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Receptor-Mediated Gene Delivery Using PAMAM Dendrimers Conjugated with Peptides Recognized by Mesenchymal Stem Cells

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Abstract: As mesenchymal stem cells (MSCs) can differentiate into multiple cell types, the delivery of exogenous genes to this type of cell can be an important tool in tissue regeneration and engineering. However transfection of MSCs using nonviral gene delivery vectors is difficult, the development of more efficient and safe DNA vehicles being necessary. Moreover, specific transfection of MSCs may be required to avoid unwanted side effects in other tissues. In this study, a novel family of gene delivery vectors based on poly(amidoamine) (PAMAM) dendrimers functionalized with peptides displaying high affinity toward MSCs was prepared. The vectors were characterized with respect to their ability to neutralize, bind and compact plasmid DNA (pDNA). The complexes formed between the vectors and pDNA were analyzed concerning their size, ζ-potential, capacity of being internalized by cells and transfection efficiency. These new vectors exhibited low cytotoxicity, receptor-mediated gene delivery into MSCs and transfection efficiencies superior to those presented by native dendrimers and by partially degraded dendrimers.

Keywords: PAMAM dendrimers; DNA; gene delivery; cell recognition; mesenchymal stem cells

Introduction

Bone marrow derived mesenchymal stem cells (MSCs) are multipotent cells that can differentiate into a variety of cell types (including cells from the osteogenic, chondrogenic, myogenic and adipogenic lineages), thus being of key importance in the field of tissue regeneration and engineering.[1,2] When devising strategies for therapeutic applications, the introduction of exogenous genes in these cells is often desirable, as their expression can influence cell mitosis and differentiation. Viral systems, because of their sophisticated machinery, are by far the most effective nucleic acids delivery carriers but, besides being refractory to repeated infections, may raise safety problems such as acute toxicity, immunogenicity, and oncogenicity.[3−5] Nonviral vectors, on the other hand, have been shown to present a limited success

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in primary cell transfection, as is the case of MSCs. It is thus necessary to develop more efficient and safe vectors for transfection of MSCs.[6,7] Additionally, specific transfection of MSCs may be required to avoid unwanted side effects in other tissues, making the design of gene delivery vectors for cell targeting an important challenge.

Dendrimers have extensively been investigated as gene delivery vectors due to their unique characteristics.[8−11] Dendrimers are hyperbranched molecules that present well-defined three-dimensional architectures (they are organized in layers named generations), molecular weights, and size, as well as a multivalent-functionalized terminal surface.[8] In particular, poly(amidoamine) (PAMAM) dendrimers, with primary amine termini and tertiary internal amines, are protonated at physiological pH and can bind, condense and deliver the anionic deoxyribonucleic acid (DNA) molecule into a variety of cells.[8−11] It is believed that the primary amine groups at the periphery bind and compact DNA, thus promoting its cellular uptake, while the buried tertiary amino groups act as a proton sponge in endosomes, thus enhancing the release of DNA into the cytoplasm.[11] However, the literature shows that PAMAM dendrimer transfection efficiency strongly varies with the type of cells.[12]

Herein, we report the synthesis, characterization and evaluation of novel gene delivery vectors based on poly(amidoamine) dendrimers conjugated to peptides recognized by receptors at MSCs membrane. Indeed, very recently, we have demonstrated that PAMAM dendrimers (generations 5 to 7), although showing low transfection efficiency, were able to deliver the hBMP-2 gene into MSCs, thus promoting in vitro osteogenesis.[13] We then hypothesized that, by functionalizing dendrimers with peptides recognized by receptors at MSCs membrane, transfection efficiency could be improved and MSCs targeting achieved. A low affinity MSCs binding (LAB) peptide and a high affinity MSCs binding (HAB) peptide were used in the studies, based on a previously published patent.[14] With this biomimetic approach, novel gene delivery vectors were designed that exhibit low cytotoxicity and receptor-mediated gene delivery into MSCs. Transfection efficiencies were superior to those presented by native dendrimers and by Superfect (SF, a commercially available gene delivery system made of partially degraded PAMAM dendrimers).

Table 1. Average Number of PDP and of Peptides per Dendrimer, Respectively, after Reactions A and B

peptide	av no. of PDP per dendrimer (after reaction A)	av no. of peptides per dendrimer (after reaction B)	mol wt[a]
NSMIAHNKTRMHGGGSC	2.1	2.1	31008
(LAB peptide)	4.0	3.9	34866
SGHQLLLNKMPNGGGSC	2.2	2.1	30830
(HAB peptide)	4.1	4.1	34510
	8.2	8.1	41870

[a] Theoretical value based on the molecular weight of dendrimers provided by the supplier.

Materials and Methods

Materials and Reagents. Generation 5 poly(amidoamine) PAMAM dendrimers (G5) ethylenediamine-cored were obtained from Dendritech Inc. (Midland, MI). Cysteine-modified low affinity binding and high affinity binding peptides[14] (LAB- and HAB-peptides, with molecular weights 1842 and 1753, respectively) were synthesized with a purity of 80−90% and characterized by HPLC and MS at the University of Virginia Biomolecular Research Facility (Charlottesville, VA). The primary structure of the peptides is shown in Table 1, and molecular structures are supplied as Supporting Information. Plasmid DNA (pDNA) encoding enhanced Green Fluorescent Protein and Firefly Luciferase (pEGFPLuc, 6.4 kb) with a cytomegalovirus promoter (CMV) was generously provided by Prof. Tatiana Segura (Department of Chemical and Biomolecular Engineering, UCLA, Los Angeles, CA). The plasmid was purified from *Escherichia coli* cultured overnight using the GenElute HP Endotoxin-Free Plasmid Megaprep Kit and stored in ultrapure water at −20 °C. All other reagents used, if not specified, were obtained from Sigma-Aldrich Co. and used without further purification. Cell culture dishes were from Nunc.

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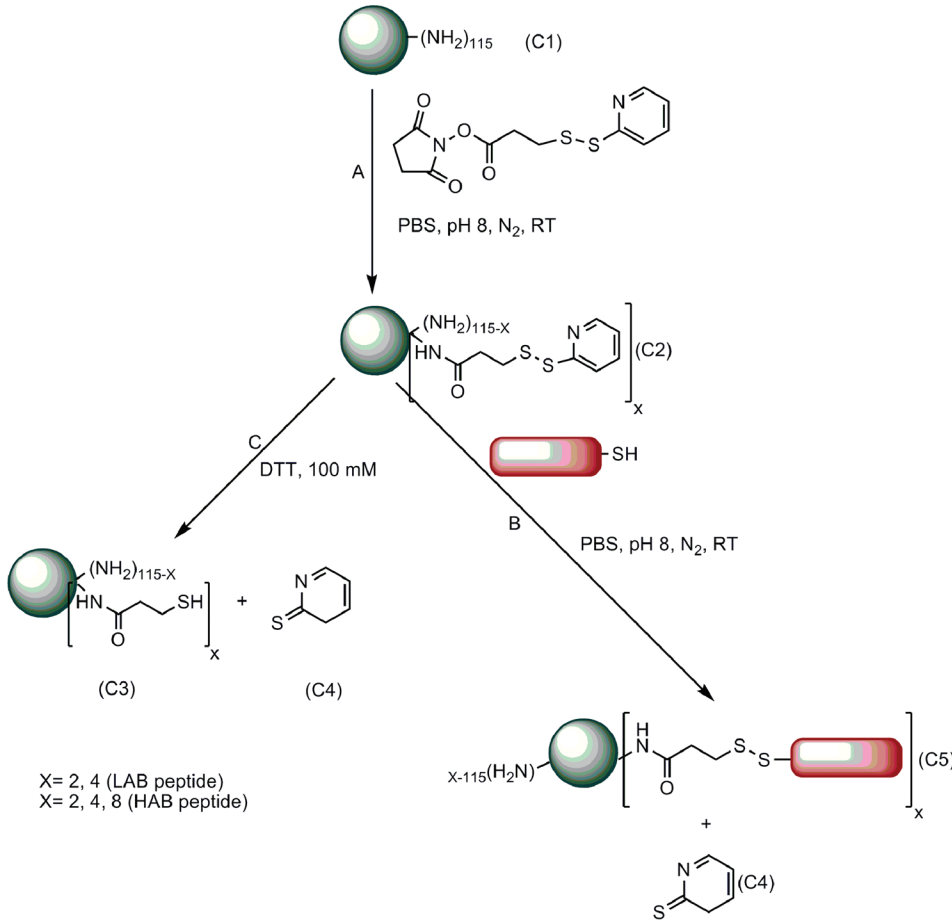

Figure 1. The two-step reaction for the synthesis of peptide-functionalized G5 PAMAM dendrimers (pathway A + B), and for indirect estimation of the number of peptide units by spectrophotometry (pathway A + C). Note: The amine content of G5 PAMAM dendrimers (115 amines/dendrimer) was previously determined experimentally.

Experimental Determination of Primary Amine Group Content of Dendrimers. The primary amine group content of G5 PAMAM dendrimers was determined by spectrophotometry after reaction of the free amine groups with 2,4,6-trinitrobenzene sulfonic acid (TNBS) as described in the literature,[15] and using glycine as standard. A total of 115 amine groups was attributed to each dendrimer, this value being in agreement with the mass spectrometry data furnished by Dendritech Inc. Based on this result, when needed, the TNBS method was always used to calculate the concentration of both nonfunctionalized and functionalized dendrimers. In both cases the standard solutions and the sample solutions were serially diluted in 0.1 M sodium tetraborate to a final volume of 1 mL (the concentration range was 0−40 μg/mL). To each standard and sample 25 μL of TNBS (0.03 M) diluted in water was added. After 15 min at rt, absorption was measured at 420 nm in a GBC-Cintra 40, UV−visible spectrophotometer.

Synthesis and Characterization of Peptide-Functionalized G5 PAMAM Dendrimers. In a first step, G5 PAMAM dendrimers (compound 1, C1) were substituted with various amounts of the bifunctional 3-(2-pyridyldithio)-propionic acid *N*-hydroxysuccinimide ester (SPDP), as described earlier by Szoka et al.[16] and schematized in Figure 1. G5 PAMAM dendrimers (0.50 μmol) in 2 mL of buffer (0.25 M NaCl, 0.1 M phosphate, pH 8) were mixed with SPDP (1−4 μmol) dissolved in 250 μL of ethanol. The reaction was carried out at rt and under a nitrogen atmosphere. Several compounds were obtained (compound 2, C2) by varying the molar ratio dendrimer/pyridyldithiol (PDP) (1/2, 1/4 and 1/8). The mixtures were allowed to react for 3 h, at rt, under nitrogen and with continuous stirring. Afterward, low molecular weight products were removed by gel permeation chromatography on a PD-10 column (GE Healthcare) equilibrated in 0.15 M NaCl, 0.1 M phosphate buffer, pH 7.4. The fractions containing dendrimers linked to PDP were pooled, concentrated, snap frozen in liquid nitrogen and stored at −80 °C. The content in C2 was estimated by TNBS assay as described previously. The degree of modification with the SPDP linker was evaluated spectrophotometrically at 343 nm by the release of pyridine-2-thione (compound 4, C4) after reduction of an aliquot with

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excess dithiothreitol (DTT, 0.1 M) which gave compounds 3 and 4. As calibration standards, known concentrations of SPDP solutions were treated with 0.1 M DTT under the same conditions, and the changes in absorbance were used as a measure of pyridine-2-thione (C4) in solution. In a second step, cysteine-modified LAB and HAB peptides were dissolved in acetic acid (10% v/v) and mixed, under nitrogen, with C2 diluted in 500 mM phosphate buffer, pH 8. An excess of peptide (1.8- to 2.2-fold) to PDP present in C2 was used to increase reaction yields. After 3 h at rt, the amount of released pyridine-2-thione (C4) was measured at 343 nm to determine the extent of reaction. Peptide-functionalized dendrimers (compound 5, C5) were purified by gel filtration using PD-10 column equilibrated in 0.5 M NaCl, 20 mM sodium acetate, pH 5. Conjugates were applied to the column and the void fractions containing the conjugates were dialyzed against phosphate buffered saline (PBS) solution, pH 7.2, using dialysis tubes with a molecular weight cutoff of 10 kDa (Spectrum Laboratories) for 3 days. After dialysis, conjugates were sterile filtered and aliquots were snap frozen in liquid nitrogen and stored at −80 °C. Conjugates were characterized by ^{1}H NMR using D_2O as solvent in a Bruker 400 MHz Avance II + NMR spectrometer.

Polyplex Preparation. Peptide-functionalized dendrimers/pDNA complexes were prepared at several *N*:*P* ratios (where *N* = number of primary amines in the conjugate; *P* = number of phosphate groups in the pDNA backbone) by mixing equal volumes of the conjugate solution and the pDNA solution. Conjugate and pDNA concentrations varied according to the experiments. Hepes-buffered glucose (HBG, Hepes 10 mM, glucose 5% w/w, pH = 7.1) was always used to prepare these solutions unless otherwise stated. Polyplex solutions were vortexed gently and allowed to incubate for 20 min at room temperature prior to experiments. The generation 5 of PAMAM dendrimers was always used as a control. Superfect (Qiagen, Germany) was also used as a reference in gene delivery experiments. Superfect polyplexes were prepared according to the manufacturer's instructions for transfection of primary cells.

Agarose Gel Electrophoresis Retardation Assay. Gel electrophoresis in agarose gels was carried out at 75 V. Agarose gel (0.7% w/v) containing ethidium bromide (0.05 μL/mL) was prepared in Tris-acetate-EDTA buffer. Polyplex solutions were prepared at different *N*:*P* ratios, as described above, using 1 μg of pDNA diluted in 50 μL of buffer. Before subjecting the samples to gel electrophoresis, 2.5 μL of Blue Loading Buffer (Fermentas, Germany) were added. Functionalized dendrimer−pDNA interaction is shown by a lack of migration of the pDNA in the electrophoretic field.

PicoGreen Intercalation Assay. 200 μL of polyplex solutions at different *N*:*P* ratios were prepared as described above using 0.1 μg of pDNA and an adequate amount of peptide-functionalized dendrimer, both diluted in 100 μL of Hepes-buffered saline (HBS, 10 mM HEPES, 150 mM NaCl, pH 7.0). Then, 200 μL of PicoGreen (PG, Molecular Probes) reagent, diluted in Tris-EDTA buffer (TE, 10 mM Tris, 1 mM EDTA, pH 7.5), was added and mixtures were further incubated for 5 min. Three independent experiments were performed. PG fluorescence (λ_{ex} = 485 nm, λ_{em} = 535 nm) was measured using a microplate reader (model Victor^3 1420, PerkinElmer). The relative fluorescence (in %) was determined using the following equation:

$$\%\,F = 100 \times \frac{F_{\text{sample}} - F_{\text{blank}}}{F_{\text{DNAonly}} - F_{\text{blank}}}$$

Dynamic Light Scattering and Zeta Potential Measurements. The size of the polyplexes was measured at 633 nm on a dynamic light scattering instrument (Zetasizer Nano ZS, Malvern Instruments). Solutions (100 μL) of polyplexes were prepared at an *N*:*P* ratio of 5, as described above, using 5 μg of pDNA diluted in 50 μL of HGB. The solutions were then diluted by adding 700 μL of HGB. Particle sizes were determined for these initial solutions at room temperature with a detection angle of 173°. Zeta potential measurements were performed using the same instrument with a detection angle of 17°. Zeta potentials were calculated using the Smoluchowsky model for aqueous suspensions. The data presented are means of three independent sample measurements.

Isolation and Culture of Rat Bone Marrow Derived Mesenchymal Stem Cells. Rat bone marrow derived MSCs were isolated from long bones of 8-week-old male Wistar rats. Following euthanasia by pentobarbital 20% (v/v), femora were aseptically excised, cleaned of soft tissue, and washed in PBS. The metaphyseal ends were cut off, and the marrow was flushed out from the midshaft with 5 mL of α-minimum essential medium (α-MEM) using a 23-gauge needle and syringe. The cells were centrifuged (600*g*, 5 min), suspended in fresh medium containing 10% heat-inactivated fetal bovine serum (FBS, Gibco), 50 μg/mL ascorbic acid, 100 U/mL penicillin and 100 μg/mL of streptomycin—the basic medium—and seeded in 75 cm^2 flasks. After removal of nonadherent cells and medium exchange at day 3, cells were harvested at day 7 by trypsinization, and used in subsequent experiments.

Cellular Uptake Studies by Fluorescence-Activated Cell Sorting (FACS). Cells were seeded in 12-well plates at a density of 1.25 × 10^4 cell/cm^2 and incubated in basic medium at 37 °C, 5% CO_2, for 24 h, to yield a cell confluency of around 60−70%. Prior to polyplex formation, pDNA was labeled with PicoGreen dye (Molecular Probes) according to the manufacturer's directions. Polyplex solutions (100 μL) were prepared at an *N*:*P* ratio of 5 as previously described. Cells in 0.5 mL of basic medium were then transfected using 100 μL of the polyplex solution (2 μg/cm^2 of pDNA was used). The cells were incubated with each solution for 2 h and then rinsed twice with PBS. The extracellular fluorescence associated with cell surface-bound nanoparticles was quenched with 0.4% (w/v) Trypan Blue (TB) for 5 min. The cells were trypsinized, pelleted, and resuspended in 400 μL of PBS containing 2% FBS for FACS analysis (Cytomics FC500, Beckman Coulter). Twenty thousand events were collected in triplicate for each sample.

Gating and analysis was performed using CXP software analysis program, using PG-labeled naked pDNA transfected cells as the primary negative control. The positive fluorescence level was established by visual inspection of the histogram of the negative control, such that less than 1% of positive cells appeared in the positive region. Results are expressed as the percentage of fluorescent cells and the fluorescence intensity per cell (the mean value). Three independent experiments were performed.

Intracellular Trafficking of pDNA. Fluorescence microscopy was used to study the intracellular trafficking of pDNA. Prior to polyplex formation, pDNA was labeled with rhodamine isothiocyanate (RITC) by a slight modification of a reported method.[17] Briefly, 0.1 mg of pDNA diluted in 297 μL of sodium carbonate-buffered solution (0.1 M, pH 9.0) was mixed with 3 μL of RITC solution (100 mM solution prepared in dimethyl sulfoxide) at room temperature, for 3 h. The RITC-labeled pDNA was separated from residual RITC by gel filtration using a PD-10 column (GE Healthcare), followed by ethanol precipitation to obtain the RITC-labeled pDNA. Twenty-four hours prior to transfection, cells were seeded at a density of 1.25×10^4 cell/cm^2 in 24-well plates containing collagen-treated coverslips. Before contact with polyplexes, medium was exchanged for fresh basic medium. Transfection was carried out with complexes prepared at an *N*:*P* ratio of 5 using 1 μg/cm^2 RITC-labeled pDNA. The distribution of pDNA inside cells was analyzed 2 and 4 h after transfection. Thirty minutes prior, the acidic late endosome and lysosome compartments were stained with the addition of LysoSensor DND-189 dye (Molecular Probes) to the medium at a final concentration of 1 μM. After quenching with 0.4% (w/v) TB, cells were washed twice with PBS and fixed with 3.7% (v/v) formaldehyde prepared in PBS at rt for 10 min. Cell nuclei were then stained using a 300 nM 4′,6-diamidino-2-phenylindole (DAPI) solution for another 10 min. Cells were washed several times with PBS and stored at 4 °C (protected from light). Fluorescence images were acquired using a Nikon Eclipse TE 2000E inverted microscope equipped with a 100× NA 0.5−1.3 Plan Fluor objective.

Gene Delivery Studies. Gene delivery was studied based on reporter gene expression (Luciferase, Luc, and Enhanced Green Fluorescent protein, EGFP).

Expression of the Luc Gene. Two independent experiments were performed, and all samples were performed in triplicate. Cells were seeded at 1.25×10^4 cell/cm^2, in 24-well plates, 24 h prior to transfection. At the time of transfection, cells reached 60−70% confluence. Before contact with polyplexes, medium was exchanged for 0.5 mL of fresh basic medium. Polyplex solutions (100 μL) were then added to the cells and, after 4 h, the culture medium was again replaced with fresh medium. Transfection was carried out at an *N*:*P* ratio of 5 and using 1 and 2 μg/cm^2 pDNA. Nontransfected cells and cells transfected with naked pDNA were used as negative controls. At different time points (48, 72, and 96 h) after transfection, the medium was removed and the cells were washed with PBS solution and treated with 100 μL of reporter lysis buffer (Promega). Cell lysates were analyzed for luciferase activity with Promega's luciferase assay reagent in triplicate (following the supplier's instructions). For each sample, the microplate reader (model Victor^3 1420, PerkinElmer) was set for 3 s delay with signal integration for 10 s. The amount of protein in cell lysates was determined using the bicinchoninic acid assay (BCA assay) with bovine serum albumin as a standard. The gene delivery efficiency of each sample was characterized by firefly luciferase expression and denoted as relative light units (RLU). Specific receptor-mediated gene delivery was confirmed by saturating cell receptors with the HAB peptide prior to transfection. Cells were incubated with a 100 μM peptide solution for 1 h. Subsequently, cells were washed twice with PBS and fresh basic medium was added. Polyplexes based on HAB peptide functionalized dendrimers were then added (a pDNA concentration of 1 μg/cm^2 was used in these assays) and allowed to incubate for 4 h. Luciferase activity was assessed 48 h post-transfection.

Expression of the EGFP Gene. Enhanced Green Fluorescent protein expression studies were carried out as mentioned above for the Luc gene expression. Twenty-four hours after transfection, cells were observed with an inverted fluorescence microscope (Nikon Eclipse TE 2000E) equipped with a cold Nikon camera. Digital image recording and image analysis were performed with the NIS Elements Advanced Research (version 2.31) software.

Cytotoxicity Studies. The cytotoxicity of the gene delivery vectors (alone) and of the polyplexes they form with pDNA was studied. Cytotoxicity was evaluated by determining the percentage of cell viability (in respect to unexposed cells) using the rezasurin reduction assay that establishes a correlation between the cellular metabolic activity and the number of viable cells in culture.[18]

Gene Delivery Vector Cytotoxicity. Cell viability was studied as a function of the gene delivery vector type and concentration. Cells were seeded in 96-well FluoroNunc plates at a density of 3×10^4 cell/cm^2. After 24 h, medium was replaced with fresh basic medium and 10 μL of each polymer (diluted in HBG) was added to achieve the final desired concentration. After 4 h, the medium was exchanged for fresh medium containing 0.1 mg/mL rezasurin and incubated for another 4 h. Resorufin fluorescence ($\lambda_{ex} = 530$ nm, $\lambda_{em} = 590$ nm) was measured in a microplate reader (model Victor^3 1420, PerkinElmer).

Polyplex Cytotoxicity. The information about polyplex cytotoxicity was obtained during the experiments performed

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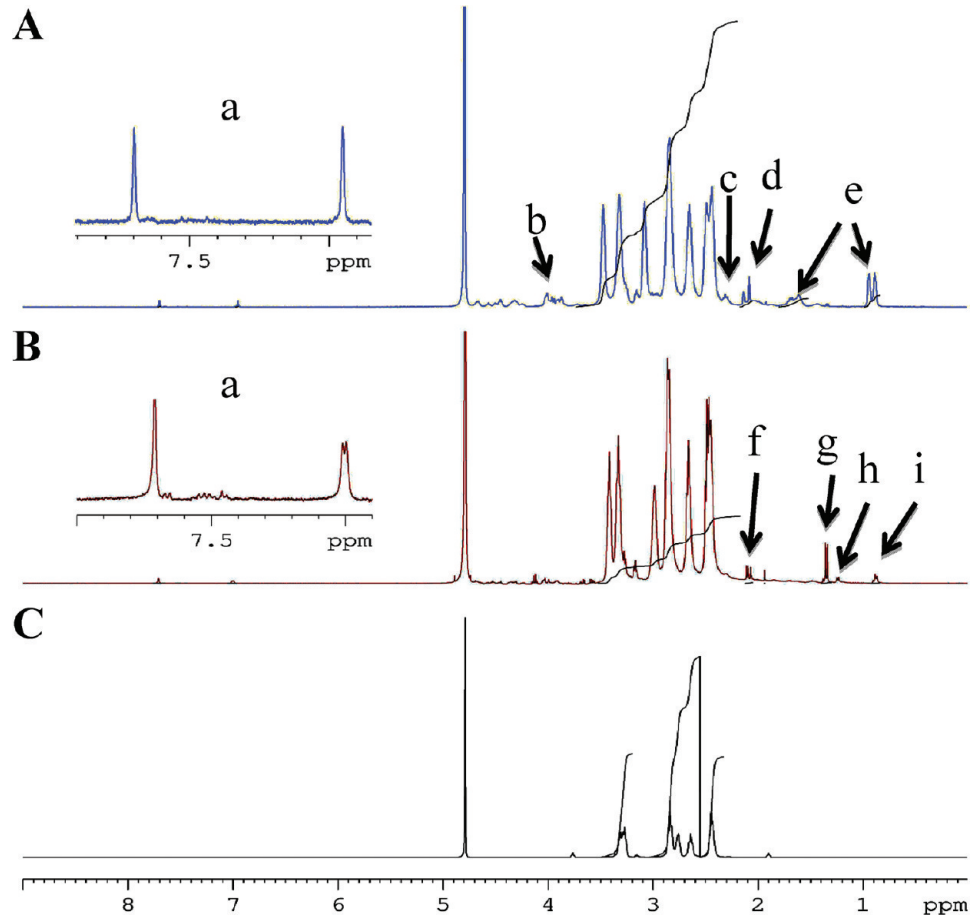

Figure 2. ^{1}H NMR spectra of (A) G5−(HAB)$_8$, (a) histidine, (b) glycine, (c) proline, (d) glutamine and (e) leucine; (B) G5−(LAB)$_4$, (f) methionine, (g) lysine, (h) threonine and (i) isoleucine; and (C) G5 native dendrimer. The insets show the imidazole proton peaks from the amino acid histidine.

to study the expression of the Luc gene (described above), being cell viability evaluated 24 h post-transfection.

Statistics. Statistical analyses were performed using GraphPad Prism 5.0 for Windows. Results are reported as mean ± standard error of mean (SEM). Unpaired, Student's *t* test and 2-way ANOVA with Bonferroni Post Hoc test were used to assess the statistical differences between the group means.

Results and Discussion

Generation 5 PAMAM dendrimers (G5) with amine termini (C1) were conjugated to LAB and HAB peptides via a two-step method using the 3-(2-pyridyldithio)propionic acid *N*-hydroxy-succinimide ester (SPDP), a heterobifunctional cross-linker which contains both amine- and sulfhydryl-reactive groups (Figure 1). For linkage, a cysteine residue was present at the end of both amino acid sequences: NSMIAHNKTRMHGGGSC (LAB peptide) and SGHQLLLNKMPNGGGSC (HAB peptide). During the synthesis, the concentration of both nonfunctionalized and functionalized dendrimers was determined by spectrophotometry, based on primary amine content. The degree of functionalization was under stoichiometric control and was indirectly estimated by spectrophotometric quantification of pyridine-2-thione (C4) that is released after reduction of the dendrimer-PDP intermediate (C2) with excess of dithiothreitol (DTT) and after reaction of C2 with the peptides (Table 1). Conjugates with two (G5−(HAB)2 and G5−(LAB)2) and four (G5−(HAB)4 and G5−(LAB)4) peptide arms were first prepared. As previous gene delivery experiments revealed promising results with conjugates bearing the HAB peptide, a conjugate with eight HAB peptide arms (G5−(HAB)8) was also synthesized to better evaluate the influence of the number of attached peptides on the behavior of conjugates as gene delivery vehicles.

The conjugates were further characterized by ^{1}H NMR spectroscopy. Figure 2 exemplifies ^{1}H NMR characterization for the conjugates G5−(HAB)8 and G5−(LAB)4. As can be seen, the ^{1}H NMR spectrum of G5−(HAB)8 showed the appearance of new proton signals such as the imidazole protons around $\delta = 6.9$ ppm (s, 8H) and $\delta = 7.6$ ppm (s, 8H) (histidine amino acid), methylene protons around $\delta = 3.9$ ppm (CH_2, s, 64H) (glycine amino acid), pyrrolidine protons at $\delta = 2.08$ ppm (CH_2, s, 16H) (proline amino acid), methylene protons at $\delta = 2.02$ ppm (CH_2, s, 32H), methyl protons around $\delta = 0.7-0.9$ ppm (CH_3, s, 144H) and methine protons (CH, s, 18H) at $\delta = 1.5$ ppm (leucine amino acid), thus confirming that HAB peptides were successfully

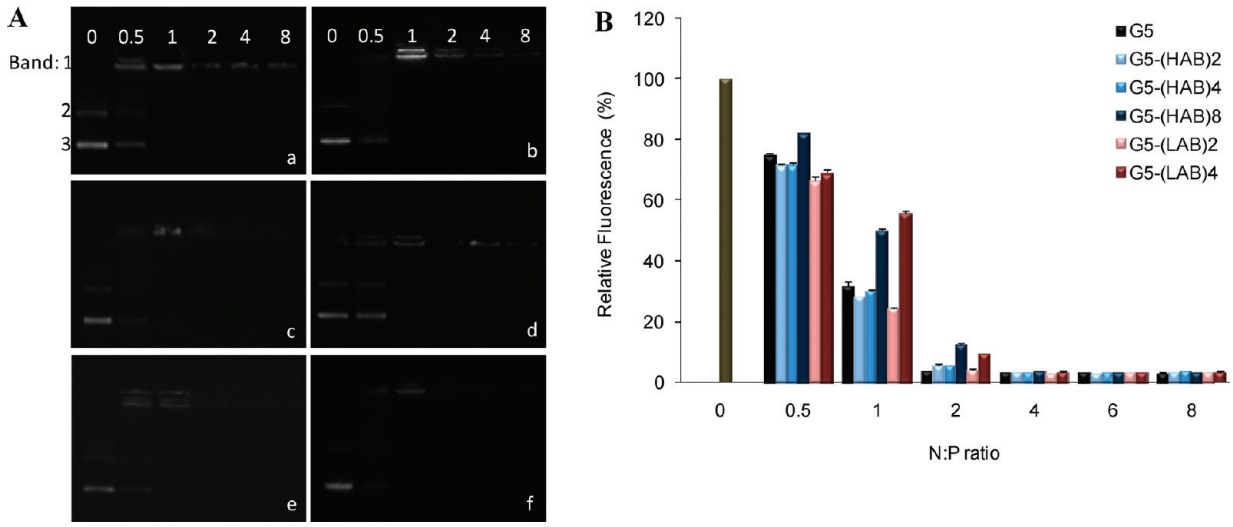

Figure 3. (A) Agarose gel retardation assay results for *N*:*P* ratios ranging from 0 (pDNA only) to 8: (a) G5; (b) G5−$(HAB)_2$; (c) G5−$(HAB)_4$; (d) G5−$(HAB)_8$; (e) G5−$(LAB)_2$; (f) G5−$(LAB)_4$. Binding is shown by the inhibition of pDNA electrophoretic mobility (band 1). Bands 2 and 3 show the relaxed and supercoiled forms of pDNA, respectively. (B) PicoGreen assay. The results are reported as the relative percentage of PG fluorescence, where 100% intensity was observed for an *N*:*P* of 0 (pDNA only). Results are expressed as the mean ± SEM obtained from three independent experiments.

conjugated to G5 PAMAM dendrimers in a ratio of 8:1. The ^{1}H NMR spectra of G5−(LAB)4 is different from the G5−(HAB)8 spectrum as evidenced by the presence of isoleucine protons around 0.9 ppm (CH_3, s, 12H) and threonine protons around 1.23 ppm (CH_3, s, 12H). Lysine protons at $\delta = 1.35$ ppm and $\delta = 1.9$ (CH_2, s, 16H), methionine protons at $\delta = 2-2.15$ (CH_2, s, 32H) and imidazole protons around $\delta = 7$ ppm (s, 8H) and $\delta = 7.7$ ppm (s, 8H) confirm the successful conjugation of 4 LAB peptides per dendrimer. ^{1}H NMR spectra are all in accordance with the results shown in Table 1.

Peptide-functionalized dendrimers were first investigated for their ability to bind, neutralize and compact plasmid DNA (pDNA). Plasmid DNA encoding for enhanced Green Fluorescent Protein and Firefly Luciferase (pEGFPLuc, 6.4 kb) was used. Prior to all studies, conjugates were mixed with pDNA at several *N*:*P* ratios in HBG for polyplex formation. Agarose gel retardation assays (Figure 3A) revealed that binding to and charge neutralization of pDNA occurred at *N*:*P* ratios of 1 and higher when native dendrimers were used, whereas for conjugated dendrimers the hampering of pDNA migration occurred at *N*:*P* ratios of 2 and higher. By using the PicoGreen (PG) dye (Figure 3B), which, when not bound to DNA, has virtually no fluorescence, we concluded that complete pDNA packaging was achieved at an *N*:*P* ratio of 2 for the native dendrimer and at an *N*:*P* ratio of at least 4 for the conjugated dendrimers. Based on these results, an *N*:*P* ratio of 5 was selected for further experiments. At this ratio, dynamic light scattering (DLS) and ζ-potential measurements were performed to examine the size and colloidal stability of the formed polyplexes (Figures 4A and 4B).

Conjugation of peptides to native dendrimers caused a significant increase in polyplex size, which, nevertheless, ranged between 110 and 160 nm in diameter. The polydispersity index (PDI) was only slightly increased by the incorporation of peptides, revealing that the level of homogeneity in the samples remained nearly constant. The incorporation of peptides also resulted in a significant decrease (about 40%, $p < 0.001$) in ζ-potential values. Even if this fact can be interpreted as an increase in colloid hydrophobicity and concomitant instability that may lead to aggregate formation, it is also challenging since the positive, low-charged polyplexes obtained might establish reduced interactions with counterions and plasma proteins, which are known to promote blood clearance and interfere with transfection *in vivo*.[9,19] Taken together, these results suggest that the peptide arms around dendrimers have a shielding effect over primary amines, reducing polyplexes' ζ-potential, conducting to higher dendrimer/pDNA ratios in polyplexes and increasing polyplex size. A similar effect was reported using poly(ethylene glycol) chains linked to PAMAM dendrimers.[20]

PicoGreen-labeled pDNA was used to compare polyplex uptake by rat bone marrow derived MSCs via fluorescence-activated cell sorting (FACS) (Figure 5). The experiments were done under the same conditions used in the following transfection assays. Cellular uptake was studied in the presence of 10% (v/v) serum, at an *N*:*P* ratio of 5 and using a seeding density of 1.25×10^4 cell/cm^2 and 2 μg/cm^2

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(20) Dan Luo, K. H.; Belcheva, N.; Han, E.; Mark Saltzman, W. Poly(ethylene glycol)-Conjugated PAMAM Dendrimer for Biocompatible, High-Efficiency DNA Delivery. *Macromolecules* **2002**, *35*, 3456–3462.

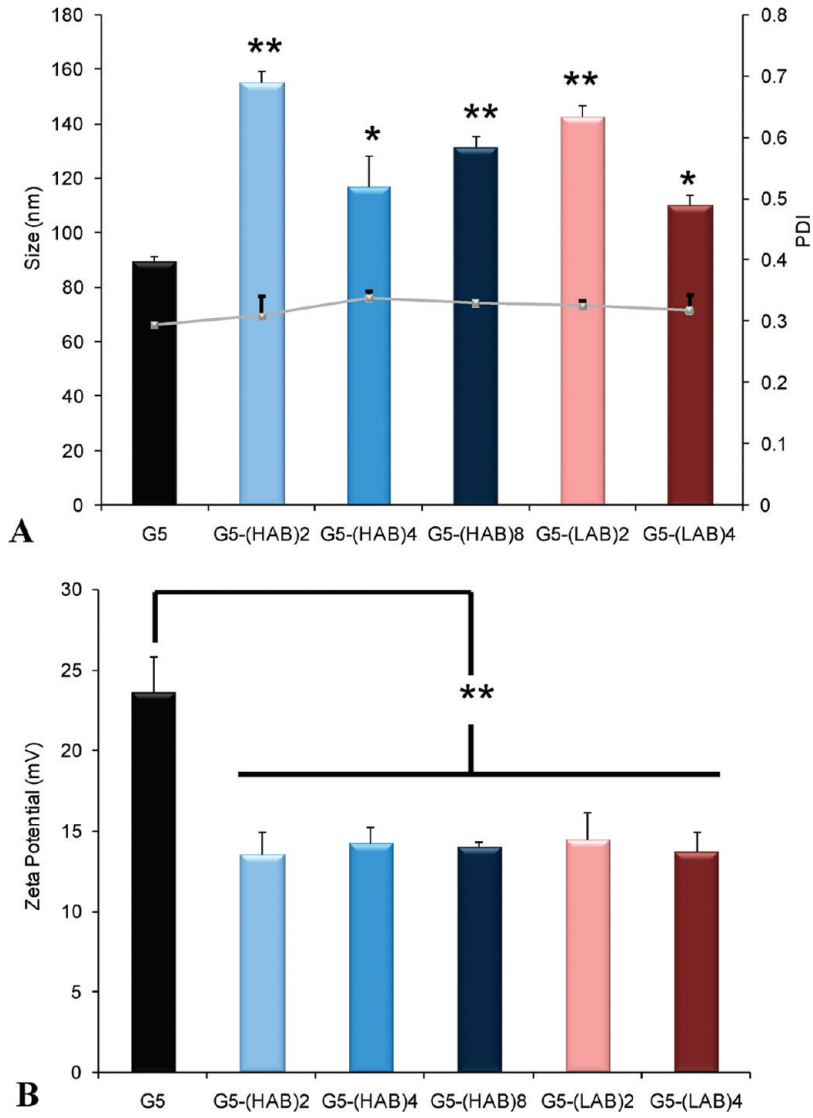

Figure 4. (A) Polyplex size (mean diameter) and polydispersity index assessed by DLS, and (B) polyplex ζ-potential. Results were obtained for an *N*:*P* ratio of 5. Results are expressed as the mean ± SEM obtained from three independent experiments. **p* < 0.05, ***p* < 0.001 when peptide-functionalized dendrimers are compare with native dendrimers.

pDNA. After 2 h of contact between polyplexes and cells, both the number of cells positive for PG-labeled pDNA and the amount of pDNA delivered per cell achieved with HAB peptide-functionalized dendrimers (G5−(HAB)2 and G5−(HAB)4) were significantly higher ($p < 0.05$) than those obtained with native dendrimers and similar to those reached using SF. However, pDNA internalization using G5−(HAB)8 was lower possibly due to the fact that this vector interacts with a higher number of receptors at the cell membrane causing a saturation-like phenomenon and hampering the cellular uptake process.[21] Cellular uptake values for LAB peptide-functionalized dendrimers were only slightly higher than those obtained with native dendrimers.

Figure 6 shows the distribution of RITC-labeled pDNA (red) inside cells after transfection using native dendrimers and peptide-functionalized dendrimers (G5−(HAB)4 and G5−(LAB)4). The acidic late endosome and lysosome compartments were stained with LysoSensor Green DND-189 (green), and the nucleus with DAPI (blue). The images were obtained using a lower concentration of pDNA (1 μg/cm^2) to diminish blur. Two hours post-transfection, a higher accumulation of pDNA could be observed inside cells when using HAB peptide-functionalized dendrimers. These data are in agreement with FACS results, which showed that HAB peptide-functionalized dendrimers were able to deliver a high amount of pDNA per cell. As time progressed (4 and 24 h), it was possible to observe the appearance of orange spots (especially for G5 native dendrimer and G5−(HAB)4) representing the colocalization of pDNA and endosome/lysosome compartments. After 48 h there was an evident accumulation of pDNA at the cell nucleus periphery for G5 native dendrimer and G5−(HAB)4. pDNA colocalization with the nucleus (pink dots) could already be observed after

(21) Schaffer, D. V.; Lauffenburger, D. A. Optimization of cell surface binding enhances efficiency and specificity of molecular conjugate gene delivery. *J. Biol. Chem.* **1998**, *273*, 28004–28009.

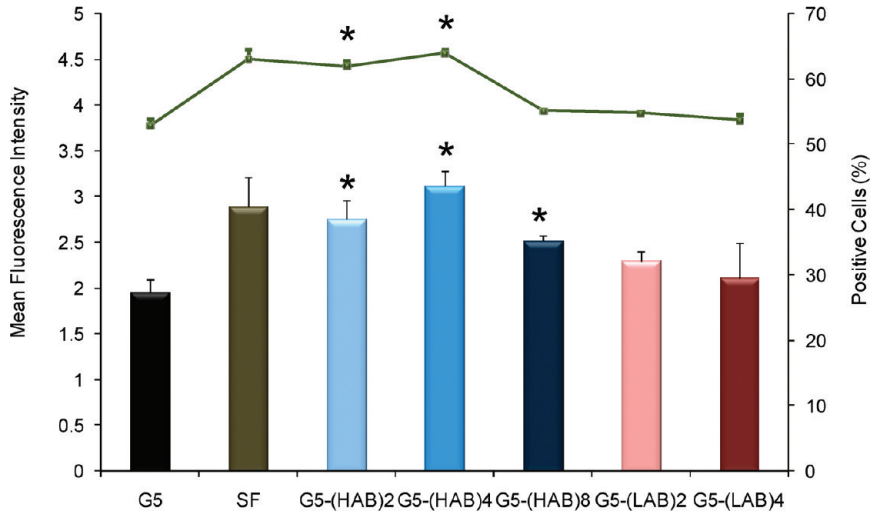

Figure 5. Polyplex uptake by MSCs after 2 h of contact. The line shows the number of cells positive for PG-labeled pDNA, whereas the bars reveal the amount of pDNA delivered per cell (displayed as the mean average fluorescence intensity). Results are expressed as the mean ± SEM obtained from three independent experiments. *$p < 0.05$, when peptide-functionalized dendrimers are compare with native dendrimers.

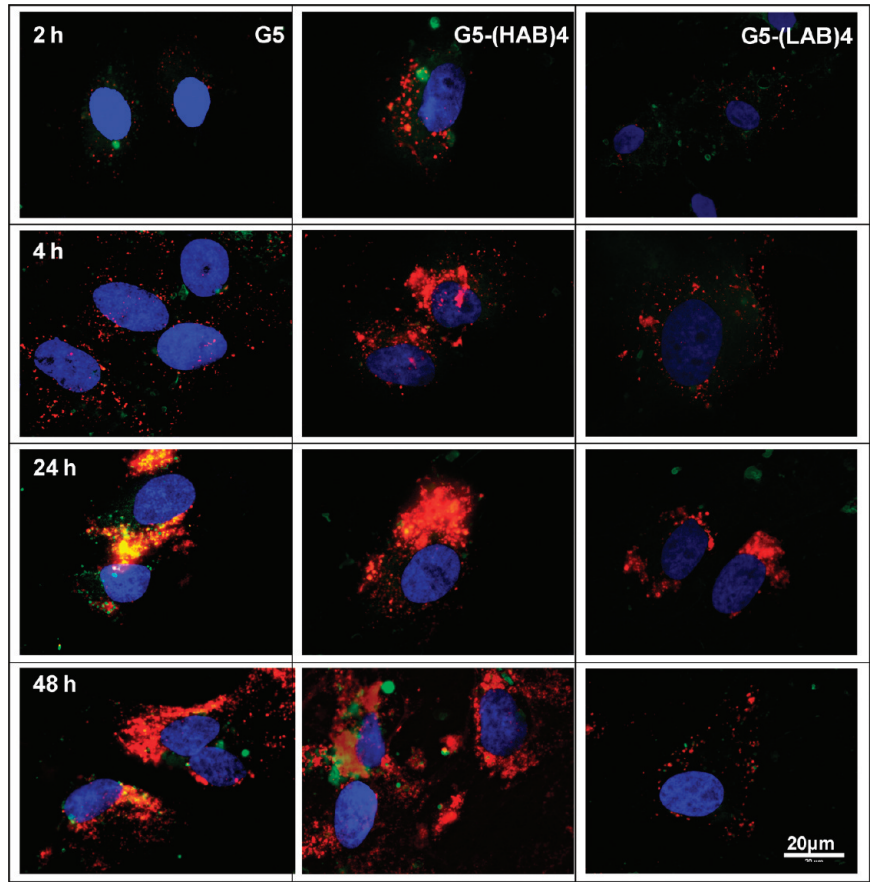

Figure 6. Cellular distribution of (RITC)-labeled pDNA (2 and 4 h post-transfection) using native dendrimers and G5−(HAB)4 and G5−(LAB)4 as vectors. The acidic late endosome and lysosome compartments were stained with LysoSensor Green DND-189 (green), and the nucleus was stained with DAPI (blue).

2 h when HAB peptide-functionalized dendrimers were used. A z-stack of the slide corresponding to a G5−(HAB)4/2 h sample is supplied as Supporting Information.

To quantitatively investigate if the conjugation of peptide to dendrimers was beneficial in terms of gene delivery, rat bone marrow derived MSCs were assayed for expression of the Luc reporter gene after 48, 72, and 96 h post-transfection (Figures 7A and 7B). Results were normalized to protein content and are shown as relative light units (RLU). A transient expression of the Luc gene was obtained in all cases

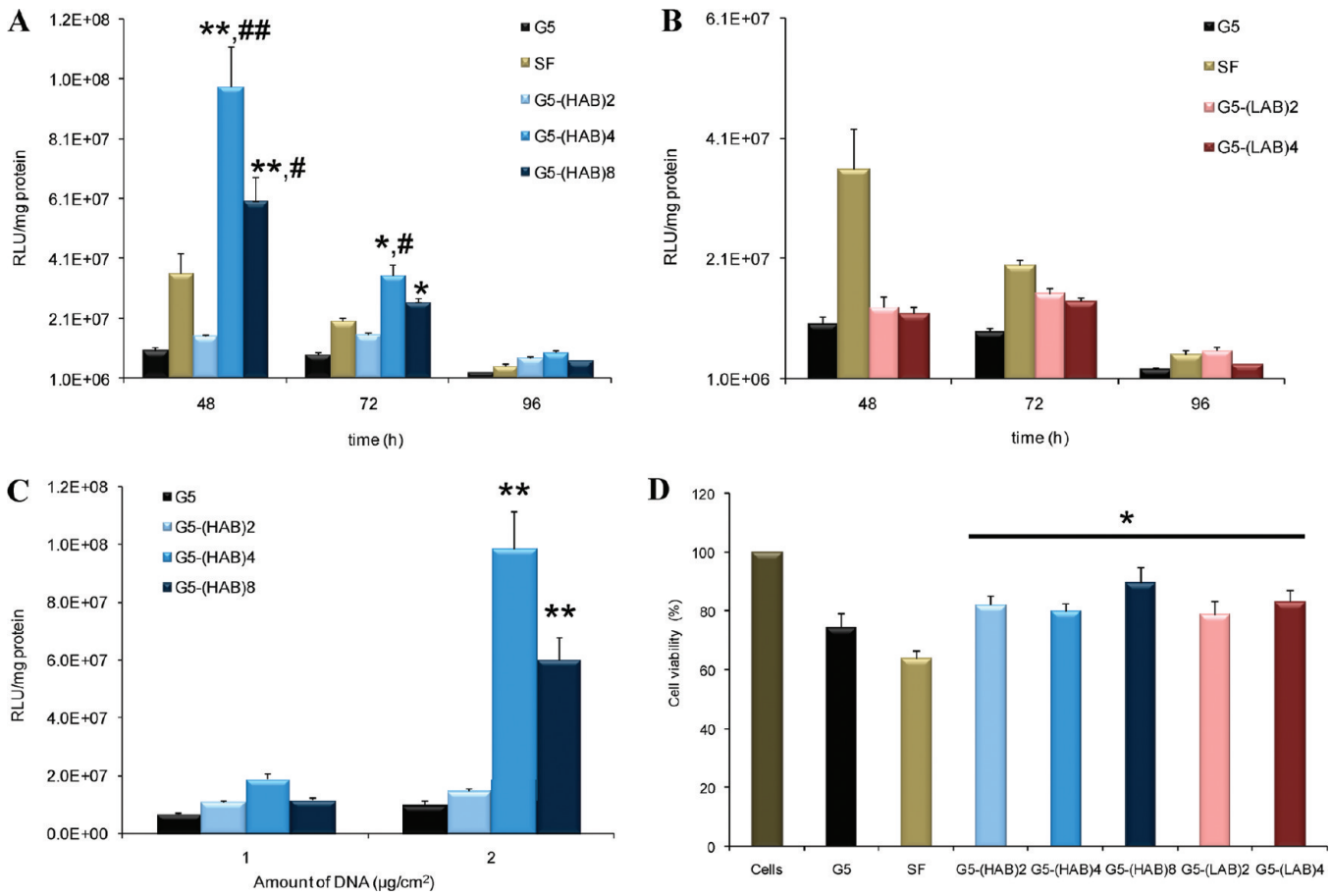

Figure 7. (A) Luc gene expression achieved with HAB peptide-functionalized dendrimers (*$p < 0.05$, **$p < 0.001$ when peptide-functionalized dendrimers are compared with native dendrimers; $^{\#}p < 0.1$, $^{\#\#}p < 0.001$ when peptide-functionalized dendrimers are compared with SF), (B) Luc gene expression achieved with LAB peptide-functionalized dendrimers, and (C) comparison of Luc gene expression 48 h post-transfection achieved with HAB peptide-functionalized dendrimers for different pDNA concentrations (**$p < 0.001$ when the two pDNA concentrations are compared). (D) Cytotoxicity evaluation 24 h post-transfection (*$p < 0.05$ when peptide-functionalized dendrimers are compared with native dendrimers). Results are expressed as the mean ± SEM and were obtained from two independent experiments.

with HAB peptide-functionalized dendrimers containing 4 and 8 peptides per dendrimer leading to an earlier gene expression possibly related with a faster polyplex cellular uptake and pDNA trafficking to the nucleus. After 48 h, HAB peptide-functionalized dendrimers containing four and eight peptides per dendrimer presented transfection levels that were, respectively, 10- and 5.5-fold higher than those obtained with native dendrimers and 2.5- and 1.5-fold higher than those achieved with Superfect. Although more moderate, a positive difference could still be noticed after 72 h post-transfection between these vectors and all the others.

Transfection efficiency was dependent on pDNA concentration as shown by decreasing it to 1 $\mu g/cm^2$ (Figure 7C). In this case, the enhancement of transfection efficiency is not so high (after 48 h, HAB peptide-functionalized dendrimers containing four and eight peptides per dendrimer presented transfection levels that were, respectively, 2.8- and 1.6-fold higher than those obtained with native dendrimers), but a positive influence of HAB peptides on gene delivery is still observed. On the other hand, after 48 h, the transfection levels attained with LAB peptide-functionalized dendrimers containing two and four peptides per dendrimer were not significantly different from that obtained with native dendrimers, reflecting the low affinity characteristics of this peptide for MSCs. Gene delivery achieved by peptide-functionalized dendrimers was also qualitatively studied, 24 h post-transfection, by visualization of Enhanced Green Fluorescent Protein expression using fluorescence microscopy. Results are shown in Figure 8 and are in accordance with the previous quantitative data.

Cell viability was assessed simultaneously to transfection experiments using the rezasurin reduction assay. As shown in Figure 7D, the enhancement of the Luc gene expression presented in particular by G5−(HAB)4 and G5−(HAB)8 vectors was accomplished with minimal cytotoxic effects to cultured cells (viability was around 80−90%). In contrast, the transfection efficiency obtained with Superfect was accompanied by a notable decrease (~40%) in cell viability. In general, polyplexes formed by native

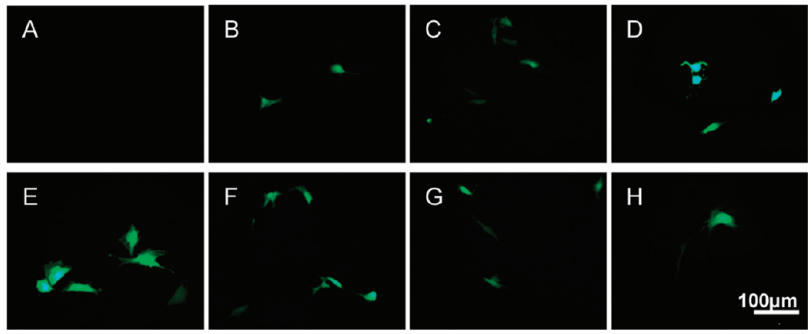

Figure 8. Fluorescence microscopy images showing Enhanced Green Fluorescent Protein expression 24 h post-transfection using: (A) naked DNA, (B) G5 PAMAM dendrimers, (C) Superfect, (D) G5−(HAB)2, (E) G5−(HAB)4, (F) G5−(HAB)8, (G) G5−(LAB)2, and (H) G5−(LAB)4.

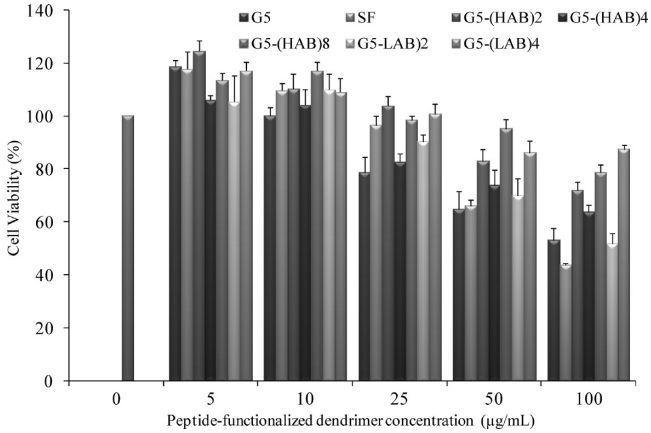

Figure 9. Cytotoxicity evaluation of peptide-functionalized dendrimers. Each data point represents the mean ± SEM of two independent measurements.

dendrimers presented a higher toxicity than those formed by the modified polymers. Studies on the cytotoxicity of vectors alone were also performed (Figure 9), revealing that functionalized dendrimers display a significantly lower toxicity than native dendrimers and SF, particularly for the highest concentrations. These facts may be explained by the partial shielding of primary amines (primarily responsible for cytotoxicity effects) achieved in the conjugates.[22]

The receptor-mediated nature of gene delivery conducted by conjugates attached to HAB peptides was confirmed by saturating cell receptors with the peptide prior to transfection (a 0.1 mM peptide solution was incubated with cells for 1 h). In this case, the level of transfection achieved with HAB peptide-functionalized dendrimers was similar to that attained with native dendrimers (Figure 10A). No cytotoxic effect associated with the previous saturation of cell receptors with the peptide was observed (Figure 10B).

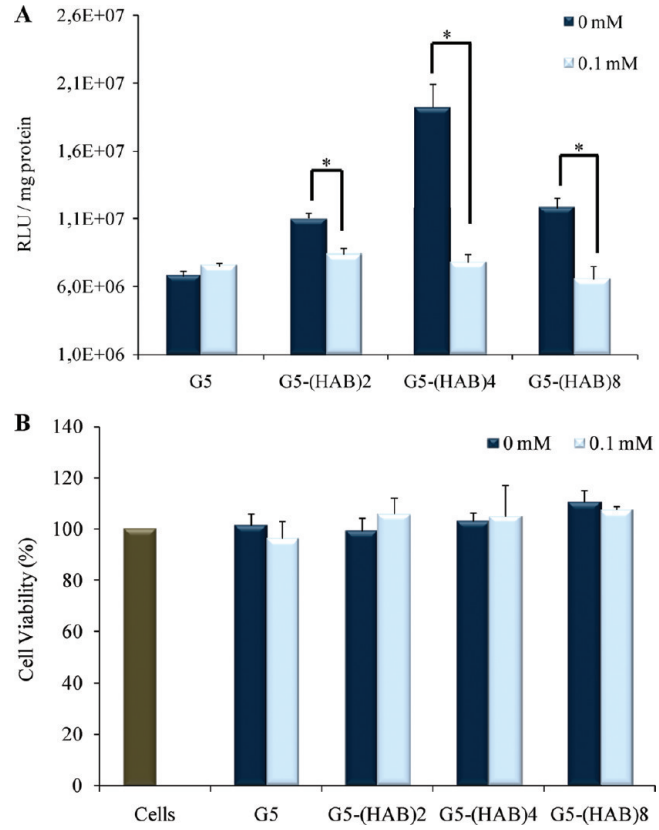

Figure 10. (A) Luc gene expression achieved with HAB peptide-functionalized dendrimers with and without saturation of cell receptors by HAB peptide prior to transfection. (B) Cytotoxicity evaluation 24 h post-transfection. Results are expressed as the mean ± SEM and were obtained from two independent experiments. A significant reduction ($p < 0.05$) on the level of gene expression was observed when transfection experiments were carried out after saturation of cell receptors.

Conclusions

In conclusion, conjugation of PAMAM dendrimers with peptides with high binding affinity for MSCs provides a mechanism for cell specific recognition, giving rise to a new family of gene delivery vectors presenting low cytotoxicity and transfection efficiencies superior to those of native

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dendrimers or partially degraded dendrimers. These systems represent a step forward in the gene delivery field and may have important applications in tissue engineering and regeneration.

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Supporting Information Available: Molecular structures of the peptides and a z-stack of the slide corresponding to a G5−(HAB)4/2 h sample. This material is available free of charge via the Internet at http://pubs.acs.org.

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