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Review

Nucleic Acid Delivery to the Vascular Endothelium

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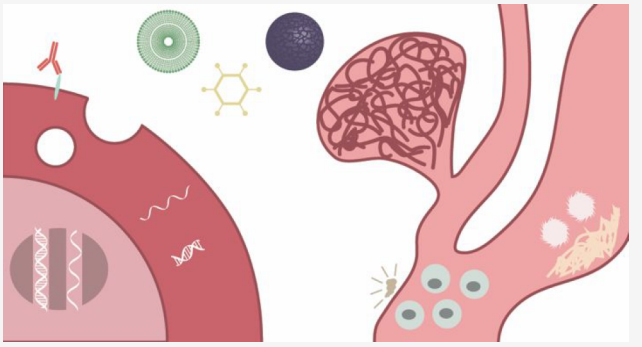
ABSTRACT: This Review examines the state-of-the-art in the delivery of nucleic acid therapies that are directed to the vascular endothelium. First, we review the most important homeostatic functions and properties of the vascular endothelium and summarize the nucleic acid tools that are currently available for gene therapy and nucleic acid delivery. Second, we consider the opportunities available with the endothelium as a therapeutic target and the experimental models that exist to evaluate the potential of those opportunities. Finally, we review the progress to date from investigations that are directly targeting the vascular endothelium: for vascular disease, for peri-transplant therapy, for angiogenic therapies, for pulmonary endothelial disease, and for the blood–brain barrier, ending with a summary of the future outlook in this field.



KEYWORDS: *nucleic acid delivery, nonviral vehicle, polymeric vehicle, nanoparticle, polyplex, endothelium*

1. INTRODUCTION

A single layer of endothelial cells (ECs) line the blood circulatory tree throughout nearly all tissues in the body with an estimated total surface area in humans of seven thousand square meters.[1] These ECs form an interface between cells and molecules circulating in the blood and the internal tissues of the body. Even in its quiescent state, the endothelium is actively signaling and reacting in order to support the basic homeostatic functions of the vascular system, delivering oxygen and nutrients, while removing carbon dioxide and waste.[2] Although often described as a single cell type and sharing some universal characteristics, a closer look reveals that the cells that make up this endothelial lining are diverse in their phenotypes and functions, varying with both the organ or tissue in which they reside and with different segments of the vasculature within the same tissue.[3,4] When activated under inflammatory conditions or in other disease states, ECs acquire new functions through altered protein expression and morphological changes. With its pivotal role in pathophysiology and its large, easily accessible surface in almost all tissues in the body, the endothelium is an attractive therapeutic target.[5]

Gene therapies enable therapeutic intervention at the level of the genome by altering gene expression. The modulation of gene expression via activation, suppression, or supplementation is a powerful way of reaching "undruggable" protein targets. This can be achieved by targeting different parts of gene expression and protein synthesis pathways. Genome editing tools such as clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) systems, peptide nucleic acids (PNAs), or restriction enzymes can permanently alter or eliminate the expression of a specific gene. The delivery of exogenous DNA or mRNA (mRNA) to cells can increase production of the encoded protein. Nucleic acid therapies can also target endogenous mRNA transcripts; small interfering RNA (siRNA) and microRNA (miRNA) reduce protein levels by targeting coding mRNAs in a sequence-specific manner through the RNA interference (RNAi) pathway, and antisense oligonucleotides (ASOs) modulate splicing or block translation of specific mRNA transcripts.

To be effective in clinical applications, therapeutic nucleic acids must have sufficient bioavailability, stability, and transfection efficiency in the desired tissue and cell type. Nucleic acids must enter the cell cytoplasm or nucleus to have an effect. Nanocarriers formulated to deliver nucleic acids to cells can improve the bioavailability and stability of therapeutic nucleic acids and can further prolong the sustained effects of altered gene expression. There are a number of endothelium-associated pathologies that are promising targets for treatment with gene therapies, including vascular diseases, inflammation, tumor microvascular beds, and peri-transplant graft injuries.

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Table 1. Summary of Nucleic Acid Therapeutics

nucleic acid therapeutic	size	function and mechanism	modifications
pDNA	∼1−200K base pairs	•increase or decrease gene expression by encoding mRNA, shRNA, or lncRNA •pDNA is transcribed by endogenous transcription machinery in the nucleus	•pDNA lacking unmethylated CpG reduces immunogenicity[30] •removal of nuclease-susceptible (e.g., homopurine-rich) sequences increases nuclease resistance[31]
mRNA	∼1−10K nucleotides	•increase expression of encoded transcript •mRNA is translated by endogenous machinery in the cytosol	•modified nucleotides reduce immunogenicity and increase stability[28] •modifications in the open reading frame (ORF), UTRs, 5′ cap, or poly(A) tail improve intracellular stability and expression kinetics[26]
PNA	20−40 nucleotides	•increase, decrease, or edit gene expression by acting as an anti-miR, antisense, or gene editing agent •PNAs bind to DNA or RNA with high affinity via Watson−Crick or Hoogsteen base pairing	•amino acid residues added to terminus reduces hydrophobicity and aggregation[32] •conjugation to PEG or to charged DNA molecules reduces hydrophobicity and aggregation
ASO	12−30 nucleotides	•increase or decrease gene expression by sequence-specific binding to miRNA, mRNA, or pre-mRNA •ASO binding to mRNA suppresses translation, binding to miRNA blocks miRNA-mediated suppression of translation, or binding to pre-mRNA modulates splicing	•replacement of phosphodiester backbone with phosphorothioate (PS) linkages protects against degradation[33] •replacement of ribofuranose rings with morpholino rings protects against degradation[33] •sugar substitution of 2′-OH with 2′-MOE or 2′-cEt or locked nucleic acid (LNA) protects against degradation[33]
siRNA	20−26 base pairs	•sequence-specific silencing of mRNA transcript •siRNA (in complex with RISC) binds to complementary sequence on target mRNA, initiating an RNA interference pathway, which blocks translation by degrading mRNA	•backbone modification with PS linkages, LNAs, or substitution of 2′-OH with 2′-OMe, 2′-MOE, or 2′-F increases nuclease resistance[34] •bioconjugation to *N*-acetylgalactosamine (GalNAc), lipids, peptides, or aptamers enhances stability and uptake[34]
miRNA	20−26 base pairs	•silencing of mRNA transcripts by partial complementary base pairing to target mRNA(s) •miRNA (in complex with RISC) binds to target mRNA, initiating an RNA interference pathway, which inhibits translation	•backbone modification with PS linkages, LNAs, or substitution of 2′-OH with 2′-OMe, 2′-MOE, or 2′-F increases nuclease resistance[35] •bioconjugation to GalNAc, lipids, peptides, or aptamers enhances stability and uptake[35]

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The accessibility of ECs by intravenous administration creates the potential for the treatment of a broad range of diseases with therapeutics that act on the endothelium. Furthermore, specific vascular beds or regions of vascular injury can be treated using EC subtype-specific targeted nanomedicines.

In this Review, we describe the current status of research in gene therapy vehicles for delivery to the vascular endothelium. We discuss newly developed nucleic acid delivery systems for reaching ECs throughout diverse tissues in the body. In addition, we describe *in vitro*, *in vivo*, and *ex vivo* models for the study of gene delivery to the human endothelium.

2. HOMEOSTATIC FUNCTIONS AND PROPERTIES OF THE VASCULAR ENDOTHELIUM

In the absence of perturbation, ECs are actively engaged in maintaining homeostasis.[6,7] ECs form a barrier that prevents platelets from contacting activating signals in the underlying vessel wall, such as collagen, while actively suppressing activation of both platelets and the coagulation cascade to maintain blood fluidity.[8,9] ECs regulate blood flow by producing vasodilators or vasoconstrictors that regulate the tone of smooth muscle cells (SMCs) in the deeper layers of the vessel wall[5] and regulate vessel wall permeability by formation and maintenance of interendothelial junctions.[7,10] Human ECs limit leukocyte capture by sequestering P-selectin in Weibel-Palade bodies and suppressing expression of E-selectin, molecules that can tether leukocytes to the EC surface.[5] ECs also regulate the expression of the intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) and the expression and display of chemokines and cytokines that mediate subsequent steps in leukocyte recruitment and activation.

In addition to these universal EC functions, organ-specific ECs carry out distinct functions as the point of exchange between materials in the bloodstream and the extravascular tissues.[4,11] This contributes to the unique EC phenotypes seen in different parts of the body. For example, the brain endothelium forms the blood−brain barrier (BBB), which is characterized by the expression of tight junctions by ECs, a high density of pericytes, and astrocyte foot processes surrounding the capillaries. Materials passing across this endothelium must be selectively transported through the ECs rather than passing through gaps or fenestrations. Conversely, in tissues such as the liver where greater exchange across the endothelium is needed, fenestrated and discontinuous sinusoidal capillaries allow some solutes to pass.[7] Further details about the functions of the vascular endothelium have been reviewed elsewhere.[7,8,10,12−14]

Quiescent ECs maintain precise regulation of the expression and production of adhesion molecules, enzymes, and other proteins and signaling molecules. Disruption of EC functions is associated with a wide range of pathological conditions including vascular diseases, inflammation, and tumor growth and metastasis, making the endothelium an important target for gene therapy. The accessibility of the endothelium to IV administered agents and the varying permeabilities and surface protein expression invites the development of gene delivery vehicles that can protect and deliver nucleic acid cargos to specific regions of the vascular system.

3. GENE THERAPIES

3.1. Nucleic Acids for Gene Therapies. Here, we briefly describe the various types of gene therapies that can be administered to the endothelium. These include genome editing, therapeutic DNA and mRNA, and oligonucleotides, many of which have been reviewed extensively elsewhere.[15,16] Key features of different nucleic acid therapeutics are summarized in Table 1. Broadly, gene therapy continues to be an exciting area of biotechnology due to rapid technology development and advances in clinical translation.

3.1.1. Genome Editing. There are several technologies designed to make permanent changes to the genome, including the widely popular CRISPR/Cas9 system and other versions utilizing various Cas family enzymes. Other nuclease-based gene editing agents include meganucleases, zinc finger nucleases (ZFNs), and TALE nucleases (TALENs). PNAs can also serve as a non-nuclease-based technology for gene editing.[17] CRISPR/Cas9 technology has become a widely used editing platform, as reagent design is relatively straightforward.[18] This system uses a guide RNA (sgRNA) to direct the Cas9 nuclease to a specific location in the genome.[19,20] Gene editing can take the form of gene knock-in/knock-out and also a precise correction if a donor DNA is delivered to serve as a template. Cas9 expression can be induced by delivering plasmid DNA, mRNA, or viral vectors encoding Cas9.[21] pDNA encoding Cas9 is ∼8−10 kilobases; Cas9 mRNA is ∼4000 nucleotides. The mRNA form is often preferred, as the duration of nuclease activity is limited, reducing the potential for off-target modifications in the genome.

Genome editing activity can also be manipulated by modifications to the Cas9 enzyme (or other Cas family enzymes) as well as sgRNAs. For example, small mutations in Cas9 can increase fidelity (i.e., retaining on-target activity while reducing off-target activity).[22] Moreover, base editors composed of nuclease-impaired Cas9 can result in precise point mutations.[23] Recently described prime editing technology involves a catalytically impaired Cas9 endonuclease fused to an engineered reverse transcriptase programmed with a prime editing guide RNA (pegRNA) that also encodes the desired edit.[24] Prime editing eliminates the need for a donor DNA and also reduces off-target effects compared to traditional CRISPR/Cas9. Chemically modified sgRNAs can also further improve editing outcomes.[25]

3.1.2. Therapeutic DNA and mRNA. If a therapeutic goal is to amplify the expression of a particular gene, the gene itself or the gene product can also be administered in the DNA or mRNA form, respectively.[26] DNA-based therapeutics must be delivered to cell nuclei, whereas mRNA-based therapeutics only need to enter the cytoplasm and can produce therapeutic effects in both dividing and nondividing cells. As plasmids, DNA therapeutics are large (several kilobases depending on the gene encoded) compared to mRNAs, though mRNA molecules are less stable than DNA and subject to rapid degradation by RNases if not modified.[27] pDNA must be translocated to the nucleus and has the potential to be integrated into the genome, whereas mRNAs do not pose any risk of insertional mutagenesis. Accordingly, pDNA delivery typically results in prolonged transgene expression while mRNA therapies produce more transient expression. The kinetics of mRNA-mediated transgene expression can be tuned by modifying the 5′ cap, untranslated regions (UTRs), or poly(A) tail.[26]

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Table 2. Nucleic Acid Delivery Vehicles

Delivery Vehicle Class	Vehicle Sub-Types	Characteristics	Encapsulation and Delivery Mechanisms	Key Features
Viral Vectors	Retrovirus, adenovirus, adeno-associated virus (AAV), herpes virus, pox virus, human foamy virus, lentivirus	• Non-essential and pathogenic viral genes are deleted, while retaining gene transfer capacity • Protein envelope on vector surface	• Therapeutic gene is packaged with viral proteins • Protein envelope mediates interaction with cell surface • Entry via membrane disruption or endocytosis; if in endosome, acidification disrupts protein envelope	• High gene transfer efficiency • Can integrate into host genome, depending on vehicle sub-type • Limited cargo size capacity, depending on vehicle sub-type (e.g., AAV capacity ≤ 4.8 kb) • Lipid or polymer coating can provide stealth or enable conjugation to cell targeting molecules
Lipids	Liposome, lipid nanoparticle (LNP), lipoplex, ionizable lipid and lipidoid, gene-lipid conjugate	• Lipids have a positively charged hydrophilic head group and a hydrophobic tail chain • Vehicles have a positive surface charge	• Electrostatic interactions condense nucleic acids into compact structures • Positive surface charge interacts with glycoproteins on cell surface to promote uptake • Lipid complex fuses with or disrupts endosomal membrane for cargo release[85]	• Reduced risk of immunogenicity relative to viral vectors • Tunable material properties – modification of lipid domains (head, backbone, or tail) • Additional components (e.g., cholesterol) can improve transfection, stability or biodistribution • Ionizable lipids promote endosomal escape • Can conjugate to cell targeting molecules
Polymers	Nanoparticle (NP), polyplex, micelle, nanocapsule, dendrimer, nanoconjugate	• Cationic polymer charge is most commonly generated from amine groups • Vehicles have a positive surface charge	• Cationic polymers condense anionic nucleic acids • Positive surface charge interacts with glycoproteins on cell surface to promote uptake • Cargo is released from endosome by osmotic lysis or pore formation[85]	• Reduced risk of immunogenicity relative to viral vectors • Tunable material properties – monomer composition, molecular weight, hydrophobicity • Blending of multiple polymers provides further customizability of vehicles • Controlled/sustained release • Biodegradable and biocompatible • Can conjugate to cell targeting molecules

mRNAs are the cargo of choice for many biotechnology applications and offer additional opportunities for improved outcomes. Recent advances in mRNA-based therapeutics include the incorporation of modified nucleotides, which can confer increased stability and reduced immunogenicity.[28] Such mRNA modifications have been incorporated into recent COVID-19 mRNA vaccines.[29]

3.1.3. Oligonucleotides. In addition to introducing exogenous mRNA transcripts, post-transcriptional modulation of gene expression can also be achieved using oligonucleotides that disrupt the processing and translation of endogenous mRNA. siRNAs and miRNAs suppress mRNA translation through RNAi, a mechanism in eukaryotic cells that silences gene expression by sequence-specific cleavage of mRNA.[36,37] RNAi is initiated by the presence of double stranded RNA (dsRNA) in the cytoplasm, which can arise from endogenous siRNA or miRNA pathways, or can be introduced for therapeutic purposes by delivering synthetic siRNA, miRNA, or dsRNA precursors into the cytoplasm.[38,39] ASOs are single-stranded, often chemically modified, oligonucleotides (12−30 nucleotides in length) that bind to sequence-specific mRNA, pre-mRNA, or miRNA targets by Watson−Crick base pairing.[40] ASOs can be designed to target mRNA in various stages of processing to either increase or reduce translation; ASOs suppress translation by binding to a target sequence on mRNA, block miRNA-mediated suppression by binding to a target sequence on miRNA, or modulate splicing (exon skipping or exon inclusion) by binding to a target sequence on pre-mRNA. ASO binding can suppress translation by steric blocking or by promoting cleavage of pre-mRNA or mRNA by RNase H1 or argonaute 2.[40]

3.2. Nucleic Acid Delivery Vehicles. A critical element of any nucleic acid-based therapeutic is designing a suitable delivery approach that protects the cargo from degradation, enables efficient uptake into cells, and delivers a sustained effect in the relevant tissue. The bioavailability of nucleic acids is limited by their size and negative charge, lack of solubility in membranes, renal clearance, and susceptibility to degradation by nucleases, which are present throughout tissues.[34] Gene therapy agents can be encapsulated in viral-, lipid-, or polymer-based delivery vehicles or conjugated to stabilization or transport molecules such as cell penetrating peptides, aptamers, or lipids.[33,41] Both of these approaches can improve the bioavailability, stability, and cellular uptake of gene therapy agents compared to administrating free nucleic acid alone, especially in the *in vivo* environment. Furthermore, delivery vehicles can be designed to target specific tissues or optimized for efficient intracellular trafficking.

Nucleic acid delivery vehicles fall into one of two categories, viral and nonviral. Viral vectors are currently the predominant delivery method for clinical applications. Around 70% of nucleic acid therapies currently in clinical trials around the world use viral vectors.[42] Nonviral lipid-based vectors have been developed due to safety concerns regarding the immunogenicity of viral vector proteins.[43] Biodegradable polymeric delivery vehicles represent an alternative approach toward producing biocompatible nanocarriers with low immunogenicity.[44,45] Because nucleic acids must act intracellularly, in either the cytosol or the nucleus, vectors used for the delivery of therapeutic nucleic acids must allow their cargo to reach the appropriate site of action. Viral vectors can take advantage of the natural mechanisms they have evolved for nucleic acid delivery. Synthetic vectors usually exploit the target cell's pathways to uptake extracellular particles. Most often, this involves internalization into membrane-coated vesicles through pathways that may be receptor-mediated or

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receptor independent. One well studied pathway involves transfer to and uptake from clathrin-coated pits, which moves the particles initially into clathrin-coated vesicles. These vesicles, in turn, are modified into early and then late endosomes in processes that are mediated by the small GTPase of the Rab subfamily. Endosomes may fuse with lysosomes forming vesicles in which the endocytosed particles are degraded within the cell or may recycle to the cell surface, resulting in exocytosis of the particle. In either case, the nucleic acid cargo must escape from the vesicle to reach the cytosol, a process that can be aided by lipid-based vectors. Here, we provide a brief overview of both viral and nonviral nucleic acid delivery vehicles, as these have been reviewed extensively elsewhere.[42,46−48] Important features of viral and nonviral nucleic acid delivery vehicles are summarized in Table 2.

3.2.1. Viral Vectors. Viral vectors are the most commonly used vehicles for gene delivery. Retroviruses, adenoviruses, adeno-associated viruses, herpes viruses, pox viruses, human foamy viruses, lentiviruses, and others have been used as carriers for genetic material.[49] Retroviruses are single-stranded RNA viruses that use reverse transcriptase to transcribe their RNA into DNA, which can then be linearly integrated into the host genome.[50] Most retroviruses used for delivery are replication defective and only active in dividing cells.[51] The inability of retroviral vectors to infect nondividing cells led to the use of lentiviral vectors, which are a subclass of retroviruses that can integrate into DNA in quiescent cells and have evolved into a powerful research tool for both *in vitro* and *in vivo* gene therapies. Lentiviral vectors have been used extensively in neurodegenerative diseases such as Parkinson's, Alzheimer's, and Huntington's disease or spinal injuries due to their natural tropism for neural stem cells.[52,53]

Adenoviral vectors have been isolated from many different species with over 100 different serotypes reported.[51] These are DNA viruses that do not integrate into the host genome and usually do not replicate during cell division. In most gene therapy applications, adenoviral vectors are applied under conditions in which the temporary expression of the protein(s) is required.[54] Adenoviral vectors have the ability to carry large segments of DNA (up to 38kb) and can infect nonreplicating cells. However, adenoviral vectors can lead to severe immune responses or other adverse side effects. The first fatality of a gene therapy clinical trial was due to an inflammatory reaction to an adenoviral vector.[55] Adeno-associated viruses (AAVs) are similar to adenoviral vectors in their composition; however, AAVs are deficient in both replication and pathogenicity in humans and, thus, assumed to be safer than adenoviruses. A unique characteristic of AAVs is their ability to site-specifically integrate on chromosome 19 with no noticeable long-term expression side effects. AAVs have been used in the treatment of cystic fibrosis, Leber congenital amaurosis, hemophilia B, and alpha-1 antitrypsin deficiency.[56−58] The greatest disadvantages of AAVs are their limited capacity (up to 4.8 kb), their immunogenicity (which prohibits multiple dosing), and their complex production process.

3.2.2. Lipids. Cationic lipid-based systems share a common chemical structure that includes a positively charged hydrophilic headgroup and hydrophobic tail chains connected by a linker. The cationic headgroup allows for electrostatic interactions with anionic nucleic acids, so that gene materials are packaged into compact structures. Lipid formulations are robust in their ability to condense nucleic acid molecules of varying sizes. The resulting nanoscale lipocomplexes have a positive surface charge that facilitates their interaction with negatively charged glycoproteins/proteoglycans on cellular phospholipid plasma membranes to promote uptake. Cationic lipids can be easily synthesized, and large libraries of materials can be generated by modifying their constituent domains (i.e., head groups, backbones, and fatty acid tails).

Among the first cationic lipids used for DNA delivery was 1,2-di-*O*-octadecenyl-3-trimethylammonium propane (DOTMA),[59,60] the success of which suggested structural features of lipid molecules important for efficient transfection. Since the discovery of DOTMA, other lipids that are useful for transfection have been identified, including 2-[3-[4-(3aminopropylamino)butylamino]propylcarbamoylamino]-ethyl-[2,3-bis[[(*Z*)-octadec-9-enoyl]oxy]propyl]-dimethylazanium (DOSPA), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-sn-glycero-3-[(*N*-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (DOGS), and dimyristyloxypropyl-3-dimethyl-hydroxyethylammonium (DMRIE). The properties of lipid nanocarriers can be modified by the inclusion of other components, such as cholesterol and its derivatives, which often improve transfection.[61−64]

Since the advent of early cationic liposomes as gene carriers, new concepts in lipid-based delivery systems have emerged including ionizable lipids and lipidoids, gene−lipid conjugates, and functionalized lipid nanoparticles (LNPs). Ionizable lipids have pK_a values that facilitate self-assembly into nanoparticles (NPs) by enabling the close contact of lipid headgroups when mixed with negatively charged nucleic acids.[65−67] Ionizable lipids can facilitate endosomal escape; lipids that have a positive charge in the endosome interact with anionic lipids in the endosomal membrane, promoting membrane disruption and the release of nucleic acid cargos into the cytosol.[68] Lipidoids consist of lipid-like materials that contain tertiary amines and are amenable to high-throughput synthesis, which allows for screening to identify compositions that lead to high transfection efficiency.[69] In gene−lipid conjugates, lipids are conjugated to their nucleic acid cargo in ways that minimize the loss of bioactivity, reduce degradation of the genetic material, and optimize biodistribution *in vivo*.[70] The use of LNPs for gene delivery and gene editing has grown substantially in the past decade. Many research groups and commercial entities are actively developing and investigating new LNP formulations for a wide range of therapeutic applications, including recently developed COVID-19 vaccines.[71−74]

3.2.3. Polymers. Polymeric vehicles are a large and versatile category of delivery materials that are highly customizable. Physical and chemical properties of polymers can be easily modified to design vehicles for a broader range of types and sizes of nucleic acid cargos than can be delivered with viral vectors.[45] Cationic polymers are effective for gene delivery because they efficiently condense nucleic acid cargos due to electrostatic interactions.[42,75] Polycations contain pendant amine groups that give the polymer a positive charge. Diethylaminomethyl (DEAE) dextran was one of the earliest cationic polymers to be used for gene delivery; however, it is highly cytotoxic.[42,76] Other polycations commonly used today, including polylysines, polyethylenimines (PEI), poly(2-*N*-(dimethylaminoethyl) methacrylate) (PDMAEMA), and polyamidoamine (PAMAM), have improved transfection efficiency over earlier methods; however, the cytotoxicity of these polymers remains a major limitation.[42,44]

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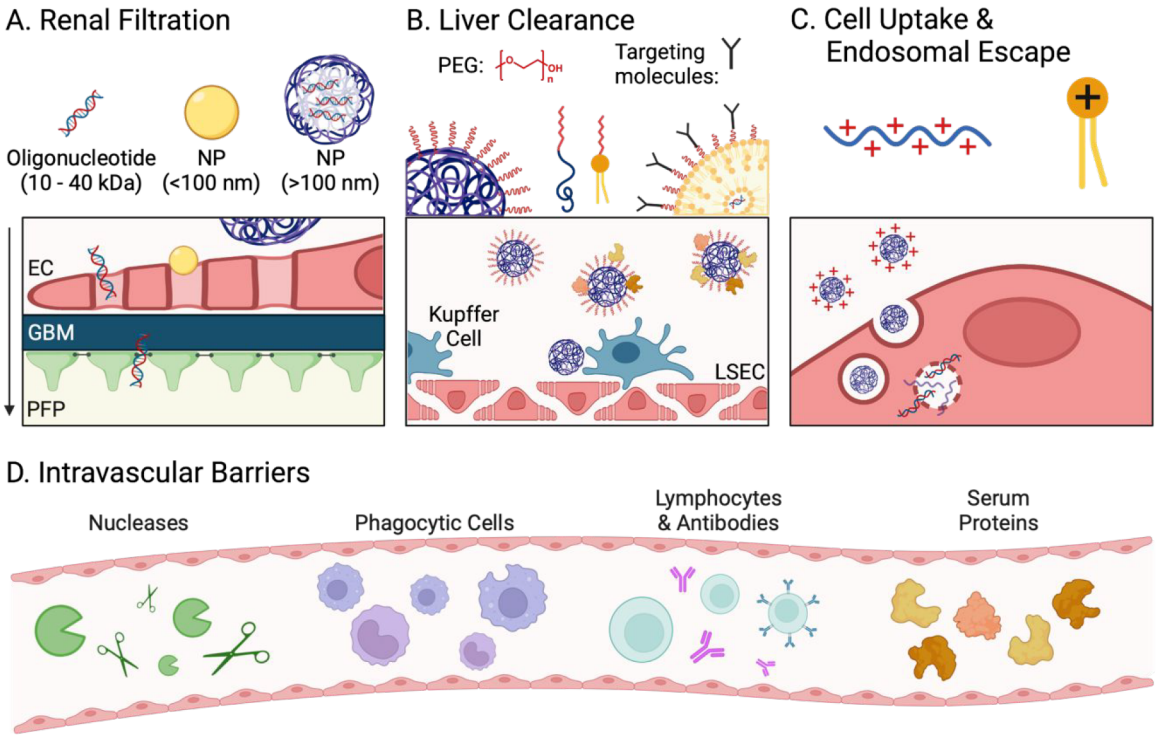

Figure 1. Challenges and approaches of delivery to the endothelium.

Poly(beta-amino ester)s (PBAEs) are biodegradable tertiary-amine containing polymers.[77] The use of PBAEs for transfecting endothelial cells has been thoroughly investigated.[78−81] From a large library of PBAE polymers generated using high-throughput combinatorial chemistry, polymers exhibited cell-type specific transfection efficiencies.[80] In this study, a high correlation in transfection performance was found between different types of ECs, whereas there was little correlation between either EC type and an epithelial cell line. Poly(amine-*co*-esters) (PACEs), another family of biocompatible polymers, are synthesized by copolymerization of an amino-diol, a diester, and a lactone. This generates mildly cationic polymers with tunable properties that can encapsulate nucleic acid cargos in the form of polyplexes, short-lifetime vehicles formed by electrostatic interactions between cationic polymers and negatively charged nucleic acids, or solid NPs.[82,83] Like PBAEs, PACEs can be synthesized with different monomer compositions and chemical end groups, which enable fine-tuning of properties that affect cellular uptake and endosomal escape.[83−85]

4. THE VASCULAR ENDOTHELIUM AS A THERAPEUTIC TARGET

4.1. Gene Delivery to the Endothelium. *4.1.1. Clearance of Delivery Vehicles from Systemic Circulation.* Delivery vehicles encapsulating nucleic acids can reach the vascular endothelium by IV administration; however, the delivery of therapeutically relevant doses to the endothelium presents unique challenges. ECs experience constant blood flow across their luminal surface, so unless the delivery vehicles adhere to the EC surface, they will not have extended contact with these cells.[86−89] The intravascular environment is dense with serum proteins and circulating cells that can affect the interaction between delivery vehicles and ECs.[90−97] Furthermore, while delivery vehicles can prolong the bioavailability of nucleic acids, materials in the bloodstream face rapid clearance from circulation by renal filtration or by intravascular mononuclear phagocytes of the liver and spleen (Figure 1).[98,99]

Naked oligonucleotides of less than 40 kDa can pass through the renal filtration barrier and exit circulation through urine output. The innermost layer of this barrier is formed by a fenestrated endothelium (∼100 nm fenestrations), after which the dense, heparan sulfate-rich glomerular basement membrane (GBM) has a more restrictive pore size of <10 nm. Larger nucleic acids as well as those encapsulated in nanoscale delivery vehicles can therefore avoid renal filtration. However, there is evidence that cationic polymer NPs of up to 100 nm in diameter can pass through the endothelial fenestrations and accumulate on the negatively charged GBM, causing disassembly of the NPs and filtration of the oligonucleotide cargo out of circulation.[100] Nucleic acids that are filtered are often reabsorbed by megalin expressed on proximal tubular epithelial cells. While renal filtration is a barrier to targeting ECs, it can be advantageous for the delivery of oligonucleotides to proximal tubular epithelial cells, which are the primary target of renal ischemic injury.

Nucleic acids, either free or encapsulated in delivery vehicles, that evade renal filtration are still subject to clearance from circulation by mononuclear phagocytic cells in the liver and spleen.[98] Uptake of NPs by liver macrophages has been exploited to deliver siRNA to treat inflammation[101] or alcoholic liver disease and fibrosis.[102] All four FDA approved siRNA drugs, patisiran, givosiran, lumasiran, and inclisiran, target hepatocytes. Patisiran is delivered in LNPs, while givosiran, lumasiran, and inclisiran are delivered without packaging and are conjugated to *N*-acetylgalactosamine (GalNAc) to target hepatocytes.[34] The shift of the biodistribution of siRNA or other nucleic acid therapies away from the liver to other tissues remains an important goal.

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Table 3. Nucleic Acid Delivery Vehicles for Endothelial Targets

Delivery Vehicle Class	Delivery Vehicle Sub-Type	Cargo	Endothelial Target	Context	Key Results	Ref
Viral Vectors	AAV2 gene delivery stents	EGFP or apoA1	Coronary Artery	*in vivo* (pigs)	Successful transduction of arterial ECs; apoA1 transduction did not reduce ISR in atherosclerosis model	112
	AAV9SLR in alginate hydrogel	EGFP	Abdominal Aorta	*in vivo* (mice)	AAV9-hydrogel transduced only aorta ECs and SMCs, whereas systemic AAV9 transduced only cells in liver	113
	Ad.MBP (myeloid-binding peptide)	EGFP	Vasculature in Multiple Organs	*in vivo* (mice)	MBP modification shifted tropism from liver cells to ECs throughout organs; greatest enhancement in lung ECs	111
	E-selectin or VCAM-1 targeted PEG Ad5IκBAA	dnIkB	Glomerular Endothelium IV	*in vivo* (mice)	E-selectin-targeted adenovirus selectively transduced glomerular ECs, suppressed adhesion molecules	127
	Ad-GFP-Cas9 and lentivirus	EGFP-tagged Cas9 and Tie2 sgRNA	Primary Human Endothelial Cells	*in vitro*	Dual viral vector system enabled gene deletion in primary human ECs without clonal expansion	140
	AAV-BR1-CRISPR	Cas9 and Ctnnbi sgRNA	Brain Endothelial Cells	*in vivo* (mice)	AAV-BR1-CRISPR system as a tool for *in vivo* identification of genes involved in BBB integrity	141
	Ad-Cas9-Col8a2gRNA	Cas9 and COL8A2 sgRNA	Corneal Endothelial Cells	*in vivo* (mice)	Knockdown of mutant COL8A2 prevented corneal EC loss	142
	rAAV1-SpCas9 and rAAV1-mK22	Cas9 and VEGFR2 sgRNA	Retinal Endothelial Cells	*in vivo* (mice)	VEGFR2 depletion decreased pathological neovascularization in retinopathy model	143
	PAMAM-coated AAV9-Cre linked to endothelial-affine peptides	Cre recombinase	Microvascular Endothelial Cells	*in vivo* (mice and pigs)	PAMAM coating on AAV9-Cre enabled peptide targeting for transduction of ECs	144
	E-selectin targeted Adluc coated with pHPMA	Luciferase	Activated Endothelial cells	*in vitro* and *ex vivo*	pHPMA coating prevented antibody recognition, liver capture; improved circulation time, vascular targeting	145
	AAV-VEGF 2	VEGF	Vasculature in Multiple Organs	*in vivo* (mice)	Increased circulating VEGF protein, reduced age-related capillary loss	146
	rAAV1	Sirt7	Vasculature in Multiple Organs	*in vivo* (mice)	Sirt7 rescue in vascular ECs increased vascular growth, extended lifespan in progeria model	147
	Ad-GFP and Ad-HSVtk with RGD4C peptide	GFP and HSVtk	Tumor Endothelium	*in vivo* (mice)	Combined transcriptional restriction, affinity targeting, and stealth properties to target tumor ECs	148
	AAVP-TNF and AAVP-HSVtk with RGD4C peptide	TNF and HSVtk	Tumor Endothelium	*in vivo* (mice)	Cyclic RGD4C peptide targeted transduction of tumor vascular ECs	149
Lipids 	DMAPAP/DOPE/Mal-PEG-DSPE Lipoplexes	P-selectin targeted RAGE-shRNA	Aorta	*in vivo* (mice)	Specific accumulation and transfection in sites of atherosclerosis in aorta of ApoE-deficient mice on high-fat diet; reduced atherosclerosis progression	114
	RGD-conjugated liposome-polycation-DNA (LPD) particles	VEGFR-2 siRNA	Angiogenic Endothelial Cells	*in vitro*	Targeting $\alpha_v\beta_3$ integrins using cyclic RGD peptide enhanced uptake and VEGFR-2 silencing in ECs	121
	PECAM-1 targeted LNPs	Luciferase mRNA	Pulmonary Endothelium	*in vivo* (mice)	PECAM-1 targeting reduced hepatic uptake of LNPs and increased mRNA delivery and protein expression in lungs compared to non-targeted	126
	VCAM-1 targeted LNPs	Luciferase and TM mRNA	Inflamed Cerebral Endothelium	*in vivo* (mice)	VCAM-1 targeting was more effective than TfR-1 or ICAM-1 targeting for transfection of inflamed cerebral vasculature	130
	E-Selectin targeted lipoplexes	VE-cadherin, CD31, and TNFR2 siRNA	Activated Endothelial Cells	*in vitro*	E-selectin targeting improved siRNA delivery, in particular in primary human ECs	133
	Thrombomodulin (TM) targeted DODAP/DSPE-PEG-VS lipid vesicles	antisense oligodeoxynucleotides	Pulmonary Endothelium	*in vitro* and *in vivo* (mice)	TM-targeted lipid vesicles selectively delivered to lung endothelium	150
	DACC lipoplexes	CD31, Tie-2, VE-cadherin, and BMP-R2 siRNA	Pulmonary Endothelium	*in vivo* (mice)	siRNA delivered to lung endothelium by systemic administration; CD31 siRNA reduced metastases and increased life span in lung cancer model	151
	GALA-modified liposomal multifunctional envelope-type nanodevice	CD31 siRNA	Pulmonary Endothelium	*in vivo* (mice)	GALA peptide targets sialic acid and promotes endosomal escape; CD31 siRNA reduced metastases in lung cancer model	152

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Table 3. continued

Delivery Vehicle Class	Delivery Vehicle Sub-Type	Cargo	Endothelial Target	Context	Key Results	Ref
Polymers 	PBAE NPs	pCMV-EGFP DNA	Macrovasculature and Microvasculature	*in vitro*	Library of PBAE polymers was evaluated to find NPs that are optimal for transfection of either macro- or microvascular ECs, or both	80
	PLGA NPs	miR-132	Endothelial Cell Transplantation	*in vitro*, transplant *in vivo* (mice)	miRNA effects were prolonged with NP delivery relative to lipid; miR-132 NP-treated HUVECs formed denser microvessels upon transplantation	122
	E/P-Selectin targeted PAMAM dendrimers	pEGFP DNA	Activated Vascular Endothelium	*in vitro* and *ex vivo*	Targeting with anti-E/P-Selectin mAb increased transfection in E-Selectin-expressing ECs	132
	PEG-*b*-PLGA/PEI NPs	Cas9 and sgRNA pDNA	Vasculature in Multiple Organs	*in vivo* (mice)	Increasing PEG size (up to 5 kDa) improved biodistribution in vascular beds outside of the liver after IV administration	109
	PACE NPs	CIITA siRNA	Vasculature in Organs for Transplant	*in vitro, ex vivo, and in vivo (mice)*	NPs reduced MHC II expression on ECs for 4-6 weeks after transplantation in immunodeficient mice; allogenic T cell responses were reduced	153
	VCAM-1 targeted PBAE NPs	anti-miR-712	Inflamed Endothelium	*in vitro* and *in vivo* (mice)	VCAM-1 targeted NPs selectively delivered functional anti-miR to inflamed endothelium	154
	CLIRRTSIC-conjugated PEI-PEG Polyplexes	ICAM-1 siRNA	Pro-atherogenic Endothelium	*in vivo* (mice)	Identified peptides that selectively bind pro-atherogenic ECs; targeted polyplexes delivered siRNA to pro-atherogenic regions of endothelium	87
	PBAE NPs	pBLAST49-hVEGF$_{165}$	Endothelial Cell Transplantation	*in vitro*, transplant *in vivo* (mice)	NPs improved VEGF expression over lipid transfection; NP delivery promoted engraftment, survival and angiogenesis in ischemic tissues	79
	PBAE NPs with PEG-lipid	Luciferase DNA and mRNA	Pulmonary Endothelium	*in vivo* (mice)	Varying monomers produced NPs optimized for either DNA or mRNA delivery; late endosomal localization was greater for DNA-NP than RNA-NP	155

NPs formulated with low molecular weight polyamines and lipids have been found to deliver siRNA to ECs more efficiently than LNPs or polycations with higher molecular weights; after IV delivery of siRNA-NPs in mice, gene silencing was achieved in ECs without significant silencing in hepatocytes or immune cells.[103] Another recent approach uses *in vivo* self-assembly of siRNA vehicles to shift the biodistribution away from the liver.[104] pDNA delivered systemically programmed hepatocytes to synthesize and package siRNAs into secretory exosomes targeting delivery to other tissues in the body.[104]

Tunable properties of lipid- and polymer-based nanocarriers such as material composition, size, shape, charge density, and stealth coatings can help overcome rapid clearance, protect nucleic acids from enzymatic degradation, and improve intracellular delivery.[86,105−110] These properties can improve the half-life of nucleic acid drugs in the bloodstream after systemic administration, which increases transfection of tissues outside the liver. In addition, expression of transgene cargo can be restricted to ECs using transcriptional regulation methods.[111] However, transcriptional regulation alone does not make up for low rates of vehicle uptake in the endothelium and cannot be applied to non-DNA-based gene therapies.[111] It is therefore often desirable to concentrate a gene therapy agent in specific regions of the vasculature. Transfection of specific vascular regions such as areas of stenosis, inflammation, or tumor-associated vascular growth can be achieved by local delivery[112,113] or by cell-specific targeting,[114] as described in the following sections. Examples of viral and nonviral vehicles designed for gene delivery to endothelial targets are summarized in Table 3.

4.1.2. Local Gene Delivery. In some cases, such as surgical interventions, ECs are accessible for local delivery of gene therapy agents directly to the site of injury. Gene therapy treatments for vascular diseases can be applied to vessels locally by embedding nanocarrier-encapsulated nucleic acids in intravascular stents or in hydrogels that can be implanted surrounding a vessel.[112,113] For patients receiving tissue allografts, the transplantation procedure provides a window of direct access to deliver gene therapies to vasculature within the organ graft.[115] Both local and systemic delivery strategies have been further enhanced by the addition of cell-surface targeting molecules on delivery vehicles to promote retention and cellular uptake.

4.1.3. Endothelial Cell-Targeting Delivery Vehicles. In addition to promoting stability and enabling sustained cargo release, nanocarriers can be functionalized to target specific cells or tissues. The heterogeneity of molecular and morphological phenotypes of ECs throughout different vascular beds motivates tissue-targeting approaches to endothelial delivery.[11] EC phenotypes are also dynamic in response to changing physiological or pathological states.[4] These properties can work against targeting efforts if surface markers are shed or internalized in response to disease states or to ligand binding or could be taken advantage of for targeting delivery vehicles to specific areas of the endothelium.[116] Targeting can be based on morphological properties of the target vasculature (for example, fenestrated or sinusoidal endothelia) or can rely on affinity-based interactions such as antibody binding to EC surface antigens.[116−118] In this section, we discuss affinity-based targeting.

In affinity-based targeting approaches, sometimes referred to as "active" targeting, ligands are typically attached to the surface of a delivery vehicle through electrostatic interactions, covalent linkages via click chemistry or thiol coupling, biotin−avidin binding interactions, or nucleic acid hybridization, among others.[119] These interactions and reactions can be used to conjugate viral vectors, LNPs, or polymer NPs to molecules that bind cell surface proteins. Monoclonal antibodies are commonly used for targeting; however, many different kinds of

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ligands can be conjugated to delivery vehicles to achieve similar effects. Arginine−glycine−aspartic acid (RGD)-containing peptides present a motif found on extracellular matrix (ECM) proteins that is responsible for binding to integrins. RGD peptides along with other cell adhesive peptides are widely used for the enhancement of cellular uptake of NPs.[111,120−123]

Proteins that are highly expressed on the EC surface relative to other cell types and abundant on all ECs throughout the vascular system are useful targets for antibody-conjugated delivery vehicles. PECAM-1 (CD31) is commonly used as an EC−surface target due to its abundant expression on ECs in culture and *in vivo*;[124,125] CD31-targeted LNPs have been shown to enhance mRNA delivery to the endothelium by reducing accumulation in the liver.[126] Several studies have performed side-by-side comparisons of two or more EC surface antigens including PECAM-1, ICAM-1 (CD54), ICAM-2 (CD102), VCAM-1 (CD106), P-selectin (CD62P), E-selectin (CD62E), and transferrin receptor-1 (TfR-1; CD71).[114,127−130]

Antibody-based targeting platforms can be used to target organ-specific or disease-specific endothelia (Figure 2). Angiotensin-converting enzyme (ACE) is enriched in alveolar capillaries relative to other vascular beds and can be used to target nanocarriers to the lung.[131] Similarly, inflamed regions of the vasculature have been targeted using antibodies that bind to selectins and other adhesion molecules that are expressed on the surface of cytokine-activated ECs; in most tissues, this is selective for ECs lining postcapillary venules.[116] Gene delivery agents targeting E-selectin have been developed using LNPs and polymer NPs conjugated to antibodies,[132−134] thioaptamers,[135] and the E-selectin binding molecule Sialyl Lewis A.[136] Additional proteins that have been used to target the inflamed endothelium include integrins,[137] cell adhesion molecules VCAM-1 and ICAM-1,[130] transglutaminase,[138] and von Willebrand factor.[139]

Microvascular beds surrounding tumors also develop specific molecular and morphological phenotypes. Angiogenic and inflammatory cytokines in the tumor environment induce the EC surface expression of $\alpha_v\beta_3$ integrin, which can be used to target gene delivery to pathologically angiogenic regions of the endothelium.[156] CD105 (endoglin) has also been identified as a target for selective delivery to breast tumor-associated ECs (BTECs) over normal ECs, possibly related to the role of CD105 in angiogenesis.[157] Cargo encapsulated in EC-targeted vehicles can be delivered to the ECs themselves to normalize their signaling and junctions or to tumor cells via transcytosis through the endothelium or by passing through gaps in the affected endothelial layer.

It is important to note that targeting via receptor−ligand binding does not work via a "homing" mechanism; binding interactions only occur at a close range (<0.5 nm).[118,158,159] A targeted NP that binds with high affinity to a specific EC marker *in vitro* will not necessarily reach and bind to the intended endothelial target *in vivo*.

4.1.4. Endothelial Intracellular Delivery. The binding of ligand-decorated delivery vehicles to EC surface molecules can affect cellular uptake and intracellular trafficking, sometimes in unpredictable ways. For example, monomolecular PECAM-1 ligands are poorly internalized, making them primarily useful for delivery to the luminal surface of the endothelium. Conversely, multivalent PECAM-1 ligands are more favorable for intracellular delivery due to their internalization by ECs via a noncanonical endocytic pathway.[160−164] Even greater enhancement of binding-induced internalization can be achieved using ICAM-1-targeted delivery vehicles.[165,166] Delivery vehicles have also been designed to target caveolae and fenestrae; vehicles targeting plasmalemmal vesicle associated protein (PV1) are internalized via caveolar endocytosis.[167]

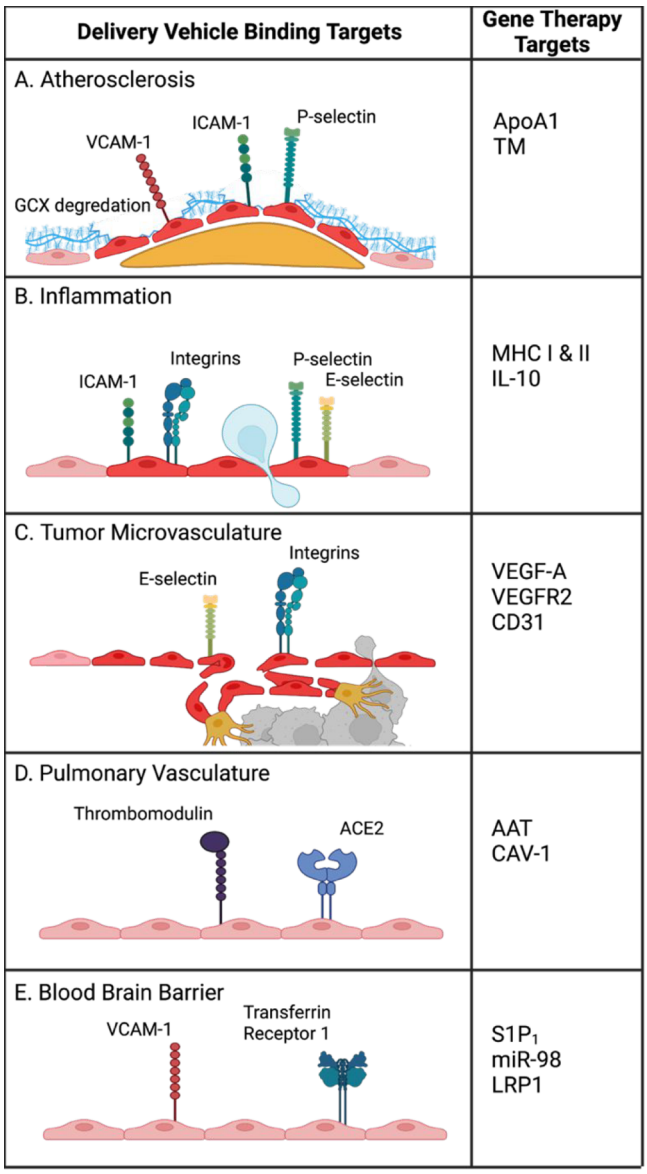

Figure 2. Pathological states of the endothelium and cell surface targets.

Internalization of targeted delivery vehicles can also be affected by biomechanical factors. Rigid delivery vehicles targeting PV1 or ICAM-1 had a smaller size limit for intracellular uptake compared to more flexible targeted vehicles.[168,169] Hydrodynamic forces and flow conditions have been found to modulate the internalization of PECAM-1 and ICAM-1 targeted vehicles.[170−172] Carrier geometry can also be used to modulate the internalization of delivery vehicles.[173−177] As described in Section 3.2, intracellular trafficking of internalized delivery vehicles and endosomal escape of nucleic acid cargos is essential for transgene expression. Epitope-specific targeting, valency, carrier shape,

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and biomechanical factors impact intracellular trafficking in addition to internalization.[178−180]

4.1.5. Gene Editing in ECs. Gene delivery vehicles, both viral and nonviral and targeted and untargeted, have been used to deliver everything from siRNA or miRNA molecules to pDNA and ECs, examples of which are described below. The past decade of gene therapies has been defined by the discovery of the use of CRISPR/Cas9 as a gene editing system. This has brought on rapid advancements in gene editing capabilities across a wide range of human and nonhuman tissues and cells. Packaging the multiple components of the CRISPR/Cas9 system for intracellular delivery has been challenging in some cell types, including primary human ECs that are difficult to transfect, have a short replicative lifespan, and cannot readily be clonally expanded. Despite challenges, gene editing of primary human ECs is a valuable tool for *in vitro* studies, as immortalized cell lines and nonhuman ECs each lack important characteristics that are integral to the physiological functions of the human endothelium. New methods, including the use of ECs derived from endothelial colony forming cells (ECFCs) from umbilical cord blood and the use of a dual adenovirus and lentivirus delivery platform, have resulted in gene editing in primary human ECs.[140,181]

CRISPR/Cas9 gene editing of ECs *in vivo* in animal models has recently been used to investigate genes that regulate BBB integrity in brain ECs[141] to suppress expression of a mutated gene associated with corneal endothelium degeneration[142] and to suppress angiogenesis in a mouse model of proliferative retinopathies.[143,182] While there have been some successes, gene editing of ECs *in vivo* has been hindered by the preferential accumulation of systemically delivered vectors in the liver, as described above, where rates of gene editing are higher than in other tissues.[109] Viral vectors are commonly used to transduce cells with Cas9 and sgRNAs *in vivo*; however, modified viral vectors, virus−lipid or virus−polymer hybrid vehicles, or entirely lipid- or polymer-based NPs have been developed with the aim of retargeting the distribution of vehicles toward the endothelium. AAVs encoding sgRNAs can be coated with a cationic polymer such as dendrimeric PAMAM, which can act as a linker to attach EC-binding peptides to the viral vector.[144] A cationic polymer coating alone improves vector stability and transfection of ECs, and the addition of targeting molecules by conjugation to the polymer coating offers further enhancement of gene transfer to ECs relative to other tissues.[144,145,183]

Polymer-based vehicles for CRISPR/Cas9 gene delivery have also been described. In one recent study, NPs were formulated using a poly(ethylene glycol)-*block*-poly(lactide-*co*-glycolide) (PEG-*b*-PLGA) copolymer core conjugated to a high molecular weight PEI (25 kDa) coating, which was added following the formulation of the PEG-*b*-PLGA NPs. The cationic outer polymer layer enabled complexation with pDNA encoding both Cas9, under control of an EC-specific CDH5 (cadherin 5 or vascular endothelial cadherin) promoter, and sgRNA on a single plasmid.[109] PEG was used to prevent accumulation in the liver, and an increase in the size of the PEG group (from 600 to 5000 Da) improved biodistribution in vascular ECs including in the lung, heart, aorta, and peripheral vessels. Nonviral delivery systems for CRISPR/Cas9 gene editing offer several advantages, including a less restrictive limitation in cargo size as well as a reduced risk of off-target effects caused by long-term expression of Cas9. As gene editing techniques in ECs continue to develop, many EC genes and pathways are being identified as potential therapeutic targets for gene therapies involving gene editing as well as RNAi, mRNA, or pDNA. Applications of systemic, local, and targeted delivery of nucleic acids to the vascular endothelium for a variety of gene therapy targets in ECs are discussed below.

4.2. Models for the Human Vascular Endothelium. Given the variation in vascular EC phenotypes across different organs and under different pathological conditions, in preclinical research, it is important to use models that accurately reflect the physiology of ECs and of entire vascular structures, including the basement membrane, SMCs, and pericytes. The human endothelium can be studied *in vitro* using cell lines or primary ECs isolated from human tissues, *in vivo* by implantation of human vascular grafts in animal models, or *ex vivo* using discarded human tissues.

4.2.1. In Vitro Culture of ECs. Primary human ECs, as opposed to transformed cell lines and ECs derived from other animals, are widely used in *in vitro* studies. The human endothelium differs from that of smaller mammals including rodents in important ways, in particular in the presentation of antigens to circulating T cells.[181,184] Primary ECs have a limited replicative lifespan but reflect endothelium physiology more closely than transformed cells, although they may not maintain all physiological properties once cultured *in vitro*. ECs from different organs present unique phenotypes and protein expression profiles, making the tissue source of primary human ECs an important consideration for *in vitro* studies.[185] Human umbilical vein ECs (HUVECs) are the most commonly used source of primary human ECs. Human arterial ECs can be isolated from the aorta or umbilical, pulmonary, or coronary arteries; however, with the exception of the umbilical artery, these tissues are less commonly available for research use.[186] Microvascular ECs can be obtained from skin, lung, heart, or uterine tissues. ECs can also be derived from endothelial progenitor cells (EPCs), which can be isolated from adult peripheral or umbilical cord blood, or derived from pluripotent stem cells (PSCs).[187−189] Characteristics of ECs isolated or derived from different tissue sources have been reviewed elsewhere.[188−191]

In *in vitro* studies, it is important to evaluate gene delivery vehicles in a setting that mimics the vascular environment where possible.[159] This is particularly important when developing targeted delivery vehicles; binding in static culture does not necessarily predict effective binding and transfection *in vivo*. Many studies employ microfluidic chambers in which ECs, and in some cases SMCs or pericytes, line the luminal walls of channels that mimic the flow conditions of the vasculature.[89,185] Microfluidic devices allow for precise control over parameters such as flow rate and vessel diameter, and many conditions can be tested simultaneously. The *in vivo* vascular environment can also be approximated *in vitro* using transwell assays, organ culture, or organoids. Transwell assays can be used to examine the effects of gene delivery to ECs on transendothelial migration, while organ culture and organoids can be used to evaluate the delivery to ECs in 3-dimensional structures with other cell types such as pericytes or SMCs.

4.2.2. In Vivo Animal Models. *In vitro* systems have the advantage of the ability to control and isolate certain parameters; however, the impact of the *in vivo* vascular environment on EC phenotypes may limit the correlation between nanocarrier efficacy *in vitro* versus *in vivo*. Animal models are nonetheless useful for the assessment of gene delivery to ECs for a wide range of applications, as discussed in

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Section 5 below, but some endothelium-associated pathologies cannot be modeled using only animal tissues. Inflammation and the immunomodulatory roles of human ECs *in vivo* can be studied using models in which explanted human vessel segments or vascular grafts assembled from human cells are implanted into immunodeficient mice.[153]

4.2.3. Ex Vivo Human Vasculature. As a complementary approach to *in vivo* models, human vasculature can be studied *ex vivo* using freshly procured human tissues that will otherwise be discarded (e.g., umbilical cord following birth by cesarean delivery) or donated tissues that have been declined for transplantation. Human umbilical arteries, which are dissected from tissues that are relatively accessible to preclinical researchers, have been used to develop an *ex vivo* isolated vessel perfusion system (IVPS).[129] For the evaluation of delivery vehicles, *ex vivo* vessel perfusion has many of the advantages of cultured EC-lined microfluidic devices, such as being able to rapidly screen vehicle formulations, while maintaining a binding environment that is truer to physiological conditions. However, explanted vessel segments are viable for only a matter of hours, so transplantation into an animal is necessary for longer term analysis of changes in gene expression. Machine perfusion of transplant-declined human organs allows for *ex vivo* evaluation of delivery vehicles in a more complex whole-organ context, including the presence of microvasculature, which is not captured using isolated arteries.[192]

5. NUCLEIC ACID THERAPIES FOR ENDOTHELIAL PATHOLOGIES

5.1. Vascular Disease. ECs have an active role in vascular diseases such as atherosclerosis, thrombosis, and ischemia-reperfusion injury (IRI). A healthy endothelium regulates blood flow and fluidity by expression of thrombomodulin (TM), plasminogen activators (t-PA, u-PA), endothelial nitric oxide synthase (eNOS), endothelins, and other molecules that are involved in coagulation and blood fluidity.[9] A buildup of plaque (atherosclerosis) or blood clotting (thrombosis) can cause obstructions in the vasculature, leading to ischemia. Coronary thrombosis can be initiated by the rupturing or erosion of atherosclerotic plaques, which disrupts the endothelial layer leading to clot formation. As atherosclerotic plaques develop, they cause a disturbance in blood flow that can alter ECs. These changes in ECs have been pursued as targets for the design of gene delivery mechanisms that specifically treat atherosclerotic regions of the vasculature.

The disturbance in blood flow associated with atherosclerosis can damage the endothelial glycocalyx (GCX), which in a healthy endothelium provides a barrier with a small pore size that allows passage of water, serum proteins, and small solutes. Small PEG-coated gold NPs (10.5 nm) have been observed to specifically accumulate in regions of the endothelium with GCX damage by passive targeting.[193] Disturbed blood flow has also been used to develop an affinity-based targeting platform for nucleic acid delivery to pro-atherogenic ECs. Chung et al. identified novel peptides that selectively bind to ECs exposed to disturbed blood flow and then conjugated these peptides to the hydrophilic PEG group of a bioreducable branched PEI−PEG polymer.[87] Through complexation of siRNA with the cationic PEI, polyplex vehicles were formed in which the PEG-peptide group was oriented outward. Peptide-conjugated polyplexes increased siRNA-mediated knockdown of ICAM-1 in ECs in pro-atherogenic regions of the endothelium, while no knockdown was observed in ECs in healthy regions.[87] Recent studies have demonstrated targeted delivery of shRNA and anti-miRNA to atherogenic ECs using PEGylated lipoplexes conjugated to a P-selectin-binding peptide[114] and PBAE NPs conjugated to a VCAM-1-binding peptide.[154]

Surgical interventions that are currently in clinical use for treatment of vascular disease present an opportunity to deliver therapeutic agents locally by incorporating the drug into an implanted material. Endovascular stenting, in which a scaffold is placed in the vessel lumen to restore blood flow, is a commonly used and minimally invasive surgical intervention. In a recent study aiming to prevent in-stent restenosis (ISR) in pigs, gene delivery stents were generated by reversible immobilization of AAV vectors to the stent surface.[112] Vectors encoding a reporter gene successfully transduced ECs in stented arteries *in vivo*; however, in-stent delivery of vectors encoding apolipoprotein A1 (apoA1), a component of high-density lipoprotein (HDL) that can prevent restenosis, did not reduce ISR in an atherosclerotic model *in vivo*.[112] Local delivery of AAV vectors to vasculature has also been pursued by suspending the vector in a biocompatible alginate hydrogel, which can be implanted around segments of the aorta. While systemically delivered AAV vectors only transduced cells in the liver, implantation of an AAV9-containing hydrogel resulted in transduction of ECs and SMCs in the adjacent section of aorta with no transduction in other organs.[113]

5.2. Peri-Transplant Injury. The endothelium plays a critical role in inflammatory responses.[194] Inflammation can be triggered in response to T cells interacting with the presentation of an antigenic peptide on the surface of an antigen-presenting cell (APC), such as a macrophage, dendritic cell, or B cell; activation is dependent on a direct interaction between the APC and the T cell receptor.[195] Nonprofessional APCs, including ECs, can also present antigens.[194−197] Being fixed in place, EC interactions with leukocytes are critical for the recruitment of circulating T cells to the site of inflammation.[198]

Alterations in the surface of the endothelium plays a key role in immunosurveillance by circulating leukocytes and presents a challenge in the context of solid organ transplantation. The endothelium of an allograft is the initial point of contact and exchange with the host circulation. Antigen presentation by ECs within an allograft can lead to inflammation and acute graft rejection.[194] Transplant recipients often receive immunosuppressants; however, these treatments weaken the immune system and leave graft recipients vulnerable to infections. As an alternative strategy to immunosuppression, gene therapies targeting graft ECs can be used to reduce the alloantigen presentation or expression of leukocyte-activating molecules.[153,197,199] An advantage of this approach is that gene therapy agents can be delivered directly to an allograft endothelium *ex vivo* prior to implantation, which prevents gene transfer in undesired tissues outside of the graft and improves retention of therapeutic doses in the graft.[200]

Lentiviral vectors have been used to deliver shRNA targeting major histocompatibility complex (MHC) molecules to graft ECs by normothermic machine perfusion (NMP) in which an organ is placed on a pump and perfused with an oxygenated red cell-based solution at 32−36 °C.[201,202] Knockdown of MHC molecules has prevented graft rejection in animal models by enabling ECs to evade detection by circulating host T cells.[203,204] Similar protection of graft ECs by MHC silencing has been observed in engineered vascular grafts

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generated from human ECs.[134] Graft EC immunogenicity has also been suppressed by delivering adenovirus encoding the anti-inflammatory cytokine IL-10 during NMP in a porcine lung transplant model.[205] Smaller nucleic acids such as ASOs have been delivered as free molecules (without a delivery vehicle) during NMP, and normothermic conditions were necessary for transfection of graft ECs with unencapsulated ASOs.[115,206] While gene delivery is restricted to the graft, NMP approaches do not always isolate delivery specifically to the endothelium. Delivery of an adenoviral firefly luciferase vector during cardiac *ex vivo* NMP resulted in transduction of myocardial cells in addition to endocardial cells and all layers of the coronary arteries.[207] On the other hand, nanoparticles (~100 nm) are confined to the intravascular space during NMP,[192] which potentially allows them to avoid delivery to parenchymal cells.

5.3. Pro- and Anti-angiogenic Therapies. *5.3.1. Neovascularization and Vascular Regeneration.* Along with delivering organ-preserving treatments via graft vasculature *ex vivo*, tissue engineering offers a parallel approach to increasing the pool of transplantable organs and tissue grafts. Engineering vascularized tissue grafts is one of several applications for pro-angiogenic therapies, which typically target ECs or mural cells to promote vascular growth.[79,122,208,209] Polymer NPs have been used to deliver pDNA encoding vascular endothelial growth factor (VEGF)[79] and proliferation inducing miRNAs[122] to ECs *in vitro* prior to incorporation into an implanted vascular graft.

Pro-angiogenic therapies can also be applied to treat age-related endothelial degeneration and dysfunction, which can affect specific vascular beds as well as the vascular system as a whole. Studies in delivering pro-angiogenic gene therapies have commonly used AAV-1 and adenoviral vectors. A recent study employed local delivery to promote tissue-specific vascular regeneration for the prevention of hearing loss in mice.[210] AAV-1 encoding VEGF was delivered locally in the ear canal and transduced cochlear pericytes, which were found to drive new vascular growth.[210]

Age-associated endothelial dysfunction is hypothesized to be a driver of systemic aging due to the integral role of the vascular system in organ homeostasis. In mice, systemic delivery of AAV encoding VEGF produced circulating levels of VEGF protein sufficient to reduce age-related capillary loss, which protected against reduced tissue oxygenation and delayed physiological and behavioral signs of aging.[146] Aging is a primary risk factor for cardiovascular diseases, which may be caused by a decline in the homeostatic functions of ECs. Age-associated decline in vascular endothelium functioning has been studied using a mouse model of Hutchinson-Gilford progeria syndrome (HGPS) in which a truncating mutation in the Lamin A gene causes premature aging.[147] Mice producing truncated Lamin A in vascular ECs were found to have suppressed expression of NAD-dependent deacylase sirtuin 7 (Sirt7) in ECs. Knock-out of Sirt7 was also associated with defective neovascularization following ischemia. AAV1-mediated Sirt7 rescue gene therapy directed at vascular ECs using an ICAM2 promoter extended the lifespan of HGPS-model mice and increased blood vessel growth.[147]

5.3.2. Vascular Normalization. In contrast to the pro-angiogenic gene therapies described above, in pathological conditions in which angiogenic signaling is elevated, such as the tumor microenvironment, antiangiogenic or vasculature-normalizing therapies have shown promise in animal models and in the clinic.[148,211−213] To this end, suppression of vascular VEGF-A or its receptor, VEGFR2, has long been an aim of therapeutic strategies targeting tumor microvasculature.[213−217] Mounting evidence supports the approach of normalizing vasculature near a tumor over blocking its growth, and therapeutic targets that allow for more finely tuned control of EC functioning are being pursued.

Adenoviral vectors and AAVs are commonly used for gene delivery to tumor-associated ECs in preclinical studies.[145,148,149] Viral vectors have been modified with polymers and lipids to reduce inflammatory properties and to shift biodistribution and gene expression to tumor ECs. Covalently coating an adenoviral vector with an amino-reactive hydrophilic polymer, based on poly[*N*-(2-hydroxypropyl) methacrylamide], successfully masked the virus to prevent receptor-mediated infection, antibody recognition of the vector, and liver capture, which improved circulation time and enabled vascular targeting.[145] Functionalization with cell surface binding molecules adds an additional method for the improvement of delivery to the tumor endothelium. As described above, tumor-associated ECs express elevated levels of cell surface proteins involved in both proliferation and inflammation relative to a quiescent endothelium. Integrins are highly expressed in proliferative tumor-associated ECs, which can be specifically targeted with a cyclic RGD peptide, RGD4C.[148,149] Monoclonal antibodies against E-selectin, which is induced under inflammatory conditions and has been found on the tumor endothelium in some forms of cancer, and TfR-1, which has been used to target tumor vasculature in the brain, have also been conjugated to viral and nonviral vehicles.[145,218]

Suppression of angiogenic signaling has also been used to address ocular pathologies including proliferative retinopathies and wet age-related macular degeneration. These are currently treated with VEGF-neutralizing antibodies; however, a gene therapy approach could reduce the number and frequency of treatments needed. Recent work has demonstrated that the depletion of VEGFR2 by CRISPR/Cas9 gene editing in retinal ECs led to a decrease in neovascularization in mouse models of retinopathies.[143] AAV1-Cas9 and AAV1-sgRNA were delivered locally to retinal ECs by intravitreal injection; ECs of pathological neovasculature were preferentially transduced, which may be attributable to underdeveloped basement membrane and intracellular junctions in newly forming vessels.[143]

5.4. Pulmonary Endothelium. Like retinal and tumor microvasculature, vascular beds in the lung are a target for gene therapies aimed at treating organ-specific vascular ECs.[150,219,220] Several properties of the pulmonary endothelium are favorable for gene delivery; 25−30% of the total endothelial surface area is in the lung, and all venous blood flow passes through pulmonary vasculature.[116,131] On the other hand, it is now known that the pulmonary vasculature is heterogeneous with cell populations expressing different surface proteins.[221−223] This discovery suggests an opportunity to target specific EC subsets but also a potential challenge to target the entire endothelial surface. In the ongoing COVID-19 pandemic, one indicator of severe illness due to SARS-CoV-2 infection has been damage to the pulmonary endothelium. Lung ECs can amplify inflammation through expression of leukocyte adhesion molecules.[224] Therapeutics that act on the lung endothelium may help treat or prevent severe cases of COVID-19.[225−232]

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Gene therapies targeting the pulmonary endothelium have been identified for the treatment of genetic disorders such as alpha-1 antitrypsin deficiency (AATD) and pulmonary arterial hypertension (PAH).[111,233−235] AATD is caused by a mutation in the AAT gene that encodes a protease inhibitor that protects against inflammatory damage. While endogenous AAT is produced in the liver, transduction of pulmonary ECs with the AAT gene may offer a more direct treatment for AATD-associated illnesses that affect the lung such as emphysema.[111,234] PAH can be caused by a number of factors including loss-of-function mutations in caveolin-1 (CAV1), which acts as a scaffolding protein in caveolae membranes and regulates signaling complexes relevant to PAH including eNOS activity. Deficiency in CAV1 is associated with both heritable and idiopathic PAH and is a potential target for gene therapies.[233] Gene therapies for tumors in the lung are also being investigated. In several studies, systemic delivery of LNP-encapsulated siRNA against CD31 to the pulmonary endothelium has prevented metastasis and increased survival in murine lung cancer models.[151,152]

The polymer or lipid composition of nonviral vehicles is important for effective transfection of the lung endothelium. In both IV and intratracheal administration routes, the addition of PEG to lipid or polymer vehicles has been found to improve transfection. However, intratracheal administration appears to primarily transfect cells in the lung parenchyma relative to ECs.[155,236] As observed in most vascular beds, differences in therapeutic nucleic acid size as well as the mechanism of genetic modulation between different types of gene therapies necessitates cargo-specific vehicle formulations for delivery to pulmonary ECs. Modification of adenoviral vectors with myeloid-binding peptides (MBP) has been shown to shift gene transfer from the liver to ECs throughout different organ systems with the greatest enhancement in transduction observed in pulmonary ECs, pointing to MBP-targeted delivery vehicles as a potential platform for the delivery of gene therapies to the lung endothelium.[111]

5.5. Endothelium of the Blood−Brain Barrier. In the brain microvasculature, the endothelium is a component of the BBB in which ECs tightly regulate the transport of molecules between the bloodstream and the brain parenchyma. Gene therapies aimed at the brain endothelium may be employed for the treatment of the endothelium itself or as a way of creating an entry point for drug delivery to the brain.[237] Gene therapy targets in BBB ECs have been identified, including $S1P_1$ or miR-98 as vascular protectants against ischemic injury[238,239] or LRP1 for maintaining EC tight junction integrity in the BBB.[240] Many recent studies of gene delivery to the brain endothelium use EC-targeted delivery vehicles; the choice of EC antigen for targeting depends on the aim of the treatment. Acute inflammation in the brain has been treated by delivering TM mRNA encapsulated in LNPs conjugated to anti-VCAM-1 antibodies to brain ECs in a mouse model.[130] For the transfection of inflammatory ECs with mRNA, VCAM-1-targeted LNPs were more effective than TfR-1 or ICAM-1-targeted LNPs.[130] Conversely, TfR-1 is frequently targeted to promote transcytosis across the BBB endothelium for the transfection of cells in the brain parenchyma.[218,241] TfR-targeted liposome NPs composed of a distearoylphosphatidylcholine (DSPC)/cholesterol bilayer and a stabilizing PEG coating accumulated most densely in capillaries; however, transcytosis of NPs from the endothelium to the brain parenchyma primarily occurred in the postcapillary venules where there is more perivascular space. Extravascular space for the movement of NPs appears to be more favorable for transcytosis, although the lack of accumulation or transcytosis of TfR-targeted NPs in the similarly sized perivascular space around arterioles suggests a model in which NP accumulation in the small diameter capillaries is important for NP transcytosis or transfection of ECs.[241] There appears to be a limit to the extent that targeting the endothelium for transcytosis can shift the balance of vehicle biodistribution to the peripheral tissues and the brain: generally, less than 1% of the injected dose goes to the brain.[242]

6. CONCLUSIONS AND FUTURE OUTLOOK

The major barrier to achieving efficient nucleic acid therapeutics in ECs is the delivery of the agents to the target cells. A chief obstacle is the rapid clearance of free nucleic acids or nucleic acids in carrier vehicles from the circulation by mononuclear phagocytic cells in the liver and spleen. A second obstacle is the challenge of achieving consistent dosing to ECs without stimulating the immune system. Several promising strategies for overcoming these two obstacles are under investigation. A third obstacle is the scale-up of these strategies to the appropriate scale for distribution as pharmaceuticals, which appears to be surmountable, given the recent successes of LNP-based vaccines. In addressing all of these obstacles, sophisticated *in vitro*, *in vivo*, and *ex vivo* models can help to elucidate barriers posed by the physiological environment. The availability of this broad experience should help facilitate the development of nucleic acid therapeutics that are directed at ECs.

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Notes

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■ ABBREVIATIONS

AATD, alpha-1 antitrypsin deficiency; AAV, adeno-associated virus; ACE, angiotensin-converting enzyme; APC, antigen-presenting cell; apoA1, apolipoprotein A1; ASO, antisense oligonucleotide; BBB, blood−brain barrier; BTEC, breast tumor-associated endothelial cell; Cas, CRISPR-associated protein; CAV-1, caveolin-1; CD105, endoglin; CD31, PECAM-1; CRISPR, clustered regularly interspaced short palindromic repeats; DEAE, diethylaminomethyl; DMRIE, dimyristyloxypropyl-3-dimethyl-hydroxyethylammonium; DOGS, 1,2-dioleoyl-*sn*-glycero-3-[(*N*-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl]; DOSPA, 2-[3-[4-(3-aminopropylamino)butylamino]propylcarbamoylamino]-ethyl-[2,3-bis[[(*Z*)-octadec-9-enoyl]oxy]propyl]-dimethylazanium; DOTAP, 1,2-dioleoyl-3-trimethylammonium-propane; DOTMA, 1,2-di-*O*-octadecenyl-3-trimethylammonium propane; DSPC, distearoylphosphatidylcholine; dsRNA, double stranded RNA; EC, endothelial cell; ECFC, endothelial colony forming cell; ECM, extracellular matrix; eNOS, endothelial nitric oxide synthase; EPC, endothelial progenitor cell; GalNAc, *N*-acetylgalactosamine; GBM, glomerular basement membrane; GCX, glycocalyx; HDL, high-density lipoprotein; HGPS, Hutchinson-Gilford progeria syndrome; HUVEC, human umbilical vein endothelial cell; ICAM-1, intracellular adhesion molecule 1; IRI, ischemia-reperfusion injury; ISR, in-stent restenosis; IVPS, isolated vessel perfusion system; LNA, locked nucleic acid; lncRNA, long noncoding RNA; LNP, lipid nanoparticle; MBP, myeloid-binding peptides; MHC, major histocompatibility complex; miRNA, microRNA; mRNA, messenger RNA; NMP, normothermic machine perfusion; NP, nanoparticle; ORF, open reading frame; PACE, poly(amine-*co*-ester); PAH, pulmonary arterial hypertension; PAMAM, polyamidoamine; PBAE, poly(beta-amino ester); PDMAEMA, poly(2-*N*-(dimethylaminoethyl) methacrylate); PEG, polyethylene glycol; PEG-*b*-PLGA, poly(ethylene glycol)-*block*-poly(lactide-*co*-glycolide); pegRNA, prime editing guide RNA; PEI, polyethylenimine; PNA, peptide nucleic acid; PS, phosphorothioate; PSC, pluripotent stem cell; PV1, plasmalemmal vesicle associated protein; RGD, arginine−glycine−aspartic acid; RISC, RNA-induced silencing complex; RNAi, RNA interference; sgRNA, single guide RNA; shRNA, short hairpin RNA; siRNA, small interfering RNA; Sirt7, NAD-dependent deacylase sirtuin 7; SMC, smooth muscle cell; TALEN, TALE nuclease; TfR-1, transferrin receptor-1; TM, thrombomodulin; UTR, untranslated region; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor; ZFN, zinc finger nuclease

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