Journal of Orthopaedic Surgery and Research

RESEARCH

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Effects of TGF-β3 on meniscus repair using human amniotic epithelial cells

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Abstract

Background Meniscus injury is one of the most common knee diseases, which is managed through conservative and surgical treatments. In recent years, biotherapy has shown great potential to treat various symptoms caused by meniscus injury repair. Human amniotic epithelial cells (hAECs), which are easy to acquire, non-tumorigenic, and high tri-lineage differentiation potential, are a promising cell source for biotherapy and tissue engineering applications. Studies have demonstrated that the Transforming Growth Factor-β3 (TGF-β3) can facilitate chondrocyte differentiation and maturation.

Methods Both in vitro test and in vivo test were employed. In the in vitro test, human amniotic epithelial cells (hAECs), human amniotic mesenchymal stem cells (hAMCs), and fibrochondrocytes (FCs) were extracted and identified by flow cytometry and immunohistochemistry (IHC). These cells were treated with TGF-β3 for one week, followed by IHC staining and qPCR to explore TGF-β3-induced fibrocartilage formation in hAECs. In the in vivo tests, a meniscus injury model was established based on rabbits, and the Sham, the control (normal saline), and the hAECs + TGF-β3 groups were used. Additionally, the meniscus was collected and checked through general examination and IHC analysis 90 d after surgery.

Results Routine transcriptome analysis confirmed that TGF-β3 induced the differentiation of amniotic epithelial cells (hAECs) into fibrochondrocytes through the Wnt signaling pathway. This finding was corroborated using Western blot (WB) and quantitative PCR (QPCR). Among the five experimental groups, the highest expression of target proteins and genes was detected in hAECs + TGF-β3 group, followed by the hAECs + hAMCs + TGF-β3 group, the hAMCs + TGF-β3 group, the hAECs + FCs group, and the FCs group. The observed differences were statistically significant ($P < 0.05$). In vivo, treatment with hAECs + TGF-β3 facilitated effective repair of damaged menisci.

Conclusions hAECs + TGF-β3 can potentially promote the healing of meniscus injuries, laying the foundation for further research to promote its clinical translation.

Trial registration Not applicable.

Keywords Human amniotic epithelial cells (hAECs), TGF-β3, Meniscus injury, Biotherapy

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Introduction

The meniscus is essential for knee homeostasis as it is key in improving tibiofemoral joint congruence, dynamic load distribution, joint stability, and proprioception [1, 2]. Meniscus injury is one of the most common knee injuries in athletes and older adults and can occur at all ages, genders, and activity levels [3]. Treatment options for meniscus tears include non-surgical interventions, meniscus repair, or meniscectomy [4]. Surgery is the primary treatment for most meniscus tears. The first open surgical repair of meniscus tears was performed by Annandale in 1885, followed by various arthroscopic techniques [4]. However, conventional treatment methods have limitations, because they often fail to effectively address the underlying pathology driving the injury [5]. By the 1970s, total meniscectomy was the gold standard treatment for meniscus tears owing to poor understanding of the significance of meniscus [4]. The long-term clinical outcome of the operation is poor, with patients presenting with degeneration of the articular cartilage, flattening of the joint surface, sclerosis of the subchondral bone, irreparable joint injury, and premature osteoarthritis [6]. Meniscectomy can lead to osteoarthritis [7, 8].

Tissue engineering, an emerging biomedical approach, accelerates the regeneration and repair of defective and damaged tissues based on an intelligent and unique combination of cells, growth factors, and scaffolds [9, 10]. Consequently, the use of biotherapy in musculoskeletal regenerative medicine has significantly increased over the past decade [11]. Animal tests have shown that mesenchymal stem cells (MSCs)-based tissue engineering is highly promising for meniscus repair owing to its multi-differentiation potential [12, 13]. Repair of damaged cartilage using stem cell-based approaches has shown promising results [14]. Being an emerging adjuvant therapy, amniotic membrane has shown significant potential in promoting the healing of orthopedic soft tissues. Derived from placental tissue, which supports fetal development during gestation, the amniotic membrane is rich in collagen, growth factors, and an extracellular matrix, all of which contribute positively to tissue repair and regeneration. Previously, amniotic membrane has been involved in various fields such as dermatology, hepatology, cardiology, and neurology, and it is now being applied to treat musculoskeletal diseases including knee osteoarthritis, spinal and hand surgeries [15]. Moreover, biologics derived from amniotic tissue have shown great potential to promote regenerative medicine [16]. Furthermore, human amniotic membrane can prevent significant degradation of knee cartilage and protect the extracellular matrix from further breakdown [17]. The epithelial cells of the amniotic membrane possess tri-lineage differentiation potential and meet two crucial criteria for clinically relevant stem cells: safety and availability [18]. Previous studies have shown that co-culturing tendon tissue with hAECs in vitro enhances its activity and cellular function [19]. As a promising stem cell type, hAECs have been widely explored in transplantation therapy for various diseases. Research has also demonstrated that TGF-β1 induces stem cells to differentiate into hyaline cartilage [20], while BMPs promote their differentiation into osteocytes [21]. TGF-β3 is a member of a family that regulates cell growth and differentiation and regulates the proliferation, development, and maturation of chondrocytes in vivo through multiple signaling pathways [22]. It plays an important regulatory role in cartilage formation and early growth and development of joints [23]. The lifecycle of chondrocytes is driven by proliferation, differentiation, maturation, and apoptosis. TGF-β3 can directly interact with the transforming growth factor beta receptor (TβR) on the external cell membrane, triggering a series of molecular changes involving SRY-related high-mobility group protein 9 (SOX9) [24]. Adipose-derived mesenchymal stem cells can be transformed into fibrochondrocytes (FCs) under the induction of TGF-β3 [25]. The hAECs were used as seed cells and TGF-β3 as a growth factor to investigate the expression of fibrocartilage markers during the differentiation process across various stem cell types. Our primary focus was to elucidate the role of TGF-β3 in facilitating fibrocartilage differentiation in hAECs. For TGF-β3 as an inducing agent, hAECs have laid the foundation for the construction of tissue engineering fibrocartilage for seed cells. It provides a theoretical and experimental basis for constructing a tissue engineering-based approach to fibrocartilage repair.

Materials and methods

In vitro test

Culture of human amniotic epithelial cells (hAECs)

The amniotic membrane samples were obtained from the Obstetrics and Gynecology Department of The First Affiliated Hospital of Jinzhou Medical University. Before sampling, fetal malformation, congenital genetic diseases of the mother, and infectious diseases such as hepatitis A, hepatitis B, HIV, syphilis, etc., were excluded. The mothers signed informed consent forms before surgery, and the study was approved by the Ethics Committee of The First Affiliated Hospital of Jinzhou Medical University (Ethics Number: KYLL202418). The amnion was peeled off bluntly from the placenta using forceps and straight forceps and washed with normal saline. It was rinsed twice with 200 mL of PBS to remove blood cells, and the specimen was processed within 2 h. The amnion was removed from PBS, excess water drained, placed on sterilized Petri dishes and cut into 3 cm^2 samples. The pieces were transferred into 10 cm Petri dishes, 20 mL of 0.25% trypsin added, cut, transferred to a cell sieve, and ground

using the bottom of the bottle for 10 min. The amnion pieces were digested for 30 min at 37℃ in a thermostatic water bath, filtered through a 200-mesh steel mesh to remove the tissue pieces, and 5–10 mL of neutralizing trypsin in FBS was added. The cell suspension was collected and centrifuged at 1200 r/min for 3 min, and the supernatant was discarded. hAECs culture solution (DMEM/F12 culture medium, containing 10 wt% BSA and 10 μg/L epidermal growth factor) was used to resuspend the precipitate. The cells were counted with Trypan blue, inoculated into a 10 cm diameter petri dish at $1 \times 10^5/cm^2$, incubated, and the liquid changed after 72 h. When the cells reached 80-90% fusion, 0.25% trypsin was used for digestion; cells were subcultured, and some were stored.

Culture of human amniotic mesenchymal stem cells (hAMCs)

The blood and mucus on the amnionic tissues were removed by repeated rinsing with phosphate buffer salt solution. The amnionic tissues were cut up, and hAMCs were isolated and cultured by enzymatic digestion. They were placed in a 37℃, 5%CO_2 incubator for 48 h. After that, the culture medium was changed to remove unattached cells, after which the culture medium was replaced every 2 d. The cells were passaged when the cell proliferation reached 90% confluence. The second-generation hAMCs were taken for preparation.

Culture of human FCs

The fibrocartilage samples were purchased from the Department of Joint Surgery, The First Affiliated Hospital of Jinzhou Medical University. The samples were taken from adolescents with an average age of 12 years old (ranging from 6 to 15 years old) who suffered from lateral meniscal injuries of the knee joint. Prior to sampling, patients were screened for infectious diseases such as hepatitis A, hepatitis B, HIV, and syphilis, and excluded if positive. Their guardians signed informed consent forms before surgery, and the study was approved by the Ethics Committee of The First Affiliated Hospital of Jinzhou Medical University (Ethics Number: KYLL202418). Fibrocartilage was obtained from the adolescent meniscus (age = 6–15 years old, with an average of 12 years old). For children with lateral disc cartilage injury of the knee joint, the disc cartilage removed through arthroscopic surgery was put into a dish with PBS solution, cut as much as possible with ophthalmic scissors, and transferred into a centrifuge tube. Five times the amount of 0.5% trypsin was added and digested at 37℃ for 30 min under oscillation. The trypsin F-12 culture solution was removed and rinsed twice. Collagenase was added (5 mL of 0.2%) and digested for 1 ~ 4 h under oscillation at 37℃. Under the microscope, the fibrocartilage appeared loose, with most cells arranged in a single layer. 4 mL of culture solution (containing 20% BSA, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL ascorbic acid) was added to the culture flask. The culture conditions were 5% CO_2 and 95% air at 37℃ with fluid change every other day. The second-generation FCs were taken for standby.

Identification of cultured cells

Identification of hAECs

Human amniotic epithelial cells rare characterized by specific positive and negative markers [26], as well as the trilineage differentiation potential [27]. The second-generation hAECs were digested using 0.25% trypsin, re-suspended in PBS at a concentration of 1×10^8 L^{-1}, dispensed into 1.5 mL EP tubes, and centrifuged at 300×g for 5 min. The supernatant was discarded. The prepared 0.3% BSA solution was added into EP with 200 μL per tube. Each tube was sealed at room temperature for 30 min, centrifuged at 300×g for 5 min, the supernatant discarded, and mouse anti-human monoclonal antibody differentiation clusters CD29, CD31, CD34, CD35, CD44, CD73, CD90, CD105, and HLA-DR were added. The tube was allowed to stand at 4 ℃ for 20 min and washed twice with PBS. Cell phenotypes were detected using a flow cytometer, and the data were analyzed using Flowjo (V10.8.1).

Identification of hAMCs

Human amniotic mesenchymal stem cells possess distinct positive and negative markers [26] and demonstrate the ability to differentiate into all three germ layers. The second-generation hAMCs were digested using 0.25% trypsin, re-suspended in PBS at a concentration of 1×10^8 L^{-1}, dispensed into 1.5 mL EP tubes, centrifuged at 300×g for 5 min, and the supernatant discarded. The prepared 0.3% BSA solution was added to the EP with 200 μL per tube. Each tube was sealed at room temperature for 30 min, the sealed EP tube centrifuged at 300×g for 5 min, and the supernatant discarded. The mouse anti-human monoclonal antibody differentiation clusters CD34, CD44, CD45, and CD73 were added, allowed to stand for 20 min at 4 ℃, and washed twice with PBS. Cell phenotypes were detected on a flow cytometer, and the results were analyzed using Flowjo (V10.8.1).

Identification of human FCs

Coverslips with FCs were fixed with paraformaldehyde, dried, and stained with Alcian blue staining. Immunohistochemistry (IHC) was performed through the dropwise addition of Type I and II collagen monoclonal antibodies (1:20), according to the conventional ABC method.

Induction and characterization of fibrocartilage

Induction of differentiation of hAECs and hAMCs to FCs

TGF-β3 was applied to induce the differentiation of stem cells into fibrocartilage [28]. The second-generation hAECs and hAMCs were made into single-cell suspensions. The cell concentration was adjusted to 1 × 109/L and inoculated in 100 mL culture flasks. The culture medium was changed to LDMEM (containing 10% fetal bovine serum, 0.1 mg of dexamethasone, 50 g of Vitamin C, and 10 mmol of sodium glycerophosphate) after the cells grew to 80%~90% fusion. In previous studies, the group treated with high-dose TGF-β3 (1000 ng/ml) demonstrated the best meniscus recovery when mesenchymal stem cells were induced with either low-dose TGF-β3 (100 ng/ml) or high-dose TGF-β3 (1000 ng/ml) for the treatment of meniscus injuries [29]. The TGF-β3-induced group continued to be cultured with TGF-β3 at a 1000 mg/mL mass concentration.

Identification of Alcian blue staining

Fibrocartilage differentiation was explored through the Alcian blue staining assay. Herein, five groups were involved, including (1) the hAECs + TGF-β3 group, (2) the hAMCs + TGF-β3 group, (3) the hAECs + hAMCs + TGF-β3 group, (4) the hAECs + FCS group and (5) the FCs group. Cell samples were washed with PBS, fixed with 40 g/L paraformaldehyde at room temperature for 1 h, and rinsed with distilled water for 15 min. Toluidine blue was added to cover the cells to stain for 4 h at room temperature. The samples were taken out of the original solution, and the cells were decolorized using anhydrous ethanol, washed with distilled water until the water was colorless, and fixed with neutral adhesive. The morphology of the Alcian blue-stained cells was observed under an inverted microscope.

IHC examination of type I and II collagen

IHC examination of Type I and II collagen was employed to investigate fibrocartilage differentiation. Five sample groups were washed with PBS, fixed with 40 g/L paraformaldehyde for 30 min, and rinsed with PBS at 4 ℃ for 20 min. Type I and II collagen was added after serum blocking for 30 min. The first antibody solution was incubated at 4 ℃ overnight, slides incubated with 200 mL of secondary antibody solution for 1 h at room temperature, and then DAB was added for coloration for 5 min. Hematoxylin counterstaining was performed for 30 s and then air-dried. The five groups of specimens were fixed with neutral adhesive, and the expression of Type I and II collagen was observed.

Differentially expressed genes (DEGs) and KEGG enrichment after transcriptome sequencing

The samples were collected and sent to Shanghai Weihuan Biotechnology Co., Ltd., where the company tested the quality of the samples. The total RNA of the samples was extracted, and the mRNA was enriched. The library was then constructed and sequenced (Illumina Novaseq 6000 PE150) after quality control. Sequencing results were obtained, and DEGs heatmap and KEGG pathway enrichment were constructed.

Total RNA extraction and fluorescence quantitative PCR

Total RNA was extracted from cells of the five groups after 1 week of culture to determine the expression levels of cartilage-related genes Aggrecan, COL1A1, COL2A1, and SOX9 was measured. Total RNA was extracted using commercial kits. cDNA was synthesized through reverse transcription reaction, and fluorescence quantitative PCR was carried out.

WB detection of protein expression

Proteins extracted from five cell groups were cultured for one week to examine the expression levels of cartilage-associated proteins (e.g., Aggrecan, COL1A1, COL2A1, and SOX9) and signaling pathway molecules (e.g., β-catenin, wnt5a, and wnt5b). Whole-cell lysate was prepared in lysis buffer with a mixture of protease inhibitors, and proteins were transferred to the membrane through electrophoresis. The membrane was then sealed with 5% skimmed milk powder at room temperature for 3 h. The corresponding first antibody was added and incubated at 4 ℃ overnight. After the membrane was washed thrice, a horseradish peroxidase-labeled secondary antibody was added and incubated at room temperature for 2 h. It was washed with TBST, developed, and photographed.

In vivo test

Rabbits (9 females and 9 males at 6 months of age) were used in this study. All animal care and experiments were performed according to the Animal Committee of Jinzhou Medical University guidelines. The preparation of hAECs was described in Sect. 1.1.1.

Establishment of meniscus injury model and grouping

Rabbits were used as the experimental subjects because their physiological metabolic processes and disease response mechanisms share certain similarities with those of humans. Furthermore, their joint size is comparable to that of humans, allowing surgical procedures to closely mimic those performed on humans. Moreover, rabbits are relatively less controversial in terms of animal ethics. The experiment was approved by the Experimental Animal Ethics Committee of Jinzhou Medical University (Ethics number: 240137). The rabbits were randomly

divided into 3 groups, with 6 rabbits in each group. The right knee lateral meniscus injury model was established as follows: The rabbit was placed supine after complete anesthesia, and the lateral para-patellar approach was used. The lateral collateral ligament was incised, and the knee was maximally flexed and rotated inward to expose the entire lateral meniscus. A drill with a diameter of 1.5 mm was inserted vertically and longitudinally into the meniscus up to the tibial plateau, resulting in two longitudinal lacerations, and the incisions were sutured layer by layer. For the hAECs + TGF-β3 group at 2, 6, and 10 weeks post-surgery, 190 μL of hAECs (10^7) + 10 μL of TGF-β3 (1000 ng/mL) suspension was injected into the molded knee joint cavity. The control group was injected with 200 μL of normal saline. The Sham group was cut layer by layer to expose the meniscus and then sutured layer by layer. After the surgery, gentamicin was administered via intramuscular injection for 3 days. No fixation device was applied to the knee joint. The wound dressing at the incision site was changed routinely, and the stitches were removed 7–10 days later. The meniscus was collected on the 90th day of the postoperative period for gross examination and IHC analysis.

Tracing

Tracing was performed one week after the rabbit's right medial meniscus was modeled; 200 μL of hAECs suspension (107) was mixed with 1 μL of DII and left in the dark for 20 min before injection into the knee joint cavity. The rabbit was sacrificed two days later, and the modeled limb and the contralateral limb were removed and placed in a small animal imaging device for detection.

Histological analysis

HE staining

The tissue was immersed in a staining solution for about 8 min, rinsed with tap water, and immersed in hydrochloric acid for color separation. Tissues were stained in concentrated ammonia and eosin staining solutions, rinsing with tap water each time. The tissues were finally dehydrated with a hexanol gradient and subjected to xylene transparent treatment.

Safranin O/fast green staining

The as-prepared paraffin sections were washed after dewaxing the sections and repairing the antigen with a citrate antigen repair solution. Safranin O/fast green staining was performed according to the standard protocol.

IHC staining of type I and II collagen

Paraffin sections were prepared and dewaxed. Antigen retrieval was performed using citrate solution (Beyotime Biotechnology), followed by a 20-min wash with PBS (Beyotime Biotechnology). To block non-specific binding, serum (Beyotime Biotechnology) was applied for 30 min, after which primary antibodies targeting type I and type II collagen (Beyotime Biotechnology) were added. The sections were incubated with the primary antibodies at 4 °C overnight. Next, 200 μL of secondary antibody reaction solution (Beyotime Biotechnology) was applied and incubated at room temperature for 1 h. Finally, the color was developed using DAB (Beyotime Biotechnology) for 5 min.

Statistical analysis

All experiments were conducted in triplicate, and results are presented as mean ± S.E.M. Quantitative data were analyzed using SPSS, ImageJ, and GraphPad Prism 8.0 software (GraphPad Software, La Jolla, CA). Normality was assessed with the Shapiro-Wilk test, considering data normally distributed when $P > 0.05$. Homogeneity of variance was tested using Levene's test, with $P < 0.05$ indicating homogeneity. Statistical significance between groups was determined by Student's t-test and one-way ANOVA, with $P < 0.05$ considered significant.

Results

Detection of surface molecules on hAECs

Flow cytometry results of the second-generation hAECs obtained from primary cells after 3 days of in vitro culture showed that third-generation adherent cells had high expressions of CD29, CD44, CD73, CD90, and CD105, and low expression of CD31, CD34, CD35, and HLA-DR (Fig. 1).

Detection of surface molecules on hAMCs

Flow cytometry results for the second-generation hAMCs obtained from primary cells after 3 days of in vitro culture showed that third-generation adherent cells expressed high levels of CD44 and CD73 and low levels of CD34 and CD45 (Fig. 2).

Identification of FCs

The second-generation FCs obtained from primary cells displayed a crawling pattern and adhered strongly to the surface. Alcian blue staining showed the cytoplasm as light blue and the nucleus as dark blue, with a well-defined nucleolus. A few heterogeneous granules were observed surrounding the cells (Fig. 3A). IHC staining of Type I and II collagen revealed positive expression. The cytoplasm was brownish-yellow, and the hematoxylin-lined nucleus was blue (Fig. 3B, C).

Alcian blue staining

Figure 4 A shows the Alcian blue staining results of the five groups (hAECs + TGF-β3, hAMCs + TGF-β3, hAECs + hAMCs + TGF-β3, hAECs + FCs, and the FCs

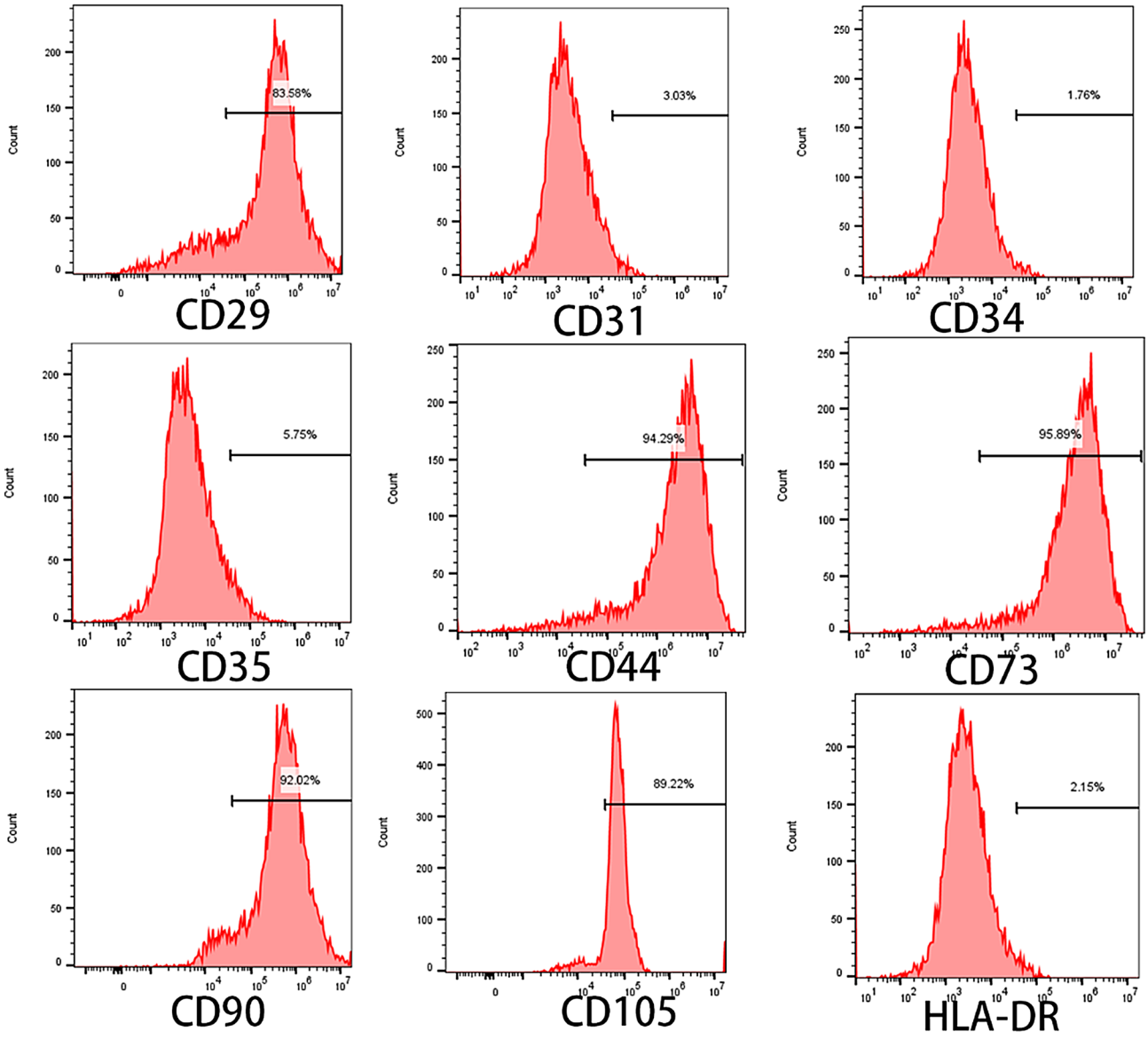

Fig. 1 Detection of surface molecules on hAECs

groups). All the experimental groups stained positive, among which the hAECs + TGF-β3 group showed the most significant positivity; the nucleus was dark blue, the nucleolus distinct, the cytoplasm bluish-purple, and there were metastatic granules in the cell and around the cell. This demonstrates that the cells secreted proteoglycans exuberantly. The quality and function of cells of the five groups followed the order: The hAECs + TGF-β3 > the hAECs + hAMCs + TGF-β3 > the hAMCs + TGF-β3 > the hAECs + FCs > the FCs group. The FCs in each group had mature fibrocartilage function and secreted FCs extracellular matrix.

IHC staining of type I and II collagen

Fibrocartilage synthesizes extracellular matrix by secreting Type I and II collagen. (Figure 4B, C): All five cell groups were stained brownish-yellow and showed positive expression, with the hAECs + TGF-β3 group showing the most distinct brown color, followed by the hAECs + hAMCs + TGF-β3 and the FCs groups, suggesting that hAECs treated with TGF-β3 3 could increase FCs function.

DEGs

RNA-seq analysis revealed significant alterations in 40 genes when comparing TGF-β3-treated hAECs to fibroblast-like chondrocytes (FCs). These genes span multiple families, including the Wnt, collagen, and

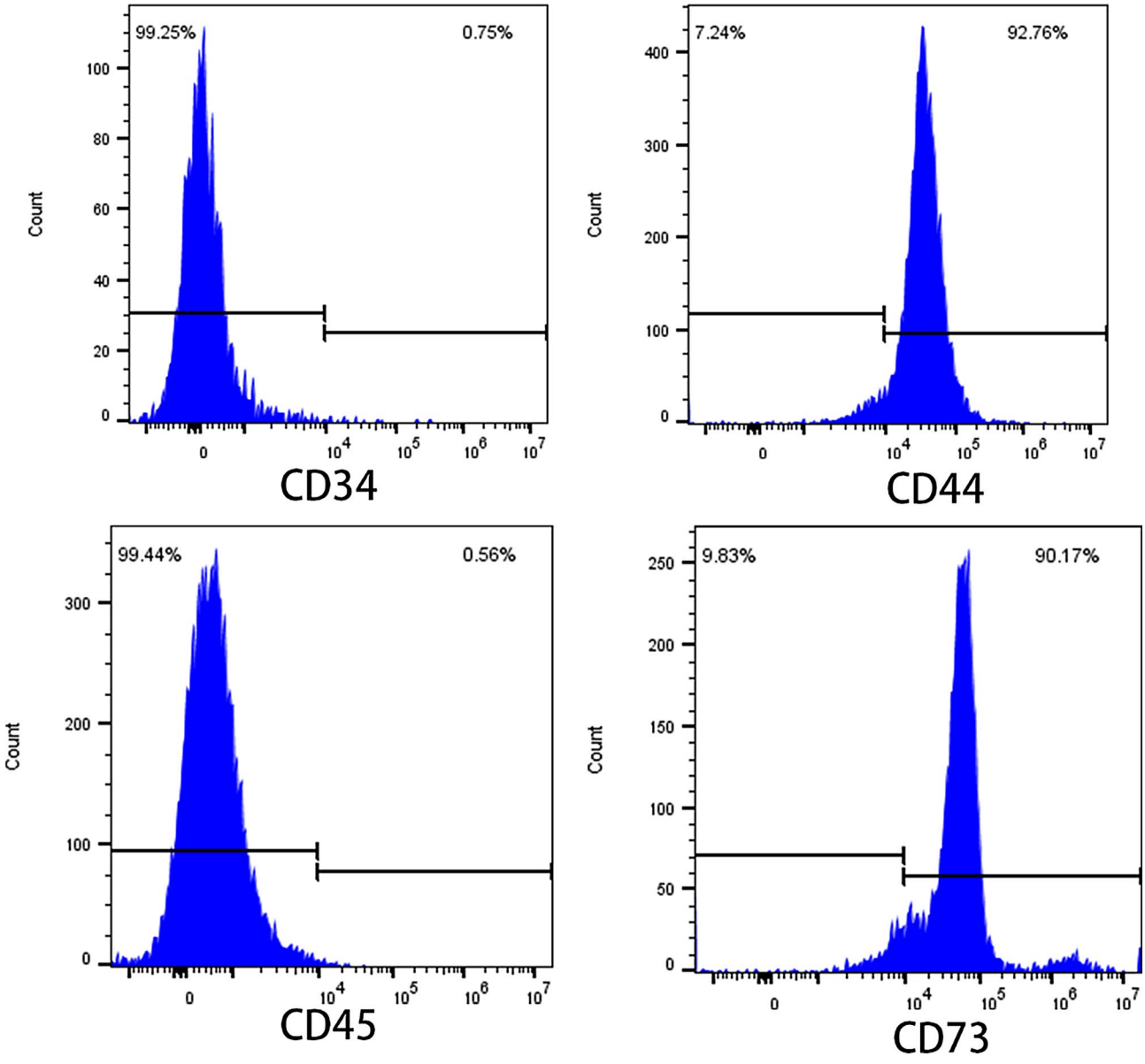

Fig. 2 Detection of surface molecules on hAMCs

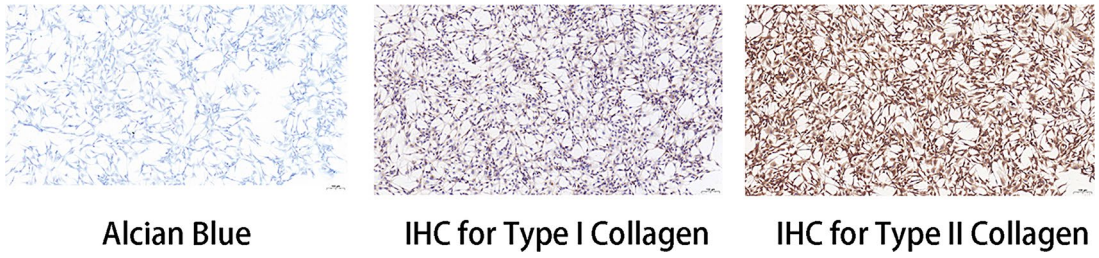

Fig. 3 Identification of human FCs

metalloproteinase families. Notably, genes related to extracellular matrix components, such as COL2A1 and SOX9, were upregulated. Furthermore, genes linked to fibrocartilage transformation and maturation, such as MMP15 and ADAMTS9, along with Wnt pathway-related genes like Wnt5A, were also upregulated (Fig. 5). These findings provide additional evidence that TGF-β3 treatment enhances fibrocartilage cell function in hAECs.

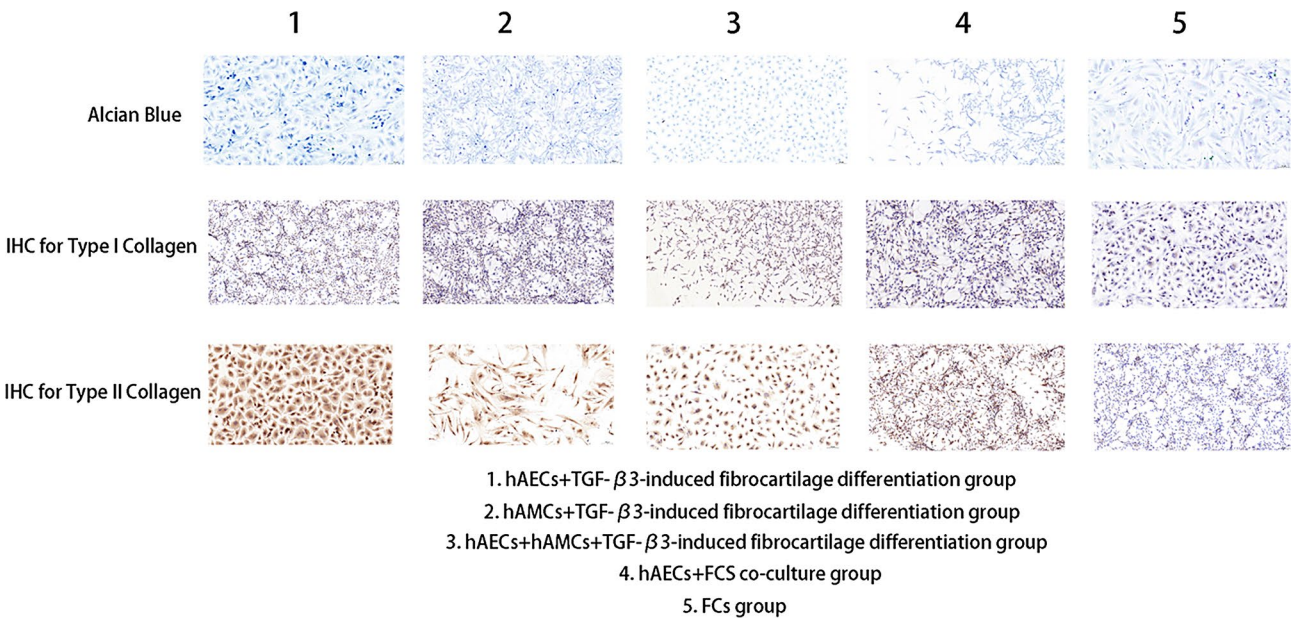

Fig. 4 (1) hAECs + TGF-β3-induced fibrocartilage differentiation group; (2) hAMCs + TGF-β3-induced fibrocartilage differentiation group; (3) hAECs + hAMCs + TGF-β3-induced fibrocartilage differentiation group; (4) hAECs + FCS co-culture group; (5) FCs group

KEGG enrichment analysis

The transformation of hAECs + TGFβ3 into FCs may occur through multiple pathways, which was confirmed through RNA-seq. The Wnt pathway was predominant, and its related genes were up-regulated during the transformation process (Fig. 6).

Fibrocartilage gene and protein expression

Fluorescence quantitative PCR, with the FCs group used as the baseline, showed that fibrocartilage-related genes of the other four groups (e.g., Aggrecan, COL1A1, COL2A1, SOX9) were highly expressed (Fig. 7 A). The expression of normal FCs protein was examined through Western blot. Overall, the TGF-β3-treated hAECs in the fibrocartilage-induced group were more capable of promoting the protein expression of fibrocartilage (e.g., Aggrecan, COLI, COLII, and SOX9) than the other four groups.

Possible mechanism

The changes of related signaling molecules after activating the Wnt pathway were analyzed to further evaluate the role of TGF-b3 in differentiating hAECs to FCs, (Fig. 8B). β-Catenin is a key member of the Wnt pathway. Western blot results showed that TGF-β3 significantly increased the cellular expression of β-Catenin protein ($P < 0.05$). The downstream wnt5a and wnt5b expression levels were quantified to clarify the Wnt pathway. As indicated, TGF-β3 significantly stimulated the expression of wnt5a and wnt5b proteins ($P < 0.05$). The results of RNA-Seq detection were confirmed.

Tracing results

Fluorescence expression was observed in the modeled area under the small animal imager. In contrast, no fluorescence was observed on the contralateral side (Fig. 9), demonstrating that stained hAECs existed in the damaged zone and also suggesting that hAECs were able to colonize in the damaged zone and further differentiate into FCs in the damaged zone, thus promoting the recovery of the damaged zone.

HE staining

Representative HE staining images of tissue sections (Fig. 10) showed that the damaged zone of the control group did not heal, and the damaged zone of the hAECs + TGF-β3 group healed, with fibers connecting in the area.

Safranin O/fast green staining

As shown in Fig. 11, the damaged zone did not heal in the control group, the fiber components were broken, and proteoglycan was lost. In the hAECs + TGF-β3 group, continuous fibers were present in the damaged zone, proteoglycan was present, and staining was enhanced compared with the control group.

IHC staining

Staining for Type I (Fig. 12A) and Type II collagen (Fig. 12B) revealed distinct differences between the groups. In the control group, broken fibers were clearly visible, and no staining was observed in the affected area. In contrast, the hAECs + TGF-β3 group exhibited

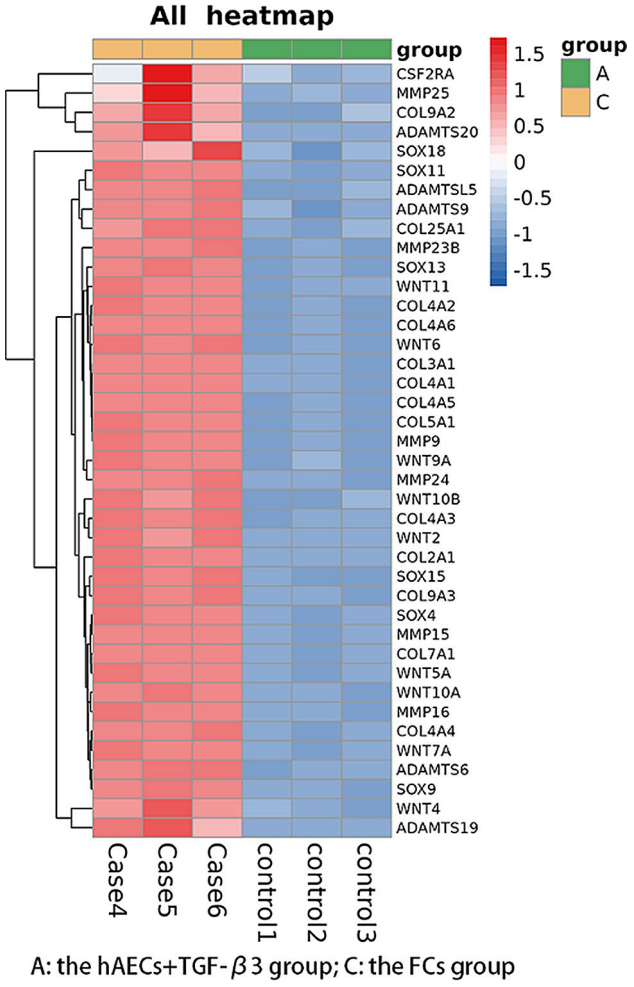

Fig. 5 A heatmap of DEGs (**A**: the hAECs + TGF-β3 group; **C**: the FCs group)

continuous fibers, with visible collagen fiber components, and positive staining in the cells.

Discussion

The meniscus is a vital knee component, and when it malfunctions, the knee becomes pathologically force-distributed and unstable, negatively affecting biomechanics [30]. Meniscus tissues have poor healing capacity as they are avascular and have few cells [31]. Partial or total meniscectomy are the most commonly used treatments for meniscus injuries; however, these procedures can negatively impact joint function and long-term health [32]. The meniscus has a high probability of developing osteoarthritis post-surgery, but the majority of surgeons still focus on surgery [33]. Biotherapy is a promising treatment. Allogeneic biotherapy involves using tissues or cells derived from the placenta, amnion, and fetal umbilical vein [34]. The hAECs seed cells are highly promising [35]. TGF-β belongs to the peptide growth factor family, which can induce the differentiation of original MSCs into cartilage tissue during embryogenesis. All members of the TGF-β family share several structural properties: They are homodimeric polypeptide molecules produced by various cells and tissues, with molecular weights of approximately 25 kD, and each polypeptide chain contains 110–140 amino acid residues [36]. TGF-β3 exhibits sequence similarity to all TGF-β-related factors. TGF-β3 exerts various physiological regulatory effects by transmitting signals to the nucleus through TGF-β receptors and the intracellular signaling system [37]. In this experiment, we demonstrated that hAECs could differentiate to FCs under induction by TGF-β3 and then realize meniscus repair.

In the in vitro tests, Alcian blue and IHC staining revealed that hAECs + TGF-β3 can transform into FCs. Indeed, MSCs can be transformed into FCs under TGF-β3 induction [25], and the co-culture of MSCs and FCs can promote the proliferation and expression of FCs [38]. DEGs heatmap analysis after transcriptome sequencing showed that compared with FCs, the genes (e.g., SOX9, COL2A1) related to the extracellular matrix of FCs in the hAECs + TGF-β3 group and some metalloenzyme genes (e.g., MMP25, ADAMT56) that promoted maturation of FCs were up-regulated. All groups' AGG, COLI, and COLII stained positive for Alcian blue and IHC, among which the hAECs + TGF-β3 group was the deepest and the most positive. Investigations using WB and qPCR demonstrated that all experimental groups could express FCs extracellular matrix (e.g., SOX9, AGG) related proteins and genes, further demonstrating the ability to convert to FCs after adding TGF-β3. The hAECs + TGF-β3 group showed the highest expression of related proteins, such as SOX9 and AGG, and genes. The KEGG enrichment analysis showed that the Wnt pathway was dominant among the associated pathways, and the DEGs heatmap showed that the downstream genes (e.g., Wnt5A) in the Wnt pathway were upregulated; WB also demonstrated that the expression of related proteins in the Wnt pathway was different from that of FCs, suggesting that transformation of hAECs + TGF-β3 to FCs was achieved through the Wnt pathway.

The tracing experiment was conducted using in vivo tests. In the small animal imager, it was found that fluorescent expression could be seen in the meniscus-associated region two days after injection of DII-treated hAECs into the affected knee joint cavity. This indicated that hAECs were capable of homing, colonizing, and repairing the damaged zone; HE, Safranin O/fast green, and IHC staining revealed that the control group showed no signs of repair in the damaged zone. The damaged zone in the hAECs + TGF-β3 group had fibrous junctions, and the associated staining increased. The related collagen expression was positive, but the damaged zone was not completely repaired.

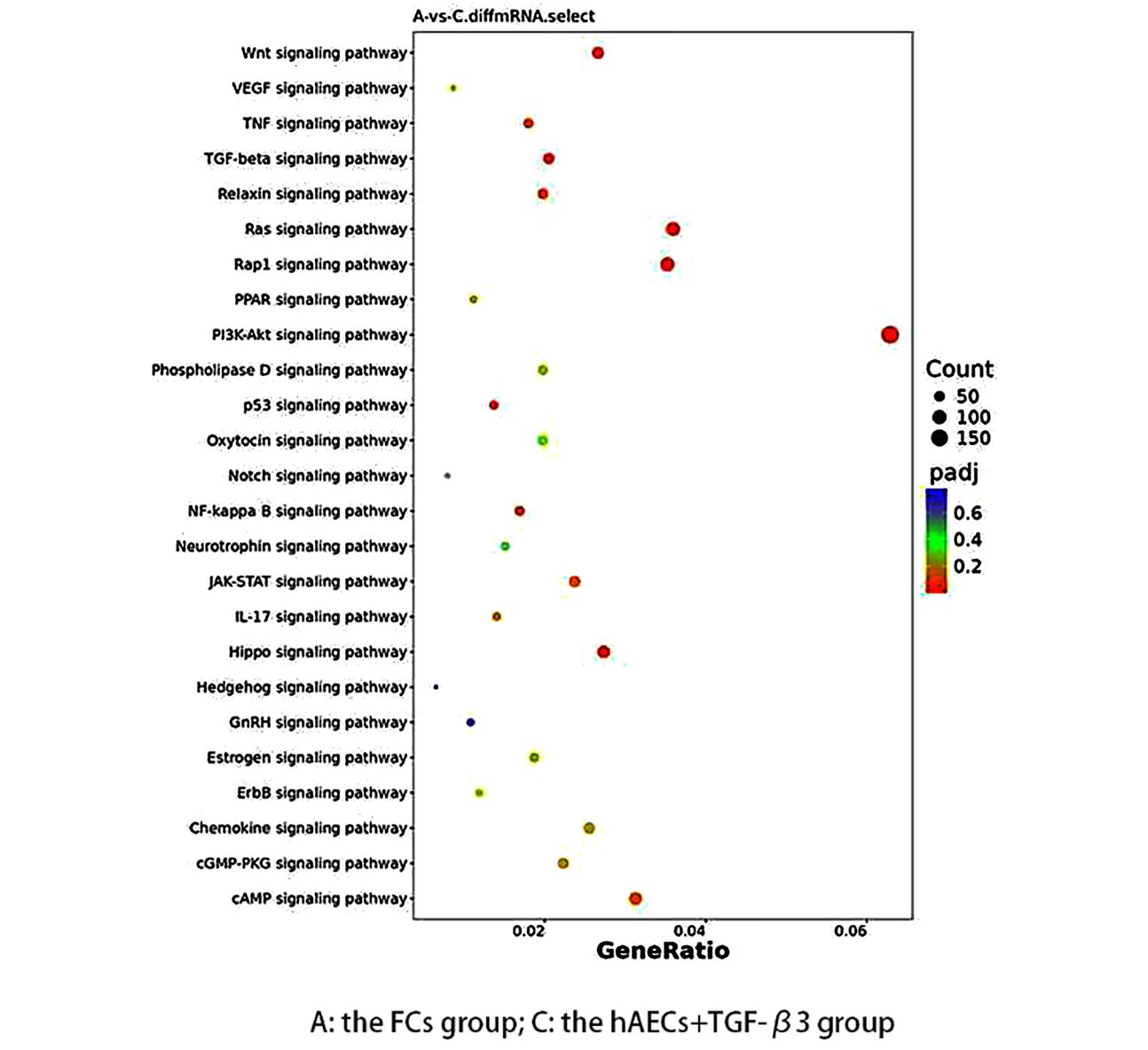

Fig. 6 KEGG enrichment analysis (A: the FCs group; C: the hAECs + TGF-β3 group)

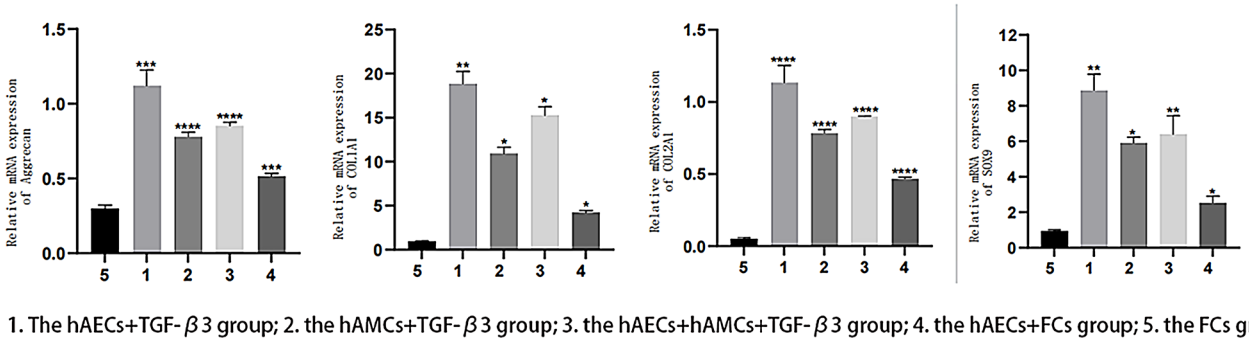

Fig. 7 The expression level of Aggrecan, COL1A1, COL2A1, and SOX9 cartilage-related genes as measured by qRT-PCR (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). (1) The hAECs + TGF-β3 group; (2) the hAMCs + TGF-β3 group; (3) the hAECs + hAMCs + TGF-β3 group; (4) the hAECs + FCs group; (5) the FCs group

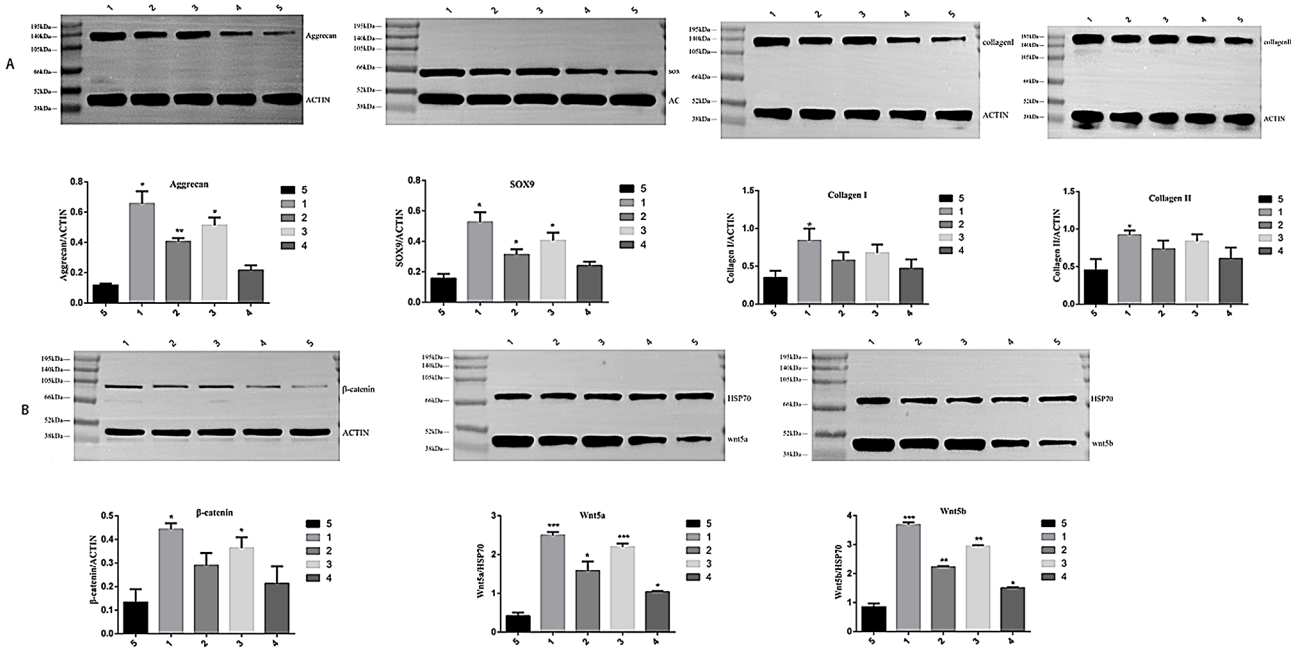

Fig. 8 The expression levels of cartilage-associated proteins (e.g., Aggrecan, COLI, COLII, SOX9) and signaling pathway molecules (e.g., β-catenin, wnt5a, wnt5b) detected by Western blot (*$P<0.05$, **$P<0.01$, ***$P<0.001$). (1) The hAECs + TGF-β3 group; (2) the hAMCs + TGF-β3 group; (3) the hAECs + hAMCs + TGF-β3 group; (4) the hAECs + FCs group; (5) the FCs group

Although the combination of hAECs and TGF-β3 has shown great potential to promote meniscus repair, it cannot be directly applied for human meniscus repair at this stage. From a safety standpoint, the use of hAECs + TGF-β3 in humans requires careful consideration of the safety of the cell source. For example, potential risks such as pathogen contamination in hAECs and the introduction of harmful impurities during extraction and culture must be addressed. Additionally, as a cytokine, excessive or abnormal expression of TGF-β3 could trigger immune responses or lead to unforeseen side effects in surrounding tissues and organs. Currently, our understanding of these potential risks associated with hAECs + TGF-β3 is limited, and further research is necessary for thorough evaluation and validation. Regarding long-term efficacy, existing studies typically have short observation periods, and there is insufficient data to support the prolonged effects of hAECs + TGF-β3 in meniscus repair. Effective meniscus repair should not only address short-term tissue healing but also consider the stability, durability, and potential long-term complications, such as reinjury at the repair site or accelerated joint degeneration. Studies with longer follow-up are needed to accurately assess its true therapeutic value.

This study had several limitations. First, the meniscus repair was not fully realized, which may be related to the quantity, quality, and number of interventions of hAECs. Meanwhile, there is no clear criterion for the optimal number of hAECs, or injection time and frequency, which can only be disposed of by comparing with the relevant literature. Additionally, the results of this experiment cannot be directly applied to the clinic as the in vivo test sample size was small, and rabbits and humans differ in genes, species, and degree of recovery. Moving forward, our team aims to carry out a series of comparative experiments with a larger sample size to determine the ideal quantity, injection timing, and frequency of hAECs. We also plan to compare hAECs with other biological agents and explore additional pathways to broaden the scope and depth of our investigation.

In summary, several key factors must be considered to enhance the efficacy of stem cell-based meniscus repair therapy and broaden its translational application. First, the safety of transplanted stem cells is critical. Long-term in vitro culturing can alter the gene expression profile of autologous stem cells, raising concerns about potential carcinogenic risks and reduced differentiation potential. Second, when using allogeneic stem cells, it is essential to remain vigilant for autoimmune reactions, which could lead to serious clinical outcomes [39]. This study highlights the conversion of hAECs + TGF-β3 into fibrocartilage cells, presenting a promising approach for the biological treatment of meniscal injuries and offering valuable insights for future research on meniscus repair using hAECs.

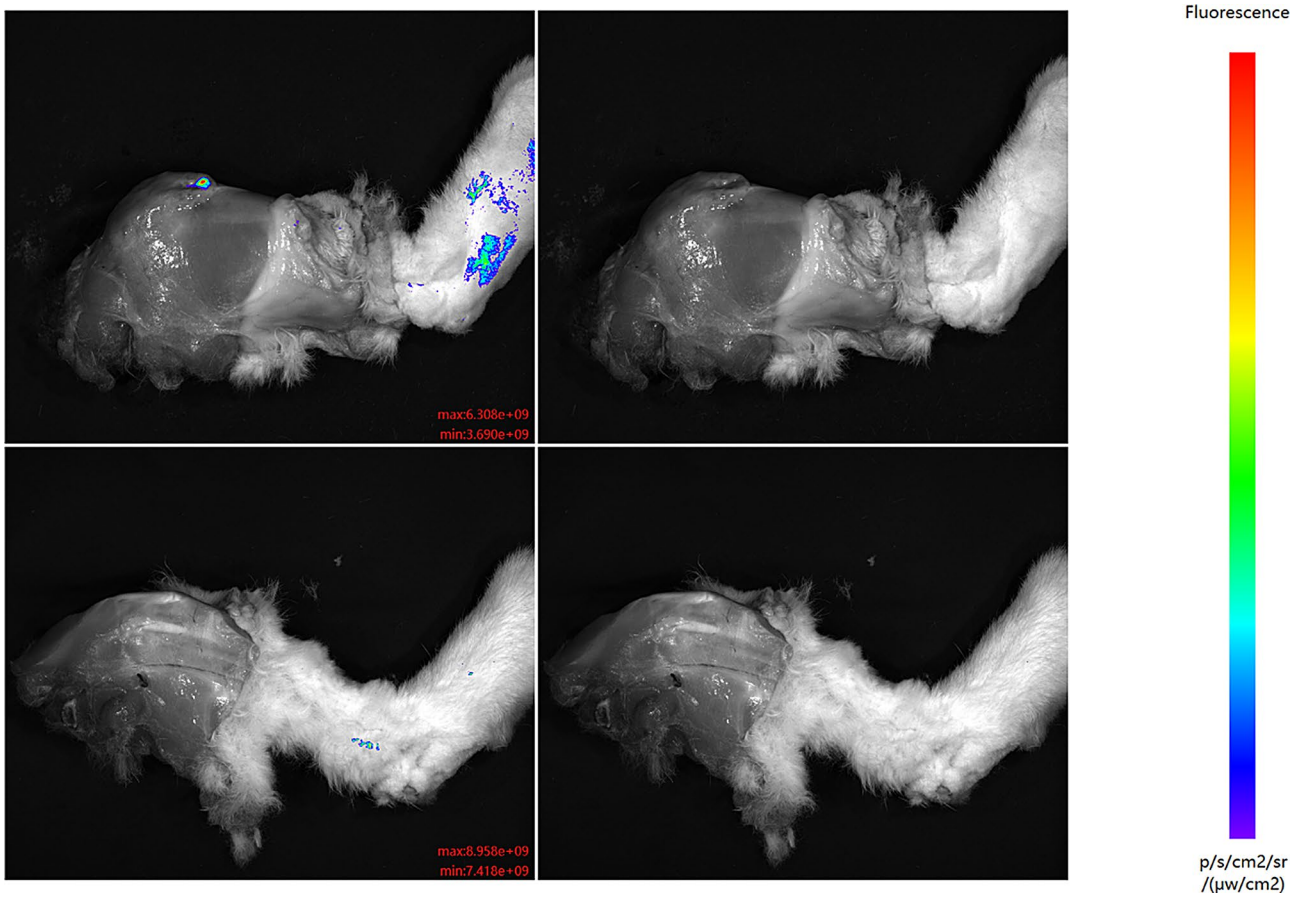

Fig. 9 Tracer detection diagram

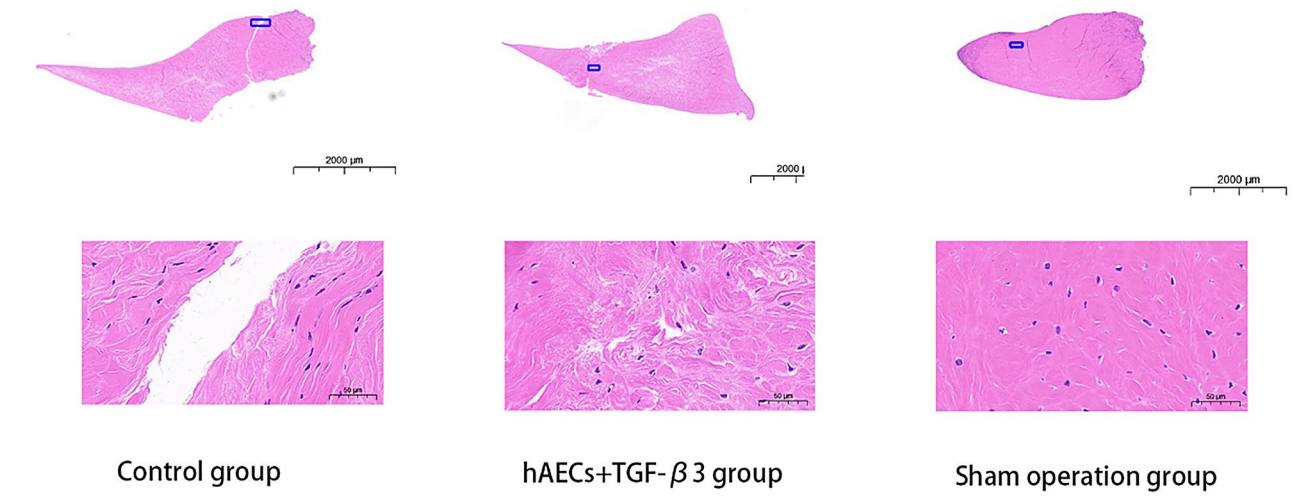

Fig. 10 Representative HE staining images

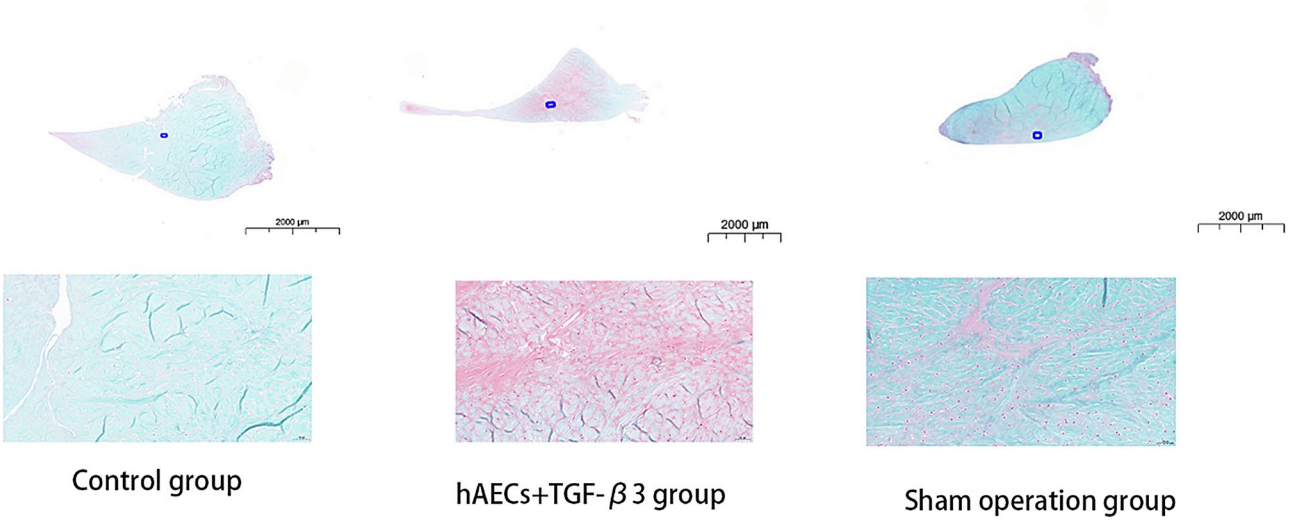

Fig. 11 Results of Safranin O/fast green staining

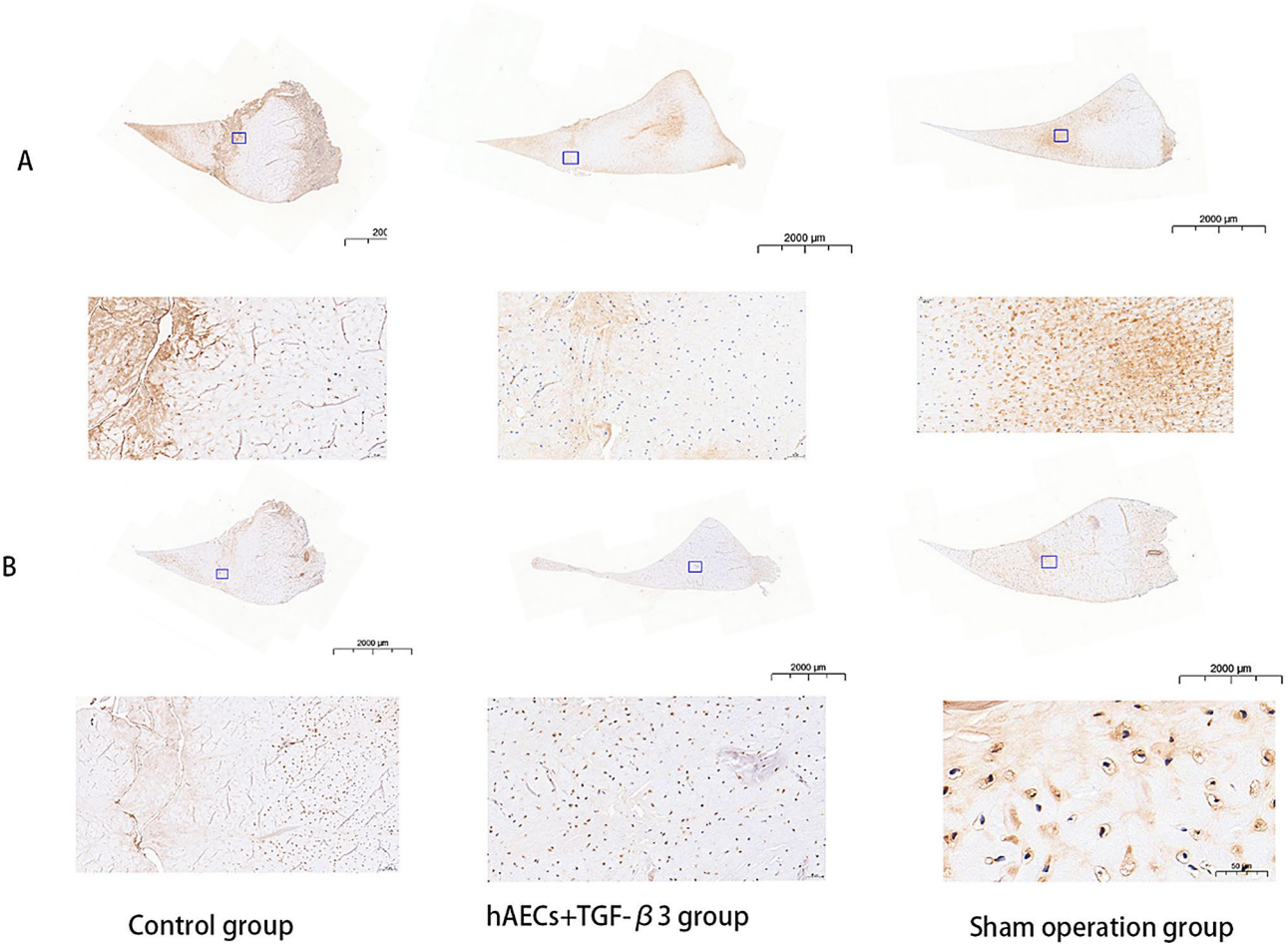

Fig. 12 Immunohistochemical staining results (A: Type I collage; B: Type II collage)

Abbreviations

hAECs	Human amniotic epithelial cells
FC	Fibrocartilage
OA	Osteoarthritis
hAMSCs	Human amniotic mesenchymal stem cells
TGF- β3	Transforming growth factor- β3
PCR	Polymerase chain reaction
ECM	Extracellular matrix
COL2A1	Collagen type Type II Alpha 1 Chain
COL1A1	Collagen type Type I Alpha 1 Chain
AGG	Aggrecan
WB	Western Blot
SOX9	SRY - box transcription factor 9
IHC	Immunohistochemistry
HE	Hematoxylin and Eosin Staining
DEG	Differentially Expressed Gene
KEEG	Kyoto Encyclopedia of Genes and Genomes

Acknowledgements
Not applicable.

Author contributions
Yupeng He: Manuscript-original, data compilation. Ya Li: methodology, formal analysis, investigation. Xiaodong Zhi: Investigation. Yuqiang Zhang: Supervision, project management. Wei Wang: Conceptualization, methodology, financing and acquisition. All authors read and approved the final manuscript.

Funding
This study was supported by the Preclinical study on the application of hAECs (hAECs EXOs) for knee joint repair via tissue engineering regenerative medicine [grant number 2020002]; and the study on the risk correlation of osteoporotic vertebral compression fractures based on radiomics combined with biomarker detection [grant number LJ212410160006].

Data availability
All data generated or analysed during this study are included in this published article.

Declarations

Ethical approval
All animal experiments were carried out according to the ethical policies and the procedures approved by the Animal Committee of Jinzhou Medical University.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 15 January 2025 / Accepted: 22 February 2025
Published online: 10 March 2025

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