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## The transplantation of particulated juvenile allograft cartilage and synovium for the repair of meniscal defect in a lapine model



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## ABSTRACT

**Background:** Synovium has been confirmed to be the primary contributor to meniscal repair. Particulated Juvenile Allograft Cartilage (PJAC) has demonstrated promising clinical effect on repairing cartilage. The synergistic effect of synovium and PJAC transplant on meniscal fibrocartilaginous repair is unclear. We hypothesize that the transplantation of synovium and PJAC synergistically facilitates meniscal regeneration and the donor cells within graft tissues still survive in the regenerated tissue at the last follow up (16 weeks postoperatively).

**Methods:** The study included 24 mature female rabbits, which were randomly divided into experimental and control groups. A cylindrical full-thickness defect measuring 2.0 mm was prepared in the avascular portion of the anterior horn of medial meniscus in both knees. The synovium and PJAC transplant were harvested from juvenile male rabbits (2 months after birth). The experimental group received synovium and PJAC transplant encapsulated with fibrin gel. The control groups received synovium transplant encapsulated with fibrin gel, pure fibrin gel and nothing. The macroscopic, imageological and histological evaluations of repaired tissue were performed at 8 weeks and 16 weeks postoperatively. The in situ hybridization (ISH) of male-specific sex-determining region Y-linked (SRY) gene was performed to detect the transplanted cells.

**Results:** The regenerated tissue in experimental group showed superior structural integrity, superficial smoothness, and marginal integration compared to control groups at 8 weeks or 16 weeks postoperatively. More meniscus-like fibrochondrocytes filled the repaired tissue in the experimental group, and the matrix surrounding these cell clusters demonstrated strongly positive safranin O and type 2 collagen immunohistochemistry staining. By SRY gene ISH, the positive SRY signal of experimental group could be detected at 8 weeks (75.72%, median) and 16 weeks (48.69%, median). The expression of SOX9 in experimental group was the most robust, with median positive rates of 65.52% at 8 weeks and 67.55% at 16 weeks.

**Conclusion:** The transplantation of synovium and PJAC synergistically facilitates meniscal regeneration. The donor cells survive for at least 16 weeks in the recipient.

**The translational potential of this article:** This study highlighted the positive effect of PJAC and synovium transplant on meniscal repair. We also clarified the potential repair mechanisms reflected by the survival of donor cells and upregulated expression of meniscal fibrochondrocytes related genes. Thus, based on our study, further clinical experiments are needed to investigate synovium and PJAC transplant as a possible treatment to meniscal defects.

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## 1. Introduction

Menisci, the semilunar wedges of fibrocartilaginous tissue are located between the femoral condyle and tibial plateau. The menisci play a critical role in load transmission, load bearing, lubrication and shock absorption during femorotibial articulation and knee movements [1]. Previous epidemiological studies have demonstrated that meniscal injuries were the most frequent knee joint-related lesions, occurring across a wide range of age groups ranging from children, young adults, elderly individuals, as well as the general population, and athletes [2,3]. In clinic, many previous studies have reported that the injuries in the anterior horn of medial meniscus could cause knee pain, clicking sensation, crepitus or even cartilage abrasion if without timely treatment [4–6]. Moreover, meniscal repair is limited to meniscal lesions, occurring in the peripheral vascular zone. However, the reported failure rates after attempted meniscal repair remain high, ranging from 24% to 50% for isolated meniscal tears [7]. Meniscectomy still remains to be the prevailing treatment for meniscal injuries in the inner avascular zone due to its limited spontaneous healing capacity [8]. The biomechanical imbalance in knee joint after meniscectomy accelerates articular cartilage degeneration or even leads to osteoarthritis (OA) [9]. However, the current therapeutic idea for meniscal injuries is to facilitate meniscal repair, retain meniscal structural integrity, prevent secondary cartilage degeneration and improve knee joint function. Thus, new technique designed to facilitate meniscal repair is urgent.

Two decades ago, synovial tissue has been demonstrated to possess the potential to repair meniscal injuries [10–14]. Cisa J et al. [10] studied the efficacy of pedunculated synovial flap transfer in repairing longitudinal incision in the avascular zone of meniscus in a lapine model. They concluded that three quarters of the menisci showed healing with vascularization of an originally avascular zone. A previous clinical study demonstrated that all 7 patients who received transplantation of vascularized synovial pedicle flaps to the suture site of meniscal tears in avascular zone showed healing [13]. Moreover, WooYoung Kim et al. [15] clarified that synovial tissue was the primary contributor to meniscal repair rather than residual meniscal tissue by the *in vivo* freeze-thaw method. They found that meniscus-like fibrochondrocytes and proteoglycan filled the meniscal defect only in the condition of intact synovium. Recently, the application of synovium-derived stem cells (SDSCs) on meniscal repair or tissue engineering have been investigated, due to its self-renewal and multipotent differentiating capacities [16,17]. Previous studies have demonstrated that SDSCs possessed superior chondrogenic capacity than other mesenchymal tissues-derived stem cells [18,19]. Many preclinical studies covering small and large animal models with duration ranging from 4 weeks to 6 months also clarified the efficacy of transplantation of autologous or allogenic SDSCs on repairing meniscal lesions [20–22], reflecting the superior capacity of cellular component of synovium in repairing meniscal injuries.

The particulated juvenile allograft cartilage (PJAC) has emerged as a therapeutic method for cartilage defects. This technique necessitates that the PJAC fragments should be harvested from donors under 13 years and stored at low temperature. Then, a mixture of PJAC fragments and fibrin glue was applied to cartilage defects [23]. The available commercial PJAC product, such as DeNovo NT, has been applied to many clinical cases and acquired promising clinical therapeutic effects [24,25]. A 2-year follow-up prospective study demonstrated that knee function improved significantly from the perspective of clinic, radiology and histology after transplantation of DeNovo NT PJAC product into knee cartilage defects [26]. PJAC has also been applied to repair cartilage defects of other joints, such as hip and ankle, and achieved good repair outcomes [27,28]. This technique relies heavily on the ability of juvenile chondrocytes [23]. One previous animal study utilizing minipig knee cartilage defect model concluded that the transplanted cells in PJAC survived for at least 3 months and expressed more higher Sry-type HMG-box 9 (SOX9) compared to autologous cartilage chips transplant [29].

Previous studies have indicated that the meniscus and articular cartilage shared a lot of similarities, including development, vascularization, innervation, composition and functions. From the perspective of development stage, vascularization and innervation, the meniscus and cartilage are all derived from a condensation of mesenchymal cells within the intermediate layer, called interzone cells [30]. Besides partial vascularization and innervation in the peripheral, the meniscus is a tissue without blood vessels and nerves like articular cartilage [31]. In terms of cell property and phenotype, the cells in avascular zone of meniscus demonstrated oval to round shaped and displayed a distinct cell-associated matrix including a mass of cartilaginous type 2 collagen and a lower but remarkable, quantity of type 1 collagen and aggrecan [32]. For articular chondrocytes, the chondrocytes also exhibited oval to round shape, and displayed rich type 2 collagen [33]. In terms of matrix composition [34], the meniscus is mainly composed of water (72% of the wet weight of mature meniscus), collagens (22% of the wet weight) and proteoglycans (1–2% of dry weight), the articular cartilage is mainly constituted with water (65–80% of wet weight), collagens (10–20% of wet weight) and proteoglycans (10–15% of wet weight). In terms of biomechanics and function, the meniscus and cartilage are all weight-bearing tissues with considerable compressive and aggregate modulus. Moreover, they share similar functions of load transmission and distribution [33,35]. Thus, PJAC should hold therapeutic potential for repairing meniscal avascular zone injuries. To our knowledge, there is no study that transplants PJAC for meniscal avascular zone repair.

For small rodent model, the robust innate regenerative potential restricts its efficacy on comparing experimental and control groups [36]. Large animal models, such as porcine and goat, have similar physiological conditions and knee joint size like humans [37], however, the high cost and complexity in operation make it difficult to ensure sufficient sample size. However, the limited inherent regenerative potential of lapine meniscus [38] compared to smaller rodents as well as low cost in feeding compared to larger animals make the rabbit an ideal animal model in investigating meniscal regeneration techniques. Thus, the lapine meniscal avascular zone defect model was applied in our study.

Previous studies indicated the transforming growth factor-beta (TGF- $\beta$ ), a key growth factor in chondrogenesis, produced by chondrocytes could facilitate the chondrogenesis process of mesenchymal stem cells (MSCs) [39] and the MSC-derived exosomes promoted proliferation and inhibited apoptosis of chondrocytes [40]. Herein, we transplanted synovium and PJAC into lapine meniscal avascular zone defect to investigate their synergistic efficacy on repairing meniscal injuries. We hypothesize that the transplantation of synovium and PJAC synergistically facilitates meniscal regeneration and the donor cells within graft tissues still survive in the regenerated tissue at the last follow up (16 weeks postoperatively).

## 2. Materials and methods

### 2.1. Study design and surgical procedure

This study was approved by the Institutional Laboratory Animal Ethics Committee of Peking university third hospital (NO. A2021154), and all experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. In order to distinguish the transplanted cells from host cells, we harvested synovium and PJAC transplant from male rabbits and transplanted them into meniscal defects of female hosts, then *in situ* hybridization for male-specific sex-determining region Y-linked (SRY) gene was performed to detect transplanted cells [41,42]. Thus, the study included 24 mature female New Zealand white rabbits (total of 48 knees). The following groups were included in the present study: Blank group, in which nothing was filled into the meniscal defect; Fibrin gel group (FG group), in which only fibrin gel was filled into the meniscal defect; Fibrin gel and synovium transplant group (FG + synovium group), in which synovium fragments encapsulated with fibrin gel were filled into the

meniscal defect; Fibrin gel, synovium and particulated juvenile allograft cartilage transplant group (FG + synovium + PJAC group), in which synovium fragments and PJAC encapsulated with fibrin gel were filled into the meniscal defect. The designed sample size for each group was 6 knees (total of 48 knees in 24 rabbits). Animals were sacrificed at 8 and 16 weeks postoperatively. We performed macroscopic, imageological and histological evaluations to determine the efficacy of synovium and PJAC transplantation in repairing meniscal defects and the donor cell fate (Fig. 1).

The animals were administered with xylazine hydrochloride (2 mg/kg) through intramuscular injection and placed in a supine position. The standard skin preparation and disinfection procedures were performed after general anesthesia. Then, a standard medial parapatellar approach was performed for all knees in each group to dislocate the patella. The medial collateral ligament was resected to expose the anterior horn of medial meniscus entirely. Then, a cylindrical full-thickness (approximate 1.0 mm) defect measuring 2.0 mm in diameter was prepared with corneal trephine in the avascular portion of the anterior horn of medial meniscus in both knees. For Blank group, nothing was filled into the defect; For FG group, only fibrin gel was filled into the meniscal defect; For FG + synovium group, synovium fragments encapsulated with fibrin gel were filled into the meniscal defect; For FG + synovium + PJAC group, synovium fragments and PJAC encapsulated with fibrin gel were filled into the meniscal defect (Fig. 2). For each animal, the two knees were treated with the same group. The medial collateral ligament was fixed with 2–0 suture. The incision was closed with a continuous suture. All surgeries were completed by the same person (W.Y.). The animals were

administered with penicillin sodium once a day for 1 week to prevent infection and were allowed to move freely in a comfortable environment.

## 2.2. Synovium and PJAC preparation and transplantation

In the present study, the synovium and PJAC transplant were harvested from juvenile male rabbits. Two juvenile male rabbits (2 months after birth) were euthanized by excessive anesthesia with intramuscular injection of xylazine hydrochloride. The knees were harvested under sterile condition. The juvenile articular cartilage was peeled off from distal femur with scalpel. The thickness of harvested cartilage block was approximate 0.5 mm. Then, the cartilage block was divided 1.0 mm interval according to the dividing rule to guarantee the homogeneity (Fig. 2). The PJAC fragments of about  $0.5 \text{ mm}^3$  were prepared with a scalpel and stored aseptically in a sterile 50 mL centrifuge tube, in which 5 mL  $\alpha$ -Minimum Essential Medium ( $\alpha$ -MEM) was added previously. The synovium was harvested from suprapatellar bursa, medial inner wall and lateral inner wall. The remaining processes were identical to the preparation of PJAC. The volume of divided synovium tissue was approximate  $0.1 \text{ mm}^3$ . They were stored in a refrigerator at  $4^\circ \text{C}$  to maintain tissue activity. The preparation proportion of fibrin gel was follows: fibrinogen (500  $\mu\text{L}$ , 50 mg/mL),  $\text{CaCl}_2$  (20  $\mu\text{L}$ , 50 mmol/L), and thrombin (500  $\mu\text{L}$ , 500 IU/mL) were separately prepared for further use. When needed, the 3 reagents alone, together with synovium fragments or plus PJAC were mixed, and then transplanted into the meniscal defects (Fig. 2). For FG group, the volume of fibrin gel was approximate  $3.14 \text{ mm}^3$ . For FG + Synovium group, two synovial tissue block (approximate  $0.2 \text{ mm}^3$ ) and approximate  $3 \text{ mm}^3$  fibrin gel were mixed and then transplanted into the meniscal defect. For FG + Synovium + PJAC group, two synovial tissue block (approximate  $0.2 \text{ mm}^3$ ), two PJAC tissue block (approximate  $1.0 \text{ mm}^3$ ) and approximate  $2 \text{ mm}^3$  fibrin gel were mixed and then transplanted into the meniscal defect. After transplantation, the knee joint was flexed and extended for 10 times to ensure the transplant was retained at the defect.

## 2.3. Sample collection and processing

Both entire left and right knees with skin integrity were harvested at 8 and 16 weeks postoperatively. The knee images were acquired by 3.0-T MRI (SIEMENS) (T1 weighted image, T1 WI). MRI scans demonstrating the maximal cross section of the meniscal defect in the sagittal plane were analyzed. After separating extra soft tissue, the distal femur was removed, while maintaining the menisci and tibia. The menisci were photographed for subsequent macroscopic evaluation. After fixation in 4% paraformaldehyde for 2 days, the menisci were cut into axial-plane segments passing through the central area of the defect. Then, the samples were embedded in paraffin after dehydration in graded ethanol solution. Subsequently,  $3 \mu\text{m}$ -thick paraffin sections were prepared by using a microtome (Leica) for histological evaluation. The prepared sections were stained using hematoxylin-eosin (HE), toluidine blue, Safranin O-Fast green, Picrosirius Red, nucleic acid in situ hybridization (ISH) and immunohistochemical stains for types 1 and 2 collagen, aggrecan and Sry-type HMG-box 9 (SOX9). The stained slices of HE, toluidine blue, Safranin O-Fast green and immunohistochemical stains for types 1 and 2 collagen, aggrecan and SOX9 were captured by a digital slide scanner (NanoZoomer, Hamamatsu). The stained slices of ISH were scanned by confocal microscope (The exact scanning parameters were demonstrated in the following ISH section). The stained slices of Picrosirius Red were analyzed by polarization microscope.

## 2.4. Meniscal repair scoring

The meniscal repair scoring system described by Zellner et al. [43] was applied for semiquantitative evaluation of healing outcomes of meniscal defects. The macroscopic and histological features of repaired meniscal tissue were included in the scoring system. Macroscopic

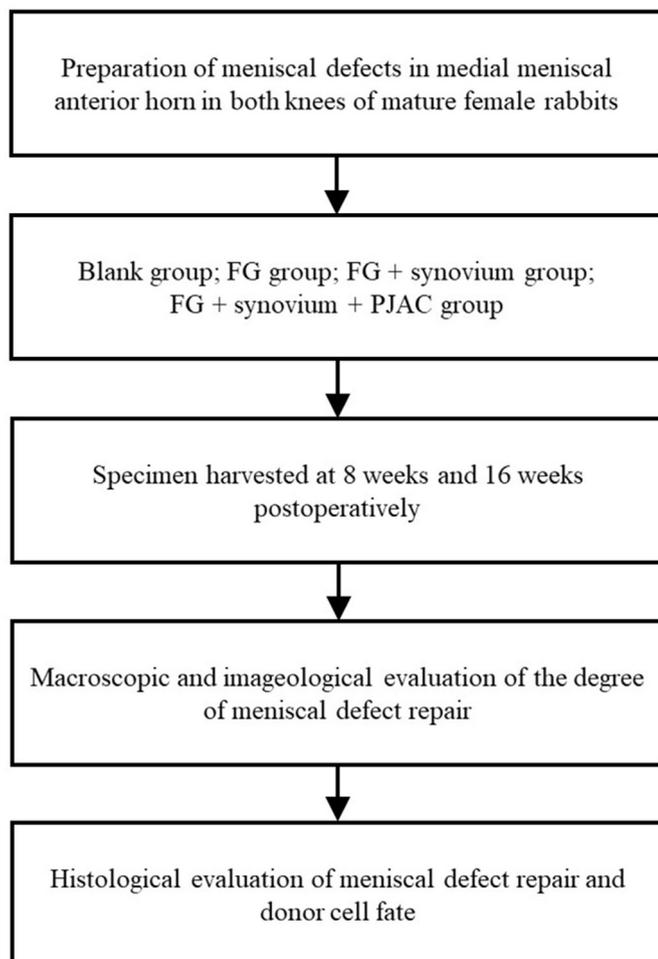


Fig. 1. The experimental flow diagram.

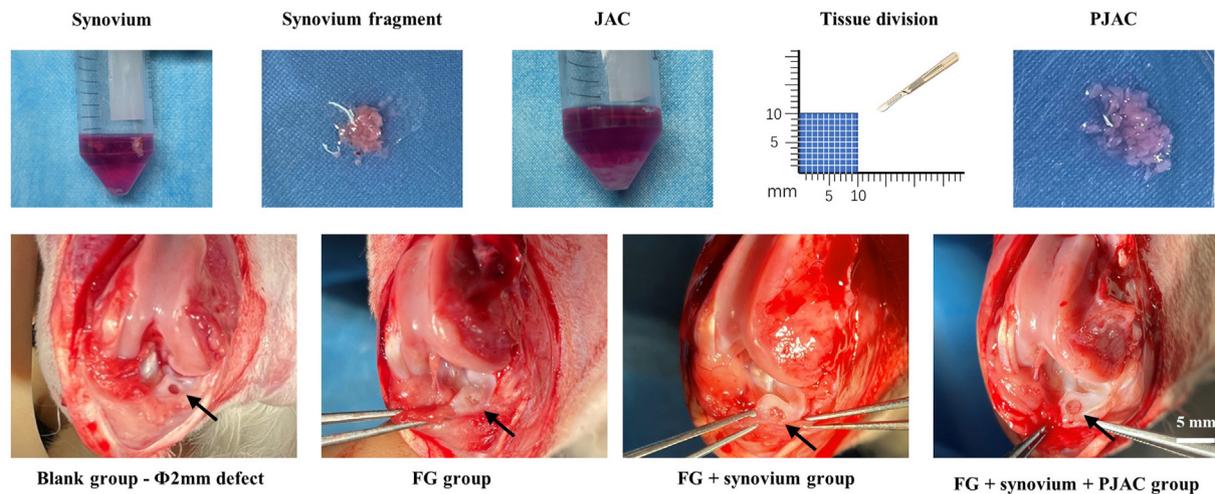


Fig. 2. The preparation and transplantation of synovium and PJAC. JAC: juvenile articular cartilage. PJAC: particulated juvenile allograft cartilage. FG: fibrin gel. Scale bar: 5 mm.

Table 1

Scoring system for evaluation of the quality of meniscal repair tissue.<sup>a</sup>

Index	0	1	2	3
Defect filling	No fill	<25%	25–75%	>75%
Surface	No surface	ruptured	Fissured/fibrillated	Meniscus-like
Integration	No integration	Partial, unilateral integration	Bilateral partial or unilateral complete integration	Bilateral complete integration
Cellularity	No cells	>10 cell cluster/slide	No cell cluster/slide, Cell-ECM-ratio > 0.5	Meniscus-like cell-ECM ratio
Cell morphology	No cells	<25% meniscus-like cells	25%–75% meniscus-like cells	>75% meniscus-like cells
Content of proteoglycan	No staining for proteoglycan	<25%	25–75%	>75%
Content of type 2 collagen	No staining for type 2 collagen	<25%	25–75%	>75%
Stability	No stability	weak	Stable in shape	Stable to pressure and pulling stress

<sup>a</sup> ECM, extracellular matrix.

features included stability and filling of the defects. The following features were included in histological evaluations: the surface area quality, integration, cellularity, cell morphology, proteoglycan and type 2 collagen content of the repaired meniscal tissue. Thus, eight individual subgroups were included in the scoring system. Scores ranging from 0 to 3 were graded into each subgroup. The final scores ranging from 0 points (no evidence of repair) to 24 points (complete repair) are demonstrated in Table 1.

### 2.5. Immunohistochemistry assessment

For immunohistochemical evaluation, primary antibodies, i.e., type 1 collagen (Arigo, ARG-21965), type 2 collagen (Invitrogen, MA5-13026), aggrecan (Sigma, C8035) and SOX9 (Millipore Sigma, HPA001758) were used. Donkey anti-goat IgG H&L (HRP) (Abcam, ab6885), Goat anti-rabbit IgG H&L (HRP) (Abcam, ab6721) and anti-mouse IgG H&L (HRP) (Abcam, ab6789) were used as secondary antibodies. Antigen retrieval was completed by incubation with 0.4% pepsin (Aladdin, P110928, Shanghai, China) at 37 °C for 1 h. Endogenous peroxidase was blocked by incubation with 3% H<sub>2</sub>O<sub>2</sub> for 15 min, and nonspecific protein binding was blocked by incubation with goat serum (Boster, AR0009, China) for 1 h. The sections were incubated with primary antibodies for 2 h at room temperature and then incubated with secondary antibodies for 1 h at room temperature. Finally, the color was developed by using diaminobenzidine (DAB) substrate kit.

### 2.6. Biotin-labeled in situ hybridization (ISH)

The male-specific sex-determining region Y-linked (SRY) gene of

male rabbit was searched. A specific probe (Qiagen) was designed based on the specific gene fragments of the rabbit's SRY gene. Probe information is as follows: SRY probe (5' and 3' biotin) TGCAAGCAG-CAAAGTGCCT. All solutions used in ISH should be free of ribonuclease. The 3 μm thick paraffin-embedded sections were immersed into fresh xylene and graded ethanol to deparaffinize and regain water. The sections were permeabilized by 20 μg/ml proteinase K solution at 37 °C for 15 min and then washed with phosphate-buffered saline (PBS). Then, the slices were incubated with prehybridization buffer at 37 °C for 1 h. The hybridization mix (hybridization buffer with SRY probe, 40 nM) was added and incubated at 54 °C for 1 h. After hybridization, rigorous wash was completed with graded sodium citrate buffer (SSC) and PBS. Nonspecific protein binding was blocked by 1% bovine serum albumin (BSA) at room temperature for 15 min. Then, the FITC-labeled anti-biotin (MilliporeSigma, F6762) was added to incubate the sections at room temperature for 1 h. Finally, the sections were incubated with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) solution for 10 min and then sealed with anti-fluorescence quenching sealing tablets after washing. The stained slices of ISH were scanned by confocal microscope (TCS-SP8, Leica) (The excitation and emission wavelength of DAPI is 358 nm and 461 nm; The excitation and emission wavelength of FITC is 490 nm and 520 nm). The SRY positive cell ratio was defined by the following formula: number of cells stained by FITC divided by number of cells stained by DAPI.

### 2.7. Semiquantitative analysis of extracellular matrix deposition and fibrochondrocyte-like cell counting in repaired menisci

Using the Image J software (US National Institutes of Health), we

calculated the average optical density (AOD) within a region of interest (ROI) (300  $\mu\text{m}$  in length, 200  $\mu\text{m}$  in width) to semiquantitatively evaluate the deposition of newly formed extracellular matrix (ECM) in repaired menisci. In this study, the toluidine blue and Safranin O stains were used to measure GAG content. The types 1 and 2 collagen and aggrecan content were measured using the same methods. For the analysis of collagen fibers stained by picrosirius red, the photographs of repaired area were captured by polarization microscope. The collagens could be distinguished, due to the different birefringence property of collagens under polarization microscope. Especially, type 1 collagen fibers: closely arranged, showing strong birefringence, yellow or red; Type 2 collagen fiber: showing weak double refraction, showing loose network distribution of various colors. The Image J software (US National Institutes of Health) was used to separate the colors, thus different collagens were divided. The optical density of each color represented the relative content of the corresponding collagen. However, the limitation was that the newly formed collagen fibers could not be distinguished from the original collagens within the graft tissues.

For fibrochondrocyte-like cell counting, a ROI (400  $\mu\text{m}$  \* 200  $\mu\text{m}$ ) was identified within the repaired area. Then, the cells demonstrating round

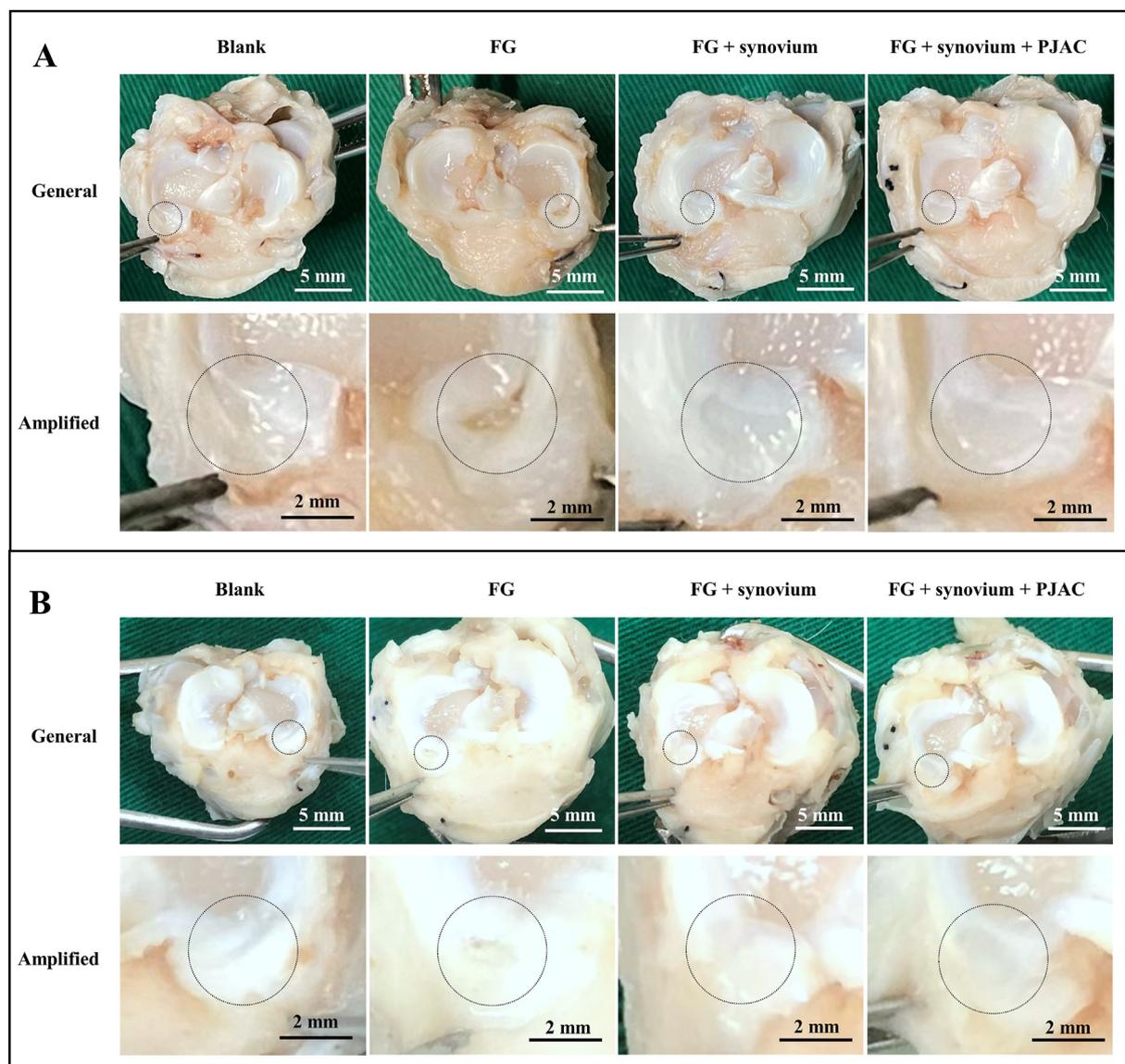
or oval appearance and surrounded by lacuna were identified as fibrochondrocyte-like cells and counted. The semi-quantitative analysis was performed in a double blinded manner and completed by Y.S.

## 2.8. Statistical analysis

A priori power analysis using the G\*Power software (G\*Power, version 3.1.9.2) was used to calculate the appropriate sample size for this study. A sample size of six menisci in each group was necessary to achieve a power of 0.8, an effect size of 0.8, at the  $\alpha$  level of 0.05. All data are demonstrated with median values with 95% confidence intervals (CIs). The ordinary two-way ANOVA with the Bonferroni multiple comparison test was used. All statistical analyses were completed using the GraphPad Prism software, version 8.0.1 (GraphPad Software). A  $P$  value < 0.05 was considered statistically significant.

## 3. Results

For all animals, no serious adverse effects were presented peri- or postoperatively, except one knee joint appeared severe infection in the



**Fig. 3.** The macroscopic findings of meniscal repair. A: Macroscopic evaluations at 8 weeks postoperatively; B: Macroscopic evaluations at 16 weeks postoperatively. FG: fibrin gel; PJAC: particulated juvenile allograft cartilage. Black dotted circles indicate the original meniscal lesions and repaired area.

blank group at the last follow up (16 weeks postoperatively). This infected knee joint has to be abandoned. Moreover, at the first week postoperatively, most of the animals in all groups exhibited signs of anorexia, knee joint swelling and resistance to flexion and extension. However, these signs disappeared gradually.

### 3.1. Meniscal score evaluation

Fig. 3 showed the best, representative and worst macroscopic findings of meniscal repair in all groups at each time point. At 8 weeks postoperatively, incomplete filling or loose connective tissue were demonstrated in the blank and FG groups. The repaired tissue demonstrated ruptured surface and poor integration in the edge. Some meniscal samples in blank and FG groups even showed obvious deterioration. The consistent findings were also observed in the MRI examinations, demonstrating abnormal MRI signals and discontinuity in the defect area (Fig. 4). Few cell clusters were detected in repaired tissue, but small cells with ovoid or circular nuclei were found (Fig. 5, A). The extracellular matrix (ECM) of the newly formed tissue was negatively or weakly stained with safranin O, toluidine blue and type 2 collagen immunohistochemistry (Figs. 6A, 7A and 8A). However, in groups FG + synovium and FG + synovium + PJAC, the meniscal defects were filled with thick connective tissue almost completely. Moreover, the newly formed tissue in FG + synovium + PJAC group demonstrated ivory white like normal meniscus, relative smooth surface and almost complete integration with peripheral meniscal tissue (Fig. 3, A). The MRI findings also demonstrated relative normal signals and continuity in the defect area (Fig. 4). A large number of meniscus-like large round or ovoid fibrochondrocytes with rich cytoplasm were seen in HE staining (Fig. 5, A). The ECM around these cells was strongly stained with safranin O, toluidine blue and type 2 collagen immunohistochemistry (Figs. 6A, 7A and 8A). The semi-quantitative analysis also showed the FG + synovium + PJAC group exhibited the highest expression of GAG and type 2 collagen (Figs. 6C, 7C and 8C).

At 16 weeks postoperatively, in groups FG + synovium and FG + synovium + PJAC, the defects were filled with thick fibrocartilage-like

tissue completely from macroscopic evaluation (Fig. 3, B). The MRI findings were consistent with macroscopic evaluations, demonstrating structural integrity and MRI signals like normal meniscus (Fig. 4). In FG + synovium + PJAC group, the regenerated tissue showed superior structural integrity, superficial smoothness, and bilateral marginal integration compared to other groups. More cell clusters showing fibrochondrocyte-like cell morphology filled the repaired tissue compared to 8 weeks postoperatively (Fig. 5, C). Moreover, the matrix surrounding these cell clusters still remained strongly positive safranin O, toluidine blue and type 2 collagen immunohistochemistry staining (Figs. 6B, 7B and 8B). However, in groups blank and FG, relative more connective tissues filled the defect compared to the corresponding groups at 8 weeks postoperatively. But, they were still inferior in defect filling, surface smoothness and bilateral marginal integration when compared to groups FG + synovium and FG + synovium + PJAC. Some menisci even appeared prominent degeneration in the defect area (Fig. 3, B). Only some small cells were observed in the repaired tissue, and the number of cells at the defect interface decreased significantly (Fig. 5, B). The matrix in the regenerated tissue was almost negatively or weakly stained with safranin O, toluidine blue and weakly stained with type 2 collagen immunohistochemistry (Figs. 6B, 7B and 8B). Semiquantitative outcomes demonstrated that the FG + synovium + PJAC group had the highest expression of GAG and type 2 collagen (Figs. 6C, 7C and 8C).

At 8 weeks postoperatively, meniscal scores were similar between blank and FG group. But, the meniscal scores were significantly higher in the groups of FG + synovium, FG + synovium + PJAC than in the groups of blank and FG and tended to be the highest in FG + synovium + PJAC group. Significant differences were observed between groups blank, FG and groups FG + synovium, FG + synovium + PJAC ( $P < 0.05$ ). At 16 weeks postoperatively, the FG + synovium + PJAC group demonstrated the highest meniscal scores compared to other groups ( $P < 0.05$ ) (Fig. 9). The detailed meniscal repair scores were summarized in Table 2.

### 3.2. Additional histological evaluation on meniscal regeneration

The type 1 collagen immunohistochemistry was used to evaluate the expression of type 1 collagen in the regenerated tissue. The FG + synovium + PJAC-16W group tended to have the highest type 1 collagen deposition. The semiquantitative analysis showed significant differences were only observed between FG + synovium + PJAC-16W group and blank group ( $P < 0.05$ ). However, no significant differences were observed among other groups (Fig. 10).

Aggrecan was another richly expressed matrix in the avascular zone of meniscus. Thus, the immunohistochemistry of aggrecan was evaluated in our study. Aggrecan was strongly expressed in the fibrochondrocyte-like cells and the surrounding matrix in FG + synovium + PJAC group and tended to be the highest in FG + synovium + PJAC-16W group. Semiquantitative outcomes demonstrated significant differences between FG + synovium + PJAC group and other groups at 8 and 16 weeks postoperatively (Fig. 11).

The positive rates of SOX9 in the blank, FG, FG + synovium and FG + synovium + PJAC group were 42.44% (30%, 52.6%), 44.02% (30.67%, 55.81%), 63.01% (49.38%, 78.13%) and 65.52% (55.56%, 86.21%) respectively at 8 weeks postoperatively. Significant differences were observed between FG + synovium + PJAC group and blank, FG groups ( $P < 0.05$ ). At 16 weeks postoperatively, the positive rates of SOX9 in the blank, FG, FG + synovium and FG + synovium + PJAC group were 48% (27.91%, 50.83%), 54.66% (28.85%, 66%), 55.92% (43.24%, 73.47%) and 67.55% (60%, 97.47%) respectively. Significant differences were observed between FG + synovium + PJAC group and blank, FG groups ( $P < 0.05$ ). FG + synovium + PJAC group demonstrated the highest positive rate, but no significant differences were observed between FG + synovium and FG + synovium + PJAC group (Fig. 12).

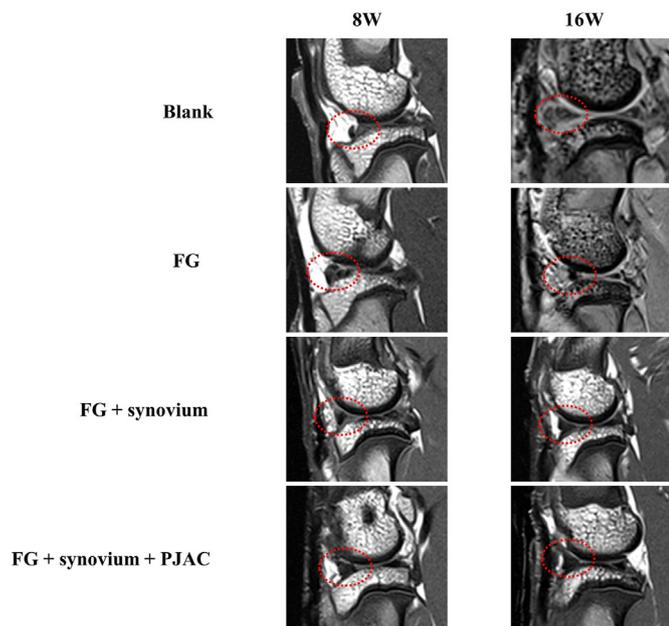


Fig. 4. The MRI findings of meniscal repair from sagittal plane. The dotted red ellipses indicate repaired tissue.

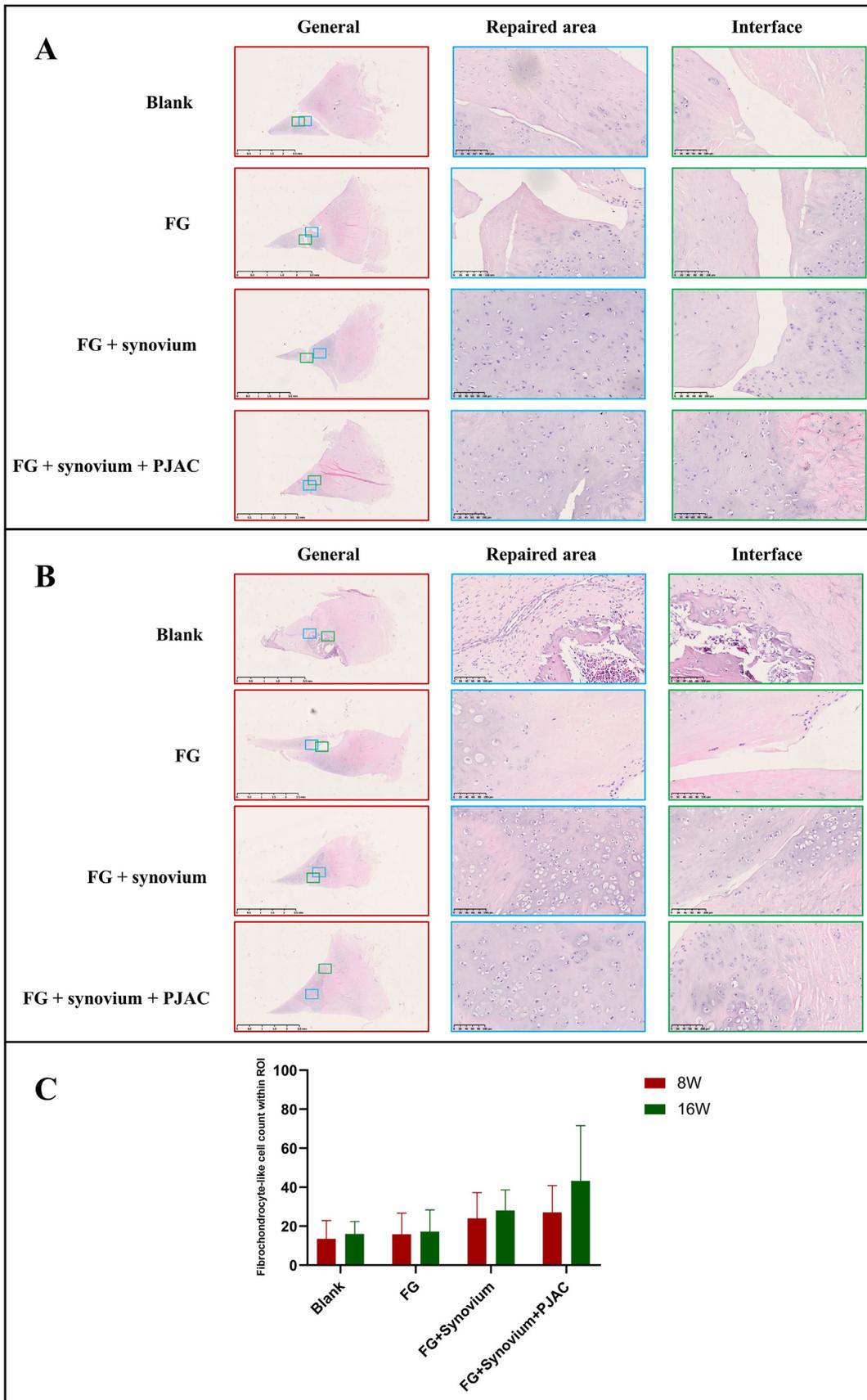
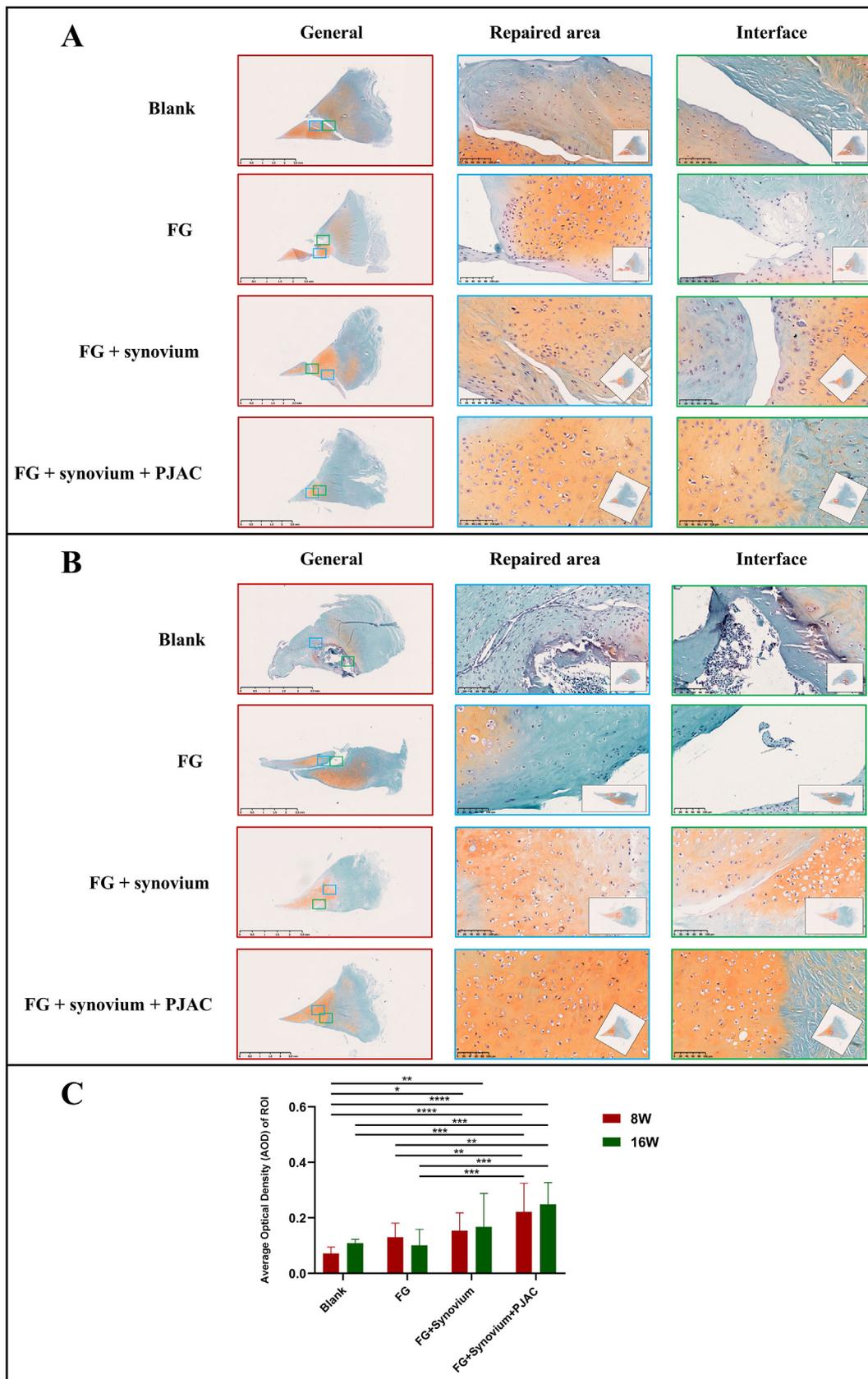
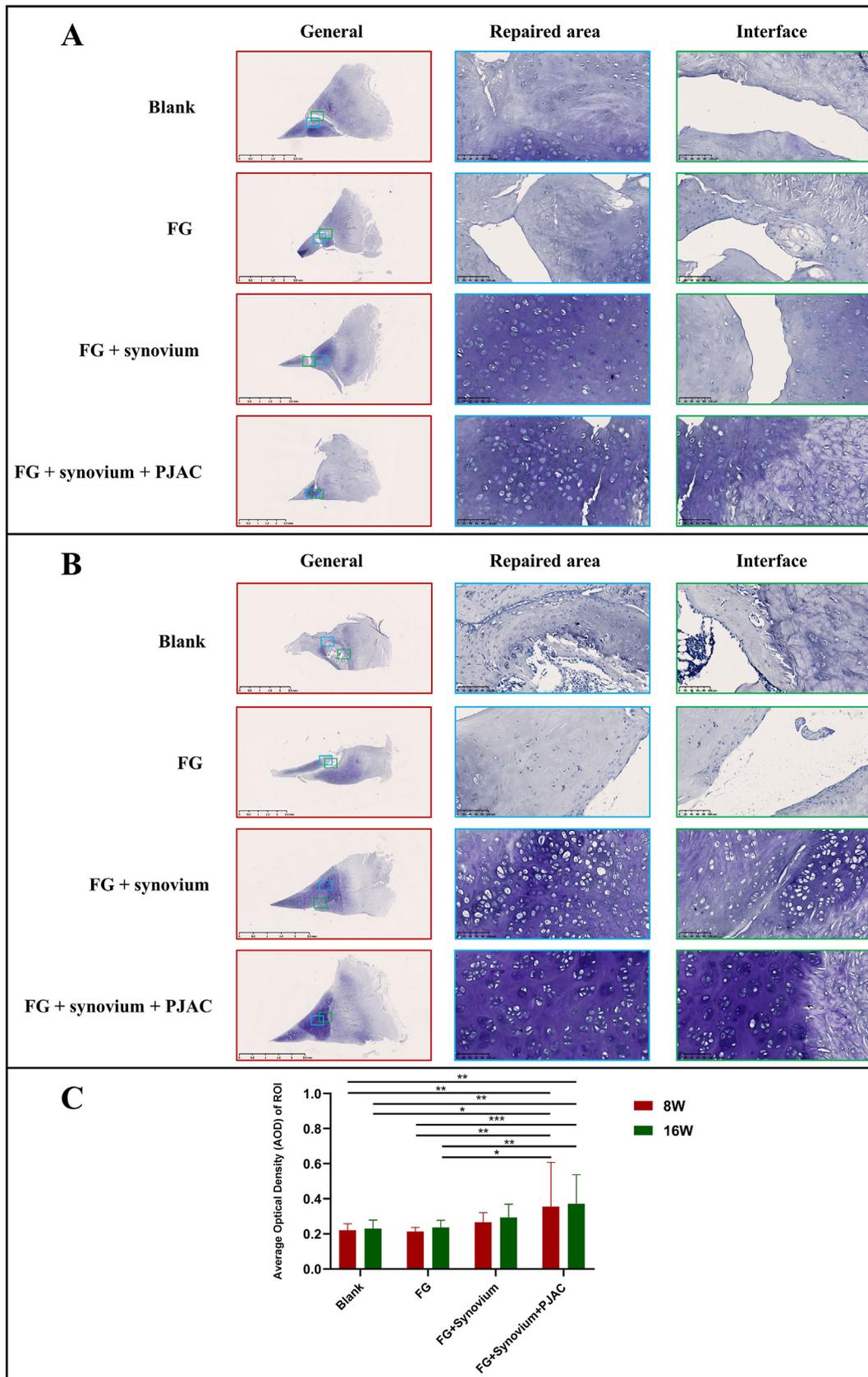


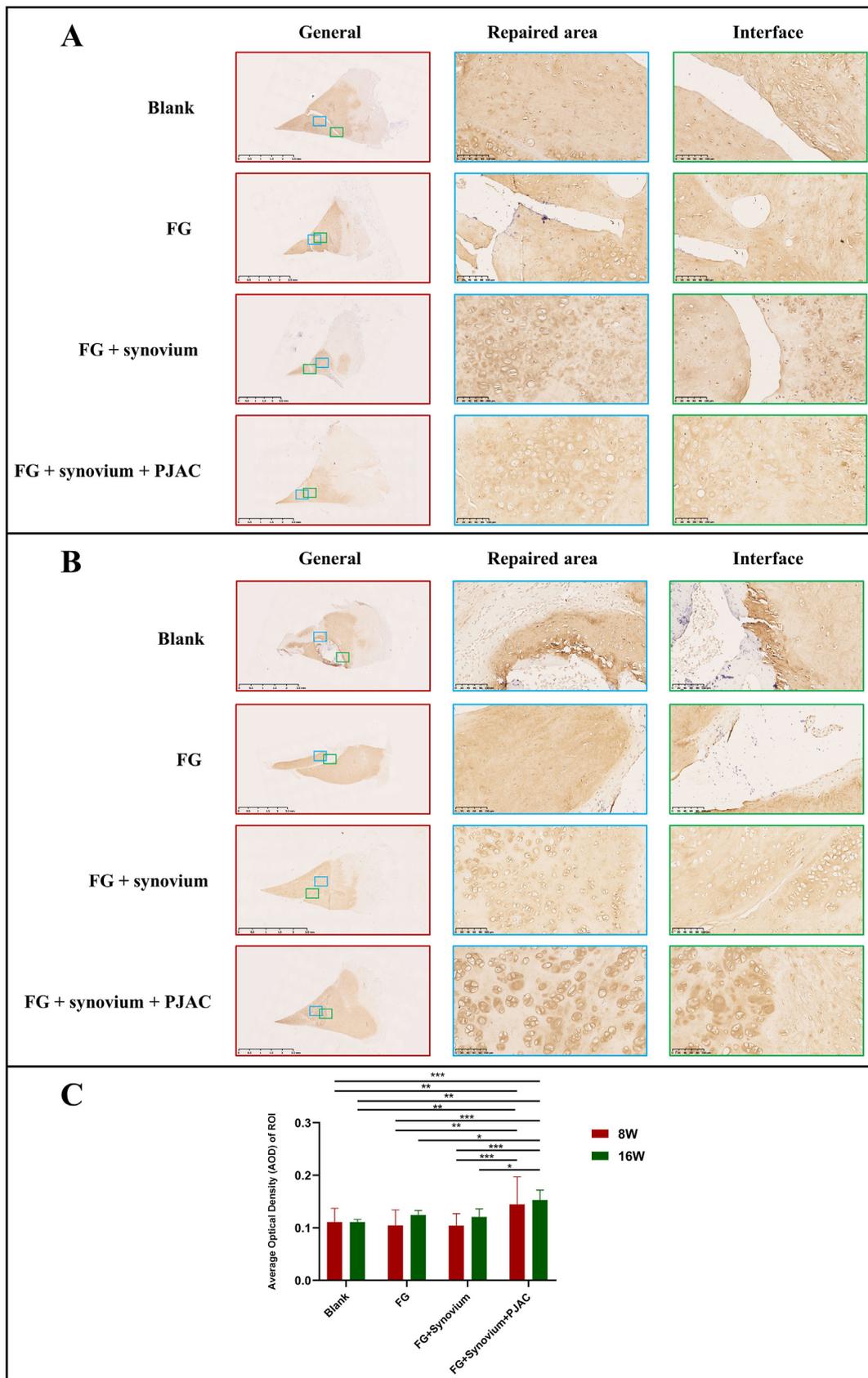
Fig. 5. HE findings of meniscal repair showing general, repaired area and interface. A: HE findings at 8 weeks postoperatively; B: HE findings at 16 weeks postoperatively; C: Fibrochondrocyte-like cell counting within a ROI (400  $\mu$ m \* 200  $\mu$ m). The scale bar of general picture is 2.5 mm; The scale bar of repaired area and interface picture is 100  $\mu$ m.



**Fig. 6.** The safranin O-Fast green findings of meniscal repair showing the general, repaired area and interface. A: Histological evaluations at 8 weeks postoperatively; B: Histological evaluations at 16 weeks postoperatively; C: Semiquantitative evaluation of GAG content reflected by safranin O staining intensity. AOD values are expressed as median values with 95% CIs.  $n = 6$  (except for  $n = 5$  in blank-16W group). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.0005$ ; \*\*\*\*,  $P < 0.0001$ . The scale bar of general picture is 2.5 mm; The scale bar of repaired area and interface picture is 100  $\mu\text{m}$ . AOD: average optical density, CI: confidence interval, GAG: glycosaminoglycans.



**Fig. 7.** The toluidine blue findings of meniscal repair showing the general, repaired area and interface. A: Histological evaluations at 8 weeks postoperatively; B: Histological evaluations at 16 weeks postoperatively; C: Semiquantitative evaluation of GAG content reflected by toluidine blue staining intensity. AOD values are expressed as median values with 95% CIs.  $n = 6$  (except for  $n = 5$  in blank-16W group). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.0005$ . The scale bar of general picture is 2.5 mm; The scale bar of repaired area and interface picture is 100  $\mu\text{m}$ . AOD: average optical density, CI: confidence interval, GAG: glycosaminoglycans.



**Fig. 8.** The type 2 collagen immunohistochemical findings of meniscal repair showing the general, repaired area and interface. A: Immunohistochemical evaluations at 8 weeks postoperatively; B: Immunohistochemical evaluations at 16 weeks postoperatively; C: Semiquantitative evaluation of type 2 collagen content reflected by immunohistochemical staining intensity. AOD values are expressed as median values with 95% CIs. n = 6 (except for n = 5 in blank-16W group). \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.0005. The scale bar of general picture is 2.5 mm; The scale bar of repaired area and interface picture is 100  $\mu$ m. AOD: average optical density, CI: confidence interval

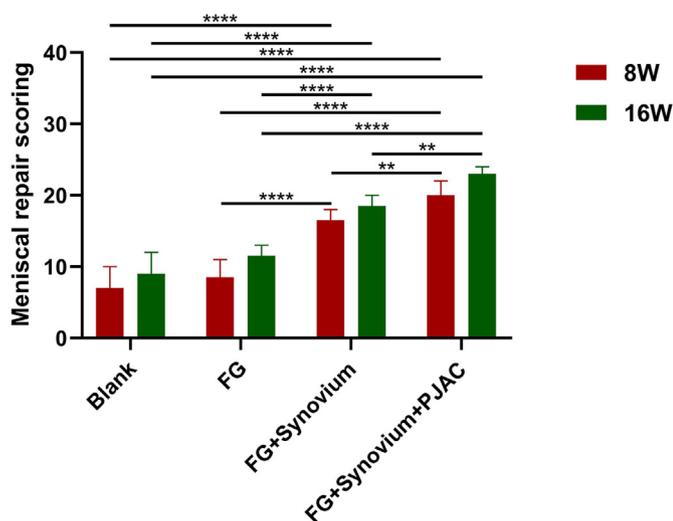


Fig. 9. The meniscal repair scoring of all groups at 8 and 16 weeks post-operatively. The values are expressed as median values with 95% CIs. n = 6 (except for n = 5 in blank-16W group). \*\*, P < 0.01; \*\*\*\*, P < 0.0001.

In Fig. 13, the picrosirius red staining was used to reflect the composition of collagen fibers in the repaired area. Under polarized light microscope, the birefringence of collagens was enhanced so as to distinguish the type of collagens. Type 1 collagen fibers: closely arranged, showing strong birefringence, yellow or red; Type 2 collagen fiber: showing weak double refraction, showing loose network distribution of various colors; Type 3 collagen fibers: thin green fibers showing weak birefringence, pale yellow. The composition and orientation of collagen fibers in the FG + synovium + PJAC group were more like normal meniscal tissue (Fig. S1) than that of other groups.

### 3.3. Donor cell survival evaluation

The outcomes of male-specific SRY gene ISH reflected the survival rate of transplanted donor cells. The positive rates of SRY in the FG + synovium and FG + synovium + PJAC group were 61.06% (45.88%, 81.82%) and 75.72% (50%, 94.74%) respectively at 8 weeks post-operatively. No significant differences were observed between these two groups. At 16 weeks postoperatively, the positive rates of SRY in the FG + synovium and FG + synovium + PJAC group were 30.39% (12.5%, 33.33%) and 48.69% (28.21%, 64.44%) respectively. These two groups demonstrated no significant differences. However, significant differences were observed between FG + synovium-8W and FG + synovium-16W as well as between FG + synovium + PJAC-8W and FG + synovium + PJAC-16W (Fig. 14). The positive and negative control for SRY gene in situ hybridization were demonstrated in Fig. S2.

Table 2  
Meniscal repair scoring results.

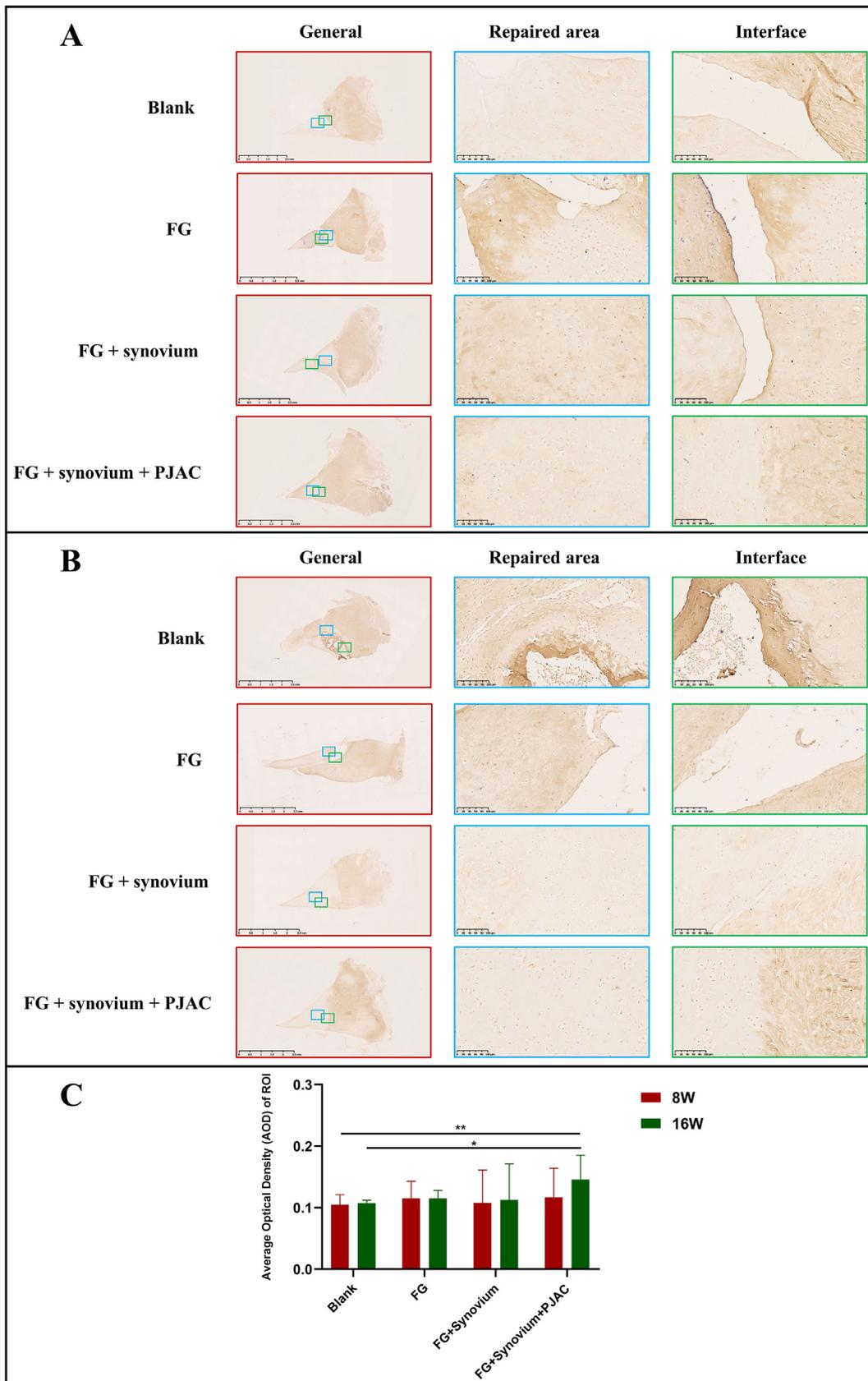
Group	8W					16W					
	5	7	10	8	6	7	9	9	12	9	9
Blank	5	7	10	8	6	7	9	9	12	9	9
FG	5	8	11	11	9	8	9	12	13	11	13
FG+Synovium	13	17	16	17	16	18	17	19	18	20	17
FG+Synovium+PJAC	18	18	22	22	21	19	22	23	24	23	21

## 4. Discussion

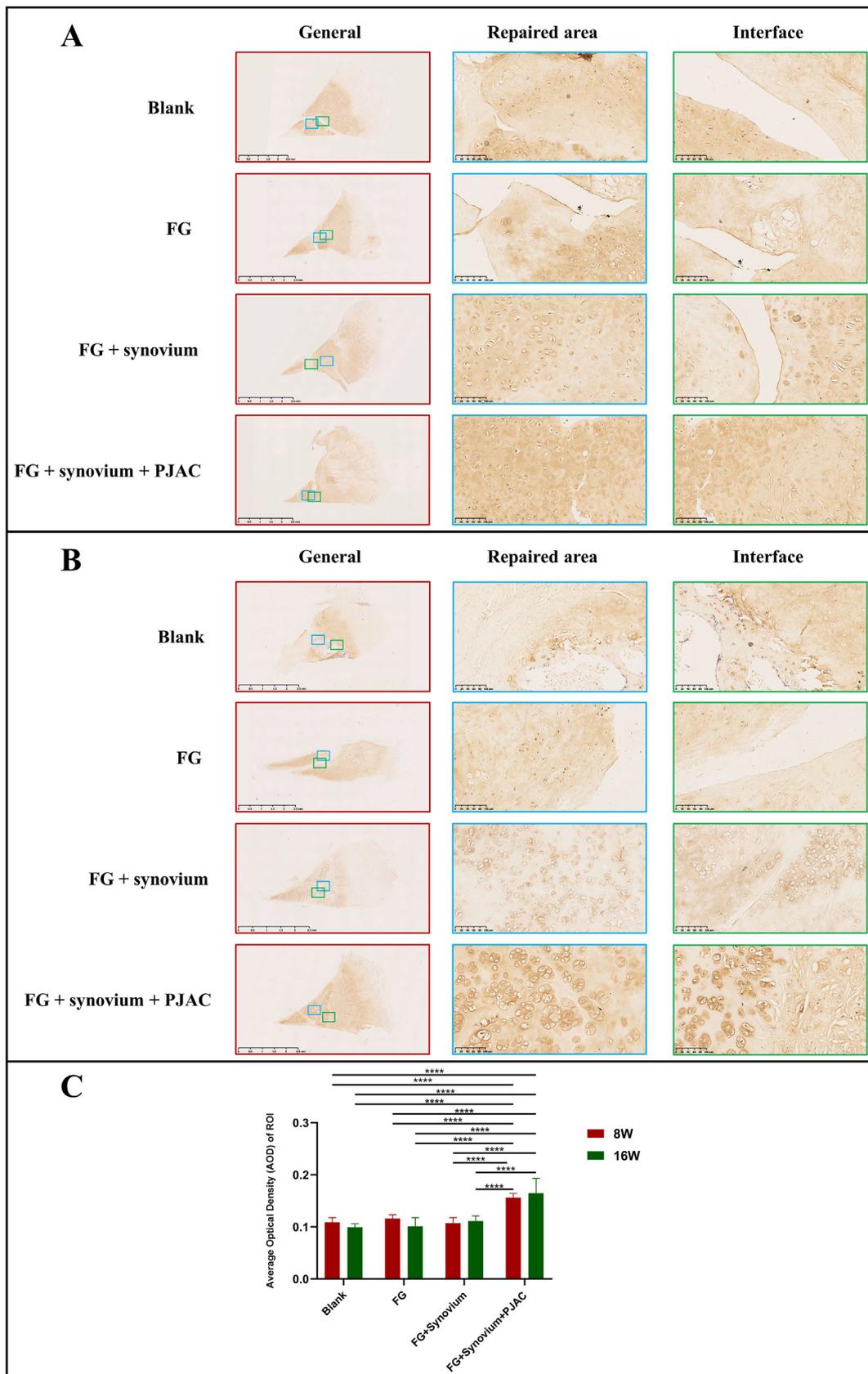
To our knowledge, the study we performed here is the first attempt to investigate the positive synergistic effect of synovium and PJAC transplant on repairing meniscal injuries and track the donor cell fate after transplanted into meniscal defects. The male-specific SRY gene in situ hybridization signals still existed at 16 weeks postoperatively, demonstrating alive donor cells. Moreover, the expression of transcription factor SOX9 was stronger in the repaired tissue when the synovium and PJAC transplanted simultaneously.

In the present study, the synovium or PJAC was encapsulated into the fibrin gel and then transplanted into meniscal defects. The fibrin gel delivery system used in our study demonstrated several advantages when compared to other scaffolds. (i) unlike synthetic hydrogel scaffold, fibrin is a biodegradable fibrinolytic system and serves as a nature temporary regeneration matrix [44]. (ii) a variety of cell integrins and binding sites were contained in the fibrin and this bioactivity makes fibrin an attractive matrix for cell proliferation and differentiation [45]. (iii) the fibrin network is among the softest substances in nature, and this allows fibrin gel to deform to a large extent and stiffen but not break [45]. This mechanical property makes fibrin gel more suitable in our study under the continuous compressive condition. However, many previous studies utilized fibrin gel as tissue adhesive to repair meniscal injuries [46–50]. In order to exclude the potential effects of fibrin gel on meniscal repair, a pure fibrin gel transplant group was included in our study. Moreover, the results showed that pure fibrin gel transplant could not facilitate meniscal defect regeneration significantly when compared to blank group.

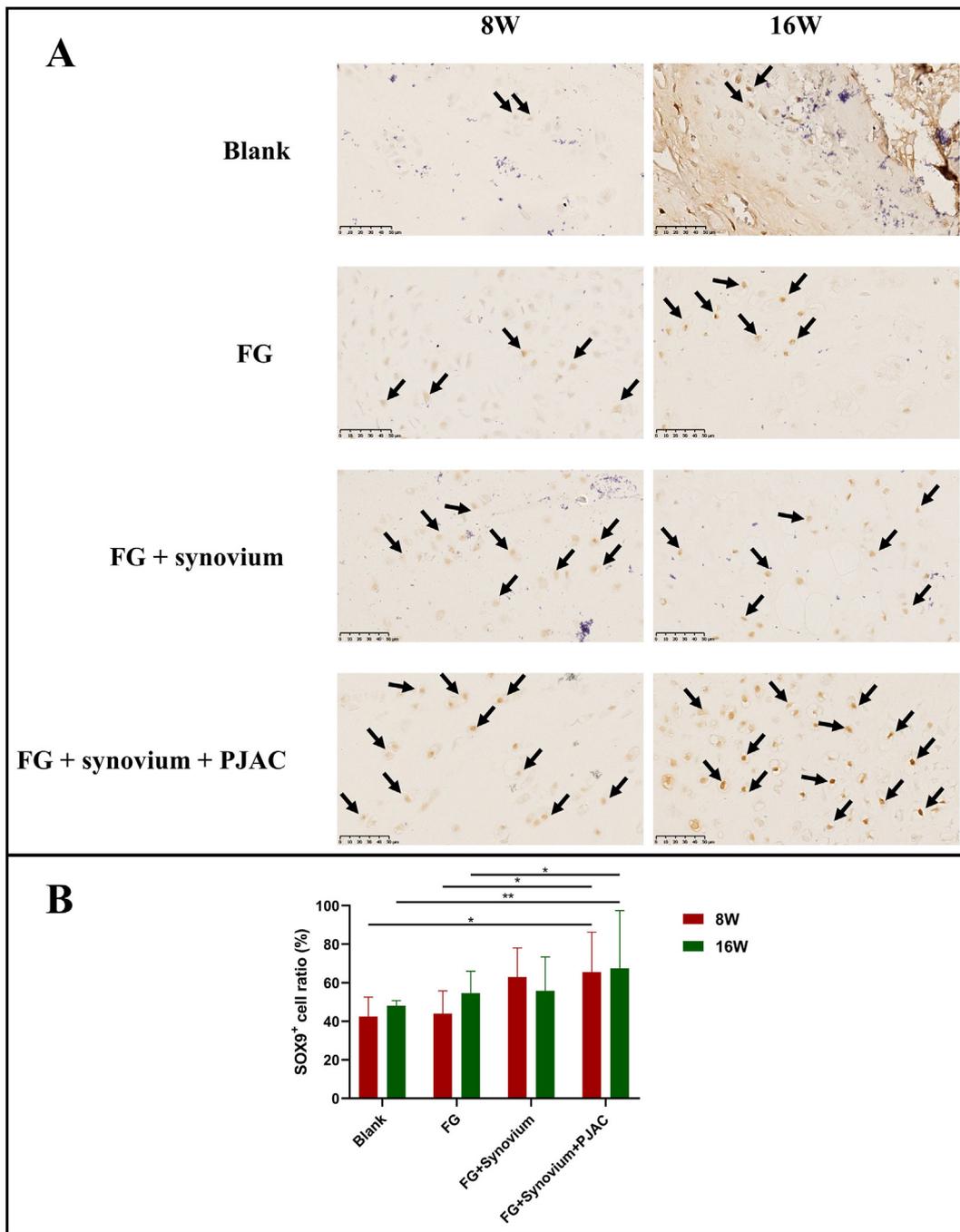
A previous study elucidated that synovium was the primary contributor to meniscal repair rather than residual meniscal tissue by the in vivo freeze-thaw method. They found that meniscus-like fibrochondrocytes and proteoglycan filled the meniscal defect only in the condition of intact synovium, while few cells recovered the meniscal defect when the synovium was freeze-thawed [15]. Mesenchymal stem cells (MSCs) also existed in the synovial fluid. The number of MSCs in synovial fluid increased significantly after injuries of cartilage [51], anterior cruciate ligament [52] and meniscus [53], and they expressed similar gene profiles compared to those of the synovium. Except for type 1 collagen, the inner avascular zone of meniscus contains rich type 2 collagen, GAG and aggrecan, which is similar to hyaline cartilage [1]. Previous studies have demonstrated that SDSCs possessed superior chondrogenic capacity than other mesenchymal tissues-derived stem cells [18,19], further reflecting the critical role of synovium in meniscal repair. Moreover, preclinical and clinical studies both demonstrated the positive effects of synovial flap transplant on meniscal healing [10–14]. Our study also showed synovium transplant facilitated meniscal regeneration, which was consistent to previous studies. Moreover, the transplantation of autologous or allogenic SDSCs has shown promising in meniscal repair [16,17,20–22]. Nevertheless, no matter for transplanted synovium or SDSCs, they were both expected to undergo a process of fibrocartilaginous metaplasia [54]. Then, the transplanted cells would transform into fibrochondrocytes and



**Fig. 10.** The type 1 collagen immunohistochemical findings of meniscal repair showing the general, repaired area and interface. A: Immunohistochemical evaluations at 8 weeks postoperatively; B: Immunohistochemical evaluations at 16 weeks postoperatively; C: Semiquantitative evaluation of type 1 collagen content reflected by immunohistochemical staining intensity. AOD values are expressed as median values with 95% CIs. n = 6 (except for n = 5 in blank-16W group). \*, P < 0.05; \*\*, P < 0.01. The scale bar of general picture is 2.5 mm; The scale bar of repaired area and interface picture is 100 μm. AOD: average optical density, CI: confidence interval



**Fig. 11.** The aggrecan immunohistochemical findings of meniscal repair showing the general, repaired area and interface. A: Immunohistochemical evaluations at 8 weeks postoperatively; B: Immunohistochemical evaluations at 16 weeks postoperatively; C: Semiquantitative evaluation of aggrecan content reflected by immunohistochemical staining intensity. AOD values are expressed as median values with 95% CIs. n = 6 (except for n = 5 in blank-16W group). \*\*\*\*,  $P < 0.0001$ . The scale bar of general picture is 2.5 mm; The scale bar of repaired area and interface picture is 100  $\mu$ m. AOD: average optical density, CI: confidence interval

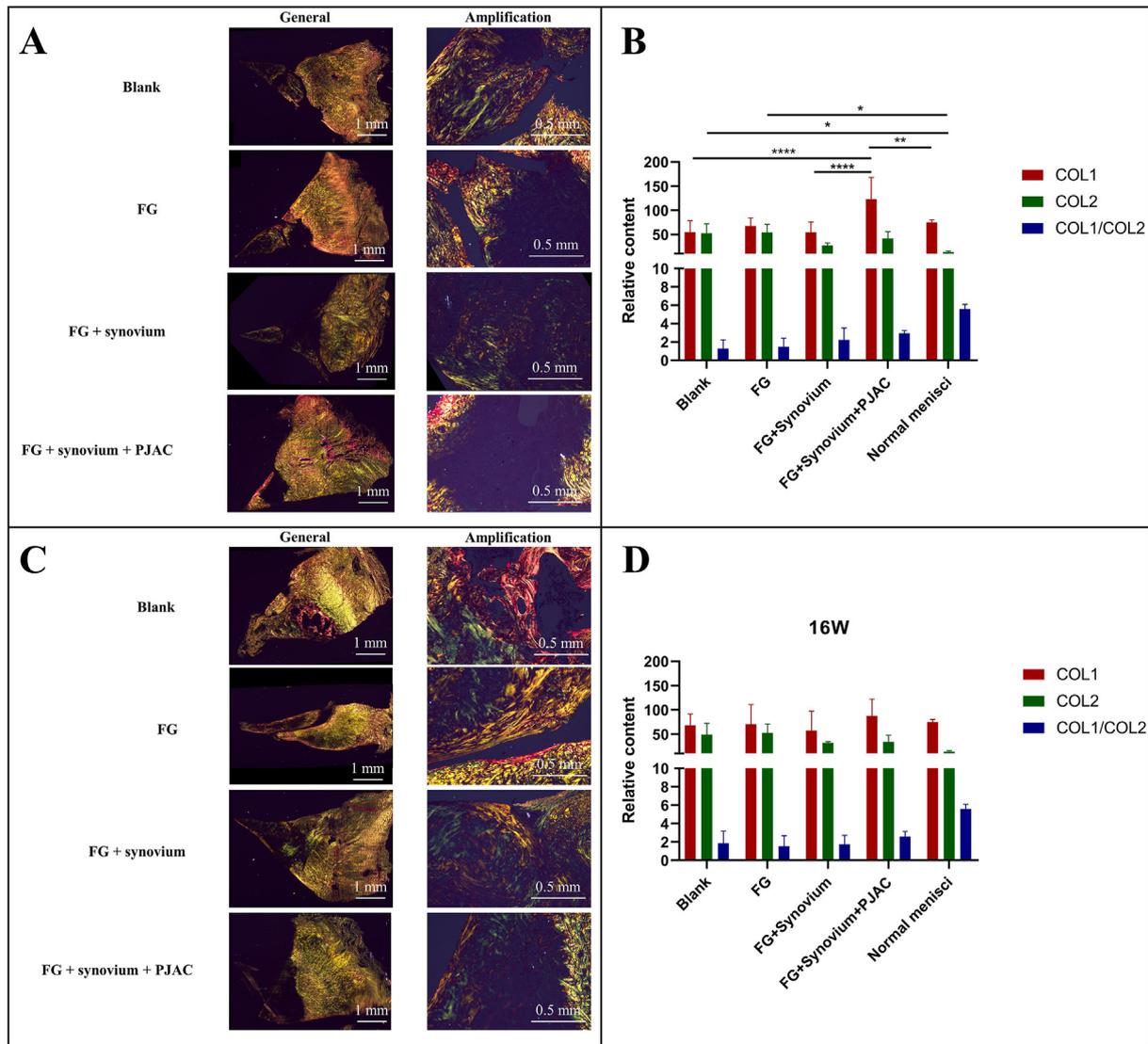


**Fig. 12.** The positive rate of SOX9 expression within the regenerated tissue. A: Immunohistochemical evaluations of SOX9 expression; B: The positive rate of SOX9 expression. Black arrows indicate SOX9 positive cells. Values are expressed as median values with 95% CIs. n = 6 (except for n = 5 in blank-16W group). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . The scale bar of general picture is 2.5 mm; The scale bar of repaired area and interface picture is 100  $\mu$ m.

produce matrix. Moreover, the transforming growth factor-beta (TGF- $\beta$ ), a key growth factor in chondrogenesis, produced by chondrocytes could facilitate the chondrogenesis process of MSCs [39]. We found that the expression of SOX9 in the repaired tissue was higher in the synovium transplant group when compared to the blank and fibrin gel group, further validating this phenotypic transformation.

Many preclinical and clinical studies have reported the positive effects of PJAC transplant on repairing hyaline cartilage defects [26–29, 55]. So far, approximate 10,000 patients have received PJAC transplant surgery worldwide, and long-term follow-up results have confirmed the promising clinical effects of PJAC surgery [56–58]. The repair process relies heavily on the ability of juvenile chondrocytes [23]. The

superiority of juvenile chondrocytes over their adult counterparts has been confirmed in multiple studies. The gene expression profiles in juvenile cartilage are more favorable for cartilage repair than those of adult cartilage [23]. Typically, the genes directing growth and expansion of chondrocytes are upregulated in juvenile cartilage, while the genes that regulate structural integrity of cartilage are upregulated in adult cartilage [59]. The capability of juvenile chondrocytes of producing and maintaining matrix is enhanced due to the increased cell density, proliferation rate, and metabolic activity [60,61]. Moreover, because cartilage is immune-privileged, the immunological rejection of PJAC transplant could be reduced greatly [62]. The matrix composition of meniscal avascular zone is similar to that of hyaline cartilage, except for abundant



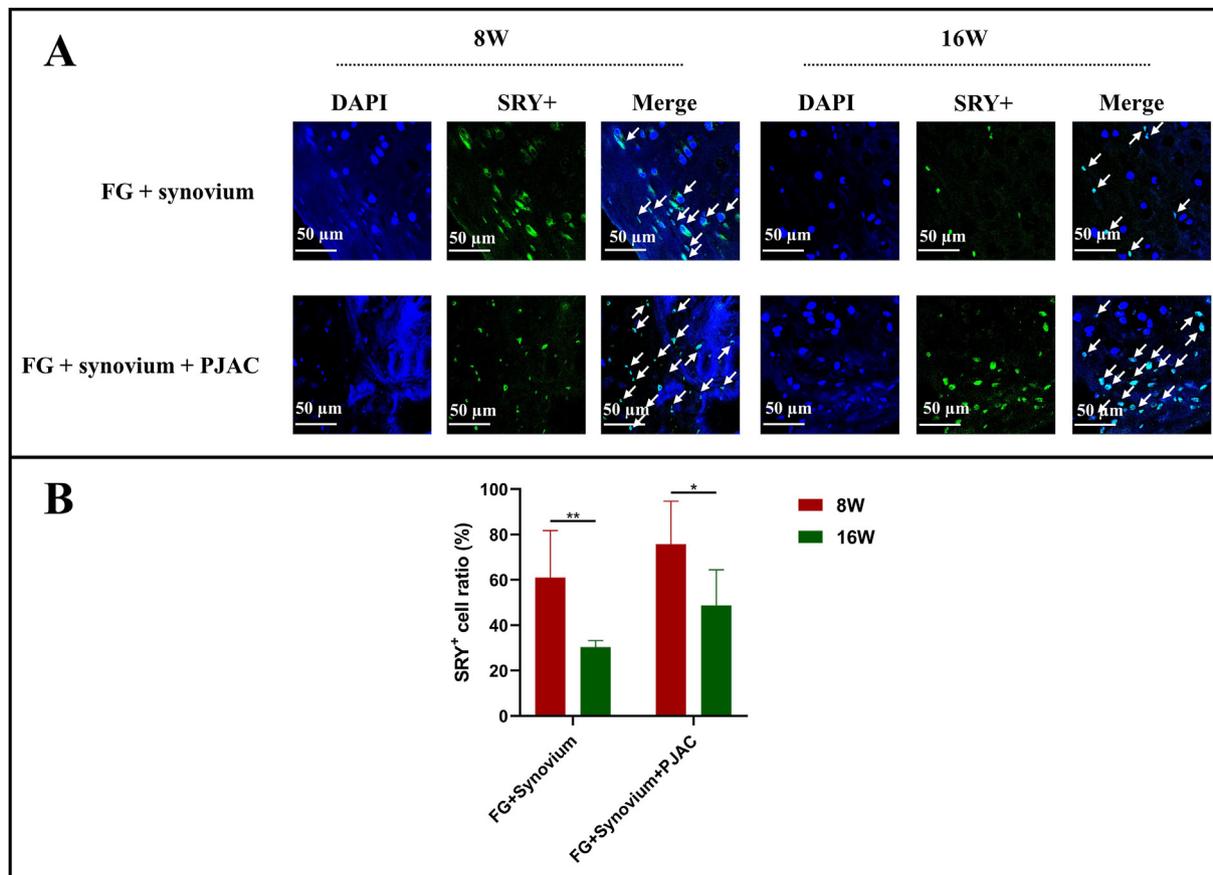
**Fig. 13.** The picrosirius red staining for collagen fibers in the repaired area evaluated at polarized light microscope. A: The picrosirius red staining of 8 weeks; B: the semi-quantitation analysis of COL1, COL2 and COL1/COL2; C: The picrosirius red staining of 16 weeks; D: the semi-quantitation analysis of COL1, COL2 and COL1/COL2.

type 1 collagen. Thus, we hypothesized that PJAC could also facilitate regeneration of meniscal defects in the avascular zone. Moreover, a previous study concluded MSC-derived exosomes promoted proliferation and inhibited apoptosis of chondrocytes [40]. Our study showed that the simultaneous transplant of PJAC and synovium enhanced meniscal regeneration significantly, thus validating our hypothesis.

When a study is evaluating the efficacy of synovium and PJAC transplant on repairing meniscal defects, it is necessary to know the survival, the key genes' function of transplanted cells. The lapine animal model described in our study could be applied to evaluate the therapeutic efficacy of synovium and PJAC on repairing meniscal injuries from the aspects of histology and related mechanisms. In our study, we evaluated whether the transplanted cells survived in the regenerated tissue of synovium transplant or synovium, PJAC transplant group. In order to distinguish the transplanted cells from host cells, we harvested synovium and PJAC transplant from male rabbits and transplanted them into meniscal defects of female hosts, then in situ hybridization for male-specific SRY gene was performed to detect transplanted cells [41,42, 63,64]. The male-specific SRY gene, a highly conserved sequence of genes in the Y chromosome was reported in 1990 [65]. Based on this specific biological characteristic, the SRY gene could be considered as a

marker for male cells [66]. Ostrander et al. [67] tracked the transplanted chondrocytes using SRY gene and found donor cells survived for at least 28 days. Pilichi et al. [68] confirmed sheep embryonic stem-like cells survived when transplanted into sheep osteochondral defects for 4 years. Those previous studies have chosen SRY gene as a marker for transplant from male, and the outcomes also validated the effectiveness and reliability of this method. A previous study concluded the donor cells in PJAC transplant survived for at least 3 months in a minipig cartilage defect model [29]. However, our results showed that SRY positive signals were detected in the regenerated tissue at 8 weeks postoperatively, and still existed at 16 weeks postoperatively in both synovium transplant and synovium, PJAC transplant group. Whereas, the positive signals in synovium, PJAC transplant group were stronger than those in synovium transplant group no matter at 8 weeks or 16 weeks postoperatively. However, the positive rates of SRY gene in synovium transplant or synovium, PJAC transplant group decreased at 16 weeks postoperatively compared to those at 8 weeks postoperatively. This may be due to the migration of host cells leading to increase in cell number or donor cell death caused by immunological rejection, apoptosis or other factors.

In our study, we performed immunohistochemical tests to evaluate the expression of transcription factor SOX9 in the repaired tissue of each



**Fig. 14.** The positive rate of male-specific SRY gene within the regenerated tissue. A: The male-specific SRY gene ISH for transplanted donor cells, cyan cells indicate alive donor cells; B: The positive rate of SRY<sup>+</sup> cells. White arrows indicate alive donor cells. Values are expressed as median values with 95% CIs. n = 6. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

group. Previous studies have confirmed transcription factor SOX9 was an upstream gene, that participated in regulating downstream genes for cartilage matrix formation or even coordinating signal transduction pathways, i.e. Wnt/ $\beta$ -catenin, TGF- $\beta$ /Smad and HIF-1 [69]. Both in vitro and in vivo studies have confirmed SOX9 was a strong transcription activator, that could activate the transcription of target genes. The Col2 $\alpha$ 1 intron is a known binding site for SOX9, and its expression is regulated directly after binding [70]. The critical role of the regulation of SOX9 has been validated in hyaline cartilage repair, and the production of Col2 $\alpha$ 1 is positively correlated with SOX9 expression [69,71]. Moreover, meniscal fibrochondrocytes generally demonstrate positive SOX9 expression, which is important for type 2 collagen synthesis and chondrogenesis [72, 73]. Type 2 collagen was richly deposited in the inner avascular zone of meniscus. Thus, the expression of SOX9 played critical role in the deposition of type 2 collagen in the inner avascular zone. Our results showed that the synovium, PJAC transplant group exhibited the highest expression of SOX9, and this indicated greater ability to produce Col2 $\alpha$ 1, which can be reflected by the results of type 2 collagen immunohistochemistry (Fig. 8).

Our study applied male-specific SRY gene in situ hybridization to confirm that transplanted cells of synovium or PJAC survive for at least 16 weeks in the recipient. Moreover, the synovium, PJAC transplant group demonstrated more robust positive SRY signals than those in synovium transplant group no matter at 8 weeks or 16 weeks post-operatively. We also validated the expression of SOX9 after synovium or PJAC transplant and the synovium, PJAC transplant group had stronger SOX9 expression compared to that of other groups. The expression of SOX9 initiated the synthesis of type 2 collagen, the primary component of meniscal avascular zone, thus facilitating meniscal repair. Moreover, the

present study demonstrated that the collagen composition (type 1 and 2 collagens, the ratio of collagen 1/collagen 2) within the repaired area was close to the native meniscal tissue in the avascular zone for FG + Synovium + PJAC group, further reflecting the phenotype of regenerated cells after transplantation was more similar to native meniscal fibrochondrocytes.

Following are the limitations of this study: (i) The synovium or PJAC transplant used in our study are fresh, whereas the PJAC particles used in clinic may be maintained at low temperature for a certain period. (ii) This study included only a 16-week observation period, without any further investigations on the repair process of meniscal defects beyond 16 weeks, and whether the newly formed tissue would degenerate as well as the utmost survival period of transplanted cells. (iii) This study did not clarify the interactions between transplanted synovium and PJAC as well as the host tissues. (iv) The present study used a lapine model instead of a large animal model (for example, a porcine or goat model) in which physiological features more closely resemble those of humans. (v) Biomechanical test of the repaired tissue was lacking in the present study. (vi) In order to distinguish transplanted cells from host cells using in situ hybridization, this study used juvenile male rabbit transplants in female rabbits. This would cause potential sexual dimorphism in phenotype or side effects.

## 5. Conclusions

Our study validated the hypothesis that the transplantation of synovium and PJAC synergistically facilitates meniscal regeneration. The male-specific SRY gene signal was still positive in the regenerated tissue at the last follow up, which indicated the donor cells survived for at least

16 weeks in the recipient. The repaired tissue demonstrated the most robust SOX9 expression after synovium and PJAC transplanted simultaneously. Thus, based on our study, synovium and PJAC transplant could be further evaluated to be a possible treatment to meniscal defects.

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### Declaration of competing interest

The authors have declared that no competing interest exists.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jot.2022.02.004>.

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