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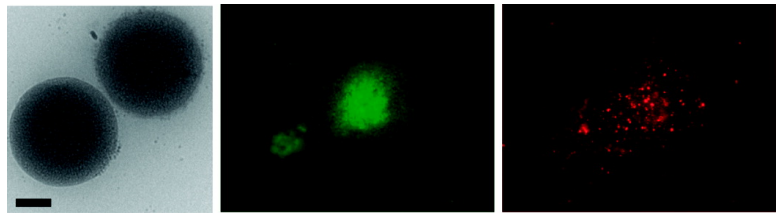

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Nanoparticles for Gene Transfer to Human Embryonic Stem Cell Colonies

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ABSTRACT

We develop biodegradable polymeric nanoparticles to facilitate nonviral gene transfer to human embryonic stem cells (hESCs). Small (~200 nm), positively charged (~10 mV) particles are formed by the self assembly of cationic, hydrolytically degradable poly(β-amino esters) and plasmid DNA. By varying the end group of the polymer, we can tune the biophysical properties of the resulting nanoparticles and their gene-delivery efficacy. We created an OCT4-driven GFP hES cell line to allow the rapid identification of nanoparticles that facilitate gene transfer while maintaining an hESC undifferentiated state. Using this cell system, we synthesized nanoparticles that have gene delivery efficacy that is up to 4 times higher than that of the leading commercially available transfection agent, Lipofectamine 2000. Importantly, these materials have minimal toxicity and do not adversely affect hESC colony morphology or cause nonspecific differentiation.

The delivery of genes to stem cells can advance cell-based therapies and the field of tissue engineering. Given the right conditions, pluripotent stem cells can potentially differentiate into any cell type of the body, which allows for wide therapeutic utilities including cardiac tissue engineering and treatments for autoimmune diseases, spinal chord injuries, and Parkinson's disease, among many others.[1] Gene delivery could allow for directed differentiation from a pluripotent stem cell into specific differentiated cell types of interest, including hematopoietic cells, neurons, cardiomycytes, and osteoblasts and could also allow a differentiated cell to be reprogrammed back into a pluripotent state.[2−4] Beyond controlled differentiation, ectopic expression of key growth and transcription factors could allow for the elucidation of fundamental cell development pathways in vitro as well as for the regulation of growth in vivo once the cells are transferred to a patient. Gene delivery can also provide a mechanism for in vivo expression of secreted therapeutic proteins.

Whereas there is promise, current approaches for gene transfer to hESCs are limited because there are safety concerns with viral approaches, and nonviral methods have low efficacy. For example, testing in the H9 hESC line showed that commonly used lipid- and polymer-based transfection agents including FuGENE, LipofectAMINE Plus, and ExGen 500 transfected less than 10% of the hESCs.[5] Electroporation resulted in similarly low transfection and, unlike in murine ESC, significantly reduced viability. Nucleofection, a modified version of electroporation that includes the addition of a nucleofactor solution, can significantly improve gene transfer to achieve 20% transfection of hESCs, whereas regular electroporation achieves only 5 to 6%.[6] However, nucleofection, like regular electroporation, can cause high cell death. Many hESC gene transfer studies lack a reliable marker to separate transfected undifferentiated cells from differentiating, differentiated, and feeder cells.[7] Studies from our group and others have shown that the differentiating cells on the periphery of cell colonies are the cells that are most likely to be transfected. Carry-over feeder cells may also be preferentially transfected over hard-to-transfect human stem cell colonies. For a quantitative analysis of transient transfection efficiency, a reliable undifferentiated hESC marker is needed.

Viral gene transfer approaches for hESCs also have limitations. Adenovirus serotype 5 has been shown to transduce just 11% of undifferentiated H9 hESCs, and adeno-associated virus (serotypes 2, 4, and 5) has been shown to transduce only 0.01% at best.[8] The most effective viral strategy to date has been lentiviral vectors, which have ~40% transduction efficiency in H1 and H9 hESCs.[9] Although the

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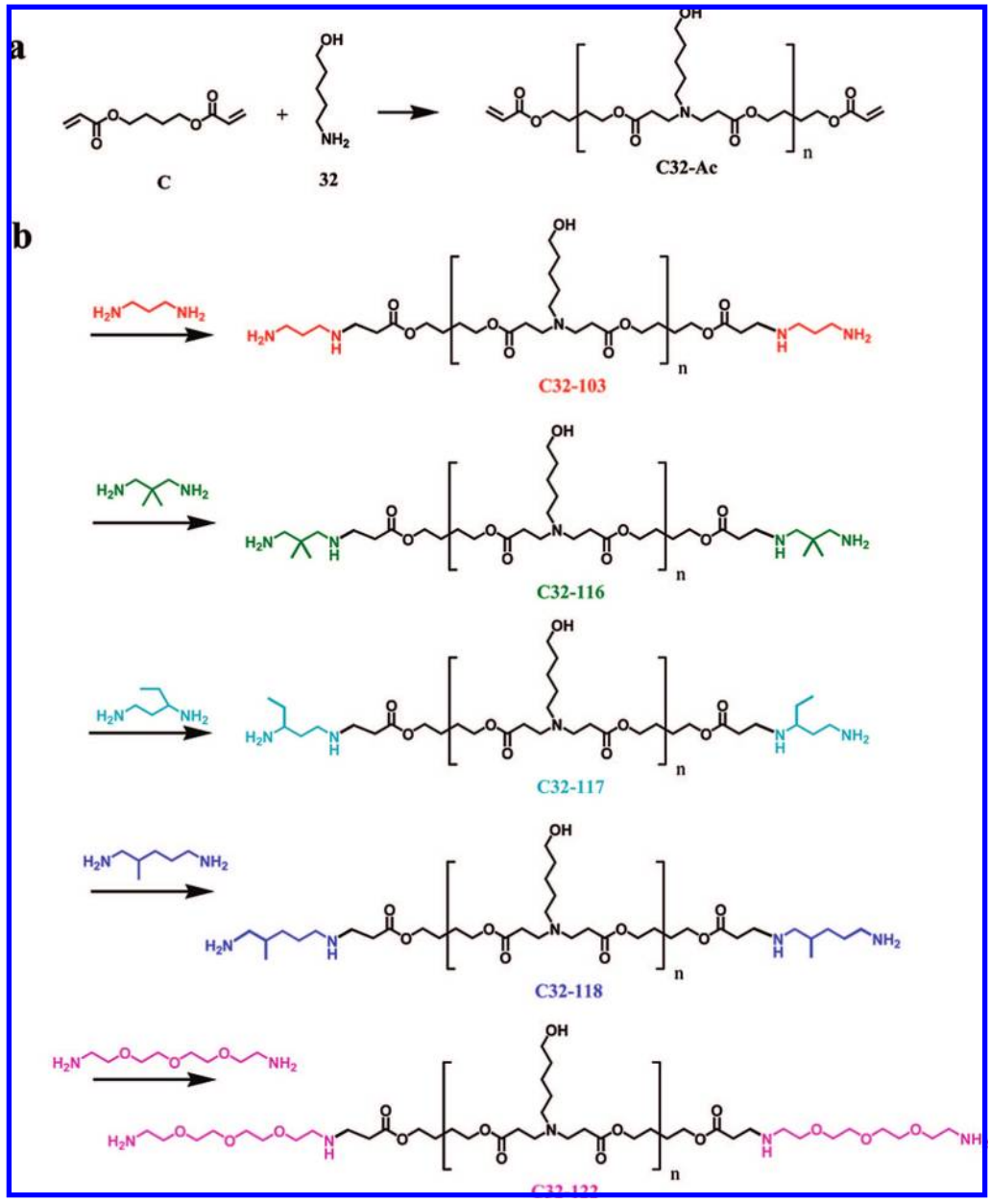

Figure 1. Synthesis of end-modified poly(β-amino esters). Each end of C32-117 and C32-118 may be in either of the shown configurations.

transgene expression can be further enhanced by concentrated virus, appropriate promoters, and drug selection after transduction, the safety concerns associated with insertional mutagenesis after viral integration are difficult to overcome. This issue limits their potential for human gene therapy. A safe and effective method for gene delivery to stem cells would be invaluable to the creation of new cell-based therapies.

We have developed a class of polymers, poly(β-amino esters), which are promising for nonviral gene delivery because of their ability to condense DNA into nanoparticles that facilitate cellular uptake and endosomal escape.[10−12] Because of the hydrolytic cleavage of the ester groups that these polymers comprise, they are biodegradable and have low toxicity.[10,13,14] These particles are also useful because they can be coated for ligand-specific delivery.[15] Chemical modification of the ends of these polymers results in improved biomaterials that can deliver DNA to human umbilical vein endothelial cells (HUVECs) at levels that are comparable to those of adenovirus.[16,17] Here we develop polymeric nanoparticles for nonviral gene transfer to undifferentiated hESCs. To ensure that the transfected hESC colonies remain in an undifferentiated state once transfected we use hESCs that are targeted to express Oct4-driven GFP stably and to monitor GFP levels as an indicator of the undifferentiated state of the hESCs. We demonstrate that these nanoparticles can be formulated to have a high efficacy for gene delivery to hESCs while also maintaining a pluripotent, undifferentiated state.

End-modified poly(β-amino esters) were synthesized in a two-step procedure. First, acrylate-terminated C32 (C32-Ac)

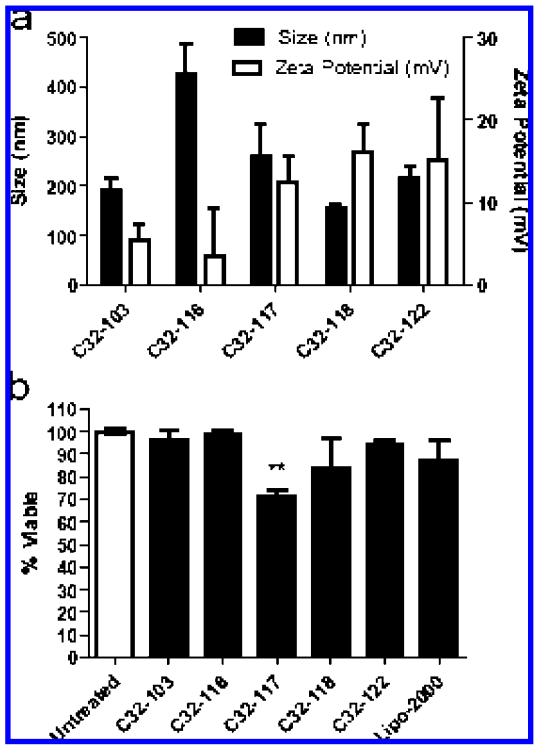

Figure 2. (a) Particle size (left axis, black bars) and zeta potential (right axis, white bars) of end-modified poly(β-amino esters)/DNA particles (mean + SD). (b) hESC viability 24 h after transfection with end-modified poly(β-amino esters) or Lipofectamine 2000 (mean + SD). Polymeric nanoparticles that are composed of C32-103, C32-116, C32-118, and C32-122 do not show cytotoxicity. C32-117 shows some cytotoxicity ($p < 0.01$) compared with untreated cells, but it is not statistically more cytotoxic than Lipofectamine 2000.

was synthesized via the conjugate addition of 5-amino-1-pentanol to 1,4-butanediol diacrylate at a 1.2:1 molar ratio of diacrylate monomer to amine monomer (Supporting Information). Next, this polymer was end-modified by the reaction of C32-Ac with an excess of various diamine monomers (Figure 1) in THF, and it was then purified by precipitation. We formed polymer/DNA nanoparticles by vortex mixing polymer and DNA solutions together in 25 mM sodium acetate and waiting 10 min for particle self-assembly (Supporting Information). All particles were formulated at a 50:1 weight ratio of poly(β-amino ester) to DNA. In general, these particles were found to have a small size (~200 nm) and a positive zeta potential (~10 mV), as measured by dynamic light scattering and phase analysis light scattering. However, slight changes to the end-group of the polymer can change theses properties (Figure 2a). For example, particles that were formed with polymer C32-116 are much larger (400 nm) and less positively charged (4 mV). Structurally, C32-116 is almost identical to C32-103 and C32-117 (Figure 1). The only difference is that C32-116 has two methyl groups near the terminal amine whereas C32-103 does not have these groups and C32-117 has an ethyl group instead. C32-118, which has a slightly longer hydrocarbon spacer between the terminal amine and the base C32 polymer, forms nanoparticles that are the smallest in size (160 nm) and the most positively charged (16 mV). C32-122, which has a slightly longer hydrophilic spacer between the terminal amine and the base C32 polymer, forms nanoparticles that are of average size but have higher than average zeta potential (15 mV).

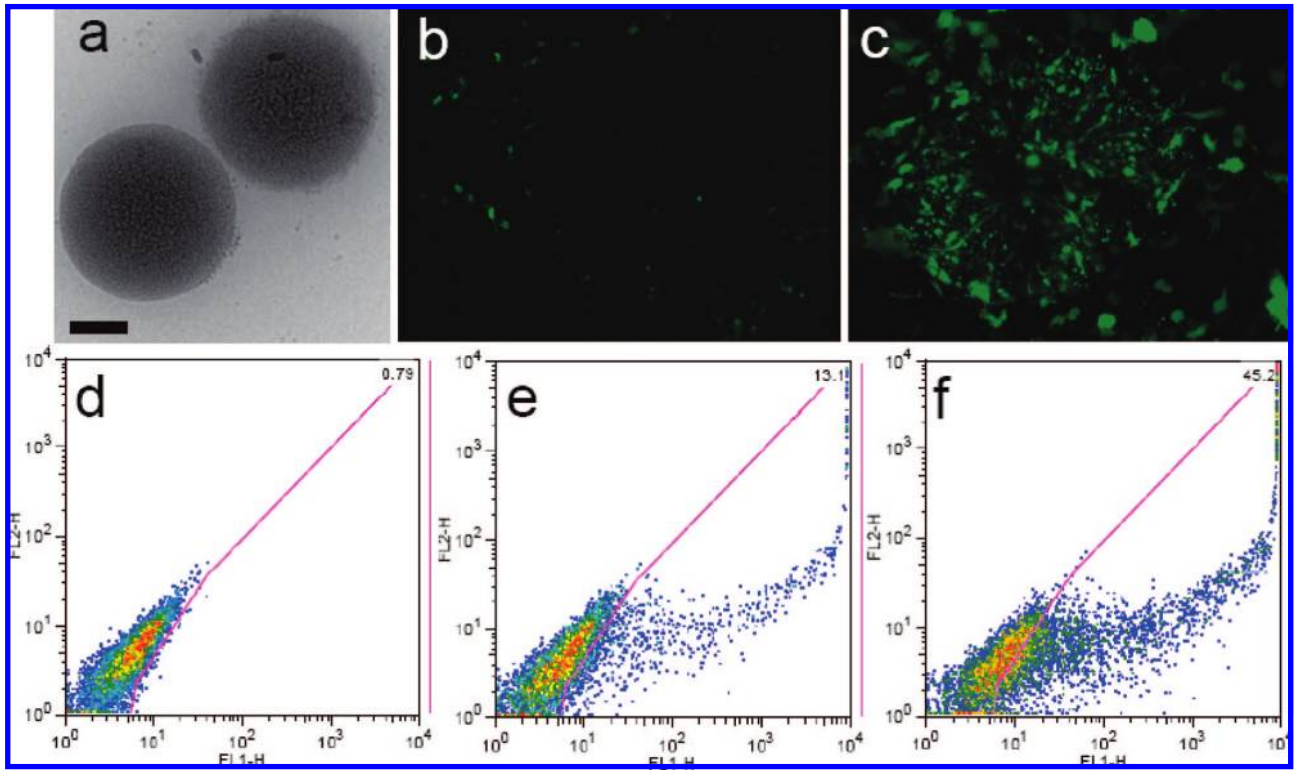

Figure 3. Poly(β-amino esters) form nanoparticles with high gene-delivery efficacy to hESC colonies. (a) Poly(β-amino ester) C32-118/DNA particles have a small, spherical shape (scale bar is 100 nm). Fluorescence micrographs are shown following treatment with (b) Lipofectamine 2000 and (c) C32-118. 2D FACS gating of GFP expression (FL1) of hESCs: (d) untreated, (e) Lipofectamine 2000, and (f) C32-118.

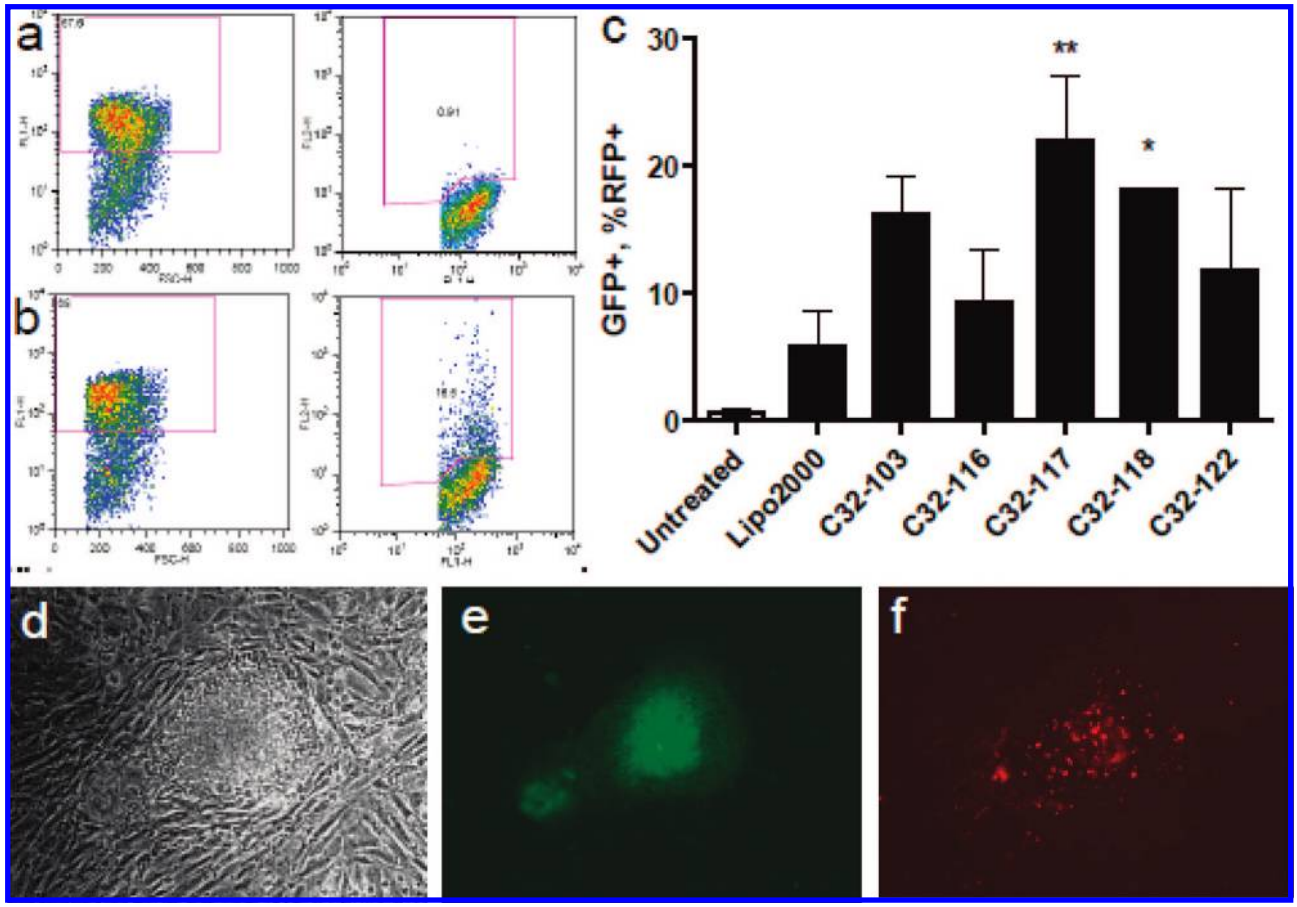

Figure 4. FACS gating of Oct4 GFP+ undifferentiated hESCs and RFP+ transfected hESCs for (a) free DNA and (b) C32-122/DNA nanoparticles. (c) Transfection of hESC colonies for different poly(β-amino esters) (mean + SD). C32-117 ($p < 0.01$) and C32-118 ($p < 0.05$) are significantly better at gene delivery than Lipofectamine 2000. Micrograph of representative hESC colony, 1 week post-transfection with C32-122/DNA nanoparticles, by (d) light microscopy, (e) GFP fluorescence (showing undifferentiation), and (f) RFP fluorescence (showing persistence of transfection).

To measure cell viability, we measured cellular metabolic activity by using the Cell Titer 96 Aqueous One Solution assay kit (Promega, Madison, WI) 24 h post-transfection (Supporting Information). When these particles are added to hESC colonies, there is generally minimal cytotoxicity. C32-103, C32-116, C32-118, and C32-122 nanoparticles each show the same viability as untreated controls (Figure 2b). However, C32-117 nanoparticles show some loss of viability compared with untreated controls (70% viable), although this is not statistically different from the small loss of viability with Lipofectamine 2000. When the levels of gene transfer are quantified by flow cytometry, the poly(β-amino ester) nanoparticles enable the transfection of up to 50% of hESC colonies. Dramatically, C32-118 nanoparticles (Figure 3a) are able to transfect hESC colonies significantly, whereas Lipofectamine 2000, the leading commercially available nonviral vector, is able to transfect cells on only the periphery. Representative fluorescent micrographs of this GFP transfection can be seen in Figure 3. For this particular transfection, it is important to note that some of these cells may be differentiated and others may be feeder cells. To avoid this, we made hES BG02-Oct4-GFP cells by introducing the hOct4-GFP-puro construct into hESCs (Supporting Information). In this construct, the GFP reporter gene is expressed by the Oct4 promoter that is active when cells are in an undifferentiated state. Upon differentiation, the Oct4 promoter is gradually inactivated; therefore, the GFP reporter is downregulated. This line expresses all pluripotent stem cell markers and forms teratomas after being grafted into severe combined immunodeficient mice (SCIS). With these cells, undifferentiated hESCs can be clearly distinguished from differentiated stem cells and feeder cells.

With this new cell system, FURW was delivered to encode red fluorescent protein (RFP) as a reporter rather than GFP. We determined the positive transfection of undifferentiated hESCs by counting cells that were positive for both RFP and GFP. The results for polymeric gene delivery to undifferentiated hESCs that remain undifferentiated following transfection are shown in Figure 4. Poly(β-amino esters) showed superior gene-delivery efficacy compared with Lipofectamine 2000. Cell viability was high, and colony morphology was normal, as shown in Figure 4d.

Interestingly, small structural changes to only the ends of the gene-delivery polymer, including single carbon changes, can significantly increase the transfection efficacy to hESC colonies. These differences may be partially due to the biophysical properties of the nanoparticles, with smaller particles (~200 nm) being more favorable than larger particles (~400 nm) and positively charged particles (~15 mV) being more favorable than weakly charged particles (~5

mV). Nanoparticles that are formed with polymers such as C32-118 may also potentially target hESCs compared with other cell types. For example, here we show that C32-118 nanoparticles transfect hESCs more effectively than do C32-103 nanoparticles. Yet, we have previously shown that in the case of differentiated human primary cells, HUVECs, C32-118 nanoparticles transfect 70% fewer cells than do C32-103 nanoparticles.[17] Therefore, the end-modification of polymers used to form gene-delivery nanoparticles may be a useful strategy in promoting cell-specific delivery when multiple cell types or cells of different differentiation states are present. In contrast, alternate polymer end-modification may create polymeric gene-delivery nanoparticles with high delivery to a range of human cell types. For example, here we show that C32-117 nanoparticles enabled the highest transfection of hESC colonies, which is also true with HUVECs.[17] Importantly, the poly(β-amino esters) that were used here had up to 4 times greater efficacy at gene transfer to undifferentiated hESC colonies than did the leading nonviral vector, Lipofectamine 2000 (22% positive with C32-117 vs 5.8% positive with Lipofectamine 2000). This facile method for gene transfer works in the presence or absence of serum and without the need for physical or electrical methods that can enhance gene transfer, but it can also significantly lower cell viability.

Compared with viral vectors, the biodegradable polymers that are used here may be safer for certain therapeutic applications. Whereas retroviruses, including lentivirus vectors, can cause nonspecific integration that may lead to mutation and cancer, the nonviral gene-delivery approach that is used here transfers an episomal plasmid to the cells that is expressed transiently. We confirmed that gene expression is transient by measuring the delivery of both fluorescent protein-reporter encoding genes and antibiotic-resistance encoding genes over time. Transient expression may also be useful for temporal control of the expression of growth factors. As a biological tool to study the roles that varying genes play in development, viral vectors are slow to prepare and screen because each gene needs to be separately cloned into each vector. In contrast, a nonviral approach that uses nanoparticles that spontaneously self assemble when mixed with DNA, as used here, facilitates high-throughput screening of gene combinations to study development, transdifferentiation, and reprogramming.

We have demonstrated the use of self-assembled, biodegradable polymeric nanoparticles as a tool for nonviral gene transfer to hESC colonies. Small modifications to the ends of the polymers were found to improve the transfection efficacy of the polymer/DNA particles significantly. Lead polymers enabled high gene expression while maintaining high viability, normal cell morphology, and an undifferentiated state. With further studies, these biodegradable, nonintegrating vectors may be a more useful alternative for high-throughput biological screening and a safer alternative to viruses for use in regenerative medicine.

Statistical calculations were performed by the use of GraphPad Prism 5 for Windows. All graphs show mean $\pm$ standard deviation. To analyze transfection efficacy, we used one-way analysis of variance to calculate statistical significance ($p < 0.01$). The Bonferroni post-test was used to measure differences between each of the polymeric nanoparticles and Lipofectamine 2000. To analyze cell viability, we used one-way analysis of variance and the Bonferroni post-test to calculate statistical significance ($p < 0.01$). Each batch of nanoparticles was compared with untreated cells as well as Lipofectamine 2000.

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Supporting Information Available: Detailed description of the materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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