and tenogenic markers (scleraxis (*SCX*) ($p < 0.001$), tenascin C (*TNC*) ($p < 0.001$), and tenomodulin (*TNMD*) ($p < 0.001$, all paired *t*-test)) was significantly higher in the hamstring tendon treated with co-culture medium than that in control medium (Figure 3b).

ACLRCs/BMSCs co-culture medium decreased apoptosis of hamstring tenocytes. The hamstring tenocytes presented a statistically significantly lower apoptosis rate after co-culture medium treatment in both early and late apoptosis than that in control medium-treated tenocytes (Figure 4a). At the same time, hamstring tenocytes treated with the co-culture medium showed: significantly decreased expression of *Bax* ($p < 0.001$) and Caspase 9 ($p = 0.004$); elevated expression of *Bcl-2* ($p < 0.001$) in the intrinsic apoptosis pathway; and lower expression of *Fas* ($p < 0.001$), Caspase 8 ($p < 0.001$), and Caspase 3 ($p < 0.001$; all paired *t*-test) in the extrinsic pathway compared with that in the control medium (Figure 4b).

ACLRCs/BMSCs co-culture medium enhanced the proliferation and migration capability of hamstring tenocytes. No statistically significant difference in tenocyte viability ($p = 0.213$, paired *t*-test) between the co-culture and control media treated hamstring tenocytes was observed (Figure 5a). However, the proliferation (Edu assay, $p < 0.001$; *Ki-67* gene expression, $p < 0.001$; both paired *t*-test) (Figure 5b) and migration capability (Transwell migration assay, $p < 0.001$; Scratch migration assay, 12 hrs, $p = 0.746$, 24 hrs, $p = 0.002$, 36 hrs, $p < 0.001$, 48 hrs, $p < 0.001$; all paired *t*-test) (Figures 5c and 5d) of the hamstring tenocytes statistically significantly increased after co-culture medium treatment compared to those after control medium treatment.

ACLRCs/BMSCs co-culture medium activated collagen synthesis, growth factor expression, and tenogenesis in the hamstring tenocytes. IHC and PCR analyses revealed that co-culture medium treated hamstring tenocytes demonstrated significantly higher expression of genes involved in collagen synthesis (collagen types I ($p < 0.001$) and III ($p < 0.001$)) and growth factor secretion (*TGF-β* ($p < 0.001$) and *VEGF* ($p < 0.001$; all paired *t*-test)) than those treated with control medium (Figure 6). The expression of tenogenic markers (*SCX* ($p < 0.001$), *TNC* ($p < 0.001$), *TNMD* ($p = 0.001$; all paired *t*-test)) also showed a significant increase in hamstring tenocytes treated with co-culture medium compared with those in the control medium (Figure 7).

Discussion

After ACL reconstruction, preservation of the ACL remnant in relation to the surrounding tissue and its effects on hamstring tendon graft maturation remain underexplored. In this study, we co-cultured ACLRCs with BMSCs, which simulate clinical conditions in ACL reconstruction with remnant preservation in vivo, to observe its effects on hamstring tendon grafts. The experimental results showed that the co-culture medium attenuated apoptosis and enhanced proliferation, collagen synthesis, growth factor secretion, and tenogenesis of the hamstring tendon compared to BMSCs-only culture medium. Furthermore, hamstring tenocytes showed substantially decreased apoptosis and increased proliferation, migration, and collagen and tenogenic gene expression after co-culture medium treatment. Although this is an in vitro study, our study results demonstrated that preservation of the ACLRCs mixed with BMSCs from the proximal tibia could improve the maturation of hamstring tendon grafts by decreasing apoptosis and enhancing the activity of the hamstring tendons and tenocytes.

Apoptosis, a regulated programmed cell death, is essential for the regulation of development and maintenance of homeostasis in tissues. However, excessive apoptosis has been demonstrated to induce tendinopathy or tears in the tendon tissue.[31,32] There are two pathways of apoptosis: the intrinsic pathway, which is mediated by mitochondria; and the extrinsic pathway, which is mediated by extracellular death receptors.[31] Immediately after ACL reconstruction, the implanted tendon graft undergoes hypovascularization, nutrition deletion, and hypoxia, leading to cell apoptosis and death.[6,33] Cell apoptosis has been speculated to induce tendon tissue degradation with a breakdown of extracellular matrix collagen, loss of its parallel arrangement, and further deterioration of its biomechanical properties.[9] In a study on cellular apoptosis during tendon healing, Wu et al[10] found that apoptosis peaks at day 3 and continues for two to four weeks. Therefore, early regulation of apoptosis of tendon cells would allow the cell to proliferate and synthesize collagen, which prevents graft microtears and enhances graft healing. In this study, the hamstring tendon treated with co-culture medium showed fewer apoptotic cells than the control medium, as demonstrated in the LIVE/DEAD and TUNEL assays. The flow cytometry results also showed statistically significantly fewer early and late apoptotic cells in the co-culture medium treatment group than in the control medium treatment group. The PCR results of the apoptotic genes in the co-culture medium-treated hamstring tendons and tenocytes showed a statistically significant reduction in expression of intrinsic and extrinsic apoptotic pathway genes. After co-culture medium treatment, the hamstring tendon showed statistically significantly higher *PCNA* and *Ki-67* expression, and the tenocytes showed a substantially increased proliferation rate. Taken together, these results demonstrate that the co-culture medium could effectively attenuate apoptosis with a robust proliferation of the hamstring tendons and tenocytes.

Following proliferation, our study also showed elevated levels of collagen synthesis, *TGF-β*, *VEGF*, and tenogenic gene expression in hamstring tendons and tenocytes after co-culture medium treatment. Collagen synthesis is fundamental for tendon or ligament healing. Growth factors are important modulators of regeneration of injured tissues and enhancement of graft healing.[34,35] TGF-β, a multifunctional cytokine, has been demonstrated to promote ACL remodelling and enhance the tendon-bone interface by increasing cell proliferation and collagen fibre formation through the TGF-β/mitogen-activated protein kinase (MAPK) signalling pathway.[36,37] Upregulation of VEGF, a key factor in angiogenesis, has been reported to promote tendon healing and tendon-bone junction regeneration.[19,38] In addition, growth factor secretion guides the peripheral cells to migrate to the graft, further proliferate to produce extracellular matrix, and induce tenogenic differentiation.[34,39] In our study, treatment with ACLRCs/BMSCs co-culture medium resulted in not only the survival and proliferation of the hamstring tendons and tenocytes, but also enhancement of the expression of collagen synthesis, growth factors, and tenogenic differentiation to promote graft maturation after ACL reconstruction.

Creating a good intra-articular microenvironment is critical for implanted graft maturation after ACL reconstruction. Increasing evidence has shown the positive effects of ACL remnant preservation in graft healing with

enhanced cell proliferation, revascularization, and regeneration of proprioceptive organs.[14-16] However, the exact mechanism remains unknown. Prior studies have demonstrated that the ACL remnant provides only stem cells and does not perform any other function. Regarding the relationship between ACL remnants and the surrounding tissue, Lu et al[20] found the ACL remnant could upregulate BMSC proliferation, migration, and collagen gene expression compared to non-ACL co-cultured BMSCs in an indirect, non-contact co-culture system. In this study, our results demonstrated two novel findings: 1) the microenvironment created by the coexistence of ACL remnants and BMSCs could effectively enhance tendon graft maturation; and 2) the ACL remnant was not only a stem cell provider but also presented the ability to modulate and cooperate with the surrounding tissue. Thus, our study results provide positive evidence for remnant preservation in ACL reconstruction.

However, the optimal time for ACL reconstruction remains debatable. Regarding postoperative arthrofibrosis, ACL reconstruction is usually performed several weeks (generally four to six weeks) after injury until the swelling subsides and range of motion is regained.[23,40] However, the current knowledge proposes that there is no increased risk and that ACL reconstruction can be performed as early as one week after injury with modern surgical techniques and an accelerated rehabilitation programme.[41-43] Few studies have investigated the effects of surgical timing on ACL remnant activity and its healing potential.[44,45] Zhang et al[44] investigated the activity of human ACLRCs, and the results showed a higher fraction of stem-like cells, expansion protection, and osteogenic capability of the tissue collected within 90 days of injury compared to that tissue collected after 90 days of injury. Inokuchi et al[45] applied an intra-articular injection model in rat ACL reconstruction to study the influence of the injury-to-surgery time interval on the activity of human ACLRCs. They found early histological healing and superior biomechanical tensile strength of the tendon-bone tunnel interface in the early operation group (less than three months after injury) compared to that in the late operation group (more than three months after injury). In this study, we selected tissues four weeks after injury to follow the clinical conventions of ACL reconstruction. In fact, the ACL remnant at four weeks after the initial injury presented the capability to cooperate with the BMSCs with decreased tendon apoptosis and enhanced graft maturation. However, the correlation between injury time and the regulating capability of ACL remnants requires further investigation.

The present study has some limitations. First, we only investigated the effects of co-culture medium on hamstring tendons and tenocytes; these findings need to be validated in future animal studies. Second, we did not examine the precise content of the co-culture medium. Third, the quantity of ACLRCs required to cooperate with BMSCs, which could synergistically enhance graft tendon healing, needs to be investigated.

In conclusion, the ACLRCs/BMSCs co-culture medium attenuated apoptosis and enhanced proliferation, migration, collagen expression, and tenogenesis in the hamstring tendons and tenocytes in this in vitro study. The study results suggest that the coexistence of ACLRCs and BMSCs can potentially reduce apoptosis and enhance regeneration of the implanted hamstring tendon with remnant preservation for ACL reconstruction.

Supplementary material

An ARRIVE checklist is included to show that the ARRIVE guidelines were adhered to in this study.

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