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RESEARCH ARTICLE

A novel bio-active microsphere for meniscus regeneration via inducing cell migration and chondrocyte differentiation

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Abstract

Meniscus injury is a common disease in clinic. If it was not treated in time, it leads to osteoarthritis which brings unbearable pain and heavy economic burden to the patients. At present, meniscectomy and meniscus suture are widely used in the treatment for meniscus injury. Nevertheless, It is not ideal for poor self-healing ability of meniscus. The recruitment of endogenous stem cells is an attractive option for wounded meniscus healing. Fully reduced high-mobility group box 1 protein (HMGB1) can accelerate the regeneration of multiple tissues by endogenous stem cell activation, migration and differentiation. Kartogenin (KGN) has shown to induce the chondrogenesis of the stem cells. However, no study has explored such effects of HMGB1 and KGN in wounded meniscus healing. Therefore, in order to improve the regeneration of meniscus, we intend to use a novel bioactive microsphere which was developed by combining fully reduced high mobility group box 1 (frHMGB1) and kartogenin (KGN) with alginate gel which slowly release high concentrations of HMGB1 and KGN to activate rat bone marrow stem cells (BMSCs) and promote cell proliferation. The results showed that this HMGB1–KGN microsphere released and kept high concentrations of HMGB1 and KGN in the wound area for more than 2 weeks. In vitro experimental results showed that the HMGB1–KGN microsphere can promote cell proliferation via recruiting rat bone marrow stem cells (BMSCs) and activating the BMSCs from G_0 to G_{Alert} stage as evidenced by cell migration testing and 5-bromo-2′-deoxyuridine (BrdU) incorporation assay. In vivo results indicated that this HMGB-KGN microsphere can recruit GFP-labeled BMSCs from tail vein to wounded meniscus and induce these GFP-labeled BMSCs to differentiate into chondrocytes. Our results demonstrated that the HMGB1–KGN-containing bioactive microsphere induced cell migration in vitro and recruited the cells to wound area to promote wounded rat meniscus healing in vivo.

Keywords Fully reduced HMGB1 (frHMGB1) · Kartogenin · Alginate microsphere · Cell migration · Meniscus regeneration

Introduction

The menisci are C-shaped fibrocartilage located between the femoral condyles and tibial plateau to transmit the load across the joint space [1]. Menisci play an important role in joint stabilization, proprioception, lubrication and protection of articular cartilage [2]. Such environment and function easily lead to meniscus injury, and damaged meniscus can cause the progression of cartilage degeneration and result in osteoarthritis [3].

Histology studies have shown that meniscus is a geometrically and biochemically complex tissue. Its outer 1/3 tissue is formed by collagen type I produced by fibroblast-like cells, while its inner 2/3 tissue is formed by collagen II and proteoglycan produced by chondrocyte-like cells [4]. Vascularization in meniscus decreases with aging. From prenatal development until shortly after birth, the meniscus is fully vascularized. Afterward, vascularization appears to subside. At 10 years of age, vascularization is present in around 10–30% of the meniscus [1, 4].

The injury in the avascular zone of the meniscus is generally more complex and broad and is often associated with a poor prognosis following repair [5]. Promoting the healing process in the avascular zone of the meniscus is an ongoing challenge for both clinical medical doctors and orthopedic researchers [6]. Recently, tissue engineering approaches that

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involve the use of adult tissue-derived multi-potent stem cells and biomaterial microspheres have gained increasing attention for enhancing the wounded meniscus healing [7]. However, the isolation and culture of stem cells not only need the donor tissue obtained by additional surgical procedure but also take long time and increase contamination risk. Therefore, recruiting endogenous stem cells to wound area to enhance wound healing is still a new challenge for orthopedic researchers.

High-mobility group box 1 protein (HMGB1) is a ubiquitously expressed, highly conserved nuclear protein [8]. In normal circumstances, HMGB1 is almost always present in the nuclei of mammalian cells and plays an important role in various biological processes including transcription, DNA repair, cell differentiation and development [9]. When the cells are stimulated or damaged, HMGB1 can be released from the nucleus to the cytoplasm [10]. The extracellular HMGB1 can interact with some cellular receptors and surface molecules to regulate the cell proliferation and cell migration [9, 11]. It has been found that HMGB1 has two different activities due to its two redox-sensitive cysteine moieties in its 215 amino acid structure [12]. When these cysteine residues in HMGB1 are in reduced thiol form, this kind of HMGB1 is called as fully reduced HMGB1 (frHMGB1) which is a chemoattractant of motile cells. However, when these thiols are oxidized to form a disulfide bond which is called oxidized HMGB1 (oxHMGB1). The oxHMGB1 is an inducer of cytokines. It has been reported that frHMGB1 binds to a chemokine protein known as C-X-C motif chemokine 12 (CXCL12) to form a HMGB1–CXCL12 heterocomplex. This HMGB1–CXCL12 complex interacts with CXCR4, a receptor of CXCL12 to promote the migration of monocytes and fibroblasts [13]. The recent study has shown that frHMGB1 transitions stem cells from G_0 to G_{Alert} [14].

Another key point for meniscus regeneration is to induce chondrogenesis differentiation of the stem cells. Recent studies have found that kartogenin (KGN), a small compound, can promote the chondrogenic differentiation of human and mouse mesenchymal stem cells (MSCs) [15]. Several studies have used KGN to enhanced wounded cartilage regeneration [16, 17]. We have shown that KGN-treated autologous tendon graft can enhance the wounded rabbit meniscus healing [18].

Finally, a suitable microsphere is needed to deliver frHMGB1 and KGN to the wound area which can release appropriate concentrations of frHMGB1 and KGN to enhance wound healing. It is well known that alginate, a naturally occurring anionic polymer, has been used for many biomedical applications due to its biocompatibility, low toxicity and low cost [19].

In this study, we developed a novel bio-active microsphere by the combination of frHMGB1 and KGN with alginate hydrogel for promoting wounded rat meniscus healing (Fig. 1a–c). Our hypotheses are that this frHMGB1–KGN-containing bio-active microsphere can deliver frHMGB1 and KGN directly to the wound area and release appropriate concentrations of frHMGB1 and KGN in the wound area to enhance wounded meniscus healing by recruiting stem cells to the wound area and inducing chondrogenic differentiation of the stem cells. This frHMGB1–KGN-containing alginate hydrogel is a suitable bioactive microsphere for promoting wounded meniscus regeneration.

Materials and methods

Animals

Thirty Sprague–Dawley (SD) rats (3 months old, female) and three GFP transgenic SD rats (3 months old, female) were used in this study. The experiments were done at Nanjing Medical University (NMU) following the approved protocol by the Institutional Animal Care and Use Committee (IACUC) of NMU.

Preparation of frHMGB1–KGN-containing bioactive microsphere

Sodium alginate obtained from Sigma-Aldrich (Catalog No. W201502, St Louise, MO, USA) was dissolved with distilled water to make a 2% of alginate solution (AS). Fully reduced HMGB1 (frHMGB1) protein (500 µg/bottle) obtained from Tecan (Catalog No. REHM114, Mannedort, Switzerland) was dissolved with 500 µl of distilled water to make a 1 µg/µl of frHMGB1 solution. KGN (25 mg/bottle) obtained from Sigma-Aldrich (Catalog No. SML0370, St Louise, MO, USA) was dissolved with 1 ml of DMSO to make a 25 mg/ml of KGN solution. All solutions were filtered through a 0.2 µm membrane under sterile conditions. The bioactive microsphere for enhancing wounded meniscus healing was prepared by adding 10 µl of frHMGB1 solution (10 µg frHMGB1 in 10 µl water) and 10 µl of KGN solution (250 µg of KGN in 10 µl of DMSO) into 80 µl of alginate solution (AS) and mixed well to get a frHMGB1–KGN–AS solution. Each 10 µl of frHMGB1–KGN–AS solution was dropped into 2% of $CaCl_2$ solution to obtain a frHMGB1–KGN-containing bioactive microsphere (frHMGB1 + KGN) which contained 1 µg of frHMGB1 and 25 µg KGN. Similarly, the control microsphere was made by adding 10 µl of distilled water and 10 µl of DMSO into 80 µl of alginate solution and mixed well to make a DMSO-AS solution. Each 10 µl of DMSO-AS solution was dropped into 2% of $CaCl_2$ to make a DMSO-containing control microsphere (DMSO). Furthermore, 10 µl of KGN solution and 10 µl

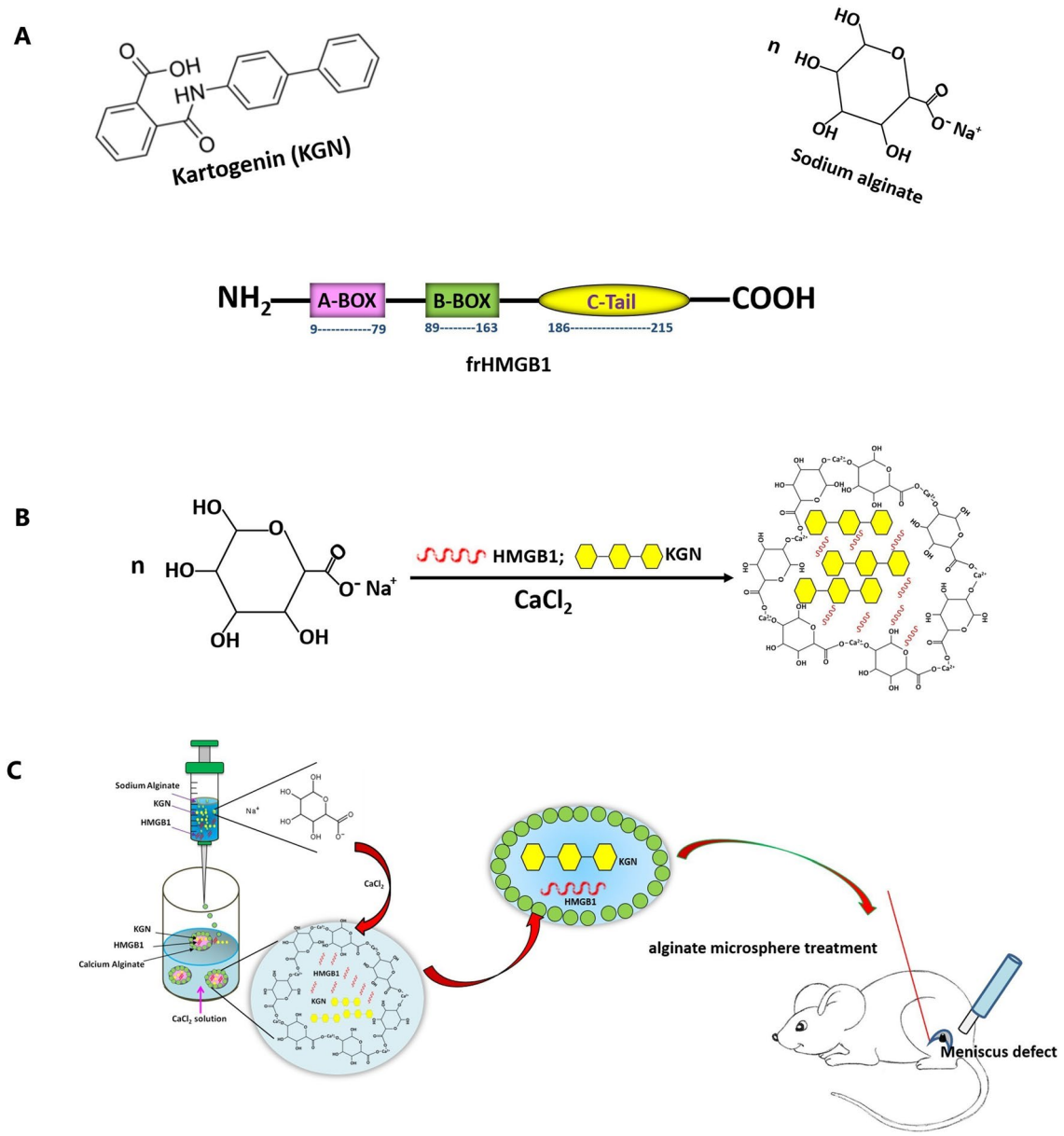

Fig. 1 Graphical abstract showing a bioactive microsphere developed by combining fully reduced HMGB1 (frHMGB1) and kartogenin (KGN) with alginate gel was used to enhance wounded rat meniscus healing. **a** Chemical structure of KGN, sodium alginate and frHMGB1; **b** Structure of frHMGB1–KGN-containing bioactive alginate microsphere; **c** Experimental procedures showing frHMGB1–KGN-containing bioactive alginate microsphere were prepared and implanted into the wounded meniscus to enhance healing

of distilled water were added into 80 µl of AS and mixed well to make a KGN-containing microsphere by dropping 10 µl of KGN–AS mixture into 2% $CaCl_2$ solution (KGN). Moreover, 10 µl of frHMGB1 solution and 10 µl of DMSO were added into 80 µl of AS and mixed well to make a frHMGB1-containing microsphere by dropping 10 µl of frHMGB1–AS mixture into 2% $CaCl_2$ solution (frHMGB1).

The morphology of frHMGB1- and KGN-containing microspheres was examined under a microscope (Nikon eclipse, TE2000-U, Melville, NY, USA), and the size of the microsphere was determined by Spot imaging software (Diagnostic Instruments, Inc., Sterling Heights, MI, USA).

Determination of frHMGB1 released from frHMGB1-containing microsphere

Each frHMGB1-containing microsphere prepared by above procedures was added into 1 ml of PBS, and the concentration of frHMGB1 in PBS was determined at different time

points using an ELISA kit (Tecan Group Ltd., Mannedort, Switzerland) according to the manufacture's protocol. Three samples were tested at each time point.

Determination of KGN released from KGN-containing microsphere

To ensure that KGN has been loaded to alginate microsphere, the KGN-containing microsphere was inspected by green fluorescence under a fluorescent microscope (Nikon Eclipse TE2000, Japan) [20], and the concentration of KGN released from KGN-containing microsphere was determined by the following procedures. Each KGN-containing microsphere prepared by above procedures was added into 1 ml of PBS, and the concentration of KGN in PBS was determined at different time points using high-performance liquid chromatography (HPLC; Hitachi, Japan) according to the published protocol [16].

Isolation and characterization of bone marrow-derived stem cells (BMSCs)

BMSCs were isolated from femur bones of GFP transgenic SD rats according to the published protocols [21]. Briefly, a needle (21-gauge) fastened to a syringe containing 0.1 ml of heparin (1000 units/ml) to aspirate 1 ml of bone marrow followed by washing the aspirates twice with phosphate-buffered saline (PBS). The bone marrow–PBS solution was centrifuged at 1500 g for 5 min. After discarding the supernatant, the cells were resuspended in growth medium consisting of 20% of fetal bovine serum (FBS)-containing Dulbecco's Modified Eagle Medium (DMEM) with 1% of penicillin and streptomycin and incubated at 37 °C with 5% of CO_2 and 95% of air atmosphere. After 3 days of culturing, MSCs adhered to plastic surface, and blood cells were removed with medium changing. The medium was changed every three days.

The stemness of BMSCs isolated from GFP rats was determined by immunostaining on stem cell marker expression including nucelostemin octamer-binding transcription factor 4 (Oct-4), and Nanog. The cells isolated from the bone marrow of GFP rats at passage 1 were seeded in 12-well plate at a density of 4×10^4/well and cultured with growth medium for 5 days. Then, the medium was removed, and the cells were washed with PBS. The washed cells were fixed with PBS-buffered 4% paraformaldehyde for 30 min and incubated with PBS-buffered 0.1% Triton-X-100 solution for another 30 min. After washing the cells three times with PBS, the cells were incubated with goat anti-nucleostemin (1:350; Neuromics, Edina, MN, USA), rabbit anti-Oct-4 antibody (1:300; Cat. No. PA1-16943, Thermo Fisher, Waltham, MA, USA), rabbit anti-Nanog antibody (1:350; Cat. No. ab106465, Abcam, Cambridge, UK) at 4 °C overnight. In the next morning, the cells were washed with PBS three times and then incubated with cyaine-3 (Cy-3)-conjugated donkey anti-goat IgG antibody (1:500, Millipore, Temecula, CA, USA) at room temperature for 2 h for nucleostemin testing, and Cy-3-conjugated goat anti-rabbit IgG antibody (1:500; Cat. No. A10520, Thermo Fisher, Waltham, MA, USA) at room temperature for 2 h for Oct-4 and Nanog testing. Finally, the cells were counterstained with Hoechst 33342 (1 μg/ml, Sigma, St. Louis, MO, USA). The positive cells were determined under a fluorescent microscope (Nikon eclipse, TE2000-U, Melville, NY, USA).

Effect of frHMGB1 on inducing the migration of rat BMSCs

The effect of frHMGB1 on inducing the migration of rat BMSCs was examined using a trans-well plate (Millipore-Sigma, St Louise, MO, USA). The rat BMSCs in DMEM at passage 2 were seeded into the upper layer of insert well of the trans-well plate. The lower layer of the trans-well plate was filled with various concentrations of frHMGB1 (0-100 ng/ml; Tecan Group Ltd., Mannedort, Switzerland)-containing DMEM or a frHMGB1-microsphere-containing (1000 ng frHMGB1/microsphere/ml) DMEM. After 24 h, the cells migrated through the membrane to the lower layer well were counted by H33342 staining (1 μg/ml, Sigma, St Louise, MO, USA) and observed under a microscope.

Effect of HMGB1 inhibitors on cell migration

The effect of frHMGB1 on inducing the migration of rat BMSCs was further examined with HMGB1 inhibitors. The rat BMSCs in DMEM at passage 2 were seeded into the upper layer of insert well of the trans-well plate. The lower layer of the trans-well plate was filled with 100 ng/ml of frHMGB1-containing DMEM with various concentrations of FSP-ZM1 and AMD3100 (Sigma, St Louise, MO, USA). After 24 h, the cells migrated through the membrane to the lower layer of each well were counted by H33342 staining (Sigma, St Louise, MO, USA) under a fluorescent microscope.

Effect of frHMGB1 on cell activation from G_0 to G_{Alert}

The effect of frHMGB1 on cell proliferation was examined by BrdU incorporation testing. The rat BMSCs at passage 2 were seeded into 12-well plate (2×10^4 cells/well) and cultured with 20% FBS–DMEM for 3 days. Then, the cells were cultured in 1 ml of serum-free medium for another 24 h, finally 10 μM of 5-bromo-2′-deoxyuridine (BrdU; Sigma, St Louise, USA) with various concentrations of frHMGB1 (1-100 ng/ml) or a frHMGB1 (1000 ng)-containing microsphere were added into 1 ml of serum-free medium and

cultured for additional 24 h. Then, the cells were washed once with PBS and fixed with PBS-buffered 4% paraformaldehyde for 20 min. The fixed cells were treated with PBS-buffered 0.1% Triton-X-100 solution for another 20 min and blocked with 3% BSA-PBS for 30 min at room temperature. The cells were incubated with mouse anti-BrdU antibody (1:250; Cat. No. MA3-071, Thermo Fisher, Waltham, MA, USA) overnight at 4 °C. In the next morning, the cells were washed with PBS three times and incubated with Cy-3-conjugated goat anti-mouse IgG antibody at room temperature for 2 h (1:500, Cat. No. AP124C, Sigma, t. Louis, MO, USA). The cell numbers-incorporated BrdU was determined under a fluorescent microscope (Nikon eclipse, TE2000-U, Melville, NY, USA) and counted by semiquantification.

Effect of frHMGB1–KGN-containing microsphere on chondrogenic differentiation of BMSCs in vitro

The effect of frHMGB1–KGN-containing microsphere on chondrogenic differentiation of BMSCs was tested in vitro according to the published protocol [22]. The rat BMSCs were seeded with 0.5 ml of growth medium in 24-well plate at a density of 6×10^5/well and centrifuged at 1500 g for 5 min. The formed cell pellet in each well was incubated at 37 °C with 5% CO_2 with four different conditions for 2 weeks. Group 1: DMSO-containing microsphere was added into the growth medium (DMSO); group 2: KGN-containing microsphere was added into growth medium (KGN); group 3: frHMGB1-containing microsphere was added into growth medium (frHMGB1); and group 4: frHMGB1–KGN-containing microsphere was added into growth medium (frHMGB1 + KGN). The medium was changed every 3 days. At day 14, the cells were fixed with 70% ethanol in an ice bath for 1 h and washed with distilled water three times. The washed cells were then stained either with Alcian blue or with safranin O for 1 h at room temperature according to the standard protocols. The stained cells were examined under an inverted microscope (Nikon Eclipse, TE2000-U, Melville, NY, USA), and a CCD camera was used to capture images which were analyzed by imaging software (Diagnostic Instruments Inc., Sterling Heights, MI, USA).

Semiquantification of positively stained cells

The positively stained cells on stem cell marker expression and BrdU incorporation were analyzed by semiquantification. Four images of each well were randomly taken, and three wells were used for each group. The proportion of positive stained cells in each image was calculated by dividing red fluorescent cell numbers by total cell numbers stained with H33342 (blue fluorescent cells). The average of 12 images from three wells for each group were used to represent the percentage of positive staining, which is the extent of either stem cell marker expression or BrdU-incorporated cells in the respective media.

To quantify the differentiation staining, three random images were taken from each well under a microscope (Nikon Eclipse, TE2000-U, Melville, NY, USA). Three wells from each group were examined from each marker. The positive stained cells in each image were measured using SPOT imaging software (Diagnostic Instruments, Inc., Sterling Heights, MI). The percentage of positive staining in each image was calculated by dividing the positively stained areas by total area viewed under the microscope (positive/total × 100%).

In vivo model for wounded rat meniscus healing

The meniscus cells were eliminated from total 30 SD rats by the treatment of the knees with 6 Gy X-ray, and three irradiated rats and three normal rats were killed at 48-h post-irradiation. The meniscus tissues were collected from these six rats (three normal and three irradiated rats) and prepared for cryosectioning by freezing at −80 °C in Neg 50 filled molds (Richard-Allan Scientific, Kalamazoo, MI). The irradiation effect on the menisci was determined by a live/dead cell viability assay kit according to the manufacture's protocol (Thermo Fisher Scientific, Cat. No.L3224, Waltham, MA).

Then, GFP-labeled BMSCs (10^6 cells) isolated from GFP rats were injected into each wounded rat via tail vein. A wound was created in each meniscus of irradiated rats with a 1-mm-diameter biopsy punch (Miltex, Inc., YorK, PA, USA). The wounded rats were divided into four groups with six rats in each group. The wound in group 1 was treated with a DMSO-containing microsphere (**DMSO**); the wound in group 2 was treated with a KGN-containing microsphere (**KGN**); the wound in group 3 was treated with a frHMGB1-containing microsphere (**frHMGB1**); the wound in group 4 was treated with a frHMGB1–KGN-containing microsphere (**frHMGB1–KGN**). Three rats from each group were killed 2 weeks post-operation, and three rats were killed 4-week post-operation. The menisci from left knee of each rat were used for cellular analysis, and the menisci from right knee of each rat were used for histology analysis.

GFP-labeled cell isolation from wounded rat menisci

The tissues collected from the wound areas of the rat menisci after irradiation, GFP-cell injection and wound healing treatments were cut into small pieces. The meniscus pieces (100 mg) were digested with 3 mg/ml of collagenase and 4 mg/ml of dispase in 1 ml PBS at 37 °C for 3 h. Then, the enzyme solution was removed by a centrifuge at 1500 g for 10 min, and the cell pellets were cultured in a tissue culture plate with growth medium (20% FBS–DMEM). The GFP

cell numbers were examined under a fluorescent microscope (Nikon Eclipse TE2000, Melville, NY, USA).

Histology analysis on wounded rat menisci

The meniscus tissues collected from each group after irradiation, GFP-cell injection and wound healing were fixed with 4% paraformaldehyde for 3 h at room temperature and then decalcified with decalcifying solution (Sigma, St Louise, MO, USA). After decalcification, the meniscus tissues were prepared for cryosectioning by freezing at −80 °C in Neg 50 filled molds (Richard-Allan Scientific, Kalamazoo, MI, USA). Each tissue block was cut into 5-μm-thick sections and stained with hematoxylin and eosin (H & E), and safranin O and fast green (S & F) according to the standard protocols. The stained tissue sections were examined under microscope. For immunostaining, the tissue sections were incubated with rabbit anti-collagen I antibody (1:500; Cat. No. PA5-95137, Thermo Fisher, Waltham, MA, USA) and mouse anti-collagen II antibody (1:500; Cat. No. MA5-12789, Thermo Fisher, Waltham, MA, USA) at 4 °C overnight. In the next morning, the tissue sections were washed with PBS three times and incubated with FITC-conjugated goat anti-rabbit IgG antibody (1:500; Cat. No. F-2765, Thermo Fisher, Waltham, MA, USA) for collagen I testing, and Cy-3-conjugated goat anti-mouse IgG antibody (1: 500; Cat. No. A10521, Thermo Fisher, Waltham, MA, USA) for collagen II testing at room temperature for 1 h. Nuclei were counterstained with H33342 (1 μg/ml, Sigma, St. Louis, MO, USA). The collagen expression in wounded meniscus was examined under a fluorescent microscope (Nikon Eclipse TE2000, Melville, NY, USA).

Statistical analysis

All data were obtained from at least three replicates and presented as mean ± SD. The statistical analyses were performed with Excel 2007 version using student *t* test or ANOVA, and when $P < 0.05$, the two groups compared are considered to be significantly different.

Results

Characterization of frHMGB1–KGN-containing bio-active microsphere

A novel bio-active microsphere for enhancing wounded meniscus healing has been developed by combining frHMGB1 and KGN with alginate gel according to the scheme shown in Fig. 2. Sodium alginate ($NaC_6H_7O_6$) is a linear polysaccharide derivative of alginic acid which can be dissolved with distilled water to make a homogenized solution (Fig. 2a). Divalent cation Ca^{2+} is a common cross-linker

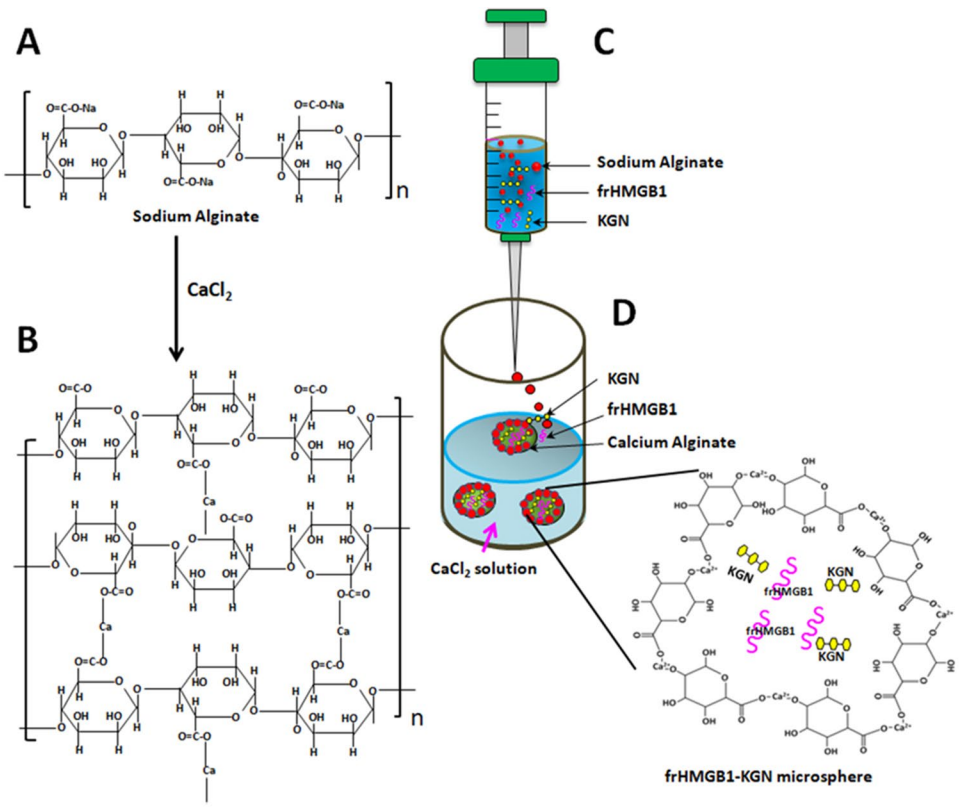

Fig. 2 Chemical structure and preparation procedures of frHMGB1- and KGN-containing bioactive microsphere. The structures indicate that sodium alginate is a water-soluble, linear polysaccharide derivate of alginic acid (**a**), while calcium alginate has an insoluble polymer structure due to the formation of ion bond between alginate and Ca^{2+} (**b**). A frHMGB1- and KGN-containing bioactive microsphere can be prepared by two steps: the first step is to add frHMGB1 and KGN into 2% of sodium alginate solution and mix well to get a frHMGB1–KGN–AS homogenized solution (**c**); the second step is to drop frHMGB1–KGN–AS solution into 2% of calcium chloride solution to form a frHMGB1–KGN-containing bio-activated microsphere (**d**)

for producing alginate microsphere by forming ion bond between alginate and Ca^{2+} (Fig. 2b). The frHMGB1- and KGN-containing sodium alginate mixtures were prepared by adding frHMGB1 solution and KGN solution into 2% of sodium alginate solution (Fig. 2c). When frHMGB1–KGN–AS solution was dropped into $CaCl_2$ solution, a solid microsphere was obtained (Fig. 2d). The control microsphere was made by adding the same volume of solvent (DMSO or/and water) instead of KGN or frHMGB1 into sodium alginate solution.

Morphological analysis indicated that the control microsphere without drug loading was a clear and empty bead (Fig. 3a) without green fluorescence (Fig. 3b), while KGN-containing microspheres (Fig. 3d) and frHMGB1–KGN-containing bioactive microsphere (Fig. 3j) were solid beads with strong-green fluorescence (Fig. 3e, k). The frHMGB1-containing microsphere was semitransparent (Fig. 3g) without green fluorescence (Fig. 3h). Microscope images showed that all microspheres have cross-linked mesh structure (Fig. 3c, f, i, l). The diameter of microsphere is 943.48 ± 123.81 for control microsphere (DMSO), 815.53 ± 116.64 for KGN-containing microsphere (KGN), 913.04 ± 106.49 for frHMGB1-containing microsphere (frHMGB1) and 934.04 ± 110.24 for frHMGB1–KGN-containing bioactive microsphere (frHMGB1 + KGN).

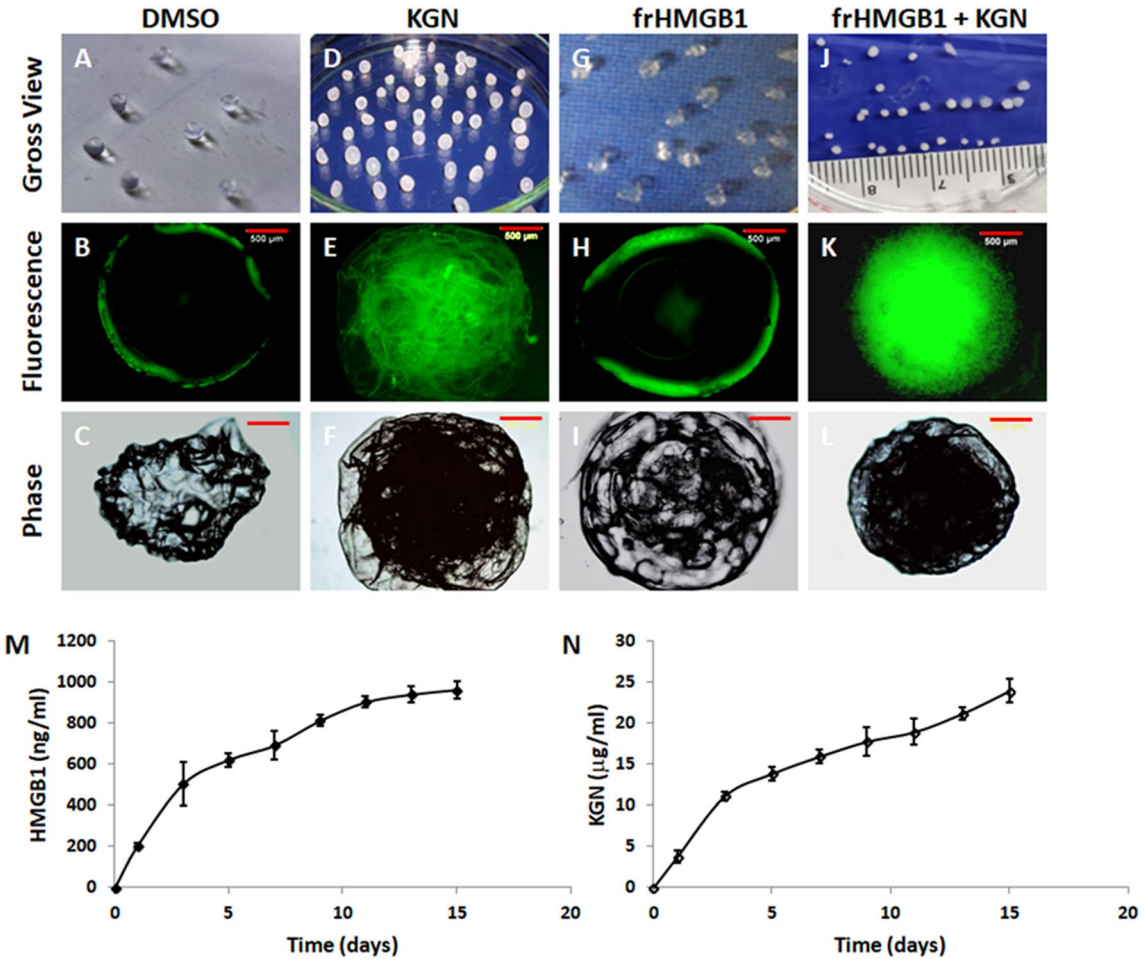

Fig. 3 Characterization of alginate microspheres. **a–c** Control microspheres without drugs (DMSO); **d–f** KGN-containing microspheres (KGN); **g–i** frHMGB1-containing microspheres (HMGB1); **j–l** frHMGB1- and KGN-containing microspheres (HMGB1 + KGN); **m** The concentrations of frHMGB1 released from frHMGB1–KGN-containing microsphere; **n** The concentrations of KGN released from frHMGB1–KGN-containing microspheres. The results indicated that alginate microspheres without drugs were clear and empty bead without green fluorescence (**a**, **b**). KGN-containing (**d**, **e**) and frHMGB1–KGN-containing microspheres (**j**, **k**) were solid beads with strong green fluorescence. frHMGB1-containing microspheres were semitransparent without green fluorescence (**g**, **h**). All alginate microspheres had cross-linked mesh structure (c, f, i, l). Both frHMGB1 (**m**) and KGN (**n**) can be released from frHMGB1–KGN-containing bioactive microsphere in a time-dependent manner. At day 15, more than 90% of frHMGB1 and KGN have been released from frHMGB1–KGN-containing bioactive microsphere. Bars: 500 μm

Determination of frHMGB1 and KGN released from frHMGB1–KGN-containing bio-active microsphere

The results obtained by the degradation of the microspheres tested in vitro indicated that both frHMGB1 and KGN were released from frHMGB1–KGN-containing bioactive microsphere in a time-dependent manner (Fig. 3m, n). After 3 days, about 51% of frHMGB1 (Fig. 3m) and 43% of KGN (Fig. 3n) have been released from frHMGB1–KGN-containing bioactive microsphere and kept high concentrations of frHMGB1 and KGN for more than 2 weeks (Fig. 3m, n). At day 15, more than 90% of frHMGB1 and KGN have been liberated from frHMGB1–KGN-containing bioactive microsphere.

Characterization of rat BMSCs

BMSCs were isolated from rat bone marrow, and the "stemness" of the BMSCs was confirmed by stem cell marker expression before they were used for in vitro and in vivo experiments. The immunostaining results showed that more than 91% of the cells isolated from rat bone marrow were positively stained with nucleostemin (Fig. 4a–d, m), more than 70% of rat BMSCs were positively stained with Oct-4 (Fig. 4e–h, m) and more than 82% of rat BMSCs were positively stained with Nanog (Fig. 4i–l, m). These

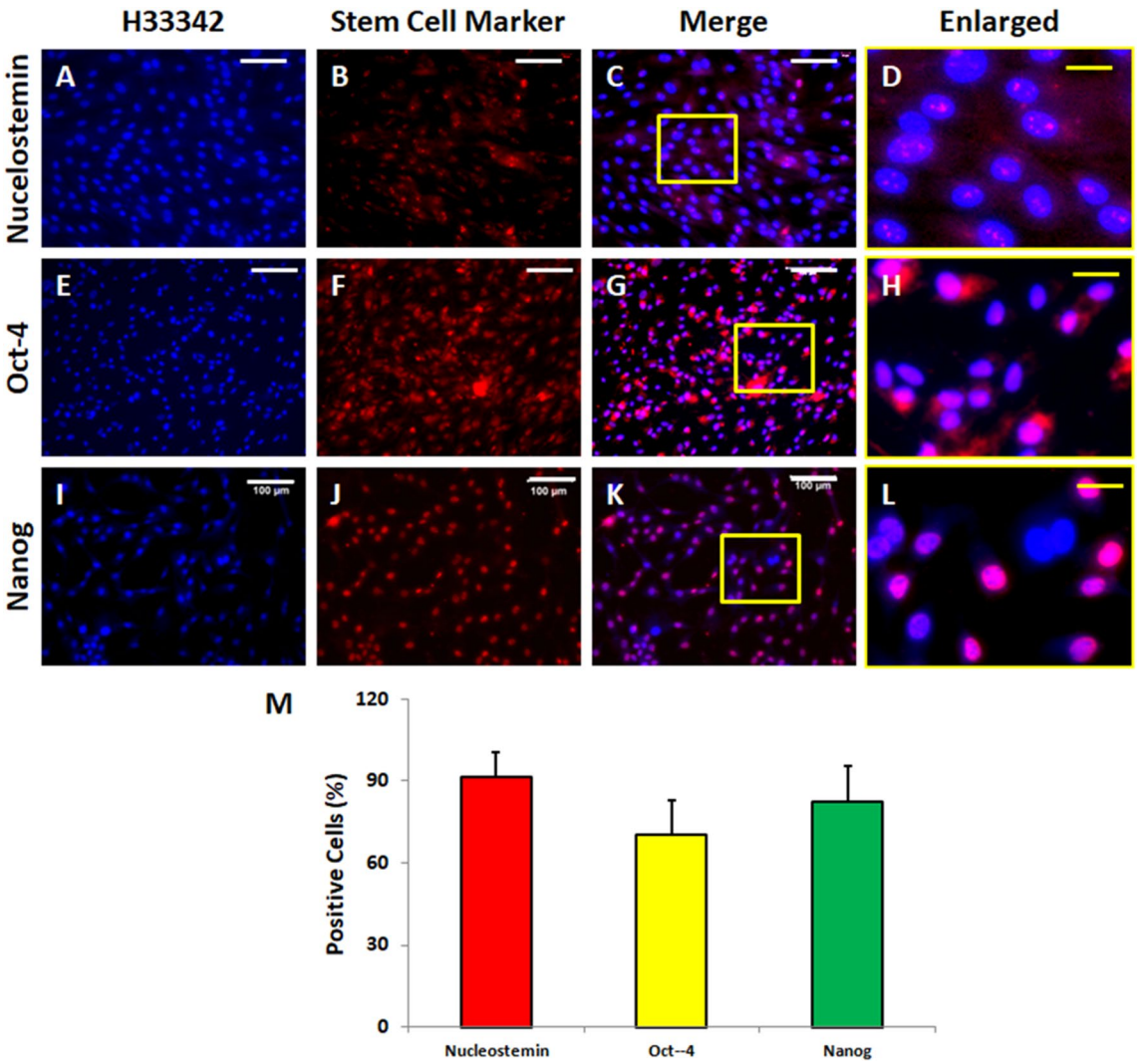

Fig. 4 Stem cell marker expression in the BMSCs examined by immunostaining. **a–d** The cells were stained with nucleostemin. **e–h** The cells were stained with Oct-4. **i–l** The cells were stained with Nanog. **m** The semiquantification of positively stained cells by each stem cell marker. The images of **D, H, L** were enlarged box areas in the images of **C, G** and **K**. The results indicated that more than 91% of the BMSCs were positively stained with nucleostemin, more than 70% of the BMSCs were positively stained with Oct-4 and more than 82% of the BMSCs were positively stained with Nanog. White bars 100 μm; yellow bars: 25 μm

results demonstrated that the cells isolated from rat bone marrow were stem cells.

Effect of frHMGB1 on cell migration

To determine the effect of frHMGB1 on recruiting cells, cell migration testing was investigated using a trans-well plate (Fig. 5). The results showed that frHMGB1 induced rat BMSC migration in a concentration-dependent manner (Fig. 5c–h). After 24 h, very few cells migrated through the membrane into the serum-free medium without frHMGB1 (Fig. 5c, h). However, more than four times of the cells have migrated through the membrane into the 100 ng/ml of frHMGB1-containing serum-free medium (Fig. 5f, h) compared to the medium without frHMGB1 (Fig. 5c, h). Similarly, more than five times of the cells have migrated through the membrane into the serum-free medium with frHMGB-containing alginate microsphere (Fig. 5g, h).

The cell migration was inhibited by adding FPS-ZM1, an inhibitor of HMGB1 into frHMGB1-containing medium (Fig. 5i–n). The results indicated that FPS-ZM1 inhibited the recruiting effect of frHMGB1 in a concentration-dependent manner (Fig. 5i–n).

Further experimental results confirmed these findings. The cell migration was also inhibited by adding another HMGB1′s inhibitor, AMD3100. Many cells have migrated through the membrane into frHMGB1-containing medium without AMD3100 (Fig. 5o, t). The migrated cell numbers decreased significantly when AMD3100 was added into frHMGB1-containing medium, the more AMD3100, the less migrated cells (Fig. 5o–t).

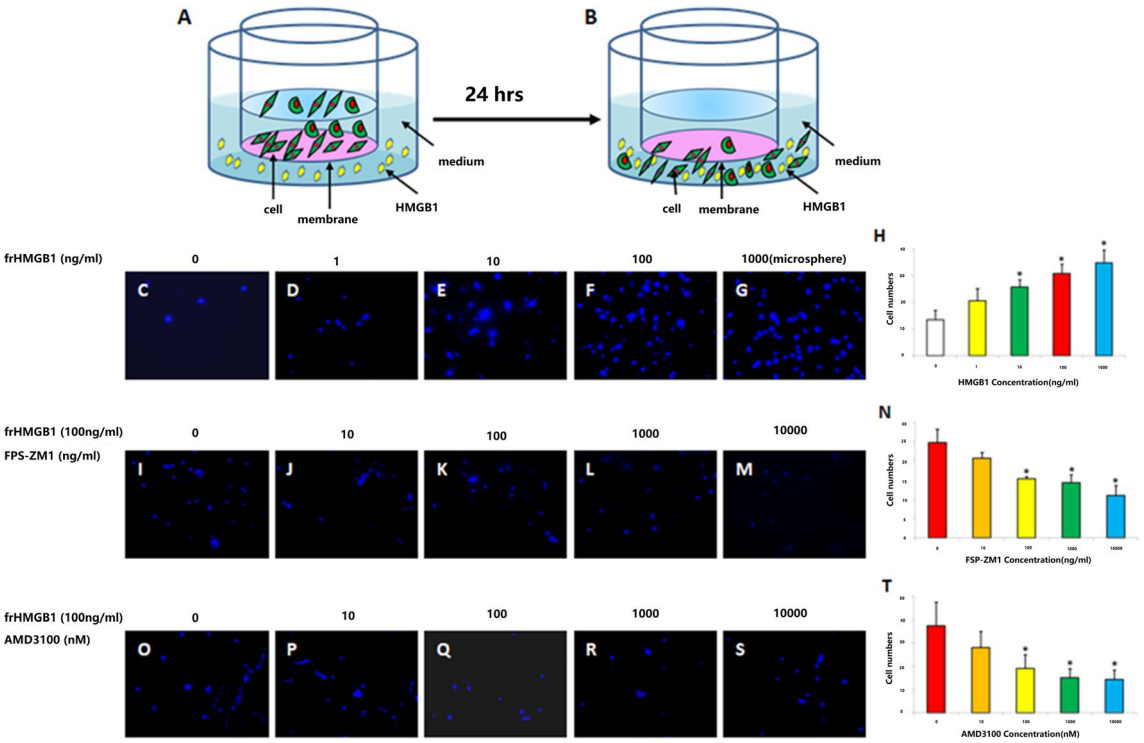

Fig. 5 Effects of frHMGB1 and its inhibitors on cell migration of rat BMSCs. **a–b** Drawing graph shows that the cell migration was determined by trans-well plate. Rat BMSCs in serum-free medium at passage 2 were seeded in the upper layer of a cell culture insert with permeable membrane of trans-well plate (5000 cells/well), and various concentrations of frHMGB1 (0–100 ng/ml)-containing serum-free medium were placed below the cell permeable membrane (**A**). After 24 h, some cells have migrated through the membrane into the lower layer (**b**). **c-f** The cells in the lower layer with various concentrations of frHMGB1 (0–100 ng/ml) were stained with H33342 and counted under microscope. **G** Cell migration was determined in serum-free medium with a frHMGB-containing microsphere (1000 ng frHMGB1/microsphere); **h** Semiquantification of the cell migration showed that frHMGB1 induced cell migration in a concentration-dependent manner. **i-m** Cell migration was determined in 100 ng/ml of frHMGB1-containing serum-free medium with 0 ng/ml of FPS-ZM1 (**i**); 10 ng/ml of FPS-ZM1 (**j**); 100 ng/ml FPS-ZM1 (**k**); 1000 ng/ml of FPS-Zm1 (**l**); and 10,000 ng/ml of FPS-Zm1 (**m**); **n** Semiquantification showed that FPS-ZM1 inhibited the cell migration induced by frHMGB1 in a concentration-dependent manner. **o-s** Cell migration was determined in 100 ng/ml of frHMGB1-containing serum-free medium with 0 nM of AMD3100 (**o**); 10 nM of AMD3100 (**p**); 100 nM of AMD3100 (**q**); 1000 nM of AMD3100 (**r**); and 10,000 nM of AMD3100 (**s**); **t** Semiquantification showed that AMD3100 inhibited the cell migration induced by frHMGB1 in a concentration-dependent manner. $*p < 0.05$ compared to control (0 ng/ml of frHMGB1 in **h**; 100 ng/ml HMGB1 and 0 ng/ml FPS-ZM1 for **n**; 100 ng/ml HMGB1 and 0 nM of AMD3100 in **t**)

Effect of frHMGB1 on cell proliferation

The effect of frHMGB1 on cell proliferation was also tested by BrdU staining. The results showed that frHMGB1 induced rat BMSC activation from G_0 to G_{Alert} in a concentration-dependent manner (Fig. 6). In serum-free condition, more than 95% of rat BMSCs were in G_0 stage as evidenced by very few cells were positively stained with BrdU (Fig. 6a, b). When frHMGB1 was added into serum-free medium, many rat BMSCs were activated from G_0 to G_{Alert} stage as evidenced by the cell numbers positively stained with BrdU (pink in Fig. 6c–h). Semiquantification results indicated that more than 38% of rat BMSCs in the medium with frHMGB1-containing alginate microsphere (1000 ng frHMGB1/microsphere) were positively stained with BrdU (Fig. 6k). These findings indicated that frHMGB1 promotes cell proliferation by activating the BMSCs from G_0 to G_{Alert}.

Effect of frHMGB1–KGN-containing bioactive microsphere on chondrogenic differentiation of BMSCs in vitro

The effect of frHMGB1–KGN-containing bioactive microsphere on chondrogenic differentiation of rat BMSCs was also tested in vitro. The results showed that this frHMGB1–KGN-containing bioactive microsphere induced the chondrogenetic differentiation of rat BMSCs as evidenced by two different chondrocyte staining reagents safranin O (Fig. 7d) and Alcian blue (Fig. 7h). High percentages of rat BMSCs grown in KGN-containing microsphere (Fig. 7b, f) and frHMGB1–KGN-containing microsphere (Fig. 7d, h) were positively stained with safranin O and Alcian blue. Although cell proliferation was increased by frHMGB1-containing microsphere, a few cells grown in frHMGB1-containing microsphere were positively stained with safranin O (Fig. 7c) and Alcian blue (Fig. 7g). Similar results were found in the cells grown in DMSO microsphere (Fig. 7a, e).

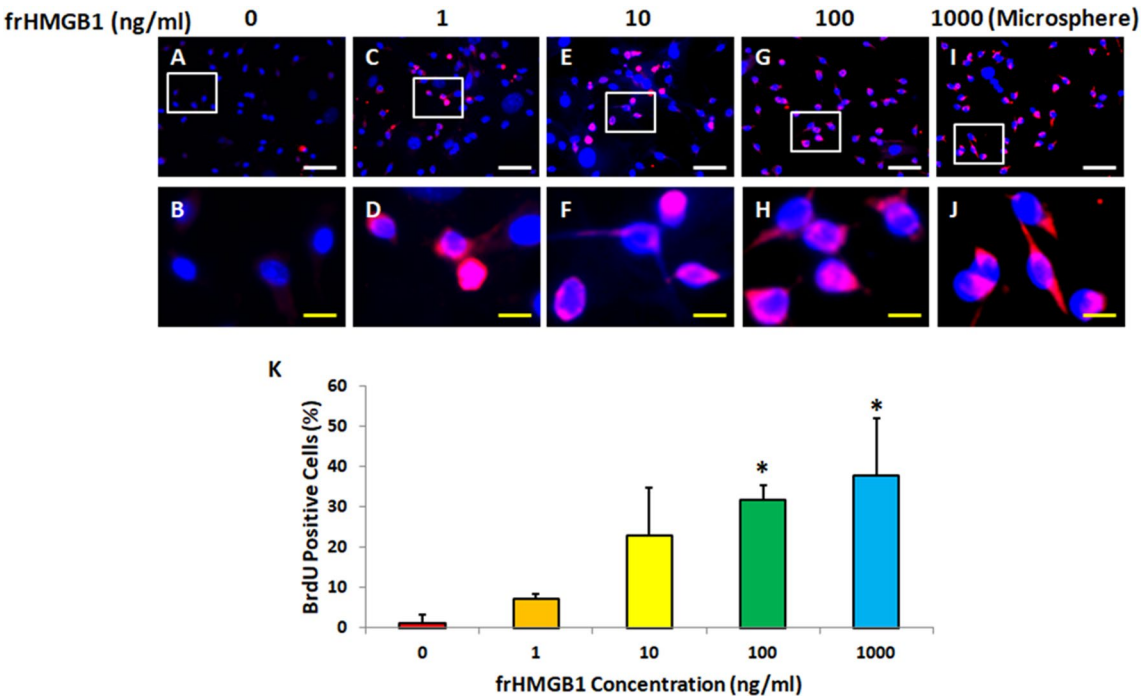

Fig. 6 Effect of frHMGB1 on cell activation of rat BMSCs determined by BrdU staining. Rat BMSCs at passage 2 were seeded in 12-well plate (20,000 cells/well) and cultured with 20% of FBS–DMEM medium for 3 days. Then, the cells were cultured with serum-free medium for 24 h, finally, various concentrations of frHMGB1 (0–100 ng/ml) and a frHMGB1-containing microsphere (1000 ng frHMGB1/microsphere) with BrdU (10 uM) were added into the serum-free medium and the cells were cultured for another 24 h. The cell proliferation was determined by anti-BrdU anti-body, and the $\mathbf{G_{Alert}}$ cells were positively stained with BrdU indicating that BrdU has been incorporated into cells. Total cell numbers were stained with H33342 and counted under microscope. **a, b** The cells were cultured in 0 ng/ml of HMGB1; **c, d** The cells were cultured in 1 ng/ml of HMGB1; **e, f** The cells were cultured in 10 ng/ml of HMGB1; **g, h** The cells were cultured in 100 ng/ml of HMGB1; **i, j** The cells were cultured in a frHMGB1-containing microsphere (1000 ng frHMGB1/microsphere) in 1 ml serum free medium; **k** Semiquantification of the positively stained cells obtained from three wells of each group. The images of **b, d, f, h, j** were enlarged box areas in the images of **a, c, e, g, i**, respectively.*$p < 0.05$ compared to control (HMGB1 0 ng/ml). White bars = 50 μm; yellow bars = 10 μm

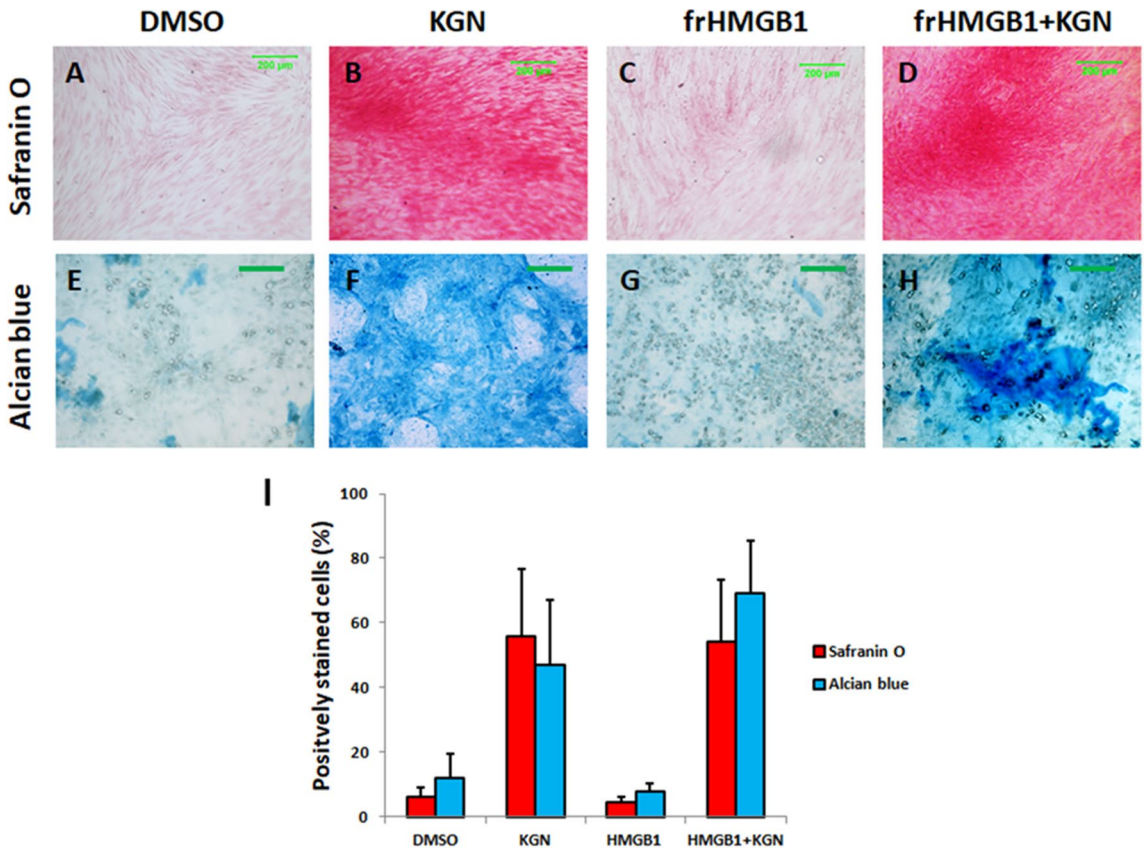

Fig. 7 Chondrogenic differentiation of rat BMSCs grown in alginate microspheres for 14 days. **a–d** safranin O staining; **e–h** Alcian blue staining. **a, e** rat BMSCs grown in control microsphere without drugs (DMSO); **b, f** rat BMSCs grown in KGN-containing microsphere (KGN); **C, G** rat BMSCs grown in frHMGB1-containing microsphere (frHMGB1); **d, h** rat BMSCs grown in frHMGB1 and KGN-containing microsphere (frHMGB1 + KGN). **I** Semiquantification of positively stained cells in each group. High percentages of rat BMSCs grown in KGN-containing microsphere (**b, f**) and frHMGB1 and KGN-containing microsphere (**d, h**) were positively stained with safranin O and Alcian blue. A few cells grown in control microsphere (**a, e**) or frHGMB1-containing microsphere (**c, g**) were positively stained with safranin O and Alcian blue. Bars: 200 μm

Our findings indicated that KGN plays an important role in chondrogenic differentiation of rat BMSCs.

Effect of frHMGB1–KGN-containing bioactive microsphere on wounded meniscus healing in vivo

Based on the above experimental results, we investigated the effect of frHMGB1–KGN-containing alginate bioactive microsphere on promoting wounded rat meniscus healing using a new in vivo rat model (Fig. 8). The native cells in the menisci of SD rats were eliminated using an irradiator (6 Gy) as shown in Fig. 8a. GFP-BMSCs were injected into tail vein of the irradiated rats (Fig. 8b), and a wound was created by a biopsy punch (1 mm diameter) in the irradiated meniscus 48-h post-irradiation (Fig. 8c). The wounds were treated with four different alginate microspheres (Fig. 8d), and the wounded meniscus healing results were examined 2- and 4-week post-operation by cellular analysis and histology analysis.

In order to eliminate native meniscus cells in wild-type rats, we irradiated the knee of adult SD rats with 6-Gy using X-ray. Previous studies have shown that general behavior of mice irradiated to 6 Gy was similar to normal mice [23, 24]. Our results demonstrated that 6 Gy is a safe and effective dosage for our experiments. No significant body weight changes were observed in normal and 6 Gy treated rats. Live/dead cell viability assay was used to compare normal and irradiated meniscus tissues, with results showing that irradiation eliminated the majority of live cells (red fluorescence in Fig. 9e, g, h) compared to the normal rat meniscus showing the majority of meniscus is populated with live cells (green fluorescence in Fig. 9b–d). The semiquantification results demonstrated that irradiation can kill more than 95% of meniscus cells (Fig. 9i).

The cellular analysis showed that frHMGB1–KGN-containing alginate bioactive microsphere can recruit cells to the wound area as evidenced by GFP cells found in the wounded menisci of SD rats (Fig. 10). The cells isolated

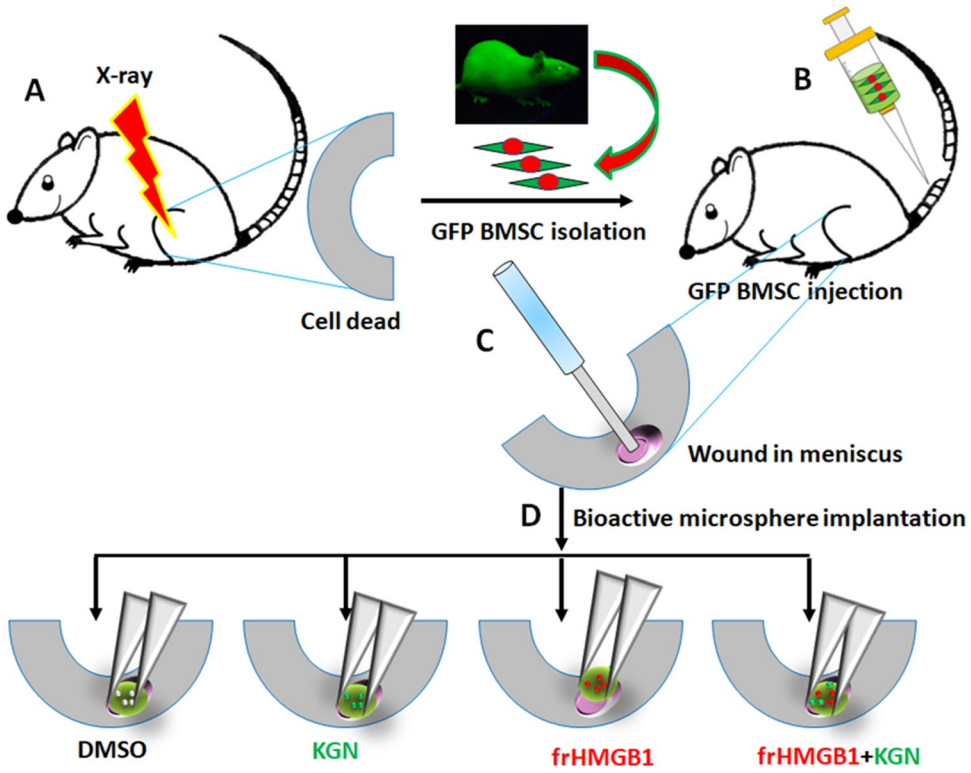

Fig. 8 In vivo animal model used for wounded meniscus healing. **a** The rat meniscus cells were removed by irradiation; **b** A wound (1 mm diameter) was created in the meniscus of the rats by a biopsy punch. **c** Green fluorescent protein-labeled rat bone marrow stem cells (GFP-BMSCs) were injected into tail vein of the rats. **d** The wounded rats were divided into four groups. Group 1: The wound was treated with a DMSO-containing alginate microsphere (**DMSO**); group 2: The wound was treated with a KGN-containing alginate microsphere (**KGN**); group 3: The wound was treated with a frHMGB1-containing alginate microsphere (**HMGB1**); group 4: The wound was treated with a frHMGB1 and KGN-containing alginate bioactive microsphere (**HMGB1 + KGN**). The effect of frHMGB1–KGN-containing bio-active microsphere on wounded meniscus healing was examined by cellular analysis and histology analysis

from wounded rat menisci of all four groups expressed green fluorescence (Fig. 10); however, the cell numbers were significantly different. After 2-week healing, the GFP cell numbers in the frHMGB1-containing alginate microsphere-treated meniscus (Fig. 10c, m) were 1.7 times, and 2.5 times in the frHMGB1–KGN-containing bioactive microsphere (Fig. 10d, m) as high as that in both DMSO-containing alginate microsphere (Fig. 10a, m) and KGN-containing alginate microsphere (Fig. 10b, m). After four-week healing, the GFP cell numbers in frHMGB1- and KGN-containing bioactive microsphere-treated meniscus (Fig. 10g, k, m) were 2.3 times more than that in DMSO-containing alginate microsphere-treated meniscus (Fig. 10e, i, m).

Histology results further demonstrated that the frHMGB1–KGN-containing bioactive microsphere not only recruited the cells to the wound area, but also induced chondrogenic differentiation of BMSCs to form cartilage-like tissue in wounded meniscus (Fig. 11g, h). The H & E staining results on tissue sections of wounded rat menisci showed that large unhealed wound areas were found in the wounded rat meniscus treated with DMSO-containing alginate microsphere for 4 weeks (Fig. 11a–c). Although some cartilage-like tissues were found in the wounded rat meniscus treated with KGN-containing alginate microsphere for 4 weeks (Fig. 11d–f), some unhealed wound areas were still presented in KGN-containing microsphere-treated meniscus (Fig. 11d, f). High cell density was found in the wound areas of the rat menisci treated either with frHMGB1-containing alginate microsphere (Fig. 11g–i) or with frHMGB1–KGN-containing bioactive microsphere (Fig. 11j, l), while more cartilage-like tissues were found in frHMGB1–KGN-containing bioactive microsphere-treated wounded rat meniscus (Fig. 11j, l).

Further histology studies on safranin O and fast green staining confirmed H & E staining results. Large unhealed areas were found in the wounded rat meniscus treated with DMSO-containing alginate microsphere (Fig. 12a–c). Many chondrocyte-like cells in the wound areas of rat menisci treated either with KGN-containing alginate microsphere (Fig. 12d–f) or with frHMGB1–KGN-containing bioactive microsphere (Fig. 12j–l) were positively stained with safranin O, while the positively stained areas

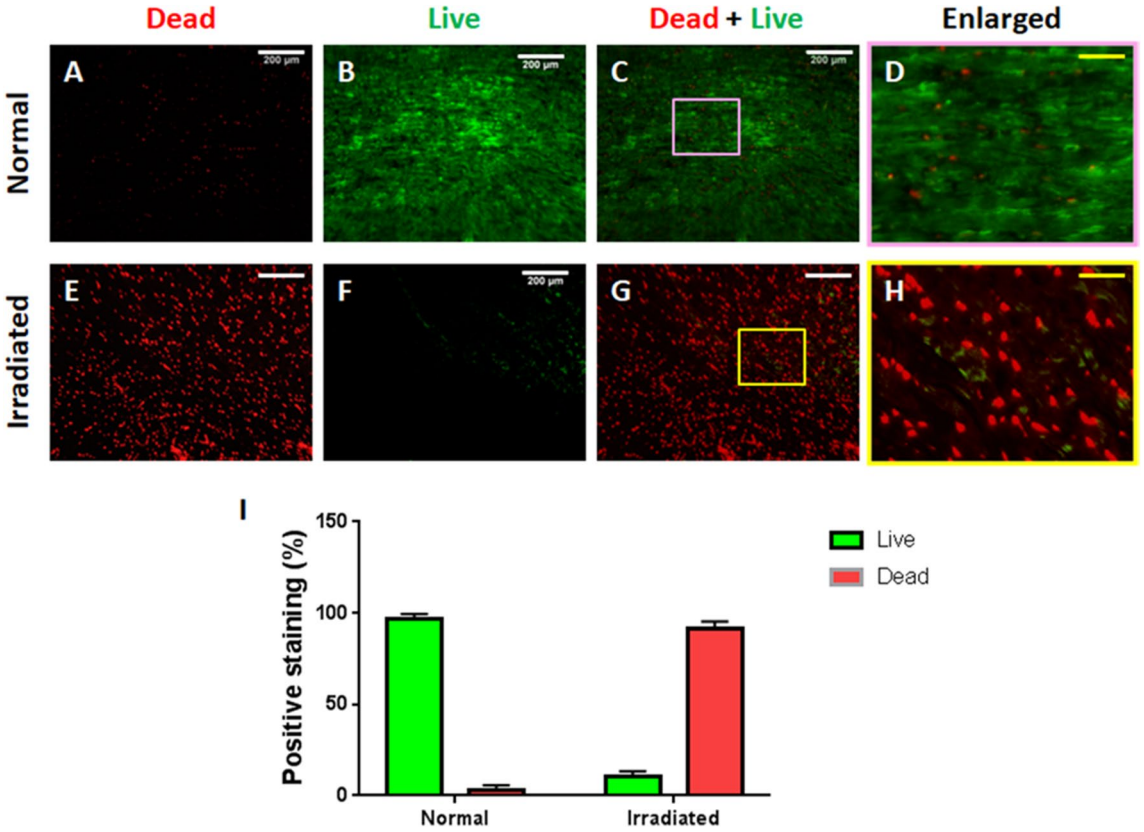

Fig. 9 Effect of irradiation on rat meniscus determined by live/dead cell viability assay kit. **a–d** normal, untreated rat meniscus; **e–h** irradiated rat meniscus; **a, e** Dead cells were stained with red fluorescence; **b, f** Live cells were stained with green fluorescence; **c** The merged images of **a** and **b**; **g** The merged images of **e** and **f**; **d** enlarged pink box area of **c**; **h** enlarged yellow box area of **g**; **i** semi-quantification of positively stained cells in each group. Live/dead cell viability assay results showed that normal rat meniscus tissue sections have more than 95% live cells as evidenced by green stained cell numbers (**b-d, i**) and a few cells are dead cells which were stained by red fluorescence (**a, c, d, i**). Irradiation has killed most of the cells as evidenced by more than 95% of the cells in irradiated meniscus stained with red fluorescence (**e, g, h, i**), and very few cells in irradiated meniscus were stained with green fluorescence (**f, g, h, i**). Yellow bars: 50 μm; white bars: 200 μm

in frHMGB1–KGN-containing bioactive microsphere-treated meniscus (Fig. 12j–l) were much larger than the wound area treated with KGN-containing alginate microsphere (Fig. 12d–f). Some unhealed wound area was found in the wounded rat meniscus treated with KGN-containing alginate microsphere (Fig. 12d–f). Although the wound treated with frHMGB1-containing alginate microsphere healed completely, very few cells were positively stained with safranin O (Fig. 12g–i).

High levels of collagen I and collagen II were found in frHMGB1–KGN-containing microsphere-treated meniscus (Fig. 13m–p). Large unhealed wound areas with weak expression of collagen I and collagen II were found in the meniscus treated with DMSO-containing microsphere (Fig. 13a-d). Although the wounds were healed with frHMGB1-containing microsphere treatment, the collagen II expression in the wound area was much lower (Fig. 13i–l) than the menisci treated either with KGN-containing microsphere (Fig. 13e–h) or with frHMGB1–KGN-containing bioactive microsphere (Fig. 13m–p).

Discussion

Meniscus injuries are extremely common with approximately one million patients undergoing surgical treatment annually in the USA alone [7]. It has been found that the injuries in the inner avascular region of the meniscus cannot be repaired due to its poor intrinsic healing capacity [25]. To date, no regenerative therapy has been proven successful for consistently promoting healing inner-zone meniscus tears [7]. Recent studies have found that the use of tissue engineering and regenerative medicine techniques may offer novel and effective approaches to repair meniscal injuries [26]. In this study, we have developed a novel frHMGB1–KGN-containing bioactive microsphere to improve avacular meniscus healing by inducing recruitment and

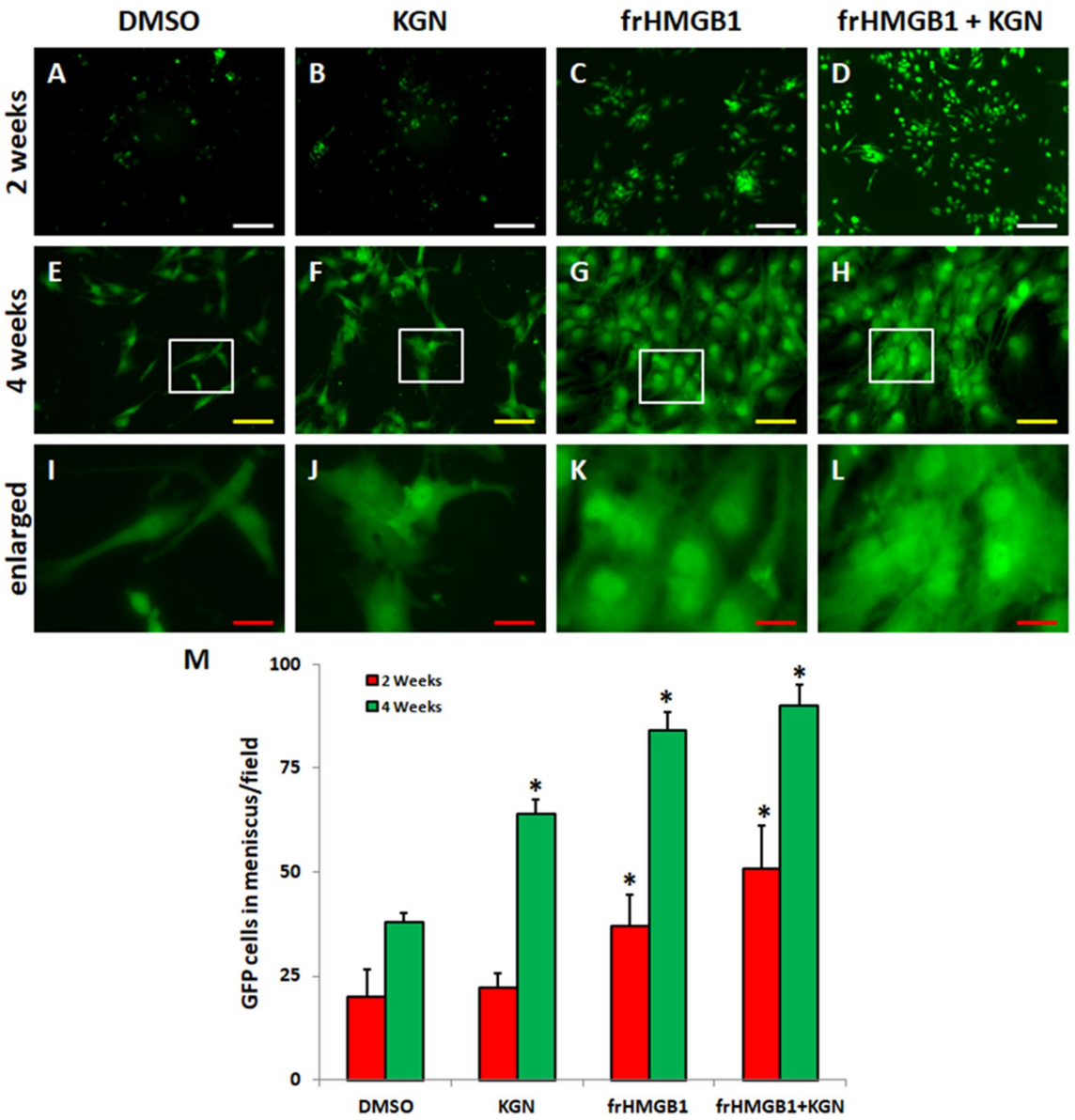

Fig. 10 GFP cells isolated from wounded meniscus of the rats treated with various alginate microspheres for 2 and 4 weeks. **a–d** 2 weeks; **e-l** 4 weeks; **a, e, i** The wound was treated with a DMSO-containing alginate microsphere (**DMSO**); **b, f, j** The wound was treated with a KGN-containing alginate microsphere (**KGN**); **c, g, k** The wound was treated with a frHMGB1-containing alginate microsphere (**frHMGB1**); **d, h, l** The wound was treated with a frHMGB1- and KGN-containing alginate microsphere (**frHMGB1 + KGN**). **m:** Semiquantification of GFP-cells calculated in each group. The meniscus was collected immediately after the rats were killed, and the cells were isolated from the meniscus and cultured for 10 days. The GFP-labeled cells in each group were examined under a fluorescent microscope. The results showed that frHMGB1-containing microsphere recruited the GFP cells to migrate to the wound area in vivo. $*p < 0.05$ compared to control (DMSC-containing microsphere treated wound). White bars: 200 μm; yellow bars: 100 μm; red bars: 25 μm

chondrogenic differentiation of BMSCs. Our results have shown that this frHMGB1–KGN-containing alginate bioactive microsphere released frHMGB1 and KGN and kept high concentrations of frHMGB1 and KGN in the system for more than 2 weeks. The frHMGB1–KGN-containing alginate bioactive microsphere successfully recruited BMSCs into the defect sites and formed cartilage-like tissue.

Our results indicated that frHMGB1 plays an important role in recruiting endogenous stem cells to the wound area as evidenced by large unhealed wound areas found in the menisci treated with DMSO-containing microsphere and KGN-containing microsphere. In vitro study also demonstrated that the cell migration was mediated by frHMGB1. When the inhibitors of HMGB1 such as FPS-ZM1 and AMD3100 were added into frHMGB1-containing medium,

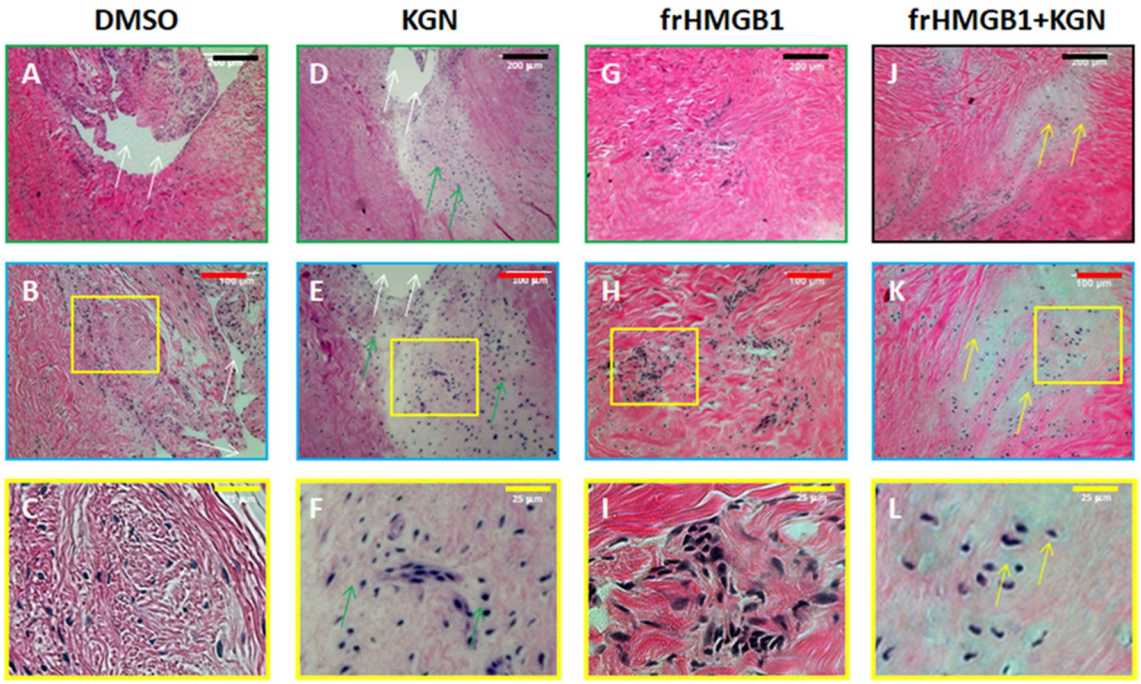

Fig. 11 H & E staining on wounded meniscus of the rats treated with various alginate microspheres for 4 weeks. **a–c** The wound was treated with a DMSO-containing microsphere (**DMSO**); **d–f** The wound was treated with a KGN-containing microsphere (**KGN**); **g–i** The wound was treated with a frHMGB1-containing microsphere (fr**HMGB1**); **J-L** The wound was treated with a HMGB1–KGN-containing bioactive microsphere (fr**HMGB1 + KGN**). The images of **c, f, i,** and **l** were enlarged yellow box areas in the images of **B, E, H,** and **K**, respectively. Large unhealed wound areas were found in DMSO-containing microsphere treated meniscus (white arrows in **A, B**). Although some cartilage-like tissues were found in KGN-containing microsphere treated meniscus (green arrows in **D–F**), large unhealed areas were still found in KGN-containing microsphere-treated wound areas (white arrows in **D, E**). The wound area was filled out with high density of the cells, but less cells were chondrocyte-like cells (**G-I**). The wound areas treated with frHMGB1–KGN-containing bioactive microsphere were filled with chondrocyte-like cells (yellow arrows in **J-L**). Black bars: 200 um; red bars: 100 μm; yellow bars: 25 μm

the cell migration was decreased in a concentration-dependent manner. It has been found that the receptor for advanced glycation end products (RAGE) is implicated in frHMGB1-mediated functions [27]. Recent studies indicated that HMGB1 promotes intraoral palatal wound healing through RAGE-dependent mechanisms [28]. More studies have shown that frHMGB1 enhances recruitment of inflammatory cells to damaged tissues by forming a complex with CXCL12 and the signaling pathway is via CXCR4 [27]. It is well known that frHMGB1 is an enhancer of the activity of CXCL12 in stimulating the migration of mouse embryonic fibroblast [29]. FPS-ZM1 is a RAGE antagonist which blocks Aβ binding to the V domain of RAGE and inhibits Aβ40- and Aβ-42-induced cellular stress in RAGE-expressing cells and in the mouse brain [30]. AMD3100 is a CXCR4 antagonist, and CXCL12-mediated engagement of CXCR4 works as an essential coreceptor signal for RAGE receptor-dependent HMGB1 migration response [31]. Our results revealed that the activity of frHMGB1 is critical for cell migration.

This study has demonstrated that both recruitment and activation of endogenous stem cells are necessary for wounded meniscus healing. Our findings indicated that BMSCs can be delivered by blood to the injured meniscus as occurs in other injured tissues [32]. The BrdU staining results showed that either frHMGB1 along or frHMGB1-containing bioactive microsphere accelerated cell proliferation by transitioning stem cells to G_{Alert} as evidenced by more BrdU-incorporating cells found in frHMGB1-treated groups. BrdU has been used to label proliferating and migrating cells in various human and animal tissues and organs [33]. It has been found that BrdU competes with thymidine for incorporation into nuclear DNA during the S-phase of the cell cycle. Therefore, BrdU serves as a marker of DNA synthesis to ensure accuracy with regard to cell division [34]. Our results indicated that the treatment of frHMGB1-containing microsphere is a good approach for promoting the wounded meniscus healing because frHMGB1 not only recruits BMSCs to the wound area but also enhances proliferation of BMSCs.

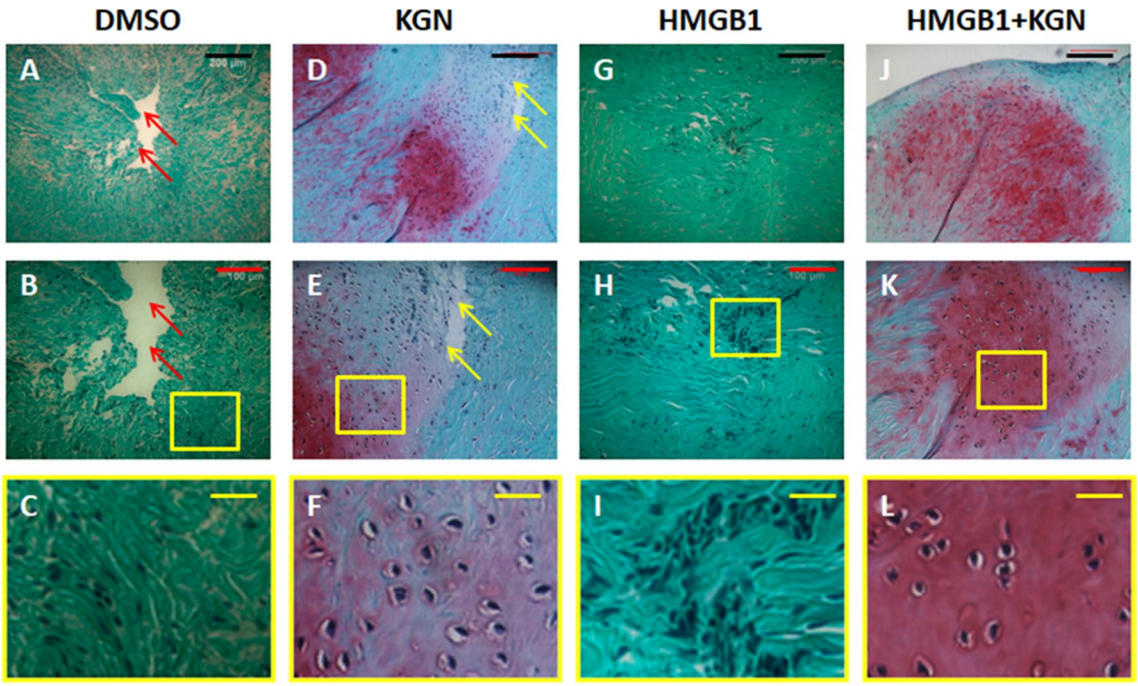

Fig. 12 Safranin O & fast green staining on wounded meniscus of the rats treated with various alginate microspheres for 4 weeks. **a–c** The wound was treated with a DMSO-containing microsphere (**DMSO**); **d–f** The wound was treated with a KGN-containing microsphere (**KGN**); **g–i** The wound was treated with a frHMGB1-containing microsphere (fr**HMGB1**); **j–l** The wound was treated with a HMGB1–KGN-containing bioactive microsphere (fr**HMGB1 + KGN**). The images of **C, F, I,** and **L** were enlarged yellow box areas in the images of **B, E, H,** and **K**, respectively. Large unhealed wound areas were found in DMSO-containing microsphere-treated meniscus (red arrows in **A, B**). Although some cartilage-like tissues were found in KGN-containing microsphere-treated meniscus as evidenced by positively stained tissues with safranin O (red in **D-F**), some unhealed areas were still found in KGN-containing microsphere-treated wound areas (yellow arrows in **D, E**). The wound area was filled out with high density of the cells, but less cells were chondrocyte-like cells (**G–I**). The wound areas treated with frHMGB1–KGN-containing bioactive microsphere were filled with chondrocyte-like cells positively stained with safranin O (red in **J–L**). Black bars: 200 μm; red bars: 100 μm; yellow bars: 25 μm

This study also indicated that KGN is necessary for wounded meniscus healing because it can induce chondrogenic differentiation of stem cells. Recent studies have found that KGN can induce chondrogenic differentiation of BMSCs via core binding factor β (CBFβ)-runt-related transcription factor-1 (Runx1) pathway [15]. Our results have shown that the wound treated either with KGN-containing microsphere or with frHMGB1–KGN-containing alginate bioactive microsphere was filled with cartilage-like tissues as evidenced by histochemical staining with safranin O and immunostaining with collagen I and collagen II (Figs. 12, 13). Although the wound treated with frHMGB1-containing alginate microsphere healed completely, there were a few chondrocyte-like cells found in the new formed tissues.

Our results indicated that a good carrier is also important for delivering bioactive reagents to the wound area to enhance the healing. In this study, we used alginate gel as a drug carrier to make bioactive microsphere due to its advantages for wounded cartilage repair including its solubility in different ionic conditions and easy manufacturing process. It is well known that sodium alginate is an injectable liquid; when sodium alginate solution was added into a calcium chloride solution, a solid microsphere was obtained. It has been reported that alginate is a biodegradable natural polymer which has a similar structure to the extracellular matrix (ECM) of chondrocytes [35]. Several studies have demonstrated that alginate provided an ideal environment to facilitate the spatial distribution of mesenchymal stem cells (MSCs), resulting in a structural organization that resembles the native in vivo cartilage microenvironment [19, 36]. Our results demonstrated that frHMGB1–KGN-containing alginate bioactive microsphere is beneficial to prospective applications in wounded meniscus repair.

Conclusion

Our results demonstrated that a novel bioactive microsphere has been developed by the combination of frHMGB1 and KGN in alginate gel. This frHMGB1–KGN-containing alginate bioactive microsphere can induce the cell migration in vitro and enhance wounded meniscus

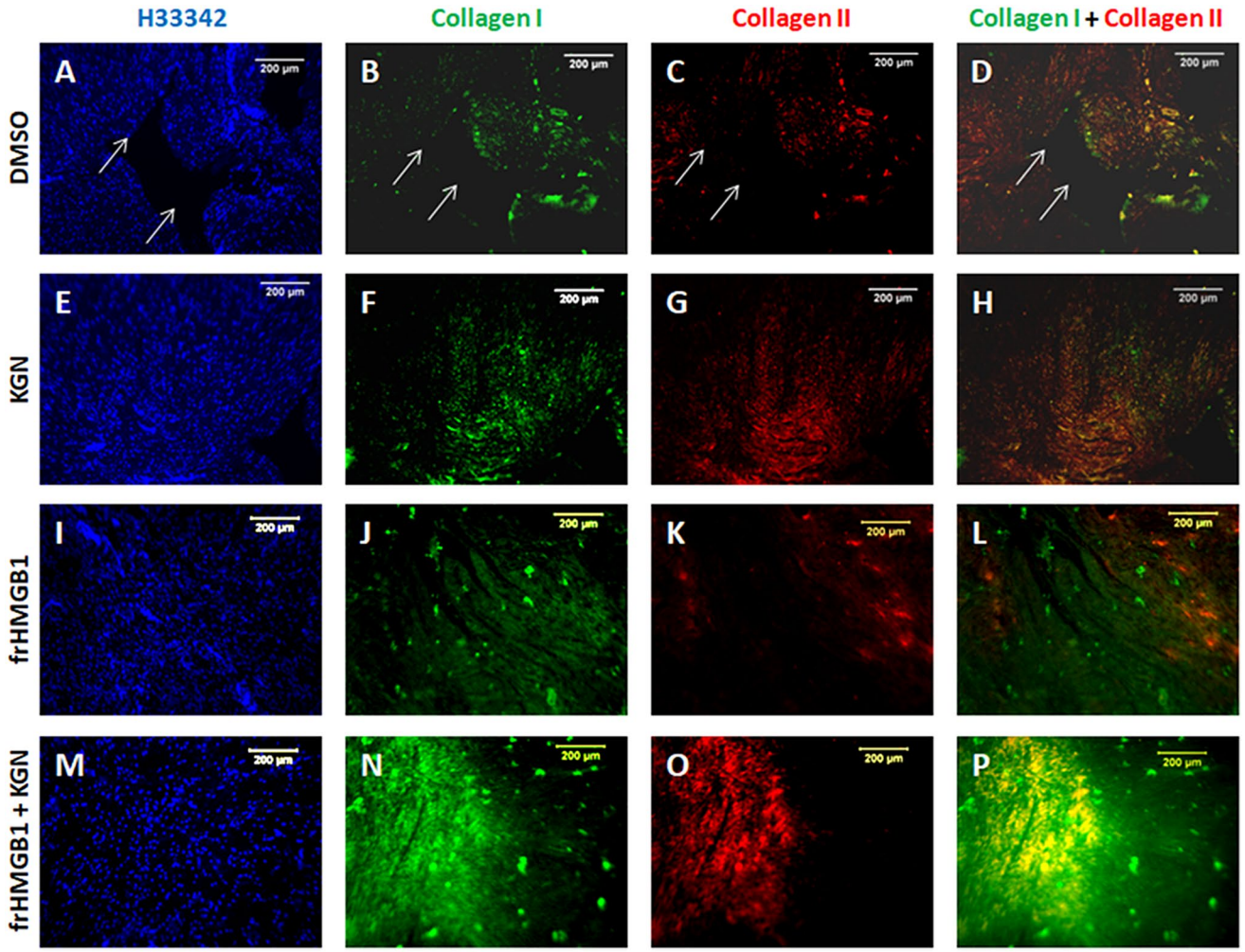

Fig. 13 Collagen I and II expression in wounded meniscus of the rats treated with various alginate microspheres for 4 weeks determined by immunostaining. **a–d** The wound was treated with a DMSO-containing microsphere (**DMSO**); **e–h** The wound was treated with a KGN-containing microsphere (**KGN**); **i-l** The wound was treated with a frHMGB1-containing microsphere (fr**HMGB1**); **m-p** The wound was treated with a HMGB1–KGN-containing bioactive microsphere (fr**HMGB1 + KGN**); **A, E, I, M** H33342 staining; **B, F, J, N** Collagen I staining; **C, G, K, O** Collagen II staining; **D, H, L, P** The images were merged images by **B, F, J, N** and **D, H, L, P**. Large unhealed wound areas were found in DMSO-containing microsphere treated meniscus (white arrows in **A–D**). Weak expression of collagen I and collagen II was found in the wound areas treated with DMSO-containing microsphere (**B-D**). High levels of collagen I and collagen II were found in the meniscus treated either with frHMGB1–KGN-containing microsphere (**N-P**) or with KGN-containing microsphere (**F–H**). Although the wounds were healed with frHMGB1-containing microsphere treatment, the collagen II expression in the wound area was much lower (**K, L**) than the menisci treated either with KGN-containing microsphere (**G, H**) or with frHMGB1–KGN-containing microsphere (**O, P**). Bars: 200 μm

healing in vivo. Our results provide the first evidence for the role of frHMGB1–KGN-containing bioactive microsphere in enhancing the wounded meniscus repair. Using frHMGB1–KGN-containing alginate bioactive microsphere to treat injured meniscus may be a useful approach for clinics.

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Author contributions HX contributed to designing the experiments and writing the manuscript. XZ and PX analyzed the data and prepared the figures. WALSF performed the histological analysis. JW helped edit the manuscript, and HH conceived the study and wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval All institutional and national guidelines for the care and use of laboratory animals were followed.

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