# GENE DELIVERY FROM STENTS FOR PREVENTION OF IN-STENT RESTENOSIS

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## ABSTRACT

The increasing sophistication of vascular stent design, especially devices that combine mechanical support with local drug delivery to the vascular wall, has resulted in major progress in the management of coronary and peripheral artery disease. This progress is reflected in expanded anatomical and clinical indications for stent angioplasty, with complementary reduction in bypass surgery rates and decreased need for target-lesion revascularisation. Nevertheless, even with second-generation drug-eluting stents (DES), the most common cause of stent failure, in-stent restenosis, while <10%, generates high numbers of cases due to the large scale of stent use (there are >1 million stent angioplasty procedures yearly in the USA alone). Gene-eluting stents (GES), the next generation of stent devices now in the preclinical phase of development, have evolved over the past two decades around the concept of localised vessel wall delivery of gene vectors attached to the stent struts. GES potentially provide several important advantages over DES, such as prolonged or even permanent anti-restenotic effect, capacity to deliver dissimilar impact on smooth muscle cells and endothelium, and fine-tuning of transgene expression and pharmacological effect with systemically administered therapeutics. Furthermore, GES can be used for treating non-occlusive lesions with the aim of slowing the underlying atherosclerotic process in the vessel wall. GES research at this time is concerned with achieving effective and safe transgene overexpression in the stented arteries, optimal vector choice, and proper techniques for vector immobilisation on the stent struts.

Keywords: In-stent restenosis, gene-eluting stents (GES), drug-eluting stents (DES), gene therapy.

## IN-STENT RESTENOSIS IN THE DRUG-ELUTING STENTS ERA

Percutaneous coronary interventions (PCI) utilising bare-metal stents (BMS) or drug-eluting stents (DES) are performed in >1 million patients in the USA[1] annually, to relieve atherosclerotic obstruction of coronary arteries. Although PCI immediately increases the flow across the narrowed segment, this gain is lost in a significant fraction of PCI patients due to in-stent restenosis (ISR) caused by a gradual build-up of neointima within the stented segment.[2] DES, especially second-generation devices which feature thinner struts, more biocompatible polymer coatings, and '-limus' drugs with a more appropriate pharmacological profile, achieve better long-term arterial patency than BMS and are associated with a reduced need for target-vessel revascularisation.[3] Unfortunately, the anti-restenotic effect of DES is less pronounced in patients with peripheral artery disease,[4] diabetes,[5] and renal failure.[5] While emerging third-generation stents, with completely biodegradable scaffolds or with resorbable coatings, hold promise of further incremental decrease of ISR rates, it is unlikely that ISR will be completely eliminated within the framework of DES technology.

## A CONCEPT OF GENE-ELUTING STENTS

The concept of gene-eluting stents (GES) has emerged at the crossroad of vascular gene therapy and DES in an attempt to overcome some inherent deficiencies of DES. Vascular gene transfer experiments pioneered by Nabel et al.[6] provided important insights regarding the vessel

wall transducibility with viral and non-viral vectors. These studies that were largely driven by a newly described human pathology, restenosis, have also established numerous potential molecular targets for restenosis prevention.[7] Performed in the pre-stent era, initial vascular gene transfer experiments exploited a straightforward 'dwell' of gene vector suspension in the temporarily isolated arterial segment, or employed catheters allowing more precise delivery of gene therapeutics to discrete vessel compartments. Arterial tissue transduction, even with advanced catheters (double balloon, microporous, Dispatch® [SCIMED Life Sciences, Minnesota, USA], Infiltrator® [Boston Scientific, Maryland, USA), has typically remained too low for clinical translation.[8] Regardless of catheter type, the short intraluminal retention time of the delivered gene therapeutics was identified as the main reason for inadequate expression of delivered transgenes in the vasculature.[8]

Compared with the scaffold-less vascular gene delivery, stents present an advantageous physical platform for local arterial gene transfer by dramatically increasing the fraction of retained vector. Better vector retention is a combined effect of physical vector association with a permanently implanted scaffold, and a shielding effect of stent struts on the vector particles located on the stent/tissue interface. As a result, vector immobilisation on the stent requires a lower vector dose and minimises the distal spread of gene vector, thus reducing inoculation of non-target tissues. Unlike fluid-phase vector delivery, stent-based delivery is possible in the presence of side branches and does not require prolonged isolation of the treated segment, obviating procedure-related ischaemic complications. Finally, stent-based gene transfer provides an optimal spatial configuration for ISR prevention, by approximating gene vector delivery to the sites of blood-borne cell recruitment and vascular cell activation that occur predominantly around stent struts.[9]

By exploiting a more basic level of intervention (genome versus druggable proteome), GES enable conceptually novel directions for stent-based prevention and treatment of ISR. Gene therapy can attain a longer lasting therapeutic modification of vascular substrate than can be achieved through sustained drug release from DES. Gene therapy can be targeted to specific cell types, thus allowing for selective inhibition of smooth muscle cell (SMC) proliferation and migration, while sparing endothelial re-growth. A fine-tuning of delivered therapy can be achieved with 'on demand' control of therapeutic transgene expression using systemically administered drugs targeting drug-regulatable promoters.[10] Finally, by targeting a much wider range of signalling pathways, GES provide means to slow or even reverse underlying atherosclerotic processes.[11]

## GENE-ELUTING STENTS TECHNOLOGIES

To date, no GES product has been evaluated in patients. The delay in clinical translation of GES is explained by the fast progress of DES technologies; by the cost and regulatory complexities of human gene therapy clinical trials; and most importantly by the remaining technical, biological, and engineering problems to be solved to fully realise GES potential. Research in the GES field has identified three major areas requiring further optimisation for moving this promising concept to clinical fruition: the nature of a therapeutic transgene, the vector type, and the delivery system (i.e. the specific way the vectors are attached to and subsequently released from the stent).

### Therapeutic Transgenes

Restenosis is a multifaceted disease with a variable contribution of inflammatory, reparative, fibrotic, and foreign body responses.[2] More than 150 molecular targets are implicated in restenosis pathogenesis, thus representing potential foci for genetic interventions.[7] Only a few of these targets were investigated in conjunction with stent-based delivery in animals.

#### Smooth muscle cell proliferation, migration, and extracellular matrix synthesis

A common pathway of ISR pathogenesis involves phenotypic modulation of SMCs to a synthetic state, their proliferation in the media and neointima, and production of extracellular matrix (ECM). These processes which are central to neointimal tissue formation were targeted by GES in several studies. AKT1 is an important signalling intermediate of a PI3K pathway shown to convey proliferative signalling in the context of restenosis. Downregulation of *AKT1* with anti-AKT1 short-interfering RNA (siRNA)-eluting stents decreased ISR by 50% compared with BMS.[12] Stent-based delivery of an anti-sense construct to a cell cycle check-point transcription factor *c-Myc*[13] has also shown the anti-restenotic effect.

Platelet-derived growth factor (PDGF) is a prototypical SMC mitogen and chemo-attractant. An anti-sense oligodeoxynucleotide sequence complementary to the 15 base conserved coding sequence of PDGF-A isoform eluted from GES showed a profound ISR inhibition in a pig model.[14] Vascular SMC proliferation pathways are subject to post-transcriptional regulation with microRNA (miRNA). A recent study reports 70% ISR inhibition in rabbits treated with miRNA-145-eluting stents in comparison with BMS-treated animals.[15] While miRNA-145 negatively regulates SMC proliferation, expression of miRNA-21 positively correlates with neointimal mass. Accordingly, anti-miRNA-21 coated stents reduced ISR in human mammary arteries transplanted into nude rats.[16]

Matrix remodelling mediated by metalloproteases upregulated by vascular injury enables migration of SMC and myofibroblasts along the gradients of specific chemo-attractants. Stabilisation of ECM achieved with GES encoding tissue inhibitor of metalloprotease-3 reduced centripetal migration of SMC through the stented pig coronaries, and resulted in 40% ISR inhibition compared with the BMS-treated arteries.[17] Transforming growth factor-α1 (TGF-α1) is the main inductor of ECM production in vasculature. In a recent pig study, ISR was attenuated with a secreted TGF-α1 receptor-encoding GES.[18] The expressed protein acted as a decoy sequestering active TGF-α1 and reducing its downstream effects on ECM production.[18]

### Enhanced endothelialisation

Early endothelialisation of denuded arteries was shown to curb neointimal formation.[19] Therefore, accelerated re-endothelialisation of stented arterial segments has been pursued as a rationale for ISR prevention. Vascular endothelial growth factor (VEGF) isoforms[20-23] and angiopoietin-1[20] were shown to be expressed following elution of respective vectors from the stent surface, with concomitant increase in endothelial re-growth and ISR inhibition in rabbit, dog, and pig models. One of these studies explored a mixed DES/GES platform and demonstrated the synergistic effect of paclitaxel/VEGF plasmid co-delivery.[23]

### Nitric oxide synthesis and bioavailability

Nitric oxide (NO) is a pleiotropic regulator of vascular quiescence.[24] Fishbein et al.[25,26] and others[27-30] have investigated GES driving upregulation of NO production through the overexpression of NO synthase 2 and 3 isoforms. While generally a marked mitigation of ISR was observed in these studies, some issues remain unclear, such as the need for arginine and tetrahydrobiopterin supplementation to prevent decoupling of NO synthase,[31] and a correlation between enhanced endothelial regrowth and inhibition of restenosis.[29]

### Inflammation

Chronic low-grade vascular inflammation aggravates restenosis,[32] therefore effectively curbing the inflammatory response represents a well-recognised anti-restenotic strategy.[33] In keeping with this idea, stents formulated with plasmid DNA encoding a dominant negative variant of monocyte chemo-attractant protein-1 reduced both macrophage infiltration of stented arteries and ISR in rabbit and non-human primate models.[34]

Nuclear factor kappa B (NF-κB) is a master transcription factor involved in the regulation of multiple inflammatory mediators. GES incorporating NF-κB decoy oligodeoxynucleotide deployed in femoral arteries of hypercholesterolaemic rabbits have demonstrated a 30% decrease of neointimal mass, with a concomitant decrease of inflammatory marker expression.[35]

### Parietal thrombus

A non-occluding (parietal) thrombus is routinely formed at the stenting site despite antiplatelet and anticoagulant premedication. If the thrombus persists, it serves as a nidus for migrating SMC, thus facilitating neointimal expansion. Localised overexpression at the site of GES implantation of the membrane-bound enzyme ectonucleoside triphosphate diphosphohydrolase, that effectively blocks platelet aggregation, prevented thrombosis after repeated interventions in rabbit femoral arteries, and decreased the extent of restenosis at Day 7 after reintervention.[36]

## Gene Vectors

Plasmid DNA possesses low transduction capacity in vascular tissue. To achieve meaningful expression levels, the therapeutic gene must be delivered in physical association with proteins, peptides, positively charged polymers, polysaccharides, or lipids, or be presented in the genome of genetically-engineered viruses. These nano-sized gene vectors provide enhanced cell entry, facilitated processing, nuclear uptake, and expression of exogenous DNA.[37] Gene vectors are categorised into viral and non-viral entities. Both types of

vectors were investigated in the context of stent-based gene transfer.

### Non-viral vectors in gene-eluting stents design

The first described GES prototypes used incorporation of expression-ready plasmid DNA dispersed in poly(lactide-co-glycolide) (PLGA)[38] or collagen[39] coatings. These seminal studies demonstrated transitory reporter expression in pig arteries, yet failed to show profound effects. Denaturation of collagen increased transfection of intimal SMC 10-fold through enhancement of actin depolymerisation.[40] Other polymers, such as polyurethane,[35,41] pullulan,[42] and chitosan,[43] were used in GES for enhancing plasmid mediated transduction with demonstration of ensuing transgene expression *in vitro* and *in vivo*.

A number of reported non-viral GES platforms utilised cationic lipids alone[29,44-46] or together with cationic polymers[27,47] for condensation and protection of plasmid DNA eluted from the stent. Immobilisation of resulting lipoplexes or lipopolyplexes on stent struts was achieved with PLGA/gelatin coating,[27,47] or using several cycles of direct application of lipoplex suspension to struts of phosphorylcholine-coated stents and air drying.[22,29,44] In some studies, plasmid was anchored to the stent surface using anti-DNA antibodies prior to inducing formation of lipoplexes with Lipofectamine™ 2000 (ThermoFisher Scientific, Massachusetts, USA).[45,46] In addition to plasmids, shorter DNA and RNA constructs, negatively affecting gene expression (anti-sense, decoy, siRNA, miRNA), were delivered from polymeric stent coatings.[13,14,16,48,49]

### Adenoviral vectors

Advantages of recombinant replication-deficient adenoviruses (Ad) as vectors used in GES are robust transduction of both dividing and quiescent cells, 8–34 kb capacity of transgene cassette allowing insertion of virtually any gene of interest, relative ease of high-titre production, and ability to tolerate chemical modifications. However, Ad elicit strong inflammatory and immune responses that are detrimental in the context of GES. Our group designed two prototypes of Ad-based GES, employing either non-covalent attachment of the vector via affinity adaptors[26] or covalent tethering of Ad particles through hydrolysable cross-linkers.[25] Both GES demonstrated durable reporter expression and therapeutic effectiveness in a rat model.[25,26] Several other groups also reported successful vascular gene transfer with Ad vector either immobilised on phosphorylcholine coating of BiodivYsio™ (Biocompatible Ltd., Farnham, UK) stents,[17,28,50] or on collagen membrane of CoverStent (Medtronik, Dublin, Ireland).[18]

### Adeno-associated virus vectors

Currently, adeno-associated virus vectors (AAV) are the most promising viral vectors for cardiovascular gene therapy, mostly due to their well-established safety profile and minimal elicitation of inflammatory and immune response. Despite this, AAV are relatively under-investigated as the vector for GES. Sharif et al.[50] demonstrated reporter gene expression for up to 28 days using GES loaded with $5x10^9$ particles of AAV-2. However, compared to the similar amount of Ad, vector transgene expression was lower, especially in the neointima. This observation contrasts our data,[51] showing that GES constructed with AAV-9 out-performs the Ad-formulated counterparts in regards to both peak levels and the duration of expression. The difference in animal model (rat versus rabbit), AAV serotype (9 versus 2), and reporter transgene (firefly luciferase versus beta-galactosidase) may explain these divergent findings.

### Recombinant baculovirus

Baculoviruses exclusively infect insect cells and thus humans do not have pre-existing neutralising antibodies to this virus. Unlike the parent virus, engineered baculoviral vectors transduce dividing and quiescent mammalian cells without eliciting marked inflammatory reactions. Recently, hybrid baculoviral vector complexed with polyamidoamine dendrimer and encapsulated into PLGA microspheres was investigated for gene transfer from a stent platform, showing uniform transduction of dog femoral arteries and therapeutic effectiveness against ISR.[20]

## General Gene-Eluting Stents Design and Delivery System

Design requirements for GES delivery system have both common and unique traits compared with DES platforms. Deliverability of both types of stents should not be compromised by the accommodation of a therapeutic moiety and its matrix on the struts. The matrix deposited on struts of DES and GES has to endure the mechanical stretch during deployment without cracking or delamination, since these post-deployment defects result in irregular release rate of a therapeutic agent

and can cause embolisation of distal vasculature. Additionally, the matrix of both DES and GES needs to be highly biocompatible. Sustained release of therapeutic moiety for 3–6 months realised in most DES platforms is unnecessary for the GES, since the stent-associated cells, after being transduced with eluted vectors, will actively secrete a therapeutic product as long as they (or their progeny, in the case of genome integration) survive. An important consideration for estimating the necessary duration of vector release from GES is that neointimal SMCs (the target cell population for most GES prototypes studied to date) are not yet present at the time of vascular injury, and will start populating the intima at 7 days after stent deployment.[52] This mandates that the transduction-competent vector is released for 2–3 weeks, posing additional requirements to GES delivery system with regard to extended vector stability *in vivo*. Gene vectors are generally more vulnerable than low molecular weight drugs, and lose their activity if not properly protected by matrix interactions. Immune response to a gene vector and expressed gene product is another specific problem of GES. Therefore, preventing vector spread to regional lymph nodes, as well as physical shielding of the vector from pre-formed antibodies and effector T cells, is crucial for sustaining therapeutic levels of encoded transgene. Lastly, the one to two orders of magnitude size difference between drugs used in DES and gene vectors dictates use of different matrices to enable release.

### Bulk immobilisation (polymer coatings)

The first reported GES[38,39] utilised polymer coatings on the surface of metallic stents in which gene vectors were dispersed. The coatings (typically 50–250 μm thick) were deposited on a primed stent surface either by a multiple dip technique or by aerosol deposition. After solvent evaporation and polymer precipitation on the stent surface, the gene vector stayed contained between the polymer fibres. The subsequent release of vector was then governed by a combination of diffusion though the matrix determined by hydrophilicity of the polymeric matrix and the pore size, chemical degradation of the matrix, and dissolution of the matrix in tissue fluid and blood. A variety of synthetic polymers (PLGA,[38] poly[beta-amino ester],[27,53,54] polyurethane,[35,41] poly[vinyl alcohol],[34] poly[phosphorylcholine-lauryl methacrylate][17,22,28,29,44,49,50]) and semi-synthetic polymers (dopamine-modified hyaluronic acid,[12,15,55] cationised gelatin,[36] styrene-modified gelatin,[56] cationised pullulan,[42] or naturally occurring macromolecules [native[39] or denatured[40] collagen, gelatin][27,47]) have been used for the bulk incorporation of gene vectors on stent struts.

The main advantage of bulk immobilisation is a high inclusion capacity for genetic material (up to 4 mg of plasmid DNA[41] and $5x10^{10}$ adenovector particles).[39] However, from a pharmacokinetic standpoint, the release profile of bulk immobilised vectors presents a poor match for cell dynamics in the injured vessel wall. Typically, 80–90% of the vector load is released within the first 24 hours,[17,27,34,35,42,43,49] thus missing neointimal SMC not yet present at the site of vascular injury. Rapid release of bulk-immobilised vector is mostly due to the 'burst release' of a fraction of the vector that has been absorbed onto the polymer layer after going out of solution during the solvent evaporation step. To an extent, the burst release can be counteracted using a super-coating with an additional layer of vector-free polymer,[27,38,47] or by a post-deposition polymer cross-linking.[56] Biologically, the central problem of the delivery systems based on bulk polymer coatings is inflammation in the treated arterial segment that negates the therapeutic effect of a transgene.[57]

### Surface immobilisation of gene vectors on coatless metal substrate

The concept of gene vectors' immobilisation on the surface of stents directly utilises the stent surface for tethering therapeutic gene vectors in an arranged pattern to facilitate transduction of tissue on the interface with the stent.

### Drug-eluting stent delivery systems based on the use of affinity binding adaptors

Studies by our group, motivated by numerous deficiencies of bulk immobilisation in the context of DES delivery systems, have investigated several strategies for reversible tethering of both viral[25,26,31,51,58] and non-viral[46] gene vectors. To chemically link the stent surface and gene vectors, we used metal surface modification with a family of poly(allylamine)-bisphosphonate (PAB) compounds that form a monolayer film (<5 nm thick) on the stent surface, through the formation of co-ordination bonds between bisphosphonic groups of PAB and metal atoms, and their oxides on the steel surface.[26] PAB can be further modified in order to provide a covalent attachment of vector binding affinity

adaptors, using N-hydroxysuccinimidyl ester-based amine conjugation and pyridyldithio-based thiol conjugation strategies.[26] High affinity vector binding proteins such as anti-Ad,[26] anti-DNA[46] antibodies, or a recombinant domain of coxsackie-adenovirus receptor[26] were used as binding adaptors, enabling high-affinity attachment of gene vectors to stents. Interestingly, both loading capacity of GES and vector release rate were shown to be modulated through using the binding adapters with different affinities to Ad vectors.[26] While fluorescently labelled Ad were observed on the interface between stent struts and the arterial wall 1 day after stent deployment, and the associated reporter (green fluorescent protein) activity was seen in all arterial layers 7 days post-stenting,[26] the release of vector *in vivo* with this affinity-based GES delivery remains poorly controllable, as the vector association with stents is determined by the antigen/antibody affinity and local pH (i.e. the factors that cannot be changed deliberately).

### Gene-eluting stent delivery systems utilising hydrolysable cross-linkers

Our later work on GES delivery systems has investigated a completely synthetic approach for reversible binding of Ad vectors to metal surfaces that obviates using protein adaptors. This strategy is based on hydrolysable cross-linker molecules that directly append vectors to PAB-activated steel.[25,58] The subsequent release of the vectors is governed by the kinetics of cross-linker hydrolysis and can be modulated by the usage of hydrolysable cross-linker molecules with variable hydrolysis rates.[58] Importantly, this linking strategy allows amplification of virus binding sites on the surface (and thus increase of vector load) through the optional expansion of the thiol group number on the metal surface.[25]

### Nanoparticulate delivery systems for magnetic stent targeting

Both bulk immobilisation and surface tethering strategies make use of GES delivery systems that are assembled prior to stent implantation in the artery. One potential downside of pre-made GES is that the insertion of a stent through the haemostatic valve of a vascular sheath, and its advancement to a deployment site through the narrowed arterial conduit, always involves some physical damage to the vector depot. Alternatively, a stent can be loaded with gene vectors *in situ* after implantation. The surface of a deployed stent can be actively targeted with vectors delivered to circulation, provided the targeting forces are strong enough for vectors' capture and retention despite the shearing effect of blood-flow.

This concept of a post-deployment stent loading with gene vectors was recently implemented by our group using a magnetic targeting paradigm.[59] Stents made of magnetisable alloys, when placed in a uniform magnetic field, generate strong highly-localised magnetic forces due to steep gradients of the magnetic field across the strut meshwork.[60] These magnetic forces enable the targeted capture of systemically or locally administered gene vectors formulated in a magnetically-responsive nanoparticle.[59] In our study,[59] aortic arch instillation of Ad-Luc containing magnetically-responsive nanoparticles in the presence of uniform magnetic field of 0.1 T resulted in sustained luciferase expression in the stented artery that vastly exceeded expression, following the delivery of an equal amount of free adenovector.

## CONCLUSIONS

Gene delivery from the surface of an intra-arterial stent provides therapeutic opportunities for ISR prevention in clinical circumstances, where current DES devices fail to provide satisfactory results. Progress alongside the transgene/vector/delivery paradigm should determine whether GES will remain a laboratory artefact, or complement the armamentarium of clinical cardiology.

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