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RESEARCH ARTICLE

Mg^{2+} containing composite scaffolds mediate macrophage polarization to enhance meniscus regeneration

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Abstract

Meniscal injury, a prevalent and challenging medical condition, is characterized by poor self-healing potential and a complex microenvironment. Tissue engineering scaffolds, particularly those made of silk fibroin (SF)/hyaluronic acid methacryloy (HAMA) and encapsulating Mg^{2+}, are promising options for meniscal repair. However, the inflammatory response following implantation is a significant concern. In this study, we prepared a composite SF/HAMA-Mg hydrogel scaffold, evaluated its physical and chemical properties, and detected its fibrochondrogenic differentiation effect *in vitro* and the healing effect in a rabbit meniscus defect model *in vivo*. Our results showed that the scaffold differentiates pro-inflammatory M1 macrophages into anti-inflammatory M2 macrophages after implantation, thereby reducing inflammation and facilitating the growth and repair of meniscus tissue. Further, the composite scaffold provided a conducive milieu for cell proliferation, anticipatory differentiation, and generation of extracellular matrix. In summary, composite SF/HAMA-Mg scaffolds display exceptional biocompatibility, anti-inflammatory properties, and superior potential for meniscus repair.

Keywords: meniscus repair, magnesium, macrophage polarization, multifunctional composite hydrogel

1. Introduction

The meniscus, an anisotropic tissue within the knee joint, significantly influences stress distribution, lubrication, and joint stability [1, 2]. A meniscus lesion compromises the positive impact of this tissue, leading to joint instability and subsequent cartilage degeneration, potentially culminating in osteoarthritis [3, 4]. In addition, the poor vascularity of the medial part of the meniscus often inhibits intrinsic recovery [5, 6]. Therefore, it is necessary to effectively reinstate the functionality of joints and hinder further degeneration by employing a tissue with the suitable structure, composition, and mechanical characteristics for rectifying impairments in the meniscus. Bioinspired gradient scaffolds for meniscus and osteochondral tissue engineering have been developed by simulating gradient characteristics, such as the pores, components, and target-inducing factors, in heterogeneous tissues, to recreate the anisotropic features of meniscus and osteochondral matrices [7-9]. However, despite numerous attempts to address this issue, current treatment options have been largely inadequate in regenerating a functional meniscus comparable to the native one [10-13].

Recently, natural hydrogels have emerged as promising candidates for treating meniscal injuries [14-17] largely due to their easy formulation, precision in targeted injection, as well as excellent biodegradability and biocompatibility. Despite their prevalence, natural hydrogels often exhibit insufficient mechanical properties and a propensity for rapid degradation within the body. To address these deficiencies, various cross-linking techniques, including physical and chemical methods, can be employed to fortify the polymer network and enhance the physical characteristics of hydrogels [18-20]. Photopolymerization has emerged as a significant area of research in the synthesis of hydrogels, primarily owing to merits [21-24], including the ability to operate at low reaction temperatures, rapid solidification, a straightforward and gentle preparation process, cost-effectiveness, and the elimination of the need for organic solvents [25, 26].

Hyaluronic acid is the fundamental constituent of the extracellular matrix within joint fluids, boasting excellent biocompatibility and biodegradability, thereby making it highly advantageous for medical applications [27-30]. Notably, an hyaluronic acid methacryloyl (HAMA) hydrogel has garnered significant interest due to its minimal immunogenicity and biological activity [31-33], and has potential applicability to the treatment of meniscal injuries. However, photocured HAMA gels typically exhibit poor mechanical properties and rapid degradation, which compromises their capacity to withstand external forces, thereby increasing the risk of rupture. Thus, there is an urgent need to develop HAMA-based composite hydrogels that improve on this factor.

Silk fibroin (SF), a naturally occurring fibrous protein derived from the cocoons of domestic silkworms, has been extensive used in the development of hydrogels due to its distinctive mechanical properties, high biocompatibility, and controlled biodegradation rate [34-37]. The incorporation of SF into HAMA hydrogels enables the replication of extracellular matrix (ECM) composition and structure, resulting in enhanced strength and regulated hydrogel degradation.

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For developing medical implants, it is critical to regulate the immune response to biomaterials implanted *in vivo*. Engineered biomaterials that stimulate macrophage behavior are essential for promoting tissue regeneration, as an increase in M2-like macrophages can be beneficial for enhancing tissue repair and healing. Magnesium, is a vital component in the human body, being involved in multiple physiological processes, including immunomodulation [38-40]. In tissue regeneration research, hydrogels incorporating Mg^{2+} recently garnered considerable interest owing to their remarkable biocompatibility and modifiability. Further, hydrogels supplemented with Mg^{2+} can enhance cell adhesion, proliferation, differentiation, ECM secretion, and immune response modulation [41]. In this study, Mg^{2+} was incorporated into SF/HAMA hydrogels to facilitate tissue regeneration by releasing Mg^{2+} gradually during scaffold degradation and investigate the regulatory impact of Mg^{2+} on mesenchymal stem cells (SMSCs) and macrophages. Furthermore, the composite SF/HAMA-Mg hydrogel was surgically implanted into a medial meniscus defect in a rabbit model to evaluate its functional effects. The underlying hypothesis is that this cell-free SF/HAMA-Mg hydrogel could enhance meniscus regeneration by promoting a macrophage shift from M1 to M2.

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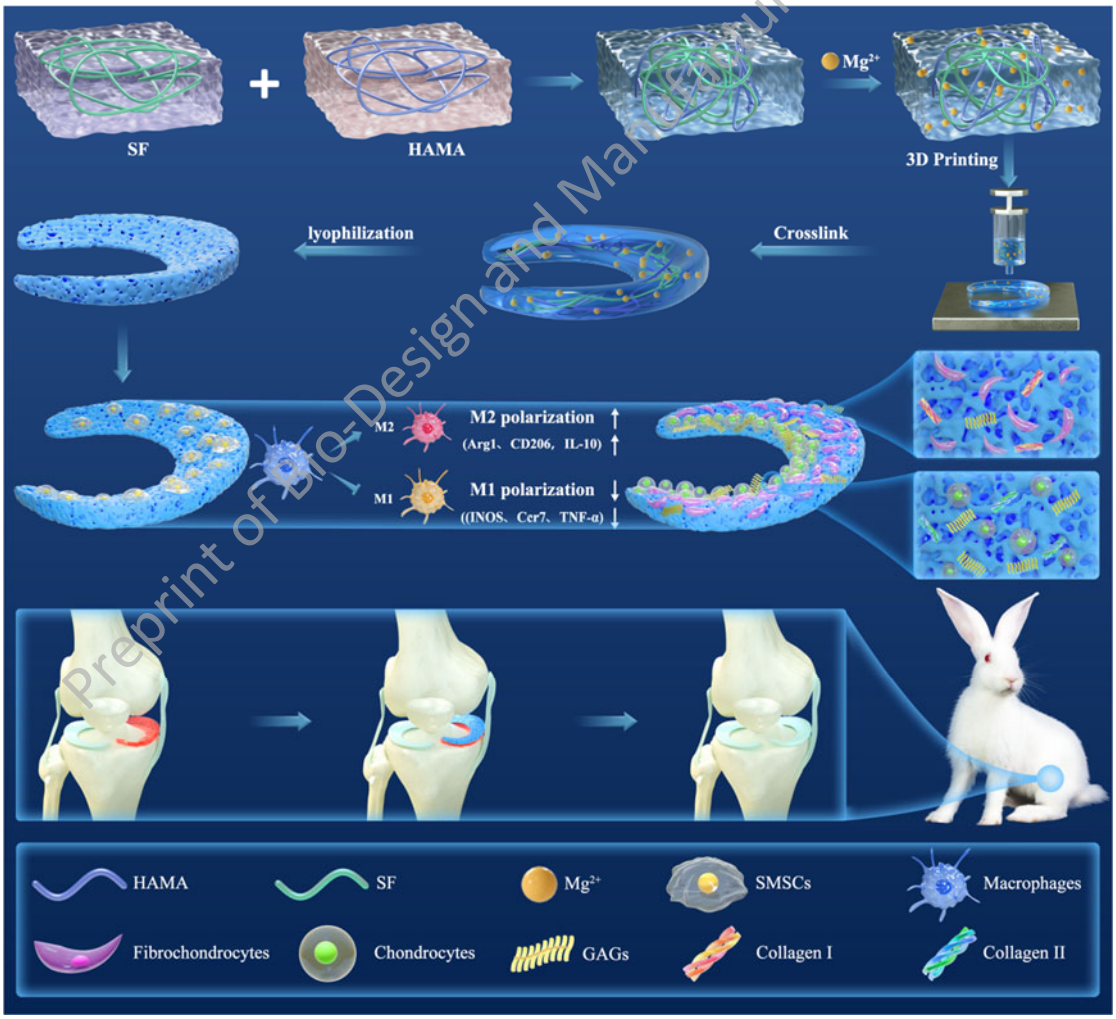

Figure 1. Schematic illustration of the use of a SF/HAMA-Mg hydrogel in meniscus regeneration

2. Materials and methods

2.1 Scaffold fabrication

Hyaluronic acid, trypsin, pepsin, Trixon X-100, methacrylic anhydride, and lithium phenyl-2,4,6-trimethylbenzoylphosphinate were purchased from Sigma-Aldrich. Cocoons were purchased from a cocoon base (Guangdong cocoon base, China). Magnesium chloride was purchased from Solarbio.

Preparation of the SF/HAMA-Mg hydrogel: SF was prepared as previously described [42]. SF purification involved the removal of sericin, a protein with adhesive properties, from the cocoons by immersing them in a boiling 0.02 M sodium carbonate solution for 1 h. Next, the cocoons were rinsed with distilled water to completely eliminate the degumming solution. The purified SF was then dissolved in a solution of lithium bromide (9.3 M), for 1 h at 70°C. The resulting solution was subjected to dialysis in distilled water for 48 h, using benzoylated dialysis tubing with a molecular weight cut-off (MWCO) of 2 kDa. To concentrate the SF, it was exposed to a 20 wt% poly (ethylene glycol) solution for ≥6 h. The SF concentration was determined by weighing the dry weight of the SF solution after placing in an oven at 70°C overnight. Additionally, the prepared SF solution was stored at 4°C until ready for use. In this study, a total volume of 500 mL of deionized water was utilized to dissolve 5 g of hyaluronic acid with a molecular weight of 1400 kDa. Dissolution was achieved through vigorous stirring. Subsequently, a dropwise addition of 20 mL of methacrylic anhydride was performed in an ice bath. The pH of the solution was maintained between 8 and 11, and adjustments were made using a 5 M NaOH solution. The reaction was carried out overnight in darkness at 0 to 4°C. Once the reaction was complete, the solution underwent centrifugation at 5000 rpm to eliminate any insoluble components. The pH of the solution was then adjusted to 7.4 by adding a 1 M HCl solution. The resulting compound was subsequently frozen and subjected to lyophilization after three days of dialysis against deionized water. In our study, the hydrogel was formulated using a combination of 10% SF, 2% HAMA, 0.12% magnesium chloride ($MgCl_2$), and 0.1% lysine amino peptidase (w/v).

To replicate the natural structure of the medial meniscus, a 3D computer-aided design model was developed. This model integrated collagen fibers aligned in both circumferential and radial orientations, resembling the organization in the native meniscus. To this end, a wedge-shaped arc disk was designed to imitate the meniscus. Hydrogel application was meticulously performed layer-by-layer, following a predetermined plan, using a 3D-Bioplotter (Envision TEC 4th generation). During printing, blue light (405 nm) was employed on the receiving platform to photocrosslink the printed hydrogel. Subsequently, the scaffolds were further crosslinked in ethanol and then lyophilized until further use.

2.2 Rheological measurement

The hydrogel's rheological characteristics were analyzed using a rheometer (ARES-G2, TA Instruments, America). The viscoelastic behavior was assessed across the angular frequency spectrum of 10^{-1}–10^{2} Hz. The shear viscosity was measured across from 10^{-2}–10^{2} shear rates.

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2.3 Fourier transform infrared spectroscopy

An Affinity-1S Fourier Transform Infrared (FTIR) spectrometer was utilized to conduct attenuated total reflection FTIR spectroscopy on hydrogels comprising SF, SF/HAMA, and SF/HAMA/Mg^{2+}. The spectral analysis was performed within the scanning range of 400 to 4000 cm^{-1}, at a resolution of 4 cm^{-1}.

2.4 Scanning electron microscopy

The scaffolds were immobilized with a 2.5% (v/v) solution of glutaraldehyde, subjected to dehydration using a graded ethanol series and dried utilizing CO_2 (EM CPD300; Leica, Wentzler, Germany), followed by gold sputtering. The scaffold microstructure was examined using a scanning electron microscope (SEM) model S-4800 with field emission technology from Hitachi, Tokyo, Japan.

2.5 Biomechanical testing

Different-sized scaffold samples were prepared to conduct biomechanical testing using an AGS-X Precision Universal Tester (SHIMADZU, Japan). Samples were kept moist with phosphate-buffered saline (PBS) during the entire testing process. The unconfined compressive strength was assessed using 15 × 15 × 15 mm^3 cubes, while the tensile strength was evaluated using 5 × 5 × 15 mm^3 specimens.

2.6 Degradation rate

The scaffolds were submerged in PBS (pH = 7.4) and a XIV protease solution at 0.5 U/mL and 37°C. At the corresponding timepoints, the scaffolds were accurately weighed (Wt). The initial scaffold weight was W0.

Degradation ratio = (W0 – Wt)/W0 × 100%

2.7 Swelling test

Swelling tests were conducted to determine the swelling properties of the samples. Freeze-dried samples were submerged in PBS (pH = 7.4) at 37°C for 24 h. Next, any excess water was eliminated, and the samples weighted. The equilibrated swelling ratios (ESR) was calculated as follows:

ESR = (Ws - Wd)/Wd × 100%

where Ws and Wd represent the hydrogel weight in the swollen and dry state, respectively.

2.8 Mg^{2+} release

To measure the concentration of released Mg^{2+}, the scaffolds were submerged in PBS for 20 days. Ion concentration measurements were carried out using ICP-OES (Optima 7000 DV, PerkinElmer, USA) and ICP-MS (ICP Q, Thermo Scientific, USA).

2.9 SMSC culture and identification

The methods for isolating and culturing SMSCs were as previously described [43]. Sprague–Dawley rats weighing 80 g were selected and synovial tissue collected from the suprapatellar bursa. The synovium was then digested with 0.2% type I collagenase (Invitrogen, Carlsbad, CA, USA) in α-MEM for 30 min at 37°C. Then, SMSCs collected by centrifugation were incubated in complete medium, which was changed every 3 days. SMSCs were evaluated for specific cell surface antigen labeling by flow cytometry. Positive markers included CD44 (ab112179) and CD90 (ab225), and negative markers included CD34 (ab187284) and CD45 (ab10558; Abcam Inc.)

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SMSCs were induced to undergo adipogenic, chondrogenic, and osteogenic differentiation according to manufacturer's instructions to characterize their pluripotency.

2.10 Biocompatibility

After 3 days of culture, the viability of SMSCs on the scaffolds was detected by LIVE/DEAD staining. In detail, scaffolds were immersed in 1 mL of working solution containing 2 mM calcein AM and 4 mM ethidium homodimer-1 reagent, incubated for 1 h at room temperature, and the fluorescence intensity of calcein-AM or ethidium homodimer-1 was detected under a confocal microscope. SMSC survival on different scaffolds was detected by the AlamarBlue method (Thermo Scientific, USA) (n = 5). OD values were then measured at 570 nm using a Varioskan Flash reader [42]. To observe the morphology of SMSCs on the scaffolds, the scaffolds were fixed with 4% paraformaldehyde for 30 min at room temperature, stained with rhodamine-phosphatidylinositol-like protein (Cytoskeleton Inc., USA) for 1 h at 37°C, and counterstained with Hoechst33258 for 10 min.

2.11 Biochemical assays

To assess the fibrochondrogenic differentiation capacity of cells on the scaffolds, we incubated them in fibrochondrogenic medium containing α-MEM, CTGF (100 ng/mlL and TGF-β3 (10 ng/mL). In detail [44], DNA, GAG and collagen secretion were detected on days 3 and 7. Then, scaffolds were immersed in papain solution (125 mg/mL papain, 5 mM EDTA, 100 mM Na2HPO4, 5 mM L-cysteine) for 16 h. DNA content was assessed by reacting 20 μL of treated samples with 200 μL of Hoechst 33258 working solution (2 μg/mL) for 1 h at 37°C shielded from light. Then, 20 μL of the above treated samples were reacted with 200 μL of DMMB reagent for 30 min at room temperature and the absorbance was measured at 525 nm. An enzyme-linked immunosorbent assay (ELISA) kit was used to determine the collagen content.

2.12 Gene expression analysis *in vitro*

Quantitative RT-PCR was performed to detect the gene expression of collagen I, collagen II, sox 9 and aggrecan on days 3 and 7 **(Table S1)**. Briefly, RNA was extracted and reverse transcribed to cDNA using the TRIzol kit and cDNA reverse transcription kit (Thermo Fisher Scientific, USA). RT-PCR was performed as previously reported [45]. Target genes were quantified using the ΔΔCt method.

2.13 Macrophage polarization on the scaffold

To assess the polarization response of macrophages on the scaffolds, RAW264.7 cells were incubated on SH and SH-Mg scaffolds for three days. Then, the gene expression of M2 markers (Arg1, CD206, and IL-10) and M1 markers (Inos, Ccr7, and Tnf-α) **(Table S2)** was assessed using qPCR after 8 h of incubation with 100 ng/mL of lipopolysaccharide (LPS) in the culture medium [46]. In addition, Arg1 and iNOS protein content was analyzed by immunofluorescence in SH group and SH-Mg group. Rabbit anti-hepatic arginase polyclonal antibody (ab96183) was used to detect Arg1, and rabbit anti-iNOS monoclonal antibody (ab209027) to detect iNOS. iNOS was then reacted with fluorophore-conjugated mouse IgG goat polyclonal secondary antibody (ab150113) against the primary antibody. Cell nuclei were co-stained using Hoechst33258.

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2.14 *In vivo* scaffold implantation

The surgery was performed according to our previous protocol [42]. All animals were 12-weeks-old New Zealand white rabbits with a body weight of 2.5 kg and randomized into four groups: a Blank group underwent meniscectomy only, a SH group underwent meniscectomy and SH scaffold implantation, a SH-Mg group underwent meniscectomy and SH-Mg scaffold implantation, and the native group which did not underwent meniscectomy. After animals were anesthetized and disinfected, the anterior medial parapatellar incision was selected to expose the medial meniscus. A medial meniscectomy was then performed, and a scaffold implanted at the location of the meniscal defect. Then, the stent was sutured and fixed to the joint capsule at the edge of the meniscus and the incision closed layer by layer. To prevent infection, penicillin was used for 3 days postoperatively. At the corresponding time points, the animals were euthanized for retrieving samples.

2.15 Evaluation of regenerated meniscus

At the corresponding time points, grafts were sampled and photographed to investigate meniscus regeneration. The implant was fixed in paraformaldehyde, dehydrated, embedded in paraffin, and sectioned for histological staining including hematoxylin and eosin (HE), Safranin O-Fast Green (SO), toluidine blue (TB), immunohistochemistry for collagen I and II, and picrosirius red (PSR).

The biomechanical characteristics of regenerated meniscus were assessed at 12- and 24-weeks post-implantation. The compressive modulus of the SH group and SH-Mg group was established utilizing a Tri-boIndenter (Hysitron Corporation, USA), as previously described [47]. Tensile tests were conducted using a mechanical measuring instrument (AG-IS; Shimadzu), following established protocols [42].

2.16 Evaluation of cartilage protection

The International Cartilage Repair Society (ICRS) classification system was introduced to assess articular cartilage condition [48]. Cartilage samples were fixed, decalcified, embedded, and cut into paraffin sections. Finally, they were stained with HE, TB and SO to grade using the Mankins score [49, 50].

2.17 Statistical analysis

All data are expressed as the mean and standard deviation. Significance was determined by one-way ANOVA analysis or Student's t-test. Statistical significance was determined as $p < 0.05$.

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3. Results and discussion

3.1 Rheological properties of hydrogels

The elastic and viscous modulus of the hydrogel flattened gradually with increasing angular frequency (**Figure 2A–B**). In addition, the hydrogels exhibited significant shear-thinning characteristics, as evidenced by the clear reduction in viscosity with increasing shear rate. These findings suggested that hydrogels possess favorable printability.

3.2 Scaffold crosslinking

In this study, FTIR spectrometry was employed to assess potential crosslinks within the composite hydrogels. As illustrated in **Figure 2C**, the peak at 1258 cm^{-1} in the

SF/HAMA and SF/HAMA-Mg group was more pronounced than in the SF group. This peak corresponds to the C-N stretching vibration. Notably, characteristic peaks approximately 1655 cm^{-1} were attributed to the α-form 3of SF. Furthermore, characteristic peaks between 1620–1635 cm^{-1} and 1230–1235 cm^{-1} indicated the presence of a β-sheet structure in SF. Further, the presence of distinct peaks at 1620 and 1517 cm^{-1} signifies the tensile vibration of the carbonyl group (C=O) in the Amide I region and the deformation vibration of the nitrogen-hydrogen bond (N-H) in the Amide II region, respectively. These observations imply a change in the structure of SF from disordered coils to a more stable β-fold conformation. Thus, these findings evidence an interaction between SF and HAMA.

3.3 Scaffold microstructure

SEM images indicated that the SF scaffold had smaller pores than SF/HAMA and SF/HAMA-Mg (**Figure 2D**). The pore size distribution analysis showed average diameters of 5.04, 8.58, and 13.47 μm for SF, SF/HAMA, and SF/HAMA-Mg hydrogels, respectively (**Figure 2E**). The incorporation of HAMA and Mg^{2+} resulted in increased pore size. However, hydrogels containing HAMA exhibited smaller porosity compared to the SF hydrogel, with the SF/HAMA-Mg hydrogel having the lowest porosity (**Figure 2F**).

3.4 Biomechanical tests *in vitro*

To evaluate their biomechanical properties, we assessed the compressive and tensile strengths of the three scaffolds. The tensile modulus for the SF, SH, and SH-Mg scaffolds was 0.369 ± 0.014, 0.376 ± 0.011, and 0.408 ± 0.024 MPa, respectively (**Figure 2G**); and the compressive modulus was 0.091 ± 0.009, 0.091 ± 0.019, and 0.118 ± 0.016 MPa, respectively (**Figure 2H**). These findings suggest that the SH-Mg scaffold displayed better biomechanical characteristics than the other scaffolds, demonstrating superior performance in both compressive and tensile strength. This may be due to the addition of metal ions. The improved mechanical properties are crucial for loading stress *in vivo* of the scaffolds.

3.5 Degradation *in vitro*

The degradation rate of hydrogels was assessed in PBS and XIV solutions. **Figure 2I** shows that the SF group exhibited a comparatively slower degradation rate compared to the other two groups. This suggests that the incorporation of SF in the composite hydrogel had a mild decelerating effect on degradation. Thus, the SF group is more appropriate for achieving a harmonious equilibrium between scaffold degradation and formation of neoplastic tissue.

3.6 Scaffold ESR

The composite hydrogel exhibited a lower ESR compared to the SF hydrogel (**Figure 2J**). This decline in ESR was attributed to increased crosslinking density from the incorporation of HAMA and Mg^{2+} into the hydrogel matrix. Despite an ESR of 101.77%, the SH/HAMA-Mg hydrogel demonstrated a remarkable ability to swiftly expand and absorb joint fluid, which was beneficial for buffering knee joint stress. Consequently, these hydrogels held promise for application in the repair of meniscus tissue.

3.7 Mg^{2+} release rate

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According to **Figure 2K**, an initial surge of Mg^{2+} ions was observed within the first week, followed by gradual deceleration over subsequent weeks. The rapid release in the early stage after implantation could effectively mitigate the local inflammatory response, while the later slow release could continue to play a role in a lower concentration for a longer period.

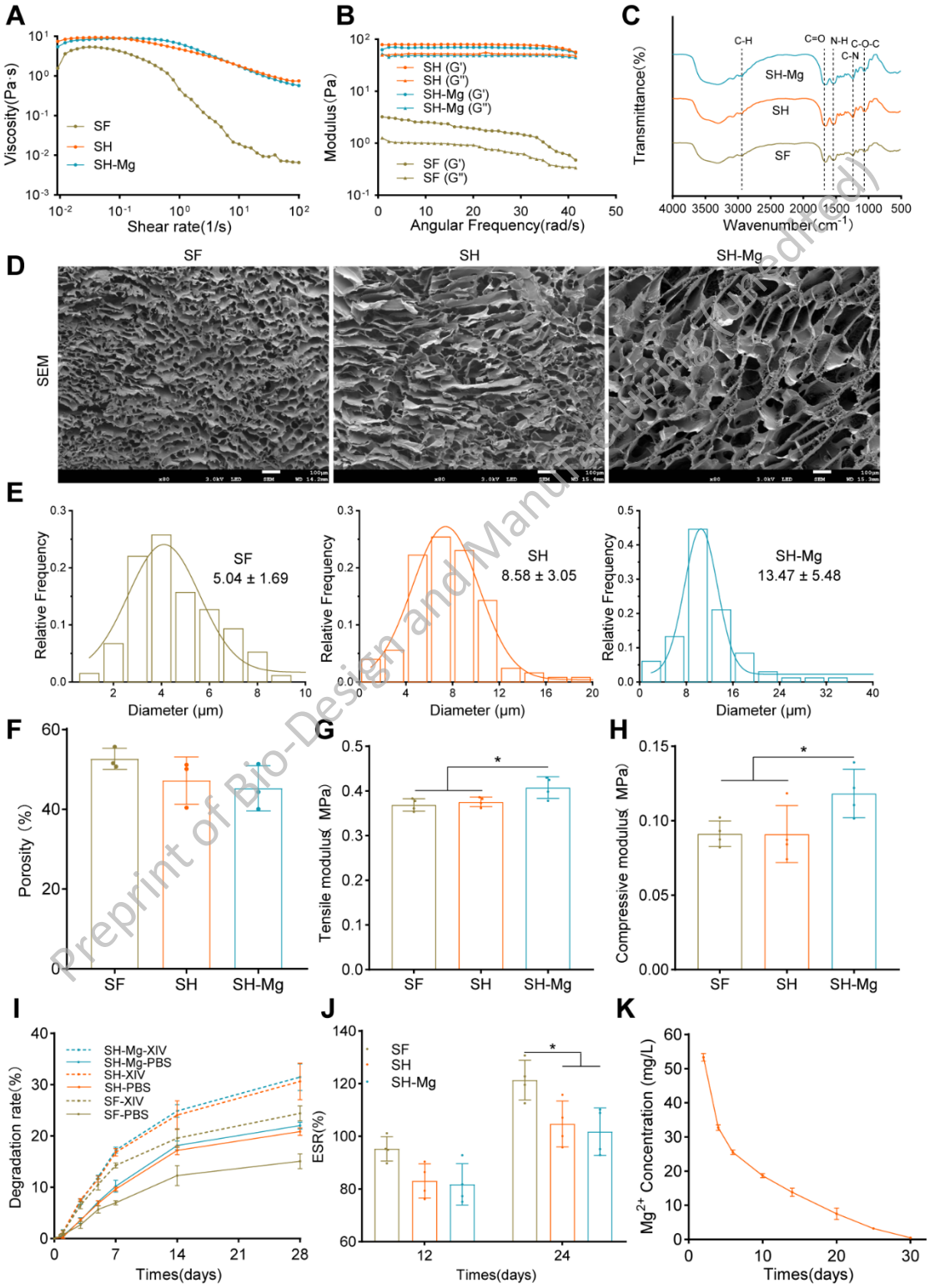

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Figure 2. Scaffold characterization. (A) viscosity and (B) modulus of SF, SH, and SH-Mg hydrogels. (C) FTIR spectra of SF, SH, and SH-Mg hydrogels. (D) Microstructure of SF, SH, and SH-Mg scaffolds after lyophilization. (scale bar = 100 μm) (E) Pore size distribution of SF, SH, and SH-Mg scaffolds. (F) Porosity, (G) tensile modulus, (H) compressive modulus, (I) degradation rate, (J) ESR, and (K) Mg^{2+} release rate of SF, SH, and SH-Mg scaffolds. ($n = 4$, $*p < 0.05$)

3.8 SMSC characterization

After the third passage, SMSCs displayed a fusiform shape and were evenly distributed following their expansion. To identify the multiple lineage differentiation capacities of SMSCs, a tri-lineage differentiation experiment was conducted. Mesenchymal stem cells (MSCs) could differentiate into three distinct cell lineages, namely adipocytes, osteocytes, and chondrocytes (**Figure 3A**). Flow cytometry analysis revealed positive overexpression of the phenotypic markers CD44 and CD90, and negative expression of lipopolysaccharide receptor CD34 and leukocyte common antigen CD45 in SMSCs (**Figure 3B**). After scaffold implantation into the site of meniscal injury, SMSCs in the knee joint migrated and adhered to the scaffold, proliferating and differentiating into fibrochondrocytes in response to the local microenvironment [51]. Previous research showed that SMSCs are well suited for meniscal repair, with gene expression profiles similar to those of meniscal cells [51, 52].

3.9 Cell viability, adhesion, and proliferation of SMSCs on scaffolds

After three days of incubation on scaffolds, the Live/Dead assay demonstrated no substantial cell death on any scaffold, indicating that MSCs survived well on both the SH scaffold and the SH-Mg scaffold (**Figure 3C, S1**). MSCs had a good stretching pattern on the scaffolds, indicating that the cells could attach to the scaffolds (**Figure 3D**). Further, the alamarBlue assay indicated a proliferative trend for SMSCs in both groups within 7 days (**Figure 3E**). However, the proliferation rate in the SH-Mg group was faster than that in the SH group, which might be due to the addition of Mg^{2+}. These results indicated that the scaffolds had good biocompatibility.

3.10 ECM production *in vitro*

Similar to previous results, the DNA content in the SH-Mg group was significantly higher than that in the SH group (**Figure 3F**). Both scaffolds could maintain cell activity and proliferation, while Mg^{2+} encapsulation could further promote SMSC proliferation.

A DMMB assay was used to measure sulfated-glycosaminoglycan (sGAG) production by SMSCs. Figure 4 showed that the sGAG content at day 7 was significantly higher than at day 3 in the SH-Mg group. Further, sGAG levels in the SH-Mg group were higher than in the SH group at day 7, which revealed that SH-Mg scaffolds could better promote sGAG secretion (**Figure 3I**). To confirm collagen production in both kinds of scaffolds, an ELISA assay was performed. The results of collagen secretion exhibited that col 1 and col 2 corrected by DNA increased over time, and SH-Mg scaffolds resulted in higher col 1 and col 2 deposition than the other (**Figure 3G, H**). Overall, these results demonstrate that SH-Mg scaffolds promoted

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superior SMSC proliferation and meniscal matrix secretion compared with SH scaffolds.

3.11 Fibrochondrogenic differentiation of encapsulated SMSCs

Next, we investigated the expression of genes associated with fibrochondrogenic differentiation, such as cartilaginous gene (col 2, sox 9, and acan), fibroblast gene (col1), and a cell hypertrophy maker (col 10). The relative mRNA level of col 1 markedly increased in the SH-Mg group compared to the SH group (**Figure 3J**). At day 3, col 2 expression was similar between groups, but in hybrid scaffolds it was significantly higher at day 7 (**Figure 3K**). The expression level of sox 9 and acan was similar that to col 1 (**Figure 3L, M**). Meanwhile, there was no significant change in the relative mRNA level of hypertrophy maker col 10 over time in both scaffolds (**Figure 3N**). These results indicate that the SH-Mg group has better potential for fibrochondrogenic differentiation.

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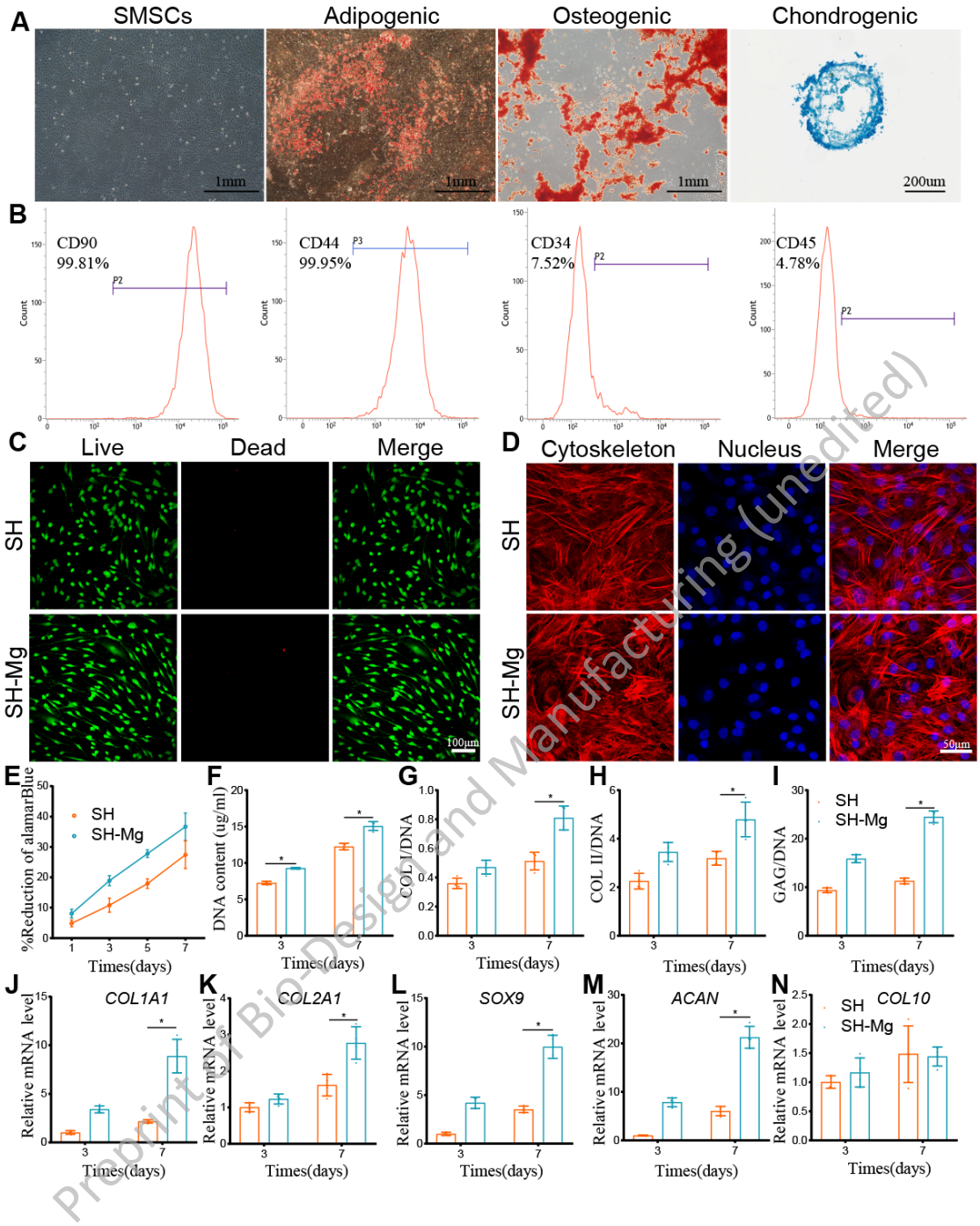

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Figure 3. (A) Adipogenic, osteogenic, and chondrogenic differentiation of SMSCs. (B) Flow cytometry analysis of SMSCs. (C) Live/Dead staining and (D) Phalloidin/Hoechst assay of SMSCs in the scaffolds. (E) AlamarBlue assay of SMSCs in the scaffolds. (F) DNA, (G) COL I, (H) COL II, and (I) GAG of fibrocartilaginous matrix production. (J-M) Fibrocartilage-specific gene expression of (J) Col I, (K) Col II, (L) SOX9, and (M) ACAN. (N) Gene expression of hypertrophy maker col 10. (n = 4, *$p < 0.05$)

3.12 SH-Mg hydrogel induced macrophage polarization toward the M2 phenotype

To simulate the inflammatory microenvironment and investigate the impact of Mg^{2+} on macrophages within the SF/HAMA hydrogel, we used an LPS-RAW264.7

cell-SH-Mg hydrogel system. To detect macrophage polarization, RAW264.7 cells were cultured on both SH and SH-Mg hydrogels. Quantitative PCR analysis showed a significant decrease in the expression of IL-6, INOS, CCR7, and TNF-α (M1 marker genes) in the SH-Mg hydrogel (**Figure 4A**), while that of Arg1, CD206, and IL-10 (M2 marker genes) significantly increased (**Figure 4B**). Immunofluorescence analysis supported the aforementioned findings, evidencing a notable decrease in M1 markers (Inos) (**Figure 4C**) and a substantial increase in M2 markers (Arg; **Figure 4D**) in the SH-Mg hydrogel.

Mg^{2+} has been found to influence macrophage polarization throughout this process. RAW cells cultured using Mg^{2+} hydrogel exhibited reduced expression of M1 genes and proteins, while showing increased expression of M2 genes and proteins. Moreover, the release of Arg-1 by M2 macrophages is consider critical for supplying crucial building blocks for collagen and fibroblast activation factors in the context of the anti-inflammatory stage. In this study, immunofluorescence staining revealed a notable increase and sustained presence of M2 macrophages (Arg 1), along with a decrease in M1 macrophages (INOS). These findings suggest that SH-Mg actively promotes M2 polarization during meniscus regeneration. In summary, a Mg^{2+} hydrogel facilitates M1-to-M2 macrophage polarization, thereby serving as a valuable approach for biomaterial-guided meniscal tissue regeneration.

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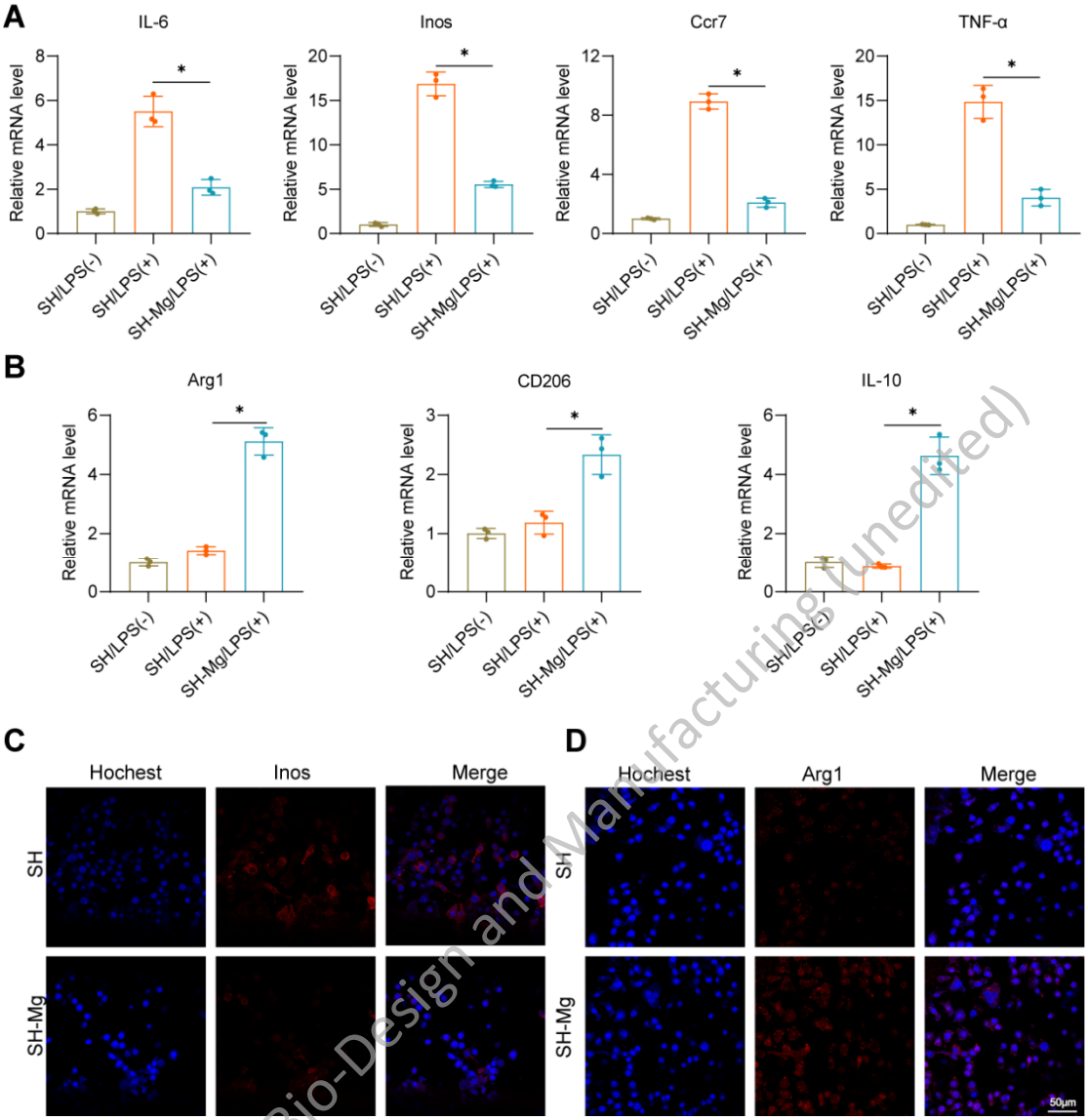

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Figure 4. (A) IL-6, INOS, CCR7, and TNF-α (M1 marker genes). (B) Arg1, CD206, and IL-10 (M2 marker genes) gene expression. (n = 3, *p < 0.05) (C) Immunofluorescence assessment of M1 markers (Inos protein). (D) Immunofluorescence analysis of M2 markers (Arg protein).

3.13 Meniscus regeneration

Meniscal regeneration was evaluated after implantation of SH-Mg scaffolds into rabbits' knee joints. Imaging of the regenerated tissues in the different groups was assessed using MRI analysis (**Figure 5B**). After 24 weeks, visible new menisci appeared in both SH and SH-Mg groups, with the regenerated tissue in the SH-Mg group closely resembling the morphology of the natural meniscus. In addition, the WORMS score in the SH-Mg group was lower than in other groups and closer to the natural meniscus, highlighting the regenerative effect of the SH-Mg scaffold (**Figure 5E**). **Figure 5A** shows the overall morphology of the regenerated meniscus, indicating that the SH-Mg group showed a more comprehensive and homogeneous

regeneration of the meniscus at 24 weeks, along with improved joint integration.

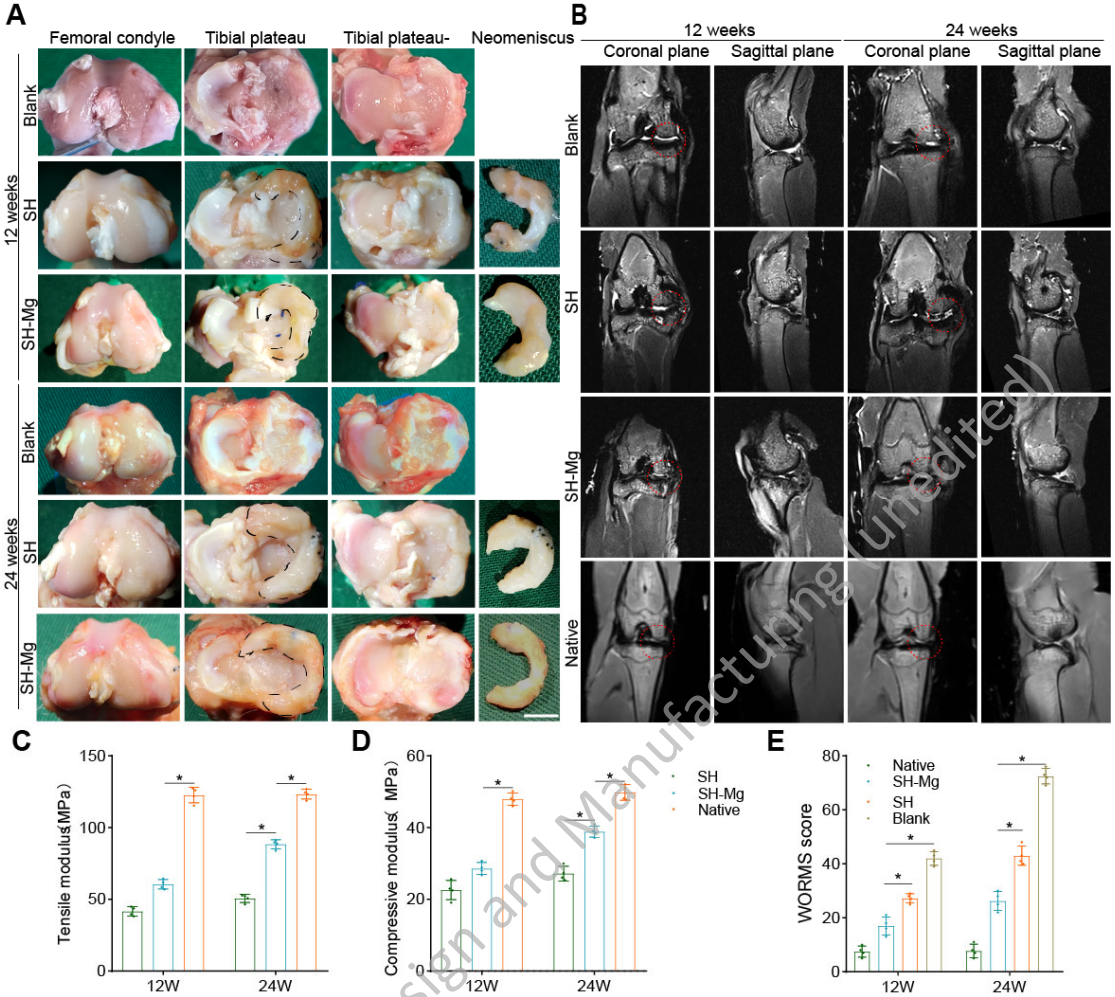

Figure 5. Macroscopic observation, MRI, and biomechanical evaluation of the regenerated meniscus *in vivo*. (A) Macroscopic observation of joints at 12 and 24 weeks after implantation. The tibial plateau column represents the tibial plateau before removing the neoplastic meniscus (In the new Figure 5, the dotted line marks the newly born medial meniscus), whereas tibial plateau- column represents the tibial plateau after the neoplastic meniscus removed. (scale bar = 10 mm). (B, E) MRI of joints and WORMS score at 12 and 24 weeks after implantation. (C) Tensile and (D) compressive modulus of the regenerated meniscus *in vivo*. (n = 4, *$p < 0.05$)

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Histological staining of the implants revealed a reduced foreign body reaction and fewer areas of cytopenia in the SH-Mg group compared to the SH group (**Figure 6A**). Furthermore, PSR, TB, SO, and immunohistochemical stainings for collagen and proteoglycans were performed to evaluate the ECM content and distribution in the implants. PSR staining was significantly increased in COL I in the outer region of the implants in the SH-Mg group compared to the SH group at 24 weeks. This suggests an orderly collagen arrangement, in accordance with COL I immunohistochemistry. Meanwhile, SO and TB staining revealed similar results for proteoglycans, with the

SH-Mg group exhibiting higher proteoglycan deposition compared to the SH group. The proteoglycans were predominantly located in the middle and inner zones of the implant, as observed in COL II immunohistochemistry. In addition, a semi-quantitative analysis of COL I (**Figure 6B**) and COL II (**Figure 6C**) was performed to assess collagen content. At 12 weeks, the relative IOD density /area was lower in COL I group and COL II groups. The SH-Mg group, however, displayed a significant increase in collagen content at 24 weeks, resembling that of the native meniscus. These results suggest that Mg^{2+}-loaded scaffolds promote meniscal regeneration. ECM deposition is closely related to the role of regenerated tissues in stress loading and shock absorption *in vivo*.

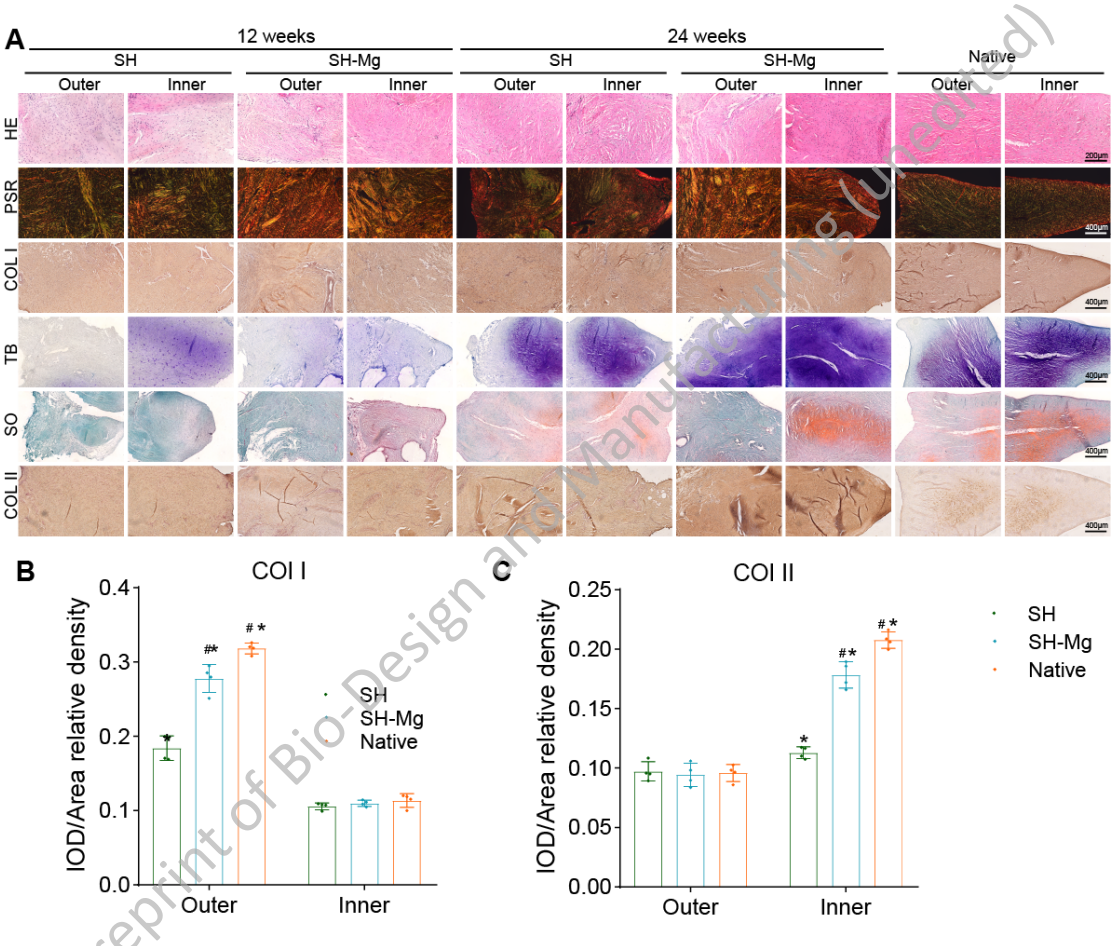

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Figure 6. Histological evaluation of the regenerated meniscus *in vivo*. The outer column represents the synovial margin (outer region) of the meniscus, and the inner column represents the free edge (inner region) of the regenerated meniscus. (A) Hematoxylin-eosin (HE), Picrosirius Red (PSR), toluidine blue (TB), safranin O-fast green (SO) staining, immunohistochemical staining for COL I and COL II. (B, C) Immunohistochemical semiquantitative analyses of COL I and COL II. (scale bar = 200 um in HE staining, and scale bar = 400 um in PSR, TB, SO, immunohistochemical staining for COL I and COL II staining) (n = 4, $^{\#}p < 0.05$ between the inner and outer region in the same group; $*p < 0.05$ between the SH, SH-Mg, and Native group in the same region).

The mechanical properties of the regenerated tissue are particularly important for meniscal repair, thus we evaluated them at 12- and 24-weeks post-implantation. At 12 weeks, both SH and SH-Mg scaffolds exhibited inferior compressive and tensile modulus than the native meniscus. Conversely, at 24 weeks, the SH-Mg group displayed a higher compressive and tensile modulus than the SH group, much closer to the native meniscus (**Figure 5C, D**). Combined with the histological staining of the ECM, collagen fibers, and proteoglycans were more favorably deposited in the regenerated meniscus of the SH-Mg group than in the SH group, resulting in greater resistance to stretch and compression, improving mechanical performance in the knee joint.

3.14 Cartilage protection

It is essential to evaluate the protective effect of articular cartilage after implantation *in vivo*. Observing the overall cartilage performance (**Figure 5A**) at 24 weeks, there was less cartilage degradation in the SH-Mg group than in the SH group. Histological analysis corroborated these findings (**Figure 7A**). Further, the ICRS and Mankins scores indicated that the SH-Mg group outperformed the SH group (**Figures 7B, C**). These results suggested enhanced cartilage protection in the SH-Mg group. Which mainly due to the morphology, properties, and texture of regenerated meniscal tissues. It also showed higher cartilage protection in the SH-Mg group, with stress loading and shock absorption *in vivo*, thus demonstrating superior abilities. The limitation of our research was not conducted in large animal models. In the future, we will actively promote the application of magnesium ion composite scaffolds in large animal models and preclinical studies, as well as its important role in meniscus injury repair.

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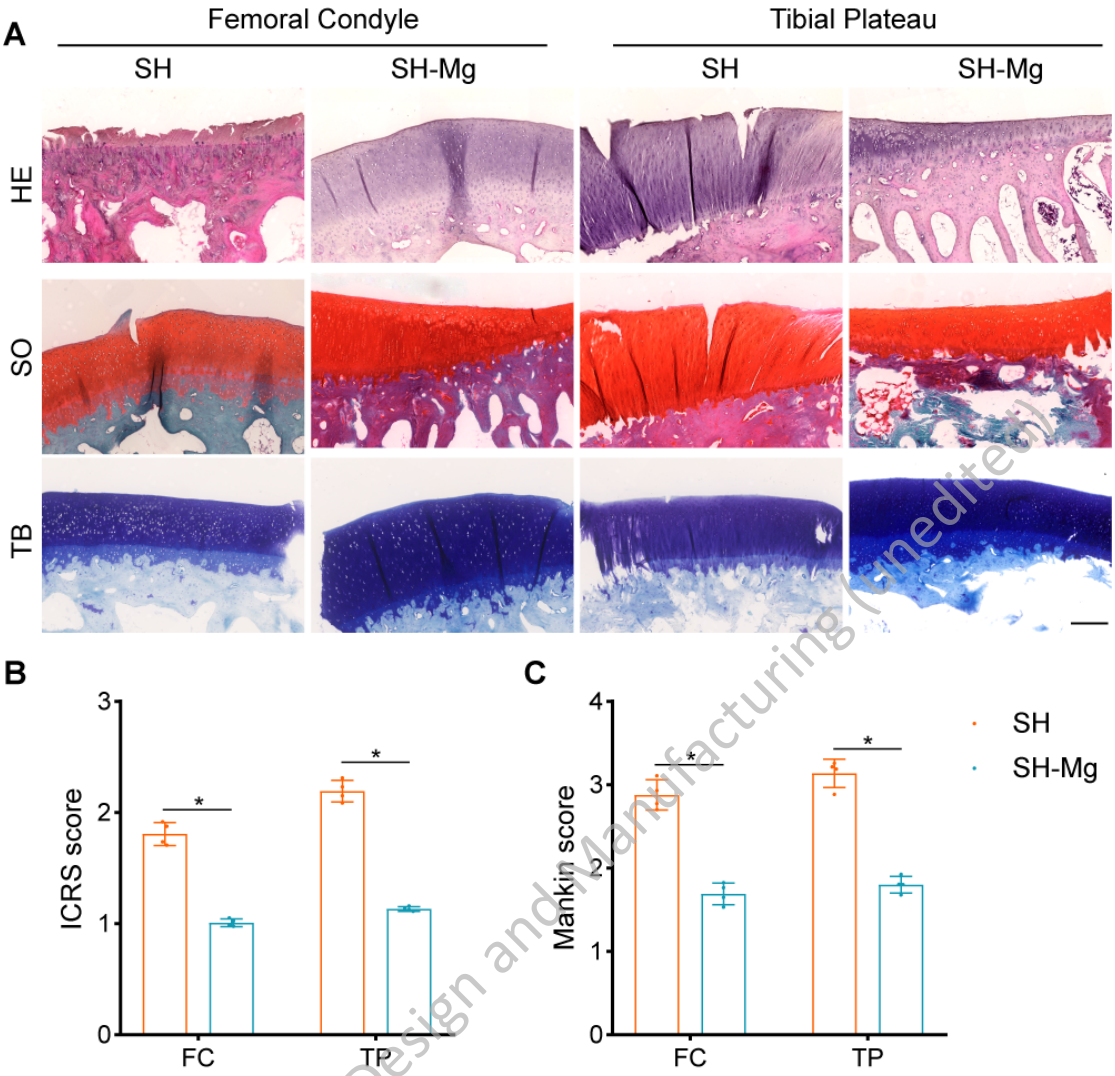

Figure 7. Cartilage histological assessment. (A) HE, SO, and Tb staining of cartilage at 24 weeks. (B) ICRS and Mankin scores (scale bar = 200 μm; n = 4, *$p < 0.05$).

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4. Conclusions

We successfully developed a composite hydrogel scaffold containing Mg^{2+} with demonstrated beneficial cell proliferation, differentiation and ECM generation. Moreover, the presence of Mg^{2+} facilitated macrophage polarization from M1 to M2, significantly improving the local inflammatory environment. Consequently, the presence of Mg^{2+} enhanced composite hydrogel scaffold, promoting meniscal regeneration and cartilage protection.

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