Supplementary Materials for

Stem cell–homing hydrogel-based miR-29b-5p delivery promotes cartilage regeneration by suppressing senescence in an osteoarthritis rat model

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Materials and Methods

Preparation and evaluation of SKP@miR

Material synthesis and preparation of hydrogels

The antisense agomir/antiagomir-29b-5p (purity > 90%) strands were synthesized and purified by GenePharma (Suzhou, China). These strands were subjected to chemical modifications, including a cholesterol modification at the 3’ end, two thio modifications at the 5’ end, four thio modifications at the 3’ end, and a full base methylation modification of the antisense strand. The purity and identity of the agomir-29b-5p were confirmed by high-performance liquid chromatography (HPLC). The agomir/antiagomir-29b-5p were used for experiments according to the manufacturer’s instructions.

The self-assembling peptides RAD (Ac-RADARADARADARA-DA-NH2) and RAD-SKP (Ac-RADARADARADARADA-GG-SKPPGTTSS-NH2) (purity > 90%) were synthesized and purified by Scilight-Peptide Co., Ltd (Beijing, China); two glycine moieties were added as a linker to increase the flexibility of the functional epitopes. The purity and identity of the peptides were confirmed by analytical HPLC and electrospray ionization mass spectrometry (ESI-MS) (fig. S10 and fig. S11). The peptide powders were dissolved in distilled water to reach a final concentration of 1% (w/v), filter-sterilized with a syringe filter (0.22 μm HT Tuffrun membrane; Pall Crop., Ann Arbor, MI, USA), and sonicated for 30 min (VCX 130PB; Sonics, Newtown, CT, USA) for subsequent use.

The SKP solution was obtained by mixing 50% pure RAD solution with 50% RAD-SKP solution. The RAD@miR and SKP@miR solutions were obtained by mixing 500 μL of RAD and SKP solutions with 1 OD of agomir-29b-5p, respectively. The hydrogels were then fabricated within cell-culture Transwell inserts (12-mm diameter; Millipore, Billerica, MA, USA) (69). Briefly, 100 μL of the solution, with or without agomir-29b-5p, was added to the inserts and incubated for 15 min at 37 °C. Sufficient Dulbecco’s modified eagle medium (DMEM, Gibco, Life Technologies, Carlsbad, CA, USA) was then carefully added to the hydrogel and incubated at 37 °C for 15 min. The medium was changed a minimum of four times to equilibrate the hydrogel to physiological pH.

Circular dichroism (CD)

The CD spectra of the solutions were measured using Chirascan plus (Applied Photophysics, Leatherhead, UK). The solutions were first diluted to working concentrations of 0.01% (w/v), and 200 μL of the diluted samples was added to a quartz cuvette with a path length of 1 mm. CD spectra were recorded over the 180 nm to 260 nm range with a 1-nm step size at room temperature. All samples were evaluated thrice, and the mean was obtained.

Atomic force microscopy (AFM)

The diluted samples (0.01%) were dropped onto a freshly cleaved mica surface and incubated for 30 s, followed by rinsing with 100 μL of distilled water. After air drying, the samples were immediately observed using an atomic force microscope (AFM, Bruker Dimension ICON; Bruker, Billerica, MA, USA) with a silicon scanning probe (OMCL-TR400PSA-1; Olympus Corp., Tokyo, Japan) in contact mode. The scan area was 5 μm × 5 μm with a scan frequency of 1.00 Hz.

Transmission electron microscopy (TEM)
The peptide solution (10 mg/mL) was applied to a copper grid. The samples were then stained with uranyl acetate (2% w/v) and observed by TEM (TECNAI Spirit, FEI, Czech Republic).

**Fourier-transform infrared (FTIR) spectrometry**

After gelation, the hydrogels were lyophilized and ground into fine powders, which were then uniformly mixed with KBr. The FTIR spectrometer (TG-MS-FTIR-X70, NETZSCH Groups, Germany) was used to record the spectra with wavenumbers ranging from 4000 to 400 cm⁻¹.

**Molecular docking**

The molecular docking between self-assembling peptides and agomir-29b-5p was assessed using ZDOCK. The peptides and agomir-29b-5p were designed using PyMOL 2.3 based on the putative miR-29b-5p structure reported previously (69). The docking energy scores and interaction force between the peptides and agomir-29b-5p were then determined.

**Distribution of agomir-29b-5p in hydrogels**

1 OD of fluorescein isothiocyanate (FITC) labeled-agomir-29b-5p was mixed with 500 μL of RAD or SKP solution. Subsequently, RAD, RAD@miR, SKP, and SKP@miR hydrogels were prepared and imaged using a laser confocal microscope (LSM980; Carl Zeiss, Jena, Germany).

To exclude the effect of FITC, 1 OD of FITC labeled-agomir-29b-5p and 1 OD of FITC were prepared and imaged with a laser confocal microscope (LSM980; Carl Zeiss, Jena, Germany). The hydrogels were washed with phosphate buffered saline (PBS, R21-040-CV; Corning, New York, NY, USA) every 5 minutes. The fluorescence radiant efficiency at each time point in each group was quantitatively analyzed.

**Scanning electron microscopy (SEM)**

After gelation, the hydrogels were fixed with 2.5% glutaraldehyde for 2 h, washed twice in PBS, and dehydrated through successive washes with 30%, 50%, 70%, 80%, 90%, 95%, and 100% ethanol (v/v) for 30 min in each bath. The samples were then dried using a CO₂ critical point dryer (Samdri-PVT-3D; Tousimis, Rockville, MD, USA). The fresh fracture surfaces of the samples were sputter-coated with a layer of platinum in a sputter-coating chamber (EM ACE600; Leica, Wetzlar, Germany) and imaged with a scanning electron microscope (SEM, Carl Zeiss, Oberkochen, Germany) at an accelerating voltage of 5 kV.

**Rheological characterization**

Rheological properties of the hydrogels (8 mm in diameter, 1 mm in height) were measured using an 8-mm diameter parallel plate at 25 °C (Physica MCR301 rheometer, Anton Paar GmbH, Graz, Austria). Stress/strain sweeps (0.01–100% at 1 Hz) were performed to identify the limits of the linear viscoelastic region of the hydrogels. Subsequently, storage (G’) and loss (G’’) moduli were recorded in a dynamic frequency sweep test (0.1–10 rad/s at 0.5% strain). Each experiment was performed in triplicate.

**Agomir-29b-5p release**
Agomir-29b-5p powder was concentrated at the bottom of the tube by centrifugation at 4000 \( \times \) g. The tube was opened gently, and 60 \( \mu \)L of RAD and SKP solutions were independently added to 1 OD agomir-29b-5p powder and gently dispersed. Subsequently, 100 \( \mu \)L of PBS was carefully added onto the surface of the solution to enable self-assembly. The samples were incubated at 37 °C. PBS was replaced and collected every two days, and agomir-29b-5p concentration was measured using Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA).

In vitro experiments

Primary articular chondrocytes and synovium-derived mesenchymal stem cells (SMSCs) culture

Rat primary articular chondrocytes were isolated from femoral condyles and tibial plateaus of 1-day-old Sprague Dawley (SD) rats. Articular cartilage was excised and shredded, and then digested with 0.25% trypsin-EDTA (Sigma-Aldrich, City of Saint Louis, USA) at 37 °C in a shaker at a speed of 200 rpm for 1 h, followed by digestion with 0.2% type II collagenase (Sigma-Aldrich) at 37 °C overnight. The supernatant was filtered through a strainer and centrifuged to collect the cell precipitate. Cells were washed twice with PBS and cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and 1% penicillin-streptomycin (Thermo Fisher Scientific). The culture was maintained at 37 °C in a humidified atmosphere containing 5% CO\(_2\). When the cells reached a confluency of 70 to 80%, they were treated with IL-1β (R&D Systems, Minnesota, USA) to induce inflammation. To induce senescence in rat articular chondrocytes, the cells were treated with 100 ng/mL doxorubicin (D1515-10MG, Sigma-Aldrich, City of Saint Louis, USA) for 14 days or were subjected to three serial passages (P3). The doxorubicin-containing medium was refreshed every 2 days.

Rat primary SMSCs and human primary articular chondrocytes were purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China).

RNA fluorescent in situ hybridization (FISH)

The Cy3-labeled locked nucleic acid miR-29b-5p probes were designed and synthesized by RiboBio (Guangzhou, China). Probe signals were detected using a fluorescent in situ hybridization kit (RiboBio), according to the manufacturer’s instructions. For in vivo FISH, tissue sections were deparaffinized, rehydrated, and permeabilized via 0.8% pepsin treatment (37 °C for 30 min) before hybridization. The cells were then stained with 4′,6-diamidino-2-phenylindole (DAPI, D3571; Life Technologies) for 10 min, washed with PBS, and observed using a confocal laser scanning microscope (LSM980) or an automatic slide scanning system (Axio Scan Z1, Zeiss, Germany).

Luciferase reporter assay

The luciferase reporter plasmid was constructed by Hanbio (Shanghai, China). In brief, the vector FireflyLuciferase-RenillaLuciferase was selected, and XbaI (NEB) was used to obtain a purified linearized vector. Target gene fragments were amplified and ligated with the linearized vector, followed by transformation of DH5α competent cells. After culturing the DH5α competent cells with LB medium in culture plates for 12–16 h, the amplified sequence was detected by Sanger Sequencing to verify the presence of the target genes. The plasmid was
extracted and stored at -80 °C. HEK-293T cells were seeded into 24-well plates and cultured until they reached a confluency of approximately 70%; then, the luciferase reporter plasmid, agomir-29b-5p, and negative control (RiboBio, Guangzhou, China) were co-transfected into HEK-293T cells using Lipofectamine 3000 (Thermo Fisher Scientific), in accordance with the manufacturer’s instructions. Forty-eight hours after co-transfection, a dual luciferase reporter assay (Promega, Madison, WI) was performed to measure the luciferase activity. The firefly luciferase activity was normalized to Renilla luciferase activity for quantification.

**Cell live/dead assay**

After being cultured for 2 days, the cells were stained using a LIVE/DEAD viability/cytotoxicity assay kit (Invitrogen) in accordance with the manufacturer’s protocol. In brief, the cells were rinsed three times with PBS (5 min each), and incubated with 0.3 mL of PBS containing 2 mM calcein-AM and 4 mM ethidium homodimer (EthD-III) (Viability/Cytotoxicity Assay Kit for Live & Dead Animal Cells, Biotium, USA) for 15 min at 37 °C. After rinsing three times (5 min each time) with PBS, the samples were observed under a laser confocal microscope (LSM980).

**Cell proliferation assay**

On day 1, 3, 5, and 7 after the rat chondrocytes and SMSCs were seeded on hydrogels, cells were treated with 200 μL of Cell Counting Kit-8 (CCK-8; Dojindo, Tokyo, Japan) working solution for 2 h at 37 °C in the dark. Next, 100 μL of the supernatant was transferred to a 96-well plate, and the absorbance was measured at 450 nm using a Multimode Plate Reader (iMark™ 168; Bio-Rad Laboratories, Inc., California, USA) (71).

**Cytoskeletal staining**

To observe the adhesion behavior of cells on hydrogels, cytoskeletal staining was performed after culturing the rat chondrocytes and SMSCs for 2 days. Briefly, the culture medium was removed, and cells were gently washed with PBS three times, and subsequently fixed with 4% paraformaldehyde (BL539A; Saiguo Biotech Co., Guangzhou, China) for 30 min. After washing, the cells were permeabilized with 0.1% Triton X-100 (Cell Signaling Technology, Massachusetts, MA, USA) in PBS for 5 min, followed by blocking with 1% bovine serum albumin (BSA, Cell Signaling Technology) in PBS for 30 min at 37 °C. After the removal of the liquid, the cells were stained with rhodamine–phalloidin (1:300; cat. no. PHDR1; Cytoskeleton, Denver, CO, USA) at room temperature for 40 min, and washed with PBS. The cells were then stained with DAPI and observed using a confocal laser scanning microscope (LSM980).

**Chondrocyte viability analysis**

The cell culture plate was equilibrated at room temperature for 30 min, after which, equal volume of reagent was added to the cells. After sufficient lysis of the cells with vigorous shaking for 5 min, luminescence was detected at room temperature (DD1102, Vazyme biotech co., ltd., Nanjing, China).

**SA-β-Gal staining**

Cells were washed twice with PBS and fixed with 2% paraformaldehyde and 0.2% glutaraldehyde for 5 min. Fixed cells were washed and incubated with SA-β-gal staining solution at 37 °C for 12 to 16 h. Cells were subsequently washed with PBS and imaged using a light
microscope. Total cells and SA-β-gal positive cells were counted in three random fields per sample.

*Chondrocyte micro-masses culture*

The chondrocyte suspension (20 μL) containing 400,000 cells was added to each well of a 24-well plate, and allowed to rest for 3 h at 37 °C. Subsequently, 1 mL of culture medium containing different hydrogels was added, which was changed every two days. After 5 days of culture, the cells were stained with toluidine blue or Alcian blue, and observed by a light microscope (71).

*SMSC migration assay*

SMSC migration was assessed using Transwell inserts containing a polycarbonate membrane (Corning, New York, NY, USA). The hydrogels were placed in the bottom chamber of the wells, and SMSCs were seeded on top of the inserts. The culture medium was added to the top insert and bottom chamber. After a 24-hour incubation at 37 °C, the cells remaining on the top of the inserts were removed, while those that had migrated to the lower side of the insert were fixed with cold methanol and stained with crystal violet (0.1% w/v). The migrated cells were then imaged by a light microscope and quantified.

*SMSC differentiation assay*

Chondrogenic differentiation was induced in commercial chondrogenic differentiation medium of synovial mesenchymal stem cells (HUXSM-9004; Cyagen Biosciences, Suzhou, China), with the supplementation of transforming growth factor-β (TGF-β). Pellets were formed by centrifuging 1×10^5 of SMSCs at 300×g for 5 min in 500 μl of differentiation medium in 15 mL centrifuge tubes (Corning), followed by 24 h incubation at 37 °C. Then the pellets were implanted to RAD, RAD@miR, SKP, and SKP@miR hydrogels and cultured for 14 days at 37 °C in a humidified incubator with 5% CO2, with media changes every 2 days. After 14 days, the pellets were assessed using biochemical analysis for glycosaminoglycan (GAG) and DNA content, qRT-PCR for gene expression analysis, and WB for protein expression analysis. The pellets were cut into 5-μm-thick sections and subjected to toluidine blue, Alcian blue, and immunofluorescence staining.

*Biochemical analysis*

After chondrogenic culture for 14 days, triplicate pellets from each group were rinsed with PBS and digested overnight at 60 °C in 4 U/ml papain solution (Sigma-Aldrich, St Louis, MO, USA). The GAG content was measured using 1,9-dimethylmethylene blue (DMMB) assays. The DNA content was determined using the CyQuant cell proliferation assay kit (C7026; Thermo Fisher Scientific), with supplied bacteriophage λDNA as standard.

*Immunofluorescence (IF)*

The chondrocytes and SMSC pellet sections were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 for 5 min, followed by blocking in 10% goat serum for 1 h at 25 °C. Then, the cells were probed overnight with primary antibodies at 4 °C, and then incubated with goat anti-rabbit IgG H&L (Alexa Fluor 594, 1:200, ab150084, Abcam) and goat anti-mouse IgG H&L (Alexa Fluor 488, 1:200, ab150117, Abcam) for 1 h at 25 °C. Nuclei were stained with DAPI, and the cells were imaged using a confocal laser scanning microscope (LSM980).
Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted and purified from rat chondrocytes or SMSCs using TRIzol (Invitrogen) and an miRcute miRNA Isolation Kit (DP501, Beijing Tiangen Biotech Co., Ltd., Beijing, China), respectively, according to the manufacturer’s instructions. The mRNA or miRNA was then reverse transcribed to cDNA using a HiFiScript cDNA Synthesis Kit (CW2569M, Kangwei, Shanghai, China) or miRNA cDNA Synthesis Kit (CW2141, Kangwei), respectively. qRT-PCR was performed using iTaq SYBR Green supermix (172-5122; Bio-Rad, Richmond, CA, USA) and a 384 real-time PCR detection system (QuantStudio 6 Flex, Thermo Fisher Scientific). Target gene expression was normalized to that of β-actin. miRNA expression was quantified using the miRNA qPCR Assay Kit (CW2142, Kangwei), and normalized to U6 levels. The 2−ΔΔCt method was used to calculate the relative expression. All experiments were performed in triplicate. All primers are listed in Table S3.

Western blot (WB)

Cells or tissues were lysed using radio immunoprecipitation assay buffer (Beyotime, Shanghai, China) supplemented with 100 mM phenylmethanesulfonyl fluoride (Beyotime) on ice. The bicinchoninic acid assay (Beyotime) was used to qualify total protein. Proteins were resolved by running on 8%, 10%, or 12% SDS-PAGE gels and transferred onto polyvinylidene fluoride membranes (Bio-Rad). The membranes were then blocked with 5% nonfat milk at room temperature for 1 h. After that, the membranes were probed overnight with primary antibodies at 4 °C and washed with Tris Buffered Saline with Tween® (TBST, 25 mM Tris, 0.15M NaCl, 0.05% Tween-20, pH 7.5, Thermo Fisher Scientific). Then, the membranes were incubated with secondary antibodies at room temperature for 1 h. Protein bands were visualized using FDbio-Femto ECL (Fudebio, Hangzhou, China) and a chemiluminescence system (ChemiDoc™ Touch Imaging System, Bio-Rad).

The following primary antibodies were used: high affinity anti-MMP13 antibody (1:1000, Abcam, London, UK), anti-MMP3 antibody (1:1000, Abcam), anti-ADAMTS4 antibody (1:1000, Abcam), anti-ADAMTS5 antibody (1:1000, Abcam), anti-COL2A1 antibody (1:1000, Abcam), anti-COL1A1 antibody (1:1000, Proteintech), anti-SOX9 antibody (1:1000, Abcam), anti-AGGRECAN antibody (1:100, ABclonal), anti-TET1 antibody (1:1000, Abcam), anti-P16INK4a antibody (1:1000, Abcam), anti-P21 antibody (1:1000, Abcam and Santa), and anti-β-actin antibody (1:2000, Cell Signaling Technology). These antibodies were diluted in primary antibody dilution solution (MB9881, Dalian Meilun Biotechnology Co., Ltd., Liaoning, China). The following secondary antibodies were used: goat anti-mouse or goat anti-rabbit secondary antibody (1:5000, Cell Signaling Technology).

RNA sequencing

Primary rat chondrocytes were transfected with 50 nM agomir-NC or agomir-29b-5p and incubated in fresh medium for 48 h. SMSCs were transfected with 50 nM agomir-NC (control group) or agomir-29b-5p (miR group), or seeded on RAD, RAD@miR, SKP, or SKP@miR hydrogels (RAD, RAD@miR, SKP, and SKP@miR groups) for 7 days. Three biological replicates were analyzed for each experimental group. Total RNA was isolated using TRIzol (Invitrogen), in accordance with the manufacturer’s instructions. RNA was sequenced by LC-Bio Technology Co., ltd. (Hangzhou, China).

Bioinformatic analysis
The mRNAs identified from RNA-seq data of rat chondrocytes transfected with agomir-NC or agomir-29b-5p were subjected to four bioinformatic tools, i.e., TargetScan (http://www.targetscan.org/), Starbase (http://starbase.sysu.edu.cn/), miRTarbase (http://miRTarbase.mbc.nctu.edu.tw/), and miRWalk (http://mirwalk.umm.uniheidelberg.de/, energy<-20, accessibility>0.01) to predict the target mRNAs.

**In vivo evaluation**

**Rat model of osteoarthritis**

All animals were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Both knee joints of eight male SD rats (aged 4 weeks and average 90 g) in the Sham group were washed with normal saline and sutured without any other treatment. Thirty-two rats were used to generate an OA model in which OA is induced by anterior cruciate ligament transection (ACLT) (72). Briefly, in each case, the knee joint was entered through the medial capsule, and the anterior cruciate ligaments were exposed and transected. An anterior drawing test was performed to verify the success of the operation: The rat knee was bent to 90 degrees, with the femoral segment fixed by the researcher using their left hand, and a back-to-front force was applied through the tibia of the rat with their right hand. The tibia of the rat could be significantly displaced relative to the femur, indicating that its anterior cruciate ligament was completely broken. The joint was then closed following irrigation with sterile saline. These rats were randomly divided into four groups (PBS, miR, SKP, and SKP@miR) after 4 weeks, and treated as follows:

- Sham group: 60 μL of PBS injected intra-articularly using a 25 μL microsyringe (Gaoge).
- PBS group: 60 μL of PBS injected intra-articularly.
- miR groups: Agomir-29b-5p powder was concentrated at the bottom of the tube by centrifugation at 4000 × g for 15 min. The tube was opened gently, and 60 μL of DEPC water were added to 1 OD agomir-29b-5p powder, and gently dispersed. The solution was then injected intra-articularly.
- SKP group: 60 μL of SKP solution injected intra-articularly.
- SKP@miR group: Agomir-29b-5p powder was concentrated at the bottom of the tube by centrifugation at 4000 × g for 15 min. The tube was opened gently, and 60 μL of SKP solution was added to 1 OD agomir-29b-5p powder, and gently dispersed. The solution was then injected intra-artically.

Samples were harvested at 3 and 6 weeks after implantation. The joints of 12-month-old male C57BL/6 mice (n = 5) were injected with agomir-29b-5p or agomir-NC (left and right) every two weeks for ten consecutive weeks.

**In vivo tracking of agomir-29b-5p**

Cy5.5 was linked to a phosphate at the 5’-end of the agomir-29b-5p, followed by a preparation of RAD@miR and SKP@miR solutions, as described above. Four-week-old male C57BL/6 mice (n = 3) were subjected to the ACLT procedure, after which, RAD@miR and SKP@miR solutions (10 μL) were injected into the joint cavity of mice, respectively. Imaging was then performed on day 1, 3, 5, 7, 9 and 14 (IVIS Lumina LT, Perkin Elmer, Waltham, Ma, USA).
**Cartilage explant penetration study**

For porcine cartilage explant penetration assay, we obtained young (2 weeks old) porcine knee joints from Zhejiang Chinese medical university laboratory animal research center, harvested cartilage explants from the trochlear groove using biopsy punch (5 mm in diameter and 2 mm in thickness), and cultured them in chemically defined medium (DMEM, 100 μg/mL streptomycin and 100 U/mL penicillin, 1% ITS+Premix) in 96-well plates. The cartilage explants were then incubated with 50 μL of Cy5.5 labeled-agomir-29b-5p (1 OD agomir-29b-5p in 500 μL DEPC water), RAD@miR hydrogel (1 OD agomir-29b-5p in 500 μL RAD) or SKP@miR hydrogel (1 OD agomir-29b-5p in 500 μL SKP) in 100 μL of culture medium for 1, 2, 3, 4 and 5 days at 37 °C under gentle agitation with medium replacement every other day. After incubation, cartilage explants were washed three times with PBS, fixed with 4% paraformaldehyde, and cut transversely using a frozen slicer (Leica, Wetzlar, Germany). Sections were mounted with DAPI Fluoromount-G Mounting Medium on glass slides and immediately observed under an automatic slide scanning system (Axio Scan Z1, Zeiss, Germany). All images are taken under the same laser intensity.

**Micro computed tomography (Micro-CT)**

Reconstructed imaging of the rat knee joint was performed using a high-resolution micro-CT instrument (InspeXio SMX-225 CT FPD HR; Shimadzu Co. Ltd., Kyoto, Japan) (71). Briefly, X-ray energy of 225 kV and a 10-μm isotropic voxel were used to image the knee articular cartilage, distal femur, and proximal tibia. Each knee joint was reconstructed using a data analyzer (VGStudio MAX; Volume Graphics, Heidelberg, Germany). Abnormally proliferating osteophytes and the following parameters were evaluated by three independent researchers.

- Trabecular separation (Tb.Sp, μm): average width of the medullary cavity between the trabeculae.
- Trabecular thickness (Tb.Th, μm): average trabecular thickness in the selected area.
- Bone volume fraction (BV/TV): fraction volume of bone in the selected area (BV) divided by the sample volume (TV).
- Trabecular number (Tb.N): number of intersections between bone and non-bone in the selected area.
- Bone surface/volume ratio (BS/BV, 1/mm): surface of bone in the selected area (BS) divided by the volume of bone (BV) (70).

**Histological analysis**

Rat and mouse knee articular cartilage specimens were fixed with 4% paraformaldehyde and decalcified before paraffin embedding. Each paraffin-embedded cartilage sample was sectioned into 5-μm-thick slices for subsequent histological analysis. To evaluate proteoglycan loss, safranin O/fast green staining (0.1% safranin O, 0.01% fast green solution; Solarbio, Beijing, China) and hematoxylin and eosin (H&E) staining were performed after deparaffinization and hydration. The sections were imaged using an automatic digital slide scanning system (Axio scan Z1, Zeiss).

**Immunohistochemistry**

The sections were incubated with primary antibodies at 4 °C overnight, washed thrice with PBST and incubated with a secondary antibody (Beyotime Institute of Biotechnology, Inc., Jiangsu, China) for 2 h at room temperature. The positively stained cells on the entire articular surface per specimen were counted, and the percentage of positive cells was calculated using Image-Pro Plus 6.0 (NIH, Bethesda, MD, USA). The number of cells positive for the marker was
quantified and expressed relative to the total number of cells, and the assessment was independently reviewed in parallel by two experienced pathologists.

*Immunofluorescence*

The sections were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 for 5 min, followed by blocking in 10% goat serum for 1 h at 25 °C. Subsequent incubation in primary antibodies against CD73 and CD90 was performed overnight at 4 °C and then the samples were incubated with goat anti-rabbit IgG H&L (Alexa Fluor 594, 1:200, ab150084, Abcam) and goat anti-mouse IgG H&L (Alexa Fluor 488, 1:200, ab150117, Abcam) for 1 h at 25 °C; nuclei were stained with DAPI. The sections were imaged using an automatic slide scanning system (Axio scan Z1, Zeiss).
Fig. S1. Small RNA sequencing reveals miR-29b-5p as potentially associated with OA development. (A) Flow chart presenting the screening method used for identifying OA-associated miRNA and conservation of miRNA across species. Validation of the miRNA expression in (B) senescent (P3) human chondrocytes, (C) senescent (P3) rat chondrocytes, (D)
OA (IL-1β) rat chondrocytes, and (E) OA (IL-1β) human chondrocytes, compared with normal chondrocytes, respectively. \( n = 3 \). Data are presented as means ± SD. Statistical analysis was performed using two-tailed Student’s \( t \)-test. \* \( P < 0.05 \), \** \( P < 0.01 \), and \*** \( P < 0.001 \).
Fig. S2. Transcriptome-wide co-expression analysis reveals that miR-29b-5p inactivates putative functional modules related to Rap1 and PI3k. (A) Heatmap representing the co-expression patterns of genes downregulated by miR-29b-5p in chondrocytes. A strong positive correlation between the corresponding gene profiles is denoted in red, while a strong negative correlation is denoted in blue. Four putative gene clusters are identified by hierarchical clustering. (B) Hypergeometric p-values for putative miR-29b-5p target genes and each gene cluster. KEGG analysis of (C) cluster 2 and (D) cluster 3. (E) qRT-PCR analysis of Rap1 and PI3K-related genes in rat chondrocytes. n = 3. Data are presented as means ± SD. Statistical
analysis was performed using two-tailed Student’s $t$-test. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. 
Fig. S3. Molecular docking and material characterization of hydrogels. (A) FTIR spectra of hydrogels. The four hydrogels have peaks at 1630 cm\(^{-1}\) and 1695 cm\(^{-1}\), which are respectively associated with C=O stretching vibration and SAP-backbone conformation, thus confirming anti-parallel \(\beta\)-sheet structures. RAD@miR and SKP@miR exhibit a peak at 840 cm\(^{-1}\). (B) CD
spectra of peptide/agomir-29b-5p solutions showing typical β-sheet structure with a positive band at 195 nm and a negative band at 216 nm. (C) AFM and TEM images of peptide/agomir-29b-5p solutions. (D) RAD and agomir-29b-5p binding site prediction, such as hydrogen bonding (Ala14, Ala6, and Arg5). (E) RAD-SKP and agomir-29b-5p binding site prediction, such as hydrogen bonding (Lys20 and Ser19). Z-rank scores of (F) RAD/agomir-29b-5p and (G) RAD-SKP/agomir-29b-5p.
Fig. S4. Properties of hydrogels and agomir uptake by cells. (A) Representative fluorescence images of hydrogels with FITC (SKP@FITC) or FITC-labeled agomirs (SKP@FITC-miR) via a live imaging system at 0, 10, 30, and 60 min. The hydrogels were washed with PBS every 5 minutes. (B) Quantitative analysis of time course fluorescence radiant efficiency of SKP@FITC and SKP@FITC-miR hydrogels. \( n = 3 \). (C) SEM images of RAD, RAD@miR, SKP and SKP@miR. (D) Rheological characterization of the hydrogels using strain sweep studies. (E) Quantitative analysis of time course fluorescence radiant efficiency within knee joints after intra-articular injection of RAD@miR and SKP@miR. \( n = 3 \). (F) FITC labeled-agomir-29b-5p uptake by rat chondrocytes (RCs) and SMSCs cultured on tissue culture plate for 2 days. Data are
presented as means ± SD. Statistical analysis was performed using two-tailed Student’s $t$-test. *$P$ < 0.05.
Fig. S5. Agomir-29b-5p penetration ex vivo and histological analysis in vivo. (A) Quantitative analysis of Cy5.5 labeled-agomir-29b-5p penetration depth into porcine cartilage explants after
5-day incubation. \( n = 3 \). **(B)** Micro-CT diagnosis of subchondral bone. **(C–E)** Trabecular separation (Tb.Sp, μm): average width of the medullary cavity between the trabeculae. Trabecular thickness (Tb.Th, μm): average trabecular thickness in the selected area. Bone volume fraction (BV/TV): fraction volume of bone in the selected area (BV) divided by the sample volume (TV). Trabecular number (Tb.N): number of intersections between bone and non-bone in the selected area. Bone surface/volume ratio (BS/BV, 1/mm): surface of bone in the selected area (BS) divided by the volume of bone (BV). BV/TV, Tb.Th, Tb.N, Tb.Sp, and BS/BV of each group after 7 and 10 weeks. \( n = 4 \). Data are presented as means ± SD. Statistical analysis was performed using one-way ANOVA. \(* P < 0.05\) and \( ** P < 0.01\). Histological scores of **(F)** enlargement of the synovial lining cell layer, **(G)** inflammatory infiltrate, and **(H)** density of the resident cells in rat synovium. \( n = 4 \). Histological scores of **(I)** cell morphology, **(J)** articular cartilage damage and ulceration, **(K)** safranin-Orange staining, **(L)** matrix staining and **(M)** surface regularity of rat cartilage. \( n = 4 \). Data are presented as means ± SD. Statistical analysis was performed using one-way ANOVA for intergroup comparison with SKP@miR at 7 or 10 weeks \(* P < 0.05\) versus SKP@miR group at 7 weeks, and \#P < 0.05 versus SKP@miR group at 10 weeks), and two-tailed Student’s \( t \)-test for comparing data at 7 and 10 weeks in SKP@miR group in **(F)** to **(M)**.
Fig. S6. Effects of hydrogels on chondrocyte senescence. (A) CCK8 results of rat chondrocytes cultured on hydrogels at 1, 4, and 7 days. $n = 3$. (B) Live/dead and cytoskeleton
staining of rat chondrocytes cultured on hydrogels at 2 days. Representative images of SA-β-gal staining of rat chondrocytes (C) treated with doxorubicin and (D) following serial passaging to P3. CCK8 results of (E) doxorubicin (100 nM)-treated rat chondrocytes and (F) P3 chondrocytes cultured on hydrogels at 1, 4, and 7 days. n = 3. (G) Cell viability analysis of P3 chondrocytes and doxorubicin-treated chondrocytes cultured on hydrogels at 7 days. qRT-PCR analysis of P21, P16, and P53 expression in rat (H) normal chondrocytes, (I) doxorubicin-treated chondrocytes, and (J) P3 chondrocytes cultured on hydrogels at 7 days. n = 3. Data are presented as means ± SD. Statistical analysis was performed using one-way ANOVA. *P < 0.05 and **P < 0.01.
Fig. S7. Gene expression of normal and OA chondrocytes on hydrogels. qRT-PCR analysis of Sox9, Aggrecan, Col2a1, Adamts4, Mmp13, Adamts5, and Mmp3 expression in rat (A) OA
chondrocytes induced by IL-1β and (B) normal chondrocytes. n = 3. Data are presented as means ± SD. Statistical analysis was performed using one-way ANOVA. *P < 0.05 and **P < 0.01.
Fig. S8. Stem cell recruitment in rat joints. Immunofluorescence staining of CD90 and CD73 in each group at 10 weeks after ACLT. SMSCs (left), synovium (right), and chondrocytes (below) are indicated.
**Fig. S9.** Effects of hydrogels on SMSC behavior. (A) CCK8 results of SMSCs cultured on hydrogels at 1, 4 and 7 days. *n* = 3. (B) Live/dead and cytoskeleton staining of SMSCs cultured on hydrogels at 2 days. (C) GO enrichment bar plot of RAD and SKP groups. (D) KEGG
analysis of RAD and SKP groups. (E) GO enrichment bar plot of RAD and RAD@miR groups.
(F) KEGG analysis of RAD and RAD@miR groups. Data are presented as means ± SD.
Statistical analysis was performed using one-way ANOVA. *P < 0.05 and **P < 0.01.
Fig. S10. Analytical HPLC trace and electrospray ionization mass spectrometry (ESI-MS) analysis of RAD peptide. (A) Analytical HPLC trace of RAD peptide. (B) ESI-MS analysis of RAD peptide.
Fig. S11. Analytical HPLC trace and electrospray ionization mass spectrometry (ESI-MS) analysis of RAD-SKP peptide. (A) Analytical HPLC trace of RAD-SKP peptide. (B) ESI-MS analysis of RAD-SKP peptide.
**Table S1. Macroscopic evaluation of cartilage repair.**

| Criteria                                      | Appearance                                                                 | Points |
|----------------------------------------------|----------------------------------------------------------------------------|--------|
| Cell morphology                              | Hyaline cartilage                                                          | 0      |
|                                              | Mostly hyaline cartilage                                                   | 1      |
|                                              | Mostly fibrocartilage                                                      | 2      |
|                                              | Mostly non-cartilage                                                       | 3      |
|                                              | Non-cartilage only                                                        | 4      |
| Matrix staining (metachromasia)              | Normal (compared with host adjacent cartilage)                             | 0      |
|                                              | Slightly reduced                                                          | 1      |
|                                              | Markedly reduced                                                          | 2      |
|                                              | No metachromatic stain                                                     | 3      |
| Surface regularity (total smooth area compared with entire area of cartilage defect) | Smooth (> 3/4)                                                            | 0      |
|                                              | Moderate (> 1/2–3/4)                                                       | 1      |
|                                              | Irregular (1/4–1/2)                                                        | 2      |
|                                              | Severely irregular (< 1/4)                                                 | 3      |
| Articular cartilage damage and ulceration    | Normal intact cartilage                                                    | 0      |
|                                              | Chondral softening and blistering, superficial lesions, fissures and cracks, soft indentation | 1      |
|                                              | Fraying, lesions and fissures extending down to <50% of cartilage depth    | 2      |
|                                              | Partial loss of cartilage thickness, cartilage defects extending down >50% of cartilage depth as well as down to calcified layer | 3      |
|                                              | Full-thickness cartilage loss with exposure of the subchondral bone        | 4      |
| Safranin-Orange staining                     | Normal                                                                     | 0      |
|                                              | Slight reduction                                                          | 1      |
|                                              | Moderate reduction                                                         | 2      |
|                                              | Severe reduction                                                           | 3      |
|                                              | No dye noted                                                               | 4      |
| Osteoarthritis Research Society International (OARSI) scores | surface intact, cartilage intact                                          | 0      |
|                                              | surface intact                                                            | 1      |
|                                              | surface discontinuity                                                      | 2      |
|                                              | vertical fissures                                                         | 3      |
|                                              | erosion                                                                    | 4      |
|                                              | denudation                                                                 | 5      |
|                                              | deformation                                                                | 6      |
Table S2. Synovitis score.

| Enlargement of the synovial lining cell layer | 0 points | The lining cells form one layer |
|---------------------------------------------|----------|--------------------------------|
|                                             | 1 point  | The lining cells form 2–3 layers |
|                                             | 2 points | The lining cells form 4–5 layers, few multinucleated cells might occur |
|                                             | 3 points | The lining cells form more than 5 layers, the lining might be ulcerated and multinucleated cells might occur |

| Density of the resident cells | 0 points | The synovial stroma shows normal cellularity |
|------------------------------|----------|---------------------------------------------|
|                              | 1 point  | The cellularity is slightly increased |
|                              | 2 points | The cellularity is moderately increased, multinucleated cells might occur |
|                              | 3 points | The cellularity is greatly increased, multinucleated giant cells, pannus formation and rheumatoid granulomas might occur |

| Inflammatory infiltrate | 0 points | No inflammatory infiltrate |
|-------------------------|----------|----------------------------|
|                         | 1 point  | Few mostly perivascular situated lymphocytes or plasma cells |
|                         | 2 point  | Numerous lymphocytes or plasma cells, sometimes forming follicle-like aggregates |
|                         | 3 points | Dense band-like inflammatory infiltrate or numerous large follicle-like aggregates |

| Sum | 0-1 | No synovitis |
|-----|-----|--------------|
|     | 2-4 | Low-grade synovitis |
|     | 5-9 | High-grade synovitis |
Table S3. List of PCR primers, miRNAs, and siRNAs.

| RAT-Adams5   | F | TCGTGGCCCGCGTCTTTGCTCAC |
|--------------|---|-------------------------|
|              | R | ACGCCGGACCTCAGACGTGGTG  |
| RAT-Col2a1   | F | CCTGGACCCCCGTGGCAGAGA   |
|              | R | GCAGGGCCAGAAGTACCCGTGTCATC  |
| RAT-Sox9     | F | GCACCAGGGTGTCAGTACA     |
|              | R | TAAATTCCCCAGTGTGCAATCC  |
| RAT-Adams4   | F | AGTTGACAGGGGTTCGGGATG   |
|              | R | CTTCGCCGTAGTGATTCCGTTG  |
| RAT-Mmp3     | F | AGTGCTTCTGAAATGTCTTCG   |
|              | R | TCTTCTGAAACTTGGGCAG     |
| RAT-Mmp13    | F | CATCATCTGGGAGCACTGAAA   |
|              | R | GCAGCTCCAAGGGCTACAA     |
| RAT-Aggrecan | F | TGGGCTTCTGGGGTCCACAAA   |
|              | R | CATTCGCCAGGGAGCAAGCCA   |
| RAT-Ccnd2    | F | CAAGTCTGCAATGTACCCCGC  |
|              | R | TAAGCAGCACAGCCTCGATT    |
| RAT-Lama5    | F | TCCCCAGGAATAAGCCTCCA    |
|              | R | GAAGGACTGTGGCTCGACAA    |
| RAT-Angpt2   | F | CATGATGTCACCCGACT       |
|              | R | TCCATGTCACAGTAGGCTCTTG  |
| RAT-Csf1r    | F | TTATCTGGGAGAAGAGTAAGGACC|
|              | R | CTGTTGCACCTGGCTCTACAA   |
| RAT-Rapgef5  | F | ACAGGAACGTCGGATGAC      |
|              | R | TTCATCAGAGTGGCGAGGC     |
| RAT-Map2k6   | F | CACGTATCCAGAGGCTTATGCAAC|
|              | R | ACTTCGCCCTGTAAACCCAC    |
| RAT-Fyb1     | F | ATGCTGTTCCACCGAGACC     |
|              | R | GTGGGAACATCCCCCTCCAATTT |
| RAT-Kdr      | F | ATCTCCATCTTTTTGGGTGGGATG|
|              | R | TCGTCACCTGGAGTAGACGTTG  |
| RAT-Pik3r3   | F | CATCTTACACGTTAAGCGCGATG|
|              | R | TCACTGGCTTGGGCTTT       |
| RAT-Lcp2     | F | TCCAACGATTAGGAGGCCCTCT  |
|              | R | TTTATGTTGGCGAGATGTGAT   |
| RAT-Itgb2    | F | AGGAGGGTTCGAGGGTTCTT    |
|              | R | CACTCCGACACAGAAACGTA    |
| RAT-Calml3   | F | AGTCTCCTCTGAACCGGAAC    |
| Gene   | Forward Primer | Reverse Primer |
|--------|----------------|----------------|
| RAT-Fgfr3 | TGTACGTGAAAACCTCCCTTC | TGGAGTCTCAGGAAGTCATC |
| RAT-Ltga7  | AACCTAACTGAAGGGGTTGC | TAAACACCCGTCCTCCGCT |
| RAT-Lbsp   | AGAACAATCCGTGGCCTCA | CGGTACTTTAAGACCCCGT |
| RAT-Bmp2   | FGAAGCCAGGTGTCTCCAAGAG | GTGGATGTCCTTTACCGTCG |
| RAT-Alp    | AGCGACACGGACAAGAAGC | GGCAAAGACGGACATC |
| RAT-Runx2  | CAGACCAGCAGCAGCTCCATA | CAGCGTCAACACCCATC |
| RAT-Ocn    | AGATTGGTTGGGCAACAAGGT | CCTTCACGCAAGGAAACCGAT |
| RAT-Opn    | GGAAGTCCGATGGCTTATGTA | TCCGACTGCTCAGTGCTC |
| RAT-Ppar   | CCTTTACCCAGGTTGACCTTC | CCTTCAACTTTTAGAGGCTC |
| RAT-Lpl    | AGCTCTACTTTGAGCAGCTT | CCTGCCTGTTCCCTCCAGCT |
| RAT-P16INK4a | GCCGTGGCCGAAAGTGAAGCCA | CGTCGTGCGGATTTGCGGAT |
| RAT-P53    | CTGGTGAGGCAAGGTTGCGT | GGAATCTTCTGGGACGGGACA |
| RAT-Tet1   | CAGACAGTGCAAGACCAGAT | TTGTGGGCCCTCCATCATCA |
| RAT-β-actin | TGTCACCAACTGGAAGGACGATA | GGGTGTGTGGAAGGCTCA |
| miR-133b  | CTTTGGTTCCCTTCAACCAGCTA |
| miR-10a-3p | CCGCGCAATTCGATCTAGGGGAATA |
| miR-137-3p | CCGCGTATTTGCTTAAGAATACCGTAG |
| miR-761   | GCAGCAGGGTGAAACTGACACA |
| miR-142-5p | GCAGCAGGTTGAAACTGACACA |
| miR-29b-5p | CGCTGGTTTTCACATGGGTGCTTA |
| miR-409-5p | CAGGTTACCCGAGCAACTTTGCTA |
| miR-568   | CGCGCGGATGTATGATAATGTATACACA |
| Gene   | Forward Primer | Reverse Primer |
|--------|----------------|----------------|
| U6-1   | CTGCTTCCGGCACACA |               |
| U6-2   | GCTTCGGCAGCACATATACTAAAAAT |               |
| RAT-Mmp9 | TTTCTTCAAGGACGTCGCT | GTACACCCACATTTTGGGCC |
| RAT-Agt  | TGGCTAAGCCTGGATTCCTT | GCAACTTGTTCTGTTGTTTCTTTTA |
| RAT-Hspb1 | CACCCGAAAATACACGCTC | TTGGCTCCAGACTGTTTCCGA |
| RAT-Bax  | ACGTCTGCGGGGAAGTCA | CTGATTCGTCATGAAACCTC |
| RAT-Lum  | GTGTCAGAGAGTAAGGGCACA | GTGCACAGTGGGTGTAGAGT |
| RAT-Trip11 | GGAGACATATTGCTCAGACAGC | CCGACGACTTATCTCTGTCAGT |
| RAT-Itgb8 | CCACAGAGCCTCAAGGATT | TGAGACATCTCCGGAGATCA |
| RAT-Hif1a | TCATCCAAGGAGCCTTAACCT | TGCTGCAGTAACGTTCCAATCC |
| RAT-Sulf1 | TATGACCCACACAGAGGACAGT | TTGATGTCGTTTGCACCGGA |
| RAT-Sfrp2 | AAGCTCCCAAGGTGTGTAA | TCACCTTGAGCTTTCAGTGCGAA |
| RAT-Creb3l2 | CTTCAGCAGCACCTGTTCA | TGATGTCGTTTTCACCCGGG |
| RAT-Tgfbr2 | TTCCCAAGTCGGTAAACAGCG | TGTCGTTCTTCCTCCCAACAG |
| RAT-Smad1 | CTCTGCAGATGCCAGCCGAC | TTCGTGTCTTCCTTCCGGC |
| RAT-Pdgfra | CTCACTTTTTCCTCCGGGCT | ATGAGGCTCCGGCCCTGT |
| RAT-Rock2 | CCAAACAAAACCAAGCTAACTGC | CACGCGCATGTTGTTGATATGA |
| RAT-Itgav  | GTGCCTATCTCCGGGATGAA | GAGTGTGTTACCTGCCGACT |
| RAT-Lamb1 | ATGTGCCGATGAGCAGAGAC | TTGCGTGCTTCCTTCCTTGCAGC |
| Gene   | Forward | Reverse |
|--------|---------|---------|
| RAT-Hgf | GCAATAAAAGCAGCTCAGAACC | CTCTTCTTCGGTCCTTCTGCATAG |
| RAT-Flnb | AAGTCACCTGCGTGATCCTC | GGCTCTTTCCGTAACCAGTAG |
| RAT-Vcl | TGCTTCTTCTCAGCTCAGATCATAT | CCTCATCCAGGAGGTGAGTT |
| RAT-Igf1r | TGATCATGTCTGCGCCTTC | TCAGGCACGTACACATCAG |
| RAT-Pik3r1 | GAGGGAGACTTTCCAGGAACT | GATCAGGAAGGGTCAACACTGGTT |
| RAT-Itga1 | CAGTCCACGAACACATCAG | TGCTGGGACTTGACGATCAG |
| RAT-Fgfr4 | TGGAGTTCTCGGAAGTGCATC | TACACGGTCAAAACAACGCT |
| RAT-Zfp36l2 | CCGCAAGCAACACATCAG | TGCTGCCTGGTCTTTG |
| RAT-Itpr1 | CCTGTGGGAAGTGGAGGTAG | CACTGAGGGCTGAAACTCCA |
| RAT-Eif4ebp1 | TCCTGATGGAGTGTCGGAAC | AACTGTGACTCTTCACCACCTG |
| RAT-Hipk1 | AGCGGAGGGTTCACATGAT | AGGCTAAAAACACTGGCCT |
| RAT-Nfatc3 | ACGTTTTACTTACCACACCAGTTTTG | TGGGCTGCATGGAACAAATCA |
| RAT-Mif | AAGCCGGACAGTACATC | CGATCTTTGGCGAGGTCTCT |
| FISH-miR-29b-5p | CY3-CTAAGCCACCATGGAACACCAG-CY3 |
| si-r-Tet1-001 | GATCCTTTCAGAATCCTA |
| si-r-Tet1-002 | GTGCTCATATTTCACAAGAG |
| si-r-Tet1-003 | CACCAGATCTGTAAGAAGA |
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