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Gene delivery nanoparticles to modulate angiogenesis

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Abstract

Angiogenesis is naturally balanced by many pro- and anti-angiogenic factors while an imbalance of these factors leads to aberrant angiogenesis, which is closely associated with many diseases. Gene therapy has become a promising strategy for the treatment of such a disordered state through the introduction of exogenous nucleic acids that express or silence the target agents, thereby engineering neovascularization in both directions. Numerous non-viral gene delivery nanoparticles have been investigated towards this goal, but their clinical translation has been hampered by issues associated with safety, delivery efficiency, and therapeutic effect. This review summarizes key factors targeted for therapeutic angiogenesis and anti-angiogenesis gene therapy, non-viral nanoparticle-mediated approaches to gene delivery, and recent gene therapy applications in pre-clinical and clinical trials for ischemia, tissue regeneration, cancer, and wet age-related macular degeneration. Enhanced nanoparticle design strategies are also proposed to further improve the efficacy of gene delivery nanoparticles to modulate angiogenesis.

Graphical Abstract

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Chemical compounds

Poly(ethylene imine); Poly(L-lysine); Poly(beta-amino ester); Poly(lactic-co-glycolic) acid; Poly(vinyl pyrrolidone); -cyclodextrin; chitosan; cholesterol; N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate

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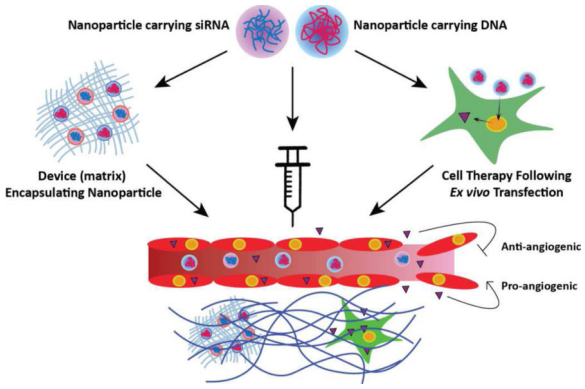

Keywords

non-viral; gene therapy; polymeric nanoparticles; ischemia; wound healing; tissue engineering; tumor; age-related macular degeneration

1. Introduction

The growth and development of higher order animals requires the establishment of adequate vasculature for nutrient and oxygen transport, waste removal, cell migration, and signal transduction. The formation and maturation of these vessels are mediated by several processes that can be classified broadly into three categories: vasculogenesis, or the *de novo* synthesis of vessels from angioblasts; angiogenesis, the formation of new blood vessels from pre-existing ones; and arteriogenesis, the maturation of existing collateral vessels to compensate for insufficiencies in primary vessels. Given the greater abundance of literature on the topic, however, most of this review will focus on the process of angiogenesis. Angiogenesis can be sub-divided into sprouting or intussusceptive mechanisms. Most of the available studies of angiogenic factors have focused on sprouting, in which select endothelial cells within a vessel, tips cells, migrate and are followed by adjacent stalk cells to form new branches, or sprouts, that eventually grow and anastomose to mature vessels. In contrast, intussusception occurs when transluminal tissue pillars develop, forming tiny holes that eventually fuse and split the vessel in two. Interestingly, several factors appear to be common to the two processes, suggesting that both should be considered during investigations of angiogenic therapeutics [1, 2].

Regulation in angiogenesis is maintained by a balance of pro- and anti-angiogenic factors. In healthy adults, this balance allows for the maintenance of the vasculature in a quiescent state under normal conditions, but poised for quick dynamic changes in response to perturbations in signaling in the microenvironment caused by situations such as wound healing, growth-related hypoxia, and inflammation. In addition, prolonged or chronic pathological conditions can shift angiogenesis equilibrium as demonstrated by the misappropriation of pro-angiogenic signals in cancer and neovascular age-related macular degeneration. Alternatively, dysfunction of existing vessels, such as atherosclerosis-induced ischemia or thrombotic complications, can lead to a sub-adequate blood supply and a demand for compensatory neovascularization.

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The molecular mechanisms of angiogenesis are quite complicated and have been reviewed in detail elsewhere [3, 4] and will be summarized only briefly here followed by more in depth discussions of specific growth factors as they apply to specific gene therapeutics. Under normal circumstances, blood vessels are maintained in a quiescent state characterized by low endothelial cell (EC) proliferation, substantial mural-cell coverage (ie. pericytes and vascular smooth-muscle cells wrap around endothelial cells and stabilize them), and well-developed vascular basement membrane. The direct contact between mural cells and endothelial cells helps to maintain this state through the secretion factors involved in vascular homeostasis, notably angiopoietin-1 (Ang1) and low levels of vascular endothelial growth factor (VEGF). Both of these factors are secreted by mural cells, which maintain vessel stabilization through paracrine and autocrine activation of their corresponding receptors on the surfaces of EC and mural cells respectively.

Following hypoxic insult, inflammatory signaling, wounding, or certain pathological conditions, however, these signals shift from maintenance to the induction of angiogenesis. Pro-angiogenic factors, such as VEGF and fibroblast growth factor (FGF) are released from the surrounding tissues and induce the expression and secretion of matrix metalloproteinases (MMPs) and angiopoietin-2 (Ang2). The MMPs begin to break down the vascular basement membrane surrounding the vessels while Ang2 inhibits the activities of Ang1, leading to a reduction in EC-EC contacts and stimulation of mural cell detachment [5, 6]. The endothelial cells then begin a directional migration to the source of the growth factor gradients. In this process, one cell takes on the lead role as the tip cell in the migration and signals to adjacent ECs via delta-Notch to follow as stalk cells to proliferate and form the body of the growing vessel. Throughout this migration adjacent cells can overtake and replace the tip cell [7]. Upon meeting another vessel or sprout, the vessels are able to merge via anastomosis and form a continuous lumen to establish blood flow. Additional growth factors, such as platelet-derived growth factor (PDGF), are then released from endothelial cells to stimulate the proliferation and recruitment of new mural cells and to re-establish a quiescent state [8].

This review focuses on the advancement and applications of non-viral nanoparticles for gene therapy to modulate angiogenesis to treat diseases involving aberrant vasculature (Figure 1, Table 1).

2. Nanoparticle technology for gene therapy

Many therapeutics, such as small molecules, proteins, peptides, and nucleic acids, are identified and tested against molecular targets in diseased cells. However, frequently due to their short half-life without modification, they are degraded and/or cleared from the systemic circulation and the body quickly, often before an effective dose can accumulate at the target site. Poor pharmacokinetics requires repeated administration and, sometime due to a narrow therapeutic window, significant detrimental side effects. Some direct modifications to drugs, including binding of albumin and conjugation of poly(ethylene glycol) (PEG) or other macromolecules to the drug molecule, have shown improvement and have been approved by the FDA [9]. The use of particles to carry drugs has also increased bioavailability of the

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therapeutics by mitigating enzymatic degradation, evading clearance by reticuloendothelial system (RES), and promoting controlled release [10].

Drugs classified as small molecules, antibodies, and peptides can treat diseases by directly interacting with or blocking key molecular signaling molecules and targets to induce therapeutic effect. For example, aflibercept is a recombinant fusion protein that directly traps vascular endothelial growth factor (VEGF) to inhibit angiogenesis [11]. Gene therapy, which introduces nucleic acids as the drug, such as DNA, siRNA or miRNA, has particular advantages compared to other drug therapies. Insertion of a therapeutic gene or deletion of a malfunctioning gene goes beyond tackling the symptoms of a disease and can potentially cure diseases of genetic origin, including cancer [12, 13]. Also, the use of a cell's own machinery to continuously produce active biomolecules encoded from exogenously delivered DNA can obviate the need for frequent drug dosing and hit targets that are otherwise "undruggable." However, the challenge with a gene therapy approach is that the nucleic acid cargo has to successfully transduce or transfect the host cells through intracellular barriers in addition to crossing extracellular barriers to reach the target cells, requiring sophisticated engineering of the delivery vehicle.

Gene therapy has seen much growth in two parallel tracks: viral and non-viral. The viral gene delivery method takes advantage of the evolutional ability of viruses to transfer genetic material to the cell. Different types of viruses, such as adeno-associated virus, have been employed and modified to deliver nucleic acids with high and prolonged efficacy [14]. The major hurdles to clinical translation of viral vectors have been limited cargo size, large-scale production, and safety. Because many viruses can integrate exogenous genetic material into a host's genome, there is a significant concern over insertional mutagenesis and oncogenesis [15]. Similarly, there are safety issues associated with potential immunogenicity and toxicity of certain viruses. In non-viral gene therapy, both synthetic and natural polymers and lipids are utilized to form nanoparticles. While these vectors have advantages of being designed non-toxic, mass producible, and flexible to modification, they generally have poor transfection efficiency. The field of viral gene therapy - including approaches for *ex vivo* transduction of cells for cellular therapy - has advanced to reduce safety risks and there have been more than 1,500 worldwide viral gene therapy clinical trials [16, 17]. The non-viral community has also advanced gene therapeutics to the clinic, with more than 500 worldwide clinical trials including lipid, polymer, and naked DNA approaches [17]. Despite all of this clinical activity, the FDA has only approved a single therapy thus far as a marketed product for use in the United States, Amgen's IMLYGIC® (Talimogene Laherparepvec), which uses a modified herpes simplex virus type 1 vector as an oncolytic and to produce granulocyte-macrophage colony-stimulating factor with the goal of producing an anti-tumor immune response [18]. The two non-FDA approved gene therapy products that have been approved by other agencies wordwide are Gendicine, a recombinant adenovirus encoding wildtype-p53 for the treatment of head and neck squamous cell carcinoma and was approved by the China Food and Drug Administration [19], and alipogene tiparvovec (Glybera®), approved by the European Medicines Agency, an adeno-associated virus encoding lipoprotein lipase for treatment of the rare inherited disorder familial lipoprotein lipase deficiency [20]. Thus there is a critical need to develop new vectors or re-engineer existing vectors to have enhanced efficacy and safety and to treat a wide array of human diseases.

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Non-viral nanoparticles face several barriers in the process of gene delivery [21]. First, a vector must form a stable complex with the genetic cargo. In the case of polymeric vectors, this is most often achieved through electrostatic interaction between cationic polymer and negatively charged nucleic acids. There are two interchangeable ways to describe this type of nanoparticles in relation to the amount of polymer and genetic material used. N/P is the ratio between the number of protonatable amines in the polymer chain and the number of phosphates in the nucleic acid backbone, while w/w is the mass ratio of the polymer to the genetic cargo. Poly(ethylene imine) (PEI) and poly(L-lysine) (PLL) are two of the most widely investigated polymers that contain primary and secondary amines that impart positive charge at physiological pH to allow DNA binding and condensation of DNA into nanoparticles [22, 23]. Linear cationic polymers, such as PEI, PLL and poly(amidoamine) (PAMAM) are also often modified to a branched dendrimer structure that allows for high density of positive charge or other functionalization [24]. Liposomal formulations and some polymeric nanoparticles, such as poly(lactic-co-glycolic acid) (PLGA) and poly(vinyl pyrrolidone) (PVP) nanoparticles, physically encapsulate nucleic acid cargos. Once the nanoparticles reach the target cell, they must enter the cells through one of several endocytic pathways. The chemical structure of non-viral materials, the presence of targeting ligands, and the physical properties of resulting nanoparticles govern their cellular uptake mechanism, which has been shown to affect the efficiency of successful transfection [25, 26]. Specifically for PEI, the molecular weight as well as the polymer structure, linear versus branched, affect the efficiency of gene delivery overall [27, 28]. The mechanism by which cationic polymeric nanoparticles, such as those formed with PEI, escape the endosomes to enter cytoplasm still remains a hypothesis. The leading hypothesis for PEI has been termed the “proton sponge effect” and is governed by the ability of a vector to buffer endosomal pH, generating high osmotic pressure leading to bursting of the endosomes [29]. Polymers without tertiary amines and less efficiency at endosomal pH buffering, such as PLL, are shown to have limited transfection efficiency without an excipient that can destabilize endosomal membrane [30]. For liposomes, it was proposed that cationic lipids can fuse with endosomal membranes to facilitate endosomal escape [31]. Following endosomal escape, nucleic acids have to be released from the nanoparticles in the cytoplasm in order to be transported into the nucleus (DNA) or to suppress the translation of mRNA (RNAi). The dissociation of nucleic acids depends on unbinding kinetics between the nucleic acid and biomaterial as well as degradation of the polymer, such as through hydrolysis of ester bonds or reduction of disulfide linkages. PEI and PLL, with high charge density but without degradable bonds, are associated with cytotoxicity as well as low transfection. Disulfide linkages are especially important for the rapid release of siRNA and shRNA in the glutathione-rich cytoplasm [32].

Shortcomings of first generation vectors during in vivo evaluations and clinical trials have led to the development of more versatile delivery systems. Many synthetic approaches have been utilized, including the investigation of polymer libraries to explore structural diversity. For example, the Reineke group has synthesized new gene delivery polymers by polymerizing polycations, (*N*-[3-(*N,N*-dimethylamino) propyl] methacrylamide or *N*-(2-aminoethyl) methacrylamide), with (2-deoxy-2-methacrylamido glucopyranose) [33]. These polymers with varied molecular weights were self-assembled with plasmid DNA to optimize

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nanoparticles that could have high transfection efficacy, low toxicity, and specificity toward liver transfection. The hydrophilic 2-deoxy-2-methacrylamido glucopyranose unit enhanced particle stability in salt and the *N*-(2-aminoethyl) methacrylamide) unit, each monomer containing a primary amine, improved gene delivery. Another library approach described in the literature describes the synthesis of a family of 144 polymers that are derivatives of PEI, synthesized from PEIs and acrylates [34]. High-throughput screening of the polymeric nanoparticles formed from these polymers and DNA was utilized to discover optimal formulations that had higher efficacy and lower cytotoxicity than 22 kDa linear PEI. Improved *in vivo* activity was also observed and the chemical composition of the polymer may have led to potential organ specificity. Other polymer library approaches have also been reported in the literature. For example, 1,536 core-shell nanoparticles were formulated with structurally different, epoxide-containing block copolymers and amine monomers for the intracellular delivery of siRNA [35]. Crosslinkers with potential buffering capacity performed better, presumably to enhance endosomal escape. Poly(β-amino ester)s (PBAEs), tertiary amine-containing polymers capable of binding with nucleic acids and providing pH buffering, can also be synthesized with finely tuned structure to generate a combinatorial library with differential hydrophobicity, buffering capacity, and physicochemical properties that can meet the requirements of successful transfection [36]. Differential structures have been shown to affect DNA binding affinity [37], cellular uptake [26], and pH buffering capacity [38]. Interestingly, screening of a PBAE polymer library also enables discovery of cell type-specific transfection. For example, Shmueli *et al.* showed that PBAEs with defined structures can selectively transfect endothelial cells from macro- or microvasculature in comparison to epithelial cells (Figure 2) [39]. More recently, a bioreducible variation of PBAEs enabled efficient delivery of siRNA to cancer cells [40].

Several important aspects must also be carefully considered at the systemic level when designing next-generation non-viral nanoparticles for gene therapy. First, nanoparticles must be stable in bodily fluids to prevent aggregation and clearance, retaining a small size to facilitate transport and enable cellular uptake. PEGylation is one of the most widely used method to modify the surface of nanoparticles to promote stability [41]. In addition, nanoparticles specifically targeted to the diseased tissue and cells would have an improved therapeutic window with higher dosage at the target site, improving efficacy, and lower dosage elsewhere, reducing side effects. Cellular targeting is most often achieved by exposing diseased tissue-specific ligands on the surface of nanoparticles, but researchers have also shown that nanoparticle biomaterial composition, including the chemical structure of constituent polymers, can direct tissue or cell specificity [42]. Non-canonical peptide motifs with affinity to specific surface receptors, such as apo-transferrin on glioma capillary endothelial cells [43], integrin on endothelial cells [44], and prohibitin on adipocytes [45], have been identified via phage display and computational approaches, the use of which is also of particular interest in the field of nanoparticle targeting. Finally, other features of a gene delivery nanoparticle, such as the promoter on delivered plasmid DNA, can also drive cellular and tissue targeting [46]. Various non-viral gene delivery vectors shown in Figure 3 have been investigated with their standard structures and also in modified forms for enhanced non-viral gene delivery to various targets, including endothelial cells to modulate angiogenesis.

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3. Pro-angiogenesis

3.1 Angiogenic Factors

The diffusion of oxygen through tissues is limited to approximately 1 – 2 mm, requiring that most tissues maintain their growth close to established vasculature [47]. However, the disruption of blood flow found in ischemic and infarct regions produces a state of hypoxia in previously vascularized regions. One method by which cells attempt to compensate for this is through hypoxia-inducible factor (HIF) signaling, specifically HIF-1 or 2. Many angiogenic factors are upregulated by HIF, including vascular endothelial growth factor (VEGF), placental growth factor (PlGF), inducible nitric oxide synthase (iNOS), Ang2, and maturation factors like Ang1 and platelet-derived growth factor-BB (PDGF-BB), which are thought to play a critical role in the revascularization process of ischemic tissue. In fact, the overexpression or pharmacological stabilization of HIF-1α in mouse models of myocardial infarction was found to decrease tissue damage and improve infarct revascularization and energy levels of infarct tissue [48–50].

Nitric oxide (NO) is a short lived, pleiotropic signaling molecule involved in a variety of functions including regulation of vascular tone, inflammation, and angiogenesis to name a few. NO generation is catalyzed by homodimeric proteins known as nitric oxide synthases (NOS) [51]. In mammals, there are three isoforms of NOS involved in the formation of NO, neuronal NOS (NOS1 or nNOS), inducible NOS (NOS2 or iNOS) and endothelial NOS (NOS3 or eNOS). NOS1 and NOS3 are named for their constitutive expression in specific tissue, brain neurons for NOS1 and endothelial cells for NOS3, although expression in other tissues has also been observed [52]. In contrast, NOS2 expression is low in most tissues unless induced under specific conditions, including ischemia through HIF-1α. Inhibition to NOS3 or NOS2 was found to inhibit the angiogenesis, collateral formation, and pericyte coverage in rat hindlimb ischemia models [53–55]. NOS is also essential for the activities of several important angiogenic factors, including VEGF, fibroblast growth factor (FGF2), and Ang2, as inhibition of NO was found to ablate their angiogenic responses.

Following the initial functional description of vascular endothelial growth factor A (VEGF-A; originally vascular permeability factor (VPF)) by Senger and Dvorak in 1983, the VEGF family of growth factors quickly become known as key angiogenic factors [56]. Signaling by these growth factors is mediated by a family of receptor tyrosine kinases (RTKs) consisting of VEGFR1 (Flt1), VEGFR2 (KDR or Flk1), and VEGFR3 that show varying specificity to certain members of the VEGF family. VEGF-A is known to possess at least 13 splice isoforms that exhibit different receptor affinities and downstream signaling properties [57, 58]. $VEGF_{165}$ appears to be the most important as it is expressed to a greater amount than the other splice isoforms and alone is sufficient for the normal development of mice [59]. Given its central role in many vascular processes, it is not surprising that changes in VEGF expression can have profound consequences in ischemic diseases. As noted above, VEGF-A expression in hypoxic tissues is upregulated by the actions of the HIF transcription factor and has also been observed in cells treated with other growth factors, including FGF2, PDGF, and transforming growth factor (TGFβ) [60, 61]. Additionally, VEGF-A mRNA was found to increase within ischemic regions of myocardium from occluded pig hearts [62].

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Placental growth factor is a homolog of the VEGF sub-family with increasing implications in pathological angiogenesis [63]. Notably, the loss of PlGF was observed to decrease angiogenesis in wound sites, cancer, and ischemic myocardial, retinal, and peripheral tissues [64]. Conversely, administration of recombinant PlGF in a mouse ischemic hindlimb models was demonstrated to have a greater therapeutic response than VEGF-A by inducing the growth of second- and third-generation collateral vessels [65]. Delivery of a plasmid encoding PlGF in rat models of myocardial infarction was also found to improve revascularization, reduce infarct size, and increase the survival of cardiac myocytes [66]. In part, this effect may be attributable to the stimulation of VEGF, PlGF, Ang1, and Ang2 expression in various regions in or around the infarct zone. Moreover, PlGF-mediated angiogenesis does not produce the excessive permeability and edema associated with VEGF over-expression, allowing for the formation of lasting neovasculature [65].

The fibroblast growth factors (FGFs) consist of a family of 22 factors that share significant sequence similarities. However, FGF1 and FGF2 are well documented in angiogenesis and will remain the focus of this review. Signaling by FGF1 and FGF2 in angiogenesis is primarily mediated through its extracellular interactions with FGF receptors 1 (FGFR1) and 2 (FGFR2) [67–69]. FGFs are thought to be one of the first factors involved in the initiating phases of angiogenesis [70–72] and their activation of FGFRs has also been shown to influence EC survival, proliferation, and migration [73]. Moreover, FGF2 has also been shown to enhance the angiogenic response to VEGF by increasing the expression of VEGFR2 in mouse models and improving VEGF-mediated vessel stability [74]. The activity of FGF has also been demonstrated to increase the expression of HIF-1α, suggesting the presence of a positive feedback loop between FGF2 and HIF-1α expression during hypoxia. In contrast to VEGFR2, however, FGF receptors are expressed in a variety of tissues in addition endothelial and mural cells and, therefore, are less specific to angiogenesis [75]. Moreover, mice lacking either FGF1 or FGF2 remain viable and fertile, suggesting that individual FGFs may possess overlapping or restricted functions [76]. As such, these and other considerations have possibly contributed to the fact that no FGF-specific inhibitors have been approved by the FDA and emphasize potential challenges in targeting FGF-signaling for therapeutic applications.

Hepatocyte growth factor (HGF; or scatter factor (SF)) has been found to influence differentiation, migration, proliferation, scattering, and survival in several cell types, including endothelial cells and pericytes [77, 78]. HGF signal transduction is mediated through the c-MET receptor, the activation of which subsequently leads to the phosphorylation of several downstream effectors common to several receptor tyrosine kinases (RTKs), such as GRB2, SRC, PLCγ, PI3K, and STAT3 (reviewed in [79]). In endothelial cells, HGF signaling was found to stimulate migration, proliferation, tube formation, and rabbit corneal neovascularization, demonstrating the growth factor's potent angiogenic properties [80]. Based on these and other findings, HGF was subsequently investigated as a potential candidate for therapeutic angiogenesis. Intramuscular injection of naked HGF peptides in rat and rabbit hindlimb ischemia models was found to significantly improve collateral artery growth and blood flow to the limb [81].

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Platelet-derived growth factors (PDGFs) improve survival and reduce the permeability of new vessels by recruiting pericytes to the nascent vasculature via a chemotactic gradient [8]. The PDGF family consists of several related members, PDGF-AA, PDGF-BB, PDGF-CC, and PDGF-DD and two receptors, PDGFRα and PDGFRβ. The activities of the PDGF family have been demonstrated to be important in the healing processes of ischemic tissues. Notably, inhibition of PDGFRα and PDGFRβ in models of myocardial infarction was found significantly impart the post-infarct scar formation associated with the tissues repair [82]. Antibody blockade of PDGFRβ in this model was found to inhibit mural cell coverage and prolong the hemorrhaging of vessels within the infarct region. Interestingly, overexposure to PDGF-BB under certain conditions can have detrimental effect on angiogenesis, as the application of exogenous PDGF-BB alone has been shown to destabilize vessels by disrupting existing PDGF-BB gradients and inhibiting mural coverage of neovasculature [83]. However, when coadministered with other angiogenic growth factors, such as VEGF or FGF, PDGF-BB improved wound healing and revascularization [84, 85].

Angiopoietins are a family of secreted glycoproteins that play a variety of roles in angiogenesis. Ang1 and Ang2 are the most studied of these factors and function through their competition for the Tie2 receptor. Ang1 is primarily secreted by perivascular and mural cells and activates EC-bound Tie2 via paracrine signaling. The binding of Ang1 to Tie2 leads to downstream signals responsible for maintaining vessel stability, survival, and quiescence [86–88]. In contrast, Ang2 is a weak Tie2 agonist and is more often thought to compete with Ang1 to deactivate Tie2, which leads to a reduction in EC-EC contacts and stimulates the release of vessel-associated pericytes [5]. In combination with high levels of VEGF, these effects of Ang2 ready the vessel for the formation of VEGF-induced sprouts [89]. Without VEGF, however, the loss of cell-cell contacts and mural cells leads to the destabilization and eventual regression of the vessels. In the treatment of ischemic tissues, intra-muscular injection of plasmids encoding Ang1 in rabbit models of hindlimb ischemia was found to improve revascularization [90]. Also, co-injection of Ang1 and VEGF-A, although still potent in stimulating new vasculature, generate far less permeable vessels [91]. The co-injection of plasmids encoding VEGF-A and Ang1 into the muscles of rabbit hindlimb ischemia models was found to provide a therapeutic benefit in excess compared to either factor delivered alone [92].

The hedgehog (Hh) signaling pathway has been demonstrated to possess pro-angiogenic activity in adult animals by upregulating the expression of NOS2 and netrin-1 [93]. These effects are initiated by the binding of SHh to patched1 (PTCH1), which blocks the inhibition of another membrane protein, smoothened (SMO). Mechanistically, SHh appears to influence angiogenesis indirectly, as SHh protein had no effect on the proliferation and migration of cultured ECs. Alternatively, intramuscular injection or corneal implantation of myristoylated-SHh was found to stimulate neovascularization in mouse hindlimb ischemia and corneal neovascularization models respectively, an affect that the authors attributed to increased expression of VEGF, Ang1, and Ang2 in the mesenchymal tissue [94]. Other *in vivo* studies, however, suggest a more complicated involvement of SHh signaling in angiogenesis. Specifically, endogenous Hh signaling was found to worsen the recovery of mouse models of myocardial ischemia, as evidenced by the improved recovery observed

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following treatment with an inhibitor of SMO [95]. In contrast, exogenous SHh treatment in post-ischemia myocardium was found to decrease infarct size, and improve recovery [96].

Adrenomedullin (AM) is a pro-angiogenic, 52 amino acid peptide secreted from endothelial and mural cells in response to hypoxia, hypertension, shear stress, and other inflammatory signals [97–99]. Binding of AM to calcitonin receptor-like receptor (CRLR) leads to the activation of several downstream effectors related to the proliferation (Ras/Raf/Mek/ERK), migration (FAK), survival, (PI3K/AKT), and nitric oxide production in endothelial cells and migration (via PI3K) and vasodilation (by PKG and PKA) in the associated smooth muscle cells (reviewed in [100]). Both CRLR and AM expression is upregulated by HIF-1α and works synergistically with VEGF, demonstrating a clear importance in responding to hypoxia [101, 102]. In addition, adrenomedullin was found to increase endothelial differentiation of bone marrow monocytes and improve the mural cell coverage of new vessels in a rat hindlimb ischemia [103]. Therefore, the ability of adrenomedullin to recruit new ECs and mural cells to the sites of neovasculature implicates it as a strong candidate for angiogenic therapy.

3.2 Applications in Gene Delivery

Although a wide range of biomolecules that promote angiogenesis has been investigated as described in section 3.1, a few potent factors have been most widely used for gene therapy to promote vasculature growth. The two main applications of pro-angiogenic gene therapy utilizing non-viral nanoparticles are for treatment of ischemia and for regenerative medicine / tissue engineering.

3.2.1 Ischemia—According to the World Health Organization, circulatory diseases remain the primary cause death worldwide, accounting for nearly 31% of all fatalities [104]. The main concerns in these conditions are ischemic complications resulting from inadequate blood supply provided to a particular tissue, restricting the available nutrients and oxygen for the tissues metabolic needs. Specific conditions are named after the locations affected: peripheral arterial disease (PAD) for extremities, coronary artery disease in the heart, and cerebral artery diseases in the brain. In severe cases, this lack of blood flow leads to the death of the tissue, known as an infarction, which can be life threatening for particular organs, ie. heart attack (myocardial infarction) or ischemic stroke (cerebral infarction) [105]. Often these diseases begin with the narrowing of arterial vessels, known as stenosis, with little or no clinical manifestations. Although congenital defects can contribute to this vessel narrowing, it most often arises as a result of endothelial dysfunction, such as improper regulation of vascular tone, and eventually leads to the formation of atherosclerotic lesions [106]. These sites are characterized by the accumulation of oxidized low-density lipoprotein plaques and inflammation within the vessel intima. The eventual rupture of these plaques and increased intimal distance within atherosclerotic vessels can lead to occluded vessels or impaired gas exchanged with nearby tissues, further reducing the availability of oxygen and nutrients to affected regions.

The endogenous restoration of blood flow to ischemic tissues is primarily the function of angiogenesis and arteriogenesis. The outgrowth of collateral vessels during arteriogenesis

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appears to be independent of hypoxia and originates in non-ischemic tissues. Instead, these events are thought to be initiated by the changes in shear stress associated with the perturbed blood flow within atherosclerotic vessels [107]. In contrast, sustained hypoxia instigates the production of pro-angiogenic signals and new capillary networks within the ischemic region. To improve patient outcome, medical intervention, often in the form of angioplasties, bypasses, stents, or anti-coagulant therapy, are usually employed as well [108–110]. However, these approaches are not always sufficient to restore normal tissue function. As a potential alternative, an increasing number of investigations have looked into use of therapeutic angiogenesis in order to establish new vessels within the ischemic regions. Notably, several of these studies have focused the delivery of genes encoding pro-angiogenic factors to achieve sustained expression of these signals within ischemic and infarct sites. Here we review several examples of such treatments using non-viral delivery methods.

Many studies have utilized viruses to deliver genes to promote angiogenesis to ischemic tissue. Adenovirus-mediated delivery of HIF-1α or HGF resulted in improvement of heart function in chronic myocardial ischemia and postinfarct heart failure model in swine and rats [81, 111, 112]. Other variations include a co-delivery of VEGF164 and PDGF-BB in mice ischemic hindlimb [113] and repeated injection of HGF in rat postinfarct heart [114] using adenovirus. Another group studied improved rabbit cardiac function following myocardial infarction upon implantation of a sheet of adipose-derived stem cells overexpressing VEGF by baculovirus transduction [115].

While these therapeutic outcomes using viral vectors are notable, non-viral gene delivery has seen significant success in addressing ischemia. Different combinations of non-viral approaches and nucleic acid cargos have been tested in ischemic animal models. In the majority of studies, nanoparticles formed with polymers or lipids are delivered directly *in vivo* to transfect target cells. PEI, while widely used in its native form to deliver nucleic acids for various applications, has also been combined with polysaccharides or lipids to create new biomaterials. For example, 250 nm nanoparticles consisting of pVEGF and 1800 Da PEI conjugated with heparin at N/P 10 resulted in over a 3-fold increase in capillary density in mouse ischemic limb model than non-modified PEI [116]. In another study, chitosan-grafted PEI with eprosartan as the targeting moiety to angiotensin II type 1 receptor was used to deliver $VEGF_{165}$ intramyocardially and showed 7% improvement in ejection fraction and 1.6-fold increase in vessel density than PEI control in rat myocardial ischemia model [117]. Han *et al.* developed a new system of water soluble lipopolymer (WSLP) by combining branched PEI with hydrophobic lipid anchor cholesterol chloroformate [118]. Using this vector, Yockman *et al.* successfully delivered hypoxia-inducible $VEGF_{165}$ plasmid intramyocardially to a rabbit myocardial infarct model in a separate study and reduced the infarct size by 36% compared to ligation only control (Figure 4) [119].

Other biomaterials used to create nanoparticles by plasmid DNA condensation include poly-arginine and elastin-like polypeptide [120, 121]. Specifically, Dash *et al.* developed a hydrogel that contains hollow elastin-based spheres carrying plasmid endothelial nitric oxide synthase (NOS3) and IL-10, which showed approximately 3-fold increase in perfusion ratio of ischemic to non-ischemic limb and in blood vessel surface density in mouse hindlimb ischemia. Meanwhile, encapsulating DNA-condensed nanoparticles in hydrogels, such as

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agarose, is known to enhance the duration of gene transfection *in vivo* [122]. The advantage of sustained expression of an angiogenic gene, such as VEGF, is that the gene product can not only initiate vessel formation but also maintain vessels once they are formed [123]. Additional types of nanoparticles that encapsulate plasmid DNA are formulated with synthetic polymers such as PLGA and Pluronic® L64, which has successfully delivered $pVEGF_{165}$ and pHIF-1α to increase capillary density by approximately 38% in rabbit myocardial and 67% in mouse hindlimb ischemia, respectively [124, 125].

While these non-viral vectors are injected locally at the ischemic site and hence invasive, other studies utilized an ultrasound-targeted microbubble destruction (UTMD) method to deliver therapeutic genes systemically. Without efficient targeting, systemic delivery of VEGF may bring adverse side effects due to undesired angiogenesis in off-target sites [123]. In UTMD-mediated gene therapy, microbubbles can be visualized and destroyed at the target site by ultrasound or contrast echocardiography, releasing the loaded gene at the site of interest to enter local cells with disturbed cell membranes due to cavitation [126]. Lipid microbubbles carrying plasmid DNA encoding VEGF, stem cell factor (SCF), and stromal cell-derived factor-1 (SDF-1), or micro RNA, such as miR-126 that inhibits negative regulators of VEGF signaling, have been investigated in *in vivo* models of myocardial infarct, ischemic hindlimb, as well as ischemic brain injury after stroke [127–130].

Another popular approach of treating diseases through gene therapy is harnessing cell therapy; cells are transfected *ex vivo* with non-viral vectors and subsequently the cells are locally injected to serve as either a depot of therapeutic factors or a source of tissue regeneration (Figure 1). For an angiogenesis application, stem cells as well as somatic cells have both been genetically modified. Mesenchymal stem cells (MSCs) transfected with dextran – pAdrenomedullin complex, bile acid-conjugated PEI – hypoxia-inducible pVEGF polyplex, or hollow mesoporous organosilica – pHGF nanoparticles demonstrated increased neovascularization, reduced infarct size, and improved cardiac function in rats following transplantation to myocardial infarct tissue [131–133]. Specifically, dextran-pAdrenomedulin complexed at 2.6 w/w ratio formed nanoparticles with 200 nm size and 12 mV surface charge that not only exhibited 2.5-fold increase in capillary density and 40% reduction in infarct size, but also higher recovery rate of heart function, including left ventricle end-diastolic pressure (EDP) and fraction shortening (FS). Also, PLGA – PEI – pVEGF as well as PBAE – pVEGF nanoparticles were shown to successfully transfect human MSCs for transplantation in mouse ischemic hindlimb [134, 135]. MSCs transfected with PBAE - pVEGFP significantly increased the percentage of limb salvage to 50% while decreasing limb loss to 20%. Endothelial progenitor cells transfected with PEI-coated quantum dot – $pVEGF_{165}$ nanoparticles were intramuscularly injected in mouse ischemic limbs and resulted in higher blood perfusion level [136]. Somatic cells, such as skeletal myoblasts and endothelial cells, were also utilized as carriers of angiogenic factors. Ye *et al.* tested myoblasts transfected *ex vivo* with PEI polyplex as well as liposomes composed of cholesterol and DOTAP to deliver $VEGF_{165}$ in rat heart suffering acute infarction, and showed increased blood vessel density and ejection fraction [137, 138]. It is important to note that two different types of vessels, arterioles (smooth muscle cells) or capillaries (skeletal muscle cells), can be formed based on differential expression of VEGF from transplanted myoblasts or shear stress in the microenvironment [139]. Cho *et al.* compared

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the efficacy of nanoparticles formed with different non-viral vectors to transfect human umbilical vein endothelial cells (HUVECs) with VEGF and transplanted HUVECs to treat mouse limb ischemia. [140]. HUVECs transfected with PBAE-pVEGF showed 50% limb salvage in contrast to 30% with lipo-pVEGF and 12.5% with PEI-pVEGF. As described earlier, the difference in particle size, surface charge, cellular uptake, and endosomal escape efficiency between PBAE, PEI, and lipid nanoparticles could affect the overall transfection efficiency and thus the therapeutic outcome of limb salvage rate. The optimal PBAE nanoparticles used in this study were formulated at 30 w/w ratio of PBAE to pVEGF, which resulted in a 200 nm in size and a −1.26 mV zeta potential, in contrast to PEI nanoparticles that are typically formulated to have a highly positive zeta potential. Meanwhile, two separate studies evaluated PLGA nanoparticles with micro RNA (miR132) using different methods to transfect endothelial cells in order to cell survival and vessel growth. Gomes *et al.* coated 170-nm PLGA nanoparticles with protamine sulfate that can complex with miR132 and facilitate cellular uptake, and transfected endothelial cells showed 3-fold higher proliferation of cells and blood flow than those transfected with negative control nanoparticles following transplantation in mouse hindlimb ischemia model [141]. In another approach, Devalliere *et al.* used a double emulsion method to encapsulate spermidine – miR132 into PLGA nanoparticles, which were utilized to transfect HUVECs [142]. When these transfected HUVECs were placed within a subcutaneous collagen gel plug in mice, they mediated 2-fold vessel growth compared to a lipofection control.

3.2.2 Tissue Regeneration—New tissue formation requires sufficient level of oxygen and nutrients. Limitations in diffusional distance of oxygen and nutrients necessitate the matrix to have a highly vascularized network. Hence, tissue growth in a natural matrix, *in situ* scaffold, or *ex vivo* artificial organ must be accompanied by angiogenesis. Skin regeneration during the wound healing process and bone regeneration are examples where an increased level of angiogenesis could accelerate and enhance the outcome while preventing necrosis [143].

Viral vector-mediated gene transfer has been shown to be effective in promoting angiogenesis and enhancing wound healing and osteogenesis in numerous studies. For example, adenovirus expressing insulin-like growth factor (IGF-1) and retrovirus expressing cyclooxygenase-2 (Cox-2) induced vessel growth through VEGF- and prostaglandin-dependent pathways in murine wound and rat femoral fracture model, respectively [144, 145]. Adenovirus-mediated $VEGF_{121}$ expression in rabbit femur re-vascularized the necrotic region and resulted in bone formation [146]. Applications of *ex vivo* gene transfer combined with cell therapy include adenovirus-mediated angiopoietin-1 gene-modified bone marrow MSCs for use in a rat skin wound model, osteoblasts expressing adenovirus-mediated VEGF and seeded to a chitosan/hydroxyapatite scaffold, and bone marrow cells expressing lentiviral-mediated HIF-1α in a rat bone regeneration model [147–149].

However, lentivirus and retrovirus suffer from potential mutagenesis or uncontrolled angiogenesis from gene insertion into host’s genome while adenovirus does not overcome the limitation of transient expression. Recent efforts are underway to expand the repertoire of non-viral nanoparticles for angiogenic gene therapy in tissue regeneration. Direct intradermal injection of nanoparticles formulated with PBAE and sonic hedgehog (SHh)-

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encoding plasmid resulted in 100% wound closure area at day 10, significantly greater than 85% for PBS and nonfunctioning plasmid controls [150]. SHh is a morphogen involved in tissue regeneration via activation of angiogenic signaling pathways, as the study shows 2~3-fold increased expression level of VEGF and SDF-1α.

Additionally, *ex vivo* transfection followed by cell transplantation has also been a popular route of gene therapy for tissue regeneration, similar to applications in ischemic disease models. One of the studies by Dr. Fan Yang's group utilized adipose-derived stromal cells (ASCs) in a mouse wound-healing model [151]. ASCs transfected with pVEGF using PBAE polymer at 30 w/w ratio of polymer to DNA were injected intradermally at the four quadrants of the wound. The PBAE-pVEGF-transfected ASCs treated wound showed not only accelerated angiogenesis and 20% greater wound closure at day 8 than non-modified ASCs, but also increased cellularity and collagen deposition (Figure 5). Nanoparticles formed at 30 w/w with the specific PBAE structure used were able to transfect less than 10% of ASCs, which was still greater than lipoplexes. Different PBAE structure may transfect ASCs with higher efficiency to enhance therapeutic effect, as shown by transfection screening of PBAE library on ASCs from a separate study [152]. A recent study reported that using PEI-pVEGF nanoparticles at N/P 7 in hyaluronic acid hydrogels with 60-μm pore led to 50% decrease in open diabetic wound area than non-porous hydrogels, possibly due to increased surface area contact for infiltrating host cells with released nanoparticles [153]. In bone tissue engineering, a recent work using a collagen scaffold containing branced PEI (bPEI) complexed with a single gene encoding PDGF-B at N/P 10 ratio showed 40% bone formation in the defect by volume and 50-fold increase in connective density compared to control, leading to bone repair in rats [154]. However, Curtin *et al.* showed that among collagen scaffolds carrying two genes expressing bone morphogenetic protein 2 (BMP2) and VEGF by bPEI only, nanohydroxyapatite (nHA) only, or mixed vectors, nHA only scaffold resulted in greater than 2-fold increase in bone nucleation area over bPEI (N/P 7) only scaffold condition *in vivo* [154, 155]. Although branched PEI (bPEI) has greater buffering capacity and charge density than linear PEI, which could lead to higher transfection at the risk of potential cytotoxicity (Figure 3), most studies followed a similar N/P between 7 and 10 to formulate nanoparticles. For many polymeric gene delivery systems, an intermediate N/P ratio is found optimal, where an N/P ratio that is too low (close to neutrality) results in polyplex nanoparticles that are unstable and that aggregate. On the other hand, as the amount of biomaterial is increased, nanoparticles that are more stable and that have improved uptake and transfection form. Yet, at still higher N/P ratios, biomaterial-mediated toxicity can become apparent, especially with highly charged, non-degradable biomaterials such as PEI [156]. While N/P ratio represents a true charge ratio when the polymer amine groups are fully charged, for certain cationic polymers the majority of the amines are tertiary amines rather than primary amines and the polymer is not highly charged at neutral pH. In these cases, the N/P ratio required for charge neutralization of anionic DNA and for the formation of compact polyplex nanoparticle formation can be much higher. These examples demonstrate the potential effectiveness of gene delivery nanoparticles using multiple types of non-viral vectors.

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4. Anti-angiogenesis

4.1 Anti-angiogenic Factors

The vascular basement membrane (vBM) is a collection of extracellular macromolecules, including collagen, fibronectin, laminin, and heparan sulfate that stabilizes the structure of vasculature. Under angiogenic conditions, however, the vBM is partially degraded in a tightly regulated fashion to allow for formation of new angiogenic sprouts while still maintaining enough structure to function as a substrate for adhesion and migration of the growing vessel. In addition to its structural role, the proteolytic degradation of the vBM generates various bioactive fragments called matricryptins to regulate endothelial cell proliferation, migration, and survival as well as to help limit excessive angiogenesis during wound healing, inflammation, and disease processes. Several extracellular matrix (ECM) components can contribute to the generation of these fragments, notably, collagens IV (arresten; canstatin; tumstatin) and XVIII (endostatin) and heparan sulfates (endorepellin) [157].

Type IV collagen is a non-fibrillar component that makes-up almost half of all basement membranes and is the source of several anti-angiogenic matricryptins, including arresten (26 kDa), canstatin (24 kDa), and tumstatin (29 kDa) [158]. Treatment of endothelial cells *in vitro* with any of these three fragments inhibits the migration and proliferation of these cells as well as the ability to form tube-like structures in matrigel substrate. The fragments also demonstrated *in vivo* efficacy by inhibiting the vascularization of injected matrigel plugs and the growth and metastasis of xenografts in nude mice [159–162]. Endostatin (20 kDa) is another matricryptin derived from collagen, specifically type XVIII. Similar to the collagen type IV matricryptins, endostatin was found to inhibit the migration, but not the proliferation, of endothelial cells *in vitro* and disrupt tumor vascularization and growth in mice [163].

In addition to the collagens, the breakdown of perlecan, major heparan sulfate proteoglycan, and plasminogen releases the anti-angiogenic fragments endorepellin and angiostatin respectively. Endorepellin is an 80 kDa fragment derived from the C-terminal domain V of perlecan that was found to inhibit EC migration and tube formation [164]. Angiostatin (38 kDa) is a potent anti-angiogenic cleavage fragment derived from plasminogen. Treatment of endothelial cells with angiostatin was found to inhibit migration and induce apoptosis [165, 166].

Mechanistically, all of the above molecules are thought to mediate their effects, at least in part, through interactions with various integrin heterodimers. Arresten binds integrin $\alpha_1\beta1$ [162], canstatin binds integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ [167], angiostatin and tumstatin bind $\alpha_v\beta_3$ [161, 168], endostatin binds $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_5\beta1$ [169], and endorepellin binds $\alpha_2\beta1$ [170]. The disruption of integrin-ECM interactions disrupts cellular adhesion and migration and has been shown to regulate the trafficking and signal duration of growth factor receptors [171]. In addition to the large protein fragments described above, a large number of short anti-angiogenic peptides have been discovered, both endogenous (ie, having natural sequences as in the proteins of origin) and biomimetic. Bioinformatics-based systems biology methodology lead to the discovery of over 100 novel peptides that inhibit

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proliferation and migration of endothelial cells in vitro [44]. These peptides were derived from different protein domains, such as collagen IV, thrombospondin-1, CXC chemokines, serpin, somatotropin, and tissue inhibitors of matrix metalloproteinases (TIMP). Structure-activity relationship (SAR) investigation yielded biomimetic peptides with optimized anti-angiogenic activity [172]. Selected peptides and their biomimetic derivatives were tested in vivo in cancer xenograft models [173, 174] and in models of ocular neovascularization [175]. Some peptides from different classes exhibit synergy that could be explored therapeutically [176]. Anti-angiogenic peptides for cancer applications have been reviewed in [177]. Importantly, some of the peptides exhibit anti-lymphangiogenic activity and thus could be useful as anti-metastatic agents or immuno-suppressive agents [178, 179]. Each of these peptides and proteins are potential agents that can be used for anti-angiogenesis therapy and that can also be genetically encoded in DNA. Through non-viral nanoparticle-mediated transfection, novel combinations of these therapeutics and others could be expressed at target tissue sites and over time. An advantage of non-viral gene delivery that can facilitate combination therapy with the factors described is that multiple plasmids and large plasmids can be delivered within a single non-viral nanoparticle [180], unlike with viral gene therapy where DNA carrying capacity is more stringently limited.

As mentioned, one of the initial hallmarks of angiogenesis is the breakdown of the vascular basement membrane to allow for the invasion of neovasculature into adjacent tissue. In humans, this activity is catalyzed by a tightly regulated family of 23 zinc-dependent endopeptidases known as matrix metalloproteinases (MMPs) which are secreted in response to growth factor signaling and are essential for initiating the process of angiogenesis (reviewed in [6]). MMP pro-angiogenic functions are controlled by the generation of anti-angiogenic matricryptins (described above) as well as endogenous inhibitors of MMP activity in the form of TIMPs [157, 181]. TIMPs are a family of four proteins (TIMP 1–4; 20–29 kDa) that bind to active sites of MMPs and inhibit their proteolytic activities. As such, TIMPs have been investigated as natural sources of anti-angiogenic therapies and have been shown to inhibit MMP-related vascular effects [182, 183]. TIMPs also possess non-MMP dependent inhibition of angiogenesis through the disruption of growth factor induced signaling [184, 185]. The anti-angiogenic properties of TIMPs have led to investigations of their use in cancer, with several studies demonstrating an inhibition of tumor metastasis [186–188]. However, other reports indicate a poorer prognosis associated with higher levels of TIMPs in some cancers. For instance, higher levels of TIMP1 has been shown to inhibit MMP9-mediated tumor regression by disrupting a pro-inflammatory response, while TIMP4 over-expression in breast cancers was associated with reduced patient survival [189, 190]. TIMPs represent promising novel therapeutic entities that can be genetically encoded for intracellular delivery and expression by gene delivery nanoparticles to treat angiogenesis-dependent diseases.

As described above, the weak kinase activity observed for VEGFR1 has led to the widespread hypothesis that VEGFR1 primarily functions as a sink for VEGFs, thereby attenuating their angiogenic signaling. Interestingly, a soluble form of VEGFR1 (sVEGFR1) secreted by both ECs and monocytes has been observed in human plasma and serum from healthy individuals [191–193]. sVEGFR1, however, still retains its high affinity for VEGF-A and has been shown to inhibit angiogenesis through the regulation of VEGFR2 activation

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and inhibition of downstream mitogenic activities [194]. Additionally, sVEGFR1 is able to form nonproductive heterodimers with bound VEGFR1 and VEGFR2, further reducing VEGF-A signaling [195]. sVEGFR1 levels were found to decrease in estrogen receptor positive breast cancer through an estrogen-dependent mechanism and this decrease correlated with an increase in angiogenic activity and tumor progression [196]. Furthermore, sVEGFR1 was found to be overexpressed in some patient samples of colorectal cancer and was associated with recurrence-free survival [197].

Pigment epithelial derived factor (PEDF) is a 50 kDa neurotrophic serine protease inhibitor (serpin) originally isolated from retinal pigmented epithelial cells but has since been identified in a variety of tissues [198]. PEDF was also identified as one of the most potent endogenous inhibitors of angiogenesis discovered thus far [199]. PEDF's inhibition of angiogenesis targets many aspects of endothelial cell biology through interactions with several cellular surface receptors, including a specific transmembrane protein known as PEDFR. Binding of PEDF to PEDFR activates several downstream signaling pathways involved in the stimulation of apoptosis in ECs through NF-κB-, PPARγ-, and p53-mediated processes while inhibiting their proliferation and migration [199–201]. Additionally, PEDF has been found to regulate the signaling of other growth factor receptors by stimulating the cleavage of their receptors or altering factor expression. Consistent with its anti-angiogenic activity, expression of PEDF is low in most tumor tissues. As such, much interest has surrounded its use as a possible cancer therapeutic. In particular, PEDF not only indirectly disrupts tumor growth through its anti-angiogenic properties, it has also been found to directly inhibit cells from a variety of tumors, including prostate, ovarian, and pancreatic carcinomas, melanomas, glioma, and osteosarcomas (reviewed in [202–204]). Specifically, PEDF was found to reduce tumor cells proliferation and invasion through changes in genes expressions (ie Notch and Wnt signaling) and disruption of MMP activity.

4.2 Applications in Gene Delivery

As described in section 4.1, a number of anti-angiogenic factors can be expressed by exogenous DNA to directly inhibit vessel formation. In addition, the process of suppressing angiogenesis can also be achieved by silencing genes that are responsible for vascularization. In such cases, siRNA or shRNA (collectively RNAi) is delivered [205, 206]. Although DNA and RNAi could have the same end goal, they may require different biomaterial design from the delivery perspective. Two significant and widespread diseases where anti-angiogenic therapy would be of great benefit are cancer and neovascular (wet) age-related macular degeneration (AMD). The utility of gene delivery nanoparticles to treat these angiogenesis-dependent diseases is described in the following sections.

4.2.1 Cancer—In order to maintain the necessary nutrient and oxygen concentrations for survival, tissues in excess of a certain volume (typically 1–2 mm^3) must establish an adequate blood supply [47]. This a particularly important for tumors, which are often characterized by rapid, misregulated growth and unique metabolic requirements. As such, extensive neovascularization is often a hallmark of many solid tumors and can be present at various stages of the cancer's development. This shift to a highly angiogenic state, known as the angiogenic switch, occurs when populations of cells within the tumor acquire the ability

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to bypass normal regulatory mechanisms and shift the tumor microenvironment in favor of excessive angiogenesis [207]. In addition to its contribution to tumor growth, the angiogenic switch is also a critical step in the progression to metastasis. Notably, newly formed vasculature is characterized by low mural coverage, poorly established basement membrane, reduced EC-EC contacts, and hyper-permeability, all of which can facilitate the intravasation and dissemination of tumor cells into circulation [208].

In 1971 Judah Folkman proposed a hypothesis suggesting that inhibition of angiogenesis could maintain tumor dormancy and reduce the occurrence of metastases [209]. Since then, anti-angiogenic therapy has become a significant arm of patient care in the treatment of solid tumors. A major benefit of vascular-based therapies is that EC rarely undergo transformation relative to their malignant counterparts and thus remain a valid target in a variety of cancer types [210]. Additionally, anti-angiogenic therapy has demonstrated promise in the neoadjuvant setting by restricting tumor growth prior to surgical resection [211]. Many of these agents directly target angiogenic growth factors (bevacizumab or aflibercept) or their receptors (tyrosine kinase inhibitors (TKIs); ie. sunitinib and sorafenib). In several vascular tumors, such as colorectal cancer, non-small lung cancer, and renal cell carcinoma, these treatments have been successful in extending disease free progression and overall survival. Moreover, sorafenib remains the only approved therapeutic for the treatment of hepatocellular carcinoma [209]. Even in these cases, however, anti-angiogenic therapy provides only modest benefits with median durations typically on the order of several months and rarely exceeding a year [212, 213].

In order to facilitate the *in vivo* delivery of anti-angiogenic genetic cargo, several different types of non-viral nanoparticles have been evaluated. PEI, similar to numerous reports of its application in therapeutic angiogenesis, is one of the most widely studied polymers in cancer therapy targeting angiogenesis. Structural modifications and targeting properties are varied to enhance the delivery efficacy of nanoparticles. PEG-grafted PEI with RGD as the targeting ligand (bPEI-g-PEG-cRGD) was developed by Kim *et al.* to form tumor endothelial-targeted nanoparticles [214]. cRGD motif has been widely used as a targeting ligand that specifically binds to overexpressed integrins on tumor vasculature, which allows higher accumulation of nanoparticles in tumor by systemic delivery [215]. Intravenous injection of DNA encoding sFlt-1 (sVEGFR1) complexed with bPEI-g-PEG-cRGD at N/P 10 in 5% glucose solution showed 7% of the injected dose accumulating in tumor, more than 3-fold inhibition of tumor growth, and increased survival in colon carcinoma model [214]. The same polymer was also used to form 160-nm nanoparticles with siRNA against VEGFR1 at N/P 10 to colon carcinoma in mice, and suppressed tumor growth by 42% at day 11 compared to control plasmid and non-targeted (PEI-g-PEG) nanoparticles [216]. A similar nanoparticle (cRGD-PEG-PEI) synthesized with a different chemistry was also used to complex with pPEDF at N/P 10 in Opti-MEM™ solution for suppressing colorectal cancer growth [217]. These sub-100 nm nanoparticles were able to decrease microvessel density by 2-fold and tumor volume by 67.4%. Another variant of bPEI polymer, Tween®-SS-bPEI, was developed to enhance stability of nanoparticles by amphiphilic property of Tween® and to release shRNA rapidly in the cytoplasm by reduction of disulfide bond. At 14 N/P ratio, the polymer condensed p65 shRNA to block NF-κB pathway, which is also known to facilitate angiogenesis, to 130 nm size, and successfully inhibited the tumor

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volume of breast cancer xenografts to 6% of saline group at 5 weeks [218]. Based on these studies, both addition of targeting moiety and varying formulation parameters to form smaller nanoparticles, such as N/P ratio or buffer used in nanoparticle formation, improve the efficiency of DNA delivery with PEI, while introducing disulfide bonds to the polymer enhances shRNA delivery to the site of action, the cytoplasm. A dendrimer system has also been investigated as a delivery platform for antiangiogenic plasmid DNA. In a study by Vincent et al., a PAMAM-based dendrimer complexed with pAngiostatin and/or pTIMP-2 between 12 – 36 w/w polymer to nucleic acid mass ratio was intratumorally injected into a subcutaneous human breast carcinoma model in mice and showed 96% inhibition of tumor growth compared to an empty plasmid control [219].

Other non-viral nanoparticles have also shown therapeutic efficacy against primary and metastatic tumors in animals. For example, synthetic polymer PVP can form hydrogen bonds and intercalate with DNA to form positively charged nanoparticles. Intramuscular injection of PVP-pEndostatin nanoparticles showed potent anti-angiogenic activity and retardation of metastatic brain tumor growth by 60% compared to control as measured with MRI [220]. Another example is PEG-conjugated poly(epsilon-caprolactone) tethered to Tat cell-penetrating sequence by disulfide bond to facilitate cytoplasmic plasmid release (mPEG-PCL-SS-Tat) [221]. Tat, having arginine-rich sequence, can be used to calculate N/P ratio for nanoparticle formulation. mPEG-PCL-SS-Tat complexed with siVEGF at N/P 30 to form 40 nm nanoparticles that could take advantage of EPR effect [222] following systemic delivery, and demonstrated 50% decrease in VEGF secretion and approximately 60% suppression of tumor growth in mouse sarcoma.

One of the earliest polypeptides used for gene condensation, PLL, was used in a copolymer structure conjugated with PEG to systemically deliver plasmid DNA sFlt-1 to subcutaneous pancreatic tumor [223]. Specifically, thiolated PLL was conjugated to PEG to form disulfide cross-linked micelles that stabilize the structure as well as enable plasmid release in response to reducing environment. Various degree of thiolation was tested against *in vivo* antitumor activity, and the optimal 11% thiolated micelles resulted in 40% tumor volume suppression compared to control. A related non-viral vector is highly branched, cationic, dendrimer-based PLL (DGL) modified with PEG [224]. As noted above, the disadvantage of PLL is its limitation to escape endosomes once endocytosed, but cell-penetrating peptides (CPPs) facilitate cellular internalization to the cytoplasm. Huang *et al.* conjugated pH- and MMP-activatable CPP (dtACPP) to DGL-PEG (dtACPPD) that could induce selective internalization of nanoparticles to cells residing in tumor microenvironment [224]. dtACPPD was complexed with doxorubicin (DOX)-intercalated shVEGF at 6 w/w ratio to form near-neutral, 150 nm nanoparticles for drug and gene co-delivery to orthotopic glioma (Figure 6). The nanoparticles successfully inhibited VEGF mRNA expression by 65% *in vivo* and increased median survival by more than 2-fold compared to untreated, free DOX, and constitutive-CPP bearing DGL-shVEGF.

Polysaccharides, such as chitosan and β-cyclodextrin, have also been explored as nanoparticulate gene carriers to tumor vasculature. Low molecular weight chitosan and cyclodextrin provide stability in biological fluids and membrane permeation enhancement capability [225]. Other key features, such as molecular inclusion of cyclodextrin, also

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simultaneously affect the efficiency of gene delivery by these carbohydrates. Mark Davis' group developed a targeted cyclodextrin-based nanoparticles for siRNA delivery to tumor (CALAA-01), which was the first of its kind to enter phase 1 clinical trial in 2008 [226]. Low molecular weight (21 kDa) Chitosan-shVEGF complex of 220 nm size formulated at N/P 3 demonstrated effective inhibition of tumor angiogenesis and tumor growth via intratumoral injection or intravenous injection to subcutaneous ectopic and orthotopic hepatocellular carcinoma models, respectively [227]. Guo *et al.* synthesized PEGylated β-cyclodextrin (βCD**)** with anisamide (AA) as the targeting moiety against sigma receptor overexpressed in prostate cancer [228]. Specifically, guanidine was first conjugated to βCD to generate a cationic cyclodextrin (G-CD), which was then further modified with PEG and the targeting ligand (G-CD-PEG-AA). Stable 200 nm nanoparticles were formed with G-CD-PEG-AA and siRNA at 75 w/w ratio. A 3-fold suppression of tumor growth was achieved with G-CD-PEG-AA-siVEGF relative to PBS-treated group, as well as greater than 3.5-fold reduction in VEGF mRNA level relative to non-targeted G-CD-PEG-siVEGF (Figure 7).

Lipid-based nanoparticle formulations have also seen success in animal models to target tumor angiogenesis. A modified lipid termed YSK05, which contains a tertiary amine for pH buffering along with greater ability to fuse with membrane to facilitate endosomal escape, is used to formulate cRGD-coated, PEGylated liposomes [229]. Systemic injection of the targeted liposome inhibited renal carcinoma tumor growth and reduced vessel area by approximately 50% compared to siLuciferase control. Another variation of lipid was developed using disulfide bond and tertiary amine containing lipid as a base. Retinoic acid, which has intrinsic property to transport to peri-nuclear site, maintains nanoparticle structure until releasing at the last step for nuclear transport. Using liposomes formulated with the vector and plasmid DNA sFlt-1, tumor growth was suppressed by 53% and area of endothelium decreased by 50% compared to PBS control in renal cell carcinoma model [230]. In another study, integrin-targeted liposomes containing micro RNA miR132 antagonist were able to decrease the volume of a human breast cancer orthotopic xenograft in mice by approximately 2-fold via restoring p120RasGAP expression in endothelium and decreasing neovascularization [231]. Interestingly and expectedly, water-soluble lipopolymer (WSLP) [119] as well as ultrasound-targeted microbubble destruction (UTMD) of liposomes [126] that were shown to be effective against ischemic diseases were also used in anti-angiogenic gene therapy against prostate [232] and mammary [233] adenocarcinomas, respectively. WSLP-mediated siVEGF delivery resulted in more than 50% reduction in VEGF concentration and tumor growth compared to bPEI-siVEGF nanoparticles, while UTMD-mediated shVEGFR2 delivery also reduced tumor volume by 40% compared to no treatment control.

This highlights an advantage of non-viral gene delivery nanoparticles in that once optimized for delivery to one cell type following a particular route of administration, a plasmid's sequence, including its encoded gene of interest, can be varied without changing the physicochemical properties of the nanoparticle and its intracellular delivery efficacy to that cell type. Similarly, nanoparticles optimized to deliver siRNA or miRNA to a particular cell type will have similar nanoparticle physicochemical properties and intracellular delivery

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even if the RNA sequence, and the genetic target for the siRNA or miRNA, was modified. Thus, the same optimized biomaterial nanoparticle formulations for a particular type of nucleic acid cargo, DNA, siRNA, miRNA, or antisense, can be used to deliver a range of different genetic medicines for different physiological effects, without necessarily requiring any re-optimization of the nanoparticle as gene delivery vector. However, when administered to a different microenvironment or following a different route of administration, due to the effect of extracellular delivery barriers, the nanoparticle may need further engineering for enhanced efficacy, such as PEGylation to resist serum proteins in the blood or smaller size to penetrate through certain tissues.

4.2.2 Neovascular Age-related Macular Degeneration (NVAMD)—Age-related macular degeneration is a complex disease that can have two phenotypes leading to vision loss: non-neovascular (or dry) AMD, in which accumulation of extracellular deposits (drusen) results in gradual death of photoreceptors and retinal pigment epithelial (RPE) cells, or neovascular (wet) AMD (NVAMD), in which new blood vessels grow into subretinal space from the choroid. Because vessels in subretinal neovasculature lack tight junctions, fluid can leak out into the retina, resulting in edema, fluid pooling, and vision loss [234]. Anti-angiogenic factors, namely anti-VEGF molecules ranibizumab (Lucentis®) and aflibercept (Eylea®), are currently injected intravitreally in patients with NVAMD to inhibit neovascularization and leakage.

Tissues and RPE cells at the posterior of the eye, which is the target site for NVAMD, can be accessed and targeted by specific routes of administration [235]. Intravitreous injection is a common delivery method, however engineered biomaterial design can enable enhanced nanoparticle diffusion to the posterior and accumulation away from the visual axis to increase therapeutically effective concentration. Subretinal injection can precisely deliver the therapeutics to the RPE at high concentration, but carries a risk of retinal detachment [236]. More recently, injection into suprachoroidal space between choroid and sclera has been shown to be safe, and merits further investigation for therapeutic delivery of nanoparticles, including gene delivery nanoaprticles [237].

Viral vectors have been widely investigated for gene therapy due to their efficiency and potential for long-term expression of transduced genes. Adeno-associated virus (AAV/AAV2) [238, 239], lentivirus [240], and hybrid AAV with serotypes that targets specific cell populations (rAAV) [241] have all shown positive results against CNV in preclinical animal studies. These viruses have transferred genes encoding endogenous angiogenic inhibitors such as soluble VEGF receptor (sFlt-1) [238, 239], pigment epithelium-derived factor (PEDF) [242, 243], endostatin and angiostatin [240], and tissue inhibitor of metalloproteinases-3 (TIMP3) [244]. However, long-term suppression of neovascularization at distant sites due to viruses that made their way into the systemic circulation is a potential safety concern [245].

Studies evaluating *in vitro* and *in vivo* transfection of the RPE cell layer with non-viral gene delivery nanoparticles have led to therapeutic efficacy against CNV. The utility of polymeric nanoparticle library approach for ocular gene therapy was demonstrated by Sunshine *et al.* [246]. 180 nm nanoparticles formed from a library of PBAEs with varying structures were

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screened and analyzed for transfection against RPE cells *in vitro*. When the best performing formulation was injected into the subretinal space, high expression was observed 72-h post injection by both RT-PCR and fluorescence microscopy of a genetically-encoded reporter in both retina and RPE/choroid. The specificity and efficiency of PBAE nanoparticles were enhanced through the polymer library screening approach. In a laser-induced CNV model in rats, PVP polymer carrying pVasostatin was able to decrease 32.5% and 48% CNV incidence as well as lesion area for 42 days compared to saline and empty vector controls [247]. In another study using the same model, shHIF-1α was encapsulated in PLGA for intravitreal injection [248]. PLGA allows for controlled release of the nucleic acids for prolonged duration as the material degrades via hydrolysis. At day 14, PLGA - shHIF-1α reduced leakage by 50% and lesion thickness by 40%. Also, PEGylated liposomes modified with hyaluronic acid to reduce immunotoxicity were formulated to deliver siVEGF intravitreally. As a result, no significant retinal toxicity was observed 2 weeks post injection, while CNV area was reduced by more than 50% compared to no treatment control [249]. Other targeted nanoparticles were designed for gene delivery following intravenous injection. RGD-labeled liposomes containing a dominant negative Raf mutant gene successfully accumulated in the CNV lesion via integrin targeting and reduced CNV area by 42% compared with no treatment control [250]. A dendrimer system composed of lipidic α–aminocarboxylic acids and a poly-lysine head was also used to complex with anti-VEGF oligonucleotide at a molar charge ratio of 6 and was administered intravitreally in a laser-induced rat CNV model [251]. The particles penetrated to all retinal cell layers and significantly inhibited CNV by approximately 2-fold at 4 months. RGD/transferrin double-labeled PLGA nanoparticles carrying plasmid for Flt23k intraceptor had a larger size of approximately 400 nm, yet accumulated in the laser-induced CNV eye and not the fellow eye [252]. The accumulation was higher with RGD ligand alone than dual RGD/transferrin ligands. RGD and dual-functionalized nanoparticle treatment exhibited 73% and 56% lower CNV areas compared to non-targeted nanoparticles. More recently, Luo *et al.* explored the anti-angiogenic functional effect of systemically delivered plasmid Flt23k with PLGA nanoparticles in a different AMD model generated by inducing CNV via knockdown of sFlt-1 [253]. This model is used because laser injury could result in other complications such as laser's acute injury and retinal burn in addition to pathological state mimicking AMD. As demonstrated by previous studies described above, RGD was an effective targeting ligand against CNV for intravenously injected PLGA nanoparticles of size 400 nm. However, in this study, Luo *et al.* formed smaller 160 nm RGD-coated PLGA nanoparticles containing plasmid Flt23k using a double emulsion technique that had greater sonication power. These nanoparticles also targeted the retina of the laser-induced CNV eye and regressed 53% CNV area in primates, while regressing 43% in a murine sFlt-1-knockdown model without any significant toxicity (Figure 8). Interestingly, this gene therapy was able to regress CNV lesions more significantly than intravitreal anti-VEGF treatment. The promising results from several polymeric systems in AMD models motivates further research in the field to optimize these non-viral nanoparticles to deliver other anti-angiogenic factors as well.

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5. Clinical trials

Clinical trials of gene therapy targeting angiogenesis have mostly consisted of injection of naked plasmid or using viral vectors, and the number of non-viral approaches is gradually increasing (Table 2). The majority of therapeutic angiogenesis clinical studies focus on the use of plasmid VEGF, which is not surprising based on the large number of preclinical studies that have demonstrated efficacy with this approach. The Regional Angiogenesis With Vascular Endothelial Growth Factor (RAVE) trial was the first large-scale phase 2, placebo-controlled study testing the efficacy of intramuscularly injected adenovirus-mediated $VEGF_{121}$ gene in patients with peripheral arterial disease (PAD; i.e. ischemic limb) [254]. Another clinical study of 54 patients with critical limb ischemia treated with intramuscular administration of $pVEGF1_{65}$ showed no amputation reduction, but significant improvement in hemodynamics, skin ulcers, and claudication [255]. Liposomal delivery of $pVEGF_{165}$ and adenovirus-$VEGF_{165}$ resulted in increased vascularity in a phase 2 clinical trial based in Finland on patients with lower limb ischemia [256], but a 10-year follow-up showed no significant difference in the number of amputations or causes of death [257]. Other clinical trials with VEGF have also demonstrated mixed results. In one phase 2 study of 13 patients, myocardial injection of a $VEGF_{165}$ plasmid demonstrated improvements with several outcomes over the standard optimal therapy, including benefits to quality of life, improved angina, and decrease in ischemic segments. However, while some of these benefits were maintained over the course of the study, myocardial perfusion progressively returned to pre-treatment conditions over the 6 and 12 month follow-ups [258]. In an additional clinical study of 93 patients with class 3 or 4 conditions (as determined by the Canadian Cardiovascular Society), there were no significant differences between high dose injections of $VEGF1_{65}$ plasmid DNA over placebo [259].

Apart from VEGF, other factors have been investigated as potential treatments for ischemia as well. In a phase 3 clinical trial, 525 patients with critical limb ischemia were randomly assigned to receive a non-viral plasmid containing the fibroblast growth factor 1 gene or placebo. No significant differences in amputation or death were observed between the two groups [260]. In contrast, significant improvements over placebo in ischemic defect size were observed in another trial of 52 patients with stable angina and reversible myocardial ischemia following intracoronary injection of adenovirus containing the gene for FGF-4 [261]. Gene therapy using HGF has also been investigated in a few small random, double-blind clinical studies of patients with critical limb ischemia. In one of these trials, 106 patients were separated into four groups, receiving either placebo or low, medium, or high doses of plasmid containing encoding for the HGF gene intramuscularly and monitored for differences in tissue perfusion, as determined by transcutaneous oxygen tension. Six months following treatment, a significant benefit to transcutaneous oxygen tension was observed at all doses relative to the placebo group; however, no differences were observed in secondary endpoint values, such as pain relief, wound healing, or major amputations [262]. In another study, 40 patients with peripheral arterial disease with a Rutherford rating of 4 (non-ulcerous) or (ulcerous) were treated with placebo or intramuscular injection of HGF plasmid. Significant changes in both resting pain (non-ulcerous) and ulcer size (ulcerous) were observed in plasmid-treated patients relative to those treated by placebo [263]. In

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addition to the standard growth factors, HIF-1α has also been investigated in a small, phase 1 clinical trial of 13 patients with coronary artery disease. Direct myocardial injection of adenovirus containing the HIF-1α gene showed no significant difference in toxicity in comparison to the placebo. However, benefits with this small sample size were minor and non-significant [264].

Whereas gene delivery-based therapies for ischemic complications focus on the administration of pro-angiogenic factors, similar treatments in cancer are primarily concerned with the inhibition and reduction of tumor vessels through the delivery of endogenous, anti-angiogenic peptide sequences. Here we describe two trials investigating the effects of intratumoral injection of adenovirus containing the gene for human endostatin. The first trial was an exploratory investigation consisting of 15 patients with various solid tumors. Noticeable serum levels of endostatin were observed following viral particle injection with tolerable toxicity at all doses. Treatment was also accompanied by a significant decrease in serum FGF2 levels on day 28 [265]. A second trial is a phase 2 study that investigated the efficacy and toxicity of adenovirus containing an endostatin gene in combination with chemotherapy in advanced head and neck cancers. The patients who received prior chemotherapy or 3–4 treatment cycles benefited from endostatin. Longer progression-free survival and increased disease control rate were seen in comparison to the group without endostatin treatment [266, 267].

As described above, wet AMD is characterized by the excessive growth of leaky, abnormal vasculature that can lead to edema and vision loss. The attenuation of this neovasculature through the inhibition of pro-angiogenic factors, such as VEGF, remain the standard of care and emphasizes the potential opportunity for gene delivery of endogenous angiogenic inhibitors. One such inhibitor, PEDF, was first identified in the pigment epithelial cells of the eye and found to possess important anti-angiogenic properties. Expression of this factor is often reduced in AMD, suggesting that the restoration of its expression would provide a therapeutic benefit. In a phase 1 trial, 28 patients with AMD were intravitreally injected with adenoviral particles containing a plasmid encoding for human PEDF. Toxicity included only minor inflammation and treatable increases in ocular pressure, while benefits consisted of inhibition of lesion growth over a period of 12 months, possibly opening opportunities for the future development of long-lasting treatments [243]. In addition, two other phase 1 trials are currently underway investigating the therapeutic benefits of sFlt-1 gene therapy delivered by intravitreal injection of adeno-associated virus particles [268, 269]. One of them had a one year follow-up report showing decreased center point thickness of 352 μm compared to 552 μm in the control group, as well as increased visual acuity [270].

6. Future directions

The short list of clinical trials for non-viral gene delivery vectors to treat angiogenesis-related diseases reveals that 1) this therapeutic approach is still nascent and 2) nanoparticles need further improvement to overcome shortcomings that have hindered their successful clinical translation thus far. Potential future innovations to gene delivery nanoparticle and genetic cargo design are discussed below and could lead to next generation technology able to modulate angiogenesis with enhanced safety and efficacy.

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The successful delivery of a gene of interest by a non-viral vector to the target cell is affected by several physicochemical properties of the vector used and the resulting nanoparticle. Although there have been many different approaches to nanoparticle-mediated gene delivery, certain physicochemical property trends have been observed. For example, although nanoparticles over a wide range of sizes, including up to 400 nm as described above, have been used for intracellular non-viral gene delivery, nanoparticles of approximately 100 nm in size or smaller tend to be the most promising due to enhancement in extracellular transport as well as enhanced cellular uptake via clathrin-mediated endocytosis [271] and caveolae-mediated endocytosis [272]. Similarly, neutrally charged particles [41] are found to resist non-specific interactions with off-target biomolecules and cells, best ensuring that the nanoparticles reach their desired target in vivo. When designing future nanoparticle strategies and evaluating them in vitro, an important consideration is that the nanoparticle properties that maximize in vitro transfection may make poor choices for therapeutic delivery. For example, larger particles with high positive surface charge may effectively transfect cells in vitro due to the sedimentation of the large particles and the electrostatic attraction of positively charged particles to the negatively charged cell surface. Yet, these same particles would make poor choices for therapeutic use due to their likelihood for quick aggregation and clearance. An approach that can be useful to mitigate this screening and identification challenge for the design of new nanoparticles is to make the in situ and in vitro analysis conditions as similar to the in vivo conditions as possible, such as by measuring the effect of serum on particle stability and transfection [273] and using novel inverted cell culture conditions to eliminate the role of sedimentation [274].

When considering biomaterial structure to compose next-generation gene delivery nanoparticles, degradable linkages as previously described are key to both alleviate potential cytotoxicity concerns of the biomaterial as well as facilitate nucleic acid release from the nanoparticle carrier. When designing new structures, comprehensive and systematic analysis of structure-activity relationships for the particular biomaterial can be enabling. For example, principal component analysis (PCA) and logical analysis of data (LAD) were performed in two separate studies to correlate specific properties of PBAE nanoparticles to their transfection efficacy [275, 276], demonstrating design principles for this class of materials. Of the many parameters to consider, biomaterial properties such as hydrophobicity and molecular weight have been found to be critical for a range of biomaterials, with a higher degree of hydrophobicity and a higher molecular weight often leading to both improved transfection and increased cytotoxicity, leading to a biphasic response with intermediate hydrophobicity and molecular weight being optimal [39, 275, 277]. Depending on the particular application, optimal design must also consider additional vector parameters relating to the crossing of disease- and tissue-specific barriers, cell-specific targeting and intracellular uptake, and endosomal escape and nucleic acid release rate.

Depending on the disease and treatment method, both local gene therapy and cell therapy following *ex vivo* gene transfer may require repeated administration to compensate for transient gene expression. While systemic delivery of nanoparticles may facilitate repeated treatments, nanoparticle stability in blood circulation and resistance to clearance can be a challenge. While many groups have studied the effect of PEG molecular weight and its

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surface density in order to improve nanoparticle stability and half-life [278], there are other strategies to promote resistance to clearance as well. Some groups have focused on investigating biomaterial structural and chemical properties to better condense DNA and improve particle stability [279]. Other innovative approaches to nanoparticle delivery consider aspects such as geometry and anisotropic shapes to extend circulation of the nanoparticle [280, 281]. Additional methods may focus on extending particle circulation by mimicking biology and camouflaging a synthetic particle. For example, the inclusion of "self" biological signals on particles can reduce nonspecific cellular uptake [282].

Biomimetic materials are an exciting area of future growth for enhanced nanoparticles for gene delivery. For example, biological membranes, including the plasma membrane as well as membranes from subcellular compartments such as exosomes, have been evaluated either as the sole vector to form nanovesicles encapsulating nucleic acid or as the surface coating to pre-formulated nanoparticles [283, 284]. Because this material can be salvaged from a host's cells, the resulting nanoparticles can potentially be able to efficiently evade rapid clearance or immune response from the body [285]. Further, studies have shown that membranes from specific cancer cells contain membrane proteins that can direct specific homing towards target tissues [286]. Research into these materials is still at a nascent stage, but it is already clear that loading of genetic material, such as siRNA by electroporation, can be accomplished, as can membrane coatings of hard nanoparticles. By enhancing circulation time, avoidance of the immune system, and tissue targeting, biomimetic coatings of nanoparticles for gene delivery to treat angiogenesis-related diseases is a promising approach.

From the genetic cargo side, there has been continuous effort in the field to prolong the transient nature of non-viral gene expression from DNA plasmid delivery. One of the most exciting new methods is the use of the CRISPR / Cas-9 system to insert an exogenous gene in the host's genome site-specifically [287]. This technique not only prolongs the expression of a delivered gene, but minimizes the risk of mutagenesis and tumorigenesis arising from random insertion. By achieving safe and long-term expression of exogenously delivered genes and/or knockdown of endogenous genes, non-viral nucleic acid delivery nanoparticles can be enabled to potentially cure diseases at the genetic level. However, imbalanced growth or reduction of vasculature from uncontrolled long-term gene therapy may lead to other pathological complications. Through pre-clinical and phase I clinical studies, it was shown that high level of exogenous VEGF or its viral expression can cause severe edema, limb loss, and hemangiomas [288]. This may be less of a concern for non-viral systems compared to viral systems because of the nature of transient gene expression, although it is still a concern. There must be a balanced approach in developing biomaterial and biomolecular tools that direct gene expression as the control of gene expression, spatially and over time at the therapeutic site, is paramount. In addition to endothelial cells as a target, mural cells, such as pericytes, are also of critical importance for vessel stabilization. Thus, there is significant potential for future directions that utilize combinatorial treatment strategies that act on both endothelial cells as well as mural cells for both pro-angiogenic and anti-angiogenic therapies [289–293].

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Finally, non-canonical biologics also hold promise as potential therapeutics. For example, as mentioned in anti-angiogenesis section, computational biology approaches have enabled identification of cryptic peptide motifs that have strong anti-angiogenic activity as well as biomimetic peptide analogs [177, 294, 295]. Such peptide motifs can be genetically encoded and engineered into a plasmid for delivery singly or in combination with gene delivery nanoparticles.

7. Conclusion

Taken together, the studies described in this review emphasize the potential use of non-viral gene delivery nanoparticles for the modulation of angiogenic processes. Angiogenesis is a critical component in the progression of several disease states and remains a prime candidate for their therapeutic intervention. Although the overall process is markedly complex, growing knowledge in the field has provided the identity of key factors as well as revealed their interplay in a tightly regulated system. Gene therapy allows for the manipulation of these interactions in order to shift the balance of angiogenic signaling, either positively or negatively, away from pathological abnormalities. Currently, the greatest clinical progress has been made with gene-loaded viral particles or injection of free DNA. Nonetheless, the rapid advancements in the field of nanotechnologies have opened opportunities to investigate their use as *in vivo* gene delivery vectors. Notably, non-viral nanoparticles can provide improved stability, cellular targeting, and internalization over free nucleic acid and are relatively easy to produce, are highly customizable, and can be designed for lower toxicity than viral counterparts. New approaches to biomaterial and nanoparticle construction, including biomaterial libraries as well as rationale design, are promising to further improve the efficacy of gene delivery nanoparticles. Unfortunately, despite much promise in pre-clinical animal models, angiogenesis-based gene therapy has only shown modest benefits thus far in the clinical setting. Challenges in dosing, transfection efficiency, safety, and an incomplete understanding of the biology in certain microenvironments are compromising factors. Therefore, there is a need to further develop our understanding of non-viral gene nanoparticles and the biology of angiogenesis to optimize the selection and delivery of genetic material to target tissues to treat angiogenesis-dependent diseases.

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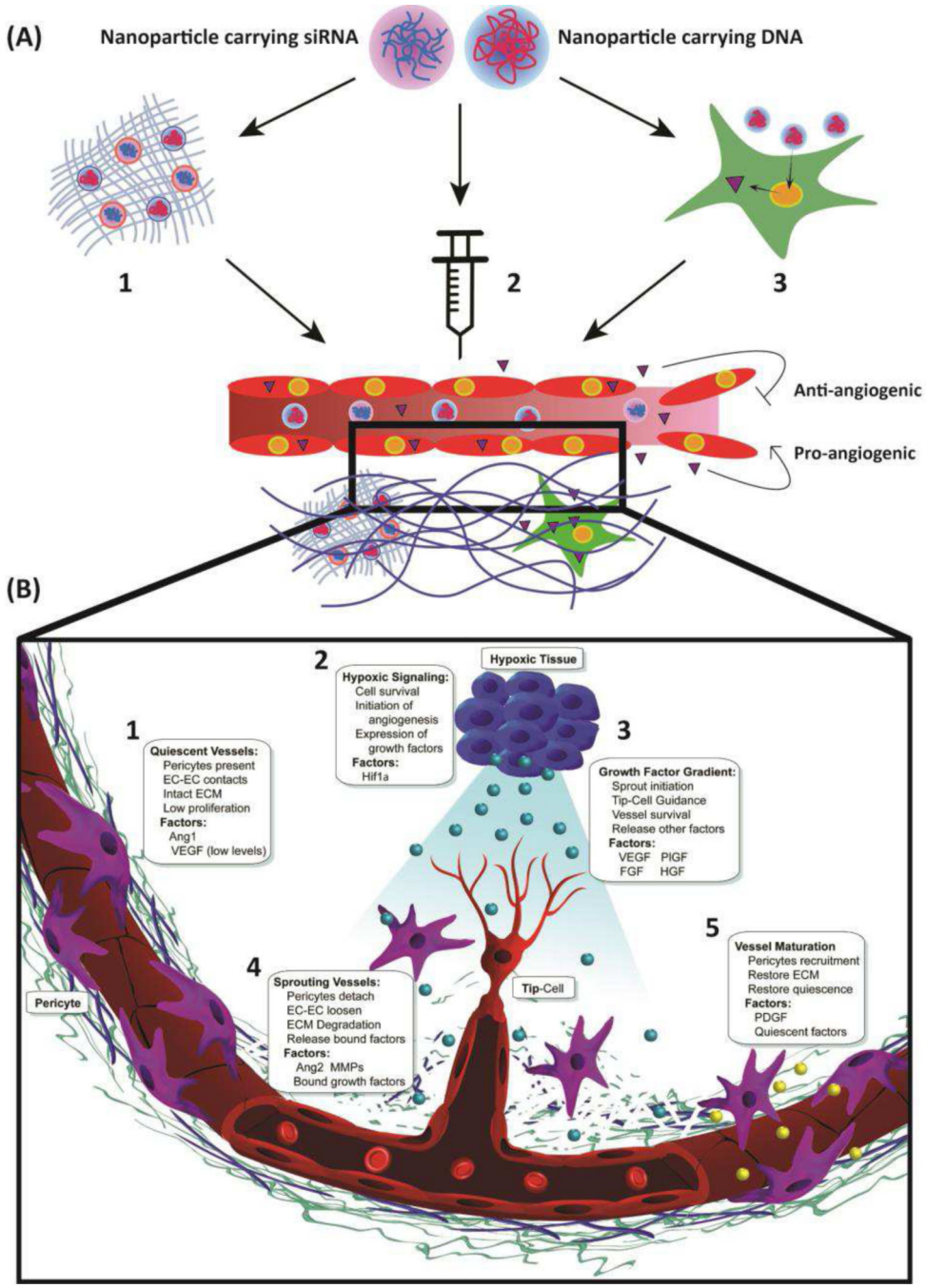

Figure 1.

(A) Schematic showing modulation of angiogenesis by gene therapy using non-viral nanoparticles. Nanoparticles containing nucleic acids related to angiogenic or anti-angiogenic signals are delivered to a target tissue by: 1) Macroscale devices encapsulating nanoparticles that carry nucleic acids (such as a nanoparticle-eluting gel); 2) Systemic administration of nanoparticles carrying nucleic acids; and 3) Cells that are transfected *ex vivo* with nucleic acid-containing nanoparticles. (B) Schematic detailing the major molecular processes related to angiogenesis using vessel growth in response to tissue

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hypoxia as an example, including 1) vessel quiescence, 2) hypoxic initiation, 3) growth factor signaling, 4) vessel sprouting, and 5) vessel maturation.

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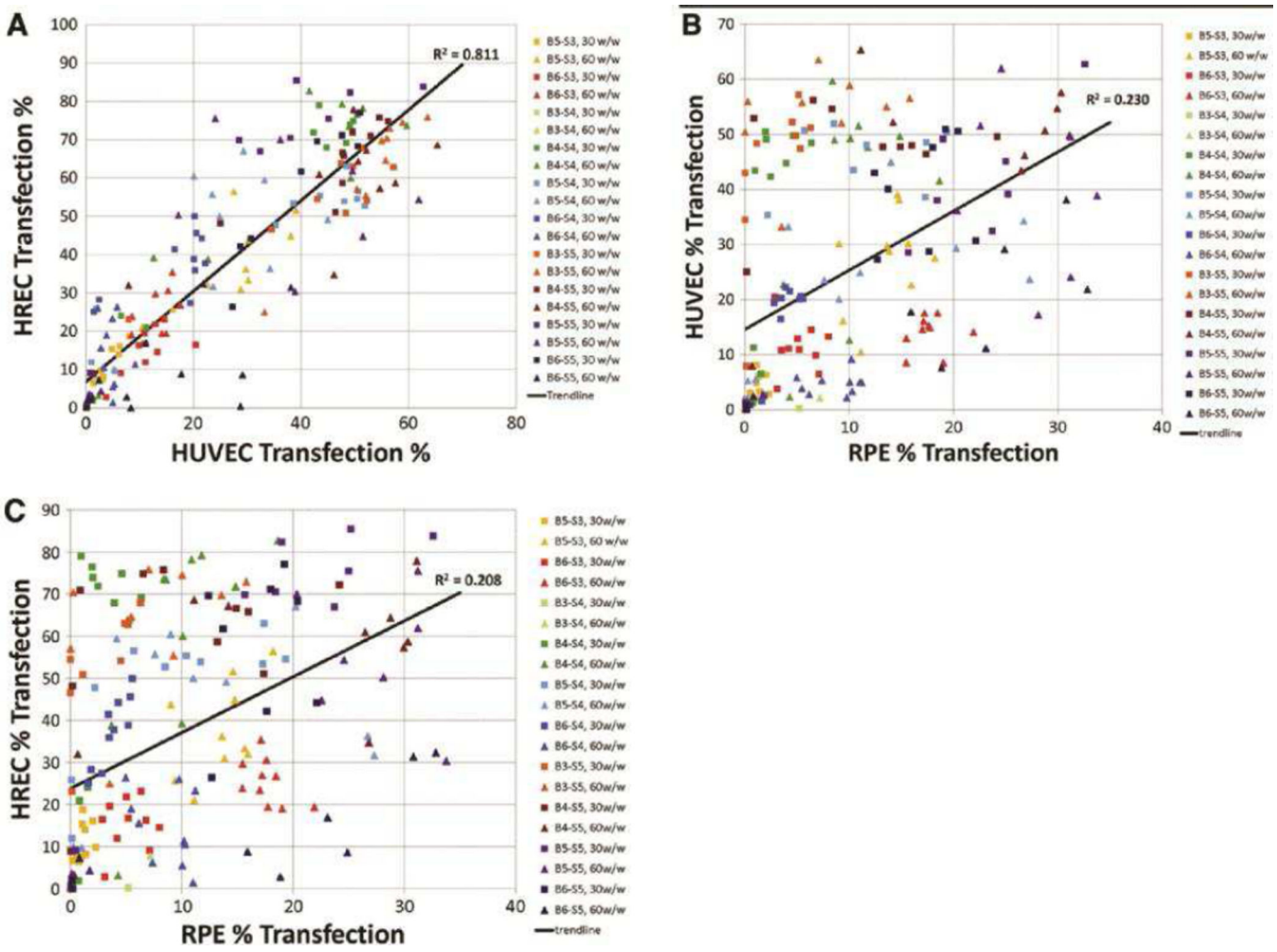

Figure 2.

Non-viral nanoparticles formulated with a library of PBAE polymers demonstrate high efficacy for transfection of human endothelial cells. Additionally, polymer structure can tune cell-type efficacy and demonstrates (A) a strong correlation in transfection between macrovasculature (HUVEC) and microvasculature (HREC), but (B/C) weaker structural correlation between endothelial and epithelial cells. Adapted from ref [39].

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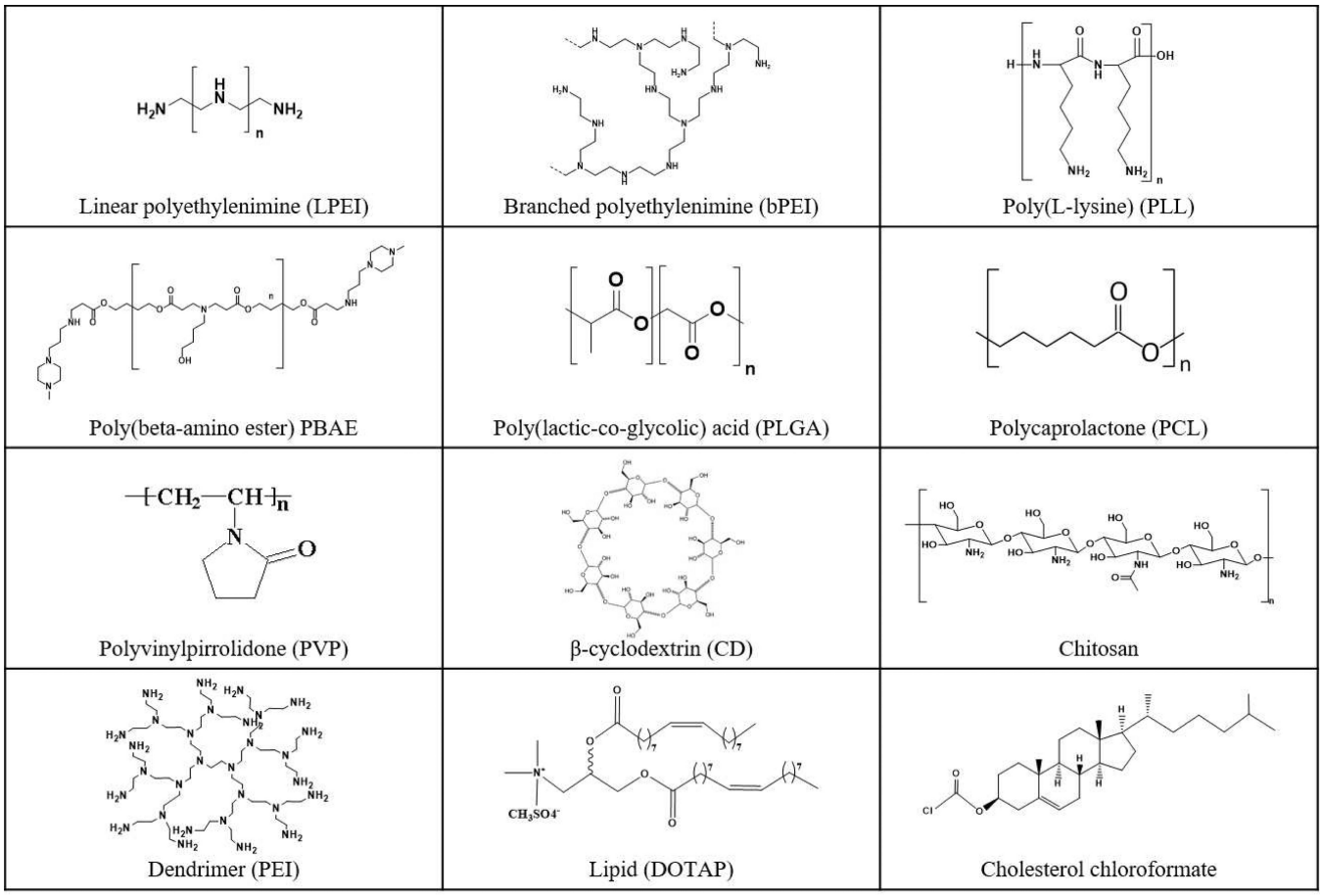

Figure 3.

Chemical structures of various non-viral vectors that can be used to fabricate gene therapy nanoparticles to modulate angiogenesis.

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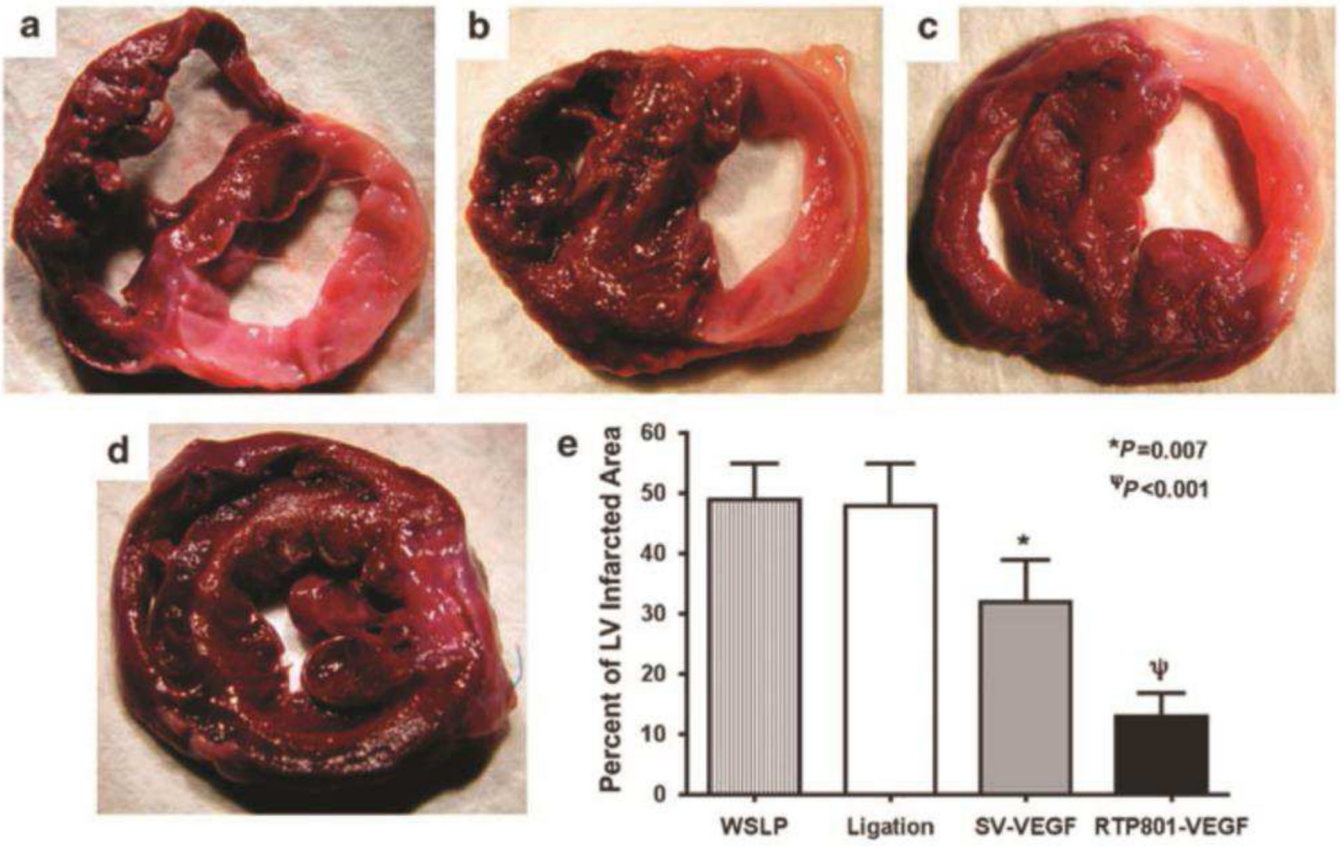

Figure 4.

Size of left ventricular infarct following (a) no injection, or injection of (b) water-soluble lipopolymer (WSLP) alone, (c) WSLP with plasmid encoding constitutively-expressed VEGF, and (d) WSLP with plasmid encoding ischemia-inducible VEGF. Infarcted fibrotic tissue appears whitish-pink. (**e**) The percentage of the ratio of infarcted to non-infarcted left ventricle. Adapted from ref [119]

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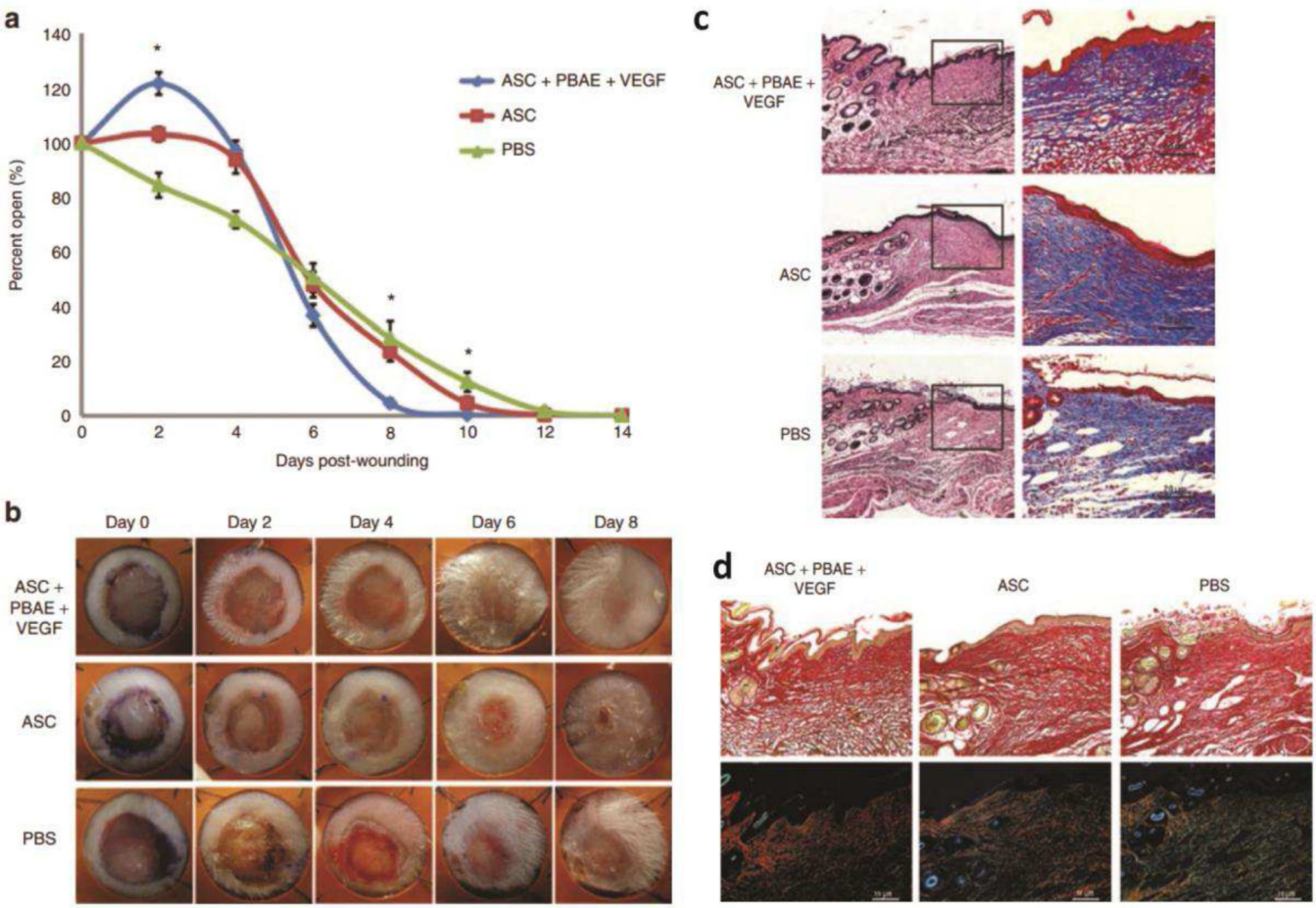

Figure 5.

(**a**) Percent of open wounds evaluated every 2 days post-wounding and treatment with PBAE/VEGF nanoparticle-transfected ASCs, non-modified ASCs or PBS control. (**b**) Wounds treated with PBAE-pVEGF nanoparticle transfected ASCs showed accelerated closure with full epithelialization observed by day 8. (**c**) H&E and Masson's trichrome staining shows increased cellularity and collagen deposition in the dermis treated with ASC-transplanted groups than PBS. (**d**) The PBAE-pVEGF-transfected ASC-treated group shows the most abundant mature collagen fibers (red-orange), whereas PBS-treated group showed the highest level of immature collagen fibers (green-yellow). Adapted from ref [151] .

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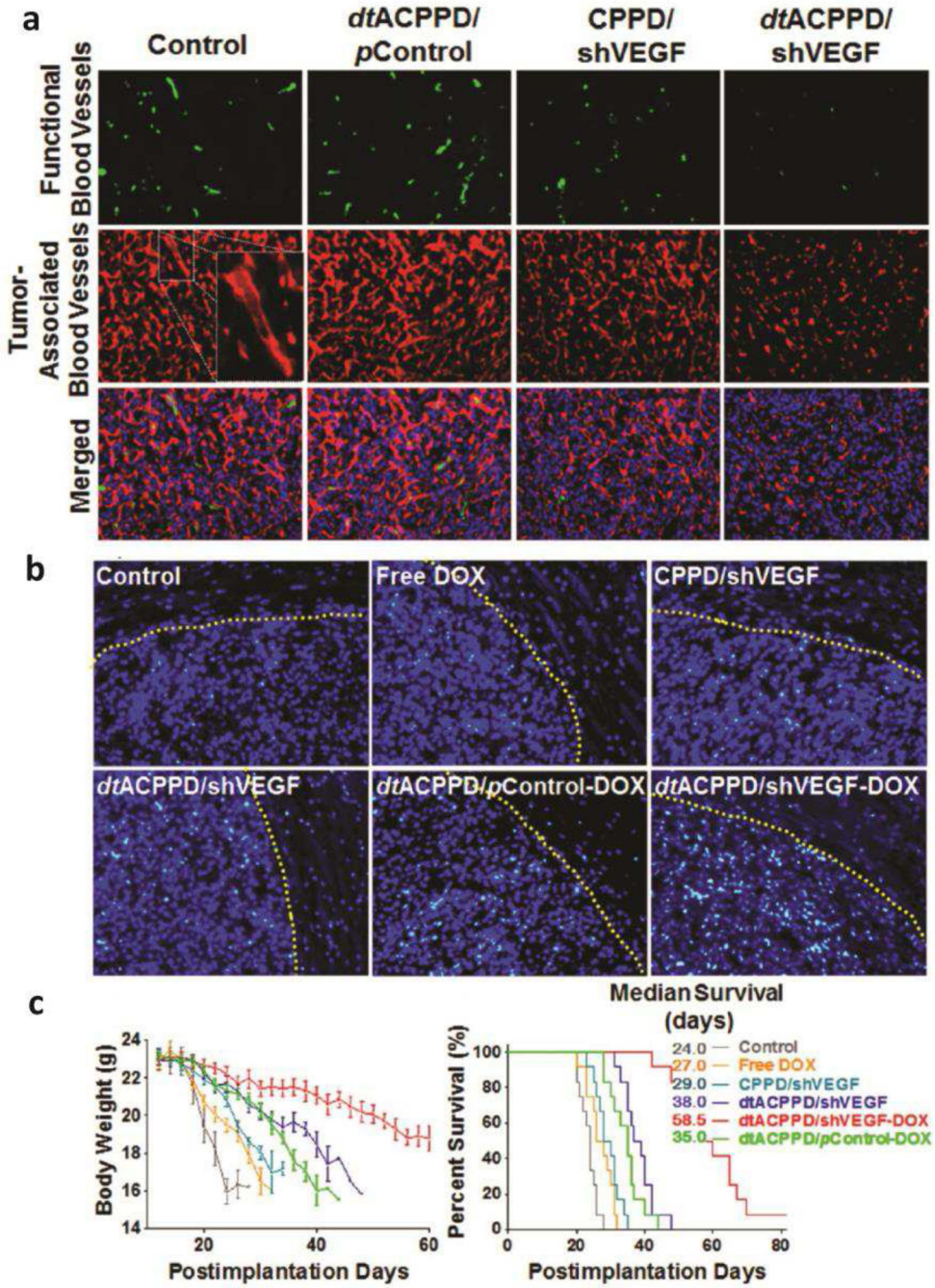

Figure 6.

pH- and MMP-activatable cell-penetrating peptide conjugated to dendrimer-based PLL-PEG (dtACPPD) successfully delivers shVEGF to enable nanoparticle-mediated anti-angiogenesis gene therapy combined with doxorubicin delivery. (a) Functional blood vessels visualized by lectin (green) and tumor-associated blood vessels by anti-CD34 (red). (b) Histological images of apoptotic cells in glioma tissues using the TUNEL assay (green: apoptotic cells, blue: DAPI). Antitumor efficacy shown by (c) average change in body

weight (left) and overall survival (right) of glioma-bearing mice. Original magnification: 200X. Adapted from ref [224]

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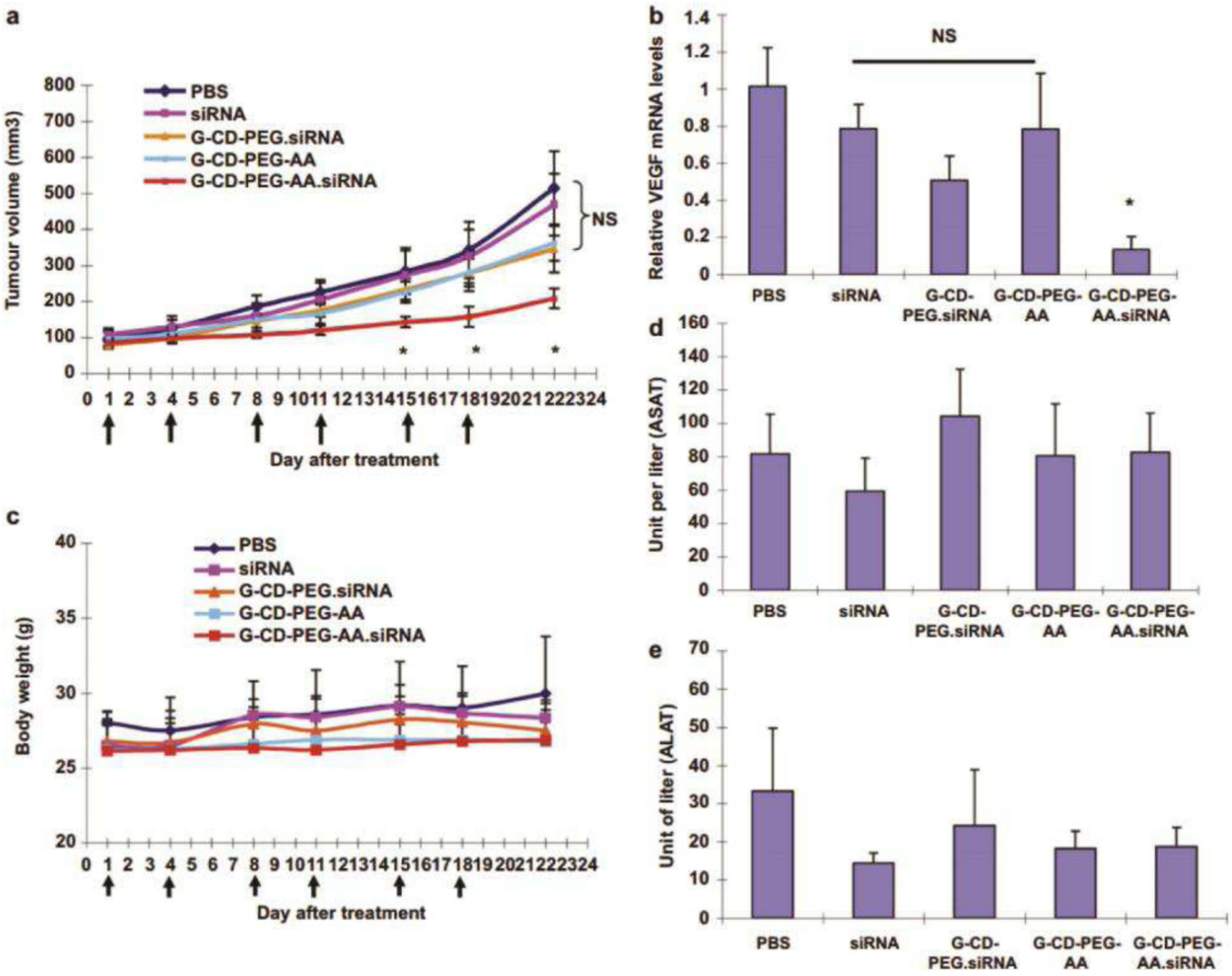

Figure 7.

Antitumor efficacy following systemic delivery of guanidine-cyclodextrin-PEG-anisamide (G–CD–PEG–AA)-siVEGF complex evaluated by (a) tumor volume, (b) VEGF mRNA knockdown, and (c) body weight concurrent with tumor treatments. Liver enzyme levels, (d) aspartate aminotransferase (ASAT) and (e) alanine aminotransferase (ALAT) (mean ± SD shown; NS = no significance, $*p < 0.05$). Adapted from ref [228]

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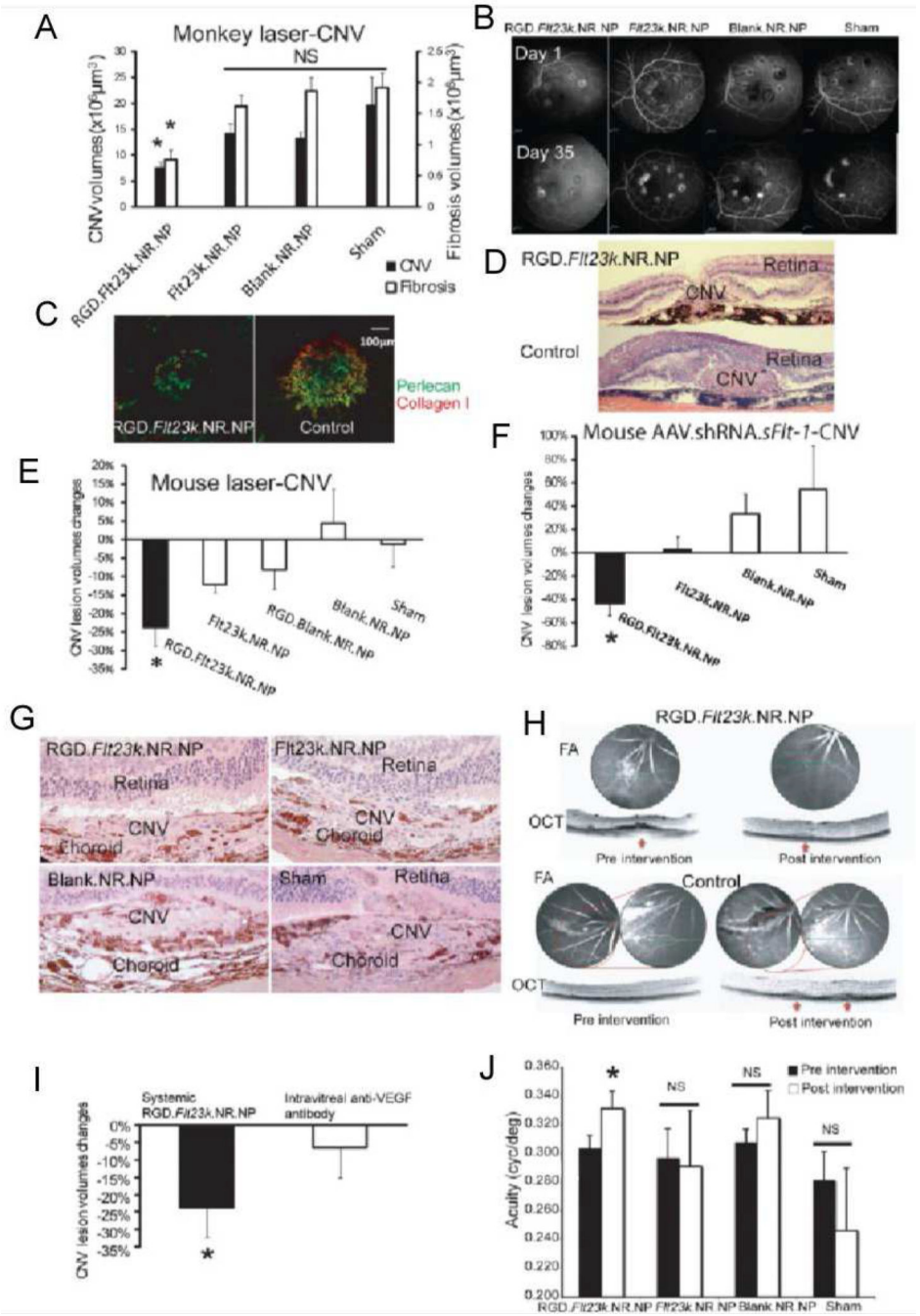

Figure 8.

(A–D) Significant reduction of CNV lesion volume (A) shown by fluorescein angiograph (FA) (B), immunohistochemistry staining (C) and H&E staining (D) after RGD-PLGA-psFlt23k nanoparticle treatment in a laser-induced CNV non-human primate model. (E–F) Regression of CNV lesion by RGD-PLGA-psFlt23k nanoparticles in a laser-induced CNV mouse model 2 weeks post treatment (E) and in the sFlt-1 knockdown induced CNV mouse model 4 weeks after treatment (F). (G–H) H&E (G) and FA/OCT (H) images of each group in panel F show the sizes of CNV lesions after treatment. (I) Single systemic injection of

RGD-PLGA-psFlt23k nanoparticles regressed CNV lesions more than intravitreal anti-VEGF antibody. (J) Partial restoration of visual acuity as determined by optomotor response 4 weeks after RGD-PLGA-psFlt23k nanoparticle treatment. Adapted from ref [253].

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Table 1

Representative examples of angiogenesis modulation via non-viral nanoparticle-based gene delivery

Angiogenesis	Disease	Factor	Delivery Vector	Animal Model	Injection	Result	Reference
Pro	*Ischemic Limb*	$VEGF_{165}$	Heparin-PEI	Mice	Intramuscular	Over 3-fold ↑ neovasulazation vs. PEI in ischemic limb	[116]
		VEGF	PBAE	Mice	exwVoHUVEC transfection	Limb salvage 50% compared to lipoplex (30%) and PEI (12.5%)	[140]
		eNOS	Elastin-based polymer	Mice	Intramuscular	~ 3-fold ↑ perfusion ratio and blood vessel density vs. control	[120]
	Myocardial In fact	Adreno-medulliln	Dextran	Rats	ex *vivo* MSC transfection	2.5-fold ↑ capillary density and 40% ↓ infarct size vs. control	[131]
		$VEGF_{165}$	Chitosan-g-PEI	Rats	Intramyocardial	1.6-fold ↑ejection fraction and vessel density vs. PEI control	[117]
		Hypoxia-inducible $VEGF_{165}$	Cholesterol chloroformate-bPEI	Rabbits	Intramyocardial	Myocardial infarct size (36% ↓) vs. vector alone without hypoxia-inducible VEGF165	[119]
		$VEGF_{165}$	PLGA	Rabbits	Intramyocardial	Capillary density (38% ↑) vs. plasmid only	[124]
	Tissue Engineering	SHh	PBAE	Mice	Transdermal	15% ↑ wound closure than PBS	[150]
		VEGF	PBAE	Mice	ex *vivo* ASC transfection	20% ↑ wound closure than non-modified ASC	[151]
		PDGF-B	PEI	Rats	collagen scaffold with PEI-pPDGF	40% bone volume and 52-fold ↑ connective density vs. control	[154]
Anti	*Cancer*	sFlt-1 or siVEGFRI	bPEI-g-PEG-cRGD	Mice	Intravenous	3-fold / 42% ↓ colon carcinoma umor growth vs. PEI-g-PEG	[214,216]
		NF-KB	Tween-SS-bPEI	Mice	Intravenous	Breast tumor volume 6% of saline	[218]
		Endostatin	PVP	Mice	Intramuscular	60% ↓ metastatic brain tumor growth vs. control by MRI	[220]
		shVEGF/ DOX	CPP-dendrimer PLL	Mice	Intravenous	VEGF mRNA (65% ↓) and median survival (2-fold ↑) vs. control in orthotopic glioma	[224]
		siVEGF	guanidine-βCD-PEG-anisamide	Mice	Intravenous	VEGF mRNA(3.5-fold ↓) / prostate tumor growth(3-fold ↓) vs. control	[228]
	Age-related Macular Degeneration	Vasostatin	PVP	Rats	Intramuscular	32.5% / 48% decrease in CNV incidence and area at day 42	[247]
		shHIF-1α	PLGA	Rats	Intravitreal	Leakage (50%↓) / lesion thickness (40%↓) vs. control	[248]

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Angiogenesis	Disease	Factor	Delivery Vector	Animal Model	Injection	Result	Reference
		Flt23k	RGD-PLGA	Monkey/ Mice	Intravenous	CNV area 53% (monkey) and 43% (mice) ↓ vs. sham	[252]

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Table 2

Clinical Trials of Gene Therapy Targeting Angiogenesis

Angiogenesis	Disease	Factor	Delivery Vector	Phase	Patient	Country	Result	Reference
Pro	*Ischemia*	$VEGF_{121}$	Adenovirus	2	105	USA	Similar end-point (peak walking time, ankle-brachial index, claudication, quality of life (QOL)) between placebo and low /high dose treatment	[254]
		$VEGF_{165}$	naked	-	54	Netherlands	No improvement in pain / amputation rate; significant improvement in hemodynamics and skin ulcers	[255]
		$VEGF_{165}$	Adenovirus / liposome	2	54	Finland	Catheter-mediated injection; anti-adenovirus increased in 61% patients; increased vascularity distal (both) and ischemic site (adenovirus); long-term (10yr) safety; no diff. in amputations	[256, 257]
		FGF-1	naked	3	525	30 countries	No significant difference between treated vs. placebo group in amputation / death rates	[260]
		HGF	naked	-	106	-	No significant difference in end-point (amputation, ankle-brachial index, wound healing, pain relief); transcutaneous oxygen tension ↑ in high dose	[262]
		HGF	naked	-	40	Japan	Safe; significant increase in rest pain, ulcer size reduction, and QOL vs. placebo	[263]
	Coronary Artery Disease	$VEGF_{165}$	naked	1/2	13	Brazil	Transitory in myocardial perfusion; 1-yr improvement in treadmill step test, oxygen consumption, and QOL vs. placebo	[258]
		VEGF	naked	-	93	Canada	Significant decrease in lesion area; no difference in myocardial perfusion, anginal symptom and treadmill time vs. placebo	[259]
		FGF-4	Adenovirus	-	52	USA	Significant ↓ in ischemic defect size; significant ↑ in reversible perfusion defect size (w/o 1 outlier). Currently phase 3; status unknown (updated 2013)	[261]
		HIF-1α	Adenovirus	1	13	-	Safe; too small sample size to make comparisons	[264]
Anti	*Cancer*	Endostatin	Adenovirus	1	15	China	Safe; anti-adenovirus antibody in serum; ↑ endostatin in serum; ↓ in FGF level vs. placebo	[265]
		Endostatin	Adenovirus	2	136	China	Combination with chemotherapy; benefit for prior chemotherapy-treated patients; longer progression-free survival; ↑ disease control rate in groups treated with endostatin	[267]
	Wet AMD	PEDF	Adenovirus	1	28	USA	No severe adverse effects at high dose of 10^a PU; no mean increase in lesion size at 6 & 12 months	[243]
		sFLT-1	AAV	1	8	Australia	Subretinal injection; no adverse effect; lack of fluid in retina; center point thickness ↓; visual acuity ↑	[269, 270]

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Angiogenesis	Disease	Factor	Delivery Vector	Phase	Patient	Country	Result	Reference
		sFLT-1	AAV	1	Up to 34	USA	Ongoing	[268]