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

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A novel bioactive macromolecule based on morpholino-functionalized phosphorus dendrimers (generation 2, G2-Mor$^+$) was developed for osteogenic differentiation of mesenchymal stem cells (MSCs). Interestingly, through *in vitro* tests, it was shown that G2-Mor$^+$ dendrimer can strongly promote the transformation of MSCs into osteoblasts, which implies the potential application of phosphorus dendrimers in bone regeneration for precision regenerative medicine.

Large bone defects, accompanied by large losses of tissue, are usually the result of exogenous factors such as fractures, disease, or surgical procedures afflicting the elderly population. This condition has become one of the most frequent cases to treat in the medical field.[1] Trauma, massive avascular necrosis, severe osteomyelitis, advanced osteoporosis, open fractures and bone tumors (*e.g.* osteosarcoma) are the main reasons why therapeutic bone regeneration strategies are required.[2–4] The use of bone grafts is the standard for the treatment of skeletal fractures, or the replacement and regeneration of lost bone can be performed.[5] Autografts, allografts, bone graft substitutes, and the incorporation of bone progenitor cells and growth factors to stimulate cell growth have been developed.[6] Due to the limitations in self-healing for bone, bone tissue engineering including, for instance, implementable bone substitutes for critical skeletal defects and multipotent progenitors, has received extensive attention, and has been considered as a promising therapeutic modality to address these important limitations.[7]

Several bone tissue engineering strategies related to the cells involved in bone formation have been developed.[8] Thus, alongside synthetic biomaterials, a strategy involving the direct placement of cells such as osteoblasts, osteocytes and multipotent stem cells (mesenchymal stem cells, MSCs), into a damaged bone to stimulate bone cell growth on its surface has been developed. Thus, the stimulation of MSCs to differentiate into preosteoblasts to begin the bone-forming process represents a powerful mechanism for the process of bone regeneration. MSCs are critical for bone tissue repair and regenerative medicine, and are a class of cells that possess the potential to self-replicate and differentiate into different phenotypes, including osteoblasts, adipocytes and myocytes.[9] However, focused differentiation of MSCs into a specific cell lineage remains an important challenge in the field of stem cell development.[10]

Adipogenic or osteogenic differentiation potential is an important criteria for the identification of MSCs.[11] In regard to bone tissue engineering, an important goal is to address the osteogenic differentiation lineage of MSCs *in vivo*, an area offering a great promise for improving the bone regeneration and repair processes.[12] Several potent inducers of the osteogenic differentiation of MSCs in osteoblasts, such as dexamethasone, bone morphogenetic proteins and nanoparticles (NPs), have been developed to enhance the levels of adipogenesis and osteogenesis.[13,14] Importantly, MSCs could be differentiated into osteoblasts through the use of chemical and physical stimuli from microenvironment, and different functional NPs have been used to promote osteogenic phenotype of MSCs.[15–17] Yi *et al.* reported that gold NPs promoted

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the osteogenic differentiation lineage of MSCs but not the adipogenic lineage of differentiation.[18] In parallel, Lee *et al.* showed that graphene-based nanomaterials could potentially stimulate the osteogenic differentiation of MSCs and display potential osteoinductive effects.[19] In contrast, Samberg *et al.* showed that silver NPs could not accelerate the differentiation of MSCs.[20] These opposite observations are likely due to the different surface properties of nanomaterials used and their differential interactions with stem cells.

Dendrimers are a specific class of three-dimensional macromolecules with hyperbranched architecture and a repetitive substructure. They have a high degree of molecular uniformity and a perfectly controlled size (1–15 nm), shape and surface chemistry. A wide range of literature reports about dendrimers are available today, showing great promise in biomedical applications, such as bioimaging, gene and drug delivery *etc*.[21–24] For several years, Majoral and Caminade and colleagues have developed biocompatible phosphorus dendrimers that possess tunable control of sizes and shapes (cores and interiors) as well surface functionality. These phosphorus dendrimers represent a versatile platform for drug and gene delivery, as well as function as drugs active *per se* in various therapeutic domains.[25]

Herein, for the first time, we report the development of an original, biocompatible, polycationic-phosphorus dendrimer, named **G2-Mor**$^+$ (generation two bearing on the surface 24 morpholino groups) as a first-in-class NP with activities for spontaneous osteogenic differentiation of MSCs, without any use of osteogenic inducing agents. To our knowledge, this is the first investigation showing that polycationic phosphorus dendrimers (generation 2) are able to enhance osteogenic differentiation of MSCs. Importantly, poly(amidoamine) (PAMAM) dendrimers (diverse generations) bearing on their surface free amine groups could not induce osteogenic differentiation of MSCs,[26] thus enhancing interest in developing phosphorus dendrimers in nanomedicine.

The preparation of **G2-Mor**$^+$ dendrimer (generation 2, 24 morpholino groups on the surface) has been done based on the previous synthetic strategy (Fig. S1, ESI†) for the preparation of the corresponding morpholino-functionalized phosphorus dendrimer (generation 1, 12 morpholino groups on the surface) developed by Majoral *et al.*[27–31] Fig. 1 shows the ^{31}P NMR spectra of phosphorus dendrimers **4** (black line) and **G2-Mor** (red line). Indeed, monitoring the reactions by ^{31}P NMR displays the disappearance of the singlet at 61.95 ppm corresponding to $P(S)Cl_2$ representing a signal corresponding to $P(S)(NHR)_2$ terminal groups at $\delta(^{31}P)$ = *ca.* 68.48 ppm for **G2-Mor**, showing the completion of the substitutions. The ^{31}P NMR chemical shifts are analogous before and after protonation of the terminal amines, thus ascertaining the integrity of the internal structure of **G2-Mor**$^+$. ^{1}H NMR and ^{13}C NMR corroborate the structure of **G2-Mor** and **G2-Mor**$^+$ (Fig. S2 and S3, ESI†). The **G2-Mor**$^+$ dendrimers having an M_w of 7912 were characterized by dynamic light scattering and ζ-potential measurements. Results reveal that the hydrodynamic size and surface potential of **G2-Mor**$^+$ dendrimers are 227 ± 18.4 nm and 23.02 ± 1.84 mV, respectively. The relatively large hydrodynamic size of the dendrimers could be due to the formation of aggregated structures in aqueous solution, while the protonation of the Mor moieties leads to the positive surface potential of the dendrimers.

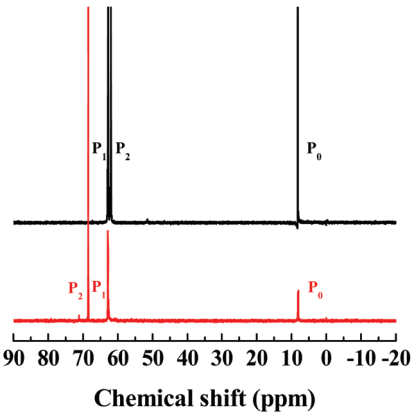

Fig. 1 ^{31}P NMR spectra of phosphorus dendrimer **4** (black line) and **G2-Mor** (red line).

The colorimetric cell counting kit-8 (CCK-8) assay was selected to quantitatively evaluate the cell viability and proliferation of MSCs in the presence of **G2-Mor**$^+$ dendrimer (Fig. S4, ESI†). The cytotoxicity assay data reveal that the **G2-Mor**$^+$ dendrimer is cytocompatible in a concentration range of 50–5000 nM, and consequently, the **G2-Mor**$^+$ dendrimer has a great potential for biomedical applications.

The osteogenic differentiation of **G2-Mor**$^+$ dendrimer was evaluated by analyzing the levels of different biomarkers such as alkaline phosphatase (ALP) activity, calcium production and osteocalcin of MSCs as well as through the formation of mineralized nodules under cell culture conditions. The expression of osteogenic biomarkers in cells cultured with **G2-Mor**$^+$ dendrimer relative to negative or positive controls allowed us to create an excellent image of the profile of the phosphorus dendrimer **G2-Mor**$^+$ on the process of osteogenic differentiation of MSCs. The effect of **G2-Mor**$^+$ dendrimer on the differentiation of MSCs was investigated based on the analysis of osteoconductive (OC) medium that lacked osteoinductive agents such as dexamethasone, but did include both β-glycerophosphate and ascorbic acid, both of which are considered as *in vitro* osteogenic factors and are important for the differentiation process of osteoblastic progenitors as well for the formation of mineralized matrix. Also, another early-stage marker of the osteoblast differentiation process, is the evaluation of the expression of the ubiquitous membrane-bound glycoprotein ALP (a cell-surface, excretory enzyme) activity. ALP catalyzes the hydrolysis of phosphate monoesters at basic pH. ALP activity, on days 7, 14, and 21 was evaluated using a spectrophotometric assay method based on the hydrolysis of *p*-nitrophenyl phosphate to the chromogenic *p*-nitrophenol (absorbance at 405 nm).

Fig. 2 shows the *in vitro* ALP activities of MSCs after they were cultured for 14 days, based on qualitative ALP staining

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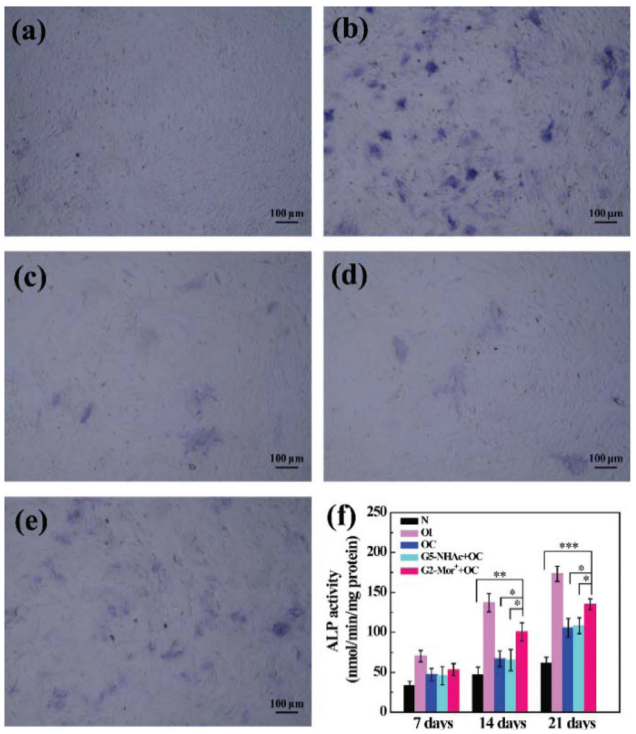

Fig. 2 ALP staining of MSCs after being cultured for 14 days and ALP activity assay. Cells with the normal growth (N) medium treatment was used as a negative control (a). The osteoinductive medium (OI) (normal medium supplemented with β-glycerophosphate, ascorbic acid salts, and dexamethasone) was used as a positive control (b). The osteoconductive (OC) medium (normal medium supplemented with β-glycerophosphate and ascorbic acid) (c) and the addition of G5-NHAc (d) in OC medium were used as control groups. The effect of **G2-Mor$^+$** on the MSCs in OC medium (e). Time course of ALP activity of MSCs (f).

and ALP enzymatic activity assays using (1) normal cell growth conditions (named N and included as a negative control that should not lead to spontaneous osteogenic differentiation of MSCs) (Fig. 2a); (2) osteoinductive medium (named OI, a normal medium supplemented with β-glycerophosphate, ascorbic acid salts, and dexamethasone that is known to be capable of inducing the osteogenic differentiation of MSCs into osteoblasts, used as a positive control) (Fig. 2b); (3) osteoconductive medium (OC, which is a normal medium supplemented with β-glycerophosphate and ascorbic acid inducing osteogenic differentiation of MSCs and also promoting the formation of mineralized matrix in the presence of bioactive materials) (Fig. 2c); (4) OC medium in the presence of acetylated PAMAM dendrimers (G5·NHAc, used as a negative control of the differentiation of MSCs) (Fig. 2d); and (5) OC medium in the presence of **G2-Mor$^+$** dendrimer (Fig. 2e). The normalized time course for the ALP activity (total protein in the cell lysates) of MSCs after 7, 14 and 21 days with or without treatment is shown in Fig. 2f.

No or low enzyme activity based on ALP expression was observed in N medium at 7, 14 and 21 days, indicating that there was no differentiation of MSCs. A slight increase in protein activity after 14 days has been observed in OC medium as a result of the effect of β-glycerophosphate and ascorbic acid. No ALP activity was noted in OC medium in the presence of G5·NHAc dendrimer, which could not promote the osteoblast differentiation of MSCs (Fig. 2d *versus* Fig. 2c). Interestingly, enhanced ALP activity was observed by the addition of **G2-Mor$^+$** dendrimer (12 μg mL^{-1}) at day 14 compared with day 7, whereas a strong effect was observed at day 21 both in OC ($p < 0.05$) and N medium ($p < 0.001$). **G2-Mor$^+$** dendrimer promoted the expression of ALP both in cultured medium and in the extracellular environment (Fig. 2e *versus* Fig. 2b–d). As expected, in the presence of the dexamethasone, which is considered to be a trigger of the osteoblast differentiation, excellent ALP activity was observed. Remarkably, **G2-Mor$^+$** dendrimer can accelerate the osteogenic differentiation of MSCs as shown in both the enzymatic ALP assay and ALP staining assay, while, another dendrimer, G5·NHAc, could not promote the differentiation of MSCs into osteoblasts. It is interesting to note that when the concentration of **G2-Mor$^+$** dendrimers was less than 12 μg mL^{-1}, we did not observe any significant osteogenic differentiation of MSCs. In the dendrimer concentration range of 12–36 μg mL^{-1}, the **G2-Mor$^+$** dendrimer-induced osteogenic differentiation of MSCs showed more or less similar levels.

Next, we aimed to evaluate the process of osteogenic differentiation of MSCs in the late stage based on the production of calcium as a representative biomarker. In order to qualify and quantify the *in vitro* mineralization of the extracellular matrix in cell culture, Von Kossa staining assays of MSCs were performed after treatment for 14 and 21 days. The formation of mineralized dark nodules was observed, along with the production of calcium phosphate crystals, which is associated with the formation of bone structures. Fig. 3a–e show the evaluation of calcium at 21 days, whereas the Fig. 3f highlights the calcium produced by both 14 and 21 days. As shown in the ALP assay (*vide supra*), we compared levels of calcium production in different cell media: (1) normal growth (N) medium as a negative control (Fig. 3a); (2) osteoinductive (OI) medium containing dexamethasone as a positive control (Fig. 3b); (3) osteoconductive (OC) medium (Fig. 3c, *vide supra*); (4) osteoconductive (OC) medium with G5·NHAc dendrimer (Fig. 3d); and (5) in OC medium in the presence of **G2-Mor$^+$** dendrimer (Fig. 3e).

Taken together, these data clearly show that the polycationic **G2-Mor$^+$** phosphorus dendrimer strongly affected the production of calcium in OC medium on both day 14 and day 21 (Fig. 3e), *versus* negative controls such as OI (Fig. 3b) and OC (Fig. 3c) medium, which included the presence or absence of the G5·NHAc dendrimer, respectively (Fig. 3d). Interestingly, the dark region of the MSCs in the presence of the **G2-Mor$^+$** dendrimer is comparable with that of the cells in the OI medium containing dexamethasone, indicating a similar level of osteoblast differentiation of MSCs. It is well known that each osteogenic factor exerts its effect through different signaling pathways. We speculate that the difference between the activities of dexamethasone and **G2-Mor$^+$** dendrimer may be attributed to different specific signaling pathways. These results are in

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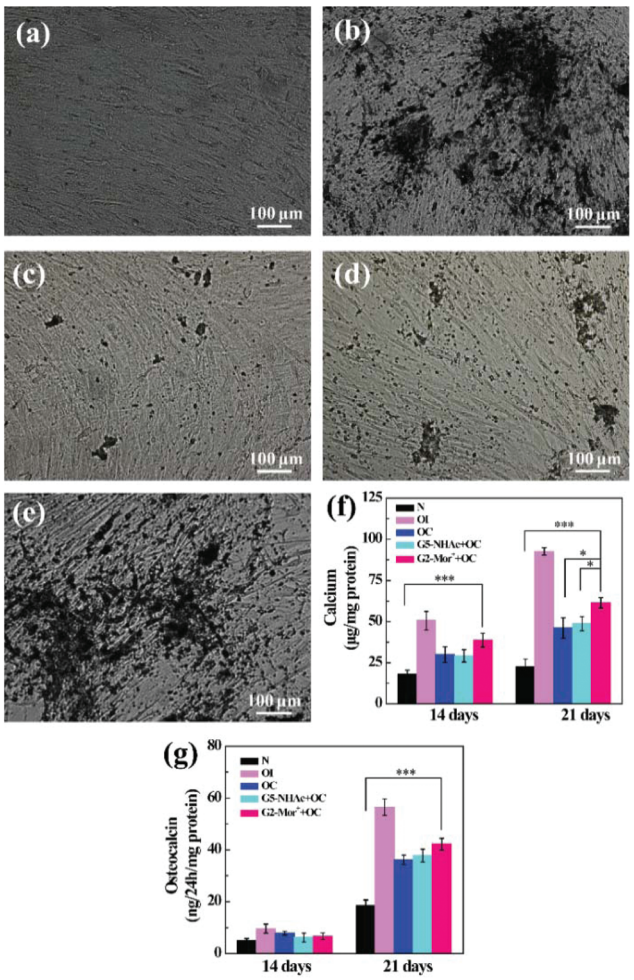

Fig. 3 Von Kossa staining of MSCs treated for 21 days. Cells with the normal growth (N) medium treatment was used as a negative control (a). The osteoinductive (OI) medium containing dexamethasone was used as a positive control (b). The osteoconductive (OC) medium (c) and the addition of G5-NHAc (d) in OC medium were used as control groups. The effect of **G2-Mor**$^+$ on the MSCs in OC medium (e). Calcium deposition of the MSCs with different treatments for 14 and 21 days (f). Osteocalcin content secreted by MSCs with different treatments at different culture time periods (g).

full agreement with those obtained using ALP enzyme assays (*vide supra*), and strongly reinforce observations regarding the osteogenic differentiation effect of the polycationic **G2-Mor**$^+$ phosphorus dendrimer on mesenchymal stem cells.

Another late stage biomarker of the osteogenic differentiation process is the bone gamma-carboxyglutamic acid-containing protein osteocalcin. It is a non-collagenous protein hormone found in the bone matrix, which is expressed by osteoblasts and promotes the maturation of bone. Interestingly, the polycationic **G2-Mor**$^+$ phosphorus dendrimer enhanced the osteocalcin secretion by MSCs in both OI and OC medium at two different time points (14 and 21 days) with and without G5·NHAc PAMAM dendrimer and **G2-Mor**$^+$ phosphorus dendrimer (Fig. 3g). On the day 14, there was no notable difference between different groups. Dramatic increases ($p < 0.05$) in osteocalcin, compared to the control (N) was detected in OC medium supplemented with **G2-Mor**$^+$ dendrimer on day 21. Upregulation of osteocalcin was observed in OC medium at day 21 in the presence **G2-Mor**$^+$ dendrimer in comparison to OC medium alone. In addition, increases in osteocalcin using **G2-Mor**$^+$ dendrimer rather than G5·NHAc PAMAM dendrimer were observed, although the latter expressed relatively more osteocalcin than the control group (the OC medium). Clearly, these results demonstrate that **G2-Mor**$^+$ dendrimer stimulates the expression of osteocalcin when OC medium is used. Also, these results confirmed that **G2-Mor**$^+$ dendrimer can enhance levels of osteoblast differentiation in MSCs without the aid of osteoinductive agents.

We developed an alternative strategy to use morpholino-functionalized phosphorus dendrimers for stem cell osteogenic differentiation. We found that the water-soluble, morpholino-functionalized G2 phosphorus dendrimer (**G2-Mor**$^+$) represents a first-in-class osteogenic differentiation agent for MSCs. Cytotoxicity assay data demonstrated that the **G2-Mor**$^+$ dendrimer is cytocompatible within a given concentration range. The data presented here clearly show that the addition of **G2-Mor**$^+$ dendrimer to a cell culture medium that does not contain any osteogenic inducing factors, leads to relevant osteogenic differentiation of MSCs. This very interesting property of **G2-Mor**$^+$ dendrimer was confirmed *via* quantitative ALP activity, calcium deposition, osteocalcin, and qualitative ALP and Von Kossa staining assays. Our results indicate that the development of phosphorus dendrimers that stimulate the transformation of stem cells into osteoblasts is feasible. The developed functionalized phosphorus dendrimer may be used for potential applications in bone tissue engineering and regenerative medicine. Extension to the use of higher generations of such dendrimers is underway. In addition, the mechanism of **G2-Mor**$^+$ on the function of MSCs needs to be extensively investigated before drawing broad and general conclusions. On the basis of the literature related to NP-involved osteogenic differentiation,[18,32] the possible mechanism behind could be that **G2-Mor**$^+$ may interact with osteogenic differentiation-associated protein located in the cytoplasm or surface of MSCs, which would activate certain cellular signaling pathways.

Conflicts of interest

There are no conflicts to declare.

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