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Effective delivery of bone morphogenetic protein 2 gene using Chitosan-polyethylenimine nanoparticle to promote bone formation

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How to treat bone defect is still a challenge in clinical practice. Recently, researchers used human bone morphogenetic protein 2 gene (hBMP-2) to induce osteoblast differentiation and promote new bone formation. However, an efficient way to deliver hBMP-2 is still needed to be created. In this study, we evaluated if chitosan-polyethylenimine (CS-PEI) nanoparticle can effectively deliver hBMP-2 locally with lower or no toxicity and promote osteoblast differentiation and new bone formation *in vitro and in vivo*. Data demonstrated that the synthesized CS-PEI/hBMP-2 nanoparticle at W/W ratio of 20 to 1, which was the smallest size (162 nm) and highest zeta potential (24 mV), effectively transfected MC3T3-E1 cells without cytotoxicity *in vitro*, and had the ability to promote cell proliferation. Interestingly, the CS-PEI/hBMP-2 nanoparticle eliminated disadvantages of lower transfection efficiency from chitosan and cytotoxicity from PEI. RT-QPCR data showed that MC3T3-E1 cells treated with CS-PEI/hBMP-2 nanoparticle dramatically expressed higher levels of BMP-2 and significantly increased gene expressions of Col 1 on days 3 and 14, Sp7 on days 3, 7 and 14, and ALP on day 14. Alizarin red staining demonstrated that CS-PEI/hBMP-2 nanoparticle-treated MC3T3-E1 cells significantly increased cell mineralization. These *in vitro* data suggest that the CS-PEI/hBMP-2 nanoparticle can effectively induce osteogenic differentiation of MC3T3-E1 cells *in vitro*. Western blot further demonstrated that transgene BMP-2 indeed phosphorylated Smad 1/5/8, which indicates that CS-PEI/hBMP-2 nanoparticle affects cell differentiation through BMP-2 signal pathway. Importantly, *in vivo* data showed that CS-PEI/hBMP-2 nanoparticle clearly promoted new bone formation at the bone defect area 12 weeks post-implantation. This indicates that synthesized CS-PEI/hBMP-2 nanoparticle has the potential to become a useful therapeutic vector for bone defect treatment with further modification.

1. Introduction

Bone defect is a very common medical situation. Many complicated conditions, such as pathological fractures and large bone defects, can cause the healing process to fail. Different therapeutic treatment, such as natural grafts, stem cells, tissue engineering, and growth factor stimulation, have their own therapeutic limitations. Therefore, each treatment of bone defect still need to improve in order to quickly and efficiently heal bone defect.

Human bone morphogenetic protein 2 (BMP-2) is an autocrine secretion protein, a member of the TGF-β superfamily and plays an important role in the development of bone and.[1,2] BMP-2 is an essential osteoinductive growth factor for the bone regeneration process, which can induce osteogenic differentiation of mesenchymal cells, healing the critical size of bone defect.[1-4] BMP-2 forms a complex with type I and II serine/threonine kinase receptors, which phosphorylate receptor-mediated SMAD1, 5, and 8 proteins; form complexes with SMAD4 protein; and further translocate to nucleus in order to regulate gene expressions related with bone regeneration.[5-7] The FDA has approved the use of human BMP-2 as a growth factor to stimulate bone regeneration clinically. In general, recombinant BMP-2 protein can be used at the bone defect area directly. Frequent application at the bone defect area may be required and is expensive and inconvenient. Many bone defects need a long time to heal. Therefore, a slow-release method could stimulate the healing process of bone defect.

Gene transfer or gene therapy could deliver hBMP2-gene to the bone defect area and transduce local cells to continually produce hBMP2 within a certain period of time. There are

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many viral or non-viral delivery systems that can be used in bone defect treatment.[8-10] Most systems, however, are still not efficient to transfect or transduce cells on the bone defect area.[11-13] Chitosan has the potential to act as a polycationic gene carrier with high biocompatibility and no toxicity profile[14]. Chitosan also has the ability to bind and protect DNA from nuclease degradation.[15] The disadvantage of chitosan is low transfection efficiency.[16] Polyethylenimine (PEI), known for its ''proton sponge effect'', has been demonstrated to be a useful non-viral carrier *in vitro* and *in vivo* for some applications.[17,18] PEI, however, has its own disadvantages, cytotoxicity and non-degradability,[18,19] The toxicity of PEI can decrease with molecular weight decrease while its transfection efficiency will also decrease.[20] Several previous studies tried to modify chitosan and PEI to overcome their disadvantages.[21-24] Further improvement is still required to effectively deliver therapeutic genes to a bone defect location.

In our current study, we created CS-PEI nanoparticle using chitosan and 1.8 KDa of PEI to carry hBMP-2 gene. Our data demonstrated that CS-PEI could effectively carry hBMP-2 and transfect MC3T3-E1 cells resulting in MC3T3-E1 differentiation *in vitro*, and local cells in the bone defect area resulting in increasing new bone formation *in vivo*.

2. Materials and methods

2.1 Synthesis of CS-PEI nanoparticles.

CS-PEI nanoparticle was synthesized according to a previous study.[25] Briefly, 0.1 M periodate-oxidized chitosan (Sigma-Aldrich, St. Louis, USA) and 0.01 M potassium periodate (Sigma-Aldrich) was dissolved in 1% sodium acetate (pH4.5) separately and degassed with N_2 for 30 min. Then, equal volume of each solution was mixed together at 4 °C for 48 h and the reaction stopped by adding ethylene glycol (10% v/v). This solution was dialyzed with 10 liters of NaCl (0.2 M, pH4.5) for 5 days, then dialyzed in deionized water (pH 4.5) for 2 days. Twenty mmol 1.8 KDa of PEI (PEI, PH 4.5, Aladdin, Shanghai, China) was added into this solution, degassed with N_2 for 30 min, and continuously mixed by magnetic stirring at 4 °C for 2 days. Thereafter the solution was neutralized by adding 0.1M sodium borohydride, magnetically stirred for 30min, and then dialyzed in the same way as mentioned above (Fig. 1a).

2.2 Characteristics of CS-PEI/hBMP-2 nanoparticle

The CS-PEI/hBMP-2 nanoparticle was prepared by mixing hBMP-2 plasmid DNA (pACCMV-hBMP-2 with CS-PEI) and then gently mixing the solution by vortex. The mixture was then kept at room temperature for 30 min.

Particle size and zeta potential of CS-PEI/hBMP-2 nanoparticle were evaluated by dynamic light scattering (Malvern Zetasizer Nano Z, UK) in triplicate. To do this, samples were placed into an analyzer chamber and measured in water. Size of particle was assessed by three cycles, and zeta potential was performed by three repeated cycles with 100 runs each. CS-PEI and hBMP-2 at various weight/weight (W/W) ratios of 1:1, 5:1, 10:1, 15:1, 20:1 and 25:1 were instantly prepared by mixing equal volumes of CS-PEI and DNA diluted with deionized water.

Agarose gel retardation assay was performed using CS-PEI/hBMP-2 nanoparticleat at different W/W ratios of 0.1:1, 0.2:1, 0.5:1, 1:1 and 2:1. Each sample was 10 µl plus 2 µl of loading buffer (6x), gel electrophoresis was run at 100 V for 20 min.

2.3 Cell culture

293T (human kidney cell line, ATCC, Manassas, VA, USA，HELA (human cervical carcinoma cell line, ATCC) and MC3T3-E1 (mouse pre-osteoblast cell line, Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco BRL), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco BRL) at 37 °C in 5% CO2 humidified atmosphere.

2.4 Cell Cytotoxicity assays

Cell viability, cell cycle and cell apoptosis were measured to evaluate *in vitro* cytotoxicity of the CS-PEI nanoparticle. Cell viability was performed with an MTT assay (AMRESCO, Solon, OH, USA) using 293T and MC3T3-E1cell lines. Cell apoptosis and cell cycle were performed using Annexin V-FITC/PI Double Staining Assay Kit (KeyGen, Nanjing, China). Cells were seeded in 96-well plate at 2×10^4 cells/well and cultured for 24 h, then 5, 10, 20, 50 and 100 µg/ml of EGFP (EGFP plasmid, pACCMV-EGFP), CS/EGFP, PEI/EGFP or CS-PEI/EGFP was added into the corresponding well to culture for 24 hours, in which time point effects of cytotoxicity were clearly indicated. Cells could float and cause inaccurate data if allowed to continue to culture beyond 24 h post-treatment. Then, we followed instructions from MTT kit, cell cycle, or the apoptosis kit to carry out assays.

2.5 Transfection efficiency assays

Next, we needed to know if the CS-PEI nanoparticle plus plasmid DNA could transfect any cells. 293T cell is a gold standard cell line to compare or test any transfection/transduction reagent and evaluate transfection/transduction efficiency. MC3T3-E1cell is a special cell line to study osteoblast differentiation *in vitro*. Therefore, to evaluate CS-PEI nanoparticle transfection efficiency, we counted the EGFP positive 293T or MC3T3-E1cells using confocal laser scanning microscopy and FACS. Cells were seeded in 6-well plates at 20×10^4 cells/well for 24 h, the medium was replaced with serum-free medium containing EGFP only, CS/EGFP, PEI/EGFP or CS-PEI/EGFP nanoparticles at W/W ratio of 20 to 1 to culture for 6 h, then the medium was replaced with normal growth medium to culture for 36 h. Transfection efficiency was then evaluated under confocal laser scanning microscopy or FACS.

2.6 Osteogenic differentiation

In this study, our purpose was to improve hBMP-2 local delivery resulting in promotion of bone defect healing. Therefore, it was important to know if the CS-PEI/BMP-2

nanoparticle could induce osteogenetic differentiation *in vitro* and *in vivo*. Osteogenetic differentiation induced by CS-PEI/BMP-2 nanoparticle was performed using MC3T3-E1 cells. MC3T3-E1 cells were seeded in a 6-well plate at 20×10^4 cells/well, then transfected as described in section 2.5. After transfection, cells were cultured in H-DMEM medium (Gibco BRL) containing 20 mM β-glycerol phosphate and 0.5 μM ascorbic acid. Cells were transfected by CS-PEI /EGFP as a control group. Cells were stimulated by recombinant hBMP-2 (20ng/ml) (R&D Systems, MN, USA) as a positive control. After 3, 7, and 14 days post-transfection, cells were collected for total RNA extraction using Qiagen RNeasy Mini Purification Kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed using iScript™ cDNA Synthesis Kit (Takara Bio, Tokyo, Japan). Real-time PCR (qPCR) was run using MxPro Mx3005P Real-Time PCR Detection System (Agilent Technologies, Santa Clara, CA, USA) with SYBR-Green Premix Ex Taq (Takara Bio). Primers used for qPCR were as follows:
β-actin, 5'-CATCCGTAAAGACCTCTATGCCAAC-3'
and 5'-ATGGAGCCACCGATCCACA-3';
ALP, 5'-CTCAACACCAATGTAGCCAAGAATG-3'
and 5'-GGCAGCGGTTACTGTGGAGA-3';
Col 1, 5'-GACATGTTCAGCTTTGTGGACCTC-3'
and 5'-GGGACCCTTAGGCCATTGTGTA-3';
Sp7, 5'AAGTTATGATGACGGGTCAGGTACA-3'
and 5'-AGAAATCTACGAGCAAGGTCTCCAC-3';

To assess mineralization, cells were stained by Alizarin red at 21 days post-transfection, in which time point MC3T3-E1 cells commonly form visible calcium deposition in osteogenetic differentiation conditions in our lab. Briefly, cells were fixed in 95% ethanol at room temperature for 10 min, washed with PBS three times, then stained with 0.1% Alizarin red solution (pH 8.3) for 20 min, washed with PBS three times and soaked in PBS for 4 h to remove non-specific Alizarin red. Quantification of mineralization was completed by adding Cetylpyridinium chloride CPC (Sigma-Aldrich) extraction and measured at 562 nm.

2.7 Western blot analysis

It is known that there is a trace of activation of Smad1/5/8 in osteoblast normally, and, that Hela cells absolutely do not have any activation of Smad1/5/8 without BMP2 stimulation. Therefore, Hela cells were used to prove if the hBMP-2 we delivered could biologically induce phosphorylation of downstream Smad1/5/8.[26,27] For this assay, Hela cells were used. The transfection procedure was the same as described in section 2.5. Cells were stimulated by recombinant hBMP-2 (20ng/ml) (R&D Systems) as a positive control. Cells were lyzed with RIPA Lysis Buffer (Beyotime, China). Thirty μg of protein was loaded onto SDS-PAGE gels for Western blots using rabbit polyclonal anti-rat phospho-Smad1(Abcam, Cambridge, MA, USA) and mouse monoclonal anti-β-actin antibody (Cell Signaling Technology, Boston, MA, USA) for detection.

2.8 *In vivo* animal experiment

Our animal protocol was approved by the Animal Care and Use Committee of Jilin University, Changchun, People's Republic of China. Twenty male Wistar rats (~200g) were used in this study. Rat is a commonly used animal model for studying critical-size defects. Advantages of using rat as compared to mouse for cranial bone defect are the size and ease for conducting craniotomies. All animal models have their limitations, but this was the best option currently available. Therefore, we used the rat model for the current study. To create the bone defect animal model,[28] rats were anesthetized using ketamine (60 mg/kg) and xylazine (8 mg/kg), the local area was shaved and sterilized with iodophor solution, a 20 mm length incision was made along the midline in the cranial parietal, and the calvarias area was exposed. Two critical bone defects (diameter 6 mm) (Fig 5a) were created on the midline with a bone trephine (3i Implant Innovation, Palm Beach Gardens, FL, USA). For vivo experiments, CS-PEI/hBMP-2 nanoparticle was created with 200 μg of CS-PEI and 10 μg of hBMP-2 at W/W ratio of 20:1, which was absorbed into a 5 mm diameter, 1.5 mm thick gelatin sponge. Gelatin sponge loaded with 10 μg recombinant hBMP-2 protein or gelatin sponge loaded with CS-PEI/hBMP-2 nanoparticle was placed in the bone defect area (Fig 5a). Treated rats were sacrificed at 6 and 12 weeks after implantation and exsanguinated via auricular dextra and perfused via ventriculus dexter with 4% paraformaldehyde solution. The cranial parietal, heart, liver, spleen, and kidney were removed and fixed in 4% paraformaldehyde solution for further examinations. Micro computed tomography (micro-CT) (micro-CT 35; Scanco Medical AG, Bassersdorf, Switzerland) was used to evaluate new bone formation. Quantification of new bone formation was calculated using Image-Pro Plus software. After imaging detection, decalcification of these samples was performed by fixing in 10% EDTA solution, replacing the decalcification solution every week for x months, and then the samples were examined using additional histological methods.

2.9 Statistical analyses

The data are presented as mean ± standard deviation (SD). One-way ANOVAs, followed by a Turkey's test were used. P value<0.05 was considered statistically significant.

3 Results

3.1 Characteristics of CS-PEI/hBMP-2 nanoparticles

Results of electrophoresis shows that migration of CS-PEI/hBMP-2 nanoparticle was retarded completely when the W/W ratio reached 1 (Fig 1b), which suggests that a W/W ratio of CS-PEI/hBMP-2 beyond 1 was sufficient for our study. Next, data in Fig. 1c shows that the size of CS-PEI/BMP2 nanoparticle quickly decreased between W/W ratios of 1 and 5, then gradually decreased with an increase of the W/W ratio (Fig. 1c). The size of CS-PEI/hBMP-2 nanoparticle was smallest at W/W ratios of 20 or 25, 162±7.9 nm (Fig 1c). Lastly, data from the zeta potential assay shows that the zeta potential of CS-PEI/BMP2 nanoparticle quickly increased between W/W ratios of 1 and 5, then basically reached a plateau with an increase of the W/W ratio (Fig. 1d). Zeta potential of CS-PEI/hBMP-2 nanoparticle was the highest at W/W ratios of 15, 20, or 25, 24±2.9 mV (Fig 1d). These data indicate that CS-PEI/BMP2 nanoparticle at a W/W ratio of 20 possesses the smallest size and highest zeta potential, which indicates that the CS-PEI/BMP2 nanoparticle is electrically stabilized and resists aggregation. Therefore, we decided to use a W/W ratio of 20 in all further *in vitro* and *in vivo* experiments.

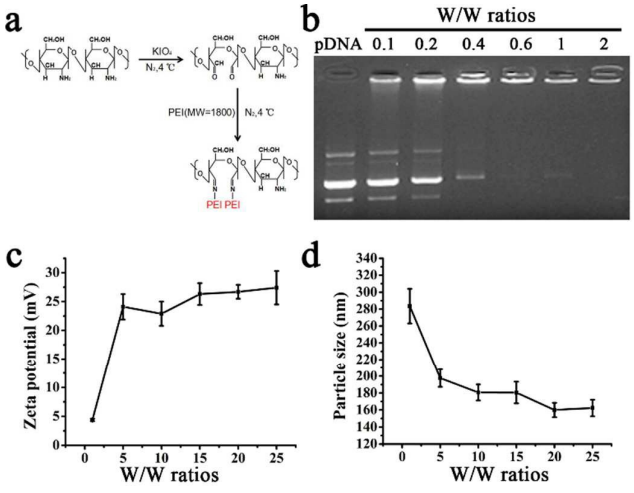

Figure 1. Synthesis and characteristics of CS-PEI nanoparticle. (a) reaction scheme of CS-PEI copolymer; (b) agarose gel electrophoresis of CS-PEI/hBMP-2 nanoparticles at various W/W ratios; (c) particle size of CS-PEI/hBMP-2 at various W/W ratios; (d) zeta potential of CS-PEI/hBMP-2 at various W/W ratios. Data are represented as means ± SD from three experiments. pDNA, plasmid pACCMV-hBMP-2.

3.2 Cytotoxicity of CS-PEI *in vitro*

Data of MTT assays showed that there was no cytotoxicity of CS and CS-PEI nanoparticle even at 100 μg/ml in either MC3T3-E1 or 293T cells (Fig. 2a and b). Interestingly, all amounts of CS-PEI nanoparticle tested herein slightly stimulated both MC3T3-E1 (~120%) and 293T cell (~120-140%) proliferation (Fig. 2a and b). Importantly, data also showed that PEI only caused severe cytotoxicity for MC3TC-E1 and 293T cells (Fig. 2a and b). Cell viability was dramatically decreased even using 5 μg/ml of PEI, 79% for MC3T3-E1 cells and 85% for 293T cells (Figs. 2a and b). When using 20 μg/ml of PEI, cell viability was only ~30% for both MC3T3-E1 and 293T cells (Fig. 2a and b). Further apoptosis and cell cycle assays showed that the CS-PEI nanoparticles did not influence apoptosis and cell cycle of MC3T3-E1cells (Fig. 2c and d). Interestingly, these data suggest that CS-PEI nanoparticles overcome toxicity effects of PEI on cells and possibly retain the ability of CS to slightly stimulate cell proliferation.

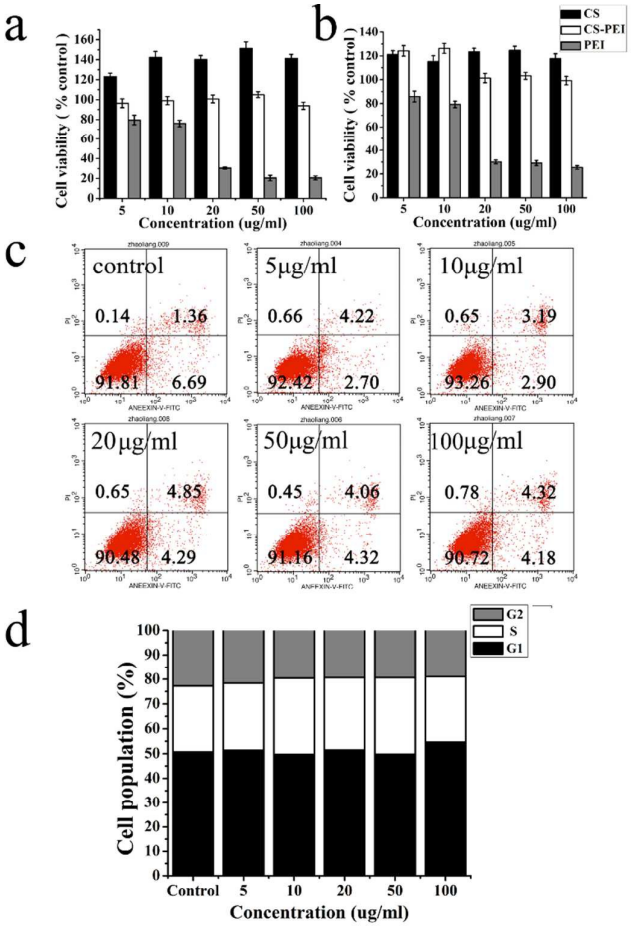

Figure 2. Cytotoxicity of CS-PEI nanoparticle *in vitro*. (a) MC3T3-E1cell viability time after 24 h post-treatment with CS, PEI or CS-PEI nanoparticle; (b) 293T cell viability after 24 h post-treatment with CS, PEI or CS-PEI nanoparticles ; (c) MC3T3-E1 cell apoptosis after 24 h post-treatment with CS-PEI nanoparticles; (d) MC3T3-E1cell cycle assay after 24 h post-

treatment with CS-PEI nanoparticles. Data are represented as means ± SD from three experiments.

3.3 Transfection efficiency of CS-PEI/EGFP nanoparticle

Data from transfection assays demonstrated that CS/EGFP had very low transfection efficiency, ~5% in 293T cells and ~0.5% in MC3T3-E1 cells (Fig. 3a, e, d and h). Transfection efficiency of PEI/EGFP was slightly, but significantly higher, than that of CS/EGFP (Fig. 3b, e, d and h). Interestingly, transfection efficiency of CS-PEI/EGFP nanoparticle was dramatically increased compared to CS/EGFP or PEI/EGFP group in both ME3T3-E1 and 293T cells (Fig. 3c, f, d and h). These data indicate that the transfection efficiency increased when we used a W/W ratio of 20 to 1 to create CS-PEI/plasmid DNA nanoparticle.

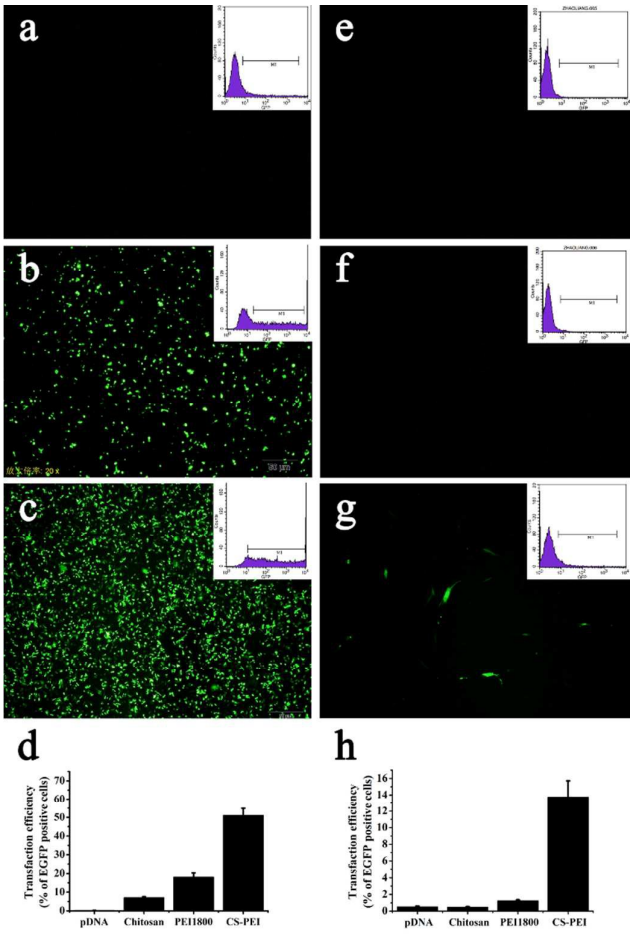

Figure 3. Transfection efficiency of CS/EGFP, PEI/EGFP and CS-PEI/EGFP nanoparticle. (a) CS/EGFP in 293T cells; (b) PEI/EGFP in 293T cells; (c) CS-PEI/EGFP in 293T cells; (d) FACS data of 293T cells; (e) CS/EGFP in MC3T3-E1 cells; (f) PEI/EGFP in MC3T3-E1 cells; (g) CS-PEI/EGFP in MC3T3-E1 cells; (h) FACS data of MC3T3-E1 cells. Data are represented as means ± SD from three experiments. pDNA, plasmid pACCMV-EGFP..

3.4 Osteogenic differentiation *in vitro*

Data from alizarin red staining showed that MC3T3-E1 cells treated with CS-PEI/hBMP-2 nanoparticle and protein BMP-2 had significantly more dark red staining than that of the control group (Fig. 4a and b) ($P<0.01$). These results indicate that CS-PEI/hBMP-2 nanoparticle-treated MC3T3-E1 cells differentiate into osteoblast with calcium deposition.

To further understand osteogenic differentiation, gene expression of BMP-2, Sp7, Col1 and ALP were evaluated. Fig. 4 shows MC3T3-E1 cells treated with CS-PEI/hBMP-2 nanoparticle significantly expressed higher levels of hBMP-2, Sp7, Col1 and ALP at almost all three time points, days 3, 7 and 14 except Col1 on day 7 and ALP on day 3 (Fig. 4b). Interstingly, ALP expression was dramatically higher than that of the control group on day 14 (Fig. 4b). Recombinant hBMP-2 treated group didn't affect hBMP-2 expression, could increase gene expressions of Sp7, Col1 and ALP at early time point and had less effect on day 14 (Fig. 4B), which indicate that CS-PEI/hBMP-2 nanoparticle can retain gene expression of hBMP-2 for longer time, and induce MC3T3-E1 cell differentiation to become osteoblast in vitro.

To understand if delivered hBMP-2 biologically affects cells through the BMP-2 signal pathway, phosphorylation of Smad 1/5/8 was checked in Hela cells. Phosphorylation of Smad 1/5/8 is a downstream signaling pathway of hBMP-2.[29] Phosphorylation of Smad1/5/8 is the symbolic signal which indicates that BMP is involved in specific biologic effects.[30] Indeed, Western blotting showed that Hela cells treated with CS-PEI/hBMP-2 nanoparticle or recombinant protein or from CS-PEI/hBMP-2 had a clear positive band for phosphorylation of Smad 1/5/8 (Fig 4d). This data indicates that both kinds of hBMP-2 can play a biological effect through BMP-2 signal pathway.

3.5 Bone formation in vivo

Fig. 5a is a representative image of cranial bone defect used in this study. In vivo data showed that new bone formation (~40% of new bone formation in the bone defect area) was not significantly different between both gelatin sponge and gelatin sponge loaded with CS-PEI/hBMP-2 nanoparticle or recombinant hBMP-2 6 weeks post-implantation (Fig. 5c, e and g). New bone formation, however, was significantly increased in the CS-PEI/hBMP-2 nanoparticle treated rats or recombinant hBPM-2 (61.2 % or 57.3% of new bone formation in the bone defect area,) 12 weeks post-implantation than that of gelatin sponge only treated rat (40.9 % of new bone formation in the bone defect area, see yellow arrow) (Fig. 5, $P < 0.05$).

Data from H&E staining further demonstrated that new bone formation was dramatically higher in the CS-PEI/hBMP-2 nanoparticle treated bone defect area than in the gelatin sponges treated bone defect area 12 weeks post-implantation (Fig. 5i, j and k). Cuboidal shaped osteoblasts were observed at the new bone area in the CS-PEI/BMP-2 nanoparticle treated bone defect. Furthermore, there were no significant histological changes in the liver, spleen, kidney or heart, which were obtained from the same rats 12 weeks post-implantation

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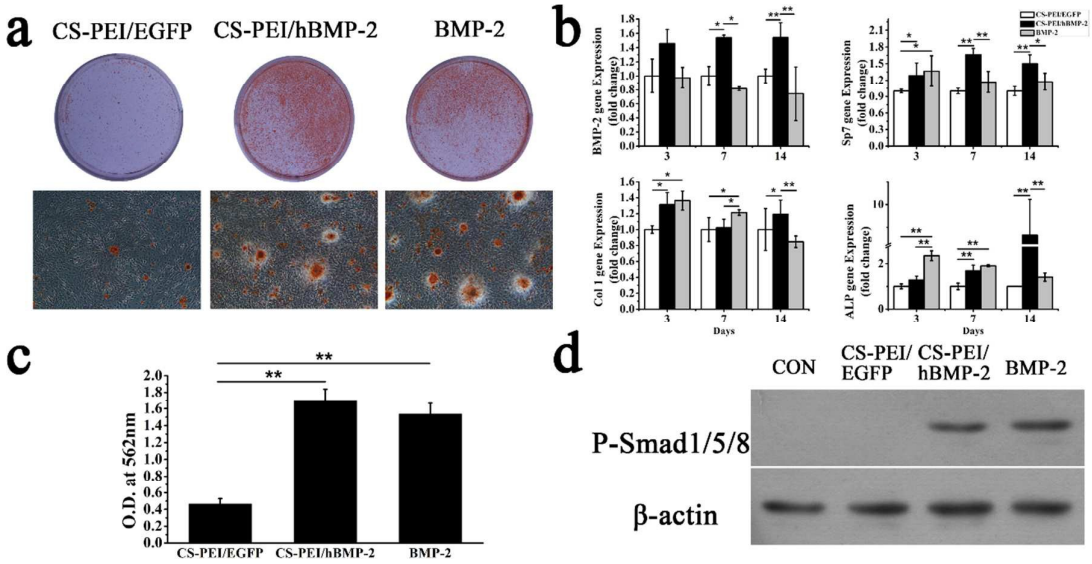

Figure 4. Effects of CS-PEI/hBMP2 nanoparticle on osteogenic differentiation. (a) Alizarin red staining of MC3T3-E1 cells after 21 days post-transfection; (c) semi quantificational analysis with CPC extraction; (b) relative gene expression of hBMP-2, SP7, Col 1 and ALP in MC3T3-E1 cells after 3, 7 or 14 days post-transfection with CS-PEI/EGFP as control group and CS-PEI/hBMP2; (d) phosphorylation of Smad 1/5/8 in HELA cells after 48 h post-transfection with CS-PEI/EGFP , CS-PEI/hBMP-2 or recombinant hBMP-2 from four experiments. Data are represented as means ± SD from two experiments. *: $P < 0.05$, **: $P < 0.01$.

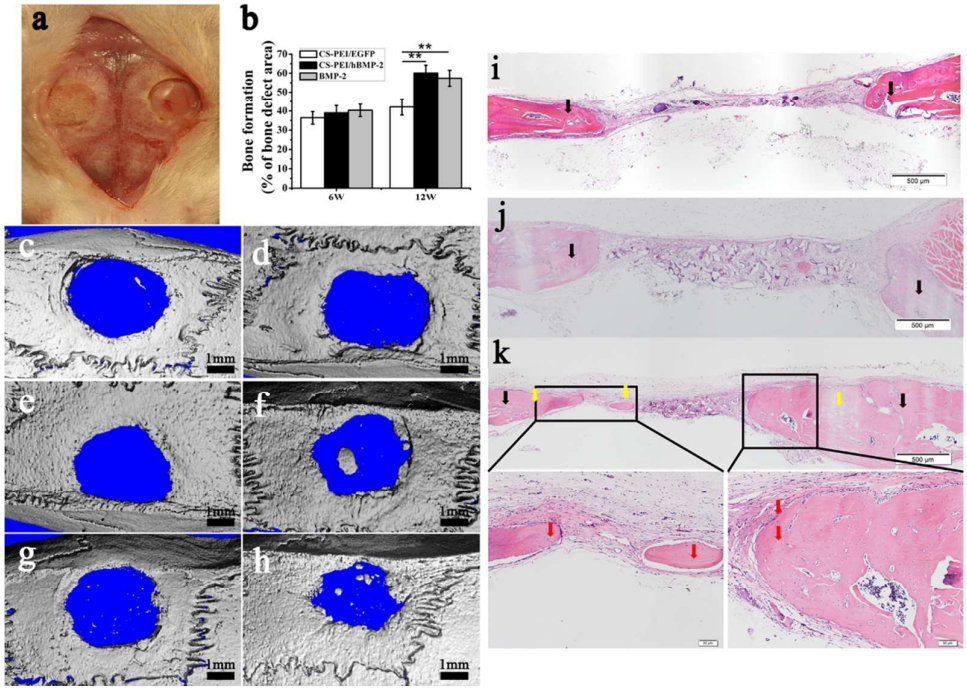

Figure 5. Data from *In vivo* animal experiments**.** (a) a representative image for bone defect (diameter of 5 mm) on skull of rats; (b) quantitative analysis of micro CT new bone formation data in the bone defect site using Image-Pro Plus software ; (c) image

of micro CT analysis 6 weeks post-implantation with gelatin sponge; (d) image of micro CT analysis 12 weeks post-implantation with gelatin sponge; (e) image of micro CT analysis 6 weeks post-implantation with gelatin sponge loaded with recombinant hBMP-2; (f) image of micro CT analysis 12 weeks post-implantation with gelatin sponge loaded with recombinant hBMP-2; (g) image of micro CT analysis 6 weeks post-implantation with gelatin sponge loaded with CS-PEI/hBMP-2; (h) image of micro CT analysis 12 weeks post-implantation with gelatin sponge loaded with CS-PEI/hBMP-2; (i) H&E staining of rat cranial defect area 12 weeks post-implantation with gelatin sponge; (j) H&E staining of rat cranial defect area 12 weeks post-implantation with gelatin sponge loaded recombinant hBMP-2; (k) H&E staining of rat cranial defect area 12 weeks post-implantation with gelatin sponge loaded with CS-PEI/hBMP2. There were 5 rats/group for *in vivo* experiments. Black arrows indicate the broken ends of fractured bone, yellow arrows indicate new bone, and red arrows indicate osteoblast.

that received both gelatin sponge and gelatin sponge loaded with CS-PEI/hBMP-2 (Fig 6). These results indicate that CS-PEI/hBMP-2 nanoparticle can also promote new bone formation at the local bone defect area *in vivo* without cytotoxicity in the liver, spleen, kidney or heart.

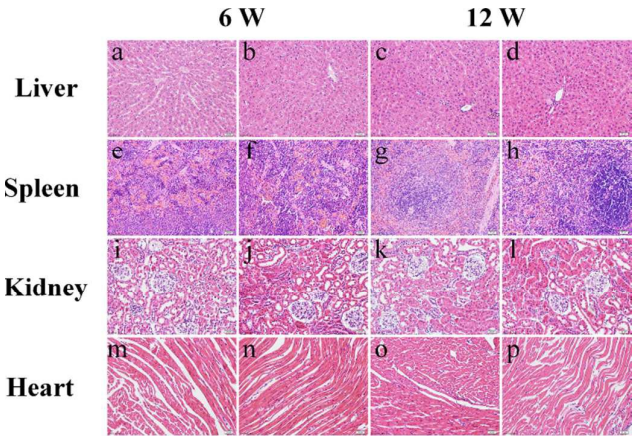

Figure 6. Histological examination of liver, spleen, kidney and heart 6 or 12 weeks post-implantation with gelatin sponge (a, e, i, m ,c, g, k, q) or gelatin sponge loaded with CS-PEI/hBMP-2 (b, f, j, n, d, h, i, p) by H&E staining. There were 5 animals/group for these *in vivo* experiments.

4. Discussion

There are several ways to treat bone defect in current clinical practice. Most treatments, however, still need to be improved. Development of efficient treatment is necessary. A delivery system of low toxicity, high efficiency, and good biodegradability is required to deliver BMP-2 locally. In this study, we created a novel nanoparticle, CS-PEI nanoparticle with CS and PEI to locally deliver a therapeutic gene, hBMP-2, in order to effectively treat bone defect.

DNA condensation is one of the necessary factors needed as a qualified gene delivery vector.[31] Synthesized CS-PEI/hBMP-2 nanoparticle was retarded completely when the W/W ratio of CS-PEI/hBMP-2 reached 1 (Fig. 1b). This indicates that the W/W ratio of CS-PEI/hBMP-2 beyond 1 will meet requirements as a qualified gene vector. Surface properties of nanoparticle, such as particle size and zeta potential, are also important factors for gene delivery vector because they can influence cell uptake, stability, and aggregation of nanoparticles.[30] In general, smaller nanoparticles can be more efficient to cross cell membrane, and higher zeta potential with stronger positive charges can increase cell binding and stability of nanoparticles and resists nanoparticle aggregation.[32] The size of CS-PEI nanoparticle synthesized in this study clearly decreased with the increase of the W/W ratio of CS-PEI/hBMP-2 (Fig. 1c). At a W/W ratio of 20 to 1, the CS-PEI/hBMP-2 nanoparticle possessed the smallest particles (162nm ± 9.7 nm)(Fig. 1c) and the highest zeta potential (24mV ±2.9 mV) (Fig. 1d), which provided effective ionic interactions and stronger positive charge to efficiently bind to anionic cell surfaces leading to active uptake by the cell.

293T cells are a gold standard cell line to compare different transfection reagents in the gene transfer field. Fig. 3 shows that CS-PEI/hBMP-2 nanoparticles are highly efficient to transfect 293T cells. The transfected positive cells are ~90% if we count by direct observation (data not shown). Therefore, the transfection efficiency of CS-PEI/hBMP-2 nanoparticles can be compared with many commercial transfection reagents. MC3T3-E1 cells are a cell/tissue specific cell line, with expected relative lower transfection efficiency normally.

Chitosan and chitosan derivatives have the ability to effectively condense plasmid DNA leading to the protection DNA from degradation. Previous studies have used chitosan as a vector to deliver genes.[14, 33, 34] The transfection efficiency of chitosan, however, was lower in order to limit its application. Our study herein also demonstrated that transfection efficiency of chitosan was much lower than that of PEI *in vitro* (Fig. 3). On the other hand, chitosan caused no cytotoxicity whatever in both tested cell lines, 293T and MC3T3-E1 cells, and even slightly promoted both cell proliferations (Fig. 2).

PEI is one of the most common non-viral vectors because it is a cationic polymer. Its cationic property allows PEI to bind nucleic acids, like plasmid DNA and attach to cell membrane resulting in efficiently delivering plasmid DNA into the cells. Branched PEI exhibits high transfection efficiency.[18, 35, 36] In general, low molecular weight PEI has less toxicity side effect.[31] Therefore, we selected relative low molecular weight, PEI 1.8 Kda. Data in Fig. 3 show that PEI had significantly higher transfection efficiency than that of CS (Fig. 3). The disadvantage of PEI, however, is that it can cause severe cytotoxicity in both 293T and MC3T3-E1 cells (Fig. 2). Indeed, previous studies also found PEI's side effect of cytotoxicity through disturbing functions of cell membrane as well as interfering with intracellular processes of cells.[22, 36-38]

Our data clearly showed that the W/W ratio of 20 to 1 for the CS-PEI nanoparticle not only retained no cytotoxicity from chitosan (Fig. 2) and higher transfection efficiency from PEI,

but also increased transfection efficiency compared to CS/EGFP or PEI/EGFP only (Fig. 3). These data suggest that we created a good biocompatibility nanoparticle which overcomes the lower transfection efficiency from chitosan and cytotoxicity from PEI.

Importantly, CS-PEI/hBMP-2 nanoparticle transfected MC3T3-E1 cells expressed much higher hBMP-2 resulting in increasing gene expressions of Col1 on days 3 and 14, Sp7 on days 3, 7 and 14, and ALP on days 7 and 14 through BMP-2 signal pathway in vitro (Fig. 4). Interstingly, recombinant hBMP-2 increased gene expressions of Sp7, Col1 and ALP on day 3, these effects gradually decreased, and had much less effects on day 14 (Fig. 4), which indicate that the CS-PEI/hBMP-2 can mediate sustained hBMP-2 synthesis. BMP-2 protein is a secreted protein which can induce osteogenic differentiation though direct effects and paracrine way to influence adjacent neighbor cells.[39, 40] It is known that Col 1 is a major protein for bone formation and repair.[41] SP7 is a transcription factor and an important indicator for osteogenic differentiation.[42] ALP activity is another powerful indicator for osteogenic differentiation.[43] Alizarin Red staining is a useful biochemical assay to quantitatively determine calcific deposition, an important step towards formation of calcified extracellular matrix to convert real bone. The increased hBMP-2 herein clearly induced osteogenic differentiation and calcium deposition increase in the MC3T3-E1 cells (Fig. 4). This indicates that CS-PEI/hBMP-2 nanoparticles effectively deliver hBMP-2 into MC3T3-E1 cells, and induce MC3T3-E1 cells to differentiate into osteoblast *in vitro*.

It is well-known that BMP-2 can induce stem cell homing and differentiation of osteoblasts, and even induce ectopic ossification.[1, 30] In our current study, new bone formation was significantly increased at the bone defect area of CS-PEI/hBMP-2 nanoparticle treated rats 12 weeks post-implantation without any observable histological change in liver, spleen, kidney or heart (Fig. 5). Our *in vitro* data indicate that chitosan has the potential to stimulate cell proliferation (Fig. 2). As seen in Fig. 5, we also noticed that there were some cuboidal shaped osteoblasts around the new bone area 12 weeks post-implantation with CS-PEI/BMP-2 nanoparticles (Fig. 5). Taken together, our data suggest that with CS-PEI/BMP-2 nanoparticle it is possible to directly promote osteoblast proliferation and also transfect local stem cells/osteoblast cells to promote new bone formation although more experiments need to be performed in order to understand *in vivo* mechanisms directly.

Our *in vivo* data (Fig.5) showed that that new bone formation (~40% of new bone formation in the bone defect area) was not significantly different between gelatin sponge and gelatin sponge loaded with CS-PEI/hBMP-2 nanoparticle or recombinant hBMP-2 6 weeks post-implantation, but was significantly different 12 weeks post-implantation. The reason for this delayed effect is not known. One possible reason may be due to two bone defects in each rat, which could cause a much higher burden to repair leading to delay healing. Second is that the size of the gelatin sponge may be not appropriate. The bigger gelatin sponge could cause early inflammatory responses and longer degradation time. On the other hand, the longer degradation time could meet a slow release requirement. Therefore, we will consider both factors to further modify our rat model and gelatin sponge to search for the optimum condition in our future study.

We understand that our current version of CS-PEI/hBMP-2 still needs further modification because it cannot fully repair bone defect on week 12. In this study, we used PEI 1.8 KDa, which is a much smaller molecular weight, to create CS-PEI/hBMP-2 nanoparticle with no cytotoxicity. Therefore, we will use different higher molecular weights of PEI to create CS-PEI/hBMP-2 in future experiments to evaluate if it still has no or lower cytotoxicity and significantly increases transfection efficiency.

5. Conclusion

Our newly synthesized CS-PEI/hBMP-2 nanoparticle, 162 nm at size with 24 mV of zeta potential, effectively transfects MC3T3-E1 cells *in vitro* without any cytotoxicity resulting in induced MC3T3-E1 cell differentiation *in vitro*. Interestingly, CS-PEI/hBMP-2 nanoparticle does not retain disadvantages of lower transfection efficiency from chitosan and cytotoxicity from PEI. More importantly, bone defect area is significantly decreased after 12 weeks post-implantation with CS-PEI/hBMP-2 nanoparticles. Our data suggest that CS-PEI/hBMP-2 nanoparticle has potential application in future bone defect treatment.

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