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ORIGINAL ARTICLE

Gene delivery to human adult and embryonic cell-derived stem cells using biodegradable nanoparticulate polymeric vectors

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Gene delivery to stem cells holds great potential for tissue regeneration and delivery of therapeutic proteins. The major barrier is the lack of safe and efficient delivery methods. Here, we report enhanced gene delivery systems for human stem cells using biodegradable polymeric vectors. A library of poly (β-amino esters) end-modified derivatives was developed and optimized for high transfection efficiency and low cytotoxicity for three human stem cell lines including human mesenchymal stem cells (hMSCs), human adipose-derived stem cells (hADSCs) and human embryonic stem cell-derived cells (hESCds). In the presence of 10% serum, leading end-modified C32 polymeric vectors exhibited significantly high transfection efficiency in hMSCs (27 ± 2%), hADSCs (24 ± 3%) and hESCds (56 ± 11%), with high cell viability (87–97%) achieved in all cell types. Our results show that poly(β-amino esters) as a class, and end-modified versions of C32 in particular, are efficient polymeric vectors for gene delivery to both adult and embryonic-derived stem cells.
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Introduction

Stem cells are promising alternative cell sources for tissue regeneration and as delivery vehicles for therapeutic proteins. In general, stem cells can be grouped into two major classes: adult stem cells and embryonic stem cells.[1–3] Adult stem cells are multipotent and have been isolated from many adult tissues, such as bone marrow,[1] fat,[4,5] nervous tissues[6,7] and muscle.[8] Human embryonic stem cells are derived from the inner cell mass of the blastocyst of embryos and possess the potential to differentiate into derivatives of all three germ layers.[3]

Before stem cells can be used to regenerate three-dimensional tissues, methods must be developed to direct and control their differentiation. It is clear that genetic signals can promote lineage-specific differentiation.[9] Recently, stem cell-based tissue engineering has been used in conjunction with gene therapy to enhance tissue regeneration by providing the stem cells with an environment of optimal protein expression.[10,11] Virus-mediated delivery of osteogenic genes, such as *bone morphogenetic protein 2*, to adult stem cells have been shown to promote bone repair.[12,13]

In addition to the applications in tissue engineering, stem cells can also serve as cell carriers to deliver therapeutic proteins for treatment of both inherited and acquired diseases.[14] The potential of genetically modified adult or embryonic stem cells to correct various genetic disorders including muscle dystrophy, hemophilia and cystic fibrosis has been explored.[15] For example, lentiviral expression of human factor IX in hematopoietic stem cells has been shown efficacious in the treatment of hemophilia B, a bleeding disorder caused by the absence of the secreted blood coagulation factor IX.[16] Furthermore, stem cell-based gene therapy approach are also being developed to treat acquired diseases, such as cancer, AIDS, chronic vascular ischemia, osteoarthritis, diabetes, Parkinson's and Alzheimer's diseases.[14] Despite the promise of stem cell-based gene therapy for making an impact on human health, this approach has rarely found its way into the clinic, and technical challenges remain to be addressed.

To become broadly useful therapeutically, methods to deliver genes to stem cells in a safe and efficient manner must be developed. Most current methods of genetic modification of stem cells utilize modified viruses, such as adenovirus, lentivirus and retrovirus.[14,17,18] Although these viral approaches may be effective at transferring DNA to stem cells, safety issues, including mutagenesis and toxicity, remain considerable concerns. In contrast,

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non-viral systems offer safety advantages including low immunogenicity and toxicity, as well as ease of production.[19] However, despite these advantages, existing polymer-based gene delivery systems have significantly lower transfection efficiency.[20] Other physical methods, such as electroporation and nucleofection, have been explored for gene delivery to stem cells.[21,22] Although relatively higher transfection efficiency may be obtained, these procedures are associated with cell death.

To improve the biocompatibility and DNA release, recent research efforts in polymer-based gene delivery have incorporated biodegradable components, such as hydrolyzable ester bonds, into the structural design.[23–28] Among these, poly(β-amino esters) are particularly attractive due to their facile synthesis, low toxicity, potential of high chemistry diversity and biodegradability. Previously, the development of a large library of 2350 structurally unique poly(β-amino esters) using high-throughput combinatorial chemistry was reported.[29] High throughput screening identified a number of polymers that show better transfection efficiency than polyethylenimine, a leading commercially available polymer transfection reagent.[29] Structure/property analyses showed polymer C32 as the most effective transfection agent (Figure 1a).[30] End modification of C32 with various amine structures has been shown to significantly enhance its *in vitro* transfection efficiency to COS-7 cells and human umbilical vein endothelial cells.[31,32]

Here, we develop poly(β-amino esters) for gene delivery to stem cells. We synthesized unmodified and end-modified C32 polymers and evaluated their transfection efficiency in three stem cell types including bone marrow-derived human mesenchymal stem cells (hMSCs), human adipose-derived stem cells (hADSCs) and human embryonic stem cell-derived cells (hESCds). Promising vectors were optimized for high efficacy and low cytotoxicity. The biophysical properties of nanoparticles after DNA complexation were also examined to better understand structure/function in relation to gene delivery. Our results show that end-modified C32/DNA polymeric nanoparticles are highly efficient biodegradable nonviral polymeric vectors for gene delivery to both adult and embryonic-derived stem cells. These vectors exhibit significantly higher transfection efficacy and cell viability than a leading commercially available nonviral transfection reagent, Lipofectamine 2000.

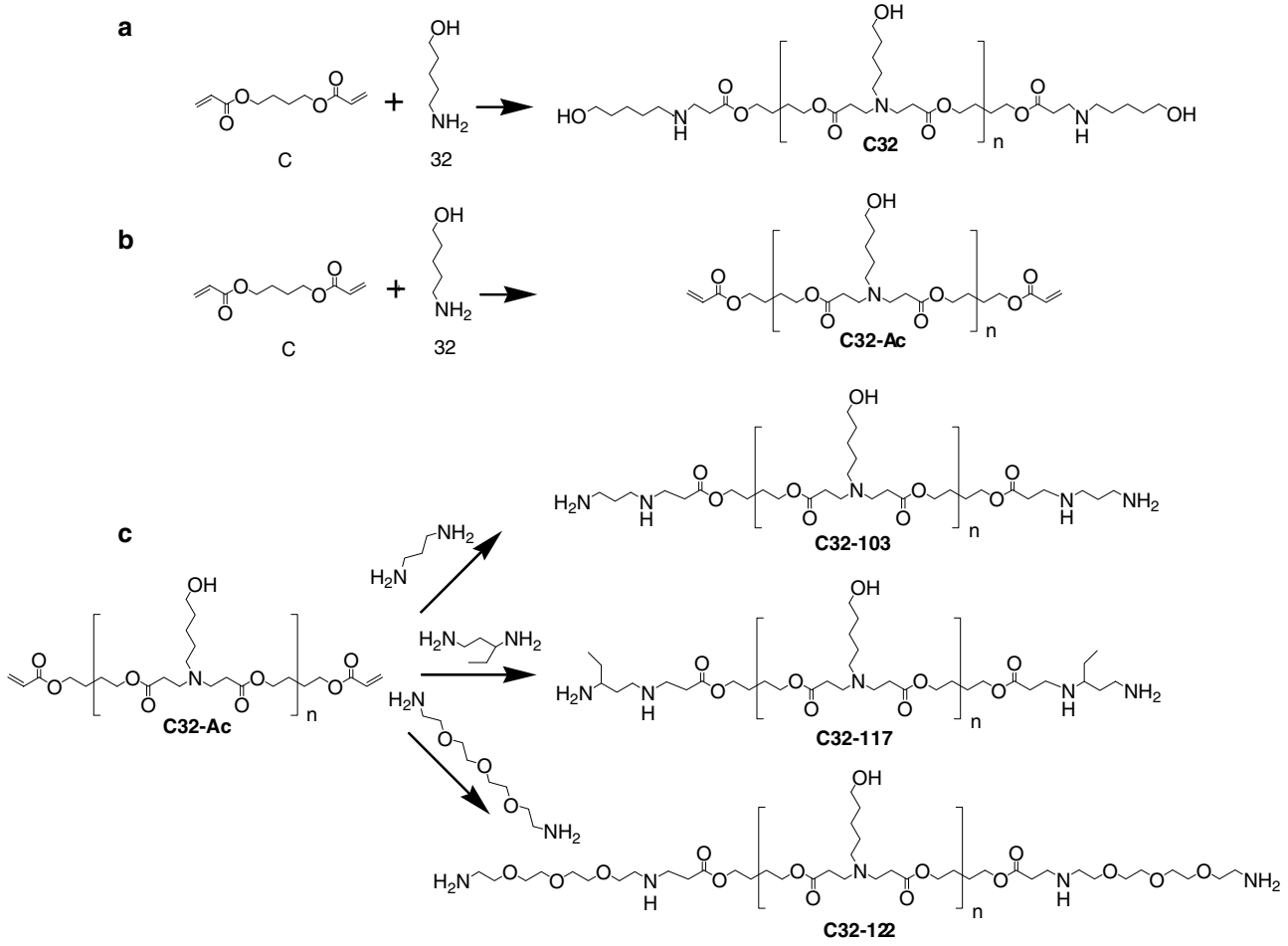

Figure 1 Synthesis of biodegradable poly(β-amino ester)s. (**a**) Synthesis of unmodified C32 polymer by 1.2:1.0 amine:diacrylate polymerization. (**b**) Synthesis of acrylate-terminated C32 polymer (C32-Ac) by 1.2:1.0 diacrylate:amine polymerization. (**c**) End-modification of acrylate-terminated C32 with three different amine groups (103, 117 and 122). Each end of a C32–117 polymer may contain either of the two conformations shown.

Results

Polymer synthesis

Poly(β-amino esters) were synthesized by the conjugate addition of amine monomers to diacrylates. For the synthesis of aminopentanol-terminated C32 polymer, aminopentanol (32) was mixed in a 1.2:1.0 molar ratio with butanediol diacrylate (C) (Figure 1a). For the synthesis of acrylate-terminated C32 (C32-Ac), butanediol diacrylate (C) was mixed in a 1.2:1.0 molar ratio with aminopentanol (32; Figure 1b). End-modified C32 polymers were synthesized by subsequently reacting C32-Ac individually with three different diamine monomers (labeled 103, 117 and 122) to yield end-modified C32 polymers: C32–103, C32–117 and C32–122 (Figure 1c).

Efficiency of gene delivery to hMSCs using end-modified C32 polymers

After 72 h of transfection, the transfection efficiency was evaluated using fluorescence microscopy and flow cytometry. A leading commercially available nonviral transfection reagent, Lipofectamine 2000, was used as a positive control and prepared using an optimized formulation. Preliminary screening has identified three end-modified polymers (C32–103, 117 and 122) as the lead polymers for transfection into stem cells. For passage 5 hMSCs, the end-modified polymers (C32–103, 117 and 122) showed significantly higher transfection efficiency than Lipofectamine 2000 (Figure 2a; $P<0.05$). Optimal transfection is dependent on DNA loading, types of polymer and polymer/DNA weight ratio. At low polymer/DNA ratio (20:1) and low DNA dosage (3 μg per well), C32–103 was the most effective polymer and transfected hMSCs 3.5-fold higher ($23\pm1\%$) than Lipofectamine 2000 ($5\pm1\%$). For C32–103, increasing DNA dosage from 3 μg per well to 6 μg per well did not further increase the transfection efficiency, and transfection efficiency significantly decreased when polymer/DNA weight ratio increased from 30 to 40. For C32–117 and C32–122, the maximal transfection efficiency was achieved with a high DNA dosage (6 μg per well) and low polymer/DNA weight ratio (20:1). For all three end-modified C32 polymers, the transfection efficiency to hMSCs dropped significantly when the polymer/DNA weight ratio was higher than 40:1 (data not shown). Under the optimal transfection conditions, C32–117 and C32–122 transfected hMSCs fourfold higher (25 ± 2 and $27\pm2\%$ positive, respectively) than Lipofectamine 2000 ($5\pm1\%$, $P<0.001$). Increasing polymer/DNA weight ratio only increased transfection efficiency at low DNA dosage (3 μg per well), but not high DNA dosage (6 μg per well). Fluorescence-activated cell sorting data were interpreted by using a two-dimensional density plot, which compares the ratio of green fluorescent protein (GFP) channel fluorescence (X-axis) to yellow channel autofluorescence (Y-axis; Figures 2b and c). GFP expression was also confirmed by fluorescence microscopy (Figure 2d).

The transfection profile of end-modified C32 polymers changed for later passage hMSCs (passage 7), and different optimal transfection conditions were observed compared to those for passage 5 hMSCs (Figure 2e). At low DNA dosage (3 μg per well), the transfection efficiency of C32–103 and C32–117 dropped significantly at low polymer/DNA weight ratio (20:1) ($P<0.01$ compared to hMSCP5). At higher polymer/DNA weight ratio (30:1), the transfection efficiency of C32–103 increased significantly to $33\pm2\%$ ($P<0.01$ compared to hMSCP5). C32–117 worked better with high DNA dosage (6 μg per well) and its transfection efficiency reached the maximum ($37\pm3\%$) at polymer/DNA weight ratio of 30:1. The transfection efficiency of Lipofectamine to hMSCP7 increased up to $14\pm2\%$, which is ~100% higher than its transfection efficiency for passage 5 hMSC cells ($P<0.01$). In contrast, C32–122 did not transfect hMSCP7 cells as well as hMSCP5, and its maximal transfection efficiency dropped to $18\pm3\%$ (3 μg per well, 30 w/w).

Efficiency of gene delivery to hMSCs using unmodified C32 polymers

Unmodified C32 polymer was less efficient in serum conditions (Figure 3), and the maximal transfection efficiency (10%) was achieved with higher DNA dosage (6 μg DNA per well) and 40:1 ratio. The transfection efficiency of C32 markedly increased in the serum-free OptiMem, and C32 polymer reached maximal transfection efficiency of 39% (Figures 3b and c) with minimal cytotoxicity (Figure 3d), using higher DNA loading (6 μg DNA per well) and lower polymer/DNA weight ratio (30:1). Further increase in weight ratio (40:1 and above) decreased the transfection efficiency (Figure 3a) as well as the cell viability (Figure 3d).

Efficiency of gene delivery to hADSCs using end-modified C32 polymers

In the presence of 10% serum, the end-modified polymers (C32–103, 117 and 122) showed significantly higher transfection efficiency for passage 4 hADSCs than Lipofectamine 2000 (Figure 4a; $^*P<0.05$). All three end-modified polymers showed a similar transfection profile, with the maximum efficiency (22–24%) occurring at lower DNA dosage (3 μg per well) and the polymer/DNA ratio of 30:1. Further increase of weight ratio to 40:1 did not have significant effects on transfection efficiency, whereas higher DNA dosage (6 μg per well) led to the decreased transfection efficiency. Under the optimal transfection condition, C32–103 transfected hADSCs threefold higher ($22\pm2\%$) than Lipofectamine 2000 ($6\pm5\%$; Figures 4a–d). For later passage hADSCs (passage 6), the transfection profile of all end-modified C32 polymers remained statistically similar, whereas the transfection efficiency of Lipofectamine 2000 increased ($11\pm0.1\%$) compared to that for passage 4 hADSCs (Figure 2e). For all three end-modified C32 polymers, the transfection efficiency to hADSCs dropped significantly when the polymer/DNA weight ratio was higher than 40:1 (data not shown).

Efficiency of gene delivery to hESCds using end-modified C32 polymers

Compared to Lipofectamine 2000, the end-modified polymers C32–103, 117 and 122 showed significant higher levels of transfection efficiency for human embryonic stem cell-derive cells (Figures 5a–c). For passage 1 hESCds , all three end-modified C32 polymers showed similar transfection profile, with the maximum

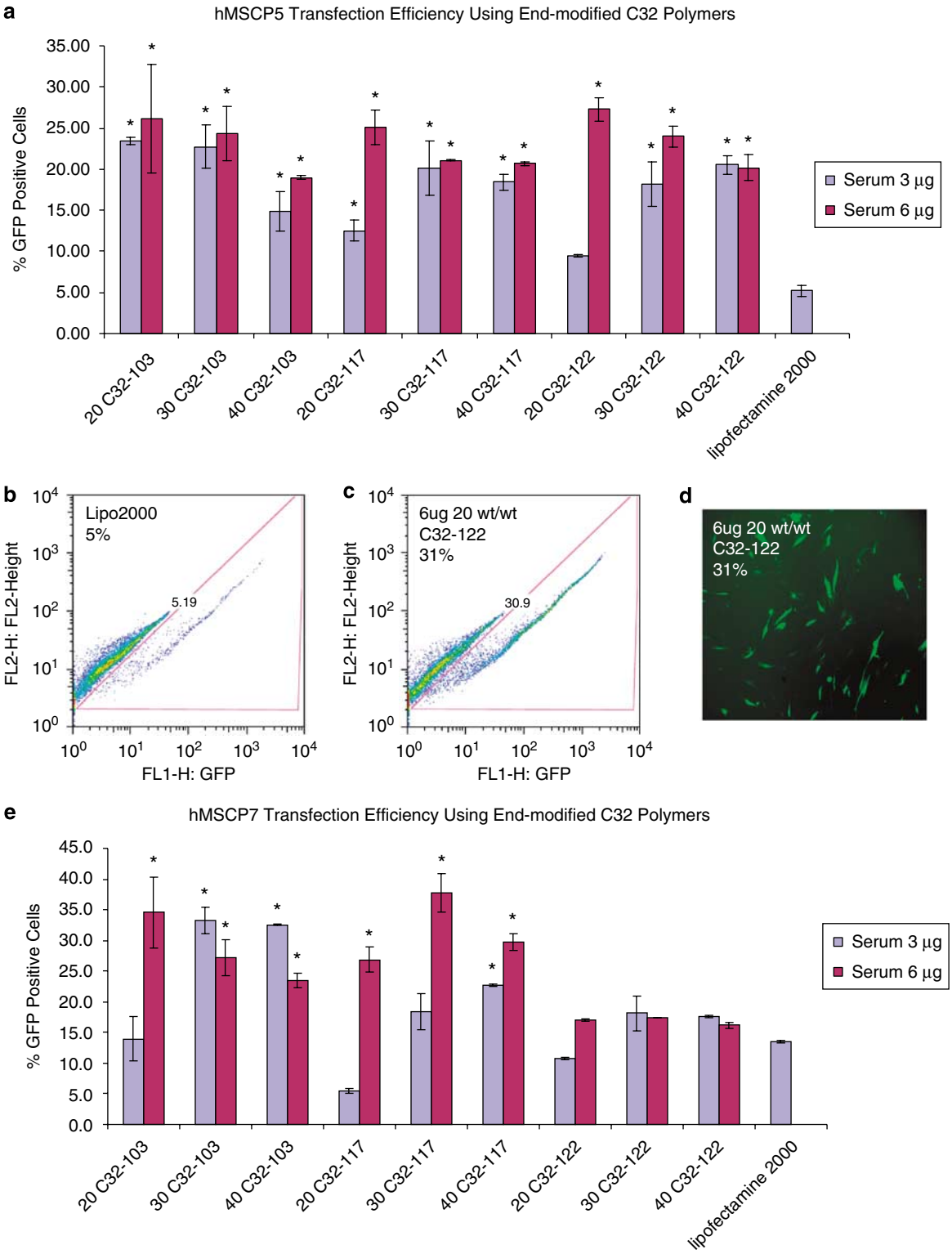

Figure 2 Efficiency of gene delivery to human mesenchymal stem cells (hMSCs) in 10% serum using end-modified C32 polymers. End-modified C32 polymers showed significantly higher transfection efficiency for passage 5 hMSCs (**a–d**) and passage 7 hMSCs (**e**) under leading transfection conditions (*$P<0.05$ compared to Lipofectamine 2000). Error bars are s.d. ($n=3$), and it is less than 0.01 for sample 30 C32–122 6 μg in panel e.

transfection achieved at low DNA dosage (3 μg per well) and low polymer/DNA weight ratio (20:1).

C32–122 (20 w/w, 3 μg DNA per well) transfected hESCds twice as high (56 ± 11%) as Lipofectamine 2000 (29 ± 7%, $P<0.05$). For all three end-modified C32 polymers, the transfection efficiency to hESCds dropped dramatically when the polymer/DNA weight ratio was higher than 40:1 (data not shown). Consistent with the fluorescence-activated cell sorting data, fluoresence image of the C32–122 group showed significantly more cells

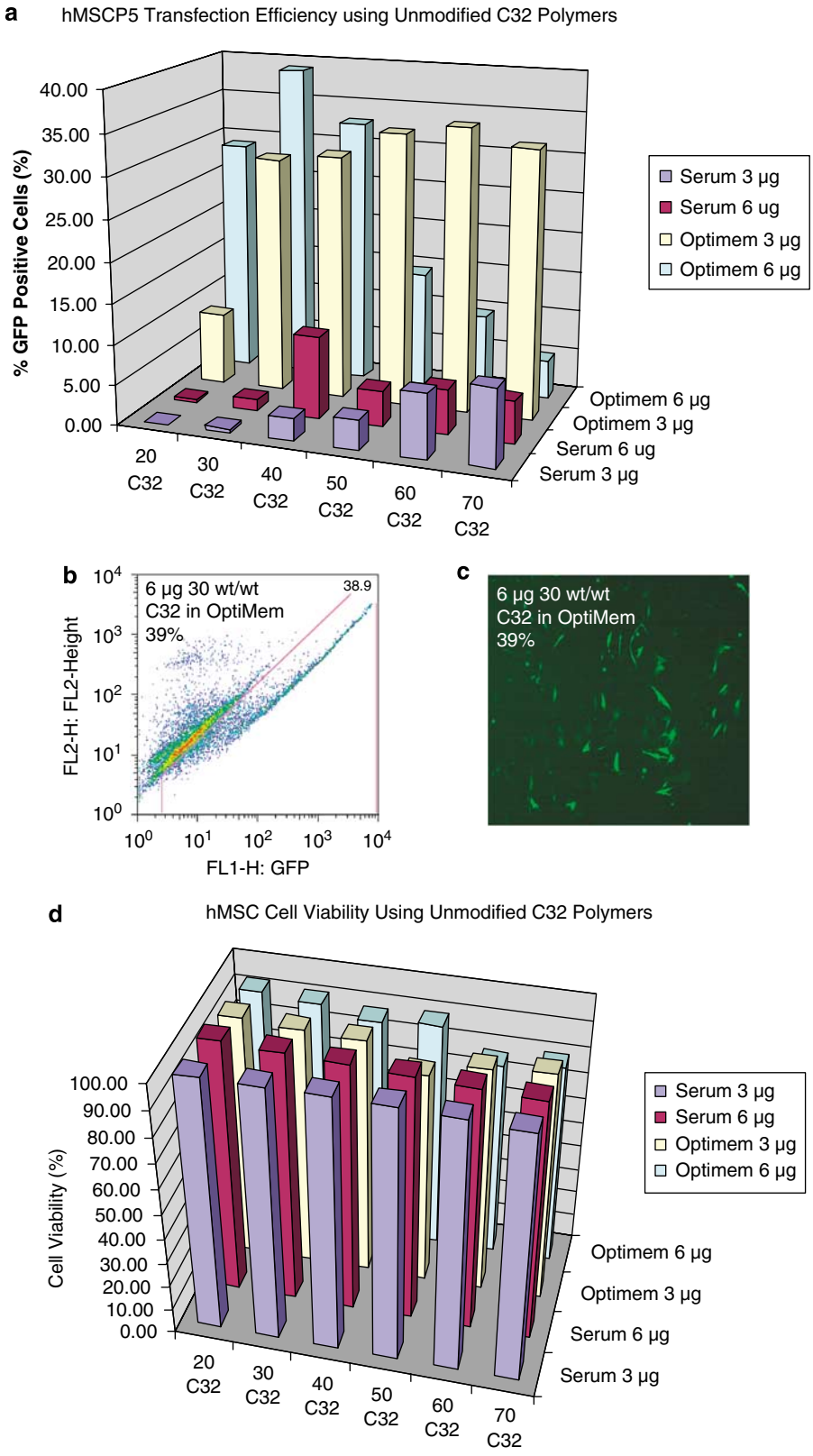

Figure 3 Efficiency and cell viability of gene delivery to human mesenchymal stem cells (hMSCP5) using unmodified C32 polymer. Unmodified C32 polymer in general performed better in serum-free OptiMem than in medium containing 10% serum (**a**–**c**). Good cell viability of hMSCP5 was observed at 72 h post-transfection (**d**).

that express GFP signals than Lipofectamine 2000 group (Figure 5d). The transfection level of both end-modified C32 polymers and Lipofectamine dropped significantly as the hESCds became more differentiated (Figure 5e). For passage 5 hESCds, C32–103 was the most effective polymer and reached its maximal transfection efficiency

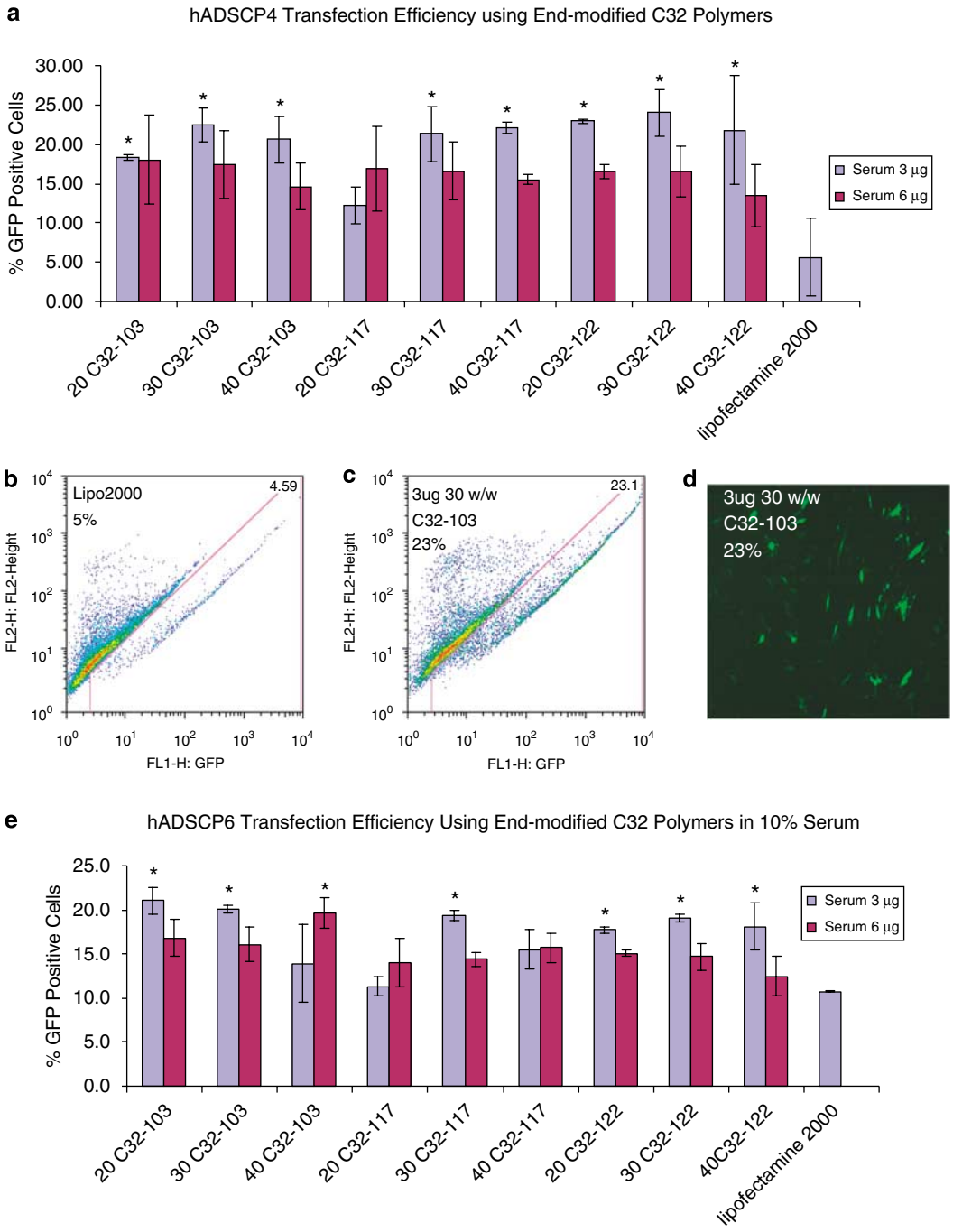

Figure 4 Efficiency of gene delivery to human adipose-derived stem cells (hADSCs) in 10% serum using end-modified C32 polymers. End-modified C32 polymers showed significantly higher transfection efficiency for passage 4 hADSCs (**a–d**) and passage 6 hADSCs (**e**) under leading transfection conditions (*$P<0.05$ compared to Lipofectamine 2000). Error bars are s.d. ($n=3$).

(29 ± 4%) at low DNA dosage (3 μg per well) and low polymer/DNA weight ratio (20:1). Further increase in DNA dosage or polymer/DNA weight ratio led to decreased transfection. The maximal transfection efficiency of C32–117 for hESCdP5 cells (23 ± 1%) was 50% higher than Lipofectamine 2000 (16 ± 4%). C32–122 transfection levels were lower than the other two polymers and were comparable to the Lipofectamine 2000.

Cell viability

The cell viability after transfection was evaluated using the Cell Titer 96 Aqueous One Solution assay kit (Promega, Madison, WI, USA). Measurements of cells transfected with polymers were converted to percentage viability in comparison with untreated controls. For hMSCs, all three end-modified C32 polymers exhibit low cytotoxicity (Figure 6a). For optimal transfection condition of C32–103, the cell viability is 87 ± 0.3%. Increasing

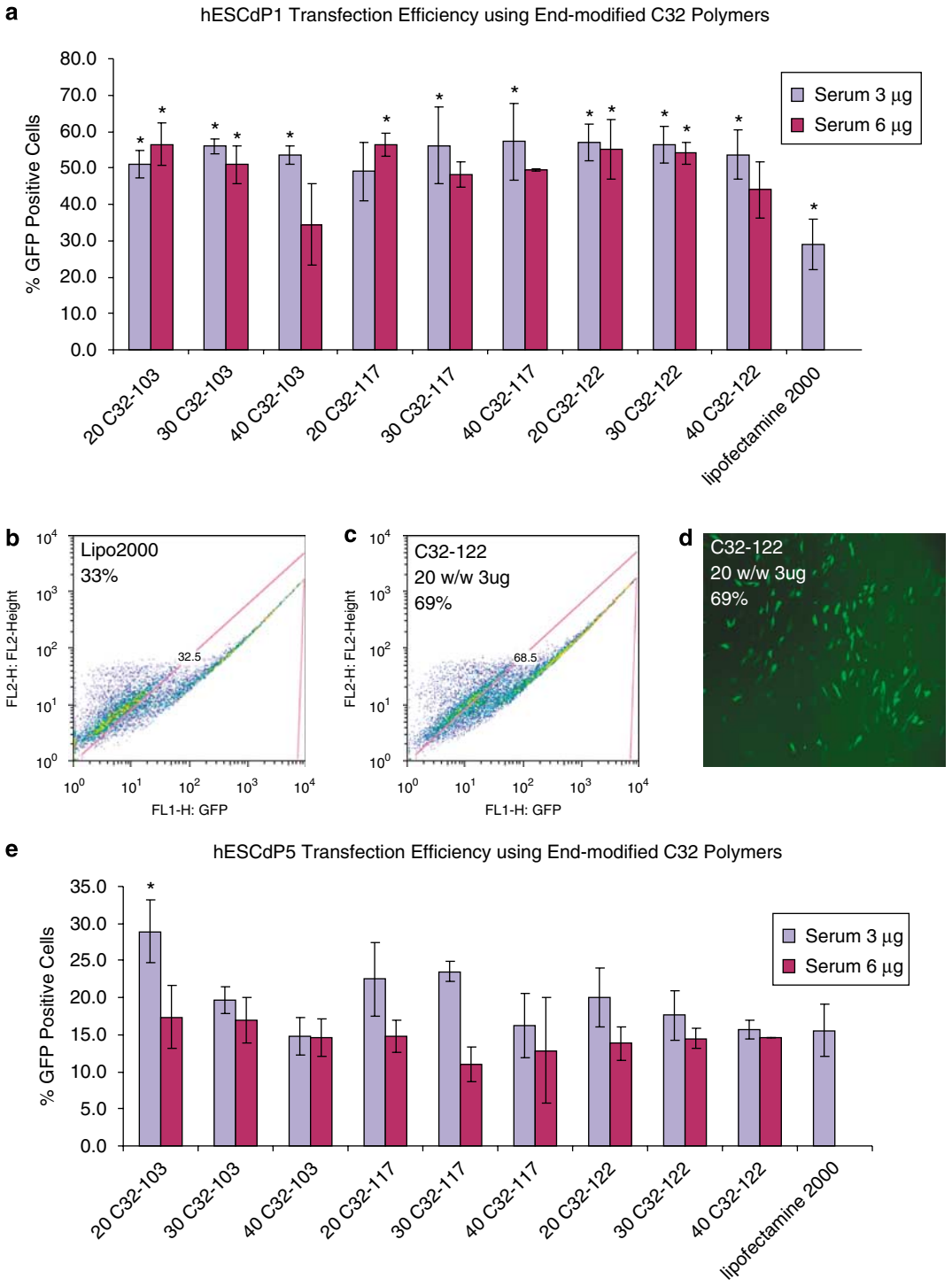

Figure 5 Efficiency of gene delivery to human embryonic stem cell-derived cells (hESCds) in 10% serum using end-modified C32 polymers. End-modified C32 polymers showed significantly higher transfection efficiency for passage 1 hESCds (**a–d**) and passage 5 hESCd cells (**e**) under leading transfection conditions (*$P < 0.05$ compared to Lipofectamine 2000). Error bars are s.d. ($n = 3$); and it is less than 0.01 for sample 40 C32–122 6 μg, in panel e.

DNA dosages or increasing the polymer/DNA weight ratio from 20:1 to 40:1 did not significantly affect the cell viability. For hADSCs, C32–103 appeared least toxic for both low- and high-dosage DNA groups (Figure 6b). Under its optimal transfection condition (3 μg per well, 30 w/w), the cell viability was 89 ± 6%. Cells transfected with C32–117 showed good cell viability (90–94%) at low DNA dosage (3 μg per well), whereas cell viability significantly decreased as the polymer/DNA weight ratio increased to 30:1 at high DNA dosage (6 μg per well). C32–122 appeared most toxic for hADSCs and cell viability dropped to 64 ± 3% under its optimal transfection condition (3 μg per well, 30 w/w). The cytotoxicity of Lipofectamine 2000 to hADSCs fell in between the polymer groups and showed a cell viability of 80.4 ± 0.3%. The hESCds appeared sensitive to the

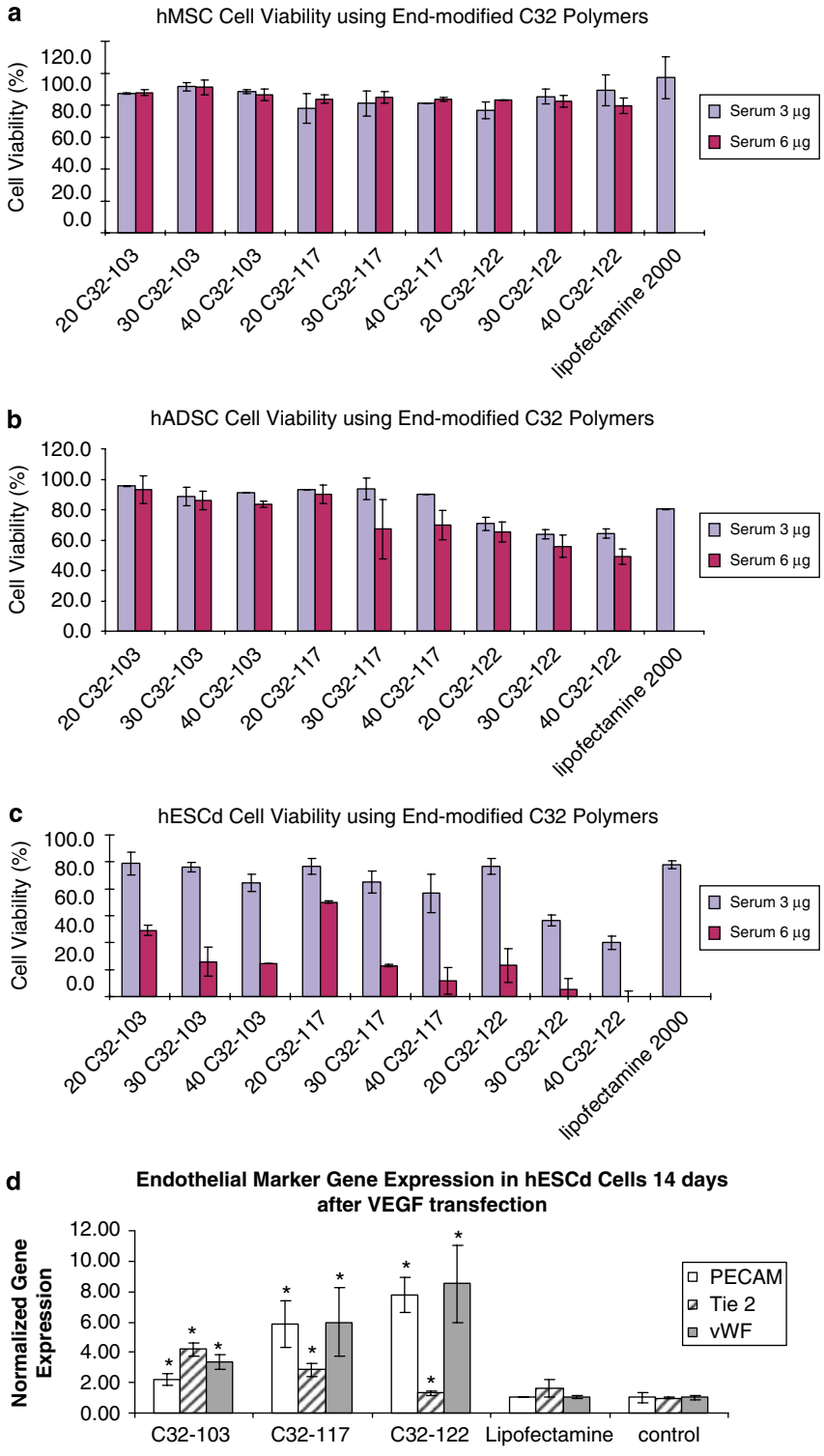

Figure 6 Cell viability of three types of stem cells using end-modified C32 polymers and gene expression of endothelial markers in human embryonic stem cell-derived cells (hESCds) after overexpression of vascular endothelial growth factor (VEGF). Cell viability was reported 72 h after transfection in 10% serum for three stem cell types including (**a**) human mesenchymal stem cells (hMSCs), (**b**) human adipose-derived stem cells (hADSCs) and (**c**) hESCds. Normalized gene expression of endothelial markers (PECAM, Tie 2 and vWF) in hESCds 14 days after transfection with VEGF (**d**) (*$P<0.05$ compared to the control group). Error bars are the s.d. ($n=3$). Error bars in the following groups are less than 0.2 and are not visible on the graph: (**a**) sample 40 C32–117 3 μg; (**b**) sample 20 C32–103 3 μg; sample 40 C32–103 3 μg; sample 20 C32–117 3 μg; sample 40 C32–117 3 μg; (**c**) sample 40 C32–103 6 μg and Lipofectamine 2000.

polymers, and good cell viability (97–99%) was observed only under low DNA dosage (3 μg per well) and low polymer/DNA weight ratio (20 w/w; Figure 6c). Lipofectamine was not toxic for hESCds and showed good cell viability (98 ± 3%). Further increase in polymer/DNA weight ratio led to a slight decrease in

viability for cell treated with C32–103 and C32–117, whereas cell viability significantly decreased down to 40 ± 5% for cells treated with C32–122. At high DNA dosage (6 μg per well), cell viability of all polymer-treated groups dropped significantly.

Enhanced endothelial differentiation of hESCds after vascular endothelial growth factor (VEGF) transfection using end-modified C32 polymers

After 2 weeks of culture in endothelial growth medium, all hESCds transfected with VEGF using end-modified C32 polymers expressed significant higher levels of endothelial markers than the untreated control group, as shown by quantitative PCR (Figure 6d). For the C32–103 transfected group, the expression levels of *Tie 2* and *Von Willebrand factor (vWF)* were, respectively, 3.2- and 2.3-folds higher than the untreated controls, with $P<0.05$. Similar trend was observed with regards to the other polymer-transfected groups. Compared to the untreated control, the gene expressions of *platelet endothelial cell adhesion molecule (PECAM)* and *vWF* in the C32–117 transfected group were, respectively, 4.7- and 4.9-folds higher; and gene expressions of PECAM and vWF in the C32–122 group were 6.5- and 7.4-folds higher ($P<0.05$). In contrast, Lipofectamine 2000 transfected group only showed a slight increase in the gene expressions of all three endothelial markers, and the differences were not statistically significant. The control group incubated with VEGF growth factor also showed some increase in the expressions of all three markers, with 83 and 62% increase in *PECAM* and *Tie-2* (endothelial-specific receptor tyrosine kinase), respectively, and 1.3-fold increase in vWF expression compared with the untreated control ($P<0.05$).

Biophysical characterization

The effective diameters of polymer/DNA nanoparticles (formed at 30 w/w) were measured using dynamic light scattering. Average diameters of the polymer/DNA nanoparticles in 10% serum-containing media are shown in Figure 7 for the unmodified C32 polymer and the three end-modified C32 polymers. Right after complexation, all end-modified C32-based nanoparticles started at a similar size range of ~150 nm (Figure 7a). In contrast, unmodified C32-based nanoparticles formed at a 30 w/w ratio started at a larger 230-nm size. The particle size of all groups gradually increased, but the increase of unmodified C32-based nanoparticles polymer was significantly higher than the increase of the end-modified C32 polymers. After i h of complexation, the particle size of unmodified C32 polymer increased to 459 nm. Particles complexed from C32–117 showed smallest particle size (185 nm) 1 h after complexation, whereas the size of C32–103 and C32–122 nanoparticles increased to 209 and 247 nm, respectively (Figure 7a). The bulk of particle size increase occurred within the first 2 h and afterwards; the particle size only increased slightly (Figure 7b). By the end of 4-h incubation, the particle size of unmodified C32 polymer (599 ± 37 nm) is 74 and 61% larger than C32–117 and C32–103, respectively, with $P<0.05$.

The surface charge of the nanoparticles were evaluated by ζ potential measurement after 1 h of complexation and shown in Figure 7c. In the presence of 10% serum-containing medium, the ζ potentials of all the polymeric nanoparticles were slightly negative. The ζ potential of three end-modified C32-based nanoparticles were more neutral than the unmodified C32-based nanoparticles (−1.79 ± 0.05 mV, $P<0.05$).

Discussion

The clinical application of stem cell-mediated gene therapy approaches rely heavily on the development of safe and efficient methods to deliver genes to stem cells. Nonviral vectors are safe and easy to produce, but their efficacy is far from satisfactory.[20] And gene delivery to stem cells under serum-containing conditions remains particularly challenging. Optimization of nonviral vectors in serum-free *in vitro* conditions may be of limited use in designing a nonviral vector for *in vivo* application. This is due to the fact that many important vector properties, such as particle size, stability and ζ potential change dramatically in the presence of serum proteins. Here, we have shown that poly(β-amino esters), and end-modified C32 polymers specifically, can transfect various types of human stem cells with high efficiency (~30% for adult-derived and ~60% for embryonic-derived, see Supplementary Figure 1) and minimal cytotoxicity in the presence of 10% serum. Unmodified C32 polymers can transfect hMSCs up to 38.9% in serum-free condition and could be useful for *ex vivo* gene delivery approaches. The efficiency we achieved using our polymers is 1–4-fold higher than the values of a leading commercially available nonviral transfection reagent Lipofectamine 2000. In addition, our results are also far better than values reported in an earlier study using electroporation, in which 12% of positively transfected hMSCs was shown with EGFP (enhanced green fluorescent protein) gene.[33] We also examined the length of GFP expression of the transfected stem cells using end-modified C32 polymers by fluorescence microscopy. Intense green fluorescence signals can be observed as early as 10 h after transfection, and the intensity reached its peak by day 2 and remained highly expressed till day 5. Afterwards, the green fluorescence intensity gradually decreased and lasted up to about 2 weeks.

Cell viability after the transfection process is another critical parameter to consider when determining the efficacy of nonviral gene delivery. Under the optimal transfection conditions, our end-modified C32 polymers led to high cell viability in all three stem cell types, with 87 ± 0.3% for hMSCs, 94 ± 8% for hADSCs, and 97 ± 6% for hESCds. The cell viability we observed is much higher than a recent study using nucleofection method for gene delivery to hMSCs, where enhanced transfection efficiency was achieved (~50%), but induced over 50% cell death postnucleofection.[22] In another study, electroporation resulted in 10% transient transfection levels in human adult progenitor cells, but this process was toxic and the transfected cell number decreased with time.[21]

The use of poly(β-amino esters) as agents for nonviral gene delivery is a significant step forward for stem cell gene transfer. Although other cationic polymers including poly-L-lysine and polyethylenimine have been recently tested for polymeric gene delivery to bone marrow stromal cells, gene delivery is relatively poor as

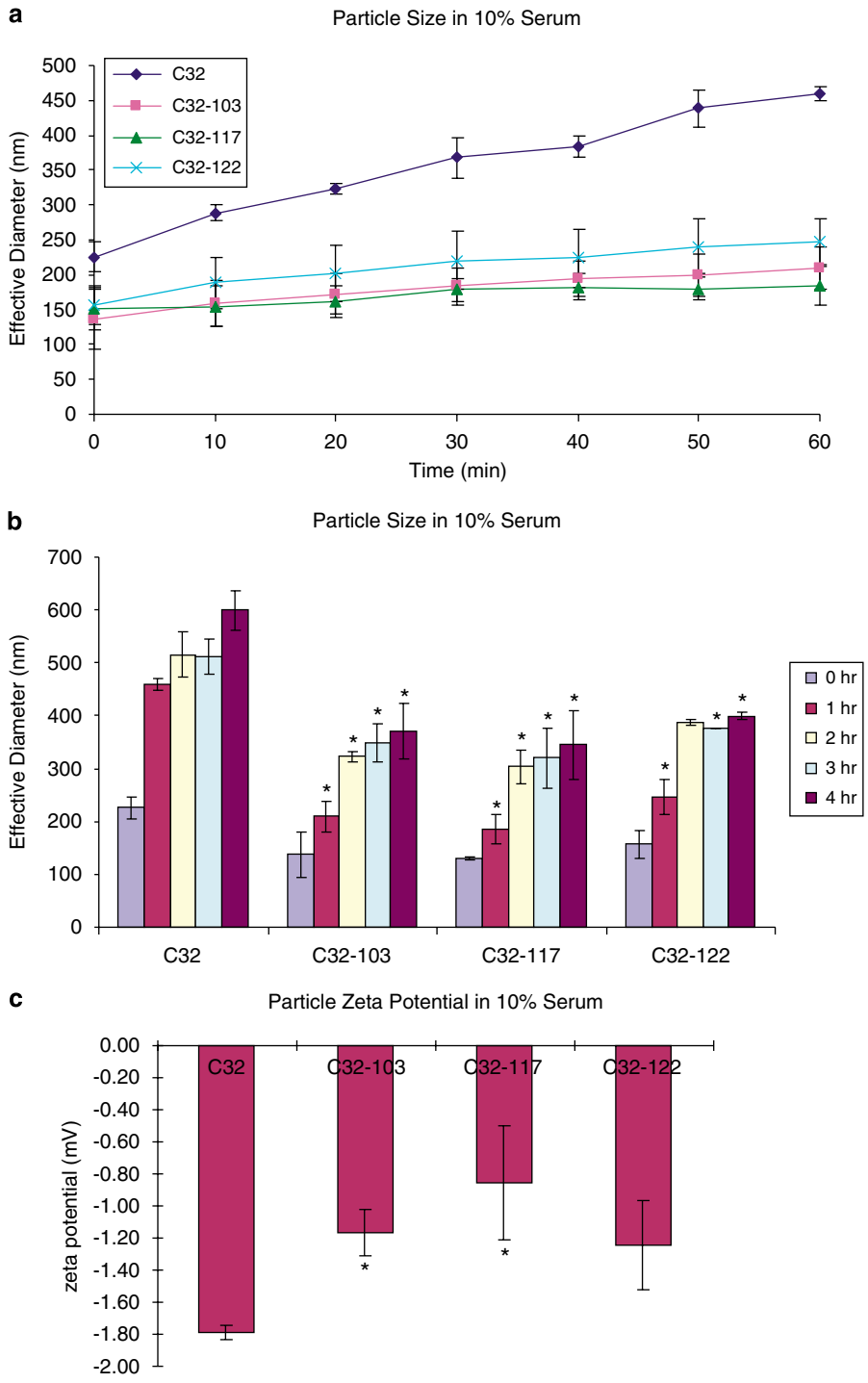

Figure 7 Biophysical characterization of nanoparticles formed using end-modified C32 polymers and DNA plasmid. In the presence of 10% serum, end-modified C32 polymers showed significant smaller particle size (**a**), better stability (**b**) and more neutral ζ potentials (**c**) compared to the unmodified C32 polymer. (*$P<0.05$ compared to unmodified C32 polymers at the same time point). Error bars are s.d. ($n=3$) and it is in panel **c,** C32–122 3 h group, is less than 0.1.

<4% of cells are positive when measured a few days after particle addition.[34] Furthermore, both of these polymers caused high cytotoxicity and reduced growth rates relative to untreated controls. In contrast, the engineered, biodegradable poly(β-amino esters) presented here are an order of magnitude more effective at delivering DNA to bone marrow-derived hMSCs, and these cells also maintain high viability following transfection. In addition, we showed this high efficacy and high viability on other clinically relevant stem cells including hADSCs and hESCds. The high transfection capability of end-modified C32 polymers in serum-containing conditions is consistent with their potential that have been shown for *in vivo* and *ex vivo* gene therapy.[31,32] In general, these modified polymers show superior performance relative to unmodified C32 polymers.

Using end-modified C32 polymers, we delivered DNA plasmid-encoding *VEGF* to the hESCds. To evaluate the influence of *VEGF* transfection on the endothelial differentiation of hESCds, we examined the expression levels of three endothelial markers including *Tie-2*, *PECAM* and *vWF*. *Tie-2* represents the earliest mammalian endothelial cell lineage marker and *Tie-2* plays a critical role during the embryonic angiogenesis.[35] *PECAM-1*, also known as *CD31*, is a type I integral membrane glycoprotein and mediates adhesive interactions between adjacent endothelial cells.[36] *vWF* mediates platelet adhesion and thrombus formation at sites of vascular injury and serves as a carrier for factor VIII in plasma.[37] *VEGF* transfection using end-modified C32 polymers led to significantly increased expressions of all three endothelial markers of the hESCds, whereas only slight increase in differentiation was noted in the group transfected with Lipofectamine 2000. These results further confirm that end-modified C32 polymers can be used as effective transfection reagents to deliver signals to direct stem cell differentiation.

Our data also suggest that the transfection efficiency of poly(β-amino esters) to embryonic stem cell-derived cells varies significantly from the transfection efficiency to adult-derived stem cells. The passage 1 hESCds are derived from embryonic origin, and we consider that these cells are the least differentiated among the three types of stem cells examined in this study. Our data showed that the transfection efficiency of end-modified C32 polymers for hESCdP1 cells (~60%) was ~100% higher than that for stem cells from adult origins (hMSCs or hADSCs) and late passage hESCds (~30%). Interestingly, the transfection profile of the polymers for late passage hESCds (hESCdP5) are similar to that of the hMSCs. We note that it has been previously reported that passage 4 hESCds become mesenchymal-like cells and express hMSC surface markers, including CD29, CD44 and CD105.[38] In general, our results suggest that poly(β-amino ester)/DNA nanoparticles are more efficient in delivering genes to stem cells that are in a less differentiated state. The transfection efficiency of Lipofectamine 2000 also depends on the stem cell differentiation stage and the transfection efficiency for hESCdP5 (~15%) is only half of that for hESCdP1 cells (~30%). In contrast, early and late passage hMSCs and hADSCs showed more comparable transfection profiles, suggesting that the capability of end-modified C32 polymers to deliver genes to adult stem cells is relatively constant.

Biophysical properties of the nanoparticles have a significant impact on transfection efficacy.[30,39–41] Our results of particle size, stability and ζ potential in serum were found to correlate well with the transfection efficacy of the polymers. In the presence of serum, negatively charged serum proteins can adsorb to the positively charged nanoparticles. Such a protein coating can lead to the changes in particle size and surface charge, and may also cause aggregation. Our results show an increase in nanoparticle size over time when the polymeric nanoparticles are incubated in serum-containing medium. The bulk of the particle size increase occurs within the 2 h after complexation, and the particle size remains relatively stable for later time points. Compared to the unmodified C32 polymer, the end-modified C32 polymer/DNA nanoparticles show smaller size and increased stability in the same medium conditions that are used for transfection. Furthermore, ζ potential measurements show that these nanoparticles have a slightly negative surface charge in the presence of serum, and end-modified C32-based nanoparticles exhibit a more neutral surface charge than nanoparticles formed with the unmodified C32 polymer. Correspondingly, end-modified polymers transfected stem cells very well in the presence of serum proteins, whereas unmodified C32 polymer worked less efficient in serum-containing medium. Thus, the formation of small (~200 nm), stable and neutral nanoparticles in serum was found to be predictive for high transfection efficacy for stem cells in serum-containing medium, and the formation of large aggregating and more negative particles in serum was found to correlate with poor transfection efficacy.

In this study, we selected stem cells from three origins (bone marrow, fat and embryoid bodies) that offer advantages such as relative abundance and ease of isolation. The fact that these end-modified C32 polymers worked well across all three stem cell types suggest that these polymers may be a general transfection reagent for other types of stem cells as well, such as neural stem cells and muscle-derived stem cells. To our knowledge, this is the first biodegradable nonviral gene delivery system that can transfect stem cells highly enough to be therapeutically useful, with minimal cytotoxicity.

In conclusion, we have shown that poly(β-amino esters), and end-modified C32 polymers specifically, are a class of highly efficient nonviral transfection vectors for gene delivery to both adult and embryonic-derived stem cells. These nanoparticles have significantly higher transfection efficacy than a leading commercially available transfection reagent Lipofectamine 2000, are stable in the presence of serum proteins, and are noncytotoxic. The fact that this system works well in serum-containing medium facilitates its use under standard *in vitro* cell culture conditions and offers great advantages for *in vivo* gene delivery applications. The biodegradability and safety of these polymers makes them readily translatable for clinical applications. Controlled delivery of DNA plasmids encoding specific growth factors, such as bone morphogenetic protein or therapeutic proteins, to stem cells using these biodegradable polymeric vectors would have great promise for various tissue engineering applications and gene therapy approaches.

Materials and methods

Materials

Monomers were purchased from Sigma-Aldrich (St Louis, MO, USA), Scientific Polymer Products Inc. (Ontario, NY, USA) and Molecular Biosciences (Boulder, CO, USA). All chemicals were used as received. Anhydrous dimethyl sulfoxide was purchased from Sigma-Aldrich (St Louis). A 25-mM sodium acetate buffer solution pH5.2 (NaAc buffer) was prepared by diluting a 3-M stock (Sigma-Aldrich). Plasmid DNA *pEGFP-N1* was obtained from Elim Biopharmaceuticals (Hayward, CA, USA).

Polymer synthesis

Unmodified C32 polymer was synthesized as described previously.[29] Briefly, butanediol diacrylate (C) and aminopentanol (32) monomers were mixed in a 1.2:1.0

amine/diacrylate molar ratio at 90 °C for 24 h to generate aminopentanol-terminated polymer chains (Figure 1a). Amine-capped polymers were prepared by the first polymerizing C32 using a 1.2:1.0 diacrylate to amine/monomer molar ratio at 90 °C for 24 h to produce acrylate-terminated C32 polymer (C32-Ac; Figure 1b).[32] Subsequently, amine-terminated C32 polymers were generated by reacting C32-Ac with diamine monomers in dimethyl sulfoxide (Figure 1c). End-capping reactions were performed overnight at room temperature using a 1.6-fold molar excess of amine over acrylate end groups.

Cell culture

Bone marrow-derived hMSCs (Lonza, Walkswille, MD, USA) were grown in MSC growth medium consisting of Dulbecco's modified Eagle Medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco), 100 mM sodium pyruvate and 100 U penicillin and 100 μg ml^{-1} streptomycin. Cells were subcultured upon confluence until passage 7. Human adipose-derived stem cells were isolated from lipoaspirated human fat tissue following the protocols as described previously.[4] The isolated cells were expanded in hMSC growth medium for further experiment. To derive hESCds, human embryonic stem cell line H9 (WiCell Research Institute, Madison, WI, USA) were grown on inactivated MEFs in hESC growth medium consisting of 80% knockout Dulbecco's modified Eagle Medium, supplemented with 20% knockout serum replacement, 4 ng ml^{-1} basic fibroblast growth factor, 1 mM L-glutamine, 0.1 mM 2-mercaptoethanol and 1% nonessential amino acid stock (Invitrogen, Carlsbad, CA, USA). For embryoid body (EB) formation, embryonic stem cell (ESC) cultures were dissociated into small clumps by incubating at 37 °C for 15 min with 2 mg ml^{-1} collagenase IV. The small ESC clumps were pelleted, resuspended in hESC medium without basic fibroblast growth factor, and cultured in Petri dishes for 10 days with a medium change every other day. The EBs were then transferred to gelatin-coated (0.1% w/v) plates. Upon 70% confluence, the cells were subcultured in MSC growth medium to induce further cell differentiation.

GFP transfections

Cells were seeded at 75 000 cells per well in clear 24-well plates 24 h before transfection. hMSCs were used by passage 5 and 7; hADSC s were used by passage 4 and 6 and hESCds were used at passage 1 and 5. Transfections using EGFP as a reporter gene were performed using DNA plasmid *pEGFP-N1* (Elim Biopharmaceuticals), which is driven by the cytomegalovirus promoter. End-modified C32 polymers (C32–103, 117 and 122) were optimized for high efficacy and low cytotoxicity in growth medium containing 10% serum. Unmodified C32 polymers were also used to transfect hMSCs both in 10% serum and in OptiMem. Vector parameters were varied including polymer type, polymer/DNA weight ratio (20, 30, 40, 50, 60 and 70), and DNA loading dosage (3 μg or 6 μg per well in a 24-well plate). Polyplexes were formed by mixing poly(β-amino esters) with DNA in sodium acetate (NaAc) buffer and waiting 10 min for the complexes to form. The polyplexes were then added to cells cultured in growth medium containing 10% serum and incubated for 4 h. Following a 4-h transfection incubation time, the complexes were removed and replaced with 1 ml fresh growth medium.

Flow cytometry

Green fluorescent protein expression was measured using fluorescence-activated cell sorting on an FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) 72 h after transfection. Propidium iodide staining was used to exclude dead cells from the analysis and 10 000 live cells per sample were acquired. Two-dimensional gating was used to separate increased autofluorescence signal from increased GFP signal to more accurately count positively expressing cells. Gating and analysis was performed using *FlowJo 6.3* software (TreeStar, Ashland, OR, USA).

Cell viability

To measure cytotoxicity and cell viability, cellular metabolic activity was measured using the Cell Titer 96 Aqueous One Solution assay kit (Promega). Metabolic activity was measured using optical absorbance on a Victor 3 Multi-label plate reader (Perkin Elmer Life Sciences, Boston, MA, USA). Measurements of treated cells were converted to percentage viability in comparison with untreated controls.

Overexpression of VEGF in hESCds

Passage 3 hESCds were seeded in 6-well plates at 300 000 cells per well 24 h before transfection. Cells were transfected with DNA plasmid-encoding human *VEGF* 165 (pBLAST49-hVEGF; provided by Johnny Huard laboratory, University of Pittsburgh). Transfection using end-modified C32 polymers (C32–103, 117 and 122) were performed using optimized condition (20 w/w, 12 μg per well in 6-well plate) for high efficacy and minimal cytotoxicity in growth medium containing 10% serum. Cells were also transfected with VEGF using Lipofectamine 2000 for comparison. Following a 4-h transfection incubation time, the complexes were removed and refilled with 3 ml endothelial growth medium (Lonza, Walkswille, MD, USA). Cells incubated with endothelial growth medium containing 2 ng ml^{-1} VEGF growth factor (R&D Systems, Minneapolis, MN, USA) and untreated cells incubated in endothelial growth medium were included as controls. All the groups were maintained in culture for 2 weeks before harvest, with medium change 3 times a week.

RNA isolation, reverse transcription and quantitative PCR

Total RNA was harvested from cells using RNeasy Kit (Qiagen, Germantown, MD, USA). The cDNA was synthesized by reverse transcriptase using the Superscript First-Strand Synthesis System (Invitrogen) and 1 μg of total RNA. Quantitative PCR was performed using Taqman Fast Universal PCR Master Mix on a StepOne Plus Realtime PCR System (Applied Biosystems, Foster City, CA, USA). Endothelial differentiations were examined using Taqman Gene Expression Assays (Applied Biosystems) including *PECAM* (Hs00169777_m1), *Tie2* (Hs00176096_m1), *vWF* (Hs00169795) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Hs02758991_g1). All samples were analyzed in triplicates, and relative mRNA level was calculated using the $\Delta\Delta C_t$ methods. In brief, the expres-

sion level of every gene was first normalized to *GAPDH*, and results were presented as relative fold changes in transfected groups using normalized mRNA level in untreated cell group as controls.

Biophysical characterization

Particle size and ζ potential of the polyplexes were measured by using a ZetaPALS dynamic light scattering detector (Brookhaven Instruments Corp., Holtsville, NY, USA; 15-mW laser, incident beam 676 nm). Samples were prepared for biophysical characterization in the same manner and at the same concentrations, as they were for transfections using 30:1 polymer/DNA weight ratio. After a 10-min complexation, 200 μl of the polymer/DNA solution was added to 1.4 ml of growth medium containing 10% serum to reach a final volume of 1.6 ml. Particle stability was determined by monitoring the changes of particle size over time. Particle size was measured every 10 min up to 1 h, and then every hour up to 4 h. Particle ζ potential was measured 1 h after complexation. Effective diameters were calculated from the autocorrelation function using the MAS option of the BIC particle-sizing software assuming a log normal distribution. The solution viscosity and refractive index were assumed equal to those of pure water at 25 °C. Average electrophoretic mobilities were measured using BIC PALS ζ potential analysis software, and ζ potential were calculated using the Smoluchowsky model for aqueous suspensions.

Statistical analysis

All experiments were performed in triplicate. The results were reported as mean ± s.d. Statistical analysis was performed using the one way analysis of variance with Dunnett's multiple comparison test as the post-test to compare all the experimental groups with Lipofectamine 2000. Two way ANOVA was performed to compare the effect of cell passage number among different polymer groups. Statistical significance was set as $P<0.05$.

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Supplementary Information accompanies the paper on Gene Therapy website (http://www.nature.com/gt)