Research Square

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PEDF peptide stimulates chondrocyte regeneration in rats with monoiodoacetate-induced articular cartilage injury

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Research Article

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Abstract

Background: Intra-articular injection of monoiodoacetate (MIA) has shown to induce extensive chondrocyte degeneration/death in articular cartilage (AC), resulting in destruction of the AC in animals. This has been used often as model of osteoarthritis (OA). Pigment epithelium-derived factor (PEDF) and its derived short peptide 29-mer have been shown to heal tissue injury by activating various types of stem/progenitor cells localized near the lesions. Here we investigated whether the 29-mer is able to evoke chondrogenesis in rats with MIA-injured AC.

Methods: AC destruction was induced in Sprague-Dawley rats by a single intra-articular injection of MIA (1 mg) in the right knee. After MIA injection for 7 days, the 29-mer infused with 5% hyaluronic acid (HA) was injected intra-articularly twice, at 4-day intervals. Also, BrdU was injected intraperitoneally into the rats to detect cell proliferation in the damaged AC. The histopathology of AC was determined by hematoxylin and eosin (H&E) staining and Safranin O staining. The regeneration of chondrocytes in the AC was detected by dual-immunostaining of BrdU and chondrocyte markers, including aggrecan, collagen type 2 and Sox9. Changes in hind paw weight distribution were measured to evaluate the MIA-induced joint discomfort. Bone marrow-derived mesenchymal stem cells (MSCs) were used to detect chondrogenic differentiation. The involvement of the PEDF receptor and STAT3 signaling on the 29-mer effects was evaluated using specific inhibitors.

Results: MIA injection caused chondrocyte death throughout the AC, with cartilage degeneration thereafter. The 29-mer/HA treatment induced extensive chondrocyte regeneration in the damaged AC, accompanied by the partial recovery of the cartilaginous matrix. The 29-mer/HA also exerted an antinociceptive effect in rats. In culture, the 29-mer promoted chondrogenic differentiation of MSCs with evidence of increased expression of chondrogenic marker genes and proteoglycans, as well as the phosphorylation of STAT3. Pharmacological inhibitors of PEDF-R and STAT3 signaling dramatically blocked the 29-mer effects on cultured MSCs and chondrogenesis in the injured AC.

Conclusions: The 29-mer/HA formulation has the ability to stimulate chondrocyte regeneration in the MIA damaged AC. The 29-mer may be a novel agent for the development of future OA treatments.

Background

Osteoarthritis (OA), the most common joint disease, is a degenerative disorder resulting from the breakdown of articular cartilage (AC) in synovial joints [1, 2]. With age, AC degenerates at a cellular level (i.e. chondocytes) and the amount of cartilaginous matrix decreases, leading to an overall loss of cartilage height [1, 3]. A breakdown in the structure of hyaline cartilage affects the load-bearing ability of AC and leads to OA, which affects millions of people worldwide [2]. However, AC has a limited ability to self-heal after trauma because of its avascular nature and the resting state of articular chondrocytes [1]. In addition, cartilage frequently may be injured, such as in sports-related trauma [1]. The pharmacologic treatment options for chondral defects are very limited; these include analgesics, anti-inflammatory drugs,

and intra-articular injection of HA to improve joint lubrication [2]. Surgical options range from arthroscopic procedures to total joint arthroplasty [2]. In addition, autologous stem cell transplantation using MSCs from various tissue sources is under development and has shown potential in improving pain relief and AC regeneration in animals and in clinical studies of knee OA [3].

Adult MSCs are able to expand in vitro and therefore provide an attractive option for the preparation of autologous chondrocytes for delivery in OA therapy [4, 5]. However, high-quality OA treatment by MSCs may be obstructed by high cost of investing in multiple steps. In addition, chondrogenic markers, including transcription factor Sox9 (SRY-type high mobility group box 9) as well as cartilage matrix proteins aggrecan and collagen type 2, may be reduced during the chondrogenic differentiation of MSCs, leading to the development of hypertrophic cartilage, instead of hyaline cartilage, in culture and in vivo [5]. Alternatively, TGFβ infused with a scaffold and implanted to cover injured AC has been documented to facilitate AC repair by recruitment of host condroprogenitor cells into the scaffold [6, 7]. The chondroprogenitor cells, which reside in the bone marrow, synovium, perichondrium, adipose tissue, tendons and superficial zone of the AC, have been proposed as potential mobile cells to heal chondral injuries [8–10].

PEDF is a 50 kDa glycoprotein of 418 amino acids and is expressed in most tissues. PEDF is effective in suppressing neovascularization [11]. It is also a neuroprotective factor, such as against glutamate neurotoxicity [12]. In addition, PEDF is known to promote the expression of osteoblastic-related genes in human MSCs, when cells are incubated in an osteogenic medium [13]. Recently, small active peptides derived from PEDF have been used to produce a PEDF effect on stem/progenitor cells. For example, a 29-mer (PEDF residues 93–121) shows the ability to stimulate the proliferation of skeletal muscle stem cells (satellite cells) and tendon stem cells in culture and in their niches in vivo [14, 15].

MIA, an inhibitor of glycolysis, is known to induce extensive chondrocyte degeneration/death in AC in a rapid and reproducible manner, after injection into the femorotibial joint space of rodents for seven days [16]. In the meantime, the inflammatory responses induced in the synovium and AC by MIA was largely resolved [17]. These features may provide a relatively simple model for the study of AC regeneration in vivo. The fundamental feature of OA is the destruction of AC. Therefore, an effective treatment would protect or regenerate cartilage at the cellular/chondrocyte level. The aim of this study was to determine whether the 29-mer, in combination with HA, is able to heal the AC defect through the induction of chondrocyte regeneration in a rat OA model induced by MIA.

Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). HA, MIA, dimethyl sulfoxide (DMSO), Percoll, insulin, hydrocortisone, 5-bromo-2'-deoxyuridine (BrdU), Hoechst 33258 dye and Alcian blue 8-GX were from Sigma-Aldrich (St. Louis, MO).

Anti-BrdU, anti-aggrecan, and anti-Sox9 were from GeneTex (Taipei, Taiwan). PEconjugated antirat CD90 antibody was purchased from Abcam (Cambridge, MA, USA). The fluorescent dye-conjugated secondary antibodies were purchased from BioLegend (San Diego, CA). Hematoxylin and eosin (H&E) dyes were purchased from Merck (Rayway, NJ, USA). Atglistatin, SC-1 and Cryptotanshinone (CPT) were from Selleckchem (Houston, TX, USA). Synthetic PEDF peptide (29-mer; residues Ser93-Leu112; SLGAEQRTESIIHRALYYDLISSPDIHGT) and a control peptide (PEDF Glu97-Ser114), were modified for stability by acetylation at the NH_2 terminus and amidation at the COOH terminus and characterized by mass spectrometry (> 95% purity) to order at GenScript (Piscataway, NJ). Each peptide was reconstituted in DMSO as stock (5 mM).

Animal Studies

All animals were housed in an animal room under temperature control (24–25°C) and a 12:12 light-dark cycle. Standard laboratory chow and tap water were available ad libitum. Experimental procedures were approved by the Mackay Memorial Hospital Review Board (project code: MMH-A-S-103-11; New Taipei City, Taiwan) and were performed in compliance with national animal welfare regulations. Adult 10-week-old male Sprague-Dawley rats (initial body wt = 312 ± 11 g) were supplied by the BioLASCO (Taiwan).

Induction of OA and 29-mer treatment

Rats were anesthetized by an intraperitoneal injection of xylazine (10 mg/kg) and ketamine (20 mg/kg); then the right knees were injected with MIA (1 mg dissolved in 25 μl of sterile saline), using a 27-gauge needle through the patellar ligament of the knee. The left knee joint (control) was injected with saline. After MIA injection for 7 days, the rats were randomly assigned to three experimental groups (n = 6, each group) as follows: (i) DMSO resolved in 25 μl of 5% HA as the vehicle control group; (ii) 29-mer/HA (final concentration 0.2 mM; 16.23 μg); and (iii) the control peptide/HA group. Treatments were given via intra-articular injections on days 8 and 12 post-MIA injection. To block PEDF-R/STAT3 signaling, the inhibitor (1 mM) was infused with HA, followed by an intra-articular injection one hour prior to 29-mer/HA treatment.

Histological examination of the knee joint

The knee joints were dissected and fixed in a 4% paraformaldehyde (PFA) solution and decalcified with Shandon TBD-2 decalcifier (Thermo Scientific, Logan, UT). The joints were sectioned mid-sagittally and embedded in paraffin blocks. Sections (5 μm in thickness) were cut longitudinally and stained with H&E. Safranin O/Fast green staining and collagen type II immunostaining were performed using a TaKaRa cartilage staining kit (#MK310; Shiga, Japan), according to the instruction manuals. Ten sections per knee joint were carefully prepared to include the most severely degenerated area. The sections were photographed using a Nikon Eclipse 80i microscope (Nikon Corporation, Tokyo, Japan) equipped with a Leica DC 500 camera (Leica Microsystems, Wetzlar, Germany).

Cartilage defects after 25 days of MIA injection were analyzed in a blinded manner by two observers and evaluated using a modified Mankin system as described previously [18, 19]. The full score included the

AC structure (a scale of 0–6): 0 = normal, 1 = irregular surface, 2 = pannus, 3 = absence of superficial cartilage layer, 4 = slight disorganization (an absent cellular row and some small superficial clusters), 5 = fissures into the calcified cartilage layer and 6 = disorganization (chaotic structure); the cellular abnormalities (a scale of 0–3): 0 = normal, 1 = hypercellularity, including small superficial clusters, 2 = clusters and 3 = hypocellularity; the matrix staining (a scale of 0–4): 0 = normal/slight reduction of staining, 1 = staining reduced in the radial layer, 2 = staining reduced in the interterritorial matrix, 3 = staining present only in the pericellular matrix and 4 = staining absent.

In vivo detection of DNA synthesis and immunofluorescence staining

BrdU was reconstituted in DMSO as stock (80 mM). 150 µl of BrdU mixed with 350 µl of PBS was intraperitoneally injected into rat. Paraffin-embedded joint specimens were deparaffinized in xylene and rehydrated in a graded series of ethanol. To detect DNA synthesis, the sections were further exposed to 1 N HCl at RT for 1 h. Subsequently, tissue sections were blocked with 10% goat serum and 5% BSA for 1 h. Immunostaining was carried out using primary antibodies against aggrecan (1:100 dilution), Sox9 (1:100 dilution) and BrdU (1:100 dilution) at 37°C for 2 h, followed by incubation with the appropriate rhodamine- or FITC-conjugated donkey IgG for 1 h at RT. Nuclei were located by counterstaining with Hoechst 33258 for 7 min. Images were captured using a Zeiss epifluorescence microscope with a CCD camera and measured from 12 randomly-selected areas in each sample, and blinded quantification was performed in triplicate by manually counting within each section.

Anti-BrdU labeling was also recognized by peroxidase-labeled goat immunoglobulin (1:500 dilution; Chemicon, Temecula, CA) for 20 min and then incubated with chromogen substrate (3,3`-diaminobenzidine) for 2 min before counterstaining with hematoxylin.

Pharmacokinetic analysis of the 29-mer in AC

29-mer (1 mg) dissolved in 25 µl of HA was intra-articular injection into normal rat knees. At time points, 0.5, 1, 2, and 4 h (n = 3 per time point), rats were euthanized and AC and synovial fluid were harvested. The AC was homogenized in CelLytic™ MT Mammalian Tissue Lysis/Extraction Reagent (Sigma, St. Louis, MO, USA) and the lysed samples were centrifuged for 10 min at 12,000 *g*. The supernatant was stored with protease inhibitor cocktail (Sigma-Aldrich) and the levels of 29-mer were measured using LC-MS as previously described [20].

Assessment of change in hind paws weight distribution

Changes in hind paw weight distribution between the right (osteoarthritic) and left (contralateral control) limbs were detected by an incapacitance tester (PM-01; Singa, Taiwan) and utilized as an index of joint discomfort in the osteoarthritic knee as described previously [17]. In brief, rats were placed in an angled plexiglass chamber positioned so that each hind paw rested on a separate force plate. The force exerted by each hind limb (measured in grams) was averaged over a 5 s period. Each data point is the mean of three, 5 s readings. The change in hind paw weight distribution was calculated by determining the

difference in the amount of weight (g) between the left and right limbs by the following equation: (1-(mean Δ weight of treated group/mean Δ weight of vehicle group))×(100).

Isolation and culture of MSCs

Sprague-Dawley rats were anesthetized and their femurs were aseptically harvested, washed in a mixture of PBS and antibiotics for 5 minutes, dissected of all soft tissue, transected at their epiphysis, and their marrow cavity rinsed repeatedly with a mixture of heparin (AGGLUTEX INJ 5000 U/ml; working conc. 100U/ml) and DMEM. The harvested cells were collected, dispersed by pipetting and centrifuged at 1000 ×*g* for 5 min at RT. Cell pellets were resuspended with DMEM and then the cell suspension was transferred to a 15 ml centrifuge tube containing 5 ml of Percoll (1.073 g/ml). After centrifugation at 1500 × *g* for 30 minutes, the mononuclear cells in the middle layer were obtained, washed three times with PBS and suspended in expansion medium (low-glucose DMEM with 10% heat-inactivated FBS, 1% penicillin/streptomycin). Cells were then placed in 75 cm^2 flasks (Corning, MA, USA) and incubated in 95% air and 5% CO_2 at 37°C. The medium was replaced every 4 d. Unattached cells were discarded and adherent cells were retained. The primary MSCs grew to approximately 80–90% confluence after culture for one week. The surface antigen of rat MSCs determined by flow cytometry showed strongly positive for CD90 (> 98%).

Chondrogenic differentiation of MSCs

The procedure was preformed as described previously [21]. Briefly, 5 × 10^3 expanded MSCs were placed at well of 96-well plate and exposed to 150 μl of chondrogenic medium (high-glucose DMEM with 100 nM dexamethasone, 0.17 mM ascorbic acid-2 phosphate, 0.35 mM proline, 10 μg/ml of insulin, 5 μg/ml of transferrin, 5 ng/ml selenium, 1 mM sodium pyruvate, 2 mM L-glutamine, and 2% FBS) supplemented with 10 ng/ml TGF-β3 (R&D Systems, Minneapolis, MN, USA) and 10 μM 29-mer by feeding three times a week.

Alcian Blue Staining and Quantification

MSCs were cultured in chondrogenic-inducing medium for 3 weeks. For Alcian blue staining, cultures were rinsed twice with PBS, fixed in 4% PFA for 15 min, and incubated in 1% (w/v) Alcian blue 8-GX in 0.1 N HCl overnight as previously described [22]. For semi-quantitative analysis, Alcian blue-stained cultures were extracted with 6 M guanidine HCl for 6 h at room temperature. The absorption of the extracted dye was measured at 650 nm in a microplate reader (Bio-Rad).

Quantitative real-time PCR

The total RNA extraction and quantitative real-time PCR were performed as in our previous study [14]. The sequences of specific PCR primers were rat aggrecan (accession number: J03485) sense, 5 $- \top GG \forall AT\mathbb{C}AG \forall \mathbb{C} \top CG - 3$; antisense, 5$-GT\mathbb{C}AGTGTGTAGCGTGTGG - 3$, rat COL2a1 (XM_006242308) sense, 5$-GG \forall GAGCGGAGACTACTGG - 3$; antisense, 5 $- \top GCAG \forall GAC \top TCATGGC - 3$, rat Sox9 (NM_080403) sense, 5 $-\mathbb{C}GGATCTG \forall G \forall GGAGAG$; antisense, 5$-C \top GACGTGTGGC \top G \top CT - 3$, and

rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH; accession number: X02231.1) sense, 5 $-AGACAG\mathbb{C}GCATC\top C\top GT-\ 3$; and anti-sense, 5 $-C\top G\mathbb{C}GTGGGTAGAGTCAT-\ 3$. All determinations were measured in triplicate. The cycle threshold (*Ct*) value of the PCR product of interest and a control mRNA (*GAPDH*) were then used to calculate relative quantities of mRNA between samples.

Western blot analysis

Cell lysis and SDS–PAGE were performed as described previously [14]. The band intensities in immunoblots were measured with a Model GS-700 imaging densitometer (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed using Labworks 4.0 software.

Statistics

The results are expressed as means ± standard deviation. Comparisons of controls and the treatment groups were performed using the Mann-Whitney U test. $P < 0.05$ was considered significant.

Results

The 29-mer prevents articular cartilage destruction induced by MIA

Rats knees were injected once with MIA in the femorotibial joint to induce extensive chondrocyte death and AC degeneration. Post-MIA treatment for 7 days, we started the 29-mer/HA treatment by way of a single intra-articular injection, twice with a 4-day interval. Post-MIA treatment for 18 days and 25 days, in the HA vehicle and control peptide/HA groups, H&E staining showed loss of cartilage integrity and superficial fibrillations throughout the AC area, and subchondral bone collapse, especially at weight bearing sites (Fig. 1a). In the 29-mer/HA group, the joint space and AC surface continuity were moderately preserved. Microscopically, vehicle sections showed that chondrocytes were lost from superficial zone of cartilage and scattered cell clusters occurred in the transitional zone and radial zone throughout the AC, 25 days post-MIA treatment (Fig. 1b). 29-mer/HA treatment revealed AC filled with large numbers of newly generated cells; the cells in the cartilage were beginning to be organized into the three cartilage-like layers: the superficial zone, consisting of spindle cells aligned parallel to the surface, a transitional zone, with accumulations of round cells and no apparent organization, and the radial/deep zone, with large chondrocytes aligned in columns.

In addition, the vehicle group showed reductions of sulfated proteoglycan and type II collagen throughout the AC region, demonstrated by safranin-O staining and immunohistochemical staining, respectively, reflecting severe cartilage degeneration (Fig. 2a). In contrast, the 29-mer/HA group showed moderately maintained cartilage matrix, compared to the sham group (normal). The modified Mankin score system, including destruction of cartilage structure, cellular abnormalities and loss of matrix staining, was used to

evaluate the AC defects. The scores were lower in the 29-mer/HA-treated group than the vehicle group (total score: 5.3 ± 0.3 versus 11.2 ± 0.5; Fig. 2b).

LC-MS analysis revealed that intra-articular injection of the 29-mer/HA was able to reach the AC and synovial fluid with $T_{1/2}$ (half-lives) of 1.82 h and 2.07 h, respectively (Fig. 3). Overall, the 29-mer/HA treatment induces cell expansion in AC and reduces loss of AC integrity induced by MIA.

The 29-mer stimulates chondrocyte regeneration in the injured AC induced by MIA

In investigating the phenotype of the newly generated cells in the AC, immunofluorescence staining of aggrecan, a marker of chondrocytes, in knee joints treated with the 29-mer/HA revealed considerable accumulations of aggrecan-positive chondrocytes in the superficial, transitional and deep zones of the AC, whereas faint aggrecan staining was seen in the vehicle group (Fig. 4a). In addition, rats were given a single intraperitoneal injection of BrdU for 24 h to detect DNA synthesis. The results were that 16.2 ± 2.2% of the cells in the superficial and transitional zones were BrdU-positive, suggesting the chondrocytes, at least in part, retained the replicative ability for expansion in the AC. Of note, the vehicle group showed pale aggrecan staining and stained negative for BrdU.

To further confirm the chondrocyte regeneration in the AC, induced by the 29-mer/HA, the BrdU injection was given simultaneously with 29-mer/HA treatment. Large amounts of BrdU-positive cells were seen in the AC (Fig. 5a); the lower BrdU intensities in some cells may be attributable to the constant replication of the cells. Dual-immunofluorescence staining further demonstrated almost the entire BrdU-positive cells stained positive for Sox9 (Fig. 5b). On the other hand, resident aticular chondrocytes retained a quiescent, slow-cycling state in the normal AC and stained negative for BrdU. Also, the AC region of the vehicle group stained negative for BrdU, confirming that there is no spontaneous chondrocyte regeneration after MIA injection. In addition, the levels of BrdU-labeled chondrocytes induced by the 29-mer (50 ~ 400 μM dissolved in HA) increased in a dose-dependent manner (Fig. 5c; 28 ± 3.1 ~ 107 ± 13.7). Saline as the 29-mer vehicle (bolus) had no such effect. Collectively, the results suggest that the 29-mer/HA effectively induces chondrogenic cell proliferation to heal the damaged AC after MIA injection.

The 29-mer reduces the shift in hind-paw weight-bearing induced by MIA

It has been established that injection of MIA into the right knee results in a dose- and time-dependent increase in joint discomfort in rats, defined by change in hind paw weight distribution [16, 17]. We also found that the vehicle group showed a significant increase of the weight-bearing shift, at day 28 post-MIA injection, whereas rats treated with a range of doses of the 29-mer (50 ~ 400 μM in HA) showed better weight-bearing ability than the vehicle group (47.6% versus 33.9 ~ 21.6%; Fig. 6a). In addition, HA vehicle alone or bolus treatment had no such antinociceptive effect. (Fig. 6b), suggesting that HA provides an

auxiliary role in supporting the pharmacologic effect of the 29-mer on the knee joint. The results suggest that the 29-mer/HA can ameliorate the MIA-induced joint discomfort.

The 29-mer promotes the chondrogenic differentiation of MSCs

The MSCs detected in several articular tissues have been proposed to have potential to act as chondroprogenitor cells in response to cartilage injury [6–10]. Therefore, we investigated the influence of the 29-mer on the chondrogenic differentiation of MSCs in culture. Rat MSCs were isolated from bone marrow, and expanded in culture for one week, and then cultured in a chondrogenic defined medium supplemented with or without the 29-mer for a further week. Real-time PCR analysis of the chondrogenic genes revealed that the 29-mer induced 3.6-, 3.3- and 2.5-fold increases of, *Sox9*, *aggrecan* (*Acan*), and *Col2a1*, respectively, compared to MSCs treated with solvent control (Fig. 7a).

To explore the molecular basis of chondrogenic differentiation of MSCs promoted by the 29-mer, MSCs were pretreated with pharmacological inhibitors that included a PEDF receptor inhibitor (atglistatin) and STAT3 inhibitors (SC-1 and CPT). All these inhibitors suppressed the expression of chondrogenic marker genes induced by the 29-mer to near basal levels. Meanwhile, western blot analysis demonstrated that the 29-mer can induce phosphorylation of STAT3 (p-STAT3) in MSCs, occurring 5 ~ 40 min after the treatment (Fig. 7b), but the effect of the 29-mer was abolished in cells pretreated with atglistatin, SC-1 or CPT. The findings imply that the 29-mer promotes chondrogenic differentiation of MSCs in vitro via activation of the PEDF-R/STAT3 signaling pathway.

Next, we investigated the biosynthesis of cartilage matrix by Alcian blue staining of glycosaminoglycans (GAGs) deposition in culture, after culture of MSCs in chondrogenic medium in the presence of the 29-mer for 21 days. There was a 2.6-fold increase in the GAGs levels, compared with the solvent control (Fig. 7c). Likewise, Safranin O staining also showed higher levels of proteoglycans induced by the 29-mer, further supporting the beneficial effect of the 29-mer on chondrogenic differentiation of MSCs in vitro. Alcian blue staining also indicated that the biosynthesis of GAGs induced by the 29-mer was blocked by inhibitors targeting PEDF-R/STAT3 signaling. Moreover, the effects of the 29-mer on healing of the AC defect and chondrocyte regeneration in OA rats were almost completely blocked by atglistatin, SC-1 and CPT (Figs. 7d and 7e). Collectively, the findings indicate that the 29-mer is able to promote the chondrogenic differentiation of BM-MSCs. The results also imply that PEDF-R/STAT3 signaling is not only essential for the effect of the29-mer on MSCs in vitro, but also crucial for its effect on the repair of the AC defects induced by MIA.

Discussion

In this study, OA induced by MIA in rats featured extensive chondrocyte degeneration/death, leading to replacement of the AC by fibrocartilaginous tissue, which has no chondrocyte regeneration, providing the conditions for the development of OA. This OA model reflects the poor self-healing ability of cartilage that

constitutes the medical burden of human OA. We demonstrate for the first time that 29-mer/HA has the ability to stimulate partial recovery of the damaged AC by the regeneration of chondrocytes. Subsequently, the increased expression of collagen type II and aggrecan show the features of hyaline cartilage in the regenerated area. Notably, the cartilage regenerative pattern is quite similar to the ex vivo, three-dimensional cartilage generated from MSC-derived chondrocytes [24]. Also, we show that the 29-mer can augment the chondrogenic potential of rat MSCs induced by TGFβ3. The 29-mer bioactivity on MSCs is likely important to achieve cartilage repair in vivo.

Cartilage wound healing by the 29-mer/HA is shown in this animal model to involve the regeneration of chondrocytes in the damaged AC. However, it is not clear the sources of the stem/progenitor cells involved in the 29-mer-mediated repair. Condrogenic stem/progenitor cells have been found to reside in several tissues of the knee joint. For example, the superficial zone of AC contains MSC-derived chondrocyte progenitors [25, 26]. In addition, a small population of chondroprogenitor cells resides in the perichondrial groove of the Ranvier region [27]. The adult synovium in the joint cavity reportedly has cell populations with the functional behavior of stem cells [9]. Given these findings, it is conceivable that MSCs may contribute to the proliferative burst of chondrogenic cells induced by 29-mer/HA. Further study of the migration of MSCs from their niches into the injured AC could provide a more comprehensive understanding of the precise therapeutic mechanism of 29-mer/HA in cartilage regeneration.

In this study, intra-articular injection of the 29-mer/HA mixture led to repair of the AC defects. Notably, the presence of HA is essential for the 29-mer effect, although HA alone (vehicle group) had no therapeutic effect on the MIA-induced AC destruction. Little is known about the role of HA in this study. Previous studies indicated that association of HA with the surface CD44 glycoprotein of MSCs facilitates the recruitment of MSCs into wound tissues [28, 29]. Therefore, it is possible that HA may act in binding the resident MSCs to promote their movement into the injured AC. In addition, HA is a common component of sustained-release formulations and may increase the retention of the injected 29-mer in the knee joint cavity.

TGFβ is known to induce chondrogenesis of MSCs by activating Smad signaling to augment the transcription of the *Sox9* gene [30]. SOX9 is a master chondrogenic transcription factor responsible for upregulation of Col2a1 and aggrecan expression, in which aggrecan can covalently link sulfated GAGs, to confer an osmotic swelling property of cartilage [31–34]. One drawback of MSCs is that they tend to differentiate towards hypertrophic cartilage instead of hyaline cartilage, leading to a reduction of the ability of the cartilage to cope with pressure and shear force [5, 35]. In this, the 29-mer stimulated the STAT3 activation that plays a critical role in augmenting the expression of chondrogenic markers in the MSCs cultured in a chondrogenic medium. Our findings support a previous conclusion that STAT3 signaling stimulates *Sox9* gene expression, following p-STAT3 nuclear translocation in chondrocytes [36, 37]. In addition, our recent study showed that a PEDF receptor inhibitor (atglistatin) can efficiently block the PEDF-R/STAT3 signaling induced by a PEDF 44-mer peptide (containing entire 29-mer) in the limbal stem cells [38]. Here, we found also that inhibition of STAT3 activation by atglistatin blocks the 29-mer effect on the induction of *Sox9* gene expression. This indicates a novel role of the PEDF-R/STAT3

signaling pathway on the regulation of chondrogenic gene expression in the chondroprogenitors. However, details of the effect of 29-mer-induced signaling on TGFβ/Smad-mediated chondrogenic differentiation signaling await further study.

Limitation of mobility induced by joint pain is the major complaint of OA patients. Thus pain reduction is one of the primary targets of therapy in OA. The source of pain is multiple. Cartilage destruction, such as dislodged cartilage, may induce pain through peripheral afferent and dorsal root ganglion (DRG) neurons [39]. In this study, the 29-mer/HA effect on blockage of AC destruction is also supported by reduction of the imbalanced hind limb weight distribution induced by MIA, implying relief of the joint pain in animals.

In summary, structural lesions confined to the AC layer do not heal spontaneously and a number of therapeutic strategies have been used to induce their repair. In the present study, we prepared a PEDF 29-mer/HA mixture and evaluated the potential for AC repair in the MIA-induced rat OA model. The 29-mer/HA effectively induced chondrogenesis in the damaged AC and inhibited the formation of fibrocartilage. We also suggest that PEDF-R/STAT3 signaling may be involved in the cartilage regeneration induced by the 29-mer. This study provides a new therapeutic strategy for the development of OA therapy.

Abbreviations

MIA
monoiodoacetate
AC
articular cartilage
OA
osteoarthritis
PEDF
pigment epithelium-derived factor
HA
hyaluronic acid
H&E
hematoxylin and eosin
MSCs
mesenchymal stem cells
STAT3
signal transducer and activator of transcription 3
Sox9
SRY-type high mobility group box 9
TGFβ
transforming growth factor beta
DMEM

Dulbecco’s modified Eagle’s medium
FBS
fetal bovine serum
DMSO
dimethyl sulfoxide
BrdU
5-bromo-2'-deoxyuridine
CPT
cryptotanshinone
PFA
paraformaldehyde
DNA
deoxyribonucleic acid
PCR
polymerase chain reaction
GAGs
glycosaminoglycans
DRG
dorsal root ganglion.

Declarations

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Availability of data and materials

Data are presented in the results section of the manuscript.

Authors’ contributions

YCL and TCH are equally contributing authors. Conceived, designed, and oversaw the experiments: YPT. Performed the experiments: YCL, TCH, CHH, and SIY. Analyzed the data: YCL, TCH, and SLC. Obtain funding: YPT. Wrote the manuscript: TCH. Revised the manuscript: YCL and YPT. All authors reviewed the manuscript. The authors read and approved the final manuscript.

Declarations

Ethics approval and consent to participate

All animal experiments were performed with the approval of the Mackay Memorial Hospital Review Board (project code: MMH-A-S-103-11; New Taipei City, Taiwan) and were performed in compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Competing interests

YCL, CHH, SIY, and SLC declare that they have no competing interests. The findings described in this manuscript have been patented by Mackay Memorial Hospital. YPT and TCH are inventors.

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Figures

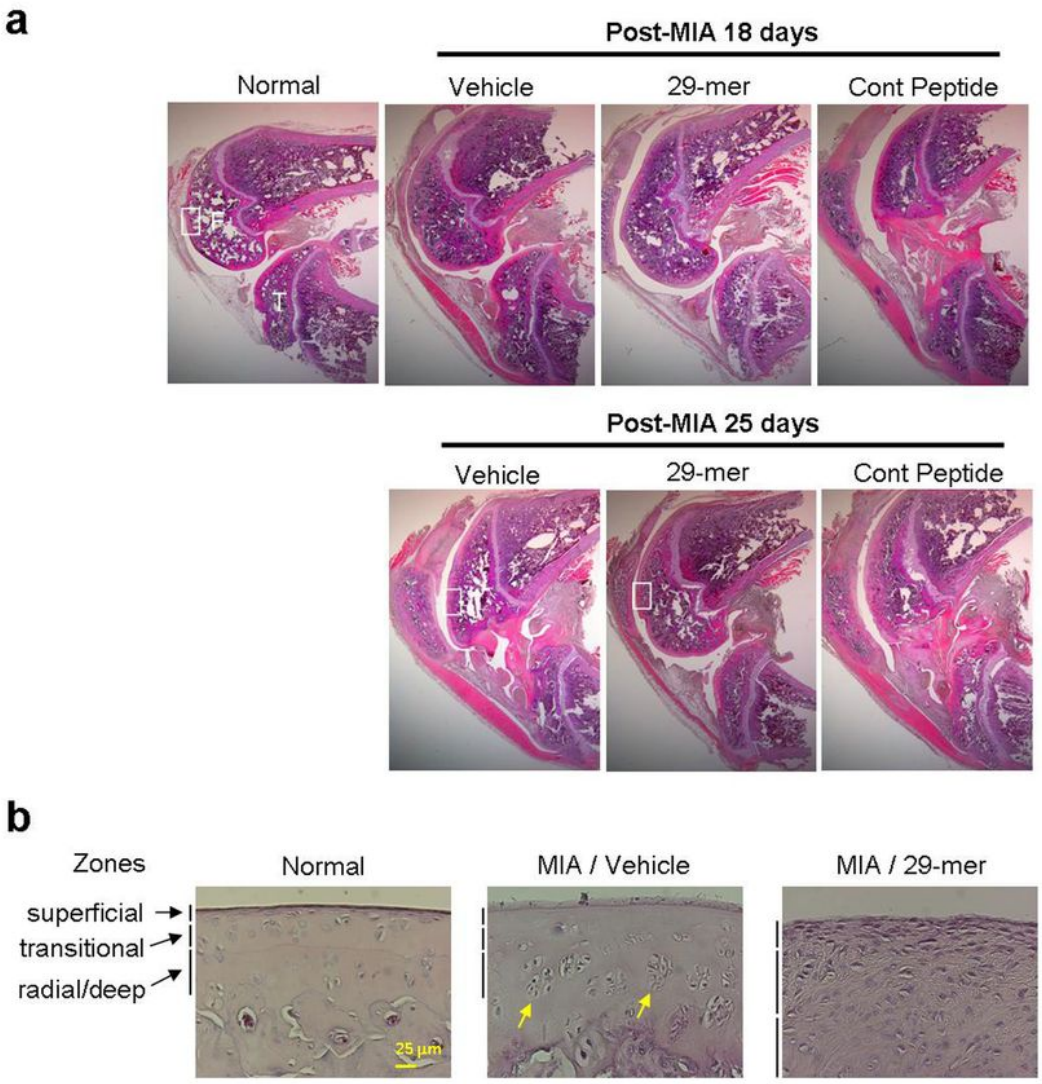

Fig. 1

Figure 1

The 29-mer is effective in against AC destruction induced by MIA. (**a**) H&E staining of longitudinal knee joint sections after MIA-induced AC damage for 18 days and 25 days. The vehicle and 29-mer/HA treatment started on day 8 post-MIA injection. Representative graphs from two independent experiments (n = 6 per group) are shown. Original magnification, × 40. (**b**) The areas surrounded by the boxes are

enlarged in B panels (× 400). Arrows indicate the chondrocyte degeneration/death with prominent pyknotic nuclei.

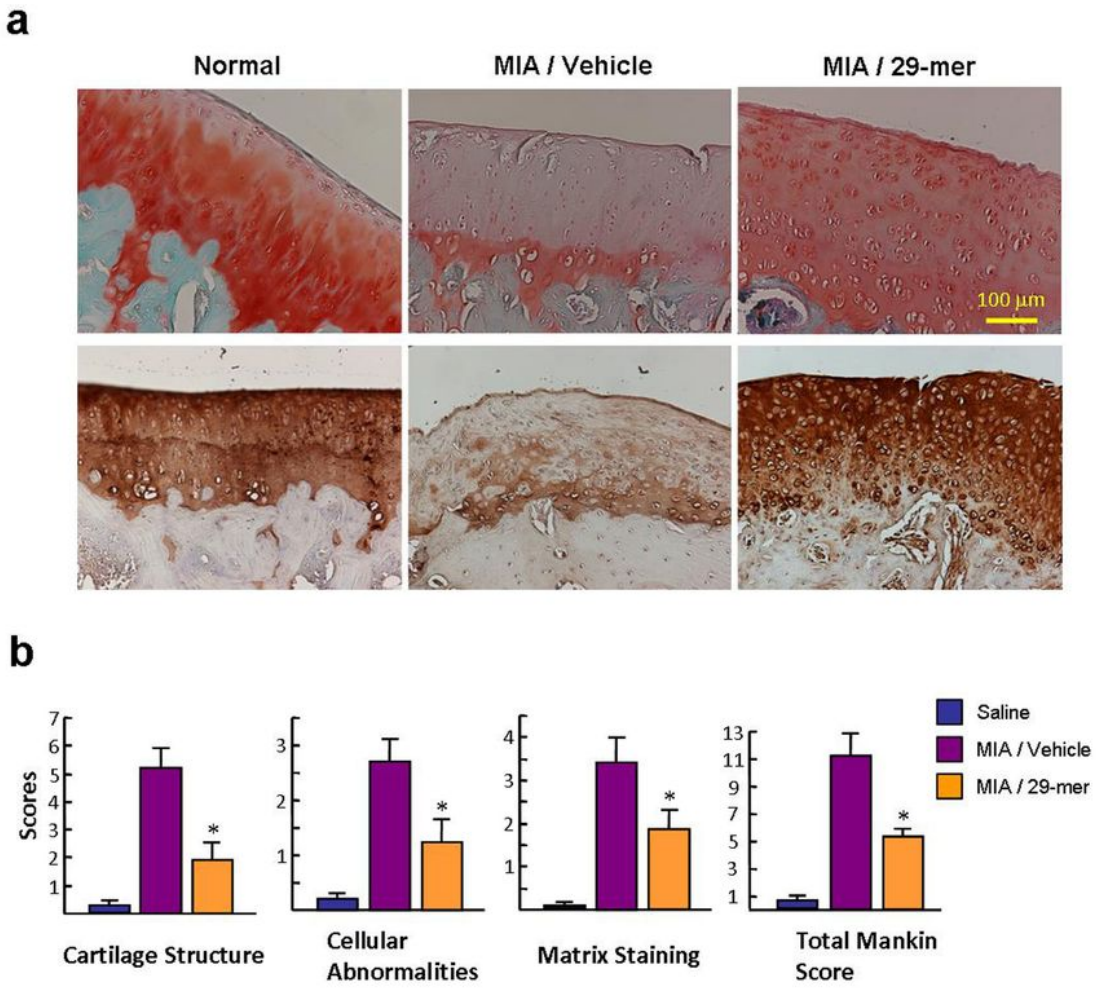

Fig. 2

Figure 2

Histological analysis of AC defects. (**a**) Knee joint sections were histochemically stained by Safranin O/Fast green (upper panel) and immunostained by type II collagen (below panel) at day 25 post-MIA

injection. Representative micrographs of femoral condyles from two independent experiments are shown (n = 3 per group). Magnification, × 200. (**b**) Evaluation of the AC defect by a modified Mankin scoring. The histological scoring was performed double-blind. *P < 0.05 versus vehicle group.

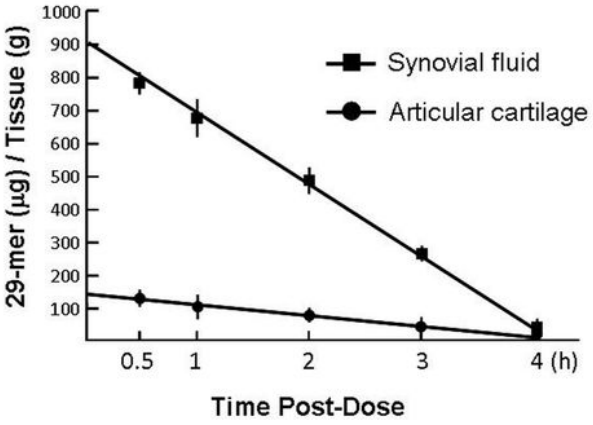

Fig. 3

Figure 3

LC-MS analysis of the 29-mer in AC and synovial fluid following intra-articular injection of 29-mer/HA. Each set of data points between 0.5-4 h was fit to a regression line to extrapolate an initial maximum level of the 29-mer in each tissue. $T_{1/2}$: elimination half-life. Error bars indicate SD (n = 3 per time point).

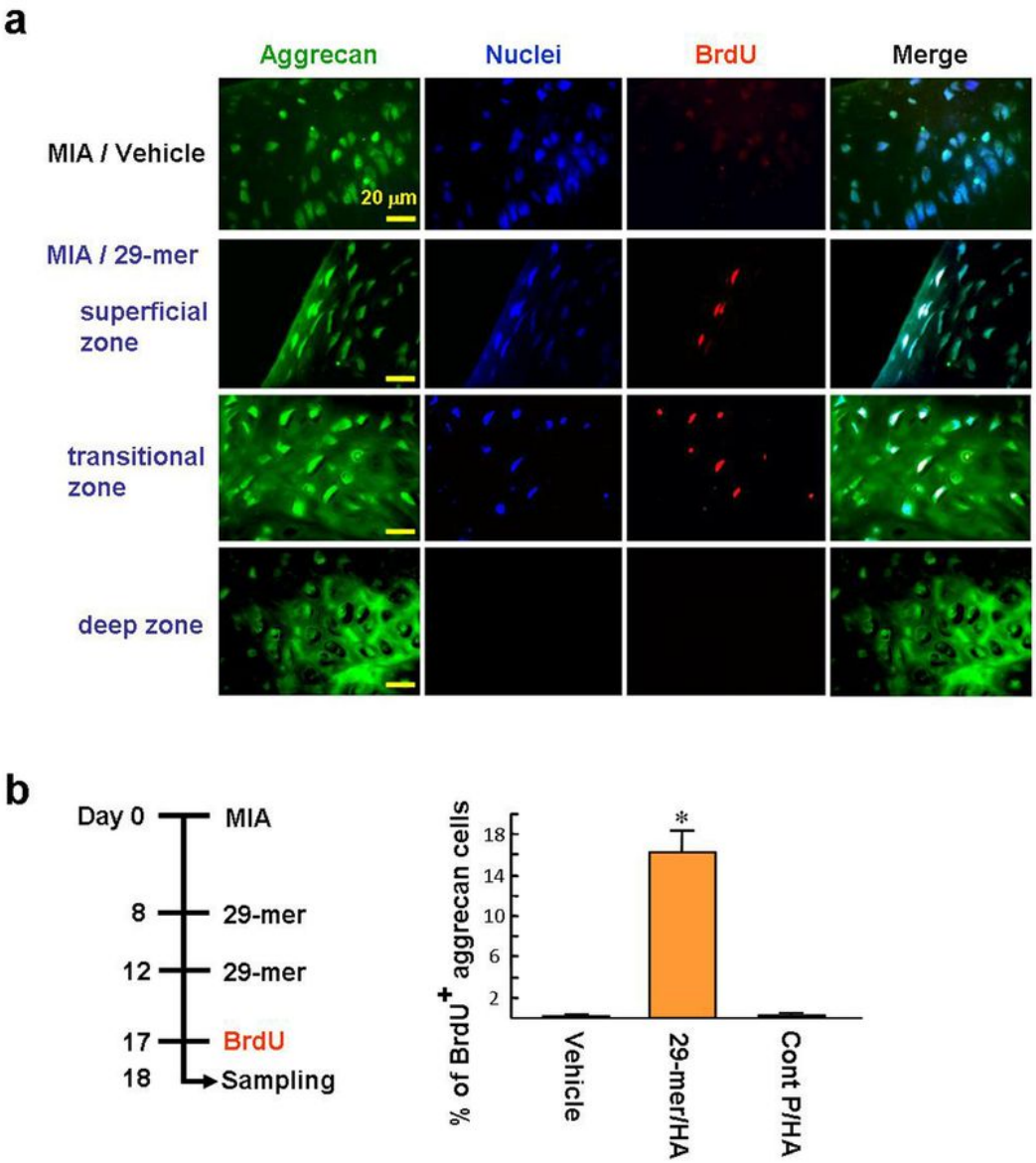

Fig. 4

Figure 4

Immunofluorescence analysis of the phenotype of newly generated cells in MIA-injured AC induced by the 29-mer/HA. (**a**) Double-immunofluorescence of aggrecan and BrdU. Nuclei were visualized with Hoechst 33258 staining. Representative images are from six sections per rat knee joint, with six rats per group. (**b**) The digital image analysis of the percentages of BrdU/aggrecan double-positive cells per total aggrecan^{+} cells was performed blinded on an average of six randomly selected × 400 magnification fields from each section, using a Zeiss epifluorescence microscope. *P = 2.41E-05 versus vehicle group.

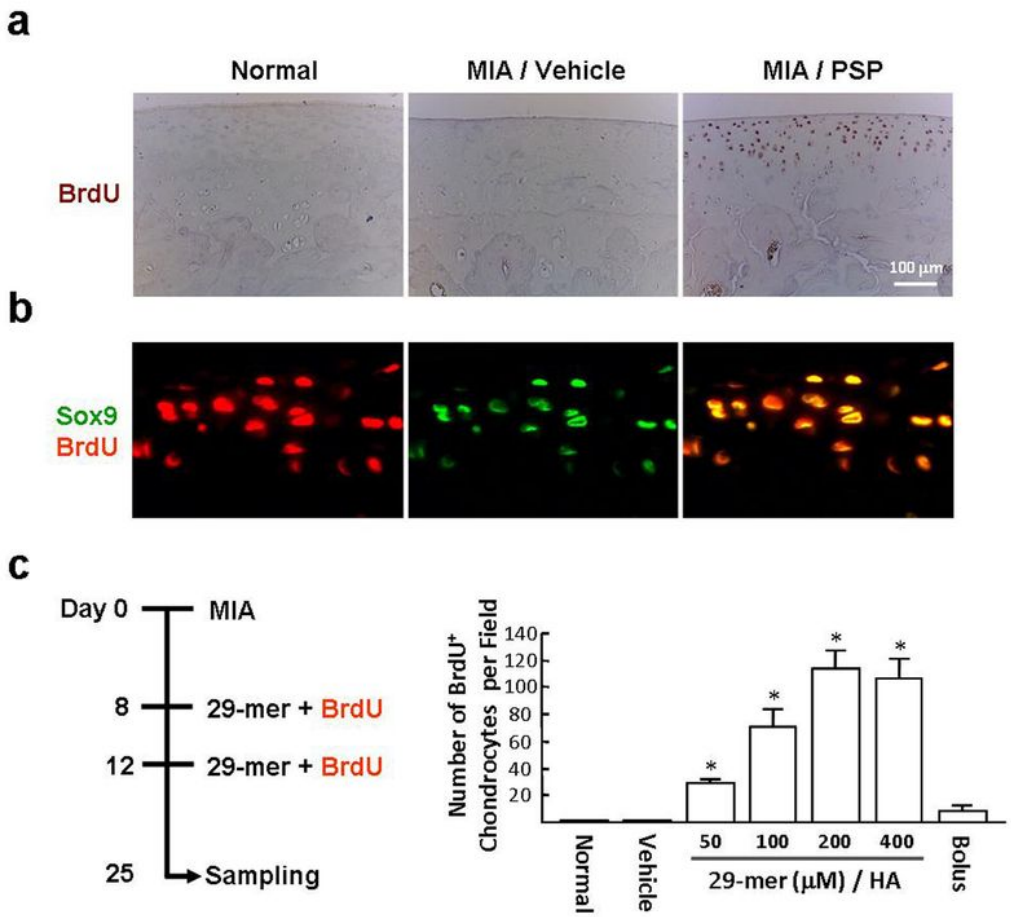

Fig. 5

Figure 5

The 29-mer/HA dose-dependently induces chondrocyte regeneration in the AC injured by MIA. (**a**) Immunohistochemical analysis of the levels of BrdU-positive cells at different groups after MIA treatment for 25 days. Rat knees were injected intra-articularly with BrdU immediately after vehicle or 29-mer/HA treatment. (**b**) Double-immunostaining of Sox9 and BrdU. Representative images from three independent experiments with six rats per group are shown. Original magnification, X 1000. (**c**) The levels of BrdU-positive cells induced by various dosages of the 29-mer/HA. The digital image analysis was performed blinded on an average of six randomly selected × 200 magnification fields from each section using a Nikon Eclipse 80i microscope equipped with a Leica DC 500 camera. *P < 0.0001 versus vehicle group.

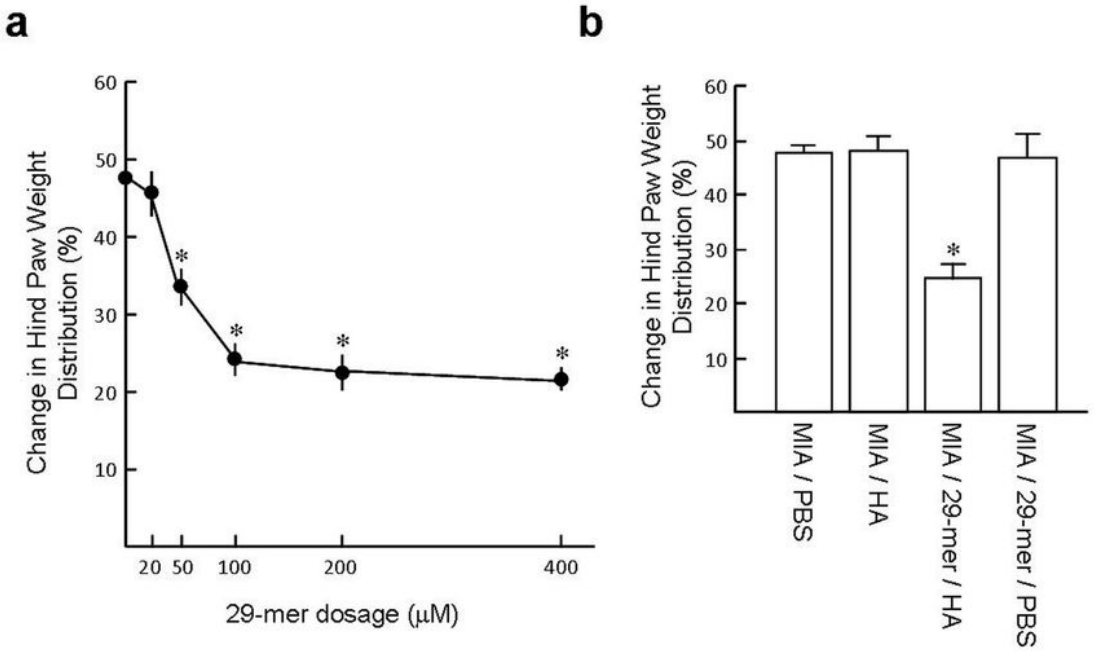

Fig. 6

Figure 6

The 29-mer/HA dose-dependently exerts an antinociceptive effect on OA rats induced by MIA. Rats injected with 1 mg of MIA in the right (osteoarthritic) knee and saline in the left (contralateral control) knee. The 29-mer and vehicle (5% HA) treatment were conducted at day 8 post-MIA injection. Change in hind paw weight distribution (weight bearing) was assessed at day 28 post-MIA injection, by use of an incapacitance tester. n = 6 per dose. *P <0.01 versus vesicle-treated rats.

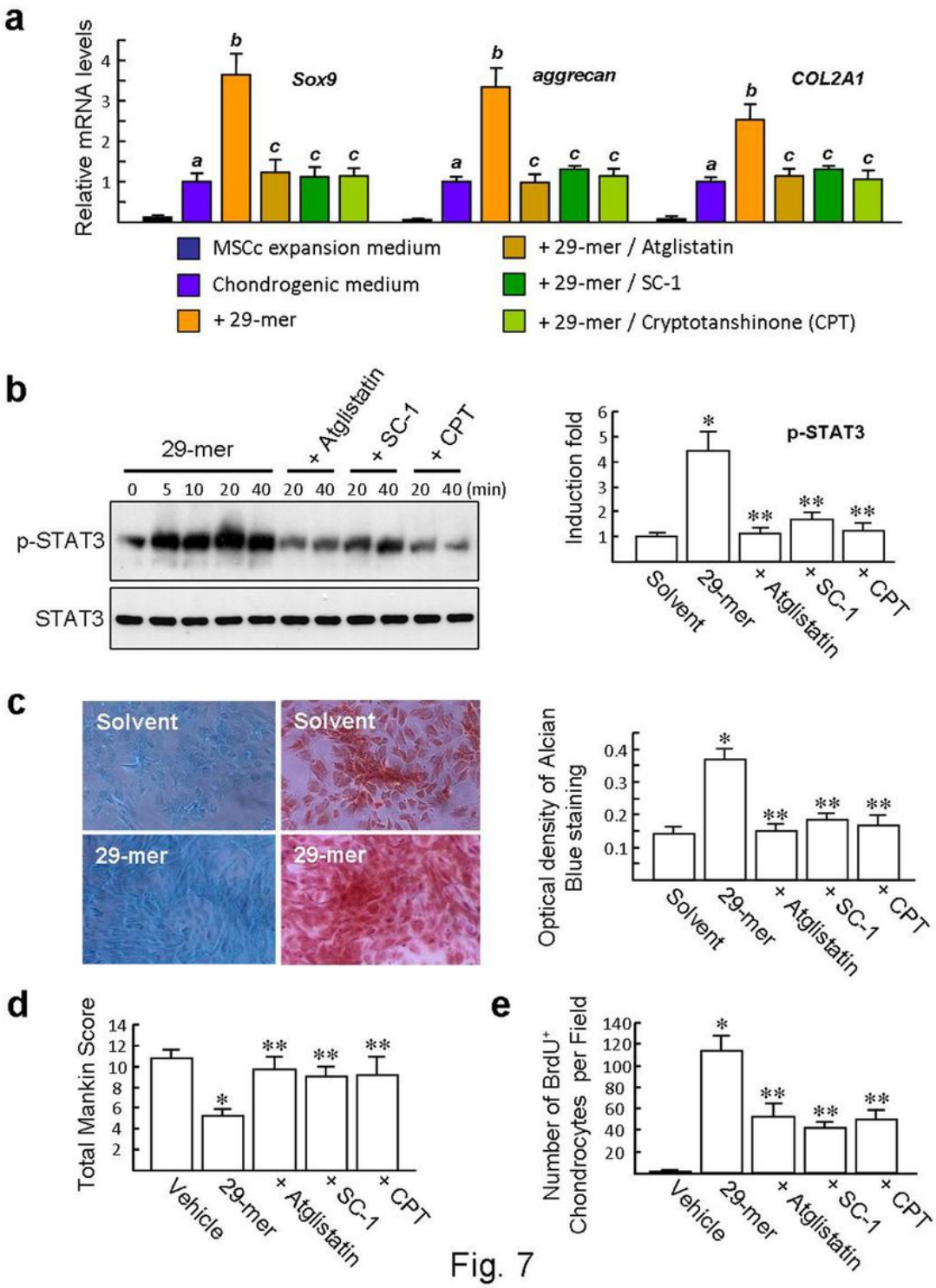

Fig. 7

Figure 7

The quality of chondrogenic differentiation of MSCs in culture is promoted by the 29-mer treatment. (**a**) MSCs cultured in chondrogenic medium were treated with the 29-mer or solvent for 7 days, the levels of chondrocyte marker genes were determined by real-time qPCR. *Gapdh* was used as a loading control. Data are representative of three independent experiments. ^{a}P <0.001 versus MSCs cultured at expansion medium. ^{b}P <0.01 versus solvent-treated MSCs. ^{c}P <0.02 versus 29-mer-treated MSCs. (**b**) Representative

immunoblots and densitometric analysis of the effect of the 29-mer on phosphorylation of STAT MSCs, the immunoblots were scanned and quantitated at sites represented cells stimulated by the 29-mer for 20 min and normalized to STAT3. *P <0.01 versus olvent-treated cells. **P <0.01 versus 29-mer treatment. (**c**) Semiquantitative analysis of GAG-rich extracellular matrix by Alcian blue staining after chondrogenic differentiation of MSCs for 3 weeks. OD values of Alcian blue are shown relative to the total protein content in culture determined by BCA assay. Representative micrographs of monolayer of chondrogenic cells cultured at chondrogenic medium for 3 weeks and stained by Alcian blue and Safranin O. *P <0.004 versus solvent-treated cells. **P <0.005 versus 29-mer treatment. (**d**) Histological analysis of AC defects as Fig. 2 description. *P <0.005 versus vehicle group. **P <0.03 versus 29-mer group. (**e**) Analysis of chondrocyte regeneration in injured AC was determined by BrdU labeling as Fig. 5 description. *P=4.91E-06 versus vehicle group. **P <0.005 versus 29-mer group.

3 in