Electronic Supplementary Information (ESI)

Morpholino-functionalized phosphorus dendrimers for precision regenerative medicine: Osteogenic differentiation of mesenchymal stem cells

Aijun Li,[a,†] Yu Fan,[a,†] Xueyan Cao,[a,†] Liang Chen,[a,e] Le Wang,[b] Carla S. Alves,[c] Serge Mignani,[c,d] Jean-Pierre Majoral,[e,*] Helena Tomás,[c,*] and Xiangyang Shi,[a,c,*]

[a] State Key Laboratory for Modification of Chemical Fibers and Polymer Materials, International Joint Laboratory for Advanced Fiber and Low-dimension Materials, College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai 201620, People's Republic of China

[b] College of Chemistry and Chemical Engineering, Shanghai University of Engineering Science, Shanghai 201620, People's Republic of China

[c] CQM-Centro de Quimica da Madeira, Universidade da Madeira, Campus da Penteada, 9020-105 Funchal, Portugal

[d] Université Paris Descartes, PRES Sorbonne Paris Cité, CNRS UMR 860, Laboratoire de Chimie et de Biochimie Pharmacologiques et Toxicologique, 45, rue des Saints Peres, 75006 Paris, France

[e]Laboratoire de Chimie de Coordination du CNRS, 205 route de Narbonne, BP 44099, F-31077 Toulouse Cedex 4, France

---

† These authors equally contributed to this work.

*Corresponding author. E-mail: jean-pierre.majoral@lcc-toulouse.fr (J. P. Majoral), lenat@uma.pt (H. Tomás) and xshi@dhu.edu.cn (X. Shi)

Experimental section

Materials and reagents. Phosphorus dendrimer (G2-P(S)Cl_2) was prepared and characterized according to the literature.[1] Morpholine was purchased from J&K Scientific (Shanghai, China). MSCs originated from rat bone marrow were friendly provided by Shanghai Sixth People's Hospital (Shanghai, China). Low-glucose Dulbecco's modified Eagle's medium (L-DMEM), fetal bovine serum (FBS), penicillin, phosphate buffer saline (PBS), and streptomycin were purchased from Gibco (Carlsbad, CA). Dexamethasone (DEX), β-glycerophosphate (β-GP), ascorbic acid (AA), silver nitrate, sodium thiosulfate, *p*-nitrophenyl phosphate, and *p*-nitrophenol standard were obtained from Sigma-Aldrich (St. Louis, MO). BCA Protein Assay Kit was purchased from Shanghai Yeasen Biotechnology Company (Shanghai, China). The QuantiChrom Calcium Assay Kit was purchased from Bioassay Systems (Hayward, CA). All other chemicals with reagent grade were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The water used in all the experiments was purified using a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA) with a resistivity higher than 18 MΩ·cm. The acetylated generation 5 poly(amidoamine) dendrimers (G5.NHAc) were synthesized and characterized according to the literature.[2]

Preparation and characterization of G2-Mor$^+$ dendrimer. G2-Mor$^+$ dendrimer was synthesized according to the literature.[3] Phosphorus dendrimer G2-P(S)Cl_2 (246 mg, 51.4 mmol) and anhydrous magnesium sulphate (2 g, 8.3 mmol) were mixed in 20 mL THF. To this cooled solution (0 °C), DIPEA (*N,N*-Diisopropylethylamine) (318.9 mg, 2467.2 mmol) was added followed by the slow addition of 4-(2-aminoethyl) morpholine (192.7 mg, 1480 mmol). The mixture was stirred overnight at room temperature, then filtered and evaporated under vacuum. The obtained solid residue was extracted with dichloromethane (100 mL) and 10% K_2CO_3 solution (w/v) in water (50

mL). Then, the organic layer was dried over anhydrous magnesium sulphate and evaporated under reduced pressure. The residue was dissolved in 10 mL of tetrahydrofuran (THF) and precipitated with 100 mL pentane. The resulting solution was stirred for 1 h, then filtered under argon to give a yellow powder. The yellow powder was dried under vacuum, affording the formation of G2-Mor dendrimer (basic form) in 90% yield (316.6 mg). Finally, G2-Mor dendrimer (315 mg, 46 mmol) was dissolved in 20 mL of THF under 0 °C. Then, hydrogen chloride solution (1 M in diethyl ether, 1104 mmol, 1.1 mL) was added under stirring overnight. The solvent was evaporated under vacuum for 6 h to give the G2-Mor^{+} dendrimer with 95% yield.

Cell culture and osteogenic differentiation of MSCs. MSCs were cultured in a humidified incubator with 5% CO_2 at 37 ^{o}C using L-DMEM supplemented with 10% FBS, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin (normal growth medium). MSCs were culture-expanded in L-DMEM with normal growth (N) medium changed every 2-3 days. For the osteogenic differentiation experiments, passages 4–6 were used for the studies. The cells were trypsinized and seeded in 24-well plates at a density of 5×10^3 cells per well in normal growth medium. After 24 h, the medium was replaced by osteoconductive (OC) or osteoinductive (OI) medium. OI medium was supplemented with 1% AA (5 mg mL^{-1} in PBS), 1% β-GP solution (1 M in PBS) and 10^{-7} M DEX to induce osteogenic differentiation of MSCs. OC medium was supplemented with β-GP and AA without DEX. Cells were then cultured for 21 days with the medium containing the G2-Mor^{+} (12 μg/mL) or G5.NHAc (12 μg/mL) being changed twice a week. We used N and OC medium without nanoparticles (NPs) as negative controls as well as OI medium as a positive control.

Cytocompatibility evaluation. CCK-8 assay was employed to evaluate the cytocompatibility of the MSCs cultured with G2-Mor^{+}. Cells were plated in 96-well plates at 8×10^3 cells per well.

Twenty-four hours after plating, the cells were treated with a 10% volume of G2-Mor^{+} at concentrations of 0, 50, 100, 500, 1000, 2000, 3000 or 5000 nM. Viability was assessed after 48 h treatment by CCK-8 assay. The CCK-8 assay was performed as described by the manufacturer's protocol (7Sea Pharmatech Co., Ltd., Shanghai, China). In brief, media were removed and replaced by 90 μL fresh culture medium and 10 μL CCK solution in each well. Samples were incubated at 37 ^{o}C for 2 h. Samples were thoroughly mixed and the absorbance was measured at 450 nm.

Protein content assay. BCA Protein Assay Kit was used to quantify the protein content of each cell sample according to the manufacturer's instructions. After 7, 14 and 21 days of culture, MSCs cultured in a 24-well plate were rinsed 3 times with PBS. Reporter lysis buffer (400 μL) was then added to each well and the cell lysis was carried out according to the manufacturer's instruction. Before analysis the cell lysates were stored at -20 ^{o}C. To analyze the protein content, 20 μL of each cell lysate or protein standards were transferred to a 96-well plate, followed by addition of 200 μL BCA working reagent to each well and incubation of each sample at 37 ^{o}C for 1 h. The absorbance at 540 nm for each well was recorded using a microplate reader (MK3, Thermo, Waltham, MA). The protein content was calculated from a standard calibration curve.

Alkaline phosphatase activity analysis. On days 7, 14, and 21, the alkaline phosphatase (ALP) activity of the MSCs was quantified using a colorimetric assay in which the hydrolysis of *p*-nitrophenyl phosphate to *p*-nitrophenol was monitored. Briefly, the cell culture medium within each well was removed, and the cells were rinsed three times with PBS. Reporter lysis buffer (400 μL) was then added to each well, and the cell lysis was carried out according to the manufacturer's instruction. The cell lysate (20 μL) was then transferred to a corresponding well of a 96-well plate. To each well was then added 200 μL of the ALP substrate (2 mM *p*-nitrophenyl phosphate in

alkaline buffer). After incubation of the samples in the dark for 60 min at 37 °C, 10 μL of 0.02 M NaOH was added to stop the reaction. Finally, the absorbance at 405 nm was read using a microplate reader. The ALP activity of the MSCs under various conditions was then quantified using a standard curve, which was prepared using serial dilutions of *p*-nitrophenol. The amount of protein in the cell lysates was determined by BCA Protein Assay Kit. Results (average of five sample measurements) are expressed as nmol of *p*-nitrophenol produced per min and per mg of protein.

Calcium deposition assay. QuantiChrom Calcium Assay Kit was used to measure the calcium content according to the manufacturer's instructions. Briefly, 5 μL of diluted standards or cell lysate were transferred to a 96-well plate. Followed by addition of 200 μL working reagent and incubation for 3 min at room temperature, the optical density at 612 nm in each well was recorded using a microplate reader (MK3, Thermo, Waltham, MA). The calcium content was calculated from a standard calibration curve.

Osteocalcin secretion assay. Rat osteocalcin ELISA kit (Cusabio Biotech Co, Ltd., Wuhan, China) was used to measure the osteocalcin secretion of MSCs on days 14 and 21. One day before the measurement, the medium was replaced with fresh growth medium or osteogenic medium without FBS. The next day, the medium was transferred to a clean Eppendorf tube, and the osteocalcin content was monitored using the rat osteocalcin EIA kit according to the instructions of the manufacturer.

ALP staining. After 14 days of cell seeding, the alkaline phosphatase (ALP) activity was detected to evaluate the osteogenic differentiation of MSCs with different treatments. An alkaline phosphatase detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to determine the ALP activity following the manufacturer’s instructions. Finally, the samples were

rinsed once with water and observed under a bright-field microscope (Carl Zeiss Axio Vert.A1, Jena, Germany).

Von Kossa staining. Von Kossa staining was used to qualitatively assess the mineralization degree achieved by cells in culture. Briefly, the culture plates were taken out on day 21. After discarding the culture medium, the cells were rinsed three times with PBS and then fixed with 3.7% formaldehyde solution for 15 min at room temperature. After removing the solution, the samples were rinsed with water several times. The cells were subsequently treated with 2.5% silver nitrate solution and exposed to ultraviolet light for 60 min. At this stage, the plates were washed with water and then treated with 5% sodium thiosulfate solution for 3 min. Finally, after washing with water, the plates were examined using a bright-field microscope.

Statistical analysis. To assess the statistical significance of the experimental data, one-way analysis of variance (ANOVA) method was used. The significance level was set at a value of 0.05, and the data were indicated as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, respectively.

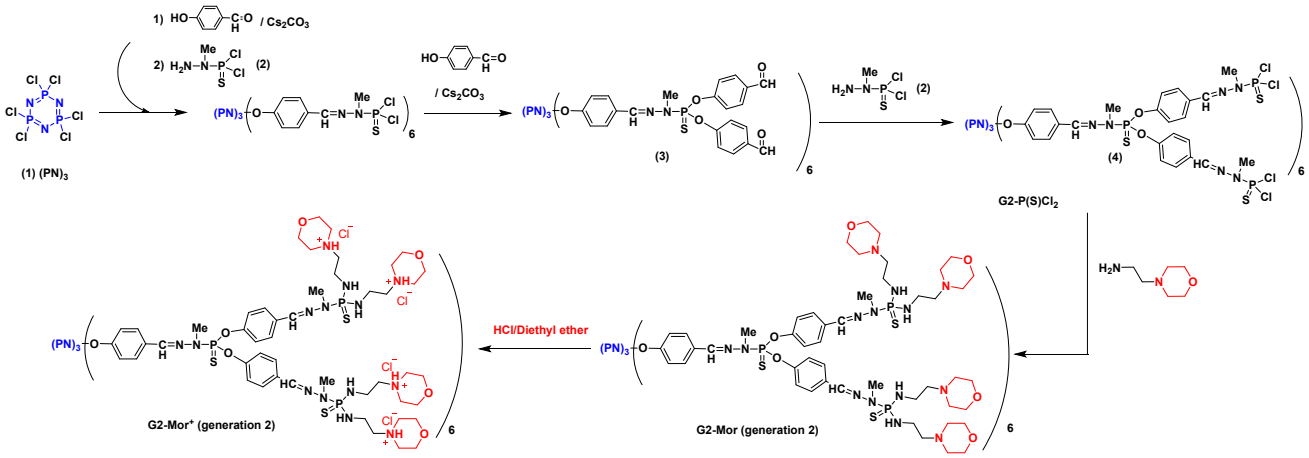

Figure S1. Schematic representation of the synthesis of **G2-Mor**$^{+}$ dendrimer.

Indeed **G2-Mor**$^{+}$ dendrimer was prepared *via* the reiteration of a sequence of two reactions from hexachlorocyclotriphosphazene (1) in 4 steps of synthesis. The first step is a nucleophilic substitution of the 6 Cl of $P_3N_3Cl_6$ (1) in basic condition in the presence of hydroxybenzaldehyde. The second step concerns the condensation of the six aldehyde groups with the dichlorophosphothiohydrazide 2, generating 6 PCl_2 functions at the periphery of the dendrimer suitable to perform again nucleophilic substitution of the Cl with hydroxybenzaldehyde to generate G1 dendrimer (3) bearing 12 aldehyde end groups. The G2 dendrimer possessing 24 Cl at the periphery was prepared by adding the dichlorophosphothiohydrazide to condensate (3). After reaction with morpholinoethanamine, **G2** dendrimer decorated with 24 morpholino units (**G2-Mor**) was formed. Final protonation in the presence of HCl afforded the formation of water-soluble **G2-Mor**$^{+}$ dendrimer. The structures and synthetic route to the polycationic phosphorus dendrimer are schematically illustrated in Fig. S1.

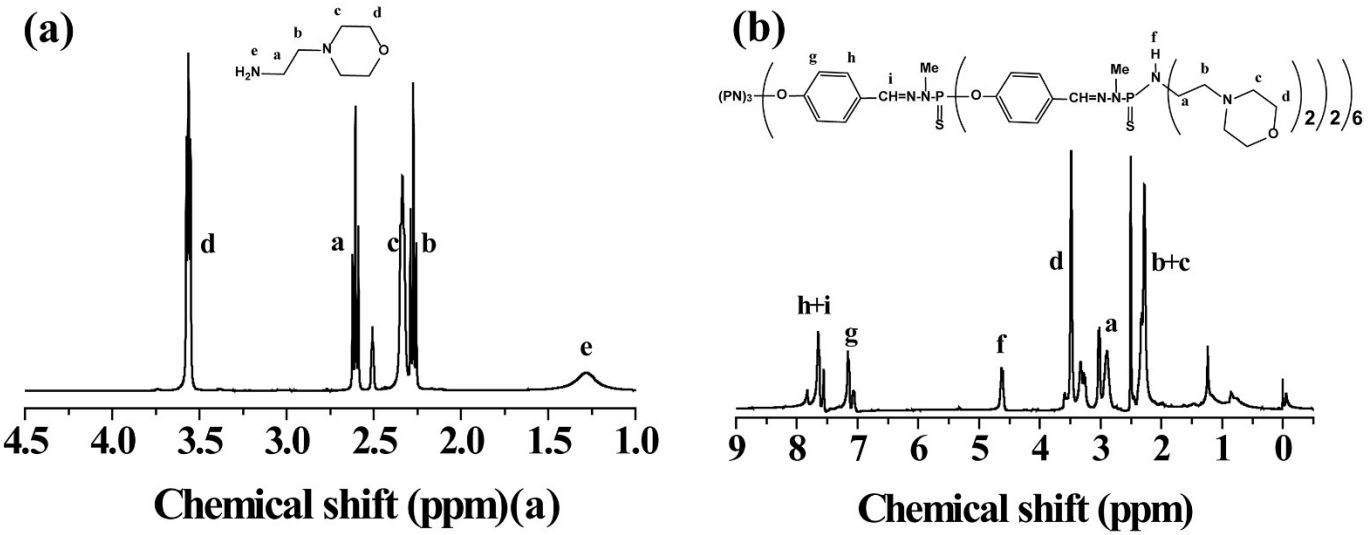

Figure S2. ^{1}H NMR spectra of Mor (a) and phosphorus dendrimers G2-Mor (b).

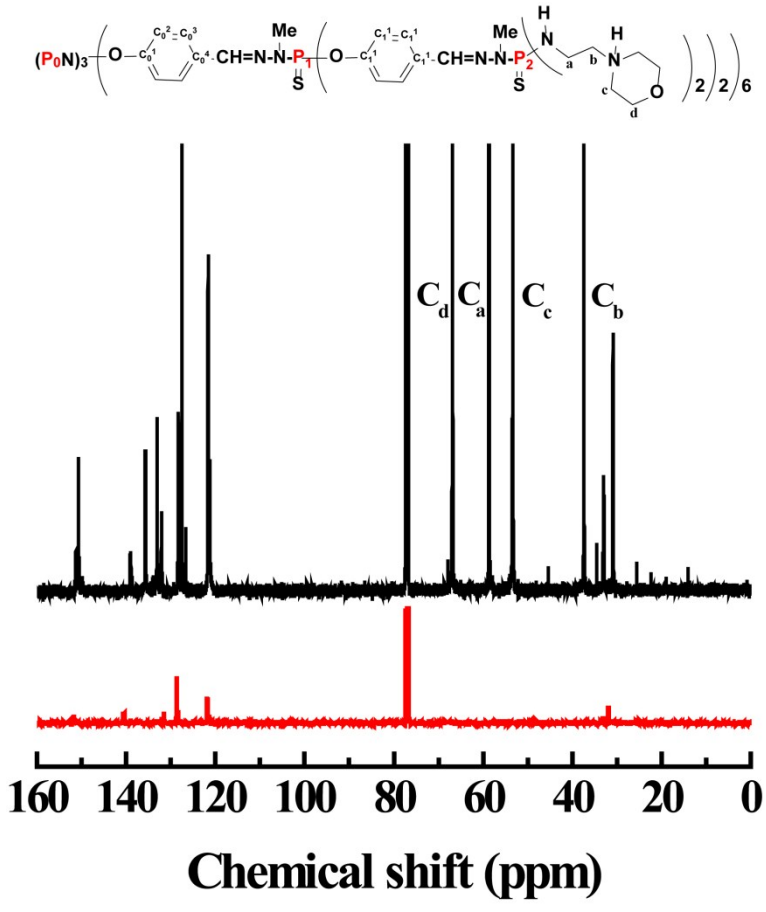

Figure S3. ^{13}C NMR spectra of phosphorus dendrimers G2-P(S)Cl_2 (red line) and G2-Mor (black line).

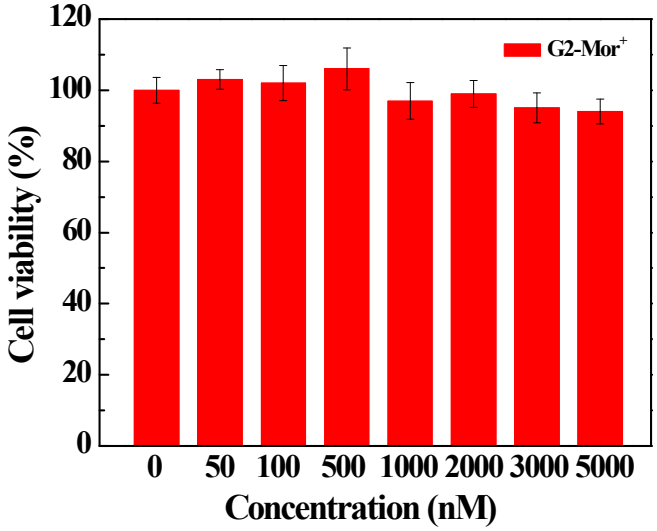

Figure S4. Cell viability of MSCs treated with **G2-Mor**$^{+}$ under different concentrations for 48 h.

Regarding bone tissue engineering applications, it is very important to investigate the cytotoxicity of the NPs in order to develop safe therapies that can be used in clinical trials. As shown in Figure S4, after 48 h incubation, no significant effects stemming from the toxicity of **G2-Mor**$^{+}$ particles were observed for dendrimer concentrations ranging up to 5 μM, at which point MSC viability is close to 100%. These results prompted us to decide to evaluate the phosphorus dendrimer **G2-Mor**$^{+}$ for its activity enhancing the osteogenic differentiation of MSCs as an inducing factor that can trigger the formation of osteoblasts.

References

1. N. Launay, A. M. Caminade and J. P. Majoral, *J. Organomet. Chem.*, 1997, **529**, 51-58.

2. R. B. Kolhatkar, K. M. Kitchens, P. W. Swaan and H. Ghandehari, *Bioconjugate Chem.*, 2007, **18**, 2054-2060.

3. C. Padie, M. Maszewska, K. Majchrzak, B. Nawrot, A. M. Caminade and J. P. Majoral, *New J. Chem.*, 2009, **33**, 318-326.