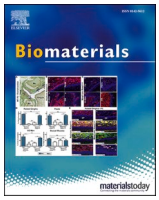
Biomaterials 275 (2021) 120994

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials



Targeted cell therapy for partial-thickness cartilage defects using membrane modified mesenchymal stem cells by transglutaminase 2

Hui Li [a,b], Yuhong Jin [c], Yu Zhao [a,b], Wenjing Li [c], Zihao He [a,b], Qingxi Zhang [a,b], Hesuyuan Huang [a,b], Jianjing Lin [a,b], Yuyang Chen [c], Dan Xing [a,b,***], Yanan Du [c,**], Jianhao Lin [a,b,*]

[a] Arthritis Clinic & Research Center, Peking University People's Hospital, Peking University, Beijing, 100044, China
[b] Arthritis Institute, Peking University, Beijing, 100044, China
[c] Department of Biomedical Engineering, School of Medicine, Tsinghua-Peking Center for Life Sciences, MOE Key Laboratory of Bioorganic Phosphorus Chemistry and Chemical Biology, Tsinghua University, Beijing, 100084, China

ARTICLE INFO

Keywords:
Partial-thickness
Cartilage defects
Transglutaminase 2
Mesenchymal stem cells
Cell membrane modification

ABSTRACT

Unlike full-thickness cartilage defects (FCD), partial-thickness cartilage defects (PCD) may still have residual healthy cartilage tissue, and therefore, the conventional clinical treatments such as microfracture and autologous chondrocyte implantation (ACI) are so traumatic that they may not be the suitable therapies for PCD. Although intra-articular injection of mesenchymal stem cells (MSCs) is a minimally invasive treatment, its therapeutic efficacy is markedly limited due to anoikis caused by failure of cell colonization in the injured area. By modifying a functional polypeptide on the MSC plasma membrane and exploiting the high expression of transglutaminase 2 (TGase2) in the regions of injured cartilage, we achieved targeted recognition and capture of modified MSCs by injured articular chondrocytes (ACs). In the *in vitro* co-culture model, MSCs improved the function of injured ACs and enhanced the chondrogenic differentiation potential of MSCs. Results of *in vitro* study also revealed that the activation of the AKT/mTOR signaling pathway may play an important role in the treatment of injured ACs by MSCs. Further, membrane-modified MSCs exhibited a better therapeutic effect than wide-type MSCs in a rabbit model of PCD. Thus, this unique cell membrane modification strategy provides a new cell-based therapeutic approach for the early treatment of articular cartilage defects and other joint diseases.

1. Introduction

Cartilage defects can be divided into full-thickness cartilage defects (FCD) and partial-thickness cartilage defects (PCD) according to the degree of articular cartilage defects. FCDs generally refer to injured superficial cartilage extending to the deep calcified cartilage, while PCD is limited to superficial cartilage injury [1]. Among the commonly used clinical treatment methods for articular cartilage defects, microfracture and autologous chondrocyte implantation (ACI) are used most commonly [2–4]. However, these methods may not be suitable for treating PCD. During the microfracture surgery or ACI, the entire layer of the articular cartilage is removed from the injured site. However, in PCD, the deep cartilage may not be seriously damaged or may even be healthy. Thus, performing microfracture or ACI for treating PCD may be unreasonable, thereby warranting the invention of more specific and efficient treatment methods.

In recent years, mesenchymal stem cells (MSCs) have been found to play an increasingly important roles in the clinical treatment of several diseases [5–7]. Since they are multi-potent stem cells and have potential to differentiate into cartilage, MSCs can not only act as seed cells to play a direct role in local repair, but also regulate metabolism and immune function through their secretory function. Given their versatility, MSCs may play key roles during different stages of local cartilage repair [8,9]. Moreover, as the articular cavity is a relatively closed space and can be easily injected into, the feasibility of injecting MSCs to treat joint diseases is markedly higher than for treating visceral diseases that require systemic injection of MSCs. Several basic science or clinical studies on the treatment of articular disorder by injecting MSCs have also

* Corresponding author. Arthritis Clinic & Research Center, Peking University People's Hospital, Peking University, Beijing, 100044, China.
** Corresponding author.
*** Corresponding author. Arthritis Clinic & Research Center, Peking University People's Hospital, Peking University, Beijing, 100044, China.
E-mail addresses: xingdan@bjmu.edu.cn (D. Xing), duyanan@tsinghua.edu.cn (Y. Du), linjianhao@pkuph.edu.cn (J. Lin).

https://doi.org/10.1016/j.biomaterials.2021.120994
Received 12 May 2021; Received in revised form 23 June 2021; Accepted 23 June 2021
Available online 27 June 2021
0142-9612/

demonstrated that this cell delivery mode is a potentially safe and reliable method for treating joint diseases, including articular cartilage defects [10,11].

Studies have suggested that PCD is similar to the lesions, such as clefts and fissures, observed in early OA. The lesions of articular cartilage grow deeper and larger during the course of the disease but never repair spontaneously [12,13]. Therefore, the reasons for the lack of self-healing ability of PCD have also been explored. The extracellular matrix of articular cartilage does not provide a favorable environment for the colonization of peripheral synovial and adipose-derived MSCs at the injured area, which may be an important factor attributable to the failure of PCD self-healing [13–16]. In contrast, this phenomenon is not observed in FCD, wherein the articular cartilage is fully damaged and the subchondral bone is exposed, which provides a suitable substrate for MSC adhesion and facilitate their role in tissue repair [12,13,17]. Although intra-articular injection of MSCs expanded *in vitro* can increase the number of cells in the articular cavity, most of the MSCs that fail to colonize the region with PCD undergo anoikis and are removed, thereby not being able to play a sustained role [18]. Thus, determining the tissue characteristics in regions with PCD and developing engineering methods for MSCs to exploit the related characteristics are crucial steps to facilitate sustained MSCs colonization at the injured site, enabling them to exert therapeutic effects.

Transglutaminase 2 (TGase2), an 80-kDa protein with multiple functions, is expressed in many cell types, and its expression level is related to pathological status in several diseases [19,20]. Studies have shown that in joint diseases such as OA, the overexpression of TGase2 in pathological articular chondrocytes (ACs) is related to the disease progression, and is regarded as a new biomarker of OA [21–23]. As one of the pathological models of early OA, the expression of TGase2 in ACs may also change when PCD occurs, thereby warranting further studies in this field. Previously, we covalently modified a peptide containing glutamine (Q)/lysine (K) on the cell membrane of MSCs with high TGase2 expression; TGase2 catalyzed the cross-linking between glutamine side chain (γ-acyl) and lysine side chain (ε-amine) to form an ε (g-glutamyl) lysine isopeptide bond. This modification enabled the specific capture of the modified MSCs by the injured endothelial cells with high expression of TGase2 [24]. In the present study, we explored the high expression levels of TGase2 in injured ACs from PCD sites and further assessed the efficiency of injured ACs to capture modified MSCs *in vitro* and *in vivo*. Based on our results, we anticipate improved targeting and therapeutic effects of modified MSCs in PCD and other joint diseases.

2. Method

2.1. Cell isolation and characterization

The articular cartilage and bone marrow blood samples were obtained from the knee joint and femur of healthy adult New Zealand white rabbits (2.5 kg). The ethics committee of Peking University People's Hospital approved this study (2019PHB098-01). The hyaline cartilage of the knee joint was cut into 1-mm^2 pieces and washed three times with phosphate buffered saline (PBS; Hyclone, USA). Thereafter, the tissue fragments were digested using 0.2% (2 mg/mL) collagenase II (Gibco, USA) at 37 °C for 4–6 h. Single cell suspensions were cultured in complete medium containing 89% DMEM/F12 (1:1) (Hyclone, USA), 10% fetal bovine serum (FBS; Gibco, USA), and 1% penicillin/streptomycin (Hyclone, USA); the medium was changed every 2 days. First-generation ACs were used for subsequent experiments. Bone marrow extracts collected from femurs of healthy rabbits were mixed with complete medium containing 89% α-MEM (Hyclone, USA) supplemented with 10% FBS and 1% penicillin/streptomycin in a 1:1 ratio. The suspension, each containing 5 mL bone marrow, was seeded in 100-mm cell culture dishes. The medium was changed every 2 days. Third-generation bone marrow-derived MSCs were used for subsequent experiments (Supplementary figure 1).

2.2. Preparation of porcine hyaline cartilage and subchondral bone plugs

The plugs, 8 mm in diameter and deep into the subchondral bone, were acquired from the femoral condyle of pigs. Cartilages with full- and partial-thickness were removed from the plugs using a scalpel. The plugs were washed three times with PBS and placed in α-MEM medium for *in vitro* MSC attachment assay. The plugs were randomly selected for histological evaluation to ensure successful establishment of the model.

2.3. Assay for MSC attachment on porcine bone plugs

MSCs (2×10^4) stained with DIO (Sigma, USA) were seeded on the surface of cartilage defects (full- and partial-thickness) and cultured at 37 °C. After 24 h, the samples were washed three times with PBS to remove the nonadherent cells. The number of adherent cells and cell morphology were observed under a fluorescence microscope. The number of cells attached to the surface was calculated in five randomly selected high-power fields (HPFs).

2.4. Generating partial-thickness cartilage defects in vivo

To verify the expression level of TGase2 in chondrocytes of the PCD region and to construct an *in vivo* repair model, we used a scalpel to create PCD in the left and right femoral trochlea of rabbits. The cartilage was removed to expose the mid-deep layer surface; the defect area was 4 mm × 4 mm (Fig. 7A). The depth of the defect was confirmed in randomly selected rabbits. The animals were sacrificed 4 weeks after the operation and the TGase2 expression level in chondrocytes from the PCD region was determined by performing immunohistochemistry.

For the evaluation of therapeutic effects, saline, wide-type MSCs, and TP-modified MSCs (tMSCs, see section 2.5; 4×10^6 cells) prepared *in vitro* were injected intra-articularly 4 weeks after establishing injury model. Samples were retrieved for analysis 8 weeks after intra-articular injection.

2.5. Surface modification of MSCs

The surface modification of MSCs with functional peptides was described, in detail, in our previous study [24]. Briefly, 1×10^5 MSCs were incubated with 200 μL of 1 mM functional targeting peptide (TP; GQLKHLEQQEG) for 25 min at 37 °C; MSCs remained suspended during the incubation period. Subsequently, TP-modified MSCs (tMSCs) were washed three times with PBS. MSCs modified by FITC-labeled TP were imaged using a confocal laser scanning microscope (Leica, Germany) and analyzed by quantitative flow cytometry (BD, USA). Cell viability, cell proliferation, and expression of stemness-related genes were assessed by performing cell viability staining, CCK8 assay, and qPCR, respectively.

2.6. Aggregation model to evaluate the ability of tMSCs to covalently bind injured ACs

Retinoic acid (ATRA) is a widely used TGase activator, which has been proved to stimulate the high expression of TGase2 in chondrocytes [25,26]. To determine the ability of MSCs to covalently bind ACs *in vitro*, we incubated an equal number (1×10^5) of MSCs (wide-type MSCs or tMSCs) and ACs (healthy ACs and ACs injured with ATRA treatment) for 30 min suspension culture in 1.5 mL tubes. MSCs in different states were marked with a green cell tracker (CellTracker Green CMFDA, Thermo Fisher Scientific, USA), while ACs in different states were marked with a red cell tracker (CellTracker Red CMTPX, Thermo Fisher Scientific, USA). Cell aggregation was observed in coverglass bottom dish by a confocal laser scanning microscope. The number of MSCs adhering to ACs was calculated in five randomly selected HPFs.

To explore the ability of PCD regions to capture tMSCs *ex vivo*, cylindrical bone plugs (4 mm diameter) were extracted from the knee joint of rabbits, immediately or 4 weeks post PCD modeling. Thereafter, 5×10^3 wide-type MSCs or tMSCs labeled with red cell tracker (CellTracker Red CMTPX, Thermo Fisher Scientific, USA) were seeded on the defective cartilage surface of bone plugs (at different defect stages) and cultured at 37 °C (Fig. 4D). After 24 h, the samples were washed three times with PBS to remove the nonadherent cells. The number of adherent cells and cell morphology were observed under a confocal laser scanning microscope. The number of cells attached to the surface was calculated in three randomly selected HPFs.

To track the colonization of tMSCs in PCD regions *in vivo*, we injected 4×10^6 wide-type MSCs or tMSCs labeled with red cell tracker (CellTracker CM-DiI, Thermo Fisher Scientific, USA) into the knee joint cavity of rabbits at 4 weeks after PCD modeling. The bone plugs were extracted from the PCD regions at 1, 2, and 4 weeks after cell injection (Fig. 4E), washed three times with PBS, and fixed with 4% paraformaldehyde for 15 min. The number of adherent cells and cell morphology were observed under a confocal laser scanning microscope. The number of cells attached to the surface was calculated in three randomly selected HPFs.

2.7. The in vitro co-culture model of tMSCs and injured ACs

To verify the effect of different numbers of tMSCs on the function of injured ACs, we used a transwell co-culture system for evaluating tMSCs and ACs *in vitro* (Fig. 5B). Different ACs (2×10^5 cells/well) were seeded onto 12-well plates. Thereafter, 1×10^4 or 4×10^4 tMSCs were seeded in transwell chambers (area: 1.1 cm^2). After 6 days of co-culture, ACs in the lower layer were evaluated by performing immunofluorescence staining, alcian blue staining, quantitative analysis of cell glycosaminoglycan (GAG), qPCR analysis of cartilage metabolism-related gene expression, and detecting changes in signaling pathways.

To clarify the effect of injured ACs on the chondrogenic differentiation potential of tMSCs, we introduced MSC pellet [27] and constructed a transwell co-culture model of tMSC pellet and ACs *in vitro* (Fig. 5F). tMSCs (5×10^5 cells) were pre-pelleted in the upper layer chambers and injured ACs (2×10^5 cells/well) were seeded onto 12-well plates. After 21 days of co-culture, pellets were evaluated by performing histological analysis and GAG quantitative analysis and subjected to qPCR for detecting the expression of cartilage metabolism-related genes.

3. Results

3.1. MSCs exhibit differential adhesion to FCD and PCD

To simulate the *in-situ* environment of cartilage defects, porcine bone plugs were employed to establish *ex vivo* models of FCD and PCD surfaces, as confirmed by Safranine O and Fast Green double staining (Fig. 1A–B). Results of the cell adhesion assay showed that more DIO-stained MSCs adhered to the surface of FCD than PCD; moreover, MSCs spread better on FCD surface. Accordingly, the number of MSCs adhered on the surface of PCD was relatively low (Fig. 1C–D). These findings highlighted the following clinical challenges: 1. MSCs lack the ability to achieve optimal colonization on PCD [12,13]; and 2. Improving the targeting ability of MSCs to colonize PCD is essential for their use as therapeutic agents.

3.2. TGase2 expression in the PCD region, injured ACs, and MSCs

As a bridge between the PCD region and targeted capture of MSCs, the expression level of TGase2 in the PCD region, chondrocytes cultured *in vitro*, and MSCs must be assessed. Accordingly, we determined the expression level of TGase2 in the ACs of PCD regions by performing immunohistochemistry. The level of TGase2 in ACs of PCD regions was significantly higher than in ACs from healthy regions (Fig. 2A). To simulate high TGase2 expression in ACs obtained from the PCD region, we used ATRA (100 nM, a TGase2 activator) treatment *in vitro* to induce injured ACs as described previously [25,26]. ATRA treatment significantly enhanced the expression of TGase2 in ACs at the protein as well as mRNA levels. Furthermore, the elevated TGase2 level in ACs treated by ATRA was significantly inhibited by the TGase2 inhibitor, cystamine (Fig. 2B and D). Finally, TGase2 expression in MSCs, which was the prerequisite for TP modification of the MSC membrane, was confirmed at the protein and mRNA levels; moreover, cystamine reduced the expression level of TGase2 in MSCs (Fig. 2C and E).

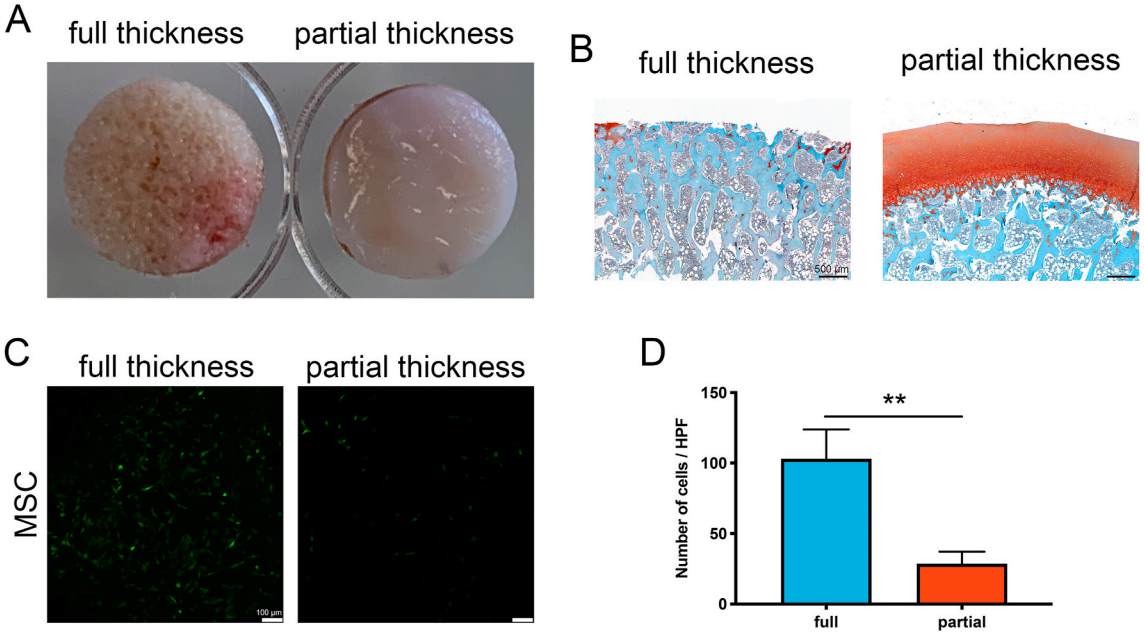


Fig. 1. Difference in MSCs adhesion between FCD and PCD. (A) FCD and PCD in porcine bone plugs. (B) Safranine O and Fast Green double staining of porcine bone plugs. (C) MSCs stained with Dio adhered to the surface of FCD or PCD. (D) Quantitative results of MSCs adhesion (**P < 0.01). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

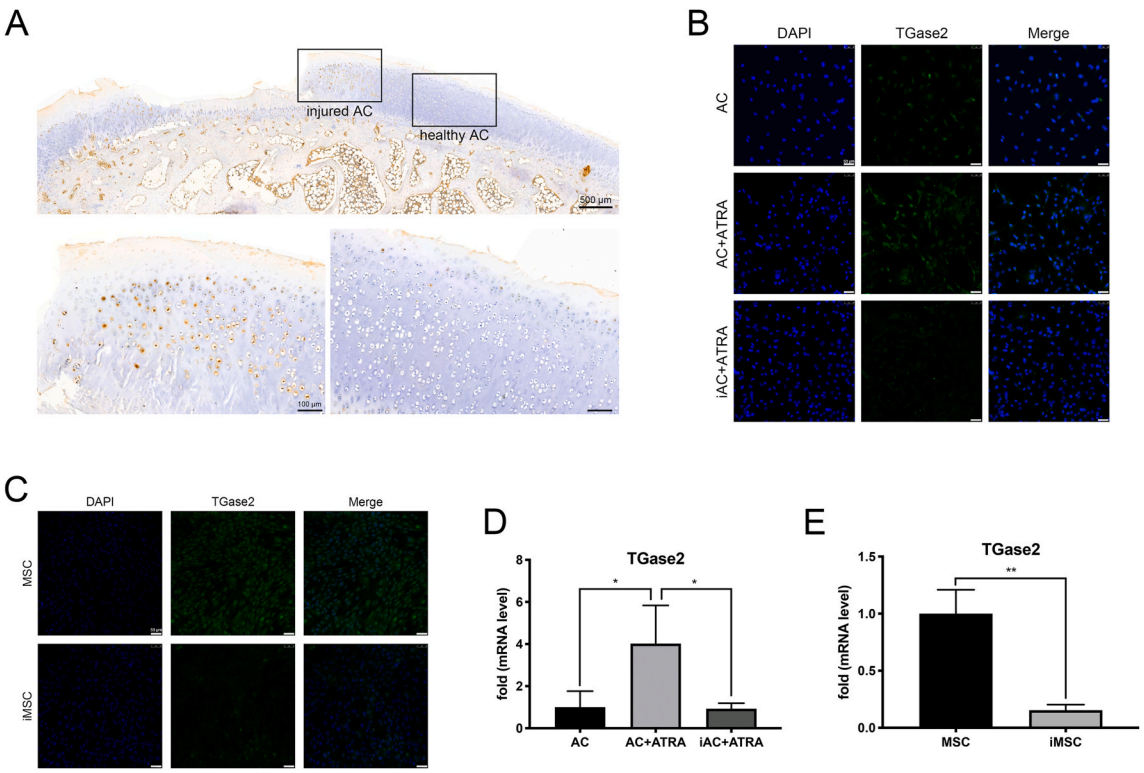


Fig. 2. The level of TGase2 in PCD area, ACs and MSCs. Cells treated with cystamine are labeled iAC or iMSC in figure. (A) The level of TGase2 in ACs from PCD areas or healthy hyaline cartilage areas in rabbit femur. (B) TGase2 level of ACs induced by ATRA *in vitro*. (C) TGase2 level of MSCs *in vitro*. (D) The mRNA level of TGase2 in ACs induced by ATRA *in vitro* (*P < 0.05). (E) The mRNA level of TGase2 in MSCs (**P < 0.01).

3.3. Characterization of TGase2-mediated cell membrane modification

Using a functional peptide with multiple glutamine (Q) or lysine (K) residues at both ends, termed "targeting peptide" (TP, GQLKHLEQQEG), we modified the cell membrane of MSCs. TP can be sequentially divided into two parts: the modifying part and the functional part. The γ-acyl on the side chain of glutamine (Q) in the modifying part can form a covalent thioester intermediate with the thiol group in TGase2 on the MSC surface. Further, the naturally-expressed substrate protein (SP) on the MSC surface contains lysine (K) residues, and the ε-amine on its side chain can react with the covalent thioester intermediate to form the ε (g-glutamyl) lysine isopeptide bond and release TGase2 (Fig. 3 A and 4 A). TP and FITC-labeled TP were successfully modified on the surface of rabbit bone marrow-derived MSCs using our method [24]. In fact, modifying MSCs with 1 mM FITC-labeled TP for 25 min did not induce significant peptide endocytosis (Fig. 3B) and ensured high modification efficiency (Fig. 3C). This is exactly the optimal modification time and TP concentration that we optimized in the previous study [24]. Moreover, analysis of cell viability, cell proliferation, and expression of genes related to stemness revealed no significant changes in the characteristics of tMSCs (Fig. 3D–F). In general, our membrane modification method successfully modified TP on the MSC surface and had little effect on cell viability, proliferation, and stemness.

3.4. Characterization of covalently aggregated injured ACs and tMSCs

After confirming that ACs cultured *in vitro* can be induced to overexpress TGase2 similar to cells in the PCD region, we verified the enhanced cell-cell adhesion between tMSCs and ACs *in vitro*. Similar to the process of TP being modified on the surface of MSC, as long as the AC surface has high expression of TGase2, the substrate protein on the AC surface containing lysine or glutamine can be crosslinked with the functional part of TP under the catalysis of TGase2 on the AC surface (Fig. 4A). First, wide-type MSCs or tMSCs were incubated with different ACs (healthy ACs and ATRA-treated ACs) for 30 min. Compared to other groups, tMSCs adhered to ATRA-treated ACs more effectively to form the largest cell cluster. Due to the limited expression of TGase2 in healthy ACs, the ability of tMSCs to form cell clusters with healthy ACs was far less than that of ATRA-treated ACs. In addition, MSCs without TP modification did not adhere to ACs (Fig. 4B–C).

After confirming the enhanced adhesion between tMSCs and ACs overexpressing TGase2 *in vitro*, we verified the adhesion of tMSCs to PCD regions *ex vivo*. Four weeks after injury (4 w post injury in Fig. 4F–G) or after fresh injury, cylindrical bone plugs (4 mm diameter) containing the injured regions were extracted to analyze the adhesion ability of wide-type MSCs and tMSCs in the PCD region (Fig. 4D). Our results showed that only tMSCs adhered extensively to the surface of bone plugs with high TGase2 expression at 4 weeks post injury; however, a lesser number of wide-type MSCs colonized the surface of bone plugs. Nonetheless, no significant difference were observed in the adhesion ability of MSCs and tMSCs on the surface of freshly injured cartilage tissue (healthy hyaline cartilage in the mid-deep layer), and adhesion ability of both cell types was significantly lower than that of tMSCs targeting 4 w post injury plugs with high TGase2 levels (Fig. 4F–G).

To further track the time-dependent changes in tMSC colonization in PCD region *in vivo*, we injected wide-type MSCs or tMSCs into the articular cavity 4 weeks after cartilage injury, and retrieved bone plugs at 1, 2, and 4 weeks after cell therapy to assess the number of colonized cells (Fig. 4E). At 1 week after intra-articular injection, the number of tMSCs in the region of cartilage injury was much higher than that of MSCs. Although the number of colonized MSCs and tMSCs decreased significantly when the period of treatment was extended, the extent of tMSC adhesion in the injured area remained significantly greater than that of MSC. Notably, after 4 weeks of cell therapy, wide-type MSCs were almost absent, while a few of tMSCs still remained at the injured site (Fig. 4H–I).

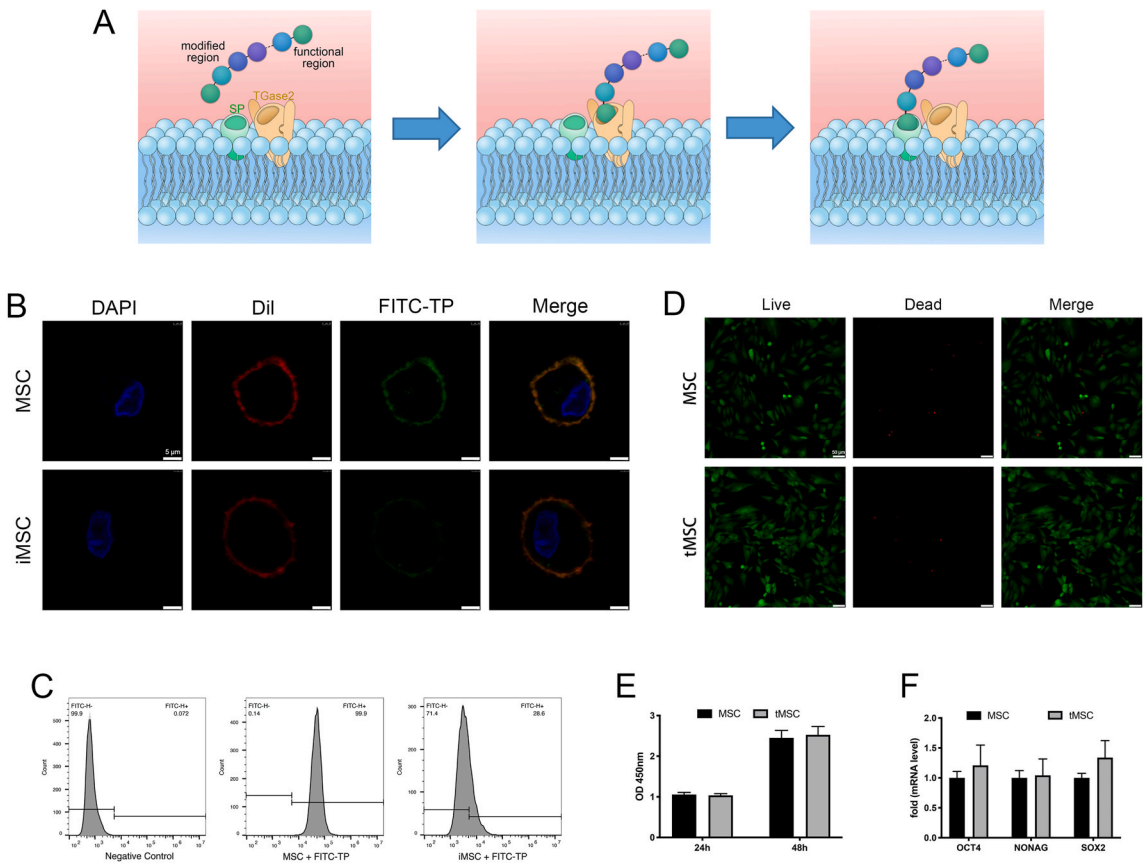


Fig. 3. Characterization of TGase2-mediated cell membrane modification. (A) Schematics of TGase2 mediated cell membrane modification process. TP can be divided into two parts: the modifying part and the functional part. The modifying part of TP can form a covalent intermediate with TGase2 on the MSC surface. The substrate protein on the MSC surface can react with the covalent intermediate to form a covalent bond and release TGase2. (B) Confocal images of FITC-labeled TP conjugation on MSCs. (C) Characterization of modification efficiency by flow cytometry analysis. (D) Cell viability staining of MSCs or tMSCs after 48 h cell culture. (E) CCK8 analysis of MSCs or tMSCs after 24 or 48 h cell culture. (F) Relative mRNA level of stemness-related genes in MSCs or tMSCs after 48 h cell culture.

These results showed that tMSCs can target ACs with high TGase2 expression *in vitro*, *ex vivo*, and *in vivo*. Further, the tMSCs colonized PCD regions for a longer duration than wide-type MSCs.

3.5. Functional changes in injured ACs and tMSCs after co-culture

The results of Safranine O and Fast Green double staining showed that the components of the cartilage matrix were more degenerated in the region with PCD than in the healthy region (Fig. 5A). Meanwhile, ATRA-treated ACs with high TGase2 expression also displayed changes in cartilage matrix metabolism including downregulation of anabolism-related genes (i.e., *COL2A1* and *ACAN*) and up-regulation of catabolism-related genes (i.e., *TIMP1* and *MMP13*) (Fig. 5E). To determine whether tMSCs could improve the cartilage matrix metabolism disorder of injured ACs *in vitro*, we used a transwell co-culture system for culturing tMSCs and ACs together (Fig. 5B). Alcian blue staining of cartilage matrix, quantitative analysis of cell GAG, and qPCR analysis of cartilage metabolism marker genes revealed that at low-as well as high-density, tMSCs could improve the pathological phenotype of injured ACs in a co-culture environment, with high density of tMSCs exhibiting better effects than low density (Fig. 5C–E). In addition, no significant differences were observed in the therapeutic effects mediated by tMSCs and wide-type MSCs (Supplementary Fig. 2A and B).

MSCs have the potential to differentiate into ACs, which is another way for MSCs to play a role in cartilage protection *in vivo*. In fact, previous studies have shown that OA chondrocytes can enhance the chondrogenic differentiation potential of MSCs [28]. Therefore, we further explored the effect of injured ACs, with high TGase2 expression, on the chondrogenic differentiation potential of tMSCs. In the transwell co-culture system, tMSCs were fabricated into pellets and placed in the upper layer (Fig. 5F). In the chondrogenic induction environment, pellets showed a better chondrogenic effect when co-cultured with injured ACs. Based on the results of pellet tissue section analysis, GAG quantitative analysis, and cartilage metabolic marker gene analysis, tMSC pellet co-culture with injured ACs exhibited a better effect than the tMSC pellet alone (Fig. 5G–I). Further, no significant differences were observed in the chondrogenic differentiation potential of tMSCs and wide-type MSCs when co-cultured with injured ACs (Supplementary Fig. 2C–D).

Thus, the matrix metabolism of ACs and the chondrogenic differentiation ability of tMSCs were improved when they were co-cultured; this synergy may play important roles in the therapeutic application of tMSCs *in vivo*.

3.6. Changes in the signaling pathway of injured ACs after co-culture with tMSCs

To further evaluate the molecular mechanism underlying tMSC-mediated improvement in matrix metabolism of ACs, a phosphor-specific protein microarray comprising 304 phosphoprotein antibodies was used to screen related signaling pathways. Among the evaluated pathways, the phosphorylation status of proteins involved in the AKT/mTOR signaling pathway was modulated significantly (Fig. 6A–C). Subsequently, western blotting was performed to verify these significant changes and identify representative protein molecules of the AKT/mTOR signaling pathway. The results of Western blot were consistent

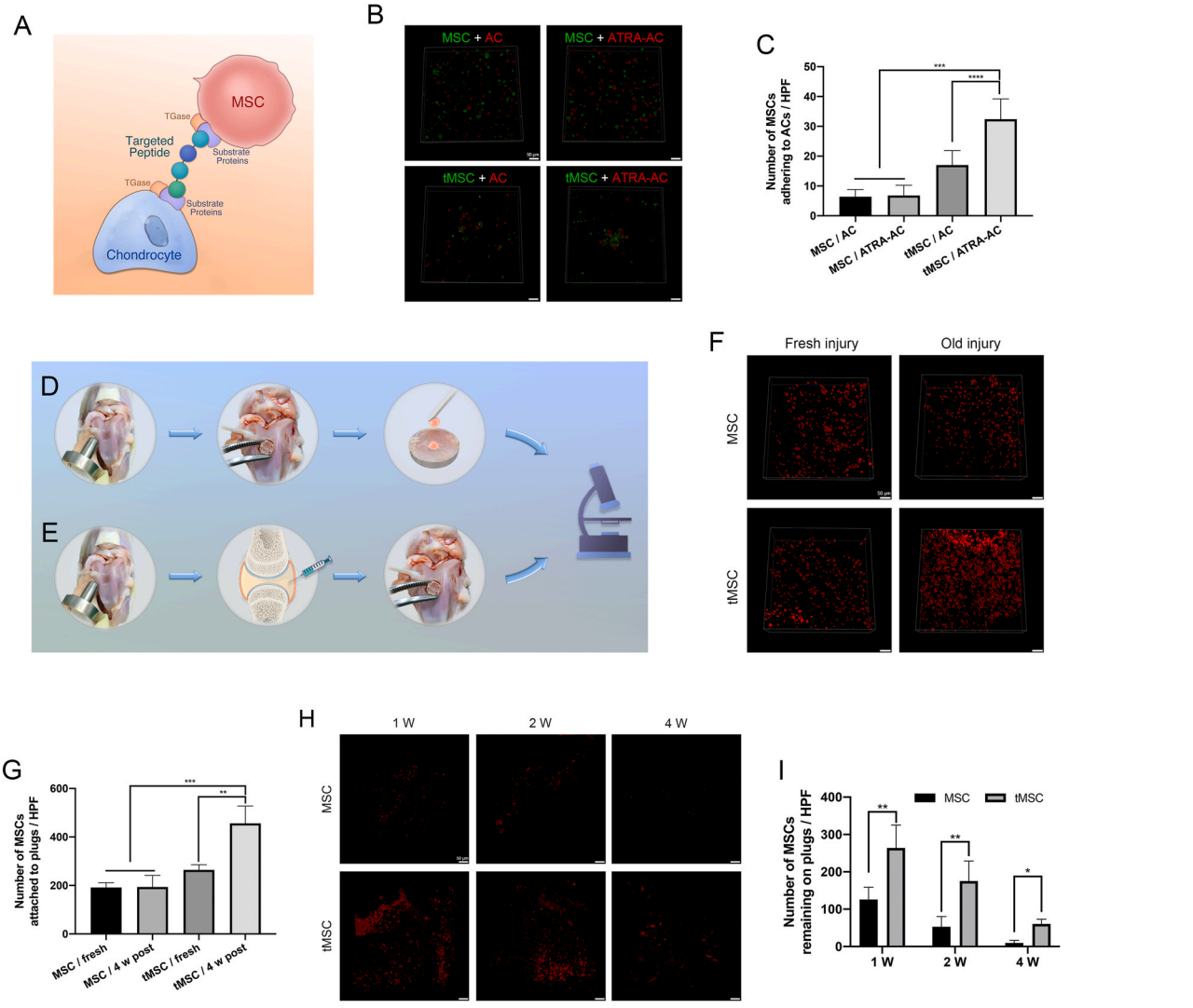


Fig. 4. Characterization of covalent aggregation of injured ACs and tMSCs. (A) Schematics of cell-cell adhesion between tMSCs and ACs. AC surface has high expression of TGase2, the substrate protein on the AC surface containing lysine or glutamine can be crosslinked with the functional part of TP under the catalysis of TGase2 on the AC surface. (B) Confocal images of cell-cell adhesion between tMSCs (green cell tracker labeled) and ACs (red cell tracker labeled). (C) Quantitative results of MSCs adhering to ACs *in vitro* (***P < 0.001, ****P < 0.0001). (D–E) Schematics of MSCs attached on PCD areas. (F) Confocal images of MSCs (red cell tracker labeled) adhering to PCD areas at different defect stages (fresh injury or four weeks after injury (4 w post injury in the figure)). (G) Quantitative results of MSCs (red cell tracker labeled) adhering to PCD areas *ex vivo* (**P < 0.01, ***P < 0.001). (H) Confocal images of MSCs remaining on PCD areas at different time points (1 w, 2 w and 4 w). (I) Quantitative results of MSCs remaining on PCD areas *in vivo* (*P < 0.05, **P < 0.01). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

with the microarray screening results. At low- and high-density, tMSCs inhibited the abnormal phosphorylation of AKT and mTOR in a dose-dependent manner. These results were in line with the previous studies, which reported that inhibition of abnormal AKT/mTOR signaling activation could protect chondrocytes [29–31]. In our study, results of Western blot analysis showed that the abnormal phosphorylation of FOXO 1/3/4 (downstream of AKT), and eIF4EBP1 (downstream of mTOR) could be inhibited by tMSCs (Fig. 6D–E); injured ACs were also treated with MK2206 (an AKT inhibitor) and rapamycin (an mTOR inhibitor) and inhibition of abnormal activation of AKT and mTOR was used as a positive control for tMSCs in the treatment of injured ACs (Fig. 6D–E).

3.7. Protective and therapeutic effect of tMSC on PCD in vivo

After confirming the targeting and therapeutic effects of tMSCs on injured ACs *in vitro*, we used tMSCs to treat partial cartilage injury *in vivo*. Saline, wide-type MSCs, and tMSCs were injected into the articular cavity of the knee joint at 4 weeks after the establishment of the PCD model. Samples were retrieved for analysis at 8 weeks after treatment (n = 6). After operation and during treatment, all animals recovered well without signs of pain or claudication, and no postoperative complications were observed.

For macroscopic evaluation of the therapeutic effects of tMSC injection on PCD, we evaluated the gross specimens according to the International Cartilage Regeneration and Joint Preservation Society (ICRS) macroscopic assessment scoring criteria. The injury site subjected to an intra-articular tMSC injection was relatively flat, and the depth of injury was not significantly deeper than that of the site with fresh injury site. Although the injured area remained visible, its boundary with healthy cartilage was relatively blurred. However, a difference was observed in the depth of the cartilage injury sites treated with wide-type MSCs; some parts were evidently damaged with enhanced deepening and the boundary with surrounding healthy cartilage was relatively clear. As a negative control, the injured area injected with saline showed progressive injury aggravation. Further, subchondral bone was exposed

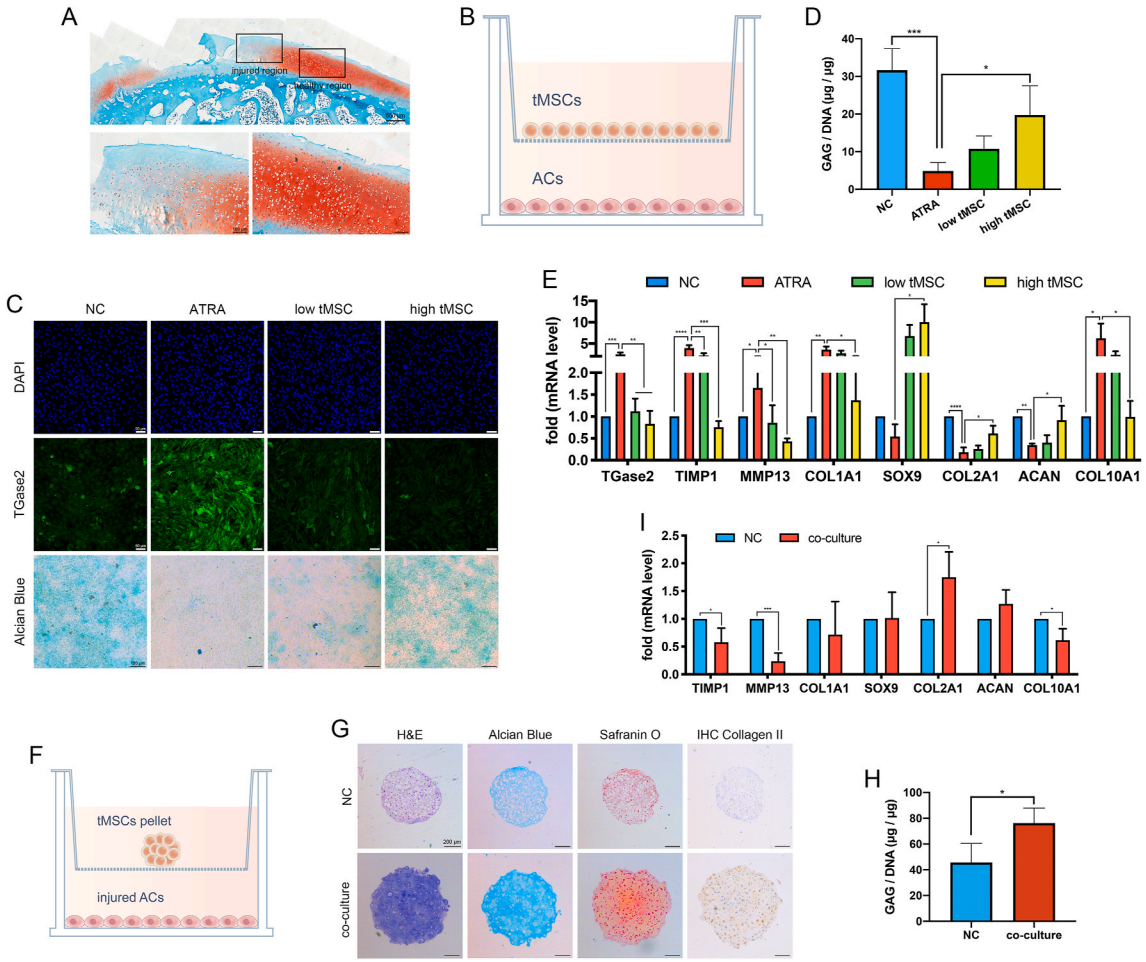


Fig. 5. Functional changes of injured ACs and tMSCs after co-cultivation. The use of low and high density tMSCs in the treatment of ATRA induced ACs was labeled as low tMSCs and high tMSCs in figure. (A) Safranine O and Fast Green double staining results of injured area and healthy area. (B) Schematics of co-culture system of tMSCs and ACs. (C) TGase2 and Alcian Blue staining results of ACs after different treatments. (D) Quantitative analysis of GAG in ACs after different treatments (*P < 0.05, ***P < 0.001). (E) The mRNA level of cartilage metabolism related genes in ACs after different treatments (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001). (F) Schematics of co-culture system of tMSCs pellet and ACs. (G) Histological (H&E, Alcian Blue and Safranine O staining) and immunohistochemistry (collagen I and II) analysis of tMSCs pellet. (H) Quantitative analysis of GAG in tMSCs pellet (*P < 0.05). (I) The mRNA level of cartilage metabolism related genes in tMSCs pellet (*P < 0.05, ***P < 0.001). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in some areas; however, in areas where the subchondral bone was not exposed, the tissue condition differed from that of healthy hyaline cartilage (Fig. 7B). The ICRS macroscopic assessment scores were significantly higher for the tMSC treatment group than for the other two groups, which could delay the progressive cartilage degeneration after PCD (Fig. 7D).

The histological and immunohistochemical changes at the injury site were assessed following different treatments. Based on the results of Safranine O and Fast Green double staining, Alcian blue staining, and type 2 collagen immunohistochemistry, the thickness of the cartilage in the tMSC-treated PCD region was lesser than that in the healthy area; however, the ECM component of hyaline cartilage appeared relatively normal. The expression level of type I collagen was relatively increased in the injured region, indicating the formation of fibrocartilage when tMSCs played a protective role (Fig. 7C). However, treatment with wide-type MSCs caused evident changes in the injured area, such as the degeneration of ECM components and the reduction of cartilage thickness. In the saline-treated PCD area, progressive degeneration was observed. Although the subchondral bone was exposed, the injured surface was covered with a thin layer of fibrocartilage-like tissue. Results of the ICRS histological score provide convincing evidence that intra-articular injection of tMSCs has a strong potential for treating PCD (Fig. 7D).

4. Discussion

Here, we used a cell membrane modification method to functionalize the plasma membrane of rabbit bone marrow-derived MSCs and utilized the pathological characteristic of high TGase2 expression in PCD regions to achieve targeted MSC delivery and treatment of cartilage defects by MSCs. To the best of our knowledge, this is the first study to achieve targeted treatment of cartilage defects by directly modifying MSCs.

MSCs display poor colonization ability at the sites of PCD [12, 13, 17] and efforts are being made to enhance the adhesion ability of MSCs at cartilage defect sites [32]. However, so far, studies have mostly focused on identifying biomaterials with stem cell recruitment properties for use in the defect area [33, 34]. Although this method can recruit MSCs from the articular cavity or bone marrow, it requires additional surgery for biomaterial implantation and thus, can increase the trauma further. This feature is the same as that of ACI, making it unsuitable for treating mild PCD. Moreover, it is difficult to determine the specific type and number of stem cells that can be recruited *in vivo*, which makes it difficult to explore the mechanism underlying the therapeutic effects. In addition, implanting adhesive biomaterials loaded with cells, such as hydrogels, at the defect sites can also induce local cell aggregation [35–38]. However, the cells cannot proliferate in the hydrogel, and may suffer damage during injection and cross-linking. These limitations

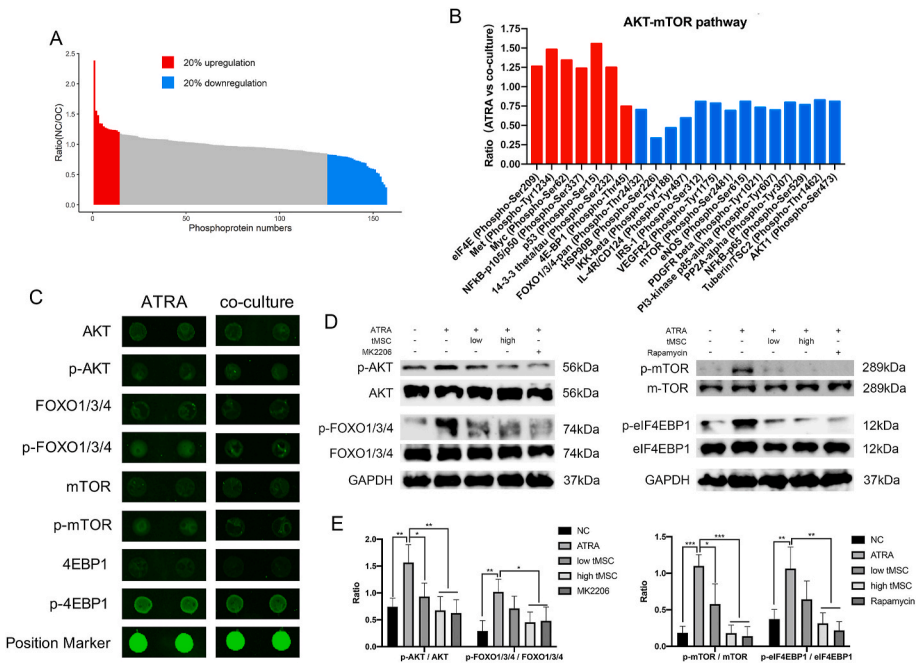


Fig. 6. Changes of signaling pathway in injured ACs after co-cultivation with tMSCs. (A) Phosphoproteome analysis of phosphoproteins changes in injured ACs and high density tMSCs treated ACs. Proteins with phosphorylation levels increased or decreased by more than 20% were labeled red and blue, respectively. (B) Phosphorylation changes in AKT-mTOR signaling pathway. (C) Representative protein in phospho-antibody array. (D) Western blot analysis results for the levels of p-AKT, AKT, p-FOXO 1/3/4, FOXO 1/3/4, p-mTOR, mTOR, p-eIF4EBP1, eIF4EBP1 and GAPDH at 6 days after treatment. (E) The quantitative assay of p-AKT/AKT, p-FOXO 1/3/4/FOXO 1/3/4, p-mTOR/mTOR and p-eIF4EBP1/eIF4EBP1 (*P < 0.05, **P < 0.01, ***P < 0.001). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

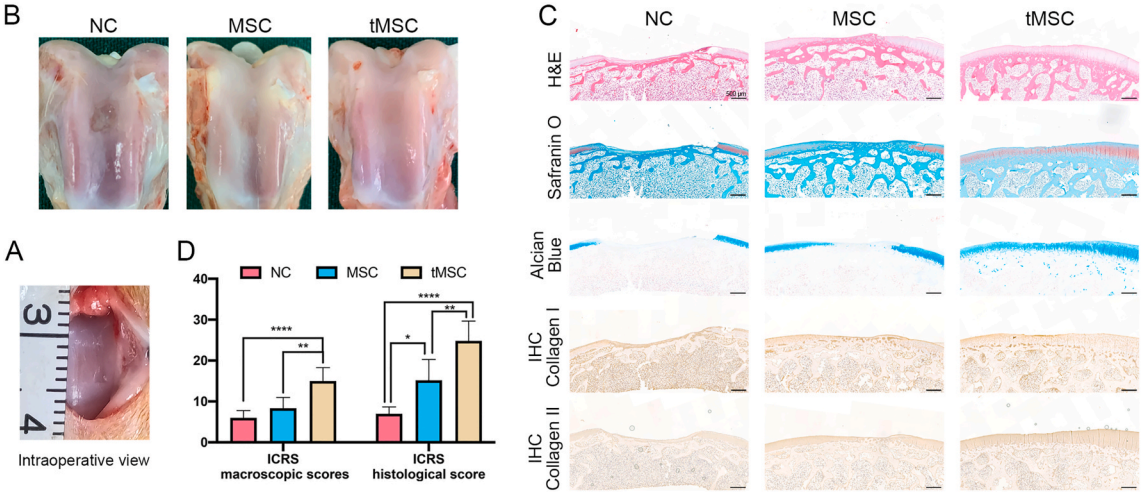


Fig. 7. Protective effect of MSC or tMSC on PCD *in vivo*. (A) Intraoperative view of constructing PCD. (B) The effect of macroscopic repair after 8 weeks of MSC or tMSC treatment. (C) Histological (H&E, Alcian Blue and Safranine O staining) and immunohistochemistry (collagen I and II) analysis after 8 weeks of MSC or tMSC treatment. (D) ICRS macroscopic evaluation and histological scoring after different treatments (*P < 0.05, **P < 0.01, ****P < 0.0001). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

restrict the suitability of this method for delivering MSCs at the target sites.

In contrast to the conventional strategies, in the present study, we directly modified the cell membrane of MSCs to achieve flexibility in the type and dose of therapeutic cells and bypass the need for cell implantation through surgery. As a new mode of cell transformation, the main application of cell membrane engineering is to achieve cell-targeted transport [39–41]. Cells subjected to direct membrane engineering differ from specific condition-stimulated cells [42,43] and genetically modified cells [44]. Direct cell membrane engineering exerts minor effects on cell function and, to the greatest extent, avoids the deleterious effects caused by changes in cell function. Previous studies, aimed at improving the targeting of MSCs via membrane modification, increased the amount of carbohydrate moieties of the sialyl Lewisx (sLex) on the surface of MSCs via a streptavidin-biotin bridge [45] and α-1,3-fucosyltransferase induction [46], so that during systemic injection, MSCs could be recognized and captured by inflammatory vascular cells overexpressing selectin. However, the immune response and cytotoxicity of these membrane modification methods limit their application. In contrast, our natural enzymatic membrane modification method follows simple operation process, is amenable to easy quality control, and causes little damage to cells. Moreover, this method showed better targeting effect of MSCs in a mouse models of ear inflammation and liver injury [24]; the present study provides evidence of the efficacy of this method in treating PCD.

Since MSCs can target the PCD region, it is important to explore their therapeutic effects and underlying mechanisms in injured areas. In previous studies, efforts to explore the therapeutic role of MSCs *in vivo* were mainly focused on two aspects: differentiation of stem cells into chondrocytes to enable cell replacement [47]; or the improving secretory function of stem cells to repair the defect area [48]. However, there must be more than one mechanism for MSCs to play a therapeutic role *in*

vivo. Few studies have systematically evaluated the repair mechanism of MSCs. Herein, we only explored whether tMSCs could change the ECM metabolic disorder of ACs through their secretory function, but also characterized the enhanced chondrogenic differentiation potential of tMSCs in an effort to interpret the multifaceted therapeutic role of tMSCs from different perspectives. Furthermore, we verified the altered signaling pathways in injured ACs after treatment with tMSCs, and identified tMSCs as the potential molecular therapeutic agents for PCD.

In the present study, we report, for the first time, the satisfactory targeted therapeutic effects of MSCs on PCD via cell membrane modification. Similar methods and strategies should be developed to achieve cell-targeted therapy in the field of cartilage defects and other joint diseases. Although in this study we used only modified MSCs, other cell subsets with better specificity for cartilage repair, such as skeletal stem cells [49] and cartilage progenitor cells [50], should be considered for similar cell membrane modification to aid in the treatment of cartilage defect. Furthermore, despite the unique advantages of intra-articular cell injection therapy, the specific application of modified MSCs in the treatment of joint diseases should not be limited to PCD. The potential of MSC modification to achieve improved therapeutic effect in other joint diseases including OA, meniscus injury, and ligament injury, should be assessed in the future studies.

5. Conclusion

Herein, we observed that MSCs do not colonize the injured PCD site easily. As a result, we used a cell membrane modification method to achieve targeted colonization of modified MSCs in the PCD region. In addition, we verified the therapeutic role and molecular mechanism of membrane modified MSCs at the site of injury and confirm their therapeutic effect *in vivo*. To the best of our knowledge, this is the first study to directly modify the MSC membrane for cartilage repair.

Funding

This study was supported by grants from the National Natural Science Foundation of China (81,973,606), Peking University People's Hospital Scientific Research Development Funds (RS2019-03, RDY2019-11), Peking University Health and Science Center Innovation Funds (BMU2020BSS001) and Beijing Science and Technology Major Projects (Z181100001818008).

Credit author statement

Conception and design: Yanan Du and Jianhao Lin. Implementation the experiments: Hui Li, Yuhong Jin, Yu Zhao and Zihao He. Analysis and interpretation of data: all authors. Drafting of the article: Hui Li and Dan Xing. Critical revision of the article for important intellectual content: all authors. Final approval of the article: all authors.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biomaterials.2021.120994.

References

[1] E.J. Jansen, P.J. Emans, L.W. Van Rhijn, S.K. Bulstra, R. Kuijer, Development of partial-thickness articular cartilage injury in a rabbit model, Clin. Orthop. Relat. Res. 466 (2008) 487–494.
[2] J.R. Steadman, W.G. Rodkey, J.J. Rodrigo, Microfracture: surgical technique and rehabilitation to treat chondral defects, Clin. Orthop. Relat. Res. (2001) S362–S369.
[3] G. Knutsen, J.O. Drogset, L. Engebretsen, T. Grontvedt, T.C. Ludvigsen, S. Loken, et al., A randomized multicenter trial comparing autologous chondrocyte implantation with microfracture: long-term follow-up at 14 to 15 years, J. Bone Jt. Surg. Am. Vol. 98 (2016) 1332–1339.
[4] L. Batty, S. Dance, S. Bajaj, B.J. Cole, Autologous chondrocyte implantation: an overview of technique and outcomes, ANZ J. Surg. 81 (2011) 18–25.
[5] A. Trounson, C. McDonald, Stem cell therapies in clinical trials: progress and challenges, Cell Stem Cell 17 (2015) 11–22.
[6] C.R. Nitkin, T.L. Bonfield, Concise review: mesenchymal stem cell therapy for pediatric disease: perspectives on success and potential improvements, Stem Cells Transl Med 6 (2017) 539–565.
[7] M. Dave, P. Jaiswal, F. Cominelli, Mesenchymal stem/stromal cell therapy for inflammatory bowel disease: an updated review with maintenance of remission, Curr. Opin. Gastroenterol. 33 (2017) 59–68.
[8] C. De Bari, A.J. Roelofs, Stem cell-based therapeutic strategies for cartilage defects and osteoarthritis, Curr. Opin. Pharmacol. 40 (2018) 74–80.
[9] D. Confalonieri, A. Schwab, H. Walles, F. Ehlicke, Advanced therapy medicinal products: a guide for bone marrow-derived MSC application in bone and cartilage tissue engineering, Tissue Eng. B Rev. 24 (2018) 155–169.
[10] D. Xing, J. Wu, B. Wang, W. Liu, W. Liu, Y. Zhao, et al., Intra-articular delivery of umbilical cord-derived mesenchymal stem cells temporarily retard the progression of osteoarthritis in a rat model, Int J Rheum Dis 23 (2020) 778–787.
[11] J.A. McIntyre, I.A. Jones, B. Han, C.T. Vangsness Jr., Intra-articular mesenchymal stem cell therapy for the human joint: a systematic review, Am. J. Sports Med. 46 (2018) 3550–3563.
[12] E.B. Hunziker, L.C. Rosenberg, Repair of partial-thickness defects in articular cartilage: cell recruitment from the synovial membrane, J. Bone Jt. Surg. Am. Vol. 78 (1996) 721–733.
[13] E.B. Hunziker, E. Kapfinger, Removal of proteoglycans from the surface of defects in articular cartilage transiently enhances coverage by repair cells, J Bone Joint Surg Br 80 (1998) 144–150.
[14] K. Lewandowska, H.U. Choi, L.C. Rosenberg, L. Zardi, L.A. Culp, Fibronectin-mediated adhesion of fibroblasts: inhibition by dermatan sulfate proteoglycan and evidence for a cryptic glycosaminoglycan-binding domain, J. Cell Biol. 105 (1987) 1443–1454.
[15] G. Schmidt, H. Robenek, B. Harrach, J. Glossl, V. Nolte, H. Hormann, et al., Interaction of small dermatan sulfate proteoglycan from fibroblasts with fibronectin, J. Cell Biol. 104 (1987) 1683–1691.
[16] A.M. Rich, E. Pearlstein, G. Weissmann, S.T. Hoffstein, Cartilage proteoglycans inhibit fibronectin-mediated adhesion, Nature 293 (1981) 224–226.
[17] W. Zhang, J. Chen, J. Tao, Y. Jiang, C. Hu, L. Huang, et al., The use of type 1 collagen scaffold containing stromal cell-derived factor-1 to create a matrix environment conducive to partial-thickness cartilage defects repair, Biomaterials 34 (2013) 713–723.
[18] D.J. Mooney, H. Vandenburgh, Cell delivery mechanisms for tissue repair, Cell Stem Cell 2 (2008) 205–213.
[19] M.V. Nurminskaya, A.M. Belkin, Cellular functions of tissue transglutaminase, Int Rev Cell Mol Biol 294 (2012) 1–97.
[20] A. Facchiano, F. Facchiano, Transglutaminases and their substrates in biology and human diseases: 50 years of growing, Amino Acids 36 (2009) 599–614.
[21] J.L. Huebner, K.A. Johnson, V.B. Kraus, R.A. Terkeltaub, Transglutaminase 2 is a marker of chondrocyte hypertrophy and osteoarthritis severity in the Hartley Guinea pig model of knee OA, Osteoarthritis Cartilage 17 (2009) 1056–1064.
[22] U. Tarantino, A. Ferlosio, G. Arcuri, L.G. Spagnoli, A. Orlandi, Transglutaminase 2 as a biomarker of osteoarthritis: an update, Amino Acids 44 (2013) 199–207.
[23] M. Adamczyk, Transglutaminase 2 in cartilage homoeostasis: novel links with inflammatory osteoarthritis, Amino Acids 49 (2017) 625–633.
[24] C. Qi, Y. Jin, Y. Chen, W. Li, Y. Li, K. Liang, et al., TGase-mediated cell membrane modification and targeted cell delivery to inflammatory endothelium, Biomaterials (2020) 120276.
[25] K.A. Johnson, D. van Etten, N. Nanda, R.M. Graham, R.A. Terkeltaub, Distinct transglutaminase 2-independent and transglutaminase 2-dependent pathways mediate articular chondrocyte hypertrophy, J. Biol. Chem. 278 (2003) 18824–18832.
[26] K.A. Johnson, R.A. Terkeltaub, External GTP-bound transglutaminase 2 is a molecular switch for chondrocyte hypertrophic differentiation and calcification, J. Biol. Chem. 280 (2005) 15004–15012.
[27] H. Jing, X. Zhang, K. Luo, Q. Luo, M. Yin, W. Wang, et al., miR-381-abundant small extracellular vesicles derived from kartogenin-preconditioned mesenchymal stem cells promote chondrogenesis of MSCs by targeting TAOK1, Biomaterials 231 (2020) 119682.
[28] A. Aung, G. Gupta, G. Majid, S. Varghese, Osteoarthritic chondrocyte-secreted morphogens induce chondrogenic differentiation of human mesenchymal stem cells, Arthritis Rheum. 63 (2011) 148–158.
[29] X. Huang, B. Ni, Z. Mao, Y. Xi, X. Chu, R. Zhang, et al., NOV/CCN3 induces cartilage protection by inhibiting PI3K/AKT/mTOR pathway, J. Cell Mol. Med. 23 (2019) 7525–7534.
[30] J. Chen, R. Crawford, Y. Xiao, Vertical inhibition of the PI3K/Akt/mTOR pathway for the treatment of osteoarthritis, J. Cell. Biochem. 114 (2013) 245–249.
[31] J.F. Xue, Z.M. Shi, J. Zou, X.L. Li, Inhibition of PI3K/AKT/mTOR signaling pathway promotes autophagy of articular chondrocytes and attenuates inflammatory response in rats with osteoarthritis, Biomed. Pharmacother. 89 (2017) 1252–1261.
[32] F.M. Chen, L.A. Wu, M. Zhang, R. Zhang, H.H. Sun, Homing of endogenous stem/progenitor cells for in situ tissue regeneration: promises, strategies, and translational perspectives, Biomaterials 32 (2011) 3189–3209.

[33] E.S. Place, N.D. Evans, M.M. Stevens, Complexity in biomaterials for tissue engineering, Nat. Mater. 8 (2009) 457–470.
[34] H. Huang, X. Zhang, X. Hu, Z. Shao, J. Zhu, L. Dai, et al., A functional biphasic biomaterial homing mesenchymal stem cells for in vivo cartilage regeneration, Biomaterials 35 (2014) 9608–9619.
[35] M.M. Hasani-Sadrabadi, P. Sarrion, S. Pouraghaei, Y. Chau, S. Ansari, S. Li, et al., An engineered cell-laden adhesive hydrogel promotes craniofacial bone tissue regeneration in rats, Sci. Transl. Med. 12 (2020).
[36] X. Xu, X. Xia, K. Zhang, A. Rai, Z. Li, P. Zhao, et al., Bioadhesive hydrogels demonstrating pH-independent and ultrafast gelation promote gastric ulcer healing in pigs, Sci. Transl. Med. 12 (2020).
[37] J. Shin, J.S. Lee, C. Lee, H.J. Park, K. Yang, Y. Jin, et al., Tissue adhesive catechol-modified hyaluronic acid hydrogel for effective, minimally invasive cell therapy, Adv. Funct. Mater. 25 (2015) 3814–3824.
[38] J. Xu, Q. Feng, S. Lin, W. Yuan, R. Li, J. Li, et al., Injectable stem cell-laden supramolecular hydrogels enhance in situ osteochondral regeneration via the sustained co-delivery of hydrophilic and hydrophobic chondrogenic molecules, Biomaterials 210 (2019) 51–61.
[39] M.T. Stephan, D.J. Irvine, Enhancing cell therapies from the outside in: cell surface engineering using synthetic nanomaterials, Nano Today 6 (2011) 309–325.
[40] S.V. Kalinin, B.J. Rodriguez, S. Jesse, K. Seal, R. Proksch, S. Hohlbauch, et al., Towards local electromechanical probing of cellular and biomolecular systems in a liquid environment, Nanotechnology 18 (2007) 424020.
[41] D. Sarkar, W. Zhao, A. Gupta, W.L. Loh, R. Karnik, J.M. Karp, Cell surface engineering of mesenchymal stem cells, Methods Mol. Biol. 698 (2011) 505–523.
[42] I. Rosova, M. Dao, B. Capoccia, D. Link, J.A. Nolta, Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells, Stem Cell. 26 (2008) 2173–2182.
[43] R. Khatiwala, C. Cai, Strategies to enhance the effectiveness of adult stem cell therapy for ischemic heart diseases affecting the elderly patients, Stem Cell Rev Rep 12 (2016) 214–223.
[44] Z. Wang, Z. Wu, Y. Liu, W. Han, New development in CAR-T cell therapy, J. Hematol. Oncol. 10 (2017) 53.
[45] R. Sackstein, J.S. Merzaban, D.W. Cain, N.M. Dagia, J.A. Spencer, C.P. Lin, et al., Ex vivo glycan engineering of CD44 programs human multipotent mesenchymal stromal cell trafficking to bone, Nat. Med. 14 (2008) 181–187.
[46] D. Sarkar, P.K. Vemula, G.S. Teo, D. Spelke, R. Karnik, Y. Wee le, et al., Chemical engineering of mesenchymal stem cells to induce a cell rolling response, Bioconjugate Chem. 19 (2008) 2105–2109.
[47] H. Yin, Y. Wang, Z. Sun, X. Sun, Y. Xu, P. Li, et al., Induction of mesenchymal stem cell chondrogenic differentiation and functional cartilage microtissue formation for in vivo cartilage regeneration by cartilage extracellular matrix-derived particles, Acta Biomater. 33 (2016) 96–109.
[48] E. Mianehsaz, H.R. Mirzaei, M. Mahjoubin-Tehran, A. Rezaee, R. Sahebnasagh, M. H. Pourhanifeh, et al., Mesenchymal stem cell-derived exosomes: a new therapeutic approach to osteoarthritis? Stem Cell Res. Ther. 10 (2019) 340.
[49] M.P. Murphy, L.S. Koepke, M.T. Lopez, X. Tong, T.H. Ambrosi, G.S. Gulati, et al., Articular cartilage regeneration by activated skeletal stem cells, Nat. Med. 26 (2020) 1583–1592.
[50] Y. Jiang, R.S. Tuan, Origin and function of cartilage stem/progenitor cells in osteoarthritis, Nat. Rev. Rheumatol. 11 (2015) 206–212.