Gene Therapy (2012) 19, 321–328

www.nature.com/gt

ORIGINAL ARTICLE

Gene-eluting stents: non-viral, liposome-based gene delivery of eNOS to the blood vessel wall *in vivo* results in enhanced endothelialization but does not reduce restenosis in a hypercholesterolemic model

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Although successful, drug-eluting stents require significant periods of dual anti-platelet therapy with a persistent risk of late stent thrombosis due to inhibition of re-endothelialization. Endothelial regeneration is desirable to protect against in-stent thrombosis. Gene-eluting stents may be an alternative allowing inhibition of neointima and regenerating endothelium. We have shown that adenoviral endothelial nitric oxide synthase (eNOS) delivery can result in significantly decreased neointimal formation and enhanced re-endothelialization. Here, we examined non-viral reporter and therapeutic gene delivery from a stent. We coated lipoplexes directly onto the surface of stents. These lipostents were then deployed in the injured external iliac artery of either normal or hypercholesterolemic New Zealand White rabbits and recovered after 28 days. Lipoplexes composed of lipofectin and a reporter *lacZ* gene or therapeutic *eNOS* gene were used. We demonstrated efficient gene delivery at 28 days post-deployment in the media (21.3 ± 7.5%) and neointima (26.8 ± 11.2%). Liposomal delivery resulted in expression in macrophages between the stent struts. This resulted in improved re-endothelialization as detected by two independent measures compared with vector and stent controls ($P<0.05$ for both). However, in contrast to viral delivery of eNOS, liposomal eNOS does not reduce restenosis rates. The differing cell populations targeted by lipoplexes compared with adenoviral vectors may explain their ability to enhance re-endothelialization without affecting restenosis. Liposome-mediated gene delivery can result in prolonged and localized transgene expression in the blood vessel wall *in vivo*. Furthermore, lipoeNOS delivery to the blood vessel wall results in accelerated re-endothelialization; however, it does not reduce neointimal formation.
Gene Therapy (2012) **19,** 321–328; doi:10.1038/gt.2011.92; published online 30 June 2011

Keywords: endothelialization; coronary artery stents; restenosis; late stent thrombosis; liposome

INTRODUCTION

Drug-eluting stents (DES) are now routinely used for occlusive atherosclerotic coronary lesions to reduce the problem of restenosis. However, DES have been associated with a higher frequency of very late stenosis and re-infarction (more than 1 year) compared with bare metal stents, 1.9% versus 0.6% per year, respectively.[1,2] In addition, animal studies have shown that DES can cause local toxicity to the vessel wall in the form of medial necrosis, intimal proliferation, chronic inflammation and delayed re-endothelialization of the stents.[1–3] Thus, although DES reduce the risk of restenosis, they are associated with delayed re-endothelialization.[4,5] An optimal therapeutic approach would involve a strategy, which inhibits intimal hyperplasia while promoting re-endothelialization and suppressing stent- or vector-related inflammatory side effects.

Gene-eluting stents have the potential to provide an alternative treatment strategy for the prevention of restenosis. The safety and feasibility of viral-mediated stent-based delivery of reporter genes to the blood vessel wall has been previously documented.[6–10] An important consideration is the choice of an effective potential therapeutic gene. Nitric oxide (NO) is a pleiotropic diatomic molecule with many diverse roles in the vasculature. It has anti-atherogenic properties including inhibition of smooth muscle cell proliferation and inhibition of platelet aggregation, as well as vasodilator effects. It is produced by nitric oxide synthase isoforms, and in particular, we have previously utilized endothelial nitric oxide synthase (eNOS) expression in the vasculature to increase NO production.[11–13] We have previously reported on successful transduction of rabbit iliac arteries with therapeutic *eNOS* gene using adenovirus-mediated gene-eluting stents, resulting in enhanced re-endothelialization with a reduction in intimal hyperplasia at 4 weeks post-gene delivery.[12] However, adenoviral-mediated gene delivery to the blood vessel wall has been associated with inflammation, which may impede progress of this potential therapy to the clinics.[14]

Non-viral and, in particular, liposome-mediated gene delivery represents an interesting alternative to viral gene delivery as they can deliver a large insert size, and are less immunogenic and have fewer bio-safety concerns. However, to date, the level of transfection achieved with these vectors is low. Several early studies examining

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Received 30 March 2011; accepted 9 May 2011; published online 30 June 2011

in vivo vascular gene delivery using plasmid DNA complexed with liposome-based carriers achieved a low level of transduction with these vectors (0.1–1%), even with modification of lipid composition (5% transduction of target cells).[15,16] The aim of the present study was to assess the efficacy and pattern of expression for this non-viral vector type, delivered from both biocompatible and bare metal stents, as well as the therapeutic potential of utilizing lipoplex-mediated delivery of eNOS to the stented vessel wall.

RESULTS

Comparison of delivery platform and coating technique for lipoplexes

Vessels were harvested at 28 days post-deployment of stents/balloon. This time point was chosen, as advanced neointimal formation is noted in the current model system at between 14 and 28 days as observed in our previous studies.[6,11] We examined delivery from both bare metal cobalt chromium stents and phosphorylcholine (PC)-coated stainless steel stents, and the difference between delivery platforms was not found to be at statistical significance (Figure 1). Further studies were carried out using PC-coated stainless steel stents. The uptake of the liposomal coating onto the surface of the stent was complete when applied using a micropipetting with air-drying method. The smooth intact coating of liposomes is demonstrated using scanning electron microscopic analysis of the stents (Figure 2). Interestingly, we were also able to demonstrate delivery from PC stents using an aerosolization system, although this was not as efficient as when the pipetting and air-drying technique were used (data not shown).

Time course for LacZ lipoplex stents

PC-coated stents with lipoplex formulations carrying a *LacZ* plasmid expressing β-galactosidase were applied using our pipetting technique with the air-drying method. This was used to examine transgene expression at 3, 7, 28 and 42 days. Reporter transgene expression was assessed by measuring the area, using image software, of tissue expressing the transgene as a percentage of the total stented area. This is used as an index for relative transgene expression between stent platforms. Our examination of these time points for transgene expression following liposomal gene delivery from PC stents revealed that relative expression peaked significantly at 28 days but also extended out to 42 days post-delivery (Figure 1). However, at 42 days, this expression had significantly decreased. Neointimal formation was found at 28 and 42 days, and expression was examined separately in both neointima and media of vessels from these time points. Relative measurements showed that the media and neointima were equally targeted at both the later time points.

Cell population targeted by lipoLacZ lipoplexes

Delivery of lipo*LacZ* from a PC stent platform and applied using a pipette resulted in expression in both the neointima and media at 28 days post-stent deployment in normocholesterolemic animals (Figure 3). When examined under light microscopy, expression was observed in media between the indentations of the struts. Moreover, as the delivery of cytoplasmic-targeted LacZ may overestimate the level of reporter transgene expression, if allowed to diffuse, we assessed representative sections from nuclear-targeted LacZ for histological comparison (Figure 3). The levels of reporter gene expression assessed

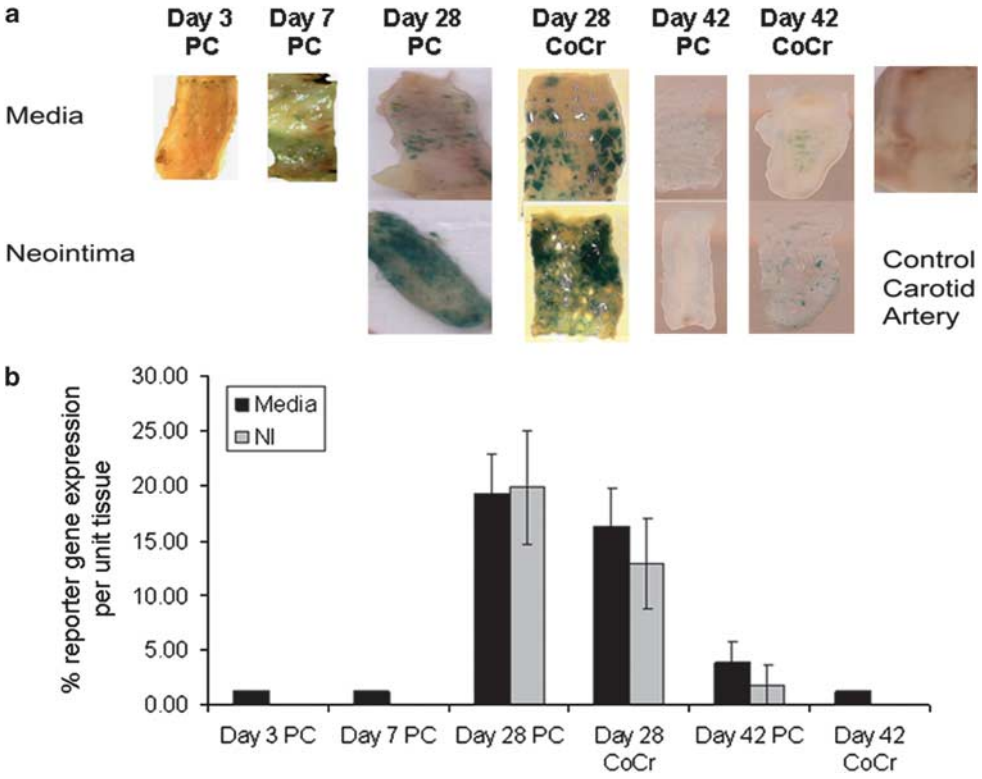

Figure 1 LacZ expression at 28 days post-deployment for both bare metal and PC stents measured at 3, 7, 28 and 42 days (for PC stents, $n=2$ at 3 days, $n=2$ at 7 days, $n=7$ at 28 days and $n=5$ at 42 days; for CoCr stents, $n=6$ at 28 days and $n=2$ at 42 days). (**a**) Representative whole vessel mounts and (**b**) mean results with error bars demonstrating s.e. are shown. Results are shown based on the percentage area of the vessel's media or neointima covered by transgene-expressing tissue. CoCr, cobalt chromium bare metal stent, PC, phosphorylcholine-coated stainless steel stent.

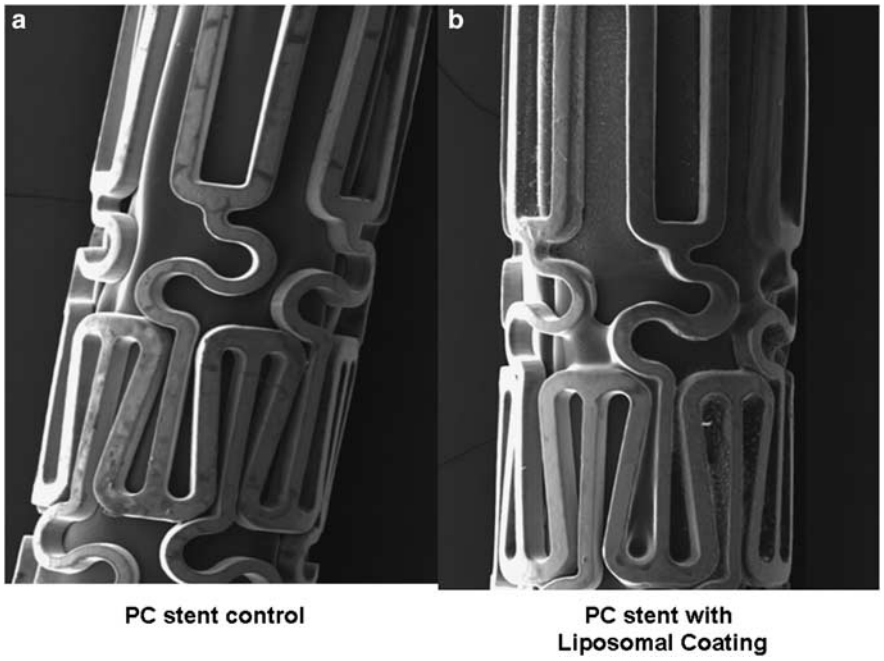

Figure 2 Scanning electron micrographs is a PC stent (**a**) and a PC stent following application of lipoplexes (**b**).

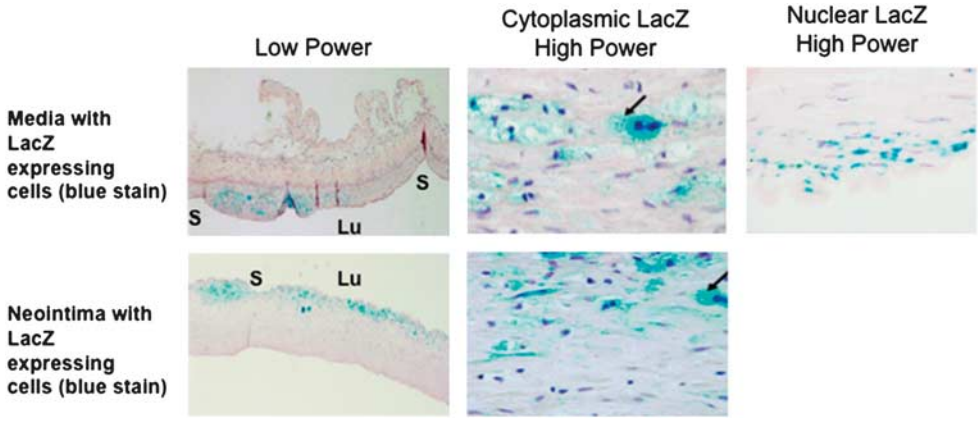

Figure 3 Expression of nuclear and cytoplasmic-targeted LacZ between stent struts at day 28 post-deployment in the common iliac of normocholesterolemic rabbits. Sections are stained using X-gal (light blue), and counterstained with hematoxylin–eosin (nuclei are deep blue). Original magnification, ×10 and ×40. Lu, lumen, S, stent strut.

using either cytoplasmic or nuclear-targeted LacZ did not differ when assessed qualitatively under the standard conditions used.

We attempted to better define the cell population targeted by the liposomal formulation (Figure 4). These experiments as with all expression studies were carried out in normocholesterolemic animals. Interestingly, β-galactosidase expression coincided with immunofluorescent-stained areas for the macrophage marker RAM-11. Surprisingly, little expression could be seen in cells stained for smooth muscle phenotype with fluorescent-labeled antibodies. Indeed, areas of reporter gene expression in the neointima were in contrast with areas staining for smooth muscle α-actin as evidenced by the overlay image in Figure 4.

Detection of eNOS expression

Stented iliac arteries transduced with lipo*eNOS* were harvested 21 days post-stent placement. To demonstrate eNOS protein expression, we transduced iliac arteries with lipostents, which carried both the *lacZ* and *eNOS* plasmids as part of the liposomal formulation. Tissues were stained for lacZ to mark the transduced cells. LacZ-positive sections were subjected to immunofluorescence with an antibody raised against human eNOS (Figure 5). eNOS expression was robustly detected in all regions in which LacZ was expressed indicating the co-expression of the two plasmids confirming *eNOS* delivery and expression from a lipo*eNOS* PC stent. Secondary antibody controls did not show any background fluorescence (data not shown).

Furthermore, *eNOS* expression was confirmed using reverse transcriptase PCR. RNA obtained from these tissue samples demonstrated the presence of the appropriate-sized band for *eNOS* in arteries tested following RT–PCR as shown in Figure 5. *Hypoxanthine phosphoribosyltransferase* was a housekeeping gene used as a control in the reverse transcriptase experiments.

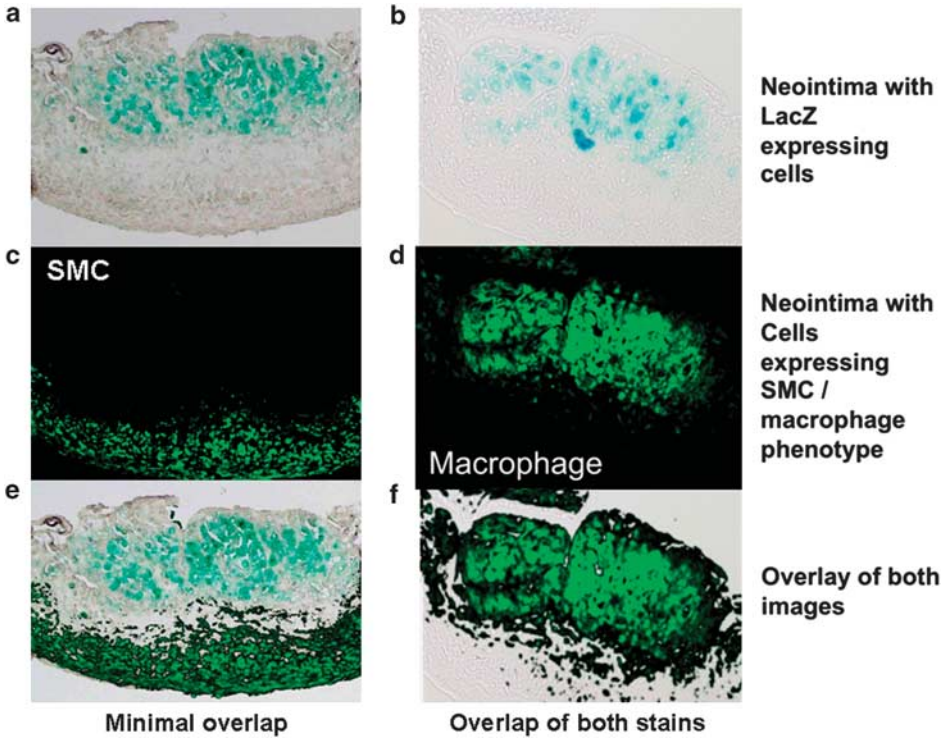

Figure 4 LacZ expression in the neointima stained with X-gal (**a** and **b**). Consecutive sections are fluorescently stained for smooth muscle cell phenotype stained using α-actin antibodies (**c**) and macrophage phenotype using RAM-11 antibodies (**d**). Minimal overlap is noted for the smooth muscle cell phenotype stained using α-actin antibodies (**e**), whereas in contrast a significant overlap is noted when macrophage phenotype is examined using RAM-11 antibodies (**f**). Sections were taken from vessels, which had lipoplex/*lacZ*/PC stent deployed for 28 days in normocholesterolemic rabbits. LacZ expression is denoted by blue stain and antibody based phenotype is denoted by green fluorescence. Original magnification, ×10. Lu, lumen, NI, neointima.

Endothelial regeneration

At 2 weeks post-intervention, blood vessels were stained before killing using Evans Blue. This stain allows areas of de-endothelialization to be viewed as areas of blue stain, whereas areas of intact endothelium remain white. We have previously shown that our angioplasty model results in complete denudation of the endothelial layer.[6,11]

At 2 weeks post-stenting, luminal Evans Blue stain revealed that endothelial regeneration was significantly enhanced ($P<0.05$, analysis of variance) in vessels ($n=4$), which had stents coated with lipo*eNOS* deployed compared with stents coated with liponull (82.48 ± 12.86% versus 49.58 ± 8.16%, Figure 6).

In addition, in a separate series of experiments at 4 weeks, histomorphometry carried out blindly and independently confirmed our Evans Blue results. Similar levels of endothelialization are noted for lipo*eNOS* and liponull controls using both Evans Blue and histomorphomtery (Figure 6). Using analysis of variance to detect global differences, a significant effect was seen on endothelialization when *eNOS* was delivered from a stent versus controls at 4 weeks post-deployment ($P<0.05$, Figure 6, Table 1). The individual groups were then compared using a *t*-test confirming that there was significantly better endothelialization for lipo*eNOS* versus lipoNull and PC stents ($P<0.05$, Figure 6, Table 1). These data also suggest that the majority of endothelialization occurs within the first 2 weeks after deployment.

Histomorphometry

Similar to the endothelialization data, full-blinded histomorphometry was undertaken at 4 weeks post-deployment of lipo*eNOS* ($n=12$) and liponull ($n=10$), as well as PC control stents ($n=5$) not carrying a vector. The parameters measured included external elastic lamina, internal elastic lamina, lumen size, media size, neointima size, % stenosis, injury score and inflammation score. No significant differences were observed between the groups for the parameters tested ($P>0.05$) with the exception of an increased number of inflammatory cells between lipo*eNOS* and liponull stents ($P<0.05$, Table 1). Fewer total occlusions were seen for lipo*eNOS* stents (2/12) versus liponull (4/10) and PC stents (2/5); however, this did not reach statistical significance (Table 1).

DISCUSSION

DES has revolutionized coronary endovascular procedures. However, once anti-platelet therapy is discontinued there remains a distinct persistent risk of late stent thrombosis. This is associated with delayed re-endothelialization of the vessel wall following stenting. Gene-eluting stents may have the potential to enhance re-endothelialization, and the use of non-viral vectors for these stents may avoid bio-safety concerns associated with viral vectors. Recent reports have described the potential for liposomal delivery of reporter genes using gelatin-based coatings.[17] Our study describes efficient therapeutic gene delivery from stents using liposomes with or without biocompatible coatings. Moreover, our study is the first to identify the cell populations targeted by lipoplexes in the context of stent-based gene delivery to the vasculature *in vivo*.

Initially, we aimed to establish the technical parameters of liposomal gene delivery from a stent. We demonstrated the advantage of pipette coating of liposomes onto stents versus aerosolization. Successful homogeneous liposome application can allow gene delivery from both PC coated as well as bare metal stents. This is in agreement with the findings of Fishbein *et al.*[18] who showed stable linkage to a

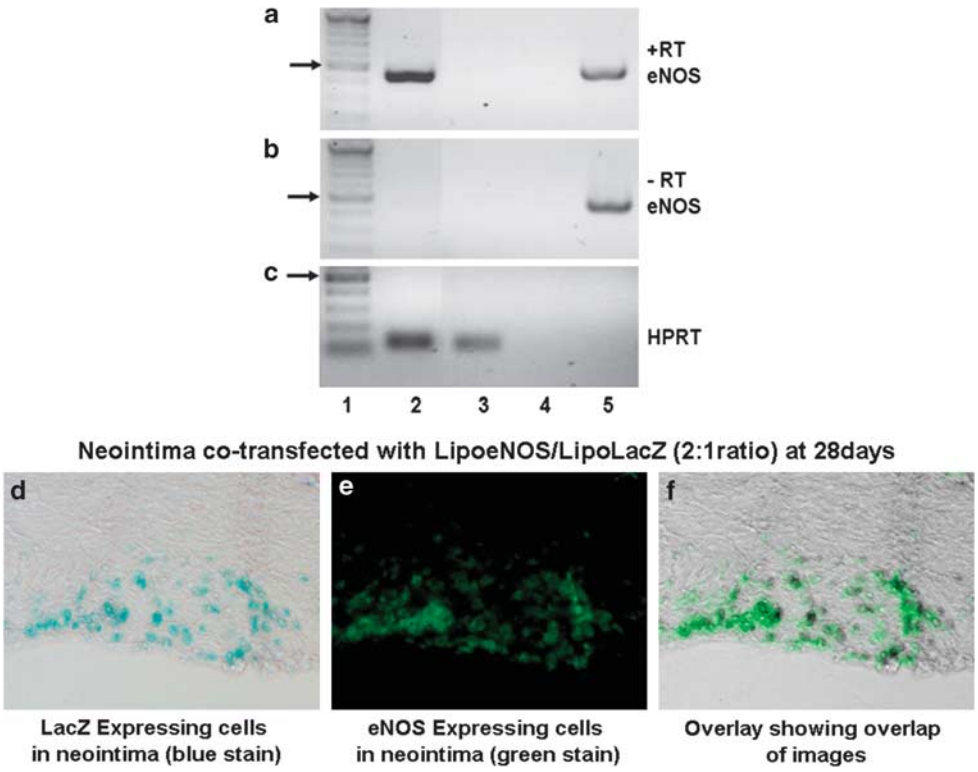

Figure 5 Detection of *eNOS* transgene expression in stented vessels harvested after 21days. Expression of the *eNOS* transgene in rabbit vessels at 21 days post-deployment detected using RT–PCR. Lane (1) 100 bp ladder (arrows indicate 500 bp size), (2) RNA from lipo*eNOS*-stented rabbit common iliac artery, (3) RNA from a control lipo*LacZ*-stented artery without *eNOS*, (4) negative control without cDNA and (5) positive control with *eNOS* plasmid. (**a**) PCR reaction with reverse transcriptase (+RT), (**b**) PCR reaction without reverse transcriptase (−RT), (**c**) PCR reaction using primers for the endogenous rabbit housekeeping gene *hypoxanthine phosphoribosyltransferase*. Histological section of a combined lipo*eNOS/LacZ*-stented artery demonstrating (**d**) LacZ expression in blue, and (**e**) immunofluorescent detection of eNOS in green with (**f**) overlap seen when graphic overlay for both stains is used.

stent allowing more focused delivery of vector. PC stents were used in further expression/therapeutic experiments for comparative purposes with our previous viral based studies. We have previously investigated the use of viral vectors from PC stents and specifically adenovirus and AAV serotype 2 for delivery of transgene to the injured vasculature.[6] Our present study shows increased expression using a non-viral vector when a PC stent was used (Table 2, note: median values are used for direct comparison with previous studies). Similar to our previous studies, transgene expression was found to be maximal at 28 days post-stenting. This is consistent with the hypothesis that liposomal efficiency is enhanced when associated with local cellular proliferation in the form of neointimal formation. We observed no significant difference between the level of expression in the neointima or media, suggesting that the entire lipoplex or the plasmid DNA was released from the stent over a prolonged period. In contrast, adenoviral vectors (the most efficient viral vector we have tested) have relatively higher levels of expression in the media versus the neointima at later time points. This implies that our non-viral vector delivered from a stent-based platform would be more beneficial for prolonged delivery of therapeutic genes to an area of increased cellular proliferation. Whether or not the observed differences in later gene release may be due to different uptake mechanisms of lipoplexes (e.g., passive, fusion) and viruses (e.g., active, endocytosis) or due to varying elution profiles of these gene delivery systems, based on their ability to adhere to biocompatible or stainless steel stents remains to be investigated.

Furthermore, our studies identified that the predominant cell type with lipoplex uptake were RAM11-positive consistent with a macrophage phenotype with little uptake in smooth muscle cells. This occurred despite our demonstration of smooth muscle transfection using lipoplex-mediated gene delivery *in vitro*. However, our *in vivo* experiments' lipoplexes targeted macrophages, and therefore may represent a vector of choice for carrying anti-inflammatory genes aiming to modulate the immune response post stenting. The observed preferred uptake of lipoplexes by macrophages is also in contrast to adenoviral uptake from stents, which resulted in transgene expression predominantly in smooth muscle cells.[6] Interestingly, we noted the presence of cells with a macrophage phenotype in sections surrounding the stent struts. This is consistent with the results of Rogers *et al.*,[19] who showed a significant increase in macrophage accumulation at stent struts following stent deployment in rabbit iliac arteries, which underwent a balloon injury before deployment. Our study similarly uses prior balloon inflation to mimic an atherosclerotic lesion, which enhances neointimal formation involving foamy macrophages.

Previous studies have shown that *in vitro* liposome-mediated gene delivery does not correlate with *in vivo* efficacy of liposome complexes when studied in the lung.[20] In addition, there are reports in the literature, suggesting that optimal *in vivo* gene delivery with liposomes can be achieved systemically when the molecular ratio of cationic liposome to nucleic acid in the lipoplex mixture (positive/negative charge ratio) is closer to 1 or greater.[21–23] This higher charge of the lipoplex complex also helps in reducing the host immune response.[23] These previous studies have dealt with gene expression/drug release following systemic delivery of liposomes and do not relate to stent-based release of liposomes. However, these observations do allow us to speculate that the prolonged and efficient gene expression seen in our study is possibly due to the intrinsic ability of the liposomes to bind

efficiently with the stent surface with stable release over time *in vivo* and due to their mechanical delivery to the blood vessel wall. Scanning electron microscopy imaging demonstrated that liposomal application to the Hi matrix PC stent resulted in complete and smooth coating of the stent (Figure 2). As pointed out by Fishbein *et al.*,[9] direct gene delivery from a stent platform is advantageous as it decreases risk of distal spread and encourages more efficient local delivery. A recent report by Brito *et al.*[17] has demonstrated the potential for liposomal gene delivery of *eNOS* when used in conjunction with PLGA and gelatin-based coatings. This showed promise in enhanced re-endothelialization and reduced restenosis. However, in contrast to our study, the model used a gentle denudation of the vessel rather than an inflation/deflation injury and was carried out in a non-disease model. Moreover, this study used a neointima/media ratio rather than full histomorphomtery results, as the standard. Our study uses liposomes without a coating in conjunction with 'off the shelf' stents, which is of practical benefit in translating this research.

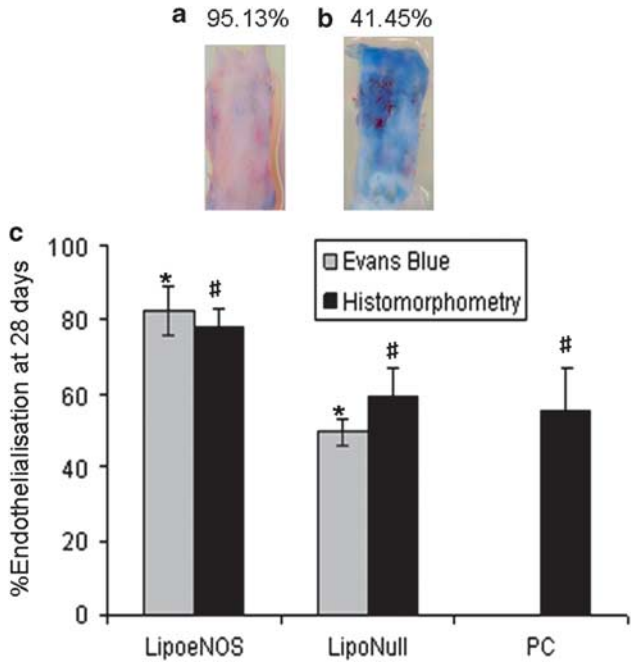

Figure 6 Endothelialization as determined using both Evans Blue at 14 days and histological analysis at 28 days post-stent deployment in the common iliac arteries of hypercholesterolemic rabbits. For Evans Blue, lipo*eNOS* and liponull controls are compared (n=4 each). For histological analysis lipo*eNOS* (n=12), liponull (n=10) and PC stents without vector (n=5) are compared. Representative whole vessel mounts of the Evans Blue-stained arteries are shown for each treatment group (**a** and **b**). In the graph, (**c**) a significantly increased level of endothelialization is noted for lipo*eNOS*-stented group compared with controls for Evans Blue analysis (*) and for histological analysis (#) using Student's *t*-test ($P<0.05$).

Our group has already demonstrated the ability of adenoviral gene delivery of *eNOS* to decrease smooth muscle cell proliferation both *in vitro* and *in vivo* in stent and injury based-models.[11,12] However, in the present study, there is a disconnect between re-endothelialization, which is enhanced and neointimal formation, which is not affected. Similar results were also noted when anti-CD34 stents were assessed.[24] Enhanced re-endothelialization due to increased bioavailability of NO has been suggested to result in enhanced endothelial cell migration and/or mobilization of endothelial progenitor cells.[25,26] We speculate that in our study this may be because of the cell type targeted, that is, macrophages. Macrophages, unlike smooth muscle cells, have their own endogenous NOS in the form of iNOS. The expression of eNOS in cells carrying iNOS may deplete the cofactors required to produce NO and thereby contribute to the recognized uncoupling of iNOS. This uncoupling can lead to an increased formation of superoxide, which may enhance the inflammatory response as seen for lipo*eNOS* versus liponull (Table 1). An enhanced inflammatory response may prevent the manifestation of potential beneficial effects on neointimal formation by liposomally delivered *eNOS*.

In conclusion, we have shown that liposome-mediated gene delivery is capable of transducing the blood vessel wall with delayed release when delivered on a stent platform with or without a biocompatible coating. Transgene expression is detected at early time points in the media and later equally in the neointima. We have also shown the potential of therapeutic gene delivery using this system. The use of non-viral-based delivery system reduces the bio-safety concerns associated with viral-based delivery systems. We suggest this method may be beneficial as an adjunct to current DES for enhancing re-endothelialization with prevention of neointimal proliferation achieved through the cytotoxic/static actions of the drug-eluting portion. Enhanced re-endothelialization may decrease the need for prolonged dual anti-platelet therapy as an important consideration in patients who cannot tolerate this regimen.[27] A rapid re-endothelialization may also decrease the risk of late stent thrombosis following cessation of anti-platelet therapy.

Table 2 Head-to-head comparisons of median levels of expression between adenoviral, AAV and liposomal-based gene delivery of LacZ as a reporter gene at 28 days post-stent deployment

Vector from PC stent	*Site*	*Median level of expression (%)*
Liposome	Media	22.42
	Neointima	16.88
Adenovirus	Media	8.87
	Neointima	1.01
AAV	Media	0.35
	Neointima	1.38

Abbreviations: AAV, adeno-associated virus; LacZ, β-galactosidase; PC, phosphorylcholine.

Table 1 Histomorphometric data from lipoeNOS, liponull and PC stents at 28 days

Stent	*EEL (mm^3)*	*IEL (mm^3)*	*Lumen*	*Media*	*Neointima*	*% Stenosis*	*Injury*	*Inflammation*	*% Endo*	*TO*	N	*%TO*
LipoeNOS	5.54 ± 0.69	5.14 ± 0.64	1.27 ± 1.16	0.40 ± 0.23	3.8 ± 1.19	75.61 ± 22.37	1.41 ± 0.35	13.61 ± 40.52	*77.76 ± 29.7	2	12	16.7
Liponull	5.41 ± 0.87	5.05 ± 0.89	1.22 ± 1.44	0.42 ± 0.43	3.77 ± 1.06	77.85 ± 22.49	1.60 ± 0.38	7.85 ± 20	*59.30 ± 43.53	4	10	40
PC	5.10 ± 0.75	4.58 ± 0.7	1.43 ± 1.24	0.53 ± 0.52	3.15 ± 0.88	71.00 ± 24.99	1.61 ± 0.52	3.44 ± 4.03	*55.69 ± 40.78	2	5	40

Abbreviations: Endo, endothelialization; EEL, external elastic lamina; IEL, internal elastic lamina; lipo*eNOS*, liposomal *endothelial nitric oxide synthase*; PC, phosphorylcholine; TO, total occlusions.
*$P<0.05$ for a Student's *t*-test.

MATERIALS AND METHODS

Construction of lipoplex complexes with plasmid *LacZ*

Plasmid DNA encoding either a nuclear-targeted (NLS) LacZ, a cytoplasmic-targeted LacZ (LacZ), pcDNA3 empty vector or *eNOS* gene driven by the cytomegalovirus promoter were grown in bacteria and prepared according to Endofree Plasmid Giga Kit manufacturer's instructions (Qiagen, Crawley, UK). For these experiments we used either control 'off the shelf', or ready-to-use liposomes (lipofectin, Invitrogen, Paisley, UK), to form lipid–DNA complexes (lipoplexes). (lipofectin is a 1:1 molar mixture of the DOTMA (*N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride and DOPE (dioleoyl phos-photidylethanolamine). DOTMA is a cationic lipid, which helps in binding the negatively charged nucleic acids, and DOPE is a so-called 'helper lipid', which allows the entrapped nucleic acid to escape the endosomes after cell entry by endocytosis.

Lipofectin–plasmid complexes: For these experiments, 10 μl of a 10 mg ml^{-1} plasmid solution (100 μg plasmid DNA-encoding LacZ or eNOS or pcDNA3 as the liponull control) was diluted to a total volume of 100 μl with endotoxin-free TE buffer (Invitrogen). A total volume of 200 μl of a 1 mg ml^{-1} lipofectin/liposome solution was added to the plasmid solution and mixed several times by inverting the tube. The components were allowed to form lipoplexes for 1 h at room temperature followed by 4 °C incubation overnight.

Cloning of human *eNOS* expression plasmid

A human *eNOS* gene was codon optimized and synthesized by GENEART (Regensburg, Germany). The human *eNOS* insert was excised and ligated into the mammalian expression vector pcDNA3 containing the cytomegalovirus promoter. Ligations were transformed and resultant bacterial colonies were screened for the inserted clone. A single positive clone was examined for protein expression by transfection of HEK-293 cells and immunoblotting (data not shown).

Preparation of liposome-coated stents

Liposomal formulations were prepared as described earlier and they were applied to cobalt chromium stents, PC-coated stents or a balloon using a pipette in 30 μl droplets followed by air-drying method. Alternatively, a nebulizing system was used to aerosolize the liposomal formulation before application to the stent. The procedures were carried out before stent deployment.

Animals

The investigation conforms to the guidelines for the care and use of laboratory animals published by the US National Institute of Health (NIH publication no. 85–23, revised 1996), and ethics approval for these experiments was obtained from the local institutional animal care committee and carried out under license, as approved by the Irish Department of Health. Male New Zealand White rabbits (Harlan Ltd, Bicester, UK) weighing 2.5–3.5 kg were used. Animals were individually housed with a 12 h light–12 h dark cycle, and fed either a standard chow or hypercholesterolemic diet at 1 month before intervention and given water *ad libitum*. All animals received low-dose aspirin for 7 days before intervention and thereafter until killing. Animals were killed at time points from 3–42 days post-stenting with a high dose of phenobarbitone, which was administered intravenously following sedation. The hypercholesterolemic diet was continued post-intervention. Animals fed with standard chow were used for lipo*lacZ* and lipo*eNOS* expression studies, whereas hypercholesterolemic animals were used for assessment of therapeutic parameters including Evans Blue and histomorphometry analysis.

In vivo catheter procedures

All procedures were performed under fluoroscopic guidance as previously described.[6,12] After administration of anesthesia, the right carotid artery was surgically exposed by blunt dissection and a 5-Fr introducer sheath (Radifocus, Terumo, Somerset, NJ, USA) was introduced into the artery and advanced to the lower abdominal aorta. All wires and catheters were passed through this sheath. A balloon injury was performed with a 2.5×14-mm commercially available balloon, which was placed in the right external iliac artery. A total of three balloon injuries were performed of 1 min duration each (six ATM for 60 s). A 1-minute interval of deflation was allowed between balloon inflations. After balloon injury a 3.0×11 mm BiodivYsio HI (Abbott, Galway, Ireland) matrix-coated stent was deployed at the injury site (six ATM for 30 s). Post-stent deployment angiography was carried out in all animals to exclude any acute thrombus formation at the site of stent deployment.

Histochemical analysis of gene expression

LacZ expression was sought in the following manner. Following killing, stented arteries were exposed, retrieved and cut longitudinally with the stent removed before staining of arteries. A significant neointimal formation inside the luminal face of the stent was noted at day 28, which was stripped from the luminal face of the stent and stained separately for LacZ expression. All stented arteries were fixed with 4% paraformaldehyde for 30 min at 4 °C and then rinsed twice with phosphate-buffered saline (PBS). Arteries were then immersed in a solution of 500 μg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (X-Gal; Boehringer-Mannheim Biochemicals, Mannheim, Germany) overnight at 37 °C. Following staining the arteries were then embedded in paraffin. Sections (5 μm) were then cut, placed on slides, heated to 60 °C overnight, deparaffinized in xylene and rehydrated in graded dilutions of ethanol. Selected sections were counterstained with hematoxylin and eosin, dehydrated and mounted. Expression was considered positive for blue cells visible under light microscopy.

Identification of transgene expressing cells

Histological sections (5 μm), which had previously been positively stained for transgene expression, as described above, were subsequently analyzed for smooth muscle or macrophage phenotype. Following X-gal staining and sectioning, slides were deparafinized and rehydrated as described above ready for immunofluorescent staining. Slides were incubated in blocking solution (5% goat serum in PBS) for 30 min. Slides were then incubated with either RAM11 antibodies (Dako, Dublin, Ireland) or α-SMA antibodies (mouse monoclonal (1 A4) anti-α smooth muscle actin) (Abcam, Cambridge, UK) diluted in 5% goat serum in PBS overnight at 4 °C. Slides were washed in PBS/1% Tween, and incubated with anti-mouse IgG-conjugated with Alexa Flour 488 (Molecular Probes, Eugene, OR, USA) diluted in 5% goat serum in PBS for 2 h minimum in the dark. Slides were washed in PBS and then mounted using Vectashield containing DAPI (Vector Laboratories Inc., Burlington, CA, USA). Transgene expression and immunostained cell types were assessed visually. All sections had secondary antibody controls run, which did not show any staining.

Detection of eNOS expression in vessels

Histological techniques. Stents (n=3) carrying liposomes, which contained two separate plasmids (*LacZ* and human *eNOS*) were deployed and harvested at 28 days. Histological sections (5 μm) were stained as previously described for *LacZ* transgene expression. Subsequently, the same sections were stained for human eNOS expression by immunofluorescence (as above) using a monoclonal antibody generated against human eNOS (BD Transduction Laboratories, Lexington, KY, USA).

Reverse transcriptase PCR

RNA was extracted from the rabbit arteries at 21 days after exposure to a PC stent with lipo*eNOS* or a PC stent alone using the RNeasy kit (Qiagen). Extracted RNA (1 μg RNA) was reverse transcribed into cDNA using random primers and the ImProm-II Reverse Transcription system (Promega, Madison, WI, USA). Generated cDNA was used as a template to perform standard PCR analysis using ReadyMix Taq PCR reaction Mix with $MgCl_2$ (Sigma, St Louis, MO, USA). PCR primers were designed to amplify the codon optimized human *eNOS* transgene without amplifying endogenous rabbit orthologues. *eNOS* primers were forward 5′-GGAGATACGAGGAGTGGAAG-3′ and reverse 5′-GCCAAACACCAGGGTCATAG-3′ with an expected product size of 449 base pairs. Primers against the rabbit housekeeping gene *hypoxanthine phosphoribosyltransferase* were used as a control with expected product size of 135 base pairs. Products were visualized on an agarose gel.

Image analysis of vessels transduced with lipo*LacZ*

The luminal surface of all the stained arteries was photographed *en face* through a dissecting microscope. Quantification of positively (blue) stained tissue was performed using Java Image processing program software (Image J)

from the National Institutes of Health, Bethesda, MD, USA. At days 3 and 7, neointimal formation could not be visualized or separated from the media, allowing only the vessel wall to be stained and quantified. However, at day 28 there was a significant neointimal formation observed, which could be removed, stained and imaged separately to the vessel media.

Detection of endothelialization

At 14 days after stent deployment, a total of eight animals were anesthetized as above (n=4 liponull and lipo*eNOS* each). A total volume of 5 ml of 1% Evans Blue (Sigma) was injected in to the left ear veins and the stents were retrieved at 30 min after injection. The animals were killed before stent retrieval with an intravenous bolus of phenobarbitone. The stented blood vessels were fixed in 4% paraformaldehyde and incised longitudinally. Photographs of the stented vessels were taken *en face* and areas of stent endothelialization (white) and non-endothelialization (blue) were analyzed using Java image software.

Histological assessment of endothelialization

In addition to stent endothelialization assessment at 14 days by Evans Blue, histological assessment of endothelialization was also performed at day 28. Stents were retrieved at day 28 and embedded in resin following local perfusion fixation. Three serial sections were taken per stent and endothelialization was assessed directly under the microscope. Independent groups of animals were analyzed for lipo*eNOS* (n=12), liponull (n=10) and PC control stents (n=5) using this method.

Morphometric analysis

Morphometric analysis was performed by CV Path Institute Inc. (Gaithersburg, MD, USA). The effects of lipo*eNOS* (n=12), liponull (n=10) and PC-coated stents (n=5) on the vessel morphology, were assessed at 28 days in hypercholesterolemic animals. After administration of anesthesia, the animals were locally perfusion fixed and the stented blood vessels were retrieved. The stented vessel segments were embedded in methyl methacrylate plastic. After polymerization, 2 to 3 mm sections were sawed from the proximal, mid and distal portions of each single stent. Sections from the stents were cut on a rotary microtome at 4 and 5 μm mounted and stained with hematoxylin, eosin and elastic Van Gieson stains. All sections were examined by light microscopy for the presence of inflammation, thrombus and neointimal formation and vessel wall injury. The cross-sectional areas (external elastic lamina, internal elastic lamina and lumen) were measured with digital morphometry. Neointimal thickness was measured as the distance from the inner surface of each stent strut to the luminal border.

Statistical Analysis

Data for all experiments were expressed and graphed either as the mean ± s.e. Statistical analysis was performed using a single analysis of variance to explore global differences with group differences explored using a Student's t-test. A $P<0.05$ was considered significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work is funded by an Enterprise Ireland Grant (CFTD 105-07). TOB is also funded by a CSET Grant to REMEDI from Science Foundation Ireland. KMC has received funding from the Health Research Board Ireland. We wish to acknowledge the help of Dr Ailish Hynes, Department of Physiology, NUI Galway, in the preparation of this manuscript.

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