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ORIGINAL RESEARCH

Ectopic chondrogenesis of nude mouse induced by nano gene delivery enhanced tissue engineering technology

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Background: Many techniques and methods have been used clinically to relieve pain from cartilage repair, but the long-term effect is still unsatisfactory.

Purpose: The objective of this study was to form an artificial chondroid tissue gene enhanced tissue engineering system to repair cartilage defects via nanosized liposomes.

Methods: Cationic nanosized liposomes were prepared and characterized using transmission electron microscope (TEM) and dynamic laser light scattering (DLS). The rat mesenchymal stem cells (rMSCs) were isolated, cultivated, and induced by SRY (Sex-Determining Region Y)-Box 9 (Sox9) via cationic nanosized liposomes. The induced rMSCs were mixed with a thermo-sensitive chitosan hydrogel and subcutaneously injected into the nude mice. Finally, the newly-formed chondroid tissue obtained in the injection parts, and the transparent parts were detected by HE, collagen II, and safranin O.

Results: It was found that the presently prepared cationic nanosized liposomes had the diameter of 85.76±3.48 nm and the zeta potential of 15.76±2.1 mV. The isolated rMSCs proliferation was fibroblast-like, with a cultivated confluence of 90% confluence in 5–8 days, and stained positive for CD29 and CD44 while negative for CD34 and CD45. After transfection with cationic nanosized liposomes, we observed changes of cellular morphology and a higher expression of SOX9 compared with control groups, which indicated that rMSCs could differentiate into chondrocyte in vitro. By mixing transfected rMSCs with the thermo-sensitive hydrogel of chitosan in nude mice, chondroid tissue was successfully obtained, demonstrating that rMSCs can differentiate into chondrogenic cells in vivo.

Conclusion: This study explored new ways to improve the quality of tissue engineered cartilage, thus accelerating clinical transformation and reducing patient pain.

Keywords: Sox9, chondrogenesis, gene enhanced tissue engineering, transfection, chondroid

Introduction

Cartilage injury, a common clinical problem, can be caused by trauma,[1] osteoarthritis,[2] rheumatoid arthritis,[3] and exfoliative chondritis.[4] Articular cartilage is a non-vascular tissue, whose regeneration ability is extremely limited.[5,6] Most believe that the damage of mature articular cartilage is partial or superficial, and cartilage cannot regenerate itself.[7–12] When the lesion is associated with the opening of the subchondral bone, cartilage repair can be completed because of the cells derived from bone marrow and blood vessels of subchondral bone.[13–15] Even so, the repair cartilage has not been found to be the same as normal cartilage structure and mechanical properties, except embryonic stem cells.[16,17] To date, no satisfactory method resulted in damaged cartilage

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repairing clinically, except when using autologous or xenogenous engrafting.[18,19] While autologous cartilage presents as feasible to graft, it is hard to obtain adequate matches.[20–22] Furthermore, all these techniques are subject to the donor site availability.[23,24] Obtaining hyaline cartilage bio-tissue is a pivotal and serious way for cartilage repair in clinical therapy.

As a natural polysaccharide polymer, chitosan has a homogeneous structure with glucosamine, which is one of the main components of the cartilage matrix, and is increasingly used in cartilage tissue engineering research.[25,26] For the hydrogel to carry seeded, chitosan has been receiving even more attention.[27,28] The chitosan hydrogel can be prepared via the interaction of ions or glutaraldehyde crosslinking,[29] and its azide derivatives could also be crosslinked under UV irradiation to fabricate hydrogel.[30–34] Therein, ions are often reacted in hydrogels.

Thermo-sensitive hydrogels made of chitosan own several attractive properties, including mild preparation conditions, a phase transition temperature and body temperature closed to or even lower than the body's temperature, and good biocompatibility and biodegradability.[35–37] Thus, chitosan has great application potential for tissue engineering and drug controlled-release carrier applications. Based on the physical and chemical as well as biological properties, chitosan can satisfy cell growth, proliferation, and differentiation, to be used as an injectable tissue engineering scaffold material.[38–40]

Tissue engineering technology, which can grow a mature tissue in vitro, is now extensively used in medicine.[41,42] A cartilage defect area repaired with tissue engineering technology has achieved a certain effect in vivo and promotes cartilage growth.[43,44] Meanwhile, engineered repair can provide the appropriate growth space for repair cells, quickly fill the cartilage defect area, prevent unwanted fiber tissue growth,[45] and provide certain biomechanical support for the early repair.[46,47] Chondrocytes and bone marrow stromal stem cells are commonly used to construct the cartilage.

However, tissue engineering technology also has its limitations, such as limited cell sources, and it is easy to cause the defects on such materials. In contrast, gene-enhanced tissue engineering technology has significantly more hope for cartilage repair.[48,49] To solve these problems, this study explored a new therapeutic approach for the treatment of cartilage defects using bone marrow stromal stem cells. Compared to the viral vectors, non-viral systems haveattracted significant attention to the cost-effectiveness and less induction of the immune system in gene delivery. A lot of nano non-viral gene vectors have been reported, which include polymer (PAMAM,[50,51] PEI[52]), protein,[53] liposomes,[54,55] and so on, due to their low toxicity, antigenicity, high entrapment efficiency, and good stability.[56,57]

The Sox gene family is a newly-discovered gene family whose main feature is a conservative base sequence HMG-box, which can be combined with DNA sequence specificity.[58] The gene family plays an important role in embryonic development, gender differentiation, nervous system, repair, and skeletal system development. Sox9 protein is considered to have an important effect on skeletal system development.[59] Animal experiments showed that the Sox9 had a high expression, and control of type II collagen synthesis in both embryonic cartilage germinal parts, thus, strongly inffecting the formation of cartilage.

Liposomes have been well established as an effective drug delivery system, due to their preparation simplicity and unique characteristics.[60] In this article, we fabricated cationic liposomes of uniform morphology. Then, rat bone marrow stromal stem cells (rMSCs) were cultivated and proliferated in vitro. The rMSCs were transfected with Sox9 gene via cationic liposomes, combined with thermal-sensitive injectable chitosan composite scaffolds for tissue engineering cartilage in vitro and implanted into nude mice to build chondroid tissue.

Materials and methods

Materials

All experimental protocols were approved by the First Affiliated Hospital of Nanjing Medical University and followed the principles of laboratory and animal care of the university. (2,3-Dioleoyloxy-propyl)-trimethylammonium (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were purchased from A.V.T. (Shanghai) Pharmaceutical Co., Ltd. Hoechst 33342 and 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Cambridge, MA, USA). Sox9 plasmid was purchased from Bioworld Technology, Inc., China (PPL00081-2b) a, and the species of the Sox9 plasmid is *Homo sapiens* (human) with vector backbone of pcDNA3 (Figure S1). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were bought from Gibco BRL (Thermo Fisher, USA). Additionally, Penicillin-streptomycin, 0.25% trypsin-EDTA, and non-essential amino acid were obtained from

Invitrogen (Thermo Fisher), anti-Collagen II (ab34712) and anti-collagen IX (ab134568) were bought from Abcam (USA), Streptavidin-Biotin Complex (SABC) kit was purchased from Boster (SA1025, USA). Other chemicals used in this work were all of the analytical pure grades and were used as received.

The fabrication and characterization of cationic liposomes

The thin membrane method was employed to prepare cationic liposomes.[61,62] Briefly, DOTAP (350 mg) was dissolved in chloroform (1 mL) and mixed with DOPC of 370 mg. The mixture was diluted to 1.0 mL in total using chloroform and vortexed for 10 minutes. The solvent was evaporated in rotary evaporator (Buchi R) at 50°C, and a thin film of dry lipid on the flask was obtained. Evaporation was continued for 1 hour after drying to remove the residue solvent. The lipid film was ground into a powder and dissolved in 4 mL water under vigorous stirring to form cationic liposomes. The formulation was further characterized by transmission elector microscopy (TEM, JEOL) and dynamic laser scattering (DLS, Malvern). The results were represented by the averages of sixmeasurements per sample with 20 seconds spent on each measurement. All measurements were performed at 25°C.

The separation, culture, and generation of MSCs in rats

Rat bone marrow was extracted from the 5–6 weeks old Sprague Dawley (SD) rat hind legs, diluted with D-Hanks for thorough incorporation, and centrifuged at 800 rpm for 5 minutes. The supernatant was abandoned, and DMEM was used to gently scatter cells into single cells suspension. The suspension was slowly added to lymphocyte density separation medium (ρ=1.077 g cm^{-3}) and centrifuged at 3,000 rpm for 20 minutes. The mononuclear cell layer was taken and washed by D-Hanks twice, and DMEM containing 20% FBS was added to the cells in the flask for further cultivation. A growth curve of rMSCs was detected by a trypan blue experiment. The flask was incubated at 37°C and 5% CO_2 in the incubator. The medium was replaced after 48 hours, and the unattached cells were abandoned. The cells were observed daily under an inverted phase contrast microscope. After being confluent, the cells were digested with trypsin and passaged. The third generation was identified by flow cytometry with CD34, CD45, CD29, and CD44.

The liposomes mediate Sox9 gene transfection with targeted chondrogenic differentiation

The transfection followed previous reports,[63] where briefly the recombinant Sox9 plasmid was diluted and mixed with cationic liposomes without FBS for 20 minutes at room temperature. The mixture was added directly to rMSCs in a petri dish and cultivated at 37°C, 95% humidity, and 5% CO_2 training. The medium was replaced with the full medium after 4 hours and continued to develop after 36 hours to detect the transfection efficiency. Cell transfection was divided into threegroups: 1) the experimental group: cationic liposomes were used to carry out the recombinant plasmid transfection of Sox9; 2) the control group: transfection of empty plasmid; 3) a blank control group: only the equivalent cationic liposomes were added; and 4) Lipofectamine 2000 was used as a positive control for the Sox9 plasmid transfection. After 7 days of culture, ELISA was used to characterize the tissue engineering materials cultured in the cells. At the 7 days transfection, confluence at 80~90% of the third generation of rMSCs was taken and Sox9 determined by ELISA for five parallel specimens from each sample. To optimize the transfection of cationic nanosized liposomes, a serial concentration of DNA plasmid (10, 50, 100, 200 ng) was used to transfect in different cell numbers for transfection (1×10^4, 2×10^4, 4×10^4, 8×10^4 cells per well). The transfected cells were collected, and the protein was extracted to detect the Sox9 protein expression via ELISA. Statistical analysis was performed on the computer, with SPSS statistical software. $\bar{X} \pm S$ was used to indicate that P=0.05.

Preparation of thermos-sensitive chitosan gel

A thermo-sensitive gel was prepared as previously described.[64] Briefly, chitosan was added into 0.1 mol L^{-1} hydrochloric acid solution to make the final concentration 2% (w/v), which formed solution A. β-sodium glycerophosphate were prepared in water at 56% (w/v), which formed solution B. Then two solutions were combined and mixed at different ratios (v/v) to form a thermosensitive hydrogel. The various ratios of the prepared hydrogels were kept at 4°C and tested at 37°C for temperature sensitive properties.

Chondrogenesis in vivo

The rMSCs were transfected and cultivated for 2 weeks, blended with thermo-sensitive chitosan hydrogel (0.2 mL), and subcutaneously injected into the back of nude mice. The

in vivo experiments with nude mice were divided into two groups: untransfected rMSCs with chitosan hydrogel (group A) and transfected rMSCs with chitosan hydrogel (group B). After 4 weeks of feeding, the subcutaneous injections were observed for the formation of cartilage in nude mice, including hematoxylin-eosin (HE) and safranin O staining. The immunohistochemistry (IHC) of collagen II and collagen IX of different groups were stained and labeled with SABC kit. Western blot (WB) of Sox9, collagen II and collagen IX were processed to quantify the chondrogenic of different groups.

Statistical analysis

Data of each group is represented by $\bar{X}\pm s$. SPSS Statistical software was used for statistical analysis. One-way ANOVA was used for multi-group data. A two sample*t*--test was used between the two groups of data. $P<0.05$ and $P<0.01$ were statistically significant.

Results

Characterization of cationic liposomes

The mean particle size of the cationic liposomes was 85.76 ±3.48 nm (Figure 1A) with a PDI value of 0.22 and a zeta potential of 15.76±2.1 mV (Figure 1B), which indicated the monodispersed nature of the nanoparticles. TEM of cationic liposomes had the same results as that of DLS, with an expected small size less than 100 nm with nearly spherical and uniform shape (Figure 1C).

Cells morphological observation of rMSCs

After inoculation, rMSCs were distributed at the bottom of the culture flask, and they were round with a bright cytoplasm and good refraction. The individual nucleus in the cells began to adhere to the wall (Figure 2A, 24 h) at 24 hours; increased spreading and the cytoplasm extending outward, similar to fibroblasts (Figure 2A, 48 h) at 48 hours, and the morphology of the adherent cells was fusiform, triangular, fan-shaped, and circular, and, after 5~8 days, the cells gradually formed a scattered colony, which is called a fibroblast colony (Figure 2A, 5–8 d). Flow cytometry was used to observe the expression of CD29 (95.34%) and D44 (85.12%), while CD34 (4.69%) and CD45 (5.12%) were negative (Figure 2B). A cell proliferation growth curve was drawn according to the determination by MTT (Figure 2C).

Cells morphological observation of chondrogenic differentiation of rMSCs after gene transfection

After rMSCs were transfected with Sox9 for 7 days, cells protrusion became longer, the body of the cell became wider, and the refractive index increased (Figure 3A). The growth curve is shown using the MTT method, and the proliferation rate of transfected cells increased (Figure 3B). The expression of Sox9 of rMSCs was detected by ELISA at different concentrations of cells (Figure 3C), which showed that the level of Sox9 expression in the experimental group was significantly higher than the other groups ($P<0.05$, Figure 3D).

Thermosensitive gel preparation

The various chitosan hydrogels kept at 4°C were incubated at 37°C for 1–5 minutes to observe the hydrogel formation process. Compared with the different gelation times of the ratio of chitosan and β-sodium glycerophosphate, we found that the gelation time[65] rapidly decreased from 15 minutes to 3 minutes with an increase of β-sodium glycerophosphate (Table 1).

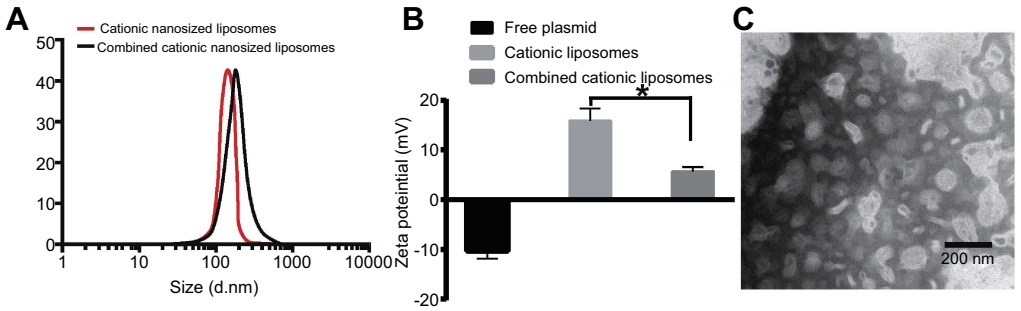

Figure 1 Characterization of cationic liposomes. (**A**) The diameter of cationic liposomes, (**B**) The zeta potential of cationic liposomes, and (**C**) TEM of cationic liposomes. * $P<0.01$.

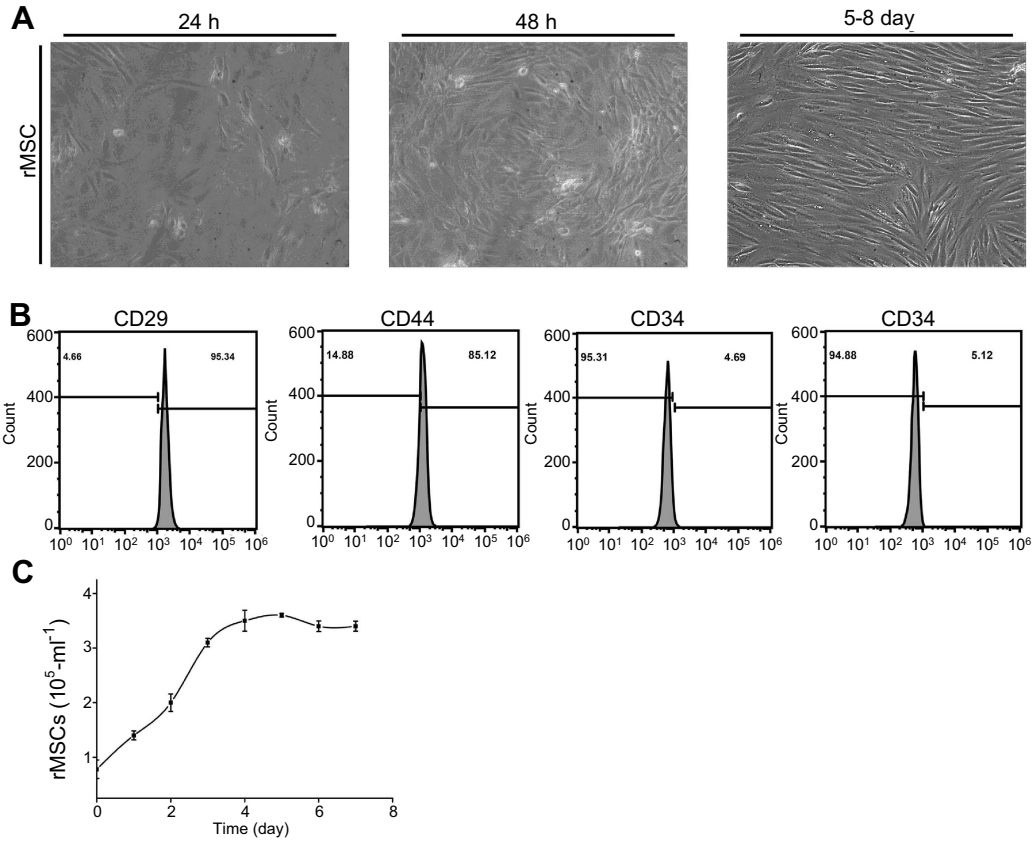

Figure 2 Isolated rMSCs cultivation and identification. (**A**) Isolated rMSCs cultivated at different times (24 hours, 48 hours, 5–8 day); (**B**) identification of rMSCs by flow cytometry; (**C**) growth curve of rMSCs.

Chondrogenesis in vivo

The transfected rMSCs and thermos-sensitive gel compound were cultured and injected into nude mice to observe chondrogenesis (Figure 4). The naked mice were sacrificed, and injection areas were anatomized after feeding for 4 weeks. We only found mass like structures in group A (untransfected rMSCs with chitosan hydrogel) and group B (transfected rMSCs with chitosan hydrogel), while no structures in group C (rMSCs) and group D (chitosan hydrogel) were observed. The mass like structures in group A and B were examined by HE, safranin O, and immunohistochemical staining of collagen II. Compared to group A, the formation of chondroid cells at the surface of tissue was observed, and the cytoplasm was brown after being dewaxed, while the nucleus was vacuolar in group B. HE staining of tissue-engineered cartilage blocks showed the aggregation of cells at the surface tissue, similar to chondrocyte expression (Figure 5). The results of immunohistochemical stain of collagen II and IX showed the significant difference between two groups. Furthermore, the WB of different groups illustrated the different protein expression of Sox9, collagen II, and collagen IX.

Discussion

The nude mice were injected with induced rMSCs and chitosan hydrogel, with the chitosan hydrogel alone disappearing under the skin while chondrocyte formation was found (Figure 4). In recent years, many have shown that rMSCs are the best choice for the seed cells of cartilage tissue engineering,[66–69] but the amount of rMSCs is extremely low for this purpose, limiting its application in tissue engineering. Proliferation rMSCs in vitro has been examined and potentially developed,[70] leading to mature protocols for isolation culture and amplification. In order to improve the quality of tissue engineering cartilage, it has become necessary to induce the differentiation of the seed cells into the chondrocytes before inserting the seed cells into the carrier materials. For the application of cells in tissue engineering technology in our study, we prepared cationic liposomes, extracted the bone marrow tissue from

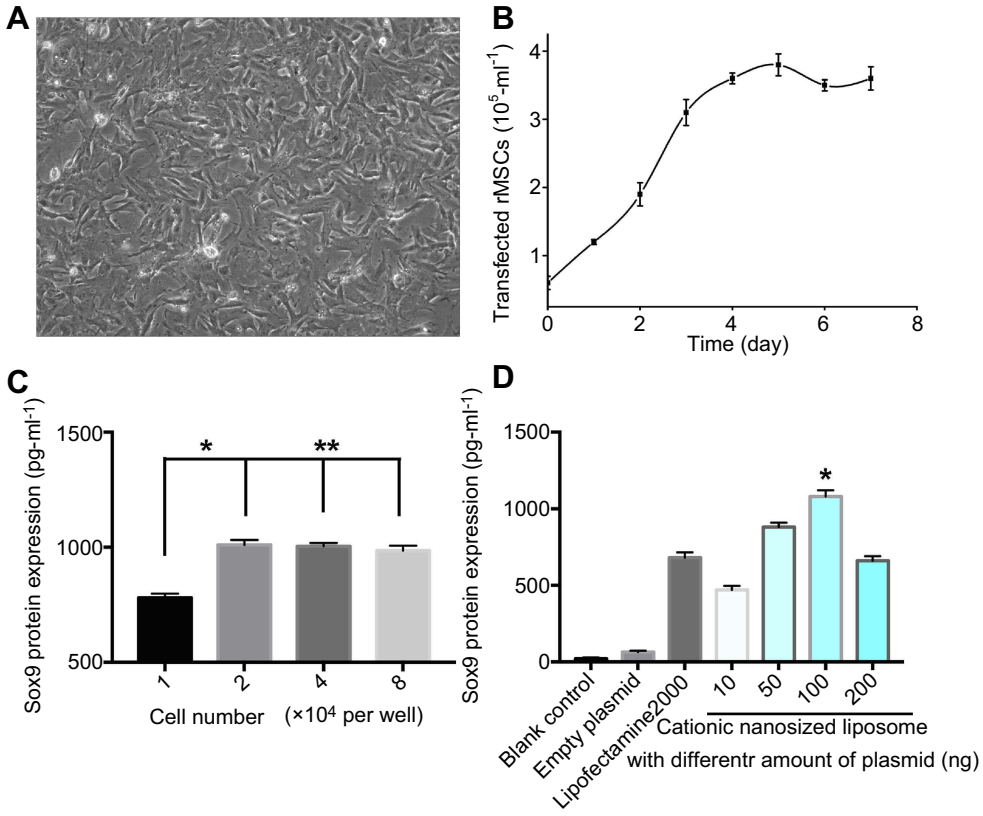

Figure 3 In vitro transfection of rMSCs. (**A**) rMSCs were transfected for 7 days; (**B**) growth curve of transfected rMSCs; (**C**) gene transfection of rMSCs with cationic nanosized liposomes at different concentrations of cells (* $P<0.05$; ** $P>0.05$). (**D**) Gene transfection of rMSCs with different formulations.

Table 1 Different ratio of chitosan and β-glycerophosphate on GT

Chitosan (2%): β-glycerophosphate (50%) (V:V)	GT (minutes)
5	3
7	15
10	50

Abbreviation: GT, gelation time.

rats, isolated rMSCs, increaded rMSCs culture cell number in vitro, and transfected them with Sox9 to differentiate into cartilage producing cells. The results showed that rMSCs were able to maintain the stem cell characteristics after proliferation and differentiation in vitro (Figure 2). The fusogenic property of DOPC plays a crucial role in macropinocytic transfer of formulation through the bilayer, resulting in enhanced cellular uptake.[71,72] However, it remains to be explored whether a specific phenotype of rMSCs can be expressed after long periods after such proliferation and permanent biochemical and directional differentiation.

Although rMSCs studies demonstrate the prospect of new cartilage repair, rMSCs should be combined with

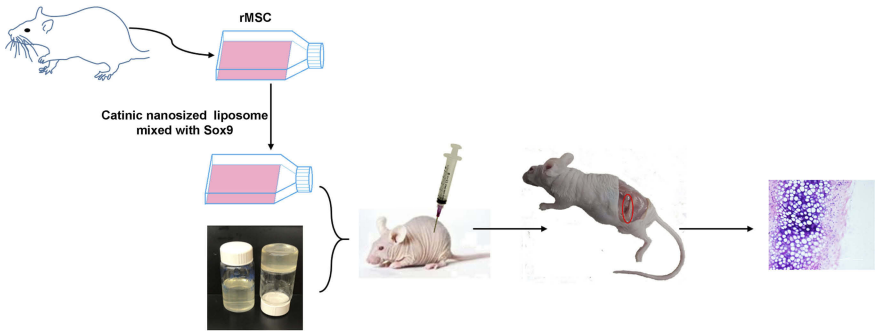

Figure 4 Sketch of Sox9 gene enhanced tissue engineering in chondrogenesis.

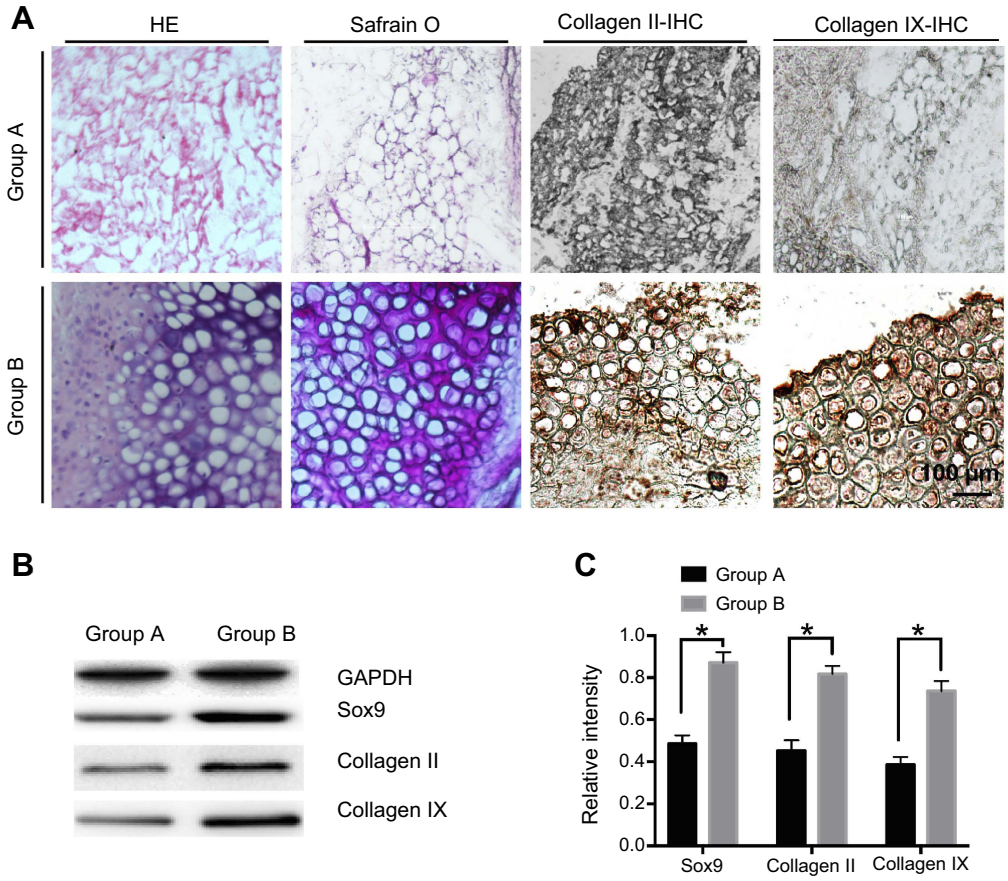

Figure 5 In vivo chondrogenesis of rMSCs. (**A**) Immunohistochemical stain of different groups. (**B**) Western blot (WB) analysis of Sox9, collagen II, and collagen IX. (**C**) Densitometric analysis of Sox9, collagen II, and collagen IX. Group A, untransfected rMSCs with chitosan hydrogel; Group B, transfected rMSCs with chitosan hydrogel. *P<0.05.

recombinant growth factors or organic combination with gene therapy if healing is to be complete. Sox9 has an effect on promoting cell proliferation effect and effectively maintains the phenotype of cartilage cells;[73–77] it also has to have biological activities at low concentrations. The direct application of Sox9 still has certain limitations, while genetic recombination and transfer technology will be available with the insertion of exogenous genes into eukaryotic cells. The expression of Sox9 can promote bone damage repair and the cartilage cell proliferation. Methods of transfection of eukaryotic cells include cationic liposome membrane fusion, electrophoresis, microinjection, calcium phosphate precipitation, etc. The present cationic liposome method is the most convenient and most commonly used method for transfection, which is suitable for transfection of multiple cells and is the only non-viral vector method approved by the FDA for clinical treatment.[78–80] In this report, we fabricated nanosized cationic liposomes, and the zeta potential reduced while combined with negative plasmids (Figure 1B). We transfected rMSCs and obtained the chondroid phenotype. With the liposome transfection technique, the expression of Sox9 was detected with ELISA method (Figure 3C). All the results showed that Sox9 can be stably expressed after induction into the chondroid phenotype from the MSCs. Meanwhile, collagen II was detected by immunocytochemistry stained collagen type II. This indicates that the rMSCs stabilized the expression of Sox9 and induced the chondrogenic phenotype. The immunohistochemical staining of tissue engineering cartilage was observed (Figure 5), and the staining of tissue-engineered cartilage blocks showed the aggregation of cells in the surface tissue, similar to chondrocyte expression in group B. All the results demonstrated that chondroid tissue formed in group B (the transfected rMSCs combined with chitosan hydrogel), which could offer a new hope for cartilage repair.

The contributions of this study are the unique combination of tissue engineering with genetic engineering to develop an effective and convenient therapeutic approach for the repair of cartilage defects. Sox9 gene transfected

with rMSCs selected in this study can continuously secrete Sox9 in rMSCs and avoid the need of Sox9 systemic or direct injection doses which can lead to unwanted side-effects. In addition, the carrier materials selected in this study can be injected with a chitosan gel and high biosafety. Furthermore, we obtained chondroid tissue in the naked mice model showing that the cartilage can be formed instead of a mixture of scaffolds and cells, which could bring a qualitative leap of clinic cartilage repair.

Conclusion

In summary, this study effectually differentiated rMSCs into chondrocytes in vivo for cartilage repair in vivo yields consistently satisfactory results. This method provides a valuable source of large numbers of MSC, which can be used for cartilage repair in vivo, with the role of Sox9 in chondrogenic differentiation via cationic liposomes. The isolation of rMSCs developed here may be useful in future investigations of stem cells for cartilage repair instead of the limited number of chondrocytes available for cartilage repair. Most importantly, this study showed that, compared with untransfected rMSCs, transfected rMSCs mixed with thermo-sensitive hydrogel could significantly increase chondroid tissue synthesis. All in all, the present gene enhanced tissue engineered artificial cartilage via cationic liposomes was successfully formed under the skin of nude mice, which should be further studied for improved clinic cartilage repair.

Atuthor contributions

Guangcheng Zhang and Weimin Fan designed experiments and acquisitioned data, Guangcheng Zhang and Mingjun Nie analyzed data, Guangcheng Zhang drafted the manuscript, Qing Zhang contributed to intellectual content and Weimin Fan contributed to final approval of the version. All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary material

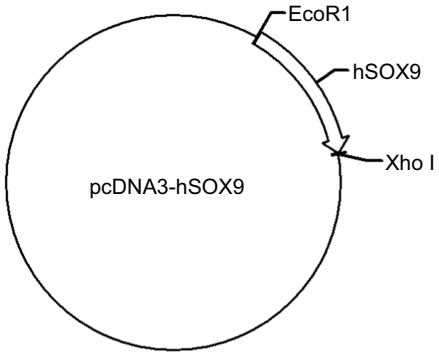

Figure S1 Homo sapiens (human) with vector backbone of pcDNA3