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Nanofibrous spongy microspheres to deliver rabbit mesenchymal stem cells and anti-miR-199a to regenerate nucleus pulposus and prevent calcification

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Abstract

Lower back pain is mainly caused by intervertebral disc degeneration, in which calcification is frequently involved. Here novel nanofibrous spongy microspheres (NF-SMS) are used to carry rabbit bone marrow mesenchymal stromal cells (MSCs) to regenerate nucleus pulposus tissues. NF-SMS are shown to significantly enhance the MSC seeding, proliferation and differentiation over control microcarriers. Furthermore, a hyperbranched polymer (HP) with negligible cytotoxicity and high microRNA (miRNAs) binding affinity is synthesized. The HP can complex with anti-miR-199a and self-assemble into “double shell” polyplexes which are able to achieve high transfection efficiency into MSCs. A double-emulsion technique is used to encapsulate these polyplexes in biodegradable nanospheres (NS) to enable sustained anti-miR-199 delivery. Our results demonstrate that MSC/HP-anti-miR-199a/NS/NF-SMS constructs can promote the nucleus pulposus (NP) phenotype and resist calcification in vitro and in a subcutaneous environment. Furthermore, injection of MSC/HP-anti-miR-199a/NS/NF-SMS can stay in place, produce

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This work was carried out in the Polymeric Biomaterials and Tissue Engineering Laboratory at the University of Michigan. GF did most of the biological and animal experiments including cell isolation, cell culture, molecular and biochemical characterizations, subcutaneous implantation, IVD injection surgery, histological analysis, radiography, microCT analysis, and drafted the manuscript. ZZ and MD did the biomaterials and controlled release studies, including polymer synthesis, scaffold and microsphere fabrication, release experiment, physical characterizations of the biomaterials, drafted the biomaterials related experiments and data. KR participated in animal and biological experiments. PXM is the director of the Polymeric Biomaterials and Tissue Engineering Laboratory, trained the participants, obtained funding, organized and supervised the project, revised and finalized the manuscript, and is the corresponding author.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

functional extracellular matrix, maintain disc height and prevent intervertebral disc (IVD) calcification in a rabbit lumbar degeneration model.

Keywords

Rabbit; mesenchymal stem cells; calcification; anti-microRNA oligonucleotides; nucleus pulposus; regeneration

Introduction

In the United States, more than \$100 billion per year is spent for the direct and indirect consequences of lower back pain (LBP) [1-3]. The lifetime prevalence of LBP is as high as 84%, afflicting all age groups. The risk that the pain will return after an initial episode is about 30%. Lower back pain is mainly caused by intervertebral disc (IVD) degeneration, with which calcification is frequently involved [4-11]. Pathologic calcification may impair nutrient supply and IVD metabolism. Calcification in an IVD may occur in 3 places: centrally in the nucleus pulposus (NP), peripherally in the endplate, and outside the disc as in the case of osteophytic outgrowth at the margins of the vertebrae [11]. Radiographic IVD calcification is correlated with the degree of morphologic degeneration [12] and calcium-containing crystal deposits could disrupt the disc's extracellular matrix [13, 14]. The pathologic calcification in degenerated IVD also negatively affects stem cell function. Overcoming the pathologic calcification is a critical challenge for intervertebral disc regeneration.

Hif-1 α is often used as a phenotypic marker of NP cells and is a key transcription factor in the NP cells [15, 16]. Hypoxia is known to modulate endochondral bone formation and Hif-1 α is likely to play a role in preventing IVD mineralization [8, 17, 18]. Our previous study also demonstrated that bone marrow mesenchymal stromal cells (MSCs) could stably retain the NP-like phenotype and resist mineralization after being primed with hypoxia [19]. However, pre-culturing MSCs under hypoxic conditions takes significant time and cannot be easily implemented in the clinic.

As endogenous small non-coding RNAs, microRNAs (miRNAs) bind to target messenger RNAs (mRNAs) at partially complementary sites to function as post-transcriptional repressors. Therefore, multiple growth factors can be simultaneously regulated by overexpressing or inhibiting one or more miRNAs. *In silico* predictions suggest that miR-199a could directly target Hif-1 α and it has been demonstrated that Hif-1 α expression is significantly down regulated by overexpressing miR-199a [20]. MiRNAs can be inhibited by anti-microRNA oligonucleotides (AMOs) and have been demonstrated in various cells or organisms [21]. We hypothesized that anti-miR-199a delivery would specifically inhibit endogenous miR-199a and enhance Hif-1 α expression to inhibit IVD calcification. However, there are significant concerns over the use of viral vectors for the clinical application of AMOs. We designed a hyperbranched polymer (HP) as a non-viral vector for miRNA delivery, where low molecular weight cationic polyethylenimine (PEI) molecules and short poly(ethylene glycol) (PEG) chains are attached to a hydrophobic hyperbranched molecular core. Such a novel HP vector has been shown to complex with miRNAs to form a

“double shell” structure and is highly efficient in delivering miRNA into cells with minimal cytotoxicity [22]. However, whether such an HP vector is suitable for AMOs delivery has not been tested.

In this study, HP-anti-miR-199a polyplexes were encapsulated in biodegradable poly(lactic acid-co-glycolic acid) (PLGA) nanospheres (NS), which were subsequently attached onto nanofibrous spongy microspheres (NF-SMS). We explore the possibility of using this system to sustained-release HP-anti-miR-199a polyplexes and support the NP differentiation of MSCs to regenerate IVD as well as to suppress calcification progression using three experimental models: *in vitro* culture, subcutaneous implantation, and rabbit lumbar spine injection.

Materials and Methods

Materials

Hyperbranched bis-MPA polyester (H40 with 64 hydroxyl groups), poly(ethylene glycol) (PEG, Mw 2000 Da), and polyethylenimine (PEI, Mw 800 Da and 25 kDa) were purchased from Sigma-Aldrich. Glycerol and tetrahydrofuran (THF) were purchased from Aldrich Chemical (Milwaukee, WI). L-lactide ((3s)-cis-3,6-dimethyl-1,4-dioxane-2,5-dione) was purchased from Sigma-Aldrich Inc. (St. Louis, MO) and purified by recrystallization from toluene. AIBN (2,2'-Azobisobutyronitrile) was purchased from Sigma-Aldrich Inc. (St. Louis, MO) and recrystallized from ethanol. HEMA (2-hydroxyethyl methacrylate) and Sn(Oct)₂ (stannous 2-ethylhexanoate) were purchased from Sigma-Aldrich Inc. (St. Louis, MO) and distilled under reduced pressure before use. 1,4-Dioxane was purchased from Aldrich Chemical (Milwaukee, WI) and dehydrated over molecular sieve prior to use.

Preparation of nanofibrous spongy microspheres (NF-SMS)

Poly(L-lactic acid)-graft-poly(hydroxyethyl methacrylate) (PLLA-g-PHEMA) was synthesized according to our previous publications [23, 24]. The PLLA nanofibrous spongy microspheres (NF-SMS) were prepared using PLLA-g-PHEMA and a phase-separation method modified from a previous publication [23], and are shown in Results. The non-porous control PLLA nanofibrous microspheres (NF-MS) and non-nanofibrous control PLLA solid microspheres (S-MS) were also prepared following a previously reported method [25, 26]. To visualize NF-SMS for confocal microscopy observation, NF-SMS were stained with rhodamine-conjugated BSA.

MicroRNA complexation

The synthesis of a non-viral hyperbranched polymer (HP) vector for miRNA delivery has been published [22]. The complexation of anti-miR-199a with HP vector followed a procedure reported previously [22, 27]. Briefly, aqueous HP solution (1.0 mg/mL) were added slowly to aqueous anti-miR-199a solution containing 60 pmol of anti-miR-199a, which was incubated at room temperature for 30 min to form HP-anti-miR-199a polyplexes (at a N/P ratio of 10). To visualize cellular uptake of anti-miR-199a, Cy3-labeled anti-miR-199a was purchased from Life Technologies (Mulgrave, Australia, Cat No 4464084).

Encapsulation of HP-anti-miR-199a using PLGA nanospheres

PLGA NS containing polymer/anti-miRNA polyplexes were fabricated using a water-in-oil-in-water (w/o/w) double emulsion method as reported before [22, 28]. Briefly, 30 mg PLGA (PLGA 50/50 4A, Mw=64 kDa, purchased from Evonik Industries) was dissolved in 1.0 ml of dichloromethane. One hundred microliters of aqueous HP-anti-miRNA polyplex solution with a N/P of 10 containing 1.8 nmol of anti-miR-199a was added into the above solution and emulsified with a probe sonicator at 50 W (Virsonic 100, Gardiner, NY). This primary w/o emulsion was then emulsified into 10 ml of aqueous polyvinyl alcohol (PVA, 87-89% hydrolyzed, Mw=85,000-124,000 from Sigma-Aldrich) solution (1% wt/vol) under sonication at 90 W to form the w/o/w emulsion. The resulting double emulsion was magnetically stirred at room temperature for 3 h to evaporate the solvent. The PLGA NS were collected by centrifugation and washed three times with water and freeze-dried. An HP-anti-miRNA encapsulation efficiency in PLGA NS of approximately 55% was achieved, similar to what reported previously for miRNAs [22].

Anti-miR-199a loading on to NF-SMS and its release

The PLGA NS containing HP-anti-miR-199a polyplexes were immobilized onto the NF-SMS using a post seeding method [27, 29]. Briefly, PLGA NS and NF-SMS were dispersed in hexane with 0.1% Span® 80 separately. Then the two suspensions were mixed together. After 2 h drying under vacuum, the power was subjected to vapor of a mixed solvent of hexane/THF (9:1 by volume) for 30 min. Then the NF-SMS loaded with PLGA NS (with or without miRNA) were dried under vacuum for 3 days to remove residual solvents. Approximately 70% PLGA NS loading efficiency on NF-SMS was achieved, similar to reported previously [27].

Two mg of NF-SMS containing 0.8 nmol anti-miR-199a (in HP-anti-miR-199a polyplex-containing PLGA NS) was incubated in 70% ethanol for 30 min and washed with RNase-free water three times. The NF-SMS were dispersed in 1 ml RNase-free PBS (pH 7.4, 0.1 M). The mixture was incubated at 37°C under gentle shaking at 60 rpm. At predetermined time intervals, 100 µl supernatant was collected and replaced with equivalent volume of prewarmed fresh RNase-free PBS for each sample. The released anti-miR-199a was detected by using the QuantiFluor RNA System (Promega, USA), following the manufacturer's instructions. The concentration of the samples was determined using a Varioskan Flash multimode reader using absorbance at 260 nm, which showed about 90% release by day 50.

Scanning electron microscopy (SEM)

Harvested MSC/microcarrier constructs were rinsed in PBS before being fixed with 2.5% glutaraldehyde and 2% paraformaldehyde overnight. These specimens were post-fixed with 1% osmium tetroxide, dehydrated in increasing concentrations of ethanol and then hexamethyldisilazane. They were then sputter-coated with gold and examined under an SEM (Philips XL30 FEG) at an accelerating voltage of 10 kV.

Transmission electron microscopy (TEM)

A negative staining method was used to visualize miRNA with heavy metal tungsten according to our previous publication [22]. The structure of HP-miRNA polyplexes was observed using a JEOL JEM-3011 TEM.

Cell culture and seeding

Bone marrow-derived mesenchymal stromal cells (MSCs) were obtained from rabbit femoral bone using an 18-gauge needle, following a published procedure [19, 23]. Briefly, 10 ml of marrow blood was collected into 1000 U of heparin. To remove fatty tissues and blood clots, the marrow blood was filtered through a cell strainer. After centrifuged at 600 g for 30 minutes, rabbit MSCs were collected and cultured in 75-cm² flasks with low-glucose α -MEM (Gibco), supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin G, and 0.1 mg/ml streptomycin in a humidified incubator with 5% CO₂ at 37°C.

The NF-SMS and various control microspheres were soaked in 70% ethanol for 30 min to sterilize and pre-wet them. They were subsequently washed with phosphate-buffered saline (PBS) three times (30 min each). These microspheres were finally washed with α -MEM containing 10% FBS for 30 min. 4×10^5 microspheres and 8×10^6 cells were mixed in 2 ml medium in a 15 mL centrifuge tube for 4 h (on an orbital shaker at 12 rpm). The MSC/microsphere mixture was then transferred into a spinner flask (Wheaton Industries Inc., Millville, NJ) containing 80 mL culture medium and stirred at 80 rpm. After 24 h of cell seeding, the culture medium was replaced with NP differentiation medium, which consists of MEM Alpha basic (C12571500BT, Gibco, USA), supplemented with 37.5 μ g/ml L-ascorbic acid (A5960, Sigma, USA), 0.1 μ mol/L dexamethasone (D4902, Sigma, USA), 10 ng/ml TGF- β 1 (H8541, Sigma, USA), 1 mmol/L Sodium pyruvate (P5280, Sigma, USA), 4 mmol/L L-Proline (P0380, Sigma, USA), 1% ITS Liquid Media Supplement (I3146, Sigma, USA). The NP differentiation medium was used for the remaining time, with medium changed twice a week.

RNA analysis

Cultured microcarrier/cell samples at predetermined times were homogenized using a Tissue-Tearor. RNeasy Mini Kit (Qiagen, Valencia, CA, USA) was used to isolate total RNA following the manufacturer's protocol. RNase-free DNase set (Qiagen) was used to digest DNA according to the manufacturer's instructions. TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA) were used to reverse-transcribe cDNA. The expression of mRNA was quantified using Real Time RT-PCR with TaqMan Universal PCR Master Mix (Applied Biosystems) and specific primers for collagen type II (pre-designed, assay ID Oc03396134_m1), Sox-9 (pre-designed, assay ID Oc04096872_m1), aggrecan (primer sequence 5'-gctacggagacaaggatgagttc-3' and 5'-cgtaaaagacctcaccctccat), collagen type X (pre-designed, assay ID Oc04097225_s1), and Hif-1 α (pre-designed, assay ID Oc03398626_m1). GAPDH was used as the internal standard.

Glycosaminoglycan (GAG) assay

After 3 weeks of culture, MSC/microcarrier constructs were harvested, washed with PBS, and digested using 200 μ l papain solution (280 mg/ml in 50 mM sodium phosphate,

containing 5 mM N-acetylcysteine and 50 mM EDTA, pH 6.5) at 65°C for 24 hrs. Reaction with dimethylmethylene blue (DMMB) was used to detect GAG. The GAG content of each construct was calculated from its optical density at 525 nm using shark chondroitin 4-sulfate as the standard.

DNA quantification assay

The amount of DNA was quantified using a DNA quantification kit (Sigma) following the manufacturer's protocol. Briefly, the MSC/microcarrier constructs were rinsed with PBS, homogenized in equal volumes of 1X DNA assay buffer and a lysis buffer (Sigma), and incubated for 1 h at 37°C. Cell lysate was centrifuged at 5000 g for 3 min at room temperature. The supernatant was used to determine DNA content. A fluorescence assay with Hoechst 33258 dye (Sigma) was carried out using a Varioskan Flash multimode reader (Thermo Scientific, Wyman Street Waltham, MA), where the excitation wavelength of 360 nm and emission wavelength of 460 nm were used.

Immunofluorescence staining

The MSC/microcarrier cultures were fixed with 4% formaldehyde solution and treated with 0.1% triton for 3 min. These specimens were blocked with PBS containing 2% BSA at room temperature for 20 min, incubated with HIF-1 α (c-19) primary antibody (Santa Cruz Biotechnology Inc) at 4 °C overnight. The specimens were then washed with PBS and incubated with fluorescein-conjugated secondary donkey anti-mouse IgG-FITC antibody (Santa Cruz Biotechnology Inc.) at room temperature for 45 min. Afterwards, the specimens were washed with PBS, incubated with Alexa Fluor 555 phalloidin (Life Technologies Inc) for 30 min, and washed with PBS 3 times. They were then mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Inc., Burlingame, CA). The specimens were imaged under a confocal microscope (Nikon TS-100, Tokyo, Japan).

Western blot

MSCs were washed with PBS and lysed in Cell Lysis Buffer (9803S, Cell Signaling Technology, USA). The lysates were centrifuged at 14,000 g at 4°C for 2 min, then separated by SDS-PAGE. Gels were electroblotted onto a PVDF membrane, blocked in 5% nonfat dry milk at 4°C, washed, and probed with rabbit sera at 1:500. Blots were then washed, incubated with biotinylated mouse anti-rabbit IgG or IgM, and transferred to Vectastain ABC (Vector Laboratories). β -Actin (Sigma, USA) was used as the internal standard.

Subcutaneous implantation

The rabbit MSC/HP-anti-miR-199a/NS/NF-SMS constructs were cultured in NP differentiation medium (containing 10 ng/mL TGF- β 1) for 3 weeks and then subcutaneously implanted into nude mice. We injected about 4×10^6 MSCs and 0.3 mg (2×10^5) NF-SMS with 0.12 nm HP-complexed anti-miR-199a into each subcutaneous pocket in mice. Rabbit MSC/HP-anti-miR-199a/NS/NF-SMS without TGF- β 1 induction (no NP differentiation culture) and rabbit MSC/HP-miR-NC/NS/NF-SMS with 3 weeks of NP differentiation culture (containing 10 ng/mL TGF- β 1) were used as controls. Six to eight-week-old male

nude mice (Charles River Laboratories, Wilmington, MA) were used for subcutaneous implantation. General inhalation anesthesia with isoflurane was used during the surgery. Two midsagittal incisions were made on the dorsa. On each side of an incision, one subcutaneous pocket was created using blunt dissection. One MSC/microcarrier construct with or without 3-week TGF- β 1 induction (NP differentiation culture) was implanted subcutaneously into each pocket at random, with 4 constructs per group. The incisions were closed with staples after placement of implants. Eight weeks after implantation, the mice were euthanized to harvest the implants. The animal procedures were performed according to a protocol approved by the Institutional Animal Care & Use Committee at the University of Michigan.

Luciferase reporter constructs

The WT and MUT reporter plasmids of Hif- α (WT-Hif- α ; MUT-Hif- α), miR-199a mimic and miR-NC were purchased from GenePharma (Shanghai, China). Co-transfections of Hif- α UTR plasmids with miR-199a mimic or miR-NC into the MSCs were accomplished by using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instructions.

Histological and immunohistological observation

The MSC/NF-SMS cultures were washed with PBS, fixed in 3.7% formaldehyde overnight, dehydrated using a graded series of ethanol, embedded in paraffin, and cut into 5 μ m sections. The sections were then deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E), Safranin O, or von Kossa method for histologic analysis. For immunohistochemical (IHC) analysis, deparaffinized slides were treated with papain solution for 15 min, incubated with antibodies for type II collagen, type X collagen or Hif-1 α at 1:100 dilutions for 1 hr, and then detected using a cell & tissue staining kit from R&D systems Inc. (Minneapolis, MN, USA). All IHC sections were counterstained with hematoxylin.

Degenerative disc model and miRNA delivery

The animal protocol was approved by the Institutional Animal Care & Use Committee at the University of Michigan. Forty New Zealand white rabbits (3 months old, 2.5Kg, male) were randomly divided into 4 groups (n=10). Rabbits were under anesthesia and placed on fluoroscopy imaging table in a lateral position. An 18-gauge spinal needle was inserted into the IVD under C-arm image guidance using a posterolateral approach at a location 1-2 cm lateral to the midline spinous process and an angle between 30 and 45 degrees. Without touching the vertebral body (to avoid osteophyte formation), the needle was carefully advanced to the lateral margin of the IVD space and gently inserted into the disc space (Supplementary Fig S1). In order to place the needle into the center of the IVD, anteroposterior and lateral views from the C-arm projection were used to guide the procedure. Once the needle was verified in the IVD, about 0.005 to 0.008 g NP was aspirated to induce disc degeneration (Supplementary Fig S1A). Under dissecting microscope, the aspiration was carefully examined to confirm only nucleus pulposus was

aspirated. Four weeks after the first surgery (to create the degenerative model), a cannula (3 mm in diameter) guided by C-arm was placed on the margin of the aimed intervertebral disc. Then an extended needle (25G, 0.5 mm in diameter) was inserted into the disc space along the guided cannula. The MSC/HP-anti-miR-199a/NS/NF-SMS suspension (1×10^6 MSCs and 0.075mg (0.5×10^5) NF-SMS with 0.03 nm HP-complexed anti-miR-199a NS, totally 20 μ l in volume) or a control treatment was injected with a 25G needle (Supplementary Fig S1B). After that, the needle was removed. The gelfoam particles loaded in another syringe were injected along the cannula to fill the puncture hole to prevent leakage (Supplementary Fig S1C). Supplementary Fig S1D shows the syringe needle with degeneration model, where the cannula was not shown in the Fluoroscopy. The injection site was carefully examined. If any leakage was found during the procedure, the animal would be removed from the experimental group.

Radiographic analysis

Radiographs were taken every 2 weeks up to 12 weeks after operation. The same level of anesthesia was carefully maintained during radiography to achieve a similar degree of muscle relaxation. Radiographs were repeated twice for each animal to avoid axial rotation of the spine and decrease the error from beam divergence. ImageJ software (NIH, Bethesda, MD) was used to analyze radiographs. Following a published method [27], disc height index (DHI) was determined for every animal. The DHI changes were expressed as %DHI that was normalized to the measured preoperative IVD height ($\%DHI = \text{postoperative DHI} / \text{preoperative DHI} \times 100$).

Micro-computed tomography (μ CT) analysis

The bone microarchitecture of the lumbar vertebrae specimens was scanned using a SkyScan 1076 Micro-CT (SkyScan, Aartselaar, Belgium) with a resolution of 36 μ m per voxel. The volume of interest of the vertebrae was restricted to an inner cylinder of 4.5 mm in diameter and 6 mm in length (1 to 4 mm proximal to the growth plate) in the cephalad level, excluding the cortex. All 3D image manipulations and analyses were performed using the Mimics v10.01 software (Materialise, Leuven, Belgium). In order to evaluate the bone reconstruction of vertebral body, osteophyte volume was separated by fixed threshold, and the bone volume (BV, mm³) before and after operation was obtained by BoneJ plug-in of ImageJ software. Finally, osteophyte bone volume was obtained by subtracting preoperative bone volume from postoperative bone volume.

IVD histological grading and endplate calcification evaluation

The histological grading system was devised by Masuda et al [30]. Briefly, the slides were graded based on the histological appearance of 4 parameters: the annulus fibrosus, the border between the annulus fibrosus and the nucleus pulposus, the cellularity of the nucleus pulposus, and the matrix of the nucleus pulposus. Each of the 4 parameters was given a grade of 1, 2, or 3. The sum of the grades for each parameter yielded a total grade. Total grades ranged from 4 to 12, with 12 representing severe degeneration.

In HE images, the boundaries of bony endplate (BEP) and internal non-mineralized areas (pore space) were identified from the anterior, central and posterior areas of slides. ImageJ software was used to quantify the ratio of pore space area (PA) to total bony endplate. The percentage of calcification area/endplate ratio was calculated as $(1-PA/BEP) \times 100\%$. An average of three measurements were made from each area on the HE slides.

Statistical analysis

All data were expressed as mean $\pm$ SD and analyzed using SPSS 15.0 software (SPSS, Chicago, IL, USA). The Shapiro-Wilk test for normality and Bartlett test for homogeneity of variance were performed. T-test was used to determine the statistically significant differences. The results of the histological and radiographical scores were analyzed using the one-way ANOVA test with Tukey's post hoc test. Significant difference was established at $p < 0.05$.

Results

MSC attachment to NF-SMS

PLLA solid microspheres (S-MS) without the NF structure (Fig. 1A), nanofibrous microspheres (NF-MS) without inter-connected large pores (Fig. 1B), and nanofibrous spongy microspheres (NF-SMS) containing inter-connected large pores throughout the microspheres (Fig. 1C) were fabricated, all with an average diameter between 30 and 60 μm . The average diameter of the inter-connected pores was approximately 15 μm and the openings between pores were about 10 μm in diameter. NF-SMS were composed of ECM-mimicking nanofibers throughout. Because of the simultaneous presentation of highly inter-connected pores and biomimetic nanofibers, NF-SMS were hypothesized to facilitate MSC seeding and attachment. S-MS and NF-MS were used as control microcarriers to test our hypothesis. After 24 h of cell seeding, the lowest number of rabbit MSCs attached on S-MS with a flatter morphology and spread over a large area on S-MS (Fig. 1D). More MSCs attached to the external surface of NF-MS than on S-MS (Fig. 1E). The highest number of MSCs attached to both the interior and exterior pores of the NF-SMS (Fig. 1F).

Nucleus pulposus (NP) tissue formation on various microspheres *in vitro*

Safranin-O staining was performed to examine the NP-like ECM deposition on the three types of microspheres after 3 weeks of culture with MSCs. More intense and uniform safranin-O staining in the NF-SMS group revealed more GAG deposition on NF-SMS than on the control microcarriers (Fig. 2A-F). Cells proliferated faster on NF-SMS than on the two control microcarriers based on DNA quantification (Fig. 2G). Importantly, there were significantly higher GAG/DNA ratios in the NF-SMS group than in the two control microcarriers at both 2 and 4 weeks (Fig. 2H). We further assessed the effect of different microcarriers on the expression of NP ECM-associated genes (Sox-9, aggrecan and type II collagen). These genes were upregulated in the MSCs cultured on NF-SMS compared to those on NF-MS and S-MS controls (Fig. 2I-K).

Two-stage anti-miR-199a release to enhance NP-like differentiation *in vitro*—

Because the *in vitro* results have demonstrated that NF-SMS enhanced NP-related gene

expression and NP-associated ECM secretion by MSCs, we selected NF-SMS as the microcarrier for cells and anti-miR-199a in subsequent studies. To sustain and efficiently deliver anti-miR-199a, HP-anti-miR-199a polyplexes were prepared (Fig. 3 A-C, D-i) and encapsulated in PLGA nanospheres (NS) with an average diameter of 300 nm (Fig. 3C & D-ii) via a double emulsion technique similarly to that reported earlier [22]. The PLGA NS (encapsulating HP-anti-miR-199a polyplexes) were subsequently attached to NF-SMS (Fig. 3C, D-iii&D-iv) using a similar process previously reported [22, 27]. The NF morphology that facilitates NP differentiation and the inter-pore connectivity that promotes cell migration were retained after PLGA NS immobilization. In order to visualize the transfection of HP-anti-miR-199a into MSCs, Cy3-labeled anti-miR-199a (orange red) was used in this experiment. DAPI was used to stain cell nuclei (blue). After transfection for 48 hours, confocal microscopic examination showed significantly higher transfection efficiency in the HP-anti-miR-199a polyplex group than in the anti-miR-199a alone group (Fig 3. E). Hif-1 α protein in MSCs cultured on NF-SMS with anti-miR-199a or miR-199a were visualized using immunofluorescence staining. Hif-1 α staining was enhanced in the anti-miR-199a group and reduced in the miR-199a group (Fig 3F).

Subcutaneous implantation of MSC/HP-anti-miR-199a/NS/NF-SMS constructs

After being cultured in NP differentiation medium with TGF- β 1 *in vitro* for 3 weeks, rabbit MSC/HP-anti-miR-199a/NS/NF-SMS constructs were implanted subcutaneously into nude mice. Eight weeks after implantation, the constructs were harvested and examined for the anti-miR-199a effect on NP-like phenotype and mineralization *in vivo* (Fig. 4). Rabbit MSC/HP-anti-miR-199a/NS/NF-SMS constructs without TGF- β 1 induction and rabbit MSC/HP-miR-NC/NS/NF-SMS constructs with TGF- β 1 induction were used as control groups. GAG was strong (SO staining, Fig. 4A) and Hif-1 α (Fig. 4B) was positive in the MSC/HP-anti-miR-199a/NS/NF-SMS constructs with TGF- β 1 induction. Importantly, they were stained negative for hypertrophy (Col X, Fig. 4B) and negative for mineralization (von Kossa, Fig. 4A). Implanted MSC/HP-anti-miR-199a/NS/NF-SMS constructs without TGF- β 1 induction were stained negative for GAG (SO staining, Fig. 4A), hypertrophy (Col X, Fig. 4B) or mineralization (von Kossa, Fig. 4A). On the contrary, implanted MSC/HP-miR-NC/NS/NF-SMS constructs were stained positive for hypertrophy (Col X, Fig. 4B) and contained large areas of mineralization (von Kossa staining, Fig. 4A). These results suggest that TGF- β 1 is important for all NP, chondrogenic and osteogenic differentiation but eventually lead to hypertrophy and mineralization without anti-miR-199a. Taken together, anti-miR-199a plays an important role in maintaining the NP-like phenotype (SO, Col II, Hif-1 α) and preventing hypertrophy (Col X) and mineralization (von Kossa) *in vivo*.

Anti-miR-199a targets Hif-1 α to functionally inhibit calcification

In order to gain mechanistic understanding of how anti-miR-199a regulates MSC calcification activity, we used miRBase (the miRNA data base at <http://www.mirbase.org>) to predict potential miR-199a targets. As one of the predicted target genes, Hif-1 α has a miR-199a-binding site in its 3' untranslated region (UTR). Enhancing Hif-1 α is known to significantly upregulate Sox-9.

To test whether miR-199a binds to Hif-1 α , the effects of miR-199a and its negative control (miR-NC) on Hif-1 α mRNA expression and protein level in rabbit MSCs were analyzed. Hif-1 α protein was downregulated by miR-199a, but the Hif-1 α mRNA was not affected by miR-199a (Fig. 5A). To test whether miR-199a targets Hif-1 α directly, a luciferase reporter with a wild-type (WT) Hif-1 α 3'UTR and a luciferase reporter with a mutated Hif-1 α 3'UTR sequence of the miR-199a-binding site were constructed (Fig. 5B). It was found that miR-199a substantially inhibited the luciferase reporter activity of the WT Hif-1 α 3'UTR, but not that of the mutated Hif-1 α 3'UTR (Fig. 5C). To examine how Hif-1 α regulates the calcification activity of MSC, Hif-1 α expression was silenced by a Hif-1 α specific siRNA (siR-Hif-1 α). The intracellular Hif-1 α protein level was substantially downregulated by siR-Hif-1 α compared to its negative control (siR-NC; Fig. 5D). In response to the reduced Hif-1 α protein level, Sox-9 and Col II protein levels were substantially downregulated. In contrast, Col X protein levels were significantly upregulated (Fig. 5E). To investigate whether miR-199a functionally targets Hif-1 α to regulate calcification, a Sox-9 specific siRNA (siR-Sox9) was used to knock down Sox-9 expression (Fig. 5F). The differences in Col X and Col II mRNA levels in response to miR-199a, anti-miR-199a and NC treatments substantially diminished by knocking down Sox-9 expression (Fig. 5G). Sox-9 and Col II protein levels were enhanced by anti-miR-199a and reduced by the miR-199a, which is consistent with the mRNA expression changes. Col X protein was reduced by the anti-miR-199a and enhanced by the miR-199a (Fig. 5H). Mechanistically, Hif-1 α inhibition by miR-199a was subsequently reduced by anti-miR-199a treatment, leading to the enhanced Sox-9 expression, which is known to inhibit calcification (Fig. 5I).

Therapeutic efficacy of MSC/HP-anti-miR-199a/NS/NF-SMS injection into IVD—

The mechanistic understanding of the role of anti-miR-199a in enhancing NP differentiation and inhibiting mineralization of MSCs led to the hypothesis that controlled release of anti-miR-199a may enhance NP regeneration and suppress mineralization in/around IVD environment *in situ*. To test this hypothesis, MSC/HP-anti-miR-199a/NS/NF-SMS constructs were injected into the rabbit IVD to treat the needle puncture-induced lumbar disc degeneration model (Supplementary Fig. S1). Six-weeks after treatment, samples from experimental and various control groups were collected and their gross appearance and x-ray images were used to assess the therapeutic efficacy (Fig. 6). In the sham group (no treatment after puncture), the NP nearly disappeared entirely, ectopic bone formation was found surrounding the IVD space (Fig. 6B, F). Notably, the MSC/HP-anti-miR-199a/NS/NF-SMS treatment group showed translucent disc matrix similar to normal disc in gross appearance (Fig. 6D). Consistently, the disc height was greater in the MSC/HP-anti-miR-199a/NS/NF-SMS experimental group than in the sham group (Fig. 6H). Based on both x-ray images and gross appearance, the discs in the MSC/NF-SMS control group were similar to the MSC/HP-anti-miR-199a/NS/NF-SMS treatment group. However, the average disc space in the MSC/NF-SMS group was found to be narrower than those in the MSC/HP-anti-miR-199a/NS/NF-SMS treatment group.

The environment in/around NP tissues are different from that in subcutaneous implantation site. The high vascularity and low/no Hif-1 β in the subcutaneous environment are anticipated to enhance mineralized bone formation. However, even in such environment, we

demonstrated that anti-miR-199a suppressed hypertrophy and mineralization. We therefore would anticipate anti-miR-199a delivery would suppress mineralization in the NP environment. To test this hypothesis, we subsequently evaluated mineralized bone formation around NP (osteophyte and end plate mineralization) and disc function in response to MSC/anti-miR-199a delivery.

Three-dimensional (3D) reconstruction of cross-sectional μ CT scans of rabbit lumbar spine confirmed the complete absence of heterotopic ossification in the normal control group. There was significantly smaller heterotopic ossification area in the MSC/HP-anti-miR-199a/NS/NF-SMS treatment group than those in the sham or MSC/NF-SMS control groups (Fig. 7A&B). The disc height index (DHI) of each group was recorded for 12 weeks (Fig. 7C). The DHI of the MSC/HP-anti-miR-199a/NS/NF-SMS group was lower than but closest to that of the normal control group during the entire time period. The DHI of the MSC/NF-SMS group was lower than that of MSC/HP-anti-miR-199a/NS/SMS group, while it was still substantially greater than that of the sham group (Fig. 7C).

The therapeutic efficacy was further evaluated using histological analysis 6 weeks after MSC/HP-anti-miR-199a/NS/NF-SMS injection (Fig. 8). In the normal control group, the large volume of the oval-shaped NP was shown in the midsagittal cross-section (Fig. 8A). The strong safranin-O staining indicated a high GAG content in the NP area (Fig. 8E, I). In the sham group, the disc space collapsed with evident fibrous tissue invasion (Fig. 8B, F, J). Although discs in the MSC/HP-anti-miR-199a/NS/NF-SMS group still displayed a certain extent of degenerative features (Fig. 8D, H, L), the therapeutic efficacy was obvious as compared to the sham group. The NP area in this treatment group was similar to the normal control and stained strongly with safranin-O (Fig. 8H, L). In the MSC/NF-SMS group, the NP was similar in volume to MSC/HP-anti-miR-199a/NS/NF-SMS treatment group (Fig. 8C, H&E), but was stained weaker by safranin-O (Fig. 8G, K) as compared to MSC/HP-anti-miR-199a/NS/NF-SMS treatment group. The thickness of bony endplate (BEP) and the calcification area were significantly higher in the sham group (Fig. 8N) and MSC/NF-SMS group (Fig. 8O) compared to the MSC/HP-anti-miR-199a/NS/NF-SMS treatment group (Fig. 8P).

The histological appearance was semi-quantitatively assessed 12 weeks after treatment using a published method [31, 32]. Briefly, the total grade ranges from 4 to 12, where 4 represents normal tissue appearance and 12 represents severe degeneration. The histological grade of the MSC/HP-anti-miR-199a/NS/NF-SMS treatment group was substantially lower than those of the MSC/NF-SMS group and sham group (Fig. 8Q). The relative area of endplate calcification was also significantly lower in the MSC/HP-anti-miR-199a/NS/NF-SMS treatment group than in either the sham group or MSC/NF-SMS control group (Fig. 8R).

Discussion

We aimed to design an injectable scaffold to regenerate NP tissue as well as to sustained release biomolecules to suppress calcification in the pathological IVD environment. Our results clearly showed that NF-SMS were advantageous in providing an environment for MSC seeding, proliferation, and differentiation towards NP phenotype. When combined

with sustained release of anti-miR-199a, MSCs were able to acquire an NP-like phenotype even in a subcutaneous *in vivo* environment. Furthermore, the percutaneous injection of MSC/HP-miR-199a/NS/NF-SMS constructs could not only regenerate NP tissue in the lumbar spine of rabbits, but also repress the calcification progression around the IVD.

Previous studies indicate the importance of 3D culture environment in maintaining round cell morphology and the phenotypic stability of NP cells [19, 33, 34]. To serve the role of an artificial extracellular matrix (ECM), a scaffolding material is often advantageous when it presents characteristic features of the natural ECM [35]. Hydrogels are widely utilized to support 3D NP cell cultures [36, 37], but this far have not reached clinically relevant IVD regeneration. More hydrogels are being explored for NP tissue engineering. Nanofibrous scaffolds in both an implantable predesigned 3D form [38-40] and an injectable microcarrier form [25, 26] made of various polymers or macromolecules and fabricated using varying techniques have been shown to promote the differentiation of mesenchymal progenitor/stem cells into a variety of mesenchymal cell types, such as osteoblasts [29, 41-45], odontoblasts [46, 47], chondrocytes [26, 48], and NP cells [19, 32]. Novel nanofibrous spongy microspheres (NF-SMS) with an interconnected pore structure have recently been developed in our lab to integrate nanofibrous (NF) structure and a highly porous microstructure [25]. On the nano-scale, NF structure mimics the structure of ECM protein fibers and has been shown to facilitate the attachment, proliferation, and NP-like MSC differentiation. On the micro-scale, the interconnected pores are required to efficiently accommodate cells and facilitate mass transfer, and new ECM formation. On the macro-scale, NF-SMS are injectable to enable minimally invasive cell delivery [26]. These multi-scale physical characteristics of NF-SMS are highly desirable for MSC delivery and NP regeneration. Indeed, the highly porous structure of the NF-SMS used in this study shows enhanced MSC proliferation and differentiation in comparison to less porous or non-fibrous control microspheres (NF-MS and S-MS). However, nanofibrous scaffolds alone may not be sufficient to specify ultimate fate of tissue regeneration, where other *in vitro* and *in vivo* microenvironmental contributions such as culture medium, growth/differentiation factors, coculture, host cells, vasculature, ECM, their biomechanical properties and so on also play important roles.

Calcification of IVD is frequently associated with aging and disease [7, 14]. Inflammatory cytokines secreted in disc tissues are known to promote osteoprogenitor differentiation and angiogenesis, which eventually activate ectopic ossification. Based on previous studies and the *in vitro* data from the current study, we hypothesized that enhanced Hif-1 α expression achieved by continuously releasing anti-microRNA oligonucleotide (AMO) from HP-AMO/NS/NF-SMS constructs could promote the differentiation of MSCs towards NP phenotype and prevent pathophysiologic ossification. Our results demonstrated that sustained release of anti-miR-199a (AMO) resulted in abundant GAG and type II collagen production *in vivo*, which was accompanied by continuous Hif-1 α expression. In contrast, the constructs without anti-miR-199a release system led to upregulation of type X collagen and ossification. In addition, rabbit lumbar degeneration model study indicated that suppression of miR-199a repressed the progression of calcification around the IVD area.

The anti-miR-199a delivered by the novel HP/NS/NF-SMS platform acts as an antagonist to block the complementary miR-199a. Previously, miR-199a was shown to be a negative regulator of MSC chondrogenesis and a positive regulator for MSC osteogenesis [20]. Based on mechanistical investigation in cell culture, we found overexpression of miR-199a to enhance calcification through functionally targeting Hif-1 α to suppress Sox9 activity. Blocking miR-199a function using highly potent and specific anti-miR-199a led to upregulation of Hif-1 α . Hif-1 α is known to bind to and activate Sox9 expression, enhancing transcriptional activity during MSC chondrogenesis [17, 49, 50]. Hif-1 α is also known to downregulate Runx2 via TWIST to inhibit MSC osteogenesis [51].

While we have identified anti-miR-199a as a potential target to enhance NP differentiation and suppress mineralization, there are concerns over the use of viral vectors for therapeutic nucleic acid delivery. Liposome-based vectors can efficiently deliver miRNA into cells [52, 53]. However, they are not stable *in vivo* or under strong mechanical forces for encapsulation [22]. Although PEI is a widely utilized polymeric non-viral delivery vehicle for miRNA, low molecular weight PEI is low in transfection efficiency and high molecular weight PEI is toxic to cells [54]. Our novel hyperbranched polymer (HP) vector has been demonstrated to be highly efficient in delivering miRNA with negligible cytotoxicity [22] and is confirmed to efficiently deliver anti-miR-199a into MSCs in this study. Furthermore, because of its stability, HP (complexed with miRNAs) can be encapsulated in biodegradable polymer nanospheres to achieve sustained release over a long period of time [22].

Stem cells and miRNAs play important roles in IVD and other degenerative diseases [52, 55-57]. Simultaneous delivery of appropriate stem/progenitor cells and target miRNAs or their antagonists is likely key to develop therapeutics to treat IVD degeneration. An injectable cell microcarrier integrated with a highly efficient non-viral vector such as the system developed in our laboratory may find applications for a broad range of miRNA-based therapeutics for IVD and many other degenerative diseases.

Taken together, the MSC/HP-anti-miR-199a/NS/NF-SMS combination integrated multiple advantageous features of the components. HP vector enables highly efficient non-viral delivery of anti-miR-199a into MSCs and PLGA NS enables controlled long-term therapeutic delivery. The NF-SMS not only serve as an advanced injectable scaffold for MSCs, but also provide high porosity to minimize potential acidity due to polymer degradation. In addition, NF-SMS enables the localization of therapeutic delivery as demonstrated previously [27]. The novel delivery platform allowed us to test the hypothesis that sustained anti-miR-199a release could sufficiently regulate Hif-1 α -mediated MSC differentiation, suppress ossification and enhance NP tissue regeneration in a rabbit IVD degeneration model. The exciting outcome warrants further investigations to optimize the delivery and NP regeneration using clinically more relevant larger animal models such as pigs, goats, sheep, canine, or monkeys [58-60].

Conclusions

In conclusion, NF-SMS presented a microenvironment to facilitate uniform MSC accommodation, proliferation, and NP-like tissue regeneration. Supported with the injectable

NF-SMS and the controlled anti-miR-199a delivery, MSCs were successfully utilized to regenerate an NP-like tissue graft *in vitro*. This system resists calcification *in vivo* and regenerates NP in rabbit lumbar spine *in situ*. The mechanistic studies suggest that the sustained *in situ* release of anti-miR-199a suppressed miR-199a, which in turn enhanced Hif-1 α and Sox-9 activities, leading to the suppression of calcification and promotion of NP regeneration. Thus, the advanced NF-SMS in combination with the sustained anti-miR-199a release system enable a very promising novel approach to clinically relevant NP regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1. SEM images of different microcarriers and rabbit mesenchymal stromal cell (MSC) attachment.

(A) solid microspheres (S-MS); (B) nanofibrous microspheres (NF-MS); (C) Nanofibrous spongy microspheres (NF-SMS). All the above microcarriers have a diameter ranging from 30 to 60 μm (Scale bars for A-C: 20 μm). MSCs were seeded on the three types of microspheres for 24h: (D) MSCs seeded on S-MS surface and spread over a large area; (E) MSCs seeded on the exterior of NF-MS; (F) MSCs seeded on both the exterior and the interior of the NF-SMS. (Scale bars for D-F: 10 μm).

Fig 2. NF-SMS provided a supportive microenvironment for nucleus pulposus-like differentiation of rabbit MSCs.

(A-F) Safranin-O staining demonstrating GAG accumulation. Both NF-SMS and NF-MS were positively stained by safranin-O for GAG, while a weak staining was found in S-MS group. (G) Proliferation curves of MSCs cultured with NF-SMS, NF-MS, and S-MS, quantified using DNA content at each predetermined time. (H) GAG/DNA ratio in the NF-SMS, NF-MS, and S-MS groups. (I-K) After 2 and 4 weeks of induction, real time RT-PCR analysis showed different expression levels of nucleus pulposus-associated genes (Sox-9,

type II collagen and aggrecan) in MSCs on different microcarriers (NF-SMS, NF-MS and S-MS). For all analyses, n=3, * indicates $p<0.05$.

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Fig 3. Two-stage delivery of anti-miR-199a.

(A-C) The HP-anti-miR-199a polyplexes were prepared, encapsulated in PLGA nanospheres (NS) via a double emulsion method, and then attached onto the PLLA NF-SMS. (D-i) TEM image showing anti-miRNA distribution (the darker rings): Anti-miRNA complexed with the positively charged outer shell of HP molecular core, the HP-anti-miRNA polyplexes self-assembled into a larger nanosized spherical shell sandwiched between an inner PEG core and an outer PEG layer. (D-ii) SEM image of PLGA NS that contain HP-anti-miRNA polyplexes. (D-iii) SEM image of a part of an NF-SMS. (D-iv) SEM image of a part of an NF-SMS with immobilized PLGA NS. (E) Confocal images of cells transfected for 2 days with Cy3-labeled anti-miR-199a (orange red) and DAPI stained nuclei (blue). A significantly higher transfection efficiency was shown in the HP-anti-miR polyplex group than in the anti-miR alone group. (F) Immunofluorescence staining for Hif-1 α in MSCs cultured on NF-SMS with HP-carried anti-miR-199a, miR-199a, or control. Green: Hif-1 α ; red: NF-SMS. Enhanced HIF-1 α staining was seen in the anti-miR-199a group, while decreased Hif-1 α staining was seen in the miR-199a group. All scale bars in E&F are 50 μ m.

A) Histological Analysis

B) Immunohistochemical Analysis

Fig 4. Histological and immunohistochemical analyses of subcutaneous implanted constructs. Rabbit MSC/HP-anti-miR-199a/NS/NF-SMS were induced by TGF- β 1 for 3 weeks and then implanted into subcutaneous pockets of nude mice ($n=4$). Note: HP and NS/NF-SMS are omitted in the images to simplify the labels). Rabbit MSC/HP-anti-miR-199a/NS/NF-SMS without TGF- β 1 induction and rabbit MSC/HP-miR-NC/NS/NF-SMS with TGF- β 1 induction were used as controls. **A) Histological Analysis:** After 8 weeks of implantation, the rabbit MSC/HP-anti-miR-199a/NS/NF-SMS constructs with TGF- β 1 induction were stained positive by safranin-O and negative by von Kossa. In contrast, rabbit MSC/HP-anti-miR-199a/NF-SMS constructs without TGF- β 1 induction were stained negative by either

safranin-O or von Kossa, while rabbit MSC/HP-miR-NC/NS/NF-SMS constructs with TGF- β 1 induction were stained positive by both safranin-O and von Kossa methods. **B)**

Immunohistochemical Analysis: Eight weeks after implantation, the rabbit MSC/HP-anti-miR-199a/NS/NF-SMS constructs with TGF- β 1 induction were stained positive by type II collagen and Hif-1 α antibodies, but stained weakly by type X collagen antibody. In MSC/HP-miR-NC/NS/NF-SMS with TGF- β 1 induction group, the constructs were stained positive by type X collagen antibody but negative by either type II collagen or Hif-1 α antibodies. Rabbit MSC/HP-anti-miR-199a/NS/NF-SMS constructs without TGF- β 1 induction were stained negative by type II collagen, type X collagen and Hif-1 α antibodies.

Fig 5. Anti-miR-199a targets Hif-1α to functionally inhibit calcification.

(A) The Hif-1α gene expression (upper panel) and protein level (lower panel) in rabbit MSCs in response to the treatment of either miR-199a or its negative control (miR-NC). (B) Schematic illustration of designing the luciferase reporters with the WT Hif-1α 3'UTR (Hif-1α 3'UTR WT) or the site-directed mutant Hif-1α 3'UTR (Hif-1α 3'UTR mutated (MUT)). (C) The effect of miR-199a or miR-NC on luciferase activity in either the Hif-1α 3'UTR WT or the Hif-1α 3'UTR MUT transfected MSCs. (D) The Hif-1α gene expression (upper panel) and the Hif-1α protein level (lower panel) in MSCs after silencing Hif-1α with a Hif-1α-specific siRNA (siR-Hif-1α). (E) Western blot analysis of Sox9, Col-II and

Col-X proteins in MSCs after siR-Hif-1 α or its NC treatment for 1 week. (F) The Sox9 gene expression (upper panel) and Sox9 protein level (lower panel) in MSCs after treatment with a Sox9-specific siRNA (siR-Sox9). (G) Real-time RT-PCR analysis of the mRNA levels of Col II and Col X in MSCs after silencing Sox-9 with siR-Sox9 and being treated with miR-199a, anti-miR-199a, or their corresponding NCs. (H) Western blot analyses of Sox-9, Col II and Col X protein contents in MSCs treated with miR-199a, anti-miR-199a, and their corresponding NCs. (I) Schematic diagram of calcification activity mediated by anti-miR-199a via modulating Sox-9 signaling pathway in MSCs. Intracellular miR-199a level was suppressed by anti-miR-199a. Subsequently, Hif-1 α activity was enhanced by reduced miR-199 expression, and therefore to promote Sox-9 activity, which resulted in inhibition of calcification. * $p < 0.05$. Triplicates were analyzed for all experiments, $n = 5$ for every group. Data are expressed as mean $\pm$ SD.

Fig 6. Representative gross images (A-D) and fluoroscopy images (E-H) of rabbit lumbar spines at 6 weeks after *in vivo* gene therapy.

In the sham group (punctured without treatment), the disc space nearly completely disappeared in the central area of the NP. Ectopic bone tissue formed as indicated by high intensity shadows in X-ray images in the sham group. Regeneration characteristics were found in the discs treated with MSC/HP-anti-miR199a/NS/NF-SMS, showing higher disc heights as compared to the sham group. Notably, this treatment group also showed more regular disc space with matrix translucency in gross appearance and less ectopic bone formation (observed under X-ray) compared to the sham group and MSC/NF-SMS groups.

Fig 7. NP regeneration and disc height maintenance after different treatments.

A) Three-dimensional reconstructions of μ CT scans of rabbit lumbar spines and representative cross-sections 6 weeks after *in vivo* MSC/HP-anti-miR-199a/NS/NF-SMS treatment along with three control groups. B) Quantification of osteophyte bone volumes after different treatments. C) The disc height index (DHI) changing over time. * $p < 0.05$, ** $p < 0.01$ for comparison against MSC/HP-anti-miR-199a/NS/NF-SMS group.

Fig 8. HE and safranin-O (SO) staining of disc samples of different treatment groups 6 weeks after injection (A-P):

In the normal control group (A, E, I, M), the volume of the oval-shaped NP was large as shown in the midsagittal cross-section of the HE staining (A), the safranin-O staining in the NP area was strong (E&I). In the sham group (B, F, J, N), there was minimal disc space (B), substantial fibrous tissue invasion and weak GAG staining (F&J). In the discs treated with MSC/HP-anti-miR-199a/NS/NF-SMS (D, H, L, P), the NP area was similar to that in the normal control and safranin-O staining was similarly strong. In the MSC/NF-SMS group (C, G, K, O), the NP area was stained positively by safranin-O, but the staining intensity was

weaker than MSC/HP-anti-miR-199A/NS/NF-SMS group. The bony endplate (BEP) thickness and calcification were substantially greater in the sham group (N) and MSC/NF-SMS (O) group compared to the MSC/HP-anti-miR-199a/NS/NF-SMS group (P).

Assessment of histological appearance and calcification (Q&R): The histological appearance of the treated discs was semi-quantitatively scored 12 weeks after treatment (Q). The calcification area/endplate area ratio was quantified at 12 weeks after treatment (R).

Scale bar: 200µm for A-H & M-P, 500 µm for I-L. ** $p < 0.01$.