

Mechanical stretching enhances the cellular and paracrine effects of bone marrow mesenchymal stem cells on diabetic wound healing

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Abstract

Background: Diabetic wounds present persistent clinical challenges characterized by disrupted extracellular matrix (ECM) homeostasis, which critically impedes tissue regeneration. While bone marrow-derived mesenchymal stem cells (BMSCs) exhibit therapeutic potential through ECM remodeling, conventional transplantation strategies are limited by suboptimal cell retention and transient therapeutic effects.

Methods: BMSCs cultured on Flexcell plates were subjected to programmable mechanical stretching using a custom-built spherical cell-stretching system. Strain rate- and duration-dependent effects on paracrine signaling and ECM secretion were longitudinally assessed through western blotting and ELISA. The optimized mechanical parameters (15% deformation, 1440 cycles, 5-s vertex residence time) were subsequently applied to generate BMSC sheets. Comparative analyses of biological activity and mechanical properties were performed between non-stretched controls and mechanically optimized groups. *In vivo* therapeutic efficacy was evaluated in diabetic rat models through wound closure kinetics, Masson's trichrome staining, and immunofluorescence detection of neovascularization markers. Mechanistic insights were obtained via transcriptomic profiling of stretch-activated signaling pathways.

Results: Mechanical stretching significantly upregulated type I collagen, type III collagen, vascular endothelial growth factor, and transforming growth factor-beta (TGF- β) secretion in BMSCs. The optimized stretching parameters (15% deformation, 1440 cycles, and 5-s vertex residence time) promoted BMSC proliferation while reducing apoptosis without compromising stemness. Mechanical stretching facilitated the formation of layered cell sheets with more organized collagen deposition and higher mechanical strength, expediting wound healing in diabetic rats through enhanced re-epithelialization and neovascularization. RNA sequencing analysis revealed that mechanical stretching significantly upregulated mechanosensitive molecules, mechanical stimulation signaling pathways, and cellular behavior regulatory pathways, particularly those associated with mechanical stimuli response, integrin binding, ECM secretion, and intercellular adhesion.

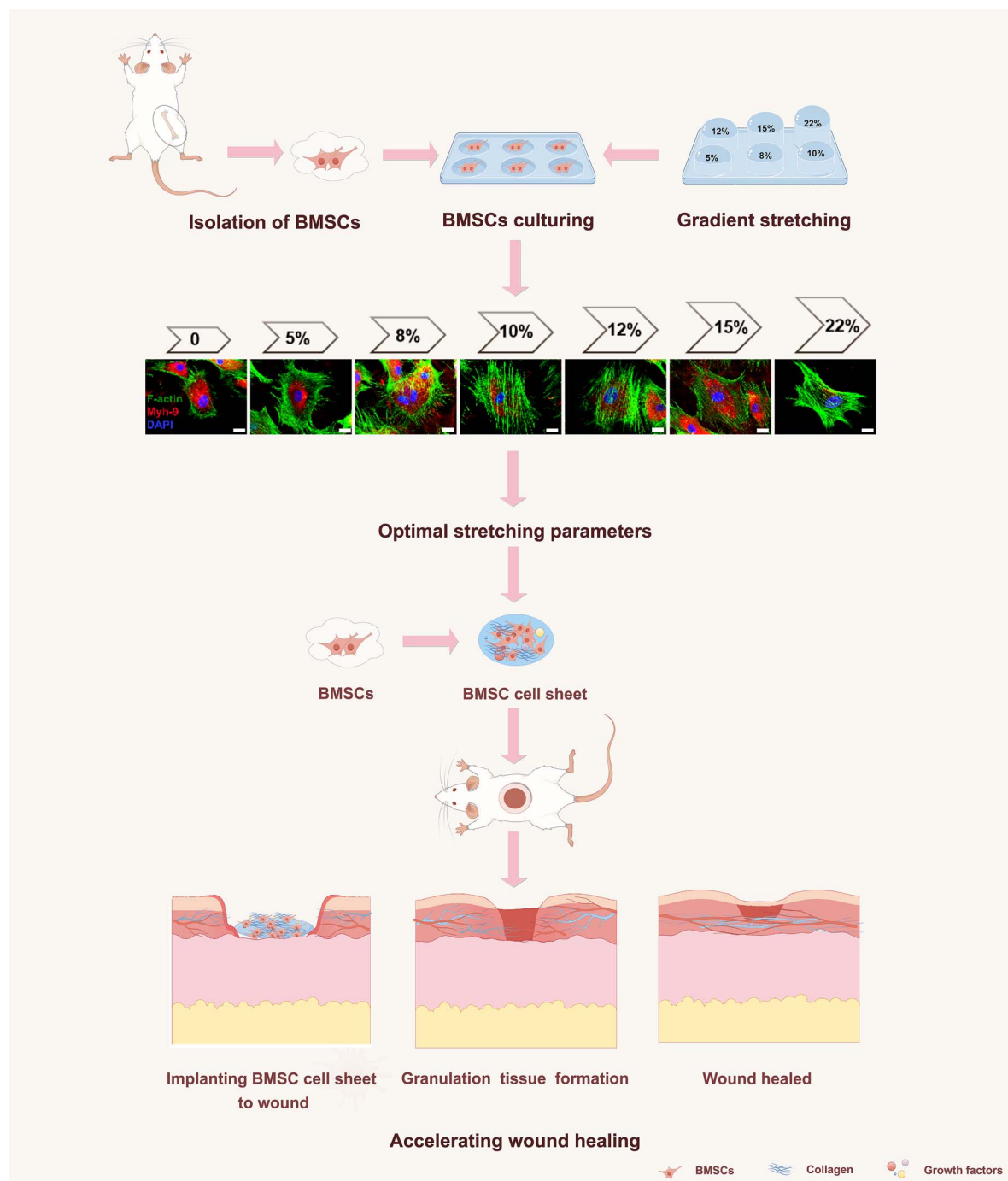
Conclusions: Mechanically stretched BMSC cell sheets can promote diabetic wound healing by enhancing cellular activity, paracrine of growth factors, and ECM components.

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Graphical Abstract



Keywords: Diabetic wound healing; Bone marrow mesenchymal stem cells; Programmable mechanical stretching; Paracrine effect

Highlights

- Mechanical stretching promotes BMSC proliferation, preserves cell stemness, and increases the expression of ECM components, including collagen and growth factors.
- Mechanical stretching optimizes the mechanical properties of BMSC cell sheets and increases the expression of growth factors and ECM components.
- BMSC cell sheets, optimized via mechanical stretching, accelerate wound closure in diabetic rats by increasing neovascularization and collagen formation.

Background

Chronic refractory wounds (CRWs) are defined as persistent skin defects that fail to progress through normal healing phases, thereby disrupting structural and functional restoration. Clinically, CRWs demonstrate no substantial healing progression after ≥ 4 weeks of standard care. Epidemiological studies indicate a CRW prevalence of 1.67–2.21 cases per 1000 population in developed nations, constituting 1%–3% of total healthcare costs [1–3]. Notably, diabetic wounds represent the predominant CRW subtype, imposing substantial socioeconomic burdens attributable to prolonged treatment requirements [4–6].

While the precise pathophysiology of CRWs remains elusive, shared pathological hallmarks include sustained inflammatory responses and ECM dysregulation [7, 8]. In diabetic wounds specifically, ischemia-hypoxia cascades impair collagen I/III biosynthesis, induce growth factor dysregulation, and provoke matrix metalloproteinase (MMP) overexpression—collectively disrupting the ECM's biomechanical support essential for tissue regeneration [9]. Concomitantly, impaired growth factor-receptor signaling induces cytoskeletal reorganization in keratinocytes and fibroblasts, thereby suppressing cell motility and proliferative capacity. These molecular perturbations culminate in failure of core repair processes: cellular repopulation, angiogenesis, and epithelial restitution [10, 11].

To address ECM dysfunction, multiple regenerative strategies have emerged, focusing on ECM reconstruction, bioactive component delivery, and biophysical modulation [12, 13]. Current modalities include acellular dermal matrices (allogeneic/xenogeneic), recombinant growth factors, bioengineered dressings, and negative pressure wound therapy [14–16]. While surpassing conventional wound care, these approaches exhibit critical limitations: Decellularized matrices lack endogenous cytokines required for cell recruitment and proliferative signaling; exogenous growth factors suffer from short half-lives and poor spatiotemporal control, restricting their utility to adjuvant applications [17, 18]. These constraints underscore the need for innovative therapeutic paradigms.

Stem cell-based therapies have emerged as a transformative approach in wound regeneration [19–23]. Beyond supplementing defective cell populations, these strategies enable functional skin reconstruction through *in vitro* expansion, directed differentiation, and genetic engineering—effectively overcoming post-repair functional deficits in tissue-engineered skin [24]. Mesenchymal stem cells (MSCs) excel in this context through multimodal mechanisms: paracrine factor secretion, multilineage differentiation, immune privilege, and ECM remodeling via collagen I/III ratio modulation [20]. Among MSC sources, bone marrow-derived MSCs (BMSCs) demonstrate superior regenerative capacity. Nammian et al. documented BMSC-mediated enhancement of endothelial migration, muscle regeneration, neovascularization, and tissue integration surpassing adipose-derived counterparts [25]. Complementarily, Fathke's group established BMSC-driven collagen matrix contraction coupled with collagen I/III regulation [26].

Recent advances focus on manipulating biochemical microenvironmental cues to direct BMSC behavior and paracrine output [19]. However, exclusive reliance on exogenous chemical signals compromises spatiotemporal control

over ECM synthesis. Notably, cellular mechanosensitivity plays an equally critical role—cell-ECM interactions and migratory dynamics profoundly influence ECM deposition patterns. Current research predominantly emphasizes biochemical stimulation, overlooking mechanical determinants. As Gilmour demonstrated, migrating cells autonomously establish cytokine gradients through receptor-mediated trafficking, challenging traditional exogenous cytokine models [27]. Therefore, biomimetic microenvironments that recapitulate native mechanobiology may better sustain BMSC-mediated ECM remodeling. Therapeutic mechanical strategies like tissue expansion and negative pressure therapy have successfully leveraged skin's inherent mechanoresponsiveness [28], highlighting mechanotherapy's potential through precise controllability and favorable safety profiles.

Despite progress, current BMSC delivery systems face persistent challenges in cell homing efficiency and retention durability—critical barriers to sustained therapeutic effects. Cell sheet engineering emerges as a promising solution to enhance cell survival and functional persistence at wound sites.

This study aims to investigate the impact of mechanical stretching on BMSC cellular activity and paracrine effects, identify the optimal mechanical stretching parameters, and assess the therapeutic efficacy of optimized BMSC cell sheets in healing full-thickness skin defects in diabetic rats. Additionally, we aim to explore the molecular mechanisms underlying the biological effects of mechanical stretching on BMSCs.

Methods

Isolation of BMSCs

One week-old Sprague-Dawley (SD) rats were obtained from the Animal Center of the Medical University. Bone marrow was extracted from both femurs of the mice with the approval of the Institutional Animal Care and Use Committee of the Air Force Medical University. BMSCs were isolated via density gradient centrifugation and then suspended in cell culture dishes with Minimum Essential Medium- α (MEM α , Procell, Wuhan China) containing 0.272 g/L of L-glutamine, 10% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin. The cell cultures were incubated at 37°C under 5% carbon dioxide for 24 h. After 24 h, nonadherent cells were washed with phosphate-buffered saline and adherent cells were passaged at 80%–90% confluence. Cells from passage two were used in subsequent experiments and maintained by replacing the entire medium every two days.

Mechanical stretch loading

A spherical automatic cell—stretching device was applied for the mechanical stretch loading of cells and cell sheets. The device comprised mainly two components: a mechanical stretch loading machine and a control system [29]. The device required 6-well Flexcell Bioflex culture plates (TTCF 5001C, Flexcell International Corporation, USA) as a carrier of cells and cell sheets for mechanical stretch loading. The mechanical stretch loading machine comprised six spherical columns corresponding to each well of the 6-well Flexcell Bioflex culture plates, which could be displaced up and down in the range of 4–8 mm. Mechanical stretching was achieved by deforming the underside of each well of the six wells of the culture plates, yielding 5%, 8%, 10%, 12%, 15%, and 22% deformation.

Thus, the mechanical microenvironment with six strain rates was constructed.

BMSCs were inoculated on culture plates at a density of $1 \times 10^5/\text{cm}^2$. The next day, BMSCs grew into a subconfluent state, the medium and nonadherent cells were washed, and 3 ml of MEM α culture medium containing 10% FBS was added quantitatively. Then, the BMSCs were subjected to mechanical stimulation at different strain rates, stretching times, and cycle times using the device.

Formation of BMSC cell sheets

BMSC cell sheet culturing was performed using 2–5 generations of cells. The cells were seeded at a density of 20 000 cells/ cm^2 on 6-well Flexcell Bioflex culture plates with MEM α containing 10% FBS and 100 $\mu\text{g}/\text{ml}$ of vitamin C (Sigma). The culture medium was completely replaced every 2 days and cultured for 2–3 weeks to harvest the formed cell sheets. The formed cell sheets were subjected to mechanical stretching before being detached from the culture plates.

Elastic modulus analysis of BMSC cell sheets

The elastic modulus of BMSC cell sheets was assessed using shear wave elastography (SWE). The cell sheets were positioned between two pieces of porcine skin and an ultrasound probe was placed on top of the skin. Continuous and focused acoustic radiation force pulses were emitted to generate lateral shear waves at the focal point within the tissue. These shear waves were then captured using an ultrahigh speed imaging system, which provided real-time, 2D elastography images. The images used color coding to reflect Young's modulus values of the tissue quantitatively. Data were collected from three different locations on each BMSC cell sheet. Young's modulus (E) was calculated using the formula $E = 3\rho C$, where C is the shear wave propagation speed and ρ represents the tissue density.

Western blot analysis

BMSCs and harvested ECMs were ground in a homogenizer with radio-immunoprecipitation assay buffer (containing phenyl methyl sulfonyl fluoride) on ice. All the samples were collected and homogenized (10 ml/mg) before centrifugation. After centrifugation at 4°C and 15 000 g for 10 min, the supernatant was collected for protein extraction. Protein concentrations were assessed using a bicinchoninic acid (BCA) protein assay kit. Equal amounts of total protein were loaded from different samples and groups for western blot (WB) analysis. The proteins were isolated via electrophoresis (110 V) on 8%–15% sodium dodecyl sulfate–polyacrylamide gel and transferred (20 V, overnight) to a polyvinylidene fluoride film (Millipore, USA), which was then sealed at room temperature with 5% defatted milk and bovine serum albumin for 1 h. The membrane was incubated with the primary antibody at 4°C overnight, and then with the secondary antibody at room temperature for 2 h. The following antibodies were used: anti-vascular endothelial growth factor (VEGF) (Servicebio, GB11034B), anti-TGF β 1 (Servicebio, GB111876), anti-collagen I (Servicebio, GB11022), anticollagen III (Servicebio, GB11023), and anti-GAPDH (Millipore, MAB374). The bands were imaged using the Bio-Rad image lab system after incubation with an electrochemiluminescence solution. The densities of specific bands were measured using Image J software (NIH, Bethesda, MD, USA).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was conducted to quantify prohealing ingredients, such as growth factors and collagens, secreted by BMSCs and BMSC cell sheets. Specifically, rat VEGF ELISA kits (Abcam, ab100751), rat TGF- β ELISA kits (Abcam, ab119557), rat Col-I ELISA kits (Abcam, ab210579), and rat COL-III ELISA kits (Cusabio, CSB-E07925m) were used to quantify the VEGF, TGF- β , Col-I, and COL-III concentrations, respectively, according to the product instructions.

Immunofluorescence of BMSCs

The cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked with 10% goat serum albumin, and stained with Ki67, CD105, MYH9, ZO-1, and filamentous actin (F-actin, 1:200; Abcam, Cambridge, UK). The cells were then incubated at 4°C overnight. Then, goat antirabbit and goat antimouse secondary antibodies (1:600; Abcam) were added and incubated at 37°C for 2 h. Finally, 4',6-diamidino-2-phenylindole (DAPI) staining was performed and the cells were examined using a microscope.

Establishment of a full-thickness skin defect model in diabetic rats

We selected 8–10-week-old SD rats in this study. After 2 weeks of adaptive feeding, the diabetic rat model was induced by a single intraperitoneal injection of 70 mg/kg of streptozotocin (STZ) on an empty stomach. One week after injection, the random blood glucose levels were measured from the tail vein. Rats with blood glucose levels of >16.7 mmol/L that maintained this level were confirmed to have successfully developed the diabetic rat model.

After the blood glucose levels had stabilized for 2 weeks, 15 rats were selected and randomly allocated into 3 groups: (i) Control group, (ii) BMSC cell sheet treatment group, and (iii) BMSC cell sheet optimized using mechanical stretching treatment group ($n = 5$). All the rats were anesthetized, and their backs were shaved. A full-thickness skin defect with a diameter of 1.5 cm was created using tissue scissors. A silicone ring with an inner diameter of 1.6 cm was attached to the surrounding skin using skin adhesive, and the outer edge of the silicone ring was further secured to the skin with 6–0 sutures to prevent skin contraction. (i) In the Control group, the BMSC cell sheet was not implanted; (ii) in the BMSC cell sheet treatment group, a 1.5-cm diameter BMSC cell sheet was implanted onto the wound; and (iii) in the BMSC cell sheet optimized by mechanical stretching treatment group, a 1.5-cm diameter BMSC cell sheet that had been optimized by mechanical stretching was implanted onto the wound. Then, all the wounds were covered with oil gauze, and finally wrapped with a self-adhesive bandage. Photographs of the wound area were taken on Days 0, 3, 5, 7, 9, 11, 13, 15, 18, and 21.

Immunohistochemistry and immunofluorescence of skin tissue

Rat skin tissue was harvested and fixed in 4% formaldehyde. The skin was sectioned into 6 μm —thick slices. Then, the tissues were subjected to Masson's trichrome, hematoxylin/eosin (H&E), and immunofluorescence staining. The fixed sections were stained with CD31, VWF antibodies (1:200; Abcam, Cambridge, UK), and DAPI (Beyotime, Shanghai, China) was

used to stain the nuclei. The images were obtained using an FSX100 microscope (Olympus, Tokyo, Japan).

Cell Labeling and tracking with PHK26

To monitor *in vivo* migration and distribution of BMSC cell sheets, the cells were labeled with the fluorescent dye PHK26 (5 μ M, Sigma-Aldrich, USA) prior to transplantation. Briefly, PHK26 was incubated with the cell sheets at 37°C for 20 minutes in the dark. Following incubation, the sheets were washed three times with PBS to remove unbound dye. Labeled sheets were then surgically implanted into the wound sites.

Tissue processing and fluorescence analysis

At days 3, 5, and 7 post-transplantation, treated skin tissues were harvested, flash-frozen, and cryosectioned into 10 μ m-thick slices using a Leica cryostat (Germany). Sections were mounted on slides, co-stained with PCNA (proliferation marker) and DAPI (nuclear counterstain), and imaged using inverted confocal laser scanning microscopy. The number of viable PHK26-labeled cells was quantified to assess cell survival and distribution.

Bioluminescence imaging for longitudinal tracking

Cell survival was assessed dynamically using an IVIS Lumina II Spectrum system (Caliper Life Sciences). Rats were anesthetized with isoflurane (EZ-Anesthesia, Euthanex) and placed in a supine position for serial bioluminescence imaging (BLI) at days 0 (baseline), 3, 5, and 7. Bioluminescent signals from the wound sites and adjacent tissues were quantified as photon flux (p/s/cm²/sr) to evaluate cell retention and migration.

RNA extraction and quality control

Total RNA was extracted from both stretched and non-stretched BMSC groups using the RNeasy Plus Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. RNA integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Only RNA samples with an RNA Integrity Number (RIN) > 8.0 were used for further analysis.

Library preparation and RNA sequencing

RNA libraries were prepared using the TruSeq Stranded mRNA Library Prep Kit (Illumina, USA) following the manufacturer's instructions. Briefly, mRNA was purified from total RNA using oligo(dT) beads, followed by fragmentation, reverse transcription to synthesize complementary DNA (cDNA), and ligation of adapters to the cDNA fragments. The libraries were then amplified and purified. The quality and concentration of the libraries were assessed using the Agilent 2100 Bioanalyzer and quantified by qPCR (KAPA Library Quantification Kit, KAPA Biosystems, USA). Sequencing was performed on an Illumina NovaSeq 6000 platform (Illumina, USA) using a paired-end 150 bp (PE150) sequencing approach, generating a minimum of 20 million reads per sample.

RNA-seq data analysis

Raw sequencing reads were first evaluated for quality using FastQC (v0.11.9). Low-quality reads and adapters were removed using Trimmomatic (v0.36). The clean reads were then aligned to the reference genome rat genome version Rnor_6.0 using STAR (v2.7.11b). Gene expression levels were

quantified as fragments per kilobase of transcript per million mapped reads (FPKM) using featureCounts (v2.03.).

Differential gene expression analysis between the stretched and unstretched groups was performed using DESeq2 (v3.2.0). Genes with an adjusted p-value < 0.05 and a fold change ≥ 2 were considered significantly differentially expressed. Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes pathway analysis were performed using the clusterProfiler package (v3.2.0) to identify biological processes and pathways associated with mechanical stretching in BMSCs.

Real-time PCR

RT-PCR analysis of Fos, Postn, and GAPDH expression was performed on three groups, with three replicates per group. PCR amplification was carried out in a 20 μ l reaction system consisting of 0.8 μ l of upstream and downstream primers, 2 μ l of cDNA, 10 μ l of Taq Pro Universal SYBR qPCR Master Mix (Vazyme, China), and 7.2 μ l of RNase-free ddH₂O. Quantitative analysis was performed using a Bio CFX real-time PCR instrument (Bio-Rad, USA). The primer sequences (5'-3') are listed in Table S1. The PCR protocol included an initial preincubation at 95°C for 3 minutes to activate the polymerase, followed by 40 cycles consisting of: denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds, and extension at 65°C for 10 seconds. Relative expression levels were calculated using the 2- $\Delta\Delta$ CT method (CT = fluorescence threshold; Δ CT = target CT—reference gene (GAPDH) CT; $\Delta\Delta$ CT = sample Δ CT—calibrator Δ CT).

Statistical analysis

Each experiment was repeated at least three times. The quantitative results were expressed as mean $\pm$ standard deviation. Statistical evaluation was performed using one-way analysis of variance, followed by Dunnett's-test for comparing the experimental and control groups and Bonferroni's test for comparing the experimental groups. The WB results were analyzed using independent sample t-tests. Significance of $P < 0.05$ was considered statistically significant.

Results

Mechanical stretching alters BMSC cytoskeletal morphology and distribution

Microscopic analysis revealed that BMSCs exhibited a monolayer morphology, characterized by spindle-shaped or multibranched cellular structures with strong adhesion to the substrate, reflecting their typical morphological traits. Flow cytometry confirmed that BMSCs expressed surface markers CD29, CD73, CD90, and CD105, while CD45 and CD34 were absent (Figure 1a, 1b).

To assess the effects of mechanical stimuli, BMSCs cultured on Flexcell six-well elastic plates were subjected to varying strain rates, stretching durations, and cycle counts. Immunofluorescence staining for F-actin was performed to evaluate cytoskeletal organization (Figure 1c). Compared to the non-stretched group, stretched BMSCs exhibited a significantly altered distribution of stress fiber bundles and an expanded cell-spreading area, indicating their mechanosensitivity to strain stimuli (Figure 1d, e, f).

As the strain increased from 5% to 10%, cytoskeletal fibers gradually thickened and elongated, demonstrating a

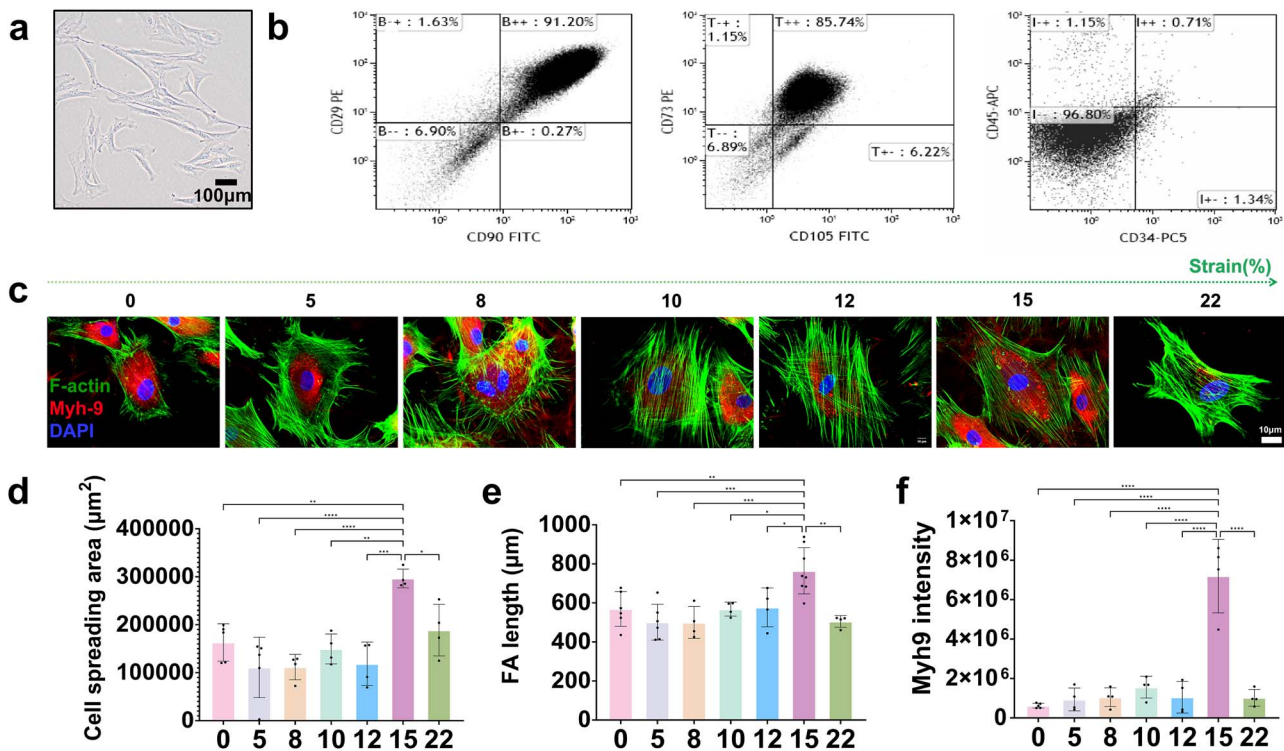


Figure 1. Cytoskeleton of BMSCs demonstrates significant responsiveness to mechanical stimuli. (a) Light microscope image of BMSCs at 20× magnification. Scale bar: 100 μm. (b) Flow cytometry data for BMSC surface markers: BMSCs express CD29, CD90, CD73 and CD105, but do not express CD45 and CD34. (c) the distribution of cytoskeletal proteins in BMSCs shows significant sensitivity in response to varying degrees of stretching stress, as evidenced by F-actin–Myh9–DAPI containing under different mechanical stretching conditions from 0% to 22% strain. Quantitative analysis of (d) cell spreading area, (e) F-actin length, and (f) relative intensity of Myh9 in each group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$

maturation trend. At 15% strain, mature microfilaments became intertwined, forming prominent stress fibers that facilitated pseudopodia formation and enhanced mechanical signal transduction capabilities. Additionally, Myh9 distribution and actin stress fiber organization were markedly different between the stretched and non-stretched groups, further highlighting the cytoskeletal adaptation to mechanical loading.

Optimal stretching conditions for increased paracrine effects and cellular activity of BMSCs

To investigate the regulatory effects of mechanical stretching on the paracrine secretion of ECM components and growth factors involved in wound healing, we selected COL-I, COL-III, VEGF, and TGF- β as key indicators. Experimental groups were established with different stretching parameters, including strain magnitudes of 5%, 8%, 10%, 12%, 15%, and 22%, stretching cycles of 720, 1440, and 2880, and strain durations of 5, 10, and 15 s.

BMSCs subjected to mechanical stretching exhibited significantly increased expression levels of COL-I, COL-III, VEGF, and TGF- β compared to the unstretched group. However, expression levels varied considerably across different stretching conditions (Figure 2). Based on these findings, the optimal mechanical stretching parameters were identified as 15% strain, 1440 cycles, and a 5-s strain duration.

To further assess cellular responses under optimal stretching conditions, we analyzed proliferation, stemness maintenance, and apoptosis at different time points (0, 12, 24, 48, and 72 h). Ki67 staining demonstrated a significantly higher proportion of proliferating cells in the stretched group

compared to the unstretched group ($P < 0.05$), suggesting that mechanical stretching enhances cell proliferation. Proliferation curves confirmed that stretched cells consistently displayed higher growth rates, peaking at 12 h post-stretching (Figure 3a).

CD105 immunofluorescence staining revealed stronger stemness maintenance in the stretched group, reaching its highest level at 12 h post-stretching (Figure 3b). Flow cytometry analysis further demonstrated a significant reduction in apoptosis rates in stretched BMSCs compared to the unstretched group (Figure 3c). These findings indicate that mechanical stimulation enhances BMSC proliferation and stemness while simultaneously reducing apoptosis.

Optimized stretching conditions enhances mechanical properties, expression of growth factors, and collagen deposition of BMSC cell sheet

To facilitate *in vivo* applications, we successfully prepared BMSC cell sheets using conditioned culture techniques and applied optimal mechanical stretching parameters. The thickness and mechanical strength of the BMSC cell sheets were significantly increased following mechanical stretching compared to the unstretched control group. Scanning electron microscopy revealed that stretched BMSC cell sheets exhibited denser and more structurally intact surface and cross-sectional microstructures compared to unstretched sheets. SWE assessment further confirmed a higher elastic modulus in the stretched group (Figure 4a).

Live/dead staining, F-actin–ZO-1–DAPI immunofluorescence staining, and Sirius red staining collectively demonstrated that optimally stretched BMSC cell sheets contained

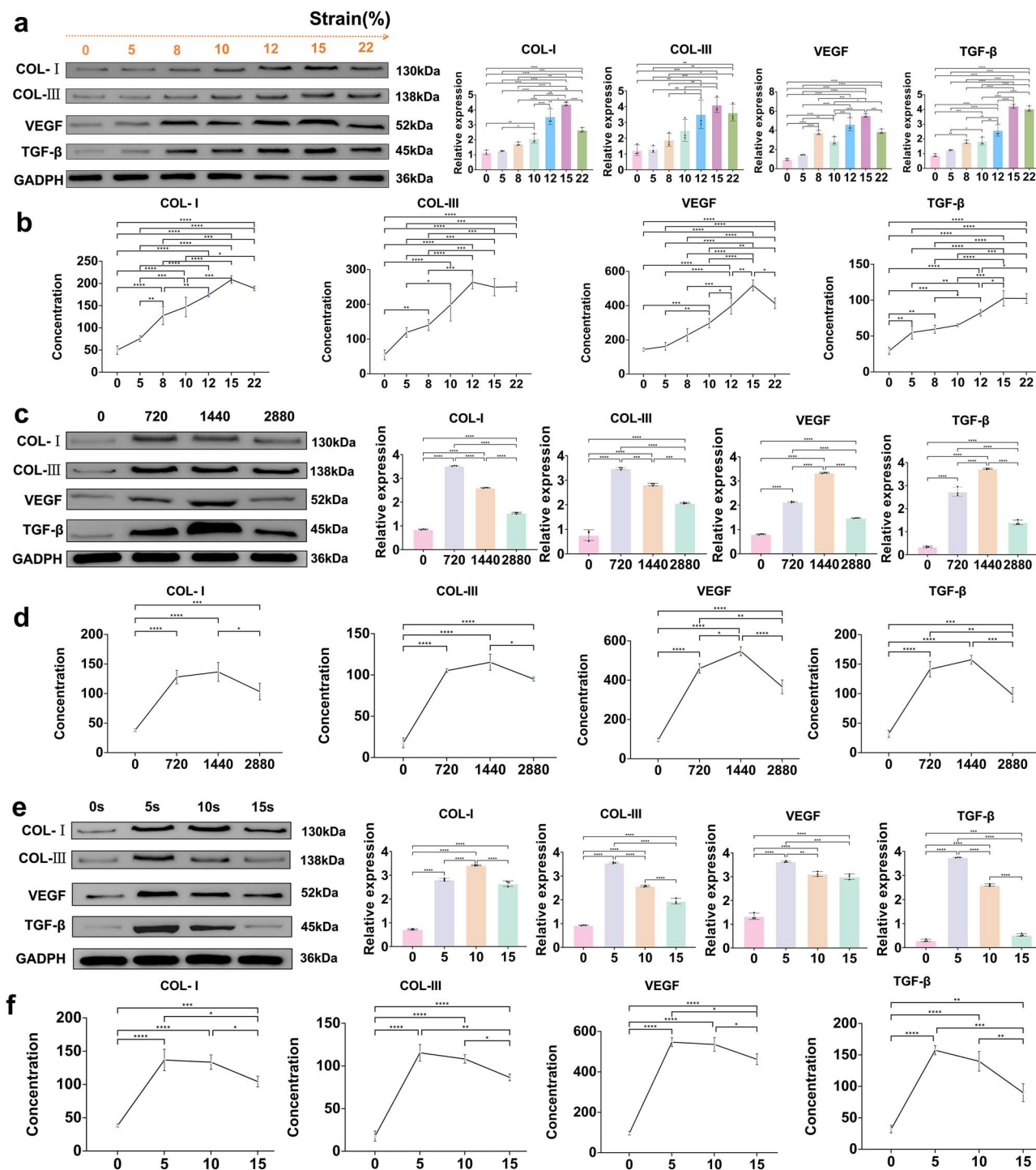


Figure 2. Mechanical stretching promotes the secretion of COL-I, COL-III, VEGF, and TGF- β in BMSCs. (a and b) quantitative analysis of relative expression levels of collagen and growth factor secretion by BMSCs under varying mechanical tensile deformation rates ranging from 0% to 22% based on (a) WB data and (b) ELISA analysis. (c) WB analysis of collagen and growth factor expression in BMSCs subjected to various mechanical stretching duration, including WB strips and gray analysis diagrams. (d) ELISA analysis of collagen and growth factor secretion in the BMSC culture medium under different mechanical stretching duration. (e) WB analysis of collagen and growth factor secretion by BMSCs subjected to varying single maximum deformation times, including WB strips and gray analysis diagrams. (f) ELISA analysis of collagen and growth factor secretion in the BMSC culture medium under different maximum deformation times of mechanical stretching. * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$

a greater proportion of viable cells and displayed well-organized cytoskeletal protein structures (Figure 4b). Moreover, these cell sheets exhibited a mature multilayered architecture with tight intercellular junctions and substantial

ECM deposition (Figure 4b). Notably, the expression levels of key structural proteins, including COL-I, COL-III, VEGF, and TGF- β , were significantly elevated in mechanically stretched BMSC cell sheets (Figure 4c, d).

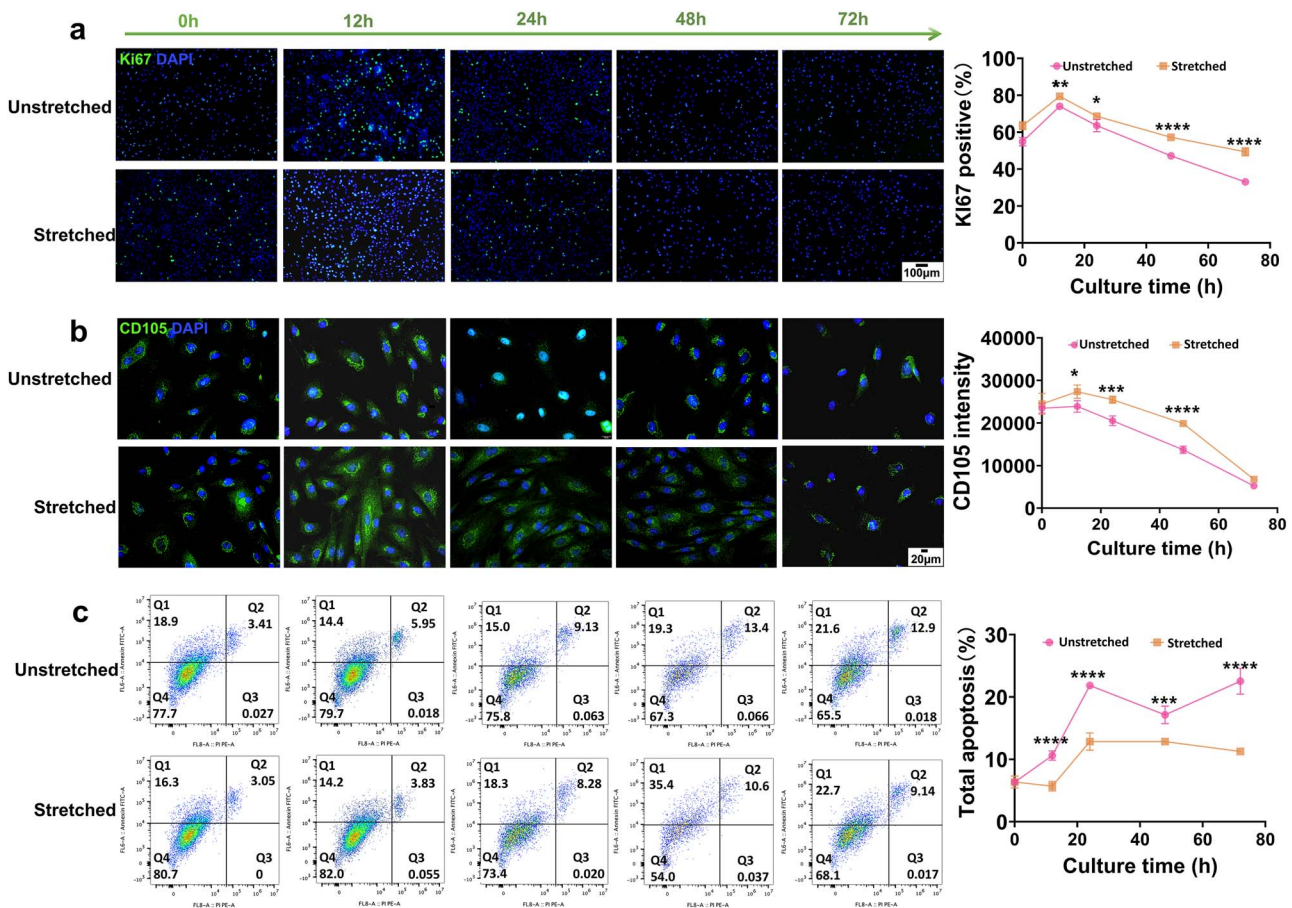


Figure 3. Effects of mechanical stretching on the cellular activity of BMSCs. **(a)** Ki67 immunofluorescence staining patterns and growth rate curves for both stretched and unstretched BMSC groups, monitored at 0, 12, 24, 48, and 72 h. Scale bar: 20 μ m. **(b)** CD105 immunofluorescence staining patterns and the corresponding growth curves for the same groups over the same time intervals. Scale bar: 20 μ m. **(c)** Apoptosis detection results and apoptosis rate curves for both groups, also measured at the aforementioned time points. Statistical significance is denoted as follows: * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$.

Mechanical stretching of BMSC cell sheets facilitates healing of diabetic skin defects in rats

The wound areas treated with mechanically stretched BMSC cell sheets were significantly smaller than those in the control groups from Day 9 to Day 21. By Day 21, wounds in the stretched BMSC cell sheet group were nearly completely healed, whereas incomplete healing was observed in the other groups (Figure 5a).

Masson's trichrome staining of wound samples collected on Day 21 demonstrated that mechanically stretched BMSC cell sheets promoted ECM formation and re-epithelialization (Figure 5b). Further histological analysis showed tight integration between the epidermal and dermal layers, a well-organized collagen fiber arrangement, and an intact basement membrane in the stretched group. Epidermal thickness measurements indicated that the newly formed epidermis in the stretched group closely resembled that of normal skin, whereas the control groups exhibited significantly thicker epidermis ($P < 0.01$), suggesting delayed remodeling (Figure 5b, 5c).

CD31-vWF-DAPI fluorescent co-localization staining revealed a higher density of blood vessels in wounds treated with stretched BMSC cell sheets, indicating enhanced angiogenesis (Figure 5b). Quantitative analysis confirmed a statistically significant increase in vascularization compared

to the control groups ($P < 0.01$; Figure 5d). Immunofluorescence staining for COL-I and COL-III further demonstrated enhanced collagen deposition in the stretched group (Figure 5b). The deposition of both COL-I and COL-III was significantly higher in the stretched group compared to the control and unstretched groups ($P < 0.01$; Figure 5e). Additionally, the COL-I to COL-III ratio in the stretched group was lower than that in the other groups ($P < 0.01$; Figure 5f), indicating that mechanically stretched BMSC cell sheets facilitated the remodeling of collagen in the wound.

Survival of BMSC cell sheets *in vivo*

To assess the *in vivo* survival of BMSC cell sheets, cells were labeled with PKH26 red fluorescent dye and transplanted into wound sites in diabetic rats. Cell survival and proliferation were monitored using BLI and PCNA immunofluorescence staining.

On Day 7 post-transplantation, BLI signals remained detectable in the wound areas of both groups, with a significantly larger live cell region in the stretched group compared to the unstretched group (Figure 6a). Confocal laser scanning microscopy of cross-sectional skin samples further confirmed the presence of BMSC cell sheets in the upper epidermis, as evidenced by red fluorescence. PCNA staining (green) indicated active cell proliferation, demonstrating that

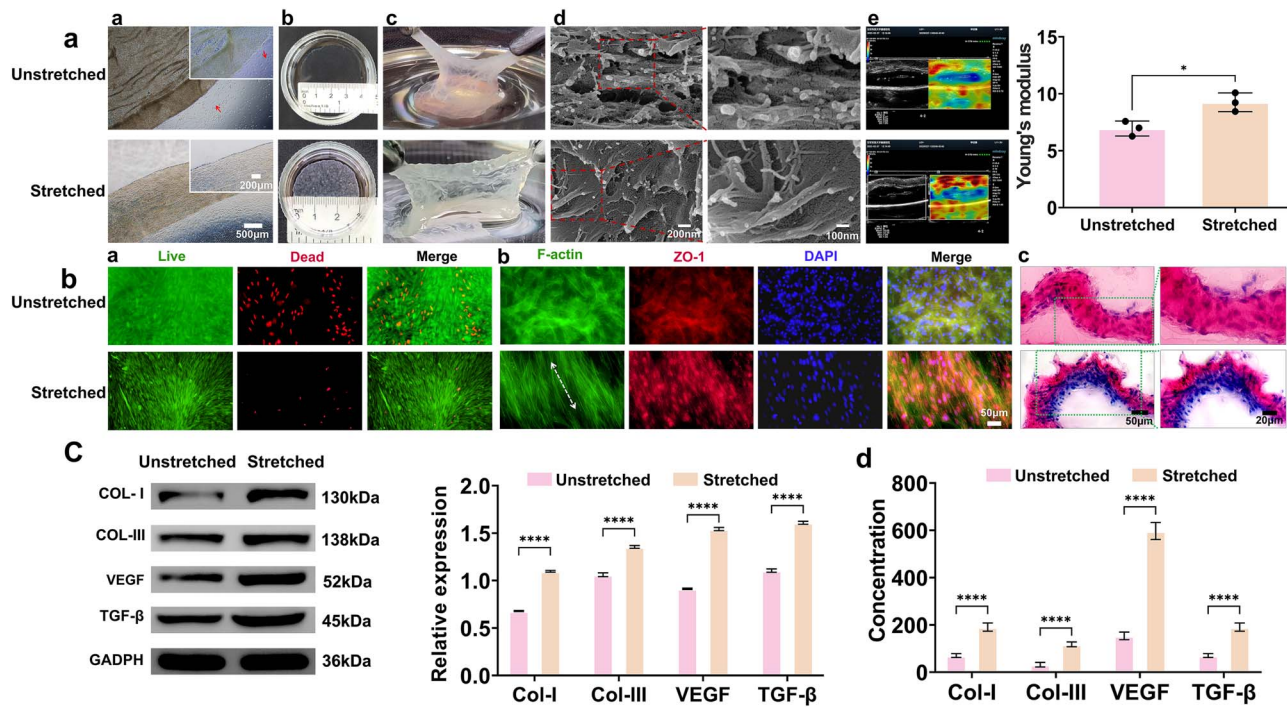


Figure 4. Effects of optimal mechanical stretching on the physical and biological properties of BMSC cell sheets. (a) Images depicting the process of handling BMSC cell sheets in unstretched group and stretched group. Scale bar: 100 μm , 200 μm , 500 μm . (b) Live/dead staining images, F-actin—ZO-1—DAPI colocalization fluorescence staining images, and Sirius red staining images of cross-sections of BMSC cell sheets for the unstretched group and the stretched group. Scale bar: 50 μm . (c) WB showing COL-I, COL-III, VEGF and TGF- β of the two groups. Scale bar: 20 μm , 50 μm ; (d) WB and (e) ELISA quantification of COL-I, COL-III, VEGF, and TGF- β in the supernatant of the two groups. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; and **** $P < 0.0001$.

the BMSC cell sheets retained biological activity throughout the implantation period (Figure 6b). The cell sheets in the stretched group were more continuous and exhibited tighter integration with the host tissue compared to the unstretched group.

Mechanosensitive receptors regulate cellular activity and paracrine effect of BMSCs

RNA sequencing analysis was conducted on unstretched and mechanically stretched BMSCs to explore the molecular mechanisms underlying the observed effects. Differential gene expression analysis revealed the upregulation of 107 genes and downregulation of 96 genes in stretched BMSCs compared to the unstretched group. Notably, mechanosensitive nuclear transcription factor Fos, which forms the AP-1 complex with Jun, was significantly upregulated in response to mechanical stretching (Figure 7a).

Further analysis indicated that mechanical stretching suppressed apoptosis-related genes while upregulating genes involved in cell proliferation, adhesion, and ECM expression, aligning with our cellular and *in vivo* findings (Figures 7b, 7c). Gene network analysis identified periostin (POSTN) as a key regulator linking mechanical stimulus responses, integrin binding, and ECM remodeling (Figure 7d).

Discussion

The ECM plays a crucial role in tissue repair and regeneration. Disruptions in its structure and function can impair key wound healing processes, including cell proliferation, migration, angiogenesis, and epithelial regeneration, all of

which are essential for the repair of chronic wounds [10, 11]. Recent research has focused extensively on therapeutic approaches aimed at enhancing ECM composition and function in wound healing, with MSCs attracting significant attention due to their multipotent differentiation ability and paracrine functions. However, optimizing the regenerative potential of BMSCs by modulating their cellular activity and paracrine effects remains a critical technical challenge.

Most existing research has focused on biochemical signaling within the cellular microenvironment to modulate stem cell behavior and paracrine effects [30]. For instance, growth factor expression in BMSCs has been enhanced by inducing a hypoxic microenvironment using copper chloride, which activates hypoxia-inducible factor-1 α (HIF-1 α). However, fewer studies have investigated how physical cues, particularly mechanical forces, influence BMSC biological activity [31].

As mechanosensitive cells, BMSCs respond dynamically to their mechanical microenvironment, which is crucial for regulating proliferation, differentiation, and ECM production [32, 33]. In this study, we applied controlled mechanical stretching to BMSCs to assess its impact on cellular function and paracrine signaling, identified optimal mechanical loading parameters, and leveraged these conditions to enhance BMSC-mediated wound healing.

Mechanotransduction, the process by which cells convert mechanical stimuli into biochemical signals, relies on cytoskeletal remodeling and nuclear deformation [34]. Mechanical stretching regulates cytoskeletal dynamics by modulating actin filament contraction, thereby influencing cell proliferation, migration, apoptosis, differentiation, and morphology [35]. Such stimuli promote interactions between

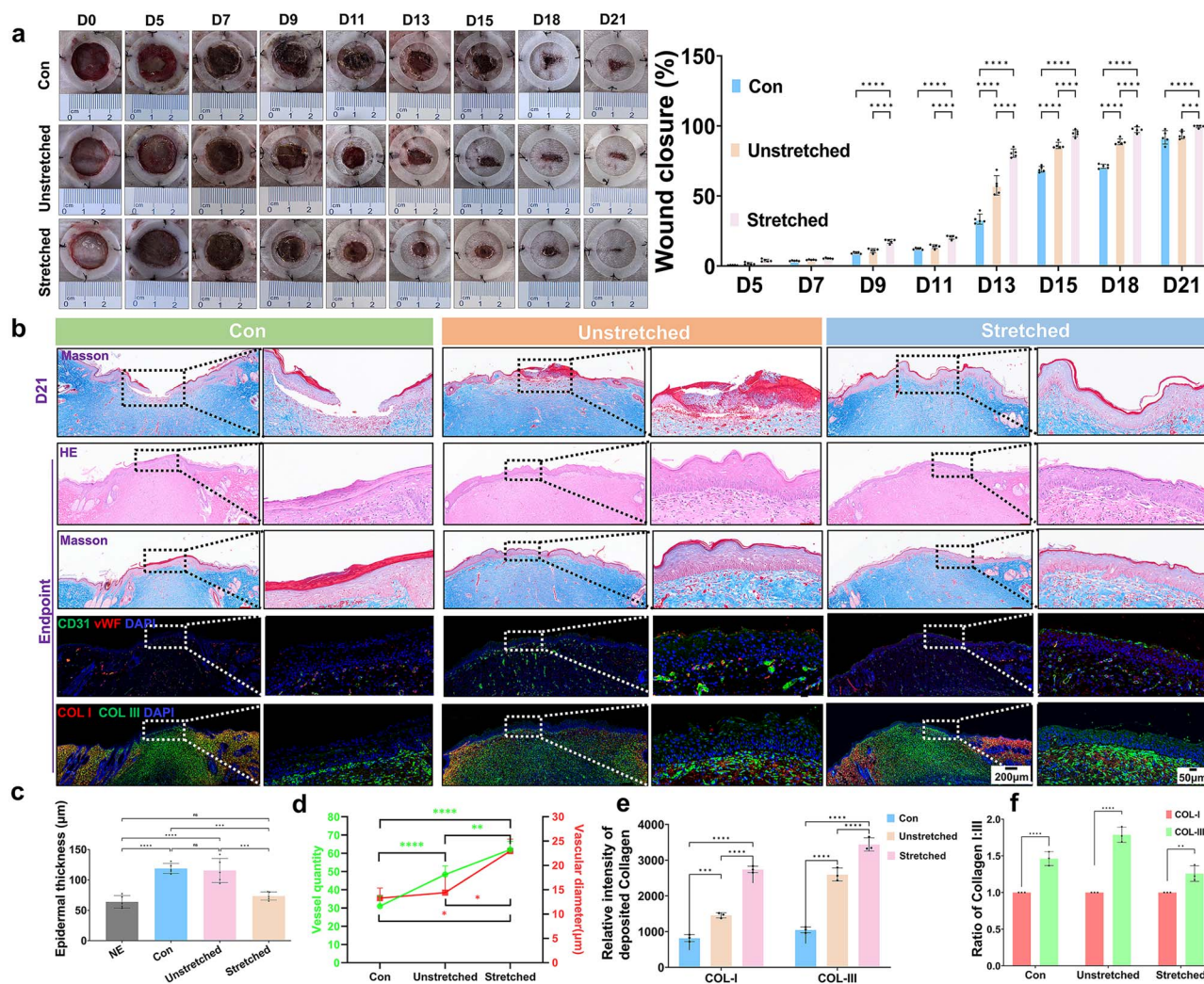


Figure 5. Mechanically stretched BMSC cell sheets promote wound healing in diabetic rats. **(a)** Macroscopic overview of wound healing across various treatment groups, and a quantitative analysis of healing rates. **(b)** Masson staining of skin wound tissues at 21 days, and hematoxylin–eosin (HE) as well as Masson staining of skin wound tissues at the healing endpoint for each group were performed. Immunofluorescent colocalization staining of CD31 (green)–vWF (red)–DAPI and collagen types I (red)–collagen types III (green)–DAPI on skin wound tissue at the endpoint of wound healing. Scale bar: 50 μ m, 200 μ m. **(c)** Quantitative analysis of epidermal thickness at the healing endpoint for the three groups and epidermis of the surrounding normal tissue. **(d)** Quantitative analysis of vascular counts and lumen diameters for the three groups. **(e)** Quantitative analysis of collagen volume fractions and **(f)** the ratio of COL-III to COL-I in the three groups. NE: Normal epidermis ** $P < 0.01$; *** $P < 0.001$; and **** $P < 0.0001$

myosin and F-actin, generating traction forces that alter cell morphology and promote spreading and pseudopodia extension [36]. Our study, using a spherical gradient cell-stretching device, demonstrated significant cytoskeletal adaptations in response to mechanical stimuli. Within a 5%–15% stretching strain, cytoskeletal fibers thickened, elongated, matured, and formed highly organized stress fibers aligned with the stretching direction, further promoting pseudopodia formation.

Stem cells possess multilineage differentiation capabilities, paracrine functions, and homing potential, enabling them to modulate tissue repair. Their paracrine effects facilitate the secretion of bioactive molecules, including growth factors, cytokines, and signaling peptides, all of which are essential for stem cell-based therapies [30].

Key growth factors, such as VEGF, fibroblast growth factor (FGF), and transforming growth factor-beta (TGF- β), are critical for endothelial cell migration, granulation tissue

formation, epithelialization, and tissue remodeling [37–39]. Additionally, collagen, the primary structural scaffold of the ECM, provides mechanical stability, adhesion sites, and a reservoir for growth factors, thereby playing a fundamental role in wound healing [40]. COL-I and COL-III, which are predominantly found in normal skin, are indispensable for the wound healing process, with COL-III dominating the early stages and gradually being replaced by COL-I as remodeling progresses [41].

Our findings indicate that mechanical stretching enhances BMSC-mediated ECM secretion, optimizing the production of VEGF, TGF- β , COL-I, and COL-III and providing an ideal set of mechanical parameters for stimulating pro-healing ECM remodeling. These findings not only provide evidence that the wound healing potential of BMSC cell sheets can be optimized but also set the foundation for further experimental studies.

Cell sheet technology, which involves harvesting confluent layers of adherent cells, preserves ECM integrity and

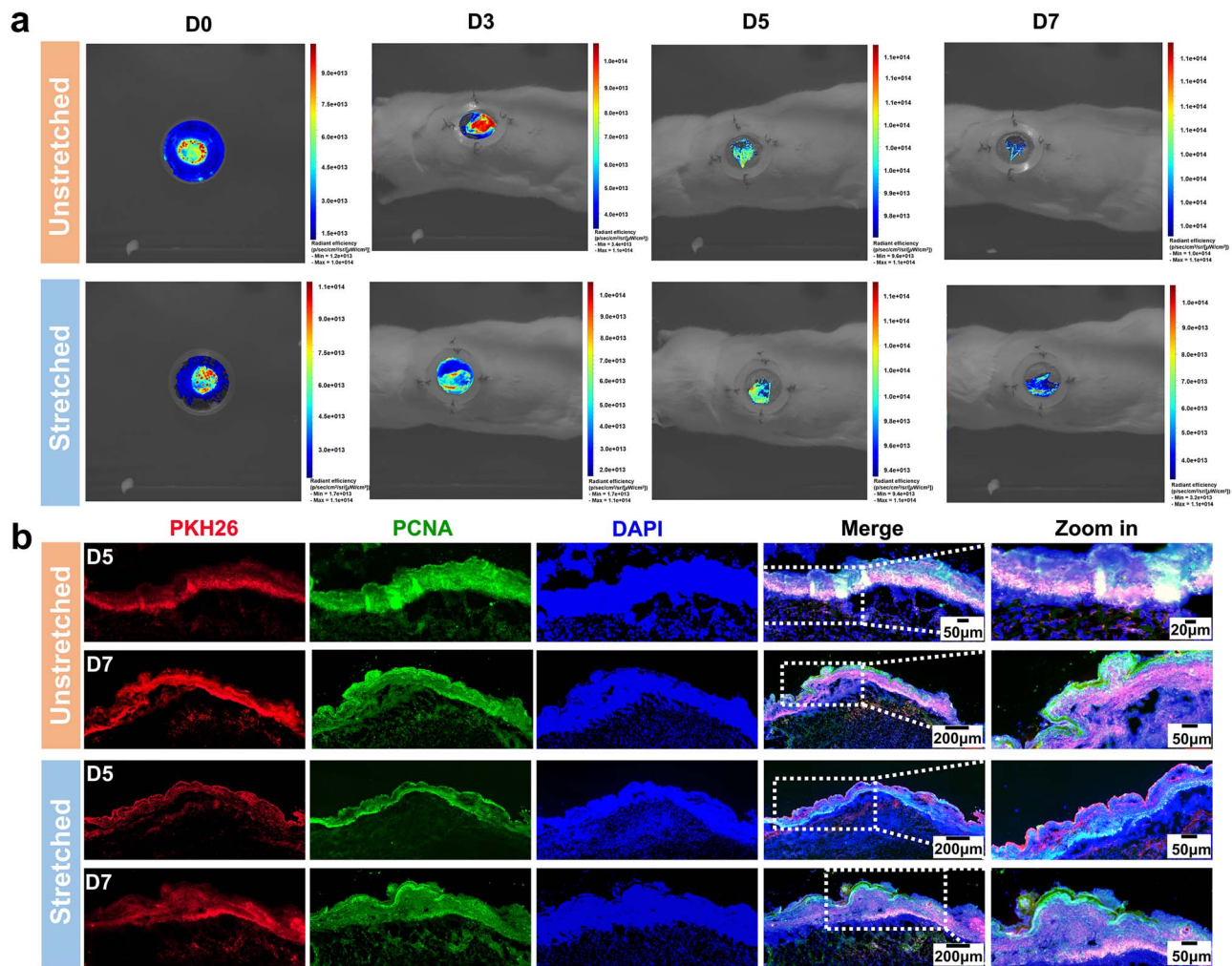


Figure 6. In vivo survival of BMSC cell sheets. (a) In vivo imaging of transplanted BMSC cell sheets labeled with PKH26 dye at D0, D3, D5, and D7 post-transplantation. The images show the survival and distribution of the cells at the wound site in both unstretched and stretched conditions. (b) Immunofluorescence staining at D5 and D7, showing PKH26 (red) for transplanted cells, PCNA (green) for proliferating cells, and DAPI (blue) for cell nuclei. Scale bar: 20 μm , 50 μm , 200 μm

intercellular connections, making it an effective strategy for tissue regeneration [42]. This technique ensures prolonged cell survival at the injury site without requiring exogenous scaffolds, while also sustaining cytokine secretion to support tissue repair [43].

Compared to conventional injection-based stem cell delivery, cell sheet technology enhances cell retention, viability, and therapeutic efficacy. Our study demonstrated that mechanical stretching enhances BMSC proliferation, stemness, and ECM secretion, thereby improving the mechanical and biological properties of BMSC cell sheets. In vivo experiments confirmed that mechanically optimized BMSC cell sheets significantly accelerated wound healing, primarily by enhancing epithelialization, neovascularization, and collagen remodeling at the injury site.

In animal experiments, we observed that collagen deposition (COL-I and COL-III) in the newly formed tissue of the stretched group was greater than that in both the control and unstretched groups (Figures 5b, e). However, the ratio of COL-I to COL-III in the stretched group was lower than in the other two groups (Figures 5b, f). Previous studies have shown that during wound healing, there is a transition from

a higher proportion of COL-III to COL-I, eventually leading to complete collagen remodeling [44]. We hypothesize that the increased collagen deposition in the newly formed tissue of the stretched group is due to a faster rate of collagen remodeling.

Moreover, *in vivo* survival experiments with BMSC cell sheets revealed detectable BLI signals in the wound areas of both groups on Day 7 post-transplantation, with BMSC cell sheets retaining a certain level of proliferative activity. These findings confirm that the observed wound healing effect is indeed mediated by the transplanted BMSCs, rather than being attributed to moisture retention alone.

Stem cell behavior, including proliferation, differentiation, and apoptosis, governs their functionality. Numerous studies have highlighted the role of mechanical forces and mechanotransduction in stem cell fate [45]. Our research showed that mechanical stretching stimulates BMSC proliferation, maintains stemness, and inhibits apoptosis. Transcriptomic analysis of mechanically loaded cells revealed upregulation of POSTN, Itga8, and the nuclear transcription factor Fos. Interaction analysis indicated that these components are closely associated with responses to mechanical stimuli, integrin binding, and ECM remodeling.

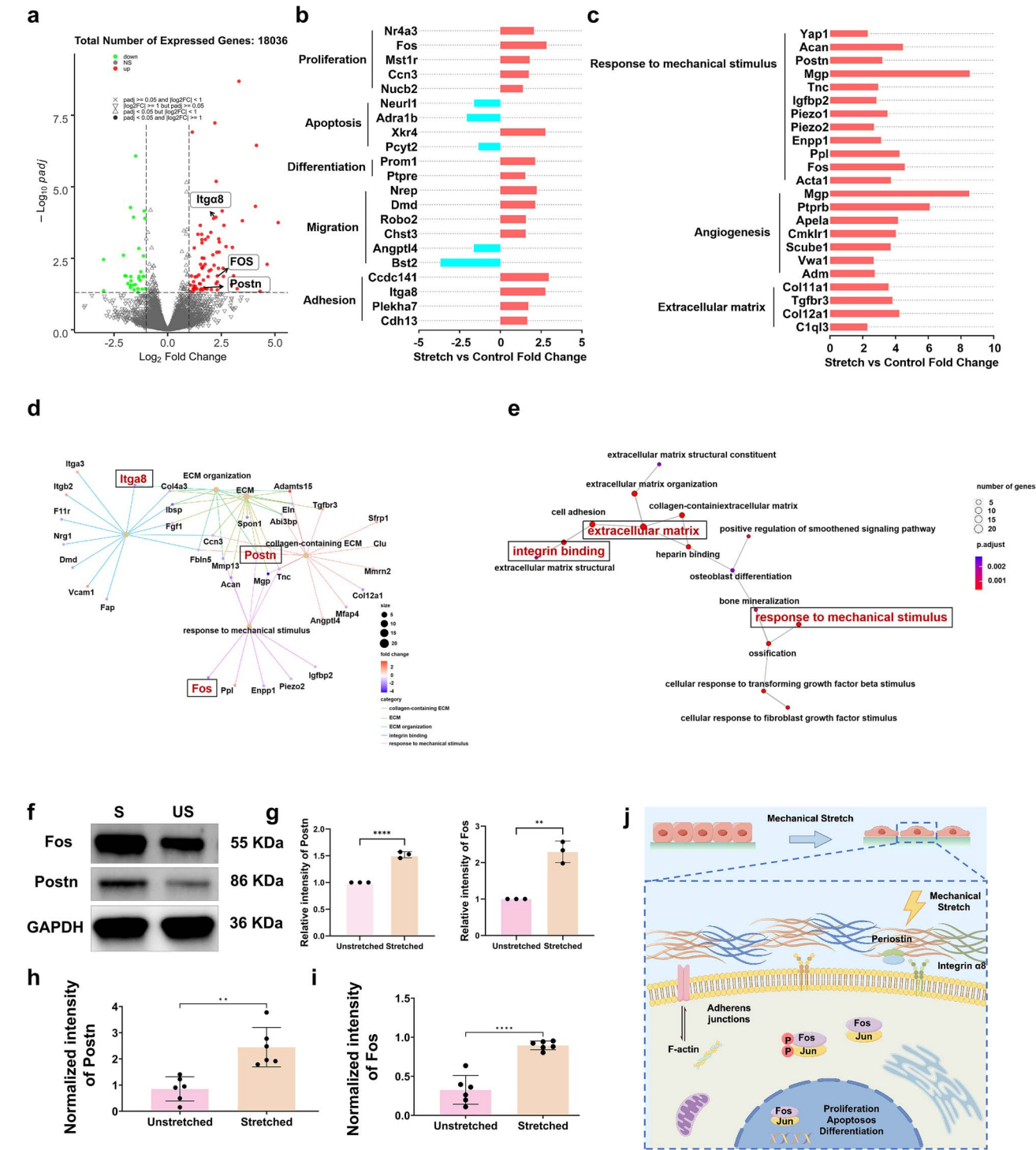


Figure 7. Transcriptomic analysis after mechanical stretching. (a) Volcano plot of differentially expressed genes. (b) Impact of stretching on BMSC proliferation, apoptosis, differentiation, adhesion, and migration functions. (c) Effects of stretching on BMSC responses to mechanical stimuli, angiogenesis promotion, and ECM secretion. (d) Network analysis diagram of differentially expressed genes. (e) Enrichment network diagram of differentially expressed genes between the two groups. (f-g) Western blot bands and grayscale analysis of Postn and Fos protein expression in BMSCs from the stretched group and the unstretched group. (h-i) Quantification of Postn and Fos mRNA expression levels in BMSCs from the stretched and unstretched groups was performed using RT-PCR. (j) Hypothetical diagram of the mechanisms by which mechanical stretching influences cellular activity and paracrine of BMSCs

POSTN, an ECM protein linked to mechanical stress, inflammation, and aging, interacts with integrins and plays significant roles in tissue development and regeneration, including wound healing and ventricular remodeling after

myocardial infarction [46, 47]. Integrins, dimeric transmembrane proteins composed of α and β subunits, act as mechanosensitive receptors on cell membranes, facilitating cell-ECM adhesion [48]. Fos, a nuclear transcription factor

and subunit of AP-1, functions downstream of the integrin signaling pathway and is crucial for signal transduction, cell proliferation, and differentiation [49]. Studies have shown that Fos is highly expressed during tendon healing promoted by mechanical stretching, possibly due to its roles in cell proliferation, differentiation, collagen production, and inflammation regulation [50]. Furthermore, it has been reported that the classical YAP/TAZ signaling pathway can bind to the Fos promoter region, stimulating its transcription, which in turn activates YAP/TAZ biological functions and enhances the expression of downstream target genes [51]. This suggests a positive feedback loop between Fos transcription and Hippo pathway activity, implying Fos' involvement in mechanotransduction. Combining the transcriptomic findings of this study with previous research reveals the relationships between POSTN, integrin, and Fos, highlighting their roles in mechanical stimulation, ECM remodeling, and tissue regeneration. In summary, we hypothesize that mechanical stretching may regulate BMSC activity and paracrine effects via the POSTN-mediated Fos pathway. While this hypothesis explains many experimental findings, further research is required for verification. Further investigation into the underlying mechanisms and specific signaling pathway targets is also needed.

Conclusions

This study investigated the effects of controlled mechanical stretching on BMSC adhesion, proliferation, and the expression of pro-healing components, including COL-I, COL-III, VEGF, and TGF- β , as well as its underlying regulatory mechanisms. We found that BMSC cell sheets subjected to optimal mechanical stretching exhibited more organized collagen deposition and enhanced mechanical strength. Furthermore, mechanically stretched BMSC cell sheets played a significant role in promoting re-epithelialization and neovascularization in diabetic chronic wounds.

These findings provide new insights into how mechanical stretching regulates stem cell behavior and paracrine functions. Additionally, they establish a theoretical foundation for the development of a novel BMSC-based bioactive wound dressing, offering a promising strategy for the clinical treatment of chronic wounds.

Abbreviations

AP-1: Activator protein 1; BCA: Bicinchoninic acid; BMSCs: Bone marrow mesenchymal stem cells; COL: Collagen; CRW: Chronic refractory wounds; DAPI: 4',6-diamidino-2-phenylindole; DNA: Deoxyribonucleic acid; DNase: Deoxyribonuclease; ELISA: Enzyme-linked Immunosorbent Assay; ECM: Extracellular matrix; EGF: Epidermal growth factor; FBS: Fetal bovine serum; GO: Gene ontology; H&E: Hematoxylin and Eosin; HIF-1: Hypoxia inducible factor-1 α ; Itg α 8: integrin α subunit 8; MEM: Minimum essential medium; MMP: Matrix metalloproteinase; PBS: Phosphate buffer saline; POSTN: Periostin; RNA: Ribonucleic acid; STZ: Streptozotocin; SWE: Shear wave elastography; TGF- β : Transforming growth factor- β ; TNF- α : Tumor necrosis factor- α ; VEGF: Vascular endothelial growth factor; VWF: Von Willebrand factor; WB: western blot; ZO-1: Zonula occludens-1.

Author contributions

Wei Dai: Project administration, writing—original draft (equal). Haowei Zhou: Conceptualization (lead), writing—review & editing (equal). Jincheng Du: Data curation (equal), validation (lead). Ruozu Xiao: Methodology (equal), data curation (equal). Junwei Su: Visualization (equal), supervision (equal). Zhe Liu: Supervision (equal), resources (equal). Rong Huang: Data curation (equal). Yuqian Li: Methodology (lead), formal analysis (lead). Jing Li: Project administration (supporting), Funding acquisition (supporting).

Supplementary data

Supplementary data are available at *Burns & Trauma Journal* online.

Ethics approval and consent to participate

All animal procedures were carried out in accordance with the principles of ARRIVE and approved by the Ethics Committee of Air Force Medical University.

Conflict of interest

The authors declare that there are no conflicts of interest. Graphical Abstract was created using Figdraw (www.figdraw.com).

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Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

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