Molecular Therapy
Nucleic Acids
Review

Gene therapy to enhance angiogenesis in chronic wounds

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Skin injuries and chronic non-healing wounds are one of the major global burdens on the healthcare systems worldwide due to their difficult-to-treat nature, associated co-morbidities, and high health care costs. Angiogenesis has a pivotal role in the wound-healing process, which becomes impaired in many chronic non-healing wounds, leading to several healing disorders and complications. Therefore, induction or promotion of angiogenesis can be considered a promising approach for healing of chronic wounds. Gene therapy is one of the most promising upcoming strategies for the treatment of chronic wounds. It can be classified into three main approaches: gene augmentation, gene silencing, and gene editing. Despite the increasing number of encouraging results obtained using nucleic acids (NAs) as active pharmaceutical ingredients of gene therapy, efficient delivery of NAs to their site of action (cytoplasm or nucleus) remains a key challenge. Selection of the right therapeutic cargo and delivery methods is crucial for a favorable prognosis of the healing process. This article presents an overview of gene therapy and non-viral delivery methods for angiogenesis induction in chronic wounds.

INTRODUCTION

The skin, the largest organ in the body, serves as a life-protective barrier at the interface between the body and external environment.[1] It plays pivotal roles in different processes such as protection and prevention of dehydration,[2] sensory functions,[3] protection against chemicals and pathogens,[4] initialization of vitamin D synthesis,[5] excretion of water and salts through the production of sweat, and thermoregulatory functions.[6] Thus, disruption of the skin integrity as a consequence of an injury can be life threatening and must be promptly restored.

Skin wound healing involves an intricate synchronization of several different cell types, growth factors, and cytokines, and can be divided into four highly integrated and overlapping phases whose outcome will result in the restoration of skin integrity and functions: hemostasis, inflammation, proliferation, and maturation (or remodeling).[7] The wound-healing phases and their biophysiological functions must occur in the proper sequence, at a specific time, and continue for a definite duration with the proper intensity.[8] An interruption in one or more phases of this process, and/or failure to progress through the normal coordinated manner of healing, could lead to the development of delayed acute and non-healing chronic wounds.[9] There are many pathologies that could lead to inadequate wound healing, including peripheral vascular disease, diabetes mellitus (DM), cancer, ischemia, or physical pressure at the wound site.[10] For example, patients with DM suffer from multiple complications during their lifetime, with diabetic foot ulcers (DFUs) being one of the most serious ones.[11] DFUs are chronic wounds that do not heal, leading in the majority of the cases to amputations, thus seriously reducing the quality of life of those patients.[12] These wounds constitute a major health burden, being the single largest reason for hospitalization and mortality among diabetic patients.[13]

In chronic and diabetic wounds, healing does not follow the normal process and is stalled at the initial phases. This stagnation commonly occurs in combination with unresolved inflammation and the presence of infections.[14,15] Impaired healing is the result of complex pathophysiologic processes, such as microbial invasion, decreased vascularization and oxygenation supply, epithelial breakdown, impaired keratinocyte proliferation and migration, elevated oxidative stress, and impaired immune function.[16,17]

Upon injury, the microvasculature is disrupted, leading to fluid accumulation, inflammation, and the development of hypoxia.[18] The formation of new blood vessels from pre-existing vessels for the provision of oxygen and nutrients in the wounded tissue during the healing process is a critical step and is known as angiogenesis.[19] An impaired angiogenic response is one of the most important factors that affects all diabetic ulcerations and plays a significant role in impaired chronic and diabetic wound healing.[20] Therefore, therapies targeted to induce or promote angiogenic pathways can be considered as a promising approach to accelerate chronic wound healing.[21]

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Evidence from numerous studies indicates that there are many possible approaches to induce angiogenesis: use of angiogenic growth factors, gene and nucleic acid-based therapies, genetic manipulation and stem cell therapies, angiogenic small molecule drugs, or even nature-inspired biomaterials and scaffolds with bespoke physical, chemical, or biological properties.[22] Among those, gene and nucleic acid-based therapies have the largest potential to become new treatments in chronic wound healing.

Gene therapy as a novel and promising therapeutic platform has the potential for a more accurate and personalized treatment of diverse life-threatening diseases.[23,24] It can offer selective correction of critical angiogenesis pathways that are dysregulated or defective at their genetic roots.[25] The most commonly explored therapeutic molecules for the modulation of gene expression include plasmid DNA (pDNA) and messenger RNA (mRNA) for overexpression of genes, and smaller short interfering RNA (siRNA), microRNA (miRNA), oligonucleotides, and aptamers for post-translational gene silencing.[26] In addition, the gene-editing tool clustered regularly interspaced short palindromic repeats (CRISPR) is boosting the development of new gene-therapy-based medicines.[27] Depending on the application, gene therapy can be used for gene augmentation (adding a gene to a cell), gene silencing (inactivation of a gene), and gene editing (revising the existing genetic code).

While topical application of gene and nucleic acid (NA) products to the skin and wounds is easy to perform,[28] they require appropriate delivery systems in order to reach their intracellular target site. This is because NAs have poor cellular uptake due to their negatively charged backbones and high molecular weight. In addition, on their way to the target cells they are prone to degradation[29] and must overcome tissue barriers.[30]

NA delivery systems are generally classified into two main groups: viral and non-viral gene delivery systems.[31,32] Viral vectors are derived from pathogenic parent viruses from which the viral-encoded genes are removed and substituted with therapeutic genetic sequences.[33] The most commonly used viral vectors in preclinical and clinical studies are the adenovirus (AV), adeno-associated virus (AAV), retrovirus (RV), and the lentivirus (LV).[34] Although viral vectors have shown considerable transfection efficacy in studies for gene delivery, their use is hampered by concerns of insertional mutagenesis and systemic inflammatory responses.[35,36]Non-viral gene delivery methods have emerged as safer alternatives to viral vectors and can be broadly classified into two main groups: chemical (vector-assisted delivery systems) and physical methods.[37,38] NAs can be packaged into chemical-based vectors, which can deliver the NAs into cells via endocytosis or membrane fusion. Non-viral chemical vectors can be prepared from a multitude of materials, including cationic lipids, cationic polymers, dendrimers, polypeptides, and inorganic nanoparticles (NPs).[39,40] Physical approaches, on the other hand, make use of a physical force or trigger to permeabilize or penetrate the cell membrane, so that externa molecules can migrate directly into the cytosol. Physical delivery methods include microinjection, ballistic gene delivery, electroporation, sonoporation, mechanoporation, and photoporation.[41,42]

In this review, we provide an overview of the different classes of NAs that, together with non-viral delivery methods, have emerged as attractive new treatment modalities to treat chronic wounds, especially under diabetic conditions. In particular, we will focus on gene therapies aimed at inducing angiogenesis signaling pathways. First, we will discuss strategies based on gene augmentation, followed by gene silencing, and finally gene editing. Special attention will go to highlighting the delivery methods that were used in the various studies as they form an intrinsic part of the therapeutic approach.

GENE AUGMENTATION-BASED THERAPEUTICS FOR WOUND ANGIOGENESIS

The most straightforward strategy for gene therapy is gene augmentation, which refers to a procedure for correcting deficiencies caused by a missing or defective gene. The objective of gene augmentation is to restore normal cellular function through the expression of selected proteins. Gene augmentation can be achieved by introducing DNA or mRNA in the target cells (Figure 1).[43] The gene augmentation approaches that were proven to specifically regulate angiogenesis within a wound-healing context are summarized in Table 1.

DNA

Protein expression through gene augmentation is commonly achieved by inserting a gene sequence of interest into an expression cassette in the form of pDNA or minicircle DNA. When delivered to the nucleus of the target cells it will lead to high and persistent protein expression.[73]

The use of DNA in gene therapy is considered one of the most common and straightforward strategies in gene augmentation therapy as only one DNA molecule can produce multiple copies of mRNAs.[74] Despite its potential, efficient delivery of DNA molecules is still challenging due to their large molecular size and the fact that they have to reach the nucleus in order to be transcribed into the corresponding mRNA.[75,76] In addition, they carry a potential risk of integration into the host genome, which may result in insertional mutagenesis.[77,78] Furthermore, non-dividing cells and several differentiated cell types are relatively resistant to the expression of transgenic DNA, probably because of limited nuclear entry.[79]

pDNA

A pDNA is a small, circular, double-stranded extrachromosomal DNA molecule that is most commonly found in bacterial cells, but is also present in some eukaryotes. In bacteria, this type of DNA carries only a small number of genes. It can replicate independently of the bacterial chromosome, which benefits the survival of the organism.[80] The possibility of designing pDNA to carry specific DNA fragments or therapeutic genes turned this macromolecule into a powerful vector in gene therapy. After internalization into the nucleus of the target cell through viral or non-viral methods, it is transcribed to

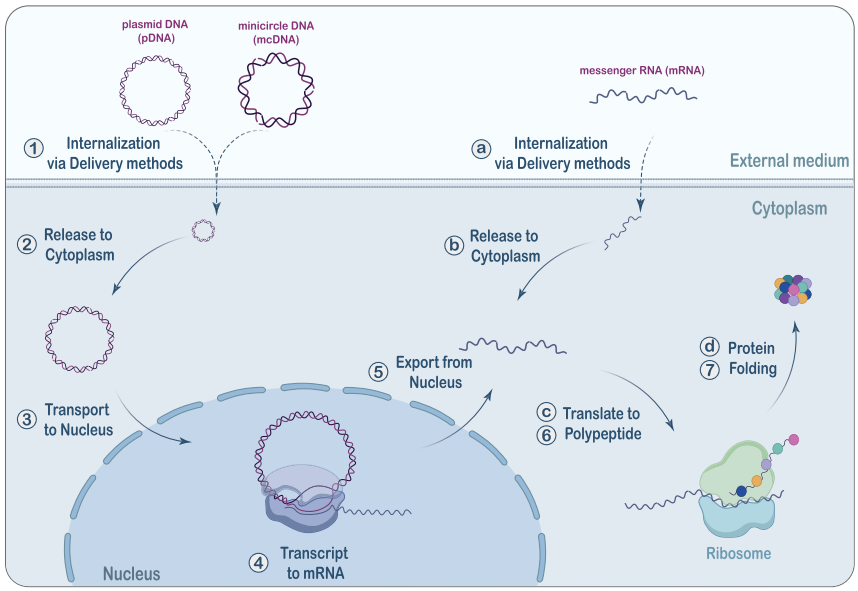

Figure 1. Schematic representation of gene augmentation and protein expression upon cell transfection with mRNA or pDNA

(1) First, therapeutic nucleic acids (pDNA or mcDNA) are internalized into the cells via viral or non-viral methods, and then (2) released into the cytoplasm. (3) The released pDNA or mcDNA is transported into the nucleus (4) where the gene expression cassette is transcribed in mRNA. (5) The transcribed mRNA is exported from the nucleus to cytoplasm (6), where the cellular translation machinery (ribosomes) is located, and translated into protein. (7) Finally, the nascent polypeptide is then folded to become the functional protein. For mRNA, (a) after cell internalization and (b) release into the cytoplasm, (c and d) it is translated directly into the corresponding protein.

mRNA, which can express the protein that is lacking in the cells (Figure 1).

Intracellular pDNA delivery for increasing angiogenesis in wounds, for instance, has been done with electroporation, in which a train of electrical pulses is applied to cells in order to induce the formation of transient pores in cell membranes.[81] For example, Lee et al. used syringe electrodes under optimal conditions (six pulses of 100 V/cm for 20 ms) for delivery of pDNA encoding for transforming growth factor β1 (TGF-β1) into a diabetic mouse excisional wound model.[44] Five to 7 days after intradermal injection of plasmid TGF-β1 followed by electroporation, increased re-epithelialization rate, collagen synthesis, and angiogenesis was observed.[44] In another study, pDNA encoding for vascular endothelial growth factor ($VEGF^{165}$) was delivered to the ischemic skin of a rat skin flap model by intradermal injection followed by electroporation.[45] This treatment significantly increased VEGF expression for 5 days after delivery, which was sufficient to induce an endogenous angiogenic response, mediate an upregulation of endothelial nitric oxide synthase (eNOS), increase perfusion, and decrease necrosis of the distal area of random skin flaps (RSFs).[45]

The angiogenic process, at the molecular level, is regulated by hypoxia-inducible factor-1 (HIF-1),[82,83] which mediates the cellular response to hypoxia, and promotes pro-angiogenic gene transcription, thus stimulating neovascularization.[84,85] HIF-1 is a heterodimeric protein that consists of a highly regulated α-subunit (HIF-1α) and a constitutively expressed β-subunit (HIF-1β).[86,87] Under normoxic conditions, the HIF-1α subunit is ubiquitinated and subsequently degraded, whereas, under hypoxic conditions, the degradation pathway of HIF-1α is suppressed and HIF-1α accumulates, dimerizes with HIF-1β, and activates transcription of a cascade of genes that enhance oxygen delivery, such as multiple angiogenic growth factors,[88] cell metabolism,[89] proliferation,[90] and the recruitment of endothelial progenitor cells.[91] In this regard, Liu et al. showed that aging of diabetic mice was associated with progressive impairment of HIF-1α mRNA expression, among other angiogenic growth factors/cytokines.[46] As a means of correcting deficient expression of HIF-1α, they applied intradermal injections of pDNA encoding for a constitutively active form of HIF-1α, followed by electroporation.[46] Levels of HIF-1α mRNA were increased at the injection site on day 3 and induced increased levels of VEGF, placental growth factor (PLGF), platelet-derived growth factor B (PDGF-B), and angiopoietin 2 (ANGPT2) mRNA on day 7 that were 8- to 37-fold higher than baseline levels. In addition, circulating angiogenic cells (CACs) in the peripheral blood were increased 10-fold and wound closure was significantly accelerated in the group treated with the HIF-1α plasmid.[46]

Intracellular delivery of pDNA to induce angiogenesis has also been widely explored by making use of chemical-based vectors such as liposomes and polymeric particles. Liposomes are small closed bilayered artificial vesicles composed of hydrated phospholipids, often together with cholesterol. Liposomes are widely used as efficient delivery systems for drug or gene delivery, including for increasing angiogenesis in wounds.[92] For example, in a study conducted by Jeschke et al., pDNA for keratinocyte growth factor (KGF) encapsulated in liposomes was administered to rats with an acute wound received weekly subcutaneous injections of liposomes complex for 4 weeks.[47] They showed that KGF increased neovascularization and concomitant VEGF concentrations and enhanced re-epithelialization of the wound by nearly 170% due to increased proliferation and decreased apoptosis of basal epithelial cells.[47] They also evaluated the synergistic effect of transfecting multiple pDNA genes, insulin-like growth factor-I (IGF-I), and KGF in comparison with using only a single pDNA (IGF-I or KGF).[48] The combination of both IGF-I and KGF pDNA had additive effects on accelerating re-epithelization, increasing proliferation, and decreasing apoptosis compared with the administration of the same growth factors individually.[48] Moreover, Pereira et al. evaluated the interaction between weekly

Table 1. Studies that investigated gene-augmentation-based therapeutics for increased angiogenesis in wound healing

Target	Therapeutic NAs	Delivery method/carrier	Model systems	Wound	Result	Reference
TGF-β1	pDNA	electroporation	*in vitro* *in vivo* female mice C57BL/6 C57BKS.Cg-m+/+Lepr^{db}	7 × 7-mm full-thickness wounds on the back of genetically diabetic mouse	increased re-epithelialization rate increased collagen synthesis increased angiogenesis	Lee et al.[44]
VEGF^{165}	pDNA	electroporation	*in vitro* *in vivo* male SD rats	8 × 3-cm full-thickness incisions on the back of rat	increased VEGF expression for 5 days upregulation of eNOS decreased necrosis of the distal area of RSFs	Ferraro et al.[45]
HIF-1α	pDNA	electroporation	*in vitro* human embryonic kidney-293 cells *in vivo* female BKS.Cg-m$^{+/+}$Lepr^{db}/J mice	full-thickness circular (5-mm diameter) on the dorsa of genetically diabetic mouse	increased levels of VEGF, PLGF, PDGF-B, and ANGPT2 mRNA increased CACs acceleration of wound closing	Liu et al.[46]
KGF	pDNA	cholesterol-containing cationic liposome	*in vitro* *in vivo* male SD rats	30% total body surface area full-thickness scald burn	increased neovascularization increased VEGF concentrations enhanced re-epithelialization	Jeschke et al.[47]
IGF-I KGF	pDNA	cholesterol-containing cationic liposome	*in vitro* *in vivo* male SD rats	30% total body surface area full-thickness scald burn	additive effects on dermal and epidermal regeneration increasing proliferation deceasing skin cell apoptosis	Jeschke and Klein[48]
KGF	pDNA	cholesterol-containing cationic liposome	*in vitro* *in vivo* male SD rats	30% total body surface area full-thickness scald burn	increases in IGF-I, IGF-BP3, FGF, and collagen IV increased re-epithelialization improved dermal regeneration increased neovascularization	Pereira et al.[49]
PDGF-B	pDNA	cationic liposomes and cholesterol	*in vitro* 3T3 mouse fibroblasts cells *in vivo* male SD rats	2.1-cm wound to the level of the loose subcutaneous tissues on the dorsal skin of STZ-diabetic rat	increases re-epithelialization increased fibrocollagen and keratin formation increased blood vessel formation	Bhattacharyya et al.[50]
SHH	pDNA	PBAE NPs	*in vitro* NIH-3T3 mouse embryonic fibroblasts cells *in vivo* Gli1^{tm2Alj}/J mice	full-thickness skin wound (1.5 cm × 1.5 cm) on the back of mouse	increased VEGF, SDF-1α improved blood vessel formation	Park et al.[51]
VEGF-A PDGF-B	pDNA	PEI-modified PLGA nanosphere	*in vitro* 3T3 mouse fibroblasts cells *in vivo* female SD rats	6-mm full-thickness skin wound on the dorsal hind foot of STZ-diabetic rat	increased the expression of VEGF-A and PDGF-B reduced the area of ulceration	Shi et al.[52]
SphK1	pDNA	sCA NPs	*in vitro* NIH-3T3 mouse fibroblasts cells *in vivo* *C57BL/6J* mice	5-mm diameter full-thickness skin punches on either side of the midline of mouse	accelerated wound closure increased angiogenesis recruitment of T cells and macrophages less scarring	Aoki et al.[53]
HIF-1α	pDNA	PLL-g-PEG nanocondensates loaded in 3D fibrin hydrogel	*in vitro* monkey kidney fibroblast cells Cos-7 cells *in vivo* SD rats	10-mm diameter full-thickness wounds on dorsal region of STZ-diabetic rat	induced expression of angiogenesis-related genes (VEGF, Pecam1, and Acta2) enhanced the number endothelial cells and smooth muscle cells	Thiersch et al.[54]
VEGF	pDNA	PEI polyplex in PHA-MMP hydrogels	*in vitro* *in vivo* female $^{db/db}$ mice female $^{balb/c}$ mice	full-thickness wounds 6 mm on genetically diabetic mouse or 4 mm on balb/c mice	porous hydrogels allowed: faster wound closure increased vessel density	Tokatlian et al.[55]
VEGF	pDNA	PEI complex in HAMA, Dex-HEAA, 526-β-CD hydrogel	*in vitro* 3T3 mouse fibroblasts cells	full-thickness of burn wounds on dorsal skin of rat	accelerated burn wound healing	Wang et al.[56]

(*Continued on next page*)

Table 1. *Continued*

Target	Therapeutic NAs	Delivery method/carrier	Model systems	Wound	Result	Reference
			human umbilical vein endothelial cells *in vivo* male SD rats		inhibiting inflammation response promoting microvascular formation	
bFGF	pDNA	PEI polyplex in PELA-PEG nanofiber	*in vitro* mouse embryo fibroblasts cells *in vivo* male SD rats	full-thickness circular wounds (about 250 mm^2) on the upper back of STZ-diabetic rat	enhanced cell proliferation higher wound recovery rate higher collagen deposition and maturation improved vascularization and complete re-epithelialization	Yang et al.[57]
KGF	pDNA	PLA-PCL nanofiber	*in vitro* 3T3 mouse fibroblasts cells *in vivo* C57BL/6J mice	6-mm full-thickness circular wounds on the dorsa of mice	enhanced wound healing improved re-epithelialization higher keratinocyte proliferation enhanced granulation response	Kobsa et al.[58]
ANG	pDNA	CMCS-PEI NP-loaded PLGA-CNCs nanofiber	*in vitro* human umbilical vein endothelial cells *in vivo* male SD rats	full-thickness burns (20 mm in diameter) on the dorsum of rats	prevented local infection promoted skin regeneration higher numbers of mature blood vessels	Mo et al.[59]
ANG	pDNA	TMC NPs in PLLA-POSS nanofibers	*in vitro* human umbilical vein endothelial cells *in vivo* male SD rats	full-thickness burns (about 20-mm diameter) on the dorsum of rats	promoted angiogenesis higher vessel densities better vascularization capability	Li et al.[60]
$VEGF^{165}$	pDNA	TMC NPs in bilayer porous collagen-chitosan/silicone equivalent	*in vitro* human umbilical vein endothelial cells *in vivo* Bama miniature pigs	full-thickness burns (30 mm) on dorsal of pig	higher number of newly-formed vessel fastest regeneration of the dermis highest expression of VEGF, CD31, and α-SMA	Guo et al.[61]
$VEGF^{165}$	pDNA	lipofectamine 2000 in bilayer porous collagen-chitosan/silicone equivalent	*in vitro* NIH-3T3 mouse fibroblasts cells *in vivo* male SD rats	full-thickness excisional wounds with the diameter of 8 mm on the mid-dorsum of diabetic rat	upregulate of VEGF expression accelerated infiltration and phenotype shift of macrophages enhanced angiogenesis deposition of oriented collagen	Lou et al.[62]
$VEGF^{165}$	pDNA	CYD-PEI complex in gelatin/β-TCP scaffold	*in vitro* epidermal stem cells *in vivo* SD rats	full-thickness excision (10 mm × 10 mm) on rat	accelerated the skin re-epithelization increased dermal collagen synthesis increased hair follicle regeneration scar inhibition stimulating angiogenesis	Peng et al.[63]
$VEGF^{165}$	pDNA	CYD-PEI complex in gelatin/β-TCP scaffold	*in vitro* bone-marrow MSCs *in vivo* SD rats	full-thickness excision (10 mm × 10 mm) on rat	higher efficacy in stimulating wound closure promoting dermal collagen synthesis higher angiogenic capacity	Peng et al.[64]
SDF-1α	pDNA	PEI NPs in collagen-chondroitin sulfate scaffold	*in vitro* bone-marrow MSCs human umbilical vein endothelial cells *in vivo* –	–	overexpression of SDF-1α mRNA in MSCs increased in viability increased tubule formation and cell migration accelerated wound closure in endothelial cells	Laiva et al.[65]
$VEGF^{165}$	mcDNA	sonoporation	*in vitro* human embryonic kidney cells Chinese hamster ovary cells	6 mm in diameter on the back of the STZ-diabetic mouse	better wound heling for mcDNA including: higher VEGF expression	Yoon et al.[66]

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Table 1. *Continued*

Target	Therapeutic NAs	Delivery method/carrier	Model systems	Wound	Result	Reference
			NIH3T3 mouse fibroblast cells *in vivo* C57BL/6J mice		better neo-angiogenesis higher blood flow faster rate of wound closing	
$VEGF^{165}$ EGF	mcDNA pDNA	sonoporation	*in vitro* human embryonic kidney-293 cells *in vivo* male C57BL/6J mice	6 mm in diameter on the back of STZ-diabetic mouse	accelerated wound closure for both EGF and VEGF increase neo-angiogenesis for VEGF enhance maturation of re-epithelization and wound heling rate for EGF	Ko et al.[67]
$VEGF^{165}$	mcDNA	PAM-RG4 dendrimer	*in vitro* *in vivo* male C57BL/6J	skin wound on STZ-diabetic mouse	complete diabetic wound closure by 12 days increased proliferation of basal cells	Kwon et al.[68]
GCP-2	mcDNA	microporation	*ex vivo* human dermal fibroblasts *in vivo* male nude mice	6-mm full-thickness wound on each side of the midline mouse	increased re-epithelialization increased capillary density increased EGF, FGF-2, and VEGF expression	Han et al.[69]
AZD8601	*mRNA*	Lipofectamine 2000 (*in situ*) citrate/saline (*in vivo*)	*in situ* human aortic smooth muscle cells *in vivo* C57BL/6BrdCrHsd-Tyr^c mice (ear microvasculature) B6.BKS(D)-$Lepr^{db}$/J mice (diabetic wound healing)	full-thickness cutaneous wounds 1 cm in diameter on the dorsum of genetically diabetic mouse	in ear of mouse upregulation of blood flow formation of neo-vessel in wound area improved the vascularization improved tissue oxygenation increased re-epithelialization	Sun et al.[70]
AZD8601	mRNA	citrate-buffered saline	*in vitro* *in vivo* men with type 2 DM	–	local functional VEGF-A protein expressed increase in basal skin blood flow	Gan et al.[71]
bFGF	mRNA	Lipofectamine Messenger Max in MCMs	*in vitro* dermal fibroblasts from human foreskin *in vivo* male C57BLKS/J mice db^+/db^+	full-thickness skin wound on genetically diabetic mouse	improved wound healing increased wound closure rate improved final wound resolution	Khalil et al.[72]

526-β-CD, β-cyclodextrin with poly(ethylene glycol) methyl acrylate 526; a-FGF, acidic fibroblast growth factor; ANG, angiopoietin; ANGPT2, angiopoietin 2; bFGF, basic fibroblast growth factor; CACs, circulating angiogenic cells; CMCS, carboxymethyl chitosan; CNCs, cellulose nanocrystals; Dex-HEAA, dextran with N-hydroxyethylacrylamide; EGF, epidermal growth factor; eNOS, endothelial nitric oxide synthase; FGF, fibroblast growth factor; GCP-2, granulocyte chemotactic protein-2; HAMA, hyaluronic acid functionalized with methacrylic anhydride; HIF-1α, hypoxia-inducible factor-1 α-subunit; IGF-BP3, insulin-like growth factor binding protein-3; IGF-I, insulin-like growth factor-I; KGF, keratinocyte growth factor; mcDNA, minicircle DNA; MCMs, mineral-coated microparticles; mRNA, messenger RNA; NPs, nanoparticles; PAM-RG4, L-arginine-grafted polyamidoamine; PBAE, poly(β-amino esters); PCL, polycaprolactone; PDGF-B, platelet-derived growth factor B; pDNA, plasmid DNA; PEG, polyethylene glycol; PEI, polyethyleneimine; PELA, poly(dl-lactide)-poly(ethylene glycol); PHA-MMP, porous hyaluronic acid-matrix metalloproteinases; PLA, poly(l-lactide); PLGA, PEI-modified polylactic-co-glycolic acid; PLGF, placental growth factor; PLL, poly-L-lysine; PLLA, poly(l-lactic acid); POSS, polyhedral oligomeric silsesquioxane; rhIGF-I, recombinant human IGF-I protein; RSFs, random skin flaps; sCA, super carbonate apatite; SD, Sprague-Dawley; SDF-1α, stromal derived factor-1 alpha; SHH, sonic hedgehog; SphK1, sphingosine kinase-1; STZ, streptozotocin; TGF-β1, transforming growth factor-β1; TMC, *N*-trimethyl chitosan chloride; VEGF, vascular endothelial growth factor; α-SMA, alpha smooth muscle actin; β-TCP, β-tricalcium phosphate.

subcutaneous injections of KGF pDNA encapsulated in liposomes with other dermal and epidermal growth factors and collagen synthesis in an acute wound model.[49] They observed that the administration of KGF pDNA increased expression of IGF-I, insulin-like growth factor binding protein-3 (IGF-BP3), fibroblast growth factor (FGF), and collagen IV. This led to increased re-epithelialization, improved dermal regeneration, and increased neovascularization, which ultimately accelerated wound healing.[49] In another investigation, a single subcutaneous injection of cationic liposomes functionalized with an integrin receptor targeting RGDK-lipopeptide 1 for specific targeting of fibroblast and carrying PDGF-B pDNA was evaluated in streptozotocin-induced diabetic Sprague-Dawley rats as a model of chronic wounds.[50] The degrees of re-epithelialization, fibrocollagen, and keratin formation as well as blood vessel formation in sectioned wound tissue of rats were found to be significantly enhanced, resulting in wound healing in 10 days.[50]

Cationic polymers are another promising candidate for non-viral delivery vectors. Positively charged polymers can form electrostatic complexes with the negatively charged NAs. Such polymer-NA complexes are often termed polyplexes.[93] For example, Park et al. used amine end-modified biodegradable cationic poly(β-amino esters) (PBAE) NPs for delivery of sonic hedgehog (SHH) pDNA into a full-thickness skin wound model.[51] SHH is a well-known prototypical

morphogen that promotes tissue regeneration by activating angiogenic signaling pathways and plays a critical role in tissue repair and wound healing.[94,95] Intradermal delivery of the SHH gene using amine end-modified PBAE NPs significantly increased the expression of VEGF and the stromal cell-derived factor-1α (SDF-1α) chemokine within the wounded regions and improved blood vessel formation.[51] In another study, Shi et al. used VEGF-A and PDGF-B pDNA incorporated into PEI-modified polylactic-co-glycolic acid (PLGA) nanospheres to upregulate genes expression in streptozotocin-induced diabetic rats for improved wound healing.[52] The authors showed that combined gene transfer of VEGF-A and PDGF-B with PLGA nanospheres could significantly increase the expression of VEGF-A and PDGF-B in full-thickness foot dorsal skin wounds and the area of ulceration was progressively and significantly reduced.[52] In another investigation, inorganic super carbonate apatite (sCA) NPs were used for topical sphingosine kinase-1 (SphK1) activation in murine skin wound healing.[53] Sphingosine-1-phosphate (S1P) is a lipid mediator generated by sphingosine kinase that, after generation, is transported out of the cell and binds in a paracrine or autocrine manner to S1P-specific G protein-coupled receptors (S1PR). This binding event regulates various physiological processes, with one of them playing a key regulatory role in vasculogenesis, angiogenesis, and blood vessel permeability.[96,97] Their results showed that topical SphK1 activation by sCA NPs accelerated wound closure, with increased angiogenesis and recruitment of T cells and macrophages.[53]

Until now, we have discussed studies that evaluated the direct application of non-viral NA formulations to wounds. However, many studies have focused on encapsulating pDNA nanocarriers in matrices such as hydrogels and nanofibers for achieving better control over the release kinetics.[98] These matrices mimic the properties of the extracellular matrix (ECM) and help preserve the activity of the therapeutic biomolecules.[99,100] For example, Thiersch et al. incorporated nanocarriers composed of HIF-1α pDNA and peptide-modified pegylated poly-L-lysine (PLL-g-PEG) into three-dimensional (3D) fibrin hydrogels for *in vivo* investigation.[54] Treatment with PLL-g-PEG-HIF-1α induced expression of angiogenesis-related genes and enhanced the number endothelial cells and smooth muscle cells as precursors for mature blood vessels.[54] In another study, Tokatlian et al. compared delivery of nanocarriers composed of VEGF pDNA and polyethylenimine (PEI) in 100- and 60-μm porous and nonporous hyaluronic acid (HA)-matrix metalloproteinase (MMP) hydrogels for local gene therapy in a diabetic wound-healing mouse model.[55] Porous hydrogels allowed significantly faster wound closure than nonporous hydrogels because the latter did not degrade and provided a mechanical barrier for wound closure. Interestingly, the pore size seemed to be the dominant factor in determining wound closure rates, with 60-μm porous hydrogels initially allowing for much faster wound closure compared with 100-μm porous hydrogels.[55] In another investigation, a sophisticated hydrogel scaffold was generated via UV irradiation from chemically modified hyaluronic acid with methacrylic acid anhydride (HAMA), dextran with N-hydroxyethylacrylamide (Dex-HEAA), and β-cyclodextrin with poly(ethylene glycol) methyl acrylate 526 (526-β-CD-526). A nanoformulation of anti-inflammatory resveratrol (Res) incorporated into the lipophilic central cavity of 526-β-CD forming inclusion complexes was included into the hydrogel.[56] PEI/pDNA-VEGF complexes as the pro-angiogenic components were also loaded into the hydrogel scaffold (Figure 2A). Through a synergistic effect of all the different components, the hydrogel embedded with Res and VEGF improved the stability and therapeutic efficacy of PEI/pDNA-VEGF complexes *in vitro* and accelerated healing of a excisional burn wound by inhibiting the inflammation response and promoting microvasculature formation.[56]

Apart from hydrogels, scaffolds made from electrospun fibers have also been investigated for localized release of pDNA nanovectors.[101,102] For example, Yang et al. incorporated basic FGF (bFGF)-encoding plasmid (pbFGF)/PEI polyplexes into core-sheath electrospun poly(dl-lactide)-poly(ethylene glycol) (PELA) fibers.[57] Also, poly(ethylene glycol) was included in the fiber sheath to allow a sustained release of pbFGF even up to 4 weeks, which was in accordance with the duration for skin wound recovery. The *in vitro* results showed that pbFGF-loaded fibrous mats enhanced cell proliferation. The gradual release of pbFGF polyplexes revealed a significantly higher wound recovery rate with collagen deposition and maturation, improved vascularization, complete re-epithelialization, and skin appendage regeneration.[57]

Another interesting strategy involves the layer-by-layer deposition of plasmids encoding for KGF and PEI on the surface of electrospun scaffolds, composed of a blend of the degradable polymers poly(l-lactide) (PLA) and polycaprolactone (PCL), as reported by Kobsa et al.[58] In this work, mice receiving the KGF plasmid-loaded nanofiber PLA/PCL scaffold exhibited significantly enhanced wound healing, which was quantified by improvements in the rate of wound re-epithelialization, keratinocyte proliferation, and granulation response.[58] In another study, electrospun PLGA, and cellulose nanocrystal (CNC) composite nanofibers have been prepared for the controlled dual delivery of plasmids encoding angiogenin (pDNA-ANG) and curcumin (Cur) as an angiogenic and anti-inflammatory agent, respectively.[59] pDNA-ANG was loaded in polyethyleneimine (PEI)-carboxymethyl chitosan (CMCS) complexes in the form of NPs. The PEI-CMCS/pDNA-ANG NPs and Cur were then encapsulated into polymeric nanofibers (PLGA/CNCs) by electrospinning (Figure 2B). *In vitro* release studies showed that the bioactivity of Cur and pDNA-ANG was preserved in the nanofibers, and a sequential release pattern was achieved in which nearly 90% of the Cur was released in 6 days and the ANG release lasted up to about 20 days. Also, *in vivo* angiogenesis and anti-infection evaluation indicated that the PLGA/CNC/Cur/pDNA-ANG composite nanofibers not only prevented local infection but also promoted skin regeneration.[59] A similar study by Li et al. incorporated polyhedral oligomeric silsesquioxane (POSS) NPs into poly(l-lactic acid) (PLLA) electrospun nanofibers to increase the strength and toughness of the matrix, while simultaneously forming a porous fiber structure.[60] Also, to drive a pro-angiogenic response, *N*-trimethyl chitosan chloride (TMC) was used to condense the pDNA encoding angiopoietin-1 (pANG) into NPs, and dispersed

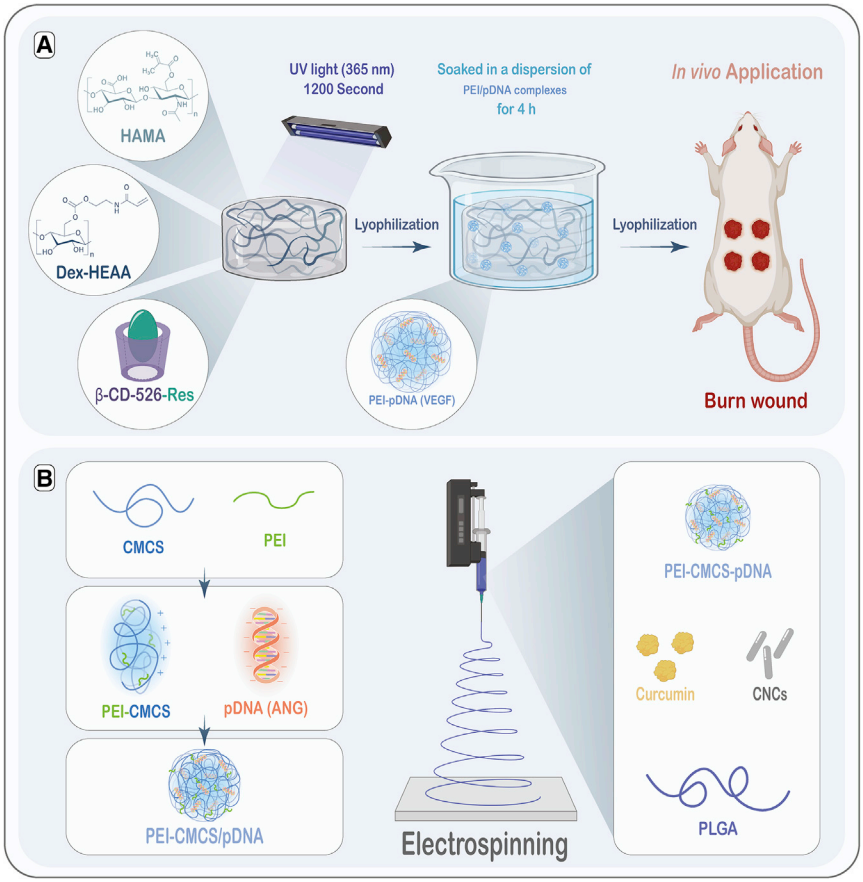

Figure 2. Schematic illustrations of scaffold formation and preparation of composite nanofibers for increased angiogenesis in a wound model

(A) Schematic illustration of scaffold formation and its application in a splinted excisional wound model. The scaffold is made of modified hyaluronic acid functionalized with methacrylic anhydride (HAMA), dextran modified with N-hydroxyethylacrylamide (Dex-HEAA), and β-cyclodextrin modified with N-hydroxyethylacrylamide, and simply mixed with resveratrol (β-CD-526-Res). Hydrogels with different ratios of HAMA, Dex-HEAA, and 526-β-CD-Res were formed by photo-polymerization under a UV initiator at 365 nm for 10 min. Then, the freeze-dried hydrogel was soaked in a dispersion of PEI/pDNA complexes for 4 h in the dark. Finally, the hydrogel was freeze dried again to obtain a scaffold with PEI/pDNA-VEGF complexes. Resveratrol and VEGF DNA plasmid are integrated into the scaffold for their anti-inflammatory and pro-angiogenic effects. (B) The general procedure employed for the preparation of the PLGA/CNC/Cur/pDNA-ANG composite nanofibers. First, the PEI-CMCS copolymer was preformed using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) as coupling reagents and then mixed with pDNA-ANG to form PEI-CMCS/pDNA complexes. Finally, PEI-CMCS/pDNA complexes and Cur were added to the PLGA/CNC electrospinning solution at room temperature to create electrospun composite nanofibers. ANG, angiogenin; CNCs, cellulose nanocrystals, CMCS, carboxymethyl carbodiimide; NHS, N-hydroxysuccinimide; PEI, polyethyleneimine; PLGA, polylactic-co-glycolic acid.

the complexes into PLLA/POSS nanofibers by electrospinning (Figure 3). The pANG-loaded PLLA/POSS scaffold showed sustained release of pANG over 35 days and effectively promoted angiogenesis and dermal wound healing with higher vessel densities and better vascularization capabilities.[60]

Besides the use of hydrogels and electrospun polymeric fibers, other groups have focused on the use of dermal equivalents, which are natural or synthetic scaffolds covered with a silicone membrane that are being used as wound dressings to cover extensive excisional wounds and burns.[103] Guo et al., for example, reported TMC/pDNA-VEGF complexes loaded into a bilayer porous collagen-chitosan/silicone membrane dermal equivalents (BDEs) for treatment of full-thickness burn wounds.[61] The *in vitro* results of TMC/pDNA-VEGF complexes loaded in BDEs depicted a significantly higher level of VEGF and showed higher viability than the other control groups. Also, the *in vivo* results on porcine full-thickness burn wound showed that the TMC/pDNA-VEGF loaded in the BDEs group had a significantly higher number of newly formed and mature blood vessels, and the fastest regeneration of the dermis that had the highest expression of VEGF, CD31, and α-SMA.[61] In a similar study, VEGF pDNA was loaded into the BDEs to form a gene-activated BDEs (Ga-BDEs) for regulating inflammation and promoting angiogenesis, with the difference that, instead of TMC, pDNA was encapsulated in Lipofectamine 2000 complexes.[62] The pDNA complexes were released in a sustained manner and showed the capacity to upregulate the expression of VEGF *in vitro*. Also, the treatment of Ga-BDEs on a rat full-thickness diabetic chronic wound showed accelerated infiltration and phenotype shift of macrophages, enhanced angiogenesis, and better regeneration outcomes, including deposition of oriented collagen and fast re-epithelialization.[62]

Scaffolds for controlled release of pDNA nanocarriers have also been combined with recombinant stem cell-based skin grafts that are then able to develop new skin layers with normal functions. For instance, Peng et al. prepared a gelatin scaffold containing β-tricalcium phosphate (β-TCP) and nanocomplexes composed of VEGF[165] pDNA and β-cyclodextrin-linked polyethylenimines (CYD-PEI). Epidermal stem cells (ESCs)[63] and bone-marrow mesenchymal stem cells (MSCs)[64] were subsequently grown in this 3D scaffold and became transfected (Figure 4). The scaffold containing the transfected stem cells was subsequently transplanted in an *in vivo* wound model, resulting in accelerated skin re-epithelization and wound closure. Furthermore, dermal collagen synthesis and hair follicle regeneration was promoted, while scar formation was inhibited. Angiogenesis was found to be stimulated at the wound site.[63,64] In another study, Laiva et al. reported

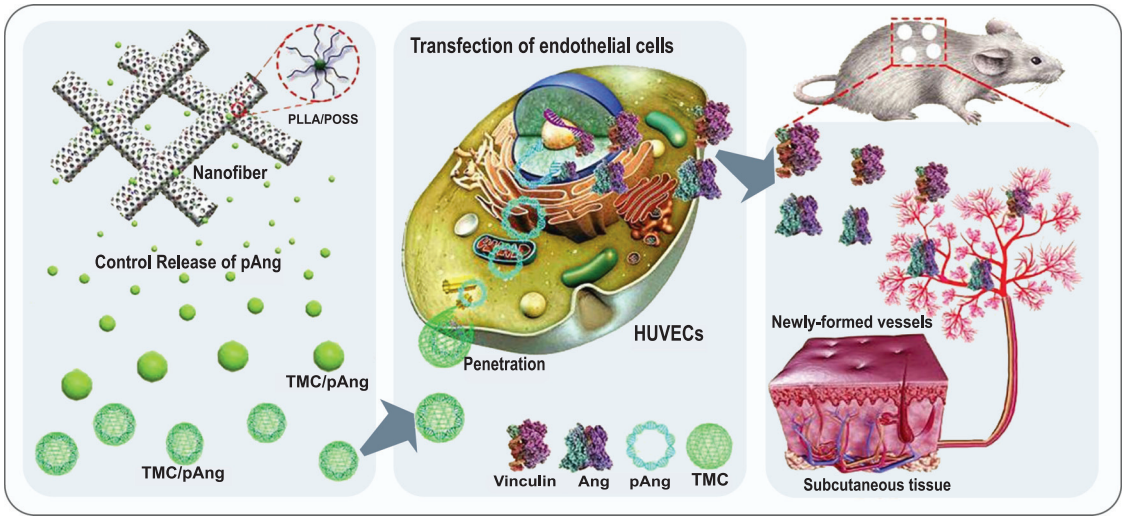

Figure 3. Schematic illustration of porous nanofibers for controlled release of pAng nanovectors

The PLLA/POSS porous nanofibers loaded with pANG-TMC could release pAng in a sustained manner. After transfection of endothelial cells by pAng, cells can express angiopoietin (Ang) and vinculin, which are important growth factors involved in angiogenesis and regulating migration, adhesion, and survival of endothelial cells. pAng, pDNA encoding angiopoietin-1, TMC, *N*-trimethyl chitosan chloride, HUVECs, human umbilical vein endothelial cells. Adapted from Li et al.[60] with permission from Elsevier.

pDNA/PEI polyplexes encoding for stromal derived factor-1 alpha (SDF-1α), which was combined with a collagen-chondroitin sulfate scaffold as a pro-angiogenic gene-activated scaffold (GAS).[65] MSCs grown on GAS exhibited an early overexpression of SDF-1α mRNA with the activation of angiogenic markers VEGF and CXC chemokine receptor 4 (CXCR4). Also, exposing endothelial cells to conditioned media collected from the MSC containing GAS scaffold promoted a 20% increase in viability, 33% increase in tubule formation, and 50% increase in cell migration and wound closure with upregulation of angiogenic genes (VEGF, CXCR4, eNOS, and SDF-1α).

Minicircle DNA

Recently, the use of minicircle DNA (mcDNA) has gained attention as a promising alternative to pDNA that can offer better biosafety and higher expression rates[104] due to its small size and the absence of unmethylated CpG motifs, which can induce immune responses.[105] In a study by Yoon et al., mcDNA encoding for human $VEGF^{165}$ ($mcDNA\text{-}VEGF^{165}$) was compared with a conventional pDNA ($pDNA\text{-}VEGF^{165}$) *in vitro* (HEK293, NIH3T3, and Chinese hamster ovary [CHO] cells) and *in vivo* (diabetic mouse with the back skin wound). Both NAs were delivered via sonoporation with microbubbles.[66] Sonoporation is another non-viral physical method that can form transient pores in the cell membrane based on acoustic pressure waves.[106] Their results showed that $mcDNA\text{-}VEGF^{165}$ resulted in 2- to 3-fold higher VEGF expression than $pDNA\text{-}VEGF^{165}$ and exhibited a better safety profile *in vitro*.[66] Also, $mcDNA\text{-}VEGF^{165}$ was proved to increase neo-angiogenesis, blood flow, and the rate of wound closing in STZ-induced diabetic mice.[66] In another investigation by the same group, sonoporation of epidermal growth factor (EGF) pDNA was compared with $mcDNA\text{-}VEGF^{165}$.[67] Both pDNA-EGF and mcDNA-VEGF accelerated wound closure compared with non-treated diabetic control mice. However, blood flow and neo-angiogenesis were better in the mcDNA-VEGF group, while wound-healing rates and the maturation of organized re-epithelization were more enhanced in the pDNA-EGF group.

Delivery of mcDNA by nanocarriers has also been reported. For example, Kwon et al. reported complexes composed of positively charged L-arginine-grafted polyamidoamine (PAM-RG4) dendrimer and mcDNA encoding for $VEGF^{165}$.[68] These complexes were able to induce complete wound closure in diabetic mice by day 12 due to the expression of VEGF in rapidly proliferating basal cells.[68]

Recently, an *ex vivo* study was reported in which granulocyte chemotactic protein-2 (GCP-2)-overexpressing human dermal fibroblasts (HDF) were transfected with mcDNA delivered via microporation technology.[69] Microporation is a electroporation technology that uses specialized pipette tips containing gold-plated electrodes.[107] It was shown that the overexpression of GCP-2 enhances the angiogenic potential of MSCs[108] and triggers the expression of wound-healing-related cytokines.[109] The *in vitro* assays demonstrated that the culture medium derived from HDF overexpressing GCP-2 accelerated wound closure and Matrigel network formation.[69] Also, injection of GCP-2-transfected HDFs in the wound area of nude mice increased re-epithelialization and capillary density, with increased levels of angiogenic factors (EGF, FGF-2, and VEGF) in the wound tissue.[69]

mRNA

The use of synthetic mRNA to produce a desired protein combines several advantages compared with plasmid and minicircle DNA: (1) synthetic mRNA must only reach the cytoplasm of cells where the cellular translation machinery is located and does not need to pass

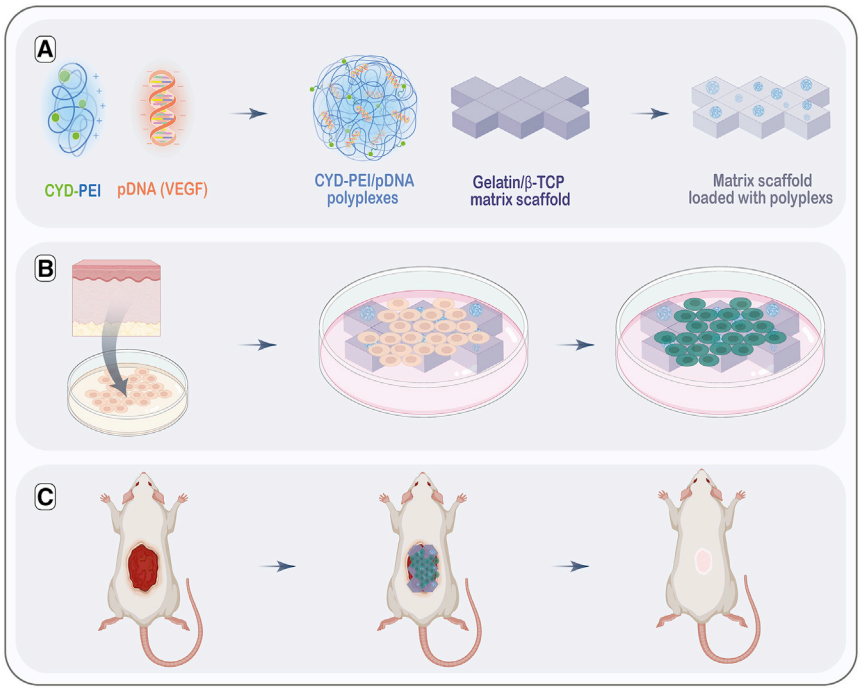

Figure 4. A schematic illustration of the animal study in rat skin wounds for ESCs transfected in a 3D scaffold

(A) Preparation of the CYD-PEI/pDNA polyplexes and gelatin/β-TCP matrix scaffold. (B) Isolation and culture of ESCs from skin tissue biopsy and then culture and transfection of the ESCs in the gene-activated matrix. (C) Establishment of rat full-thickness skin wound. Application of transfected ESCs with the 3D scaffold to the wound site. Wound healing is stimulated by the recombinant ESCs.

through the nuclear envelope (Figure 1);[110] (2) the risk of carcinogenesis and mutagenesis usually associated with DNA can be excluded because mRNA does not integrate into the host genome;[111] (3) the production process of mRNA is easier than that for the manufacturing of DNA, and it can be standardized, maintaining its reproducibility;[112] (4) mRNA gene therapy circumvents the need for selecting a specific promoter, and thus the transfection process is relatively efficient and simple, and the onset of expression is usually detected within 1 h after transfection, with a peak in expression 5–7 h later;[113] (5) mRNA-based therapeutics are effective not only in mitotic cells but also in slow or even non-dividing cells;[112,114] (6) as synthetic mRNA is transiently present in cells until it is naturally degraded due to physiological metabolic pathways, the duration of protein production is limited, which can prevent complications related to persistent protein expression.[115]

However, obstacles such as high instability and immunogenicity have hampered the development of mRNA-based therapeutics, making it less pursued than DNA-based therapies.[116,117] Naked mRNA displays a short plasma half-life (about 5 min after intravenous administration[118]), which is caused by the rapid degradation by abundant extracellular RNases. Delivery challenges are also linked to mRNA's large size (300–5,000 kDa, 1–15 kb), which is significantly larger than siRNA and miRNA (13–15 kDa), and anti-miR (4–10 kDa).[119,120] In addition, exogenous RNA can be recognized as a signal of viral infection and trigger an innate immune response.[121]

In order to overcome some of these challenges, several strategies have been proposed, including the development of chemically modified mRNAs, as well as advancements in mRNA delivery systems.[119] Chemically modified mRNA has been investigated only recently as a novel modality for efficient and transient expression of angiogenesis factors in wound healing, so little literature is available in this field. One study was performed by Sun and colleagues, in which they make use of a new, purified, and clinical-grade modified mRNA encoding VEGF-A[122] (AZD8601) optimized for minimizing the innate immune response.[123,124] In this study, AZD8601 in a citrate/saline solution was intradermally injected in the ear of mice. This resulted in dose-dependent vasodilation, upregulation of blood flow, and formation of neo-vessels. In addition, the bioactivity of AZD8601 in an established mouse model of diabetic wound healing improved vascularization and tissue oxygenation within the wound area and resulted in improved re-epithelialization.[70] Also, in a clinical trial study (phase 1), safety and potential therapeutic effects of AZD8601 mRNA were assessed in men with type 2 DM (NCT02935712). It was revealed that intradermal AZD8601 mRNA injection at the volar forearm was well tolerated with only mild reactions at the injection sites. VEGF-A protein was steadily expressed from 4 to 24 h after administration, which led to a 2-fold increase in basal skin blood flow after 4 h and a sustained increase 7 days post administration.[71]

Despite the potential advantages of synthetic mRNA, its applicability still requires of the effort of the scientific community in order to overcome several challenges, such as short cytoplasmic half-life, short-term overexpression, need for repeated dosing, the antigenicity of *in vitro*-transcribed mRNA, and high production costs. Recently, Khalil et al. used mineral-coated microparticles (MCMs) as a novel biomaterial-based approach to induce a more durable *in vivo* response.[72] bFGF mRNA was first complexed with Lipofectamine Messenger Max and adsorbed onto the MCM surface. This facilitated a sustained biological response via an "overexpress and sequester" mechanism *in situ* (Figure 5A).[72] Topical application of mRNA/MCM resulted in improved wound healing in a murine diabetic wound model compared with mRNA alone (Figure 5B). In this study, the authors also investigated co-delivery of the B18R protein, which is a vaccinia virus-encoded receptor that acts as a decoy receptor for type I Interferons and inhibits activation of interferon-mediated signal transduction. Co-delivery of the B18R protein using this

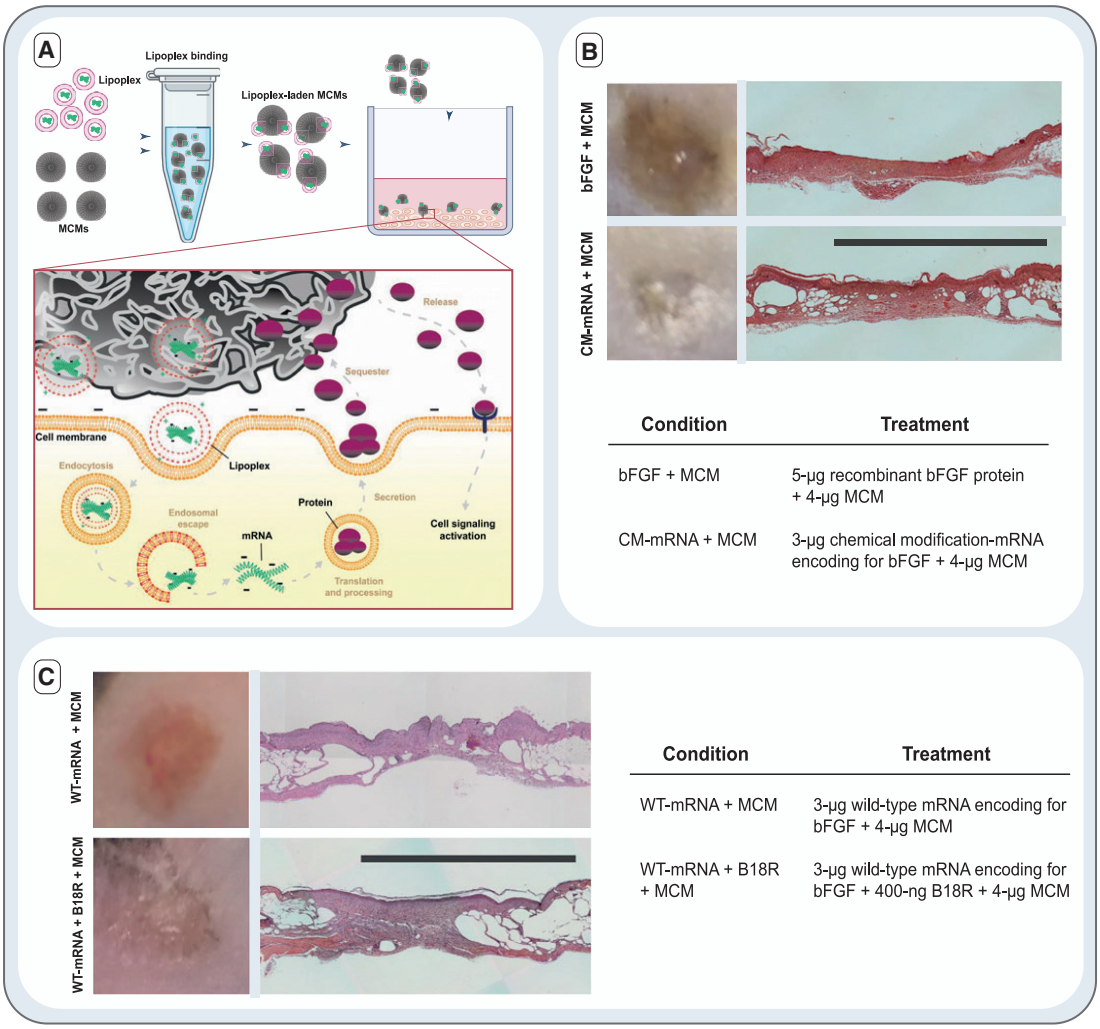

Figure 5. Effect of mRNA lipoplexes loaded onto mineral-coated microparticles (MCMs) in wound healing

(A) Schematic overview of the sequester and release mechanism of mRNA lipoplexes loaded onto mineral-coated microparticles (MCMs). Lipoplexes containing basic growth factor (bFGF) mRNA are loaded onto MCMs. After treatment, mRNA lipoplexes are released from MCMs and taken up by the cells. After the endosomal escape, the bFGF mRNAs are translated into proteins, which are secreted into the extracellular space. The secreted proteins are bound and sequestered by the MCMs, which sustain growth factor release over time and prolong the biological response. (B) MCM-mediated mRNA delivery in wound healing. Representative gross and histological images show improved wound closure and resolution after the application of chemically modified bFGF mRNA (CM-mRNA)-loaded MCMs compared with recombinant bFGF protein with MCMs to wounds in mice. Scale bar, 5 mm. (C) Co-delivery of B18R via MCM-mediated mRNA delivery in wound healing. Representative gross and histological images show improved wound closure and resolution for wild-type (WT) mRNA delivered via MCMs with B18R compared with WT-mRNA alone delivered via MCMs. Scale bar, 5 mm. Adapted from Khalil et al.[72]).

biomaterial eliminated the need for mRNA chemical modifications and resulted in an increased wound closure rate and improved wound healing (Figure 5C).[72]

GENE-SILENCING-BASED THERAPEUTICS FOR WOUND ANGIOGENESIS

Gene silencing, as an epigenetic modification, generally refers to the downregulation of gene expression by targeting specific pre-mRNA or mRNA for degradation and/or repression of its translation.[125] These effects are often mediated by RNA interference (RNAi), anti-miRNA oligonucleotides, antisense oligonucleotides (ASOs), or aptamers, and usually reduce the expression of a gene by at least 70%, but without eliminating it.[126,127] Gene silencing approaches for improving angiogenesis within the context of wound healing are summarized in Table 2.

RNAi

RNAi is a natural defense mechanism for the invasion of exogenous genes[146] based on the regulation of cellular signaling pathways through small double-stranded RNA (dsRNA) effector molecules.

Table 2. Studies that Investigated gene silencing-based therapeutics for increased angiogenesis in wound healing

Target	Therapeutic NA	Delivery method/carrier	Model systems	Wound	Result	Reference
miR-132	miRNA	PLGA NPs	*in vitro* human umbilical vein endothelial cells *in vivo* transfected cells implantation into the abdominal wall of SCID/bg mice	–	accelerated the vascularization process increased the number of microvessels improved therapeutic efficacy	Devalliere et al.[128]
miR-27b	miRNA	Dharmacon and F-127 Pluronic gel	*in vitro* bone-marrow mononuclear cells *in vivo* male BKS.Cg-m$^{+/+}$Lep^{db}/J mice	full-thickness cutaneous wounds on genetically diabetic mouse	improved proliferation improved adhesion increased tube formation delayed apoptosis	Wang et al.[129]
miR-148b	miRNA	Lipofectamine RNAiMAX (*in vitro*) Pluronic F-127 gel (*in vivo*)	*in vitro* human umbilical vein endothelial cells *in vivo* CD-1 female mice	5-mm full-thickness excisional wounds on the dorsal skin of mouse	increased cell migration increased proliferation promoted wound vascularization accelerated wound closure	Miscianinov et al.[130]
miR-302a miR-155	miRNA	gold nanorod	*in vitro* human embryonic kidney-293 cells human outgrowth endothelial cells *in vivo* male nude mice	6-mm diameter dorsal full-thickness wounds on mouse	highest cell engraftment highest wound-healing kinetics	Lino et al.[131]
PHD2	siRNA	polymeric NPs incorporated into PEUR scaffolds	*in vitro* NIH-3T3 mouse fibroblasts *in vivo* balb/c mice	–	increased local angiogenesis increased both number and size of vessels within the scaffolds	Nelson et al.[132]
PHD2	siRNA	polymeric NPs incorporated into PTK-UR scaffolds	*in vitro* A7r5 rat smooth muscle cells *in vivo* male SD rats	8-mm full- thickness wounds on the STZ-diabetic rats' dorsal skin	more robust tissue regeneration improve vascular development increased cellular proliferation increased new tissue growth	Martin et al.[133]
PHD2	siRNA	LbL AuNPs	*in vitro* NIH-3T3 mouse fibroblasts *in vivo* –	–	increased levels of VEGF and FGF-2 accelerated migration and proliferation of NIH3T3cells	Shaabani et al.[134]
FKBPL	siRNA	RALA NPs in electrospun ALG/PVA-CHT/PVA nanofiber	*in vitro* human microvascular endothelial cells *in vivo* C57BL/6J mice	5-mm full-thickness wounds	*in vitro* improved cell migration increased endothelial tubule formation *in vivo* increased in angiogenesis increased blood vessel density	Mulholland et al.[135]
PHD2	plasmid shRNA	FuGENE transfection reagent	*in vitro* fibroblasts of diabetic Lepr $^{db/db}$ mice *in vivo* female diabetic Lepr $^{db/db}$ mice	full-thickness 6-mm wounds on the dorsa of diabetic mice	accelerated wound healing increased vascular density upregulation of SDF-1, VEGFα, PDGFα, and CD31 improved perfusion increased survival of muscle bulk	Paik et al.[136]
PHD2 miRNA-210	shRNA AMO	LbL coating onto the Tegaderm mesh	*in vitro* NHEK primary keratinocytes human keratinocyte cells NIH-3T3 mouse fibroblasts *in vivo* male C57BL/6 db/db mice	6-mm wounds on the dorsum of mouse	increased proliferation and migration of keratinocytes reduced the time of wound closing increased expression of CD31 and Ki67	Dallas et al.[122]
miR-26a	AMO	–	*in vitro* human umbilical vein endothelial cells human dermal microvascular	dorsal full-thickness skin wounds (1 cm^2) on genetically diabetic mouse	induced angiogenesis increased granulation tissue thickness accelerated wound closure	Icli et al.[137]

(*Continued on next page*)

Table 2. *Continued*

Target	Therapeutic NA	Delivery method/carrier	Model systems	Wound	Result	Reference
			endothelial cells human dermal fibroblasts *in vivo* male $^{db/db}$ mice			
miR-135-3p	AMO	–	*in vitro* human umbilical vein endothelial cells *in vivo* male $^{db/db}$ mice	dorsal full-thickness skin wounds (1 cm^2) on genetically diabetic mouse	increased angiogenesis increased granulation tissue thickness accelerated wound closure	Icli et al.[138]
miR-615-5p	AMO	–	*in vitro* human umbilical vein endothelial cells *in vivo* male $^{db/db}$ mice C57BL/6 mice	dorsal full-thickness skin wounds (1 cm^2) on genetically diabetic mouse	increased angiogenesis increased granulation tissue thickness accelerated wound closure	Icli et al.[139]
miR-92a	AMO	–	*in vitro* human umbilical vein endothelial *in vivo* male $^{db/db}$ mice female domestic Yorkshire crossbred pigs	6-mm diameter full-thickness skin wounds on the dorsal skin of genetically diabetic mouse	increased granulation tissue formation increased angiogenesis increased tissue perfusion Accelerates wound closure	Gallant-Behm et al.[140]
miR-92a	AMO	light-activatable caged nucleobases	*in vitro* *in vivo* male C57/BL6N mice BKS(D)-Leprdb/JorlRj ($^{db/db}$) mice	6-mm full-thickness wounds on diabetic mouse	improved wound healing stimulating wound cell proliferation increased angiogenesis	Lucas et al.[141]
miR-26a	AMO	PEI-ceria nanocluster loaded into the collagen hydrogel	*in vitro* human umbilical vein endothelial cells HaCaTs immortalized human skin keratinocytes *in vivo* male SD rats	full-thickness excision (15 mm in diameter) on the dorsal surface of STZ-diabetic rat	reformation of oxidative wound microenvironment accelerated wound closure enhanced quality of the healed wound	Wu et al.[142]
miR-29A	AMO	HA-PEI and Alg@ori loaded in OHMPC/HA-ADH hydrogel	*in vitro* L929 mouse fibroblasts *in vivo* SD rats	2-cm wound on the backs of STZ-diabetic rat	accelerated diabetic wound healing promoted the expression of α-SMA and CD31, inhibited the expression of IL-6 and TNF-α	Yang et al.[143]
anti-VEGF-R2	aptamer	tHA-PEGDA hydrogel	*in vitro* human umbilical vein endothelial cells *in vivo* –	–	prompted cell adhesion improve cell viability stimulated cell migration and angiogenesis	Roy et al.[144]
Apt02 AptVEGF	aptamer	tFNA	*in vitro* human umbilical vein endothelial cells *in vivo* female BALB/c mice	–	promoted endothelial cell proliferation and migration increased tubule formation enhanced spheroid sprouting promoted formation of microvessels	Zhao et al.[145]

Alg@ori, oridonin alginate microspheres; ALG/PVA, alginate/poly-(vinyl alcohol); Amo, anti-miRNA oligonucleotides; anti-VEGF-R2, anti-vascular endothelial growth factor receptor-2; CHT/PVA, chitosan/poly-(vinyl alcohol); FKBPL, FK506-binding protein-like; HA-ADH, adipic dihydrazide-modified hyaluronic acid; HA-PEI, hyaluronic acid-polyethyleneimine; IL-6, interleukin 6; LbL, layer-by-layer; LPP, lipoproteoplex; miRNA, microRNA; OHMPC, oxidized hydroxymethyl propyl cellulose; PDGFα, platelet-derived growth factor subunit α; PEI, polyethyleneimine; PEUR, poly(ester urethane); PHD2, prolyl hydroxylase domain protein 2 PTK-UR, poly(thioketal urethane); SD, Sprague-Dawley; shRNA, short hairpin RNA; siRNA, short interfering RNA; STZ, streptozotocin; tFNA, tetrahedral framework nucleic acid; tHA-PEGDA, thiolated hyaluronic acid-polyethylene diacrylate; TNF-α, tumor necrosis factor α; α -SMA, alpha smooth muscle actin.

RNAi effector molecules bound to the RNA-induced silencing complex (RISC) enable sequence-specific recognition of mRNA by intermolecular base pairing, thus silencing gene expression through inhibition of protein translation or direct mRNA degradation.[147] RNAi effector molecules include miRNA, short interfering RNA, and short hairpin RNA (shRNA).[148] RNAi agents opened promising prospects for gene therapy due to their lower molecular size (19–29 bp), ease of preparation, potent knockdown of virtually any gene, and the fact that it is sufficient to be delivered to the cytosol.[149] Nevertheless, like other NAs, their use and development have been hampered due to their

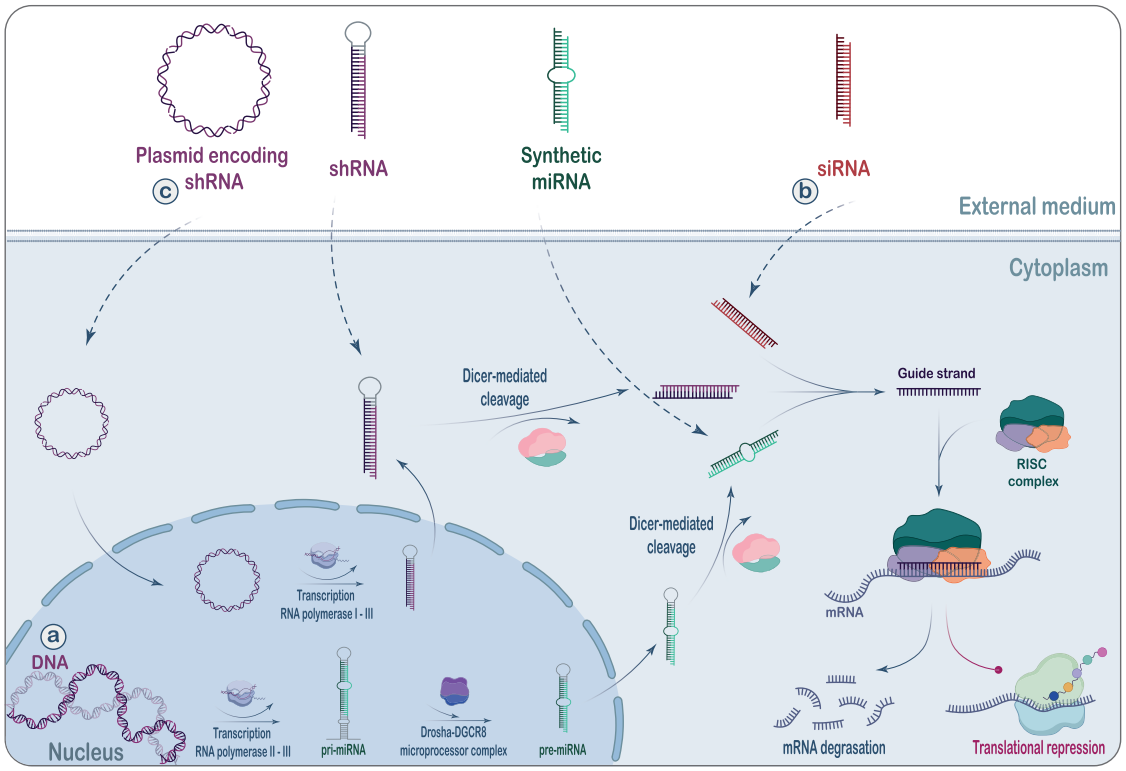

Figure 6. Schematic representation of the mechanism of RNAi

(a) miRNA is transcribed from DNA sequences into primary miRNA (pri-mRNA) and is later processed into precursor miRNA (pre-miRNA) by the Drosha-DGCR8 complex. The pre-miRNA is then actively transported to the cytoplasm by exportin-5. Next, the pre-miRNA is further processed by the Dicer-TRBP complex, usually generating two paired, partially complementary, mature miRNAs. One of the mature miRNA strands then guides the RNA-induced silencing complex (RISC) to cognate target genes and represses target gene expression by either destabilizing target mRNAs or repressing their translation. (b) When siRNA enters the cytosol, the passenger strand is removed from the guide strand and directly incorporated into RISC to guide the RISC complex to its complementary mRNA target for degradation. (c) The plasmid encoding shRNA is transcribed in the nucleus and shRNA is subsequently exported to the cytoplasm via exportin-5. Similar to miRNA, the enzyme Dicer cleaves shRNA into siRNA, which then combines with RISC to degrade the targeted mRNA sequence.

fragile nature, triggering immune responses, having insufficient therapeutic efficacy, as well as inducing off-target effects.[146,150] The use of each of them will be discussed next.

miRNA

Mature functional miRNAs are short noncoding RNA molecules, 18 to 25 nucleotides in length, that originated in the nucleus and are processed and matured in the cytoplasm. miRNAs are generated from the single-stranded primary miRNA (pri-miRNA), transcribed by RNA polymerase II and III, processed by the Drosha-DGCR8 complex, and then cleaved by an endoribonuclease named Dicer.[151] An miRNA can regulate post-transcriptional silencing of different genes by its incorporation into the RISC (Figure 6).[148] A single miRNA sequence can target hundreds of mRNA sequences because they are not specific to a single mRNA and influence the expression of many genes often involved in a functional interacting pathway.[152]

To date, several studies have been performed to increase angiogenesis using miRNA. For example, Devalliere et al. reported transfection of endothelial cells before transplantation by PLGA polymer NPs loaded with miRNA-132 and coated with cyclic RGD (cRGD) peptides for stimulating clathrin-mediated endocytosis.[128] miRNA-132 as an angiogenic switch suppressed p120RasGAP expression, increased activation of Ras signal pathway, and leads to neovascularization in response to growth factors.[153] It was found that transplantation of human umbilical vein endothelial cells (HUVECs) transfected with miRNA-132 accelerated the vascularization process in mice and increased the number of microvessels per square millimeter.[128]

In another study, the function of bone-marrow-derived angiogenic cells (BMACs) was evaluated. It demonstrated that miRNA-27b expression was decreased in diabetic BMACs, which is responsible for BMAC's functional loss in diabetic wound healing. Local miRNA-27b delivery via F-127 Pluronic gel and DharmaFECT transfection reagent I rescued impaired BMAC angiogenesis in a type 2 diabetic mice wound model by improving proliferation, adhesion, tube formation, and delayed apoptosis. The study also found that miRNA-27b protects BMAC's function through suppression of the antiangiogenic molecules (1) thrombospondin-1 (TSP-1) and (2) semaphorin 6A (Sema6A), and suppression of (3) the

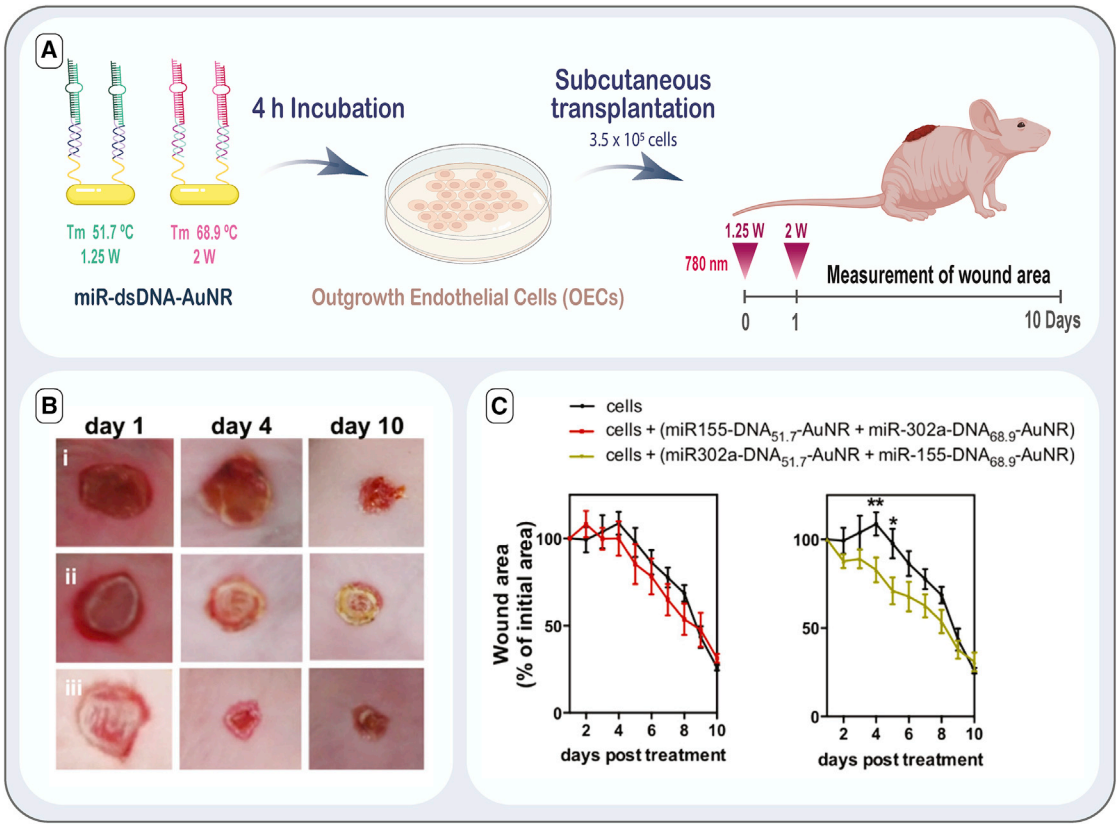

Figure 7. Effect of outgrowth endothelial cell (OEC) transplantation incubated with miR-dsDNA-AuNR and sequenced irradiation in an in vivo acute wound mouse model

(A) Schematic representation of outgrowth endothelial cell (OEC) transplantation in an *in vivo* acute wound mouse model. OECs were incubated for 4 h with a mixture of miRNA-dsDNA-AuNRs. After incubation, cells were trypsinized and transplanted subcutaneously at the border of the dorsal wounds created in nude mice. After transplantation, the site of injection was irradiated for 2 min at 1.25 W cm^{-2}. After 24 h, a second stimulus was applied (2 min at 2 W cm^{-2}). (B) Wound closure in nude mice treated with cells previously incubated with miR-dsDNA-AuNR. (i) OECs without treatment. (ii) OECs incubated with miRNA-155-dsDNA-AuNR and miR302a-dsDNA-AuNR. The site of injection was irradiated for 2 min at 1.25 W cm^{-2} to release miRNA-155 conjugated with ssDNA. After 24 h, miRNA-302a conjugated with ssDNA was released by a second stimulus (2 min at 2 W cm^{-2}). (iii) The order of delivery was inverted by changing the DNA strands conjugated to each miRNA so that OECs first incubated with miRNA-302a-dsDNA-AuNR and after 24 h miRNA-155-dsDNA-AuNR was released. Reprinted with permission from Lino et al.[131] Copyright 2018 American Chemical Society. (C) The wound-healing kinetics was monitored during 10 days by the quantification of wound area. Results are expressed as average ±SEM (n = 7, 8). Statistical significance,*$p < 0.05$ and **$p < 0.01$ assessed by unpaired t test. Reprinted with permission from Lino et al.[131] Copyright 2018 American Chemical Society.

pro-oxidant protein p66shc leading to reduced mitochondrial oxidative stress.[129] Moreover, Miscianinov et al. recently have demonstrated the role of miRNA-148b in the regulation of TGF-β signaling in endothelial cells by targeting TGF-B2 and SMAD2. The modulatory role of TGF-β in endothelial cell plasticity is represented by the endothelial-to-mesenchymal transition (EndMT), which allows the endothelium to provide an additional source of mesenchymal cells for participation in fibrosis.[154,155] They showed that endothelial cells transfected with miRNA-148b by Lipofectamine RNAiMAX increased cell migration and proliferation. On the other hand, delivery of miRNA-148b via Pluronic F-127 gel in a mouse model of skin wound healing promoted wound vascularization and accelerated the wound closure.[130] Making use of inorganic carriers, Lino et al. developed plasmonic gold nanorods (AuNRs) for light-triggered delivery of two miRNAs (miRNA-302a and miRNA-155).[131] miRNA-302a is able to promote cell proliferation by the inhibition of cell cycle regulator expression, such as cyclin D1.[156] On the other hand, miRNA-155 regulates endothelial cell survival in hypoxic conditions by increasing the pro-survival enzyme heme-oxygenase.[157] AuNRs were modified with single-stranded DNA (ssDNA) with different melting temperatures, which act as linkers for hybridization with complementary DNA-conjugated miRNAs (Figure 7A). By tuning the laser power and heating the AuNRs to a specific temperature, independent release of each miRNA could be achieved. The evaluation of miRNA-dsDNA-AuNRs in human outgrowth endothelial cells (OECs) indicated that this developed system could silence different targets sequentially and could modulate cell activity with spatiotemporal resolution. Also, 4 h of treatment of OECs with miRNA-dsDNA-AuNRs and then injection of them in an acute wound animal model showed

the highest cell engraftment and the highest wound-healing kinetics compared with other groups (Figure 7B).[131]

siRNA

siRNA is a short, double-stranded RNA molecule, composed of 19–25 base pairs, which has been designed to recruit and bind to the RISC enzyme complex.[158] It has two strands, a sense and antisense, which are separated when incorporating into the RISC complex.[159] The sense "passenger" strand is ejected, whereas the antisense "guide" strand remains inside the complex. Through Watson-Crick base pairing, the guide strand binds the RISC complex to the complementary mRNA, which is subsequently degraded by endonucleolytic catalytic cleavage, thus preventing the mRNA from translating into proteins (Figure 6).[160] There are slight differences between siRNA and miRNA, such as the higher specificity of siRNA, whereas one miRNA may compromise the expression of several different mRNA target genes simultaneously.[161] Like other NAs, one of the biggest obstacles of siRNA is its intracellular delivery without being degraded first. Here, we will focus on the siRNAs that have been used in wound healing for increasing angiogenesis.

As mentioned earlier, the angiogenic process is mainly regulated by HIF-1,[87,162] which mediates the cellular response to promote pro-angiogenic gene transcription and stimulates neovascularization. On the other hand, its activity is regulated by the oxoglutarate-dependent prolyl hydroxylase domain-2 (PHD-2) protein. Thus, in normoxia, HIF-1α is hydroxylated on proline residues by PHD-2. The hydroxylated form of HIF-1α is required for binding to the von Hippel-Lindau protein (VHL) which is part of an E3 ubiquitin ligase complex, and which targets HIF-1α for ubiquitination and proteasomal degradation (Figure 8).[163–165] Numerous studies have shown that silencing of PHD-2 with siRNA resulted in stabilization and upregulation of HIF-1α and its downstream genes, improving angiogenesis.[166–168]

The Duvall Laboratory developed strategies for controlled delivery of siRNA for knockdown of PHD2.[132,133] In their system PHD2 siRNA was loaded into polymeric micelles made from 2-(dimethylamino) ethyl methacylate (DMAEMA), 2-propylacrylic acid (PAA), and butyl methacrylate (BMA), which together form the diblock copolymer poly[$DMAEMA_{71}$-*b*-(BMA_{103}-*co*-PAA_{68}-*co*-$DMAEMA_{57}$)]. These micellar NPs were incorporated into a hydrolytically biodegradable poly(ester urethane) (PEUR) scaffold for delivery of PHD2 siRNA into the wound bed.[132] Through tuning of the quantity of trehalose added during the PEUR scaffold fabrication, and by alteration of the chemistry of the isocyanate moieties, a versatile PEUR scaffold was made. Knockdown of PHD2 by siRNA demonstrated increased local angiogenesis in a subcutaneous mouse wound model with an increased number and size of blood vessels within the scaffolds.[132]

In order to improve the PEUR scaffold further, a poly(thioketal urethane) (PTK-UR) scaffold was made that degrades at a rate that is proportional to the concentration of reactive oxygen species (ROS), thus releasing siRNA against PHD2.[133] The obtained results showed that implanted PTK-UR scaffolds promoted more robust tissue regeneration in diabetic wounds than PEUR scaffolds in terms of vascular development, cellular proliferation, and new tissue growth.[133]

In a recent study, our group also developed a tunable layer-by-layer siRNA nanoformulation for silencing PHD-2 in NIH-3T3 fibroblast as target cells. In this nanoformulation, siRNA was electrostatically complexed with chitosan-coated gold NPs, after which a final outer layer of chitosan (AuNPs@CS) or poly L-arginine (AuNPs@PLA) was coated around NPs for the protection of siRNA and to facilitate and tune the endosomal escape process (Figure 9A).[134] It was found that AuNPs@PLA not only has outstanding stability over time as a siRNA carrier but also proved to be highly effective for cytosolic release after endocytic uptake. In addition, the combination of those nanocarriers with the cationic amphiphilic drug desloratadine resulted in an augmented endosomal escape of siRNA for AuNPs@PLA, while this was not the case for AuNP@CS. Overall, in NIH-3T3 fibroblast cells, siRNA-mediated downregulation of PHD-2 resulted in increased levels of VEGF and FGF-2 angiogenesis factors (Figure 9B). These *in vitro* results could pave the way for a novel NP-based angiogenic siRNA therapy for improved healing of diabetic wounds.[134]

Another factor that could affect angiogenesis is the FK506-binding protein-like (FKBPL), a novel member of the immunophilin protein family. FKBPL is a potent secreted antiangiogenic protein that targets the CD44 pathway, and its inhibition offers a potential angiogenic therapy.[169] Mulholland et al. investigated the delivery of an siRNA targeting FKBPL (siFKBLP) by RALA NPs.[135] RALA is a modified artificially designed peptide composed of a 30-amino-acid sequence derived from KALA where the lysine residues are replaced by arginine.[170] Treatment of HMEC-1 cells with RALA/siFKBPL resulted in significant improvements in cell migration and endothelial tubule formation *in vitro*. Also, for facilitating the controlled delivery of the RALA/siFKBPL NPs *in vivo*, NPs were incorporated into an electrospun bilayered wound patch consisting of alginate/poly-(vinyl alcohol) (ALG/PVA) and chitosan/poly-(vinyl alcohol) (CHT/PVA). The nanofibers were crosslinked to improve stability before NP incorporation via soak loading. *In vivo* wound-healing assays demonstrated a significant increase in angiogenesis and blood vessel density.[135]

shRNA

shRNA is a type of comparatively long RNA molecule with a region that forms a hairpin loop. shRNA sequences are usually encoded in a DNA vector that can be introduced into cells via plasmid transfection or viral transduction.[171] shRNA needs to be transcribed in the nucleus through either RNA polymerase II or III to a hairpin-like stem-loop structure before it can enter the RNAi pathway similar to miRNA.[172] Since shRNA is constantly synthesized in the target cells, more durable gene silencing is achieved in comparison with other forms of RNAi (Figure 6).[173,174]

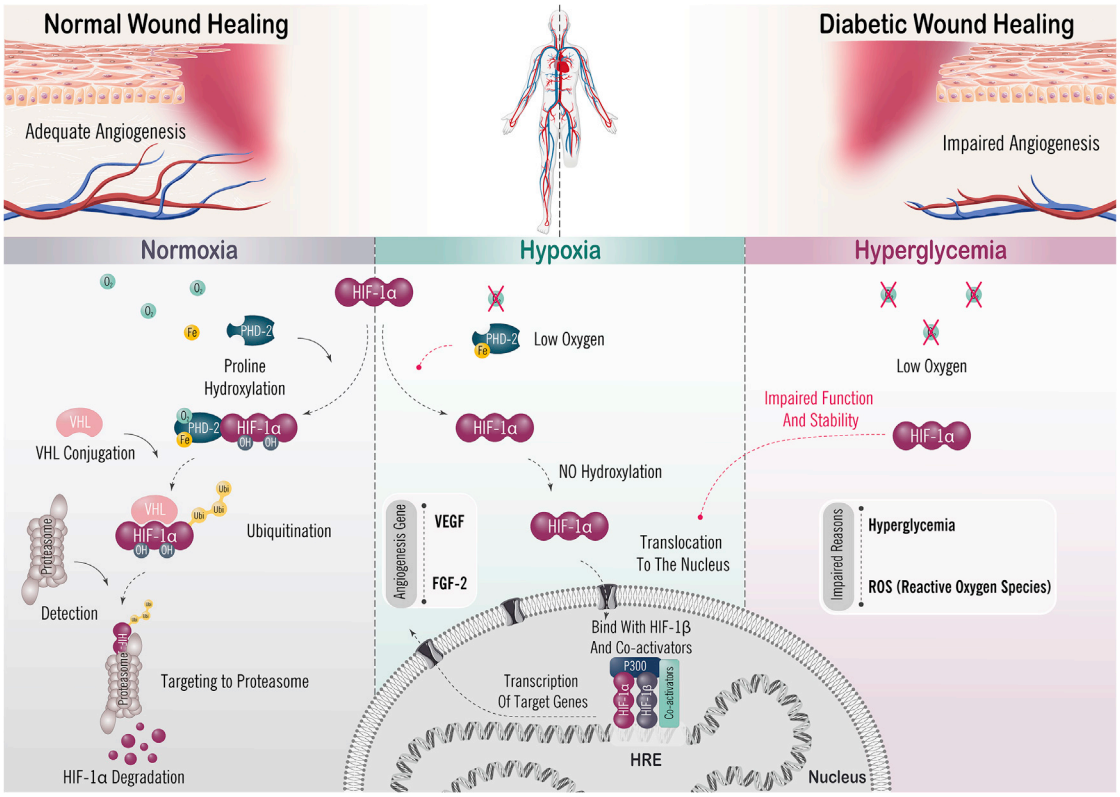

Figure 8. Schematic representation of how HIF-1 activates gene transcription in response to normoxic versus hypoxic conditions

Under normoxic conditions, HIF-1α is subjected to hydroxylation by prolyl hydroxylase domain protein 2 (PHD-2). This hydroxylation is required for binding of the von Hippel-Lindau protein (VHL), the recognition subunit of a ubiquitin protein ligase that targets HIF-1α for ubiquitination and proteasomal degradation. Under hypoxic conditions, hydroxylation is inhibited and HIF-1α is stabilized, which dimerizes with HIF-1β and binds to target genes at the consensus sequence hypoxia-responsive element (HRE). The target gene DNA sequence is then transcribed into mRNA. Under hyperglycemic conditions in the diabetic wound, the function and stability of HIF-1α are impaired by high levels of glucose and reactive oxygen species (ROS), so that fewer new vessels are formed and wound healing becomes impaired. Reproduced from Shaabani et al.[134]

Similar to siRNA, shRNA has also been explored for inhibition of PHD-2. For example, Paik et al. used shRNA to target and knock down PHD-2 expression (shPHD2) to improve angiogenesis in diabetic wounds and limb ischemia.[136] The *in vitro* results showed that shRNA knockdown of the PHD-2 transcript resulted in higher levels of HIF-1α protein, as well as upregulation of downstream angiogenic genes SDF-1 and VEGFα. In addition, shPHD-2 accelerated healing of full-thickness excisional wounds in diabetic mice with an increased vascular density and upregulation of the angiogenic transcript platelet-derived growth factor subunit α (PDGFα) in wound beds, as well as enhanced staining for CD31. Also, shPHD-2 treatment improved perfusion of ischemic hind limbs and enhanced muscle bulk survival.[136] In another investigation, the combination of shRNA and anti-miRNA was used to accelerate wound healing in diabetic conditions.[122] For this study, both shRNA targeting PHD2 and anti-miRNA that inhibits miRNA-210 were chemically modified for improved biostability and reduced immunostimulatory effects. miR-210 is upregulated by HIF-1α, and, hence, its levels are elevated when HIF-1α is stabilized, which has negative consequences for wound healing, including the attenuation of keratinocyte proliferation as well as re-epithelialization in ischemic wound healing. This combination treatment of shRNA and anti-miRNA proved to increase the proliferation and migration of keratinocytes *in vitro*. For *in vivo* applications and slow release of the encapsulated oligonucleotides into the wound bed, negatively charged NAs were co-assembled via layer-by-layer (LbL) technology with positively charged polymers into a thin multilayer coating onto the surface of a nylon woven dressing (Tegaderm mesh). The application of the LbL formulation to wounds in diabetic mice showed accelerated wound closing (4 days faster for single-oligonucleotide treatment and 4.75 days faster for the combination of both oligonucleotides), and increased the expression of neovascularization markers CD31 and cell proliferation markers Ki67.[122]

Anti-miRNA oligonucleotides

Anti-miRNA oligonucleotide (AMO) is an antagonist of miRNA relying on the complementary base pairing of the oligonucleotide sequence to its target miRNA through Watson-Crick base pairing, inhibiting its activity.[175] AMO is a synthetic single-stranded structure designed to be a perfect match to the miRNA target, especially to the seed region of the miRNA,[176] which has been shown to influence anti-miRNA specificity and activity.[177] Since miRNA reduces gene

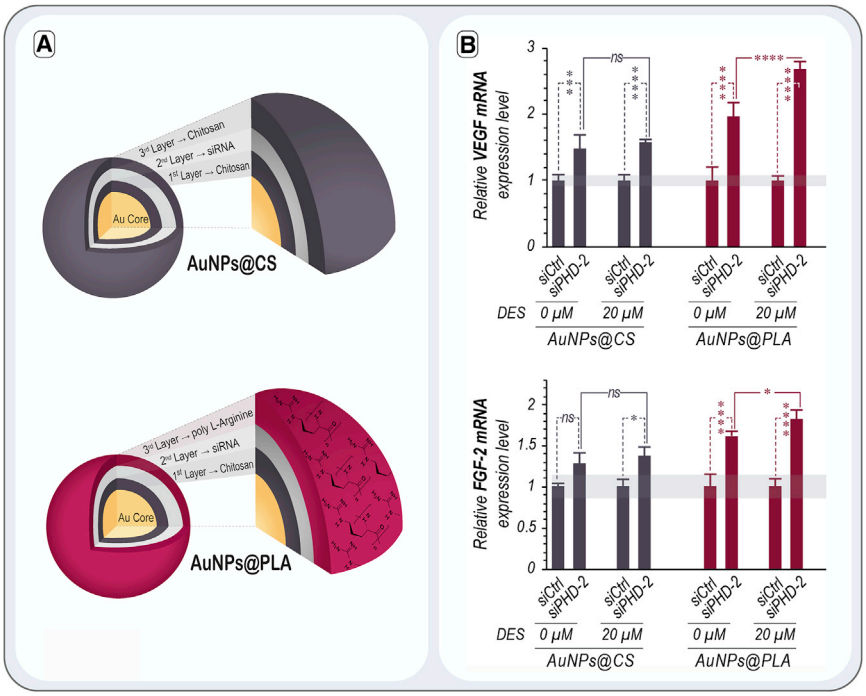

Figure 9. siPHD-2 LbL nanoformulation for the transfection of NIH3T3cells

(A) Schematic representation of the LbL nanoformulations with either Chitosan (CS) (AuNPs@CS) or PLA (AuNPs@PLA) as the third and final layer. (B) Knockdown of PHD-2 and its effect on the expression of angiogenic growth factors in NIH3T3cells. NIH3T3cells were incubated for 4 h with the nanoformulations loaded with 30 nM non-specific siRNA (siCtrl) or siPHD-2, followed by 20-h incubation with cell culture medium without or with 20 μm desloratadine (DES). After another 24-h incubation in fresh cell medium, mRNA was isolated and subjected to real-time reverse transcriptase-PCR analysis to determine expression of vascular endothelial growth factor (VEGF), and fibroblast growth factor 2 (FGF2). The results were normalized to the mRNA expression level of β-actin and shown relative to cells transfected with siCtrl. Adapted from Shaabani et al.[134] Data are represented as the mean ± the standard deviation for a minimum of three independent experiments. (ns = not significant $p > 0.05$, * $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

expression, AMO results in increased gene expression.[178] Targeting aberrant miRNAs with AMOs is a promising approach for post-transcriptional gene control in future therapies. Nevertheless, it is important to consider that, similarly to RNAi agents, they often show off-target effects due to hybridization to other, similar oligonucleotide sequences or to activation of immune responses.[179,180]

To date, many reverse genetic approaches have been explored that inhibit miRNA function by AMO for increasing angiogenesis in wound healing. In particular, the Feinberg group has shown numerous applications of anti-miRNA to increase angiogenesis in chronic diabetic wounds. For example, they showed the potential role of anti-miRNA-26a in a diabetic model of wound healing,[137] in which miRNA-26a expression was increased in response to high glucose levels in endothelial cells and in diabetic mice (∼3.5-fold) 4 days post wounding. The results indicated that local neutralization of anti-miRNA-26a induced angiogenesis (up to ∼80%), increased granulation tissue thickness (by 2.5-fold), and accelerated wound closure (53% after 9 days) through the BMP/SMAD1/Id1 signaling pathway.[137,181] In another investigation by this group, they identified the role of miRNA-135a-3p as an antiangiogenic miRNA by targeting the p38 signaling pathway in endothelial cells, which is linked to endothelial cell proliferation, migration, and vascular network formation. By local delivery of miR-135a-3p inhibitors to wounds of diabetic mice, increased angiogenesis, granulation tissue thickness, and wound closure rates were observed.[138] In addition, they found that miR-615-5p is an antiangiogenic miRNA targeting the VEGF-AKT-eNOS signaling pathway. Local delivery of miR-615-5p inhibitors markedly increased angiogenesis, granulation tissue thickness, and wound closure rates in diabetic mice.[139] It has previously been shown that miR-92a-3p is a potent antiangiogenic miRNA, and, thus, inhibition of miR-92a-3p in both acute and chronic wounds in diabetic mice and normal pigs increased granulation tissue formation, angiogenesis, tissue perfusion, and wound healing, and accelerated the wound closure.[140] It is of note that in none of those studies AMOs were formulated in nanocarriers. Instead, they were injected intradermally in their naked form. Therefore, it is to be expected that the observed effects could be substantially enhanced upon formulation of the AMOs in suitable delivery systems.

Lucas et al. reported light-activatable AMOs against miRNA-92a in diabetic mice.[141] An excellent on/off behavior upon irradiation was achieved by attaching photolabile protecting groups (cages) to the anti-miRNA, which temporarily blocks duplex formation with the target miRNA. Blue light from a light-emitting-diode (10 min, λ = 385 nm, 300 mA) breaks this photolabile bond and activates intradermally injected caged anti-miRNA-92a, which can locally inhibit miRNA-92a without substantially affecting miRNA-92a expression in other organs. This inhibition improved wound healing in diabetic mice through upregulation of the miRNA-92a targets, the integrin subunit α5 and sirtuin 1, and thus stimulating wound cell proliferation and angiogenesis.[141]

More recently, Wu et al. have presented a unique "seed-and-soil" strategy to improve diabetic wound conditions by reshaping the oxidative wound microenvironment into a pro-regenerative one (the "soil") by using anti-miRNA-26a (the seed).[142] In this study, they loaded anti-miRNA-26a (miR) in 25 kDa polyethylenimine (PEI-25K) functionalized ceria nanoclusters (PCN), which, in turn, were incorporated into a collagen (col) hydrogel (PCN-miR/Col)

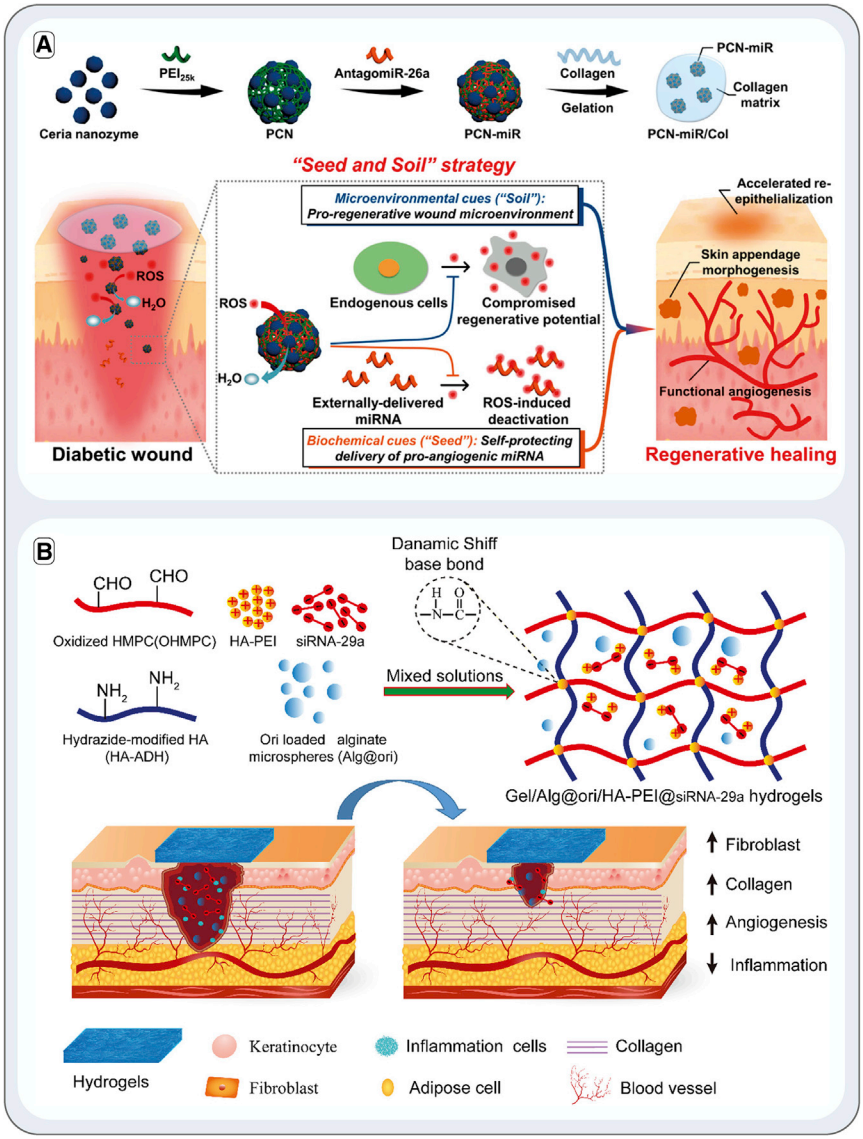

Figure 10. Schematic illustration of a hydrogel containing anti-miRNA for improvement of angiogenesis in wound healing

(A) Schematic representation of the fabrication process of the PCN-miR/Col hydrogel. A nanozyme-reinforced self-protecting hydrogel (PCN-miR/Col) composed of 25-kDa polyethylenimine (PEI25K), functionalized ceria nanoclusters (PCN), and antagomiRNA-26a (miR) nanocomplexes (PCN-miR). This PCN-miR/Col matrix is designed to simultaneously reshape the hostile wound microenvironment by creation of a pro-regenerative wound microenvironment and providing simultaneous self-protecting delivery of pro-angiogenic miRNA cues. Reprinted with permission from Wu et al.[142] Copyright 2019 American Chemical Society. (B) Schematic representation of Gel/Alg@ori/HA-PEI@siRNA-29a hydrogel preparation and wound repair. A novel hydrogel (Gel/Alg@ori/HA-PEI@siRNA-29a) prepared with a hierarchical micro/macro structure crosslinked via Schiff base bonds between adipic dihydrazide-modified hyaluronic acid (HA-ADH) and oxidized hydroxymethyl propyl cellulose (OHMPC) was combined with oridonin (ori)-loaded alginate microspheres (Alg@ori) and HA-PEI@siRNA-29a gene complexes (HA-PEI@siRNA-29a) in the interior of the hydrogels. The addition of ori and siRNA-29a is aimed at downregulating pro-inflammatory factors and enhancing the vascularization. Reproduced from Yang et al.[143] with permission from Elsevier.

(Figure 10A). Their results showed that the PCN-miR/Col not only enabled reformation of the hostile oxidative wound microenvironment by ceria nanocrystals with antioxidant enzyme-mimetic activity[182,183] but also protected the encapsulated anti-miRNA against ROS-induced damage. Furthermore, the inhibition of miRNA-26a accelerated wound closure and enhanced the quality of the healed wound, as became clear from a highly ordered alignment of collagen fibers, skin appendage morphogenesis, growth of functional new blood vessels, and oxygen saturation.[142] Following a similar strategy, Yang et al. developed another hydrogel dressing that can shorten the inflammatory stage (through the use of oridonin) and promote angiogenesis (through inhibition of miRNA-29a (siRNA-29a)[184,185]). The Gel/Alg@ori/HA-PEI@siRNA-29a hydrogel (Figure 10B) was prepared by hydrogel crosslinked Schiff base bonds between oxidized hydroxymethyl propyl cellulose (OHMPC) and adipic dihydrazide-modified hyaluronic acid (HA-ADH), and then loaded with oridonin alginate microspheres (Alg@ori) and hyaluronic acid-polyethyleneimine siRNA-29a complexes (HA-PEI@siRNA-29a). The prepared hydrogels showed excellent mechanical properties and biocompatibility *in vitro*.[143] Also, *in vivo* investigation demonstrated that the prepared Gel/Alg@ori/HA-PEI@siRNA-29a hydrogel not only significantly accelerated diabetic wound healing and promoted the expression levels of angiogenesis-related cytokines such as α-SMA and CD31 but also inhibited the expression levels of pro-inflammatory factors, including interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α).[143]

ASOs

ASOs are short (about 12–30 nucleotides long), synthetic, single-stranded nucleic acid sequences that can be employed to modulate gene expression via activating RNase H or steric blockade of the ribosome.[186,187] They have the ability to target RNA linked to human diseases and modify the production of disease-causing proteins.[188] Multiple types of modifications have been made to the nucleotides and their linkages to improve the potency, efficacy, stability, and pharmacokinetic properties of ASOs.[189] To date, three therapeutic RNase H-competent ASOs (fomivirsen, mipomersen, and inotersen), and three splice-switching ASOs (eteplirsen, golodirsen, and nusinersen)

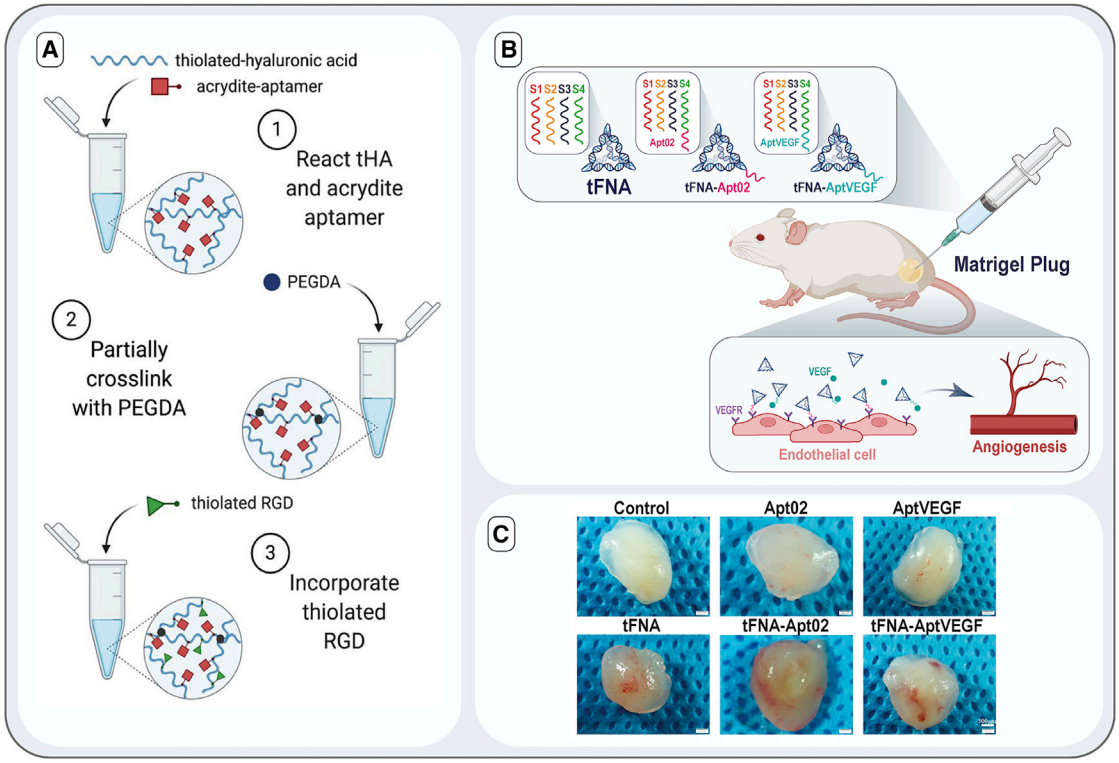

Figure 11. Aptamer approaches enhance angiogenesis in wound healing

(A) A schematic illustration of bifunctional hydrogel synthesis. (1) The thiolated hyaluronic acid (tHA) is reacted for a short time with an acrydite-modified DNA aptamer sequence to introduce the aptamer. (2) The gel is formed by the addition of the polyethylene glycol diacrylate (PEGDA) crosslinker. The PEGDA needs to be added in excess so that there are a number of free acrylate groups remaining for the final step. (3) After the gel has partially crosslinked, it is incubated in a solution of the thiol-modified arginine-glycine-aspartate (RGD) peptide to react with the remaining free acrylate groups on the PEGDA. Adapted from Roy et al.[144] with permission from Wiley Materials. (B) Angiogenic aptamer-modified tetrahedral framework nucleic acid (tFNA) stimulated angiogenesis *in vivo*. Schematic diagram of tFNA, tFNA-Apt02, and tFNA-AptVEGF formation, which can promote angiogenesis *in vivo*. For evaluation of *in vivo* angiogenesis, a Matrigel plug assay was used. The mixture of HUVECs and Matrigel was subcutaneously injected into the right ventral side of the nude mice and then, for the next 7 days, different nanoparticle treatments were injected locally into Matrigel plugs every day. Finally, the Matrigel plugs were harvested for evaluation with different methods. (C) Photographs of a Matrigel plug assay after treatment with nanoparticles (ssDNA, Apt02, AptVEGF, tFNA, tFNA-Apt02, and tFNA-AptVEGF). Scale bars, 500 μm. Reprinted with permission from Zhao et al.[145] Copyright 2021 American Chemical Society.

have received regulatory approval, while several others are in late-stage clinical development.[190] Although ASOs have been used in wound healing to increase angiogenesis,[191–195] most of them have been chemically modified to avoid degradation and were used directly, without carriers or physical delivery methods. For that reason, they will not be discussed further in this review.

Aptamers

Aptamers are a class of short single-stranded RNA or DNA oligonucleotide ligands (typically 20–100 nucleotides in length) with the capacity to fold into stable specific 3D structures.[196,197] Due to their 3D structure, they can specifically bind to various molecular targets with high specificity and affinity through electrostatic interactions, hydrogen bonding, van der Waals forces, base stacking, or a combination of those.[198] Binding often leads to the conformational change of the aptamer, which can be utilized to influence protein expression and act as a riboswitch.[199] Moreover, aptamers not only inhibit their specific target but also have the capacity to be internalized in a receptor-mediated manner. This intrinsic property makes aptamers a powerful carrier for the selective delivery of many therapeutic drugs, including chemotherapeutics, toxins, and therapeutic oligonucleotides (siRNAs, shRNA, miRNAs, or ASOs).[200,201]

Aptamers have also been explored as genetic tools for inducing wound healing. For example, Roy et al. designed a new bifunctional thiolated hyaluronic acid-polyethylene diacrylate (tHA-PEGDA) hydrogel by incorporating arginine-glycine-aspartate (RGD) peptides and anti-VEGF receptor-2 (VEGF-R2) DNA aptamers (Figure 11A).[144] They used the RGD peptide because it is the principal binding domain present within ECM proteins and can be recognized by multiple integrin receptors on the cell's surface and facilitate cell attachment and spreading.[202] On the other hand, it was shown that an anti-VEGF-R2 DNA aptamer, due to its excellent targeting properties, could simulate the VEGF pathway by attaching to vascular endothelial cell receptors on the endothelial cell surface.[203] When applying the tHA-PEGDA hydrogel containing RGD peptide and

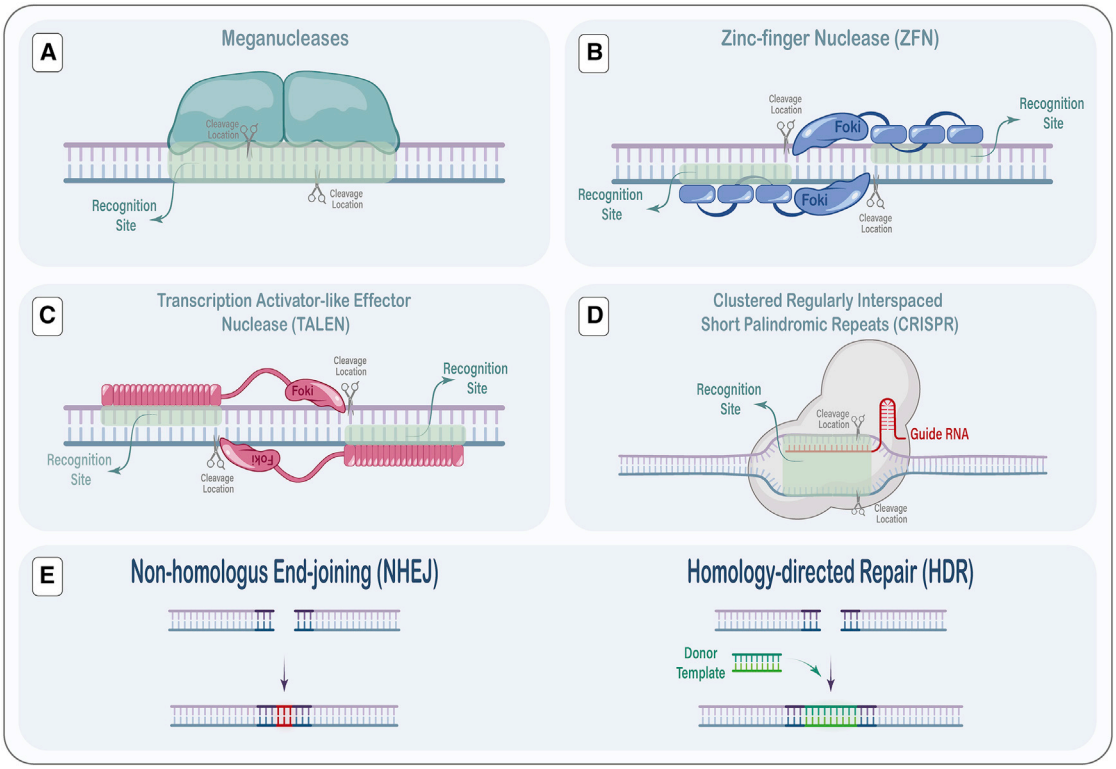

Figure 12. The four major genome-editing platforms and their working principles

(A) Homing endonucleases-meganucleases are engineered restriction enzymes that recognize long stretches of DNA sequences through protein-DNA binding. (B) Zinc-finger nuclease (ZFN) recognizes triple DNA code specificity through protein-DNA binding. (C) Transcription activator-like effector nuclease (TALEN) recognize individual base specificity through protein-DNA binding. (D) The clustered regularly interspaced short palindromic repeat (CRISPR)-associated nuclease Cas9 (CRISPR/Cas9) derives its specificity from Watson-Crick RNA-DNA base pairing. (E) All these tools result in DNA double-strand breaks, which are repaired either by error-prone non-homology end joining (NHEJ) or homology-directed repair (HDR).

anti-VEGF-R2 aptamers to endothelial cells, it was found that RGD peptides prompted cell adhesion, while anti-VEGF-R2 DNA aptamers improved cell viability and stimulated cell migration and angiogenesis.[144]

Framework NAs (FNAs) are considered promising drug delivery carriers because of their ability to pass through the cellular membrane, and their intrinsic biocompatibility, uniformity, and versatility. They have been explored as carriers for aptamers as well. FNAs are 1D to 3D nanostructures formed from DNA/RNA molecules that assemble based on the Watson-Crick base complementary pairing principle.[204–206] A tetrahedral framework nucleic acid (tFNA) is a simple 3D structure with abundant functional modification sites that is formed by four different single strands of DNA (ssDNA) through DNA origami technology.[207,208] Several studies have shown that tFNA has the function of promoting angiogenesis through activation of signaling pathways, such as Notch signaling,[209] JAK/STAT signaling,[210] and the Akt/Nrf2/HO-1 pathway.[211] Recently, Zhao et al. developed a new modified tFNA with the combination of two different angiogenic DNA aptamers (Apt02 and AptVEGF) to promote angiogenesis both *in vitro* and *in vivo*.[145] The Apt02 can mimic VEGF-A activity and it is able to combine with VEGF receptors 1 and 2 (VEGFR-1/2), functioning as an alternative to VEGF-A.[212] On the other hand, AptVEGF can bind the VEGF remaining in the cell culture medium or tissue, thus promoting angiogenesis *in vitro* and *in vivo*.[213,214] In their design, Zhao et al. formed tFNAs with DNA aptamers attached to one of the four ssDNA chains (S4-Apt02, S4-AptVEGF) (Figure 11B). By this combination, they showed tFNA-Apt02 and tFNA-AptVEGF had a stronger ability to promote endothelial cell proliferation and migration, tubule formation, spheroid sprouting, and formation of microvessels *in vivo* (Figure 11C).[145]

GENE-EDITING-BASED THERAPEUTICS FOR WOUND ANGIOGENESIS

Gene editing as a new and revolutionary genetic treatment modality is a technique that enables precise changes in the genome based on the use of programmable nucleases that induce a double-strand break (DSB) at a specific and desired location.[215,216] The four major types of gene-editing nucleases currently used for inducing these site-specific DSBs are meganucleases, zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and most recently the CRISPR-associated nuclease Cas9 (CRISPR/Cas9)[217,218]

Table 3. Studies that Investigated gene-editing-based therapeutics for increased angiogenesis in wound healing

Target	Therapeutic NA	Delivery method/ carrier	Model systems	Wound	Result	Reference
PDGF-BB	three engineered plasmids for safe-Harbor locus	nucleofection	*ex vivo* primary fibroblasts isolated from ear mice *in vivo* NOD/SCID/γc^{null} mice	6-mm full-thickness wounds on the dorsum of mouse	increased wound vascularization increased blood vessel density	Barker et al.[228]
PDGF-BB VEGF-A	Cas9 nuclease	electroporation plus AAV6	*ex vivo* human mesenchymal stromal cells *in vivo* $^{db/db}$ mice or NSG mice	6-mm full-thickness wounds on the dorsum of the genetically mouse	accelerated healing of wounds elevating secretion of growth factors improved granulation tissue formation and blood vessel density	Srifa et al.[229]
Ndrg2	Cas9/sgRNA-ribonucleoproteins	electroporation hydrogel	*ex vivo* hematopoietic progenitor cells that differentiated into DCs *in vivo* C57BL6/J wild-type mice	splinted excisional wounds on mouse	promoted angiogenesis accelerated wound healing	Henn et al.[230]

AAV6, adeno-associated virus 6; DC; dendritic cells; Ndrg2, N-myc downregulated gene 2; NSG, NOD scid gamma; PDGF, platelet-derived growth factor; sgRNA, single guide RNA; VEGF, vascular endothelial growth factor.

(Figures 12A–12D). Each of them creates a site-specific DSB in the genome of the cell that activates repair through non-homologous end joining (NHEJ) or homology-directed repair (HDR)[219] (Figure 12E). While NHEJ results in random indels and gene disruption at the target site, HDR can be harnessed to insert a specific DNA template (single stranded or double stranded) at the target site for precise gene editing.[220]

In contrast to other gene therapy strategies, gene editing provides the most versatile tool for precisely correcting disease-associated point variants within the natural locus, which would be especially important for gene products for which an exact dosage is required.[221,222] However, as with any new emerging technology, clinical translation is associated with some challenges that need to be addressed. The first one is the safe and efficient delivery of the gene-editing components to the target cells and spatiotemporal control over the expression of the nucleases.[42] The second and biggest concern with genome-editing tools is undesired editing of genomic regions, which can occur as a side effect of gene editing, resulting in undesired downstream effects.[223,224] The third one is the ethical and regulatory challenges for use in human germline manipulation and the uncertain consequences for future generations.[225–227]

As already mentioned, studies based on gene editing as a therapeutic tool are relatively new and limited in number, especially when focused on angiogenesis induction in the context of wound healing (Table 3). Cell-based therapies are one of the most promising methods for treating chronic wounds, and, to date, many efforts have been made to improve the function of these cells with genetic engineering. For example, Barker et al. showed site-specific nuclease-mediated integration of PDGF-B into the primary mouse fibroblasts, after which the engineered fibroblasts were applied into the mouse wound. The application of these engineered cells enhanced wound healing by enhancing vascularization.[228] However, after 5 months, genetically modified primary mouse fibroblasts were still present and continued to secrete PDGF-B. To avoid long-term production of growth factors that may have the potential to cause malignancy and oncogenic effects, in a follow-up study they switched to human mesenchymal stromal cells (hMSCs).[229] MSCs have a short survival time and limited distribution to other sites.[231,232] They engineered human hMSCs by delivering Cas9 nuclease via electroporation and homologous repair template via adeno-associated virus 6 (AAV6), to induce short-term platelet-derived growth factor beta (PDGF-BB)- and VEGF-A hypersecretion.[229] They succeeded in integrating DNA of up to 3.2 kb with stable transgene expression in the genome of hMSCs from multiple tissue sources without altering their *ex vivo* characteristics. Injection of engineered hMSCs into the wound bed accelerated healing of acute diabetic wounds by elevating secretion of growth factors, and improving granulation tissue formation and blood vessel density.[229] In another study, in order to increase the secretion of VEGF by dendritic cells (DCs) as a cell-based therapy for wound healing, the N-myc downregulated gene 2 (Ndrg2) was permanently knocked out in these cells by using Cas9/sgRNA-ribonucleoproteins delivered by electroporation.[230] DCs are a heterogeneous cell population that can promote peripheral immune tolerance and limit tissue damage, but, because of the high expression of Ndrg2, they have limitations on the secretion of VEGF, which is essential for wound healing. Their results showed that excisional wounds treated with Ndrg2 knockout DCs promoted angiogenesis and accelerated wound healing with strong expression of VEGF-A and antioxidant transcriptomic signatures compared with other control groups.

CONCLUSIONS AND OUTLOOK

Chronic wounds, and especially diabetic wounds, are an increasingly large global burden that is expected to become even worse in the near future due to population aging and its associated co-morbidities.[233] By understanding the pathophysiology factors that cause impairment

of the healing response in chronic wounds, it has been made clear that one of the major problems is a reduced ability to regrow microvasculature through the process of angiogenesis, in which new blood vessels form.[234] Hence, induction or promotion of the angiogenic pathways in wounds can be considered a promising approach to accelerate the healing of chronic wounds. This goal can be achieved by using angiogenic growth factors, gene therapy, cellular therapy, angiogenic small molecule drugs, and so on.[22]

Gene-therapy-based approaches are one of the most promising methods that are beginning to show encouraging results with revolutionary potential in treating diseases.[26] In this review, we summarized recent work on gene therapies for enhancing angiogenesis in wounds by using non-viral delivery methods. These studies were summarized in three categories: gene augmentation (Table 1), gene silencing (Table 2), and gene editing (Table 3). However, despite significant progress in the field, many challenges remain. A first challenge is related to the delivery of NAs. While topical administration is quite easily achieved, NAs have limited stability in the wound environment and are poorly internalized by cells. This leads to the need for the development of safe and effective delivery methods that are able to protect the genetic material from degradation and facilitate its internalization in target cells.[126,178,235] Although substantial improvement has been achieved over the past years in delivery technologies, it is a sobering fact that only a few gene therapeutics have reached clinical trials for wound healing. Delayed clinical application is related to the uncertainty of long-term toxicity and low gene transfection efficiency *in vivo* that must be overcome in the future.

A second challenge is the still-incomplete understanding of the pathology of chronic wounds, which is at least partly due to the lack of animal models that sufficiently approximate human chronic wounds. A third challenge relates to the fact that chronic wounds are often caused by other diseases and metabolic disorders, such as diabetes and vascular disease. Therefore, monotherapies to enhance angiogenesis are unlikely to be adequate to treat chronic wounds. Additional strategies should be considered to treat the underlying disorders and maximize the effect of the wound therapeutics.

After almost 30 years of promise tempered by some setbacks, gene therapies are rapidly becoming a reality for a variety of inherited and acquired human diseases, and their fully fledged clinical deployment appears to be very close. The increasing number of resources devoted to a better understanding and characterization of gene-therapy technologies demonstrates the maturation of the field. All together, we envision that wound dressings that incorporate sustained NA delivery systems for enhancing angiogenesis, combined with therapy that addresses the underlying morbidities, will enable exciting new options for treating chronic wounds in the next decade.

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AUTHOR CONTRIBUTIONS

E.S., conceptualization and writing – original draft; M.S., writing – original draft; R.F.M. and S.D.S., supervision; K.B. and J.F., writing – review & editing and supervision.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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