Capturing the RNA Castle: Exploiting MicroRNA Inhibition for Wound Healing

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Abbreviations

2′-OME	2′-O-methyl
2'-MOE	2′-O-methoxyethyl
AGO	Argonaute
db/db	leptin receptor deficient diabetic
EC	endothelial cells
HIF-1	hypoxia-inducible factor
HUVECs	human umbilical vein endothelial cells
ITGA	integrin alpha
LNA	locked nucleic acid
MAPK	mitogen-activated protein kinase
miRNA	microRNA
PS	phosphorothioate
RISC	RNA-induced silencing complex
siRNA	small/short interfering RNA
VEGF	vascular endothelial growth factor
ZEN	N,N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine

Keywords: skin; microRNA; wound healing; keratinocyte; endothelial cell.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

Abstract

The growing pipelines of RNA-based therapies herald new opportunities to deliver better patient outcomes for complex disorders such as chronic non-healing wounds associated with diabetes. Members of the microRNA (miRNA) family of small non-coding RNAs have emerged as targets for diverse elements of cutaneous wound repair, and both miRNA enhancement with mimics or inhibition with antisense oligonucleotides represent tractable approaches for miRNA-directed wound healing. In this review, we focus on miRNA inhibition strategies to stimulate skin repair given advances in chemical modifications to enhance the performance of antisense miRNA (anti-miRs). We first explore miRNAs whose inhibition in keratinocytes promotes keratinocyte migration, an essential part of re-epithelialisation during wound repair. We then focus on miRNAs that can be targeted for inhibition in endothelial cells to promote neovascularisation for wound healing in the context of diabetic mouse models. The picture that emerges is that direct comparisons of different anti-miRNAs modifications are required to establish the most translationally viable options in the chronic wound environment, that direct comparisons of the impact of inhibition of different miRNAs are needed to quantify and rank their relative efficacies in promoting wound repair, and that a standardised human ex vivo model of the diabetic wound is needed to reduce reliance on mouse models that do not necessarily enhance mechanistic understanding of miRNA-targeted wound healing.

Introduction

Two decades after RNA interference and small interfering RNAs (siRNAs) were first demonstrated in animal cells [1, 2], siRNA-based RNA drugs have finally enter the clinical market following the approval of patisiran (Onpattro®) [3, 4], givosiran (Givlaari®) [5, 6] and lumasiran (Oxlumo®) [7, 8] by the Food and Drug Administration of the USA and other regulatory bodies. These liver-targeted agents for hereditary transthyretin amyloidosis, acute hepatic porphyria and primary hyperoxaluria, respectively, typify a new era of RNA-based medicines that are likely to bring enormous benefits for patients with conditions ranging from cancer and neurological disorders to cardiometabolic disease and COVID-19 [9].

Gene silencing by siRNA is mediated by Argonaute proteins, of which there are four human paralogs (reviewed in [10]). The main effector of siRNA activity is AGO2, which is the only mammalian AGO protein that has endonucleolytic activity to enable mRNA cleavage, although limited cleavage capability has also been reported for AGO3 [11-13]. Upon delivery into the cells, siRNA duplexes are unwound by AGO2 proteins such that one strand (the guide strand) is loaded into AGO2 while the other (passenger strand) is discarded (Figure 1). The siRNA-loaded AGO2 forms the minimal effector of RNA-induced silencing complex (RISC), associates with the target mRNA through Watson-Crick base pairing and cleaves its target mRNA when there is extensive sequence complementarity, as is normally the case for siRNA [14].

Challenges with translational exploitation of siRNA-based medicines have broadly centred on delivery and function: delivery of therapeutically effective doses to the target tissue and functionalisation of siRNA to enhance stability, resistance to nuclease degradation, specificity of target binding and avoidance of immunostimulatory responses [15]. For patisiran, the siRNA is formulated in ionizable cationic lipid nanoparticles identified through extensive screening assays [16-18]. In contrast, the siRNA in givosiran is conjugated to a trivalent *N*-acetylgalactosamine moiety to promote liver uptake by binding to the asialoglycoprotein receptor on hepatocytes [5, 19]. For improved stability and function, diverse chemical modifications have been developed, often involving the 2′ position of the ribose sugar of nucleotides (Figure 2A) [20, 21]. For instance, the guide strand of patisiran has two 2′-O-methyl (2′-OME) modified ribonucleosides, while the passenger strand has nine such 2′-OME modifications, and each strand has a deoxythymidine (dT-dT) overhang at the 3′

end (Figure 2B). The use of modified nucleotides is even more thorough in givosiran, with the duplex comprised entirely of 2′-OME and 2′-fluoro modified ribonucleosides [22]. Modifications to the phosphodiester backbone are also deployed in givosiran, but only at selected ends of the duplex (Figure 2C). This contrasts with the 18-mer single-stranded nusinersen (Spinraza®) that modulates the splicing of its target pre-mRNA (SMN2) to treat spinal muscular atrophy, where a phosphorothioate backbone is used throughout [23]. These along with advances in RNA formulation and delivery have supported the growth of siRNA-based clinical trials [24]. However, it should be noted that phosphorothioate and other modifications can trigger hybridisation-independent hepatic and renal toxicities due to proinflammatory responses, interactions with cell surface or intracellular proteins and accumulation-related degenerative effects [25-27]. In addition, nucleoside modifications on siRNA can prevent efficient cleavage of the target mRNA, depending on the position of the modified nucleosides relative to the RISC cleavage site [28-30].

MicroRNAs: A brief overview

Mature microRNAs (miRNAs) are small ~22 nucleotide non-protein coding RNA molecules that repress gene expression by binding to the 3′ untranslated region (UTR) of target mRNA transcripts [31]. The mechanisms of action of miRNAs parallel those of siRNA in some ways, for instance loading of miRNAs into AGO proteins (Figure 1). However, miRNAs are only partially complementary to their targets, which expands the repertoire of target genes they can regulate, and a given miRNA typically regulates hundreds of genes [32-35]. This contrasts with siRNAs which, especially for therapeutic purposes, are designed or selected to be specific to one target gene. In the absence of extensive target complementary, mRNA decay (rather than cleavage observed with siRNA) dominates miRNA-mediated gene silencing, a process that appears to require the GW182 scaffolding protein in a way that is dispensable for siRNA function [36, 37]. Nonetheless, the development of oligonucleotides that mimic or inhibit miRNAs has capitalised on developments for siRNA therapeutics and an increasing number of clinical trials has been launched in order to exploit the translational potential of miRNA mimics and antisense miRNA inhibitors, anti-miRs [38-41]. While miRNA mimics silence the expression of target genes, much like siRNA, miRNA inhibitors bind

strongly to their miRNA targets and sequester the miRNA away from their messenger RNA target, resulting in elevated expression (derepression) of the target transcript.

Wound healing at a glance

Cutaneous wound healing is a complex physiological process orchestrated through overlapping stages comprising inflammation, proliferation, angiogenesis, epithelialization and remodelling [42], as illustrated in Figure 3. These stages are staggered yet not mutually exclusive, with significant spatiotemporal overlap, and are underpinned by the interplay of diverse cell types including keratinocytes, fibroblasts, endothelial cells and immune cells, especially neutrophils and macrophages [42-45]. As the major cellular component of the epidermis, keratinocytes are vital for barrier maintenance and also heavily involved in the restoration of the epidermis after an injury [46]. Upon injury, keratinocytes along the edge of the wound undergo membrane structure and cytoplasmic transformation. Keratinocytes become polarised and fan shaped due to a combinative effect of the cytoplasmic rearrangements [47]. Reduced adhesion to the basal lamina and increased flexibility allows for keratinocytes to migrate over the denuded area. Simultaneously, keratinocytes proliferate behind the migrating tongue. During re-epithelialisation, keratinocyte proliferation and migration is regulated by numerous agents such as cytokines, integrins and specific growth factors [46].

Targeting MicroRNAs in Cutaneous Wound Healing

Several miRNAs whose translational promise stems from deployment of miRNA mimics to enhance keratinocyte proliferation, migration and differentiation have been appraised in a separate review [48]. For an expansive complementary overview of miRNAs in diabetic wound healing, the reader is referred to a recent review by Dalgaard and colleagues [49]. Here, we focus first on miRNAs where inhibition promotes keratinocyte migration. We then review miRNAs whose inhibition in diabetic mouse models promotes wound healing through mechanisms that impinge on angiogenesis. Although the *db/db* mouse has limitations [50], it remains the preferred model among the research community, with >400 PubMed hits in 2018 and 2019, respectively. In contrast, although the NONcNZO10 diabetic mouse has been presented as superior to the *db/db* model [50], there were

only 22 reports in total returned for “NONcNZO10 diabetic mouse” in PubMed as of February 2021. The specific arm of the pre-miRNA-duplex from which the mature miRNA arises is indicated with 5p or 3p, unless, as for miR-198 and miR-4516 below, only one arm of the duplex persists, in which case we follow the miRBase convention (see http://www.mirbase.org/) and omit the 5p or 3p suffix. The miRNAs considered are summarised in Table 1. We note that long non-coding RNAs (>200 nt) have also been implicated in wound healing but as these have been appraised elsewhere recently [51, 52] will not feature in this review.

Elevated microRNA-198 impairs actin and extracellular matrix regulators in keratinocytes

In an elegant study, Sampath and colleagues uncovered a novel post-transcriptional switch underpinning reciprocal expression of miR-198 and the protein product of the miR-198 host gene *follistatin-like 1* (FSTL1) [53]. In resting primary human keratinocytes, KH-type splicing regulatory protein (KSRP) binds to the miR-198 precursor (pre-miR-198) to drive processing of mature miR-198 and keep the *FSTL1* transcript in the nucleus. However, upon wounding, KSRP and miR-198 levels are downregulated rapidly in a transforming growth factor β (TGFβ)-dependent manner and the FSTL1 transcript is exported to the cytoplasm where it is translated to FSTL1 protein. More importantly, miR-198 was constitutively elevated at the wound edge in chronic wounds from diabetes patients. This impeded wound healing by downregulation of miR-198 targets associated with regulation of the actin cytoskeleton (*DIAPH1;* diaphanous homologue 1) or the extracellular matrix (*LAMC2*; laminin γ2 chain *and PLAU;* urokinase-type plasminogen activator). Elevation of miR-198 impaired wound closure in a keratinocyte scratch monolayer and this was phenocopied by silencing of the identified miR-198 targets. Interestingly, there is some evidence that miR-198 dampens the proliferation of HaCaT keratinocytes, through silencing of cyclin D [54], suggesting that blocking miR-198 function in the wounded epidermis may also support keratinocyte proliferation. Thus, inhibition of miR-198 may represent a novel mechanism to promote re-epithelialisation in non-healing wounds and warrants studies in *db/db* mice

The microRNA-99 family inhibits HaCaT keratinocyte migration

Using full-thickness mouse wounds, Zhou and colleagues observed differential expression of 63 miRNAs at early (1 day) and late (5 days) post wounding [55]. Among these, two members of the highly conserved miR-99 family, miR-99b-5p and miR-100-5p, were modestly but significantly downregulated in human skin wounds at early time points (6 h and 1 day post-wounding), while miR-99a remained unchanged. Transfection of HaCaT keratinocytes with miR-99b and miR-100 mimics impaired migration in scratch and trans-well assays, reduced HaCaT proliferation and raised apoptosis [55]. More importantly, anti-miR-100-5p LNA boosted HaCaT keratinocyte migration 3-fold in scratch assays. Functionally, the regulation of HaCaT keratinocyte migration by miR-99 family members was associated with mechanistic target of rapamycin (mTOR) and Homeobox A1 (HOXA1) [55, 56].

Roles for microRNA-4516 in keratinocyte migration and differentiation

Potential roles for miR-4516 in the skin first emerged from studies showing upregulation of miR-4516 in HaCaT keratinocytes exposed to photochemotherapy with psolarens and ultraviolet A (PUVA), an established anti-psoriasis treatment [57]. Although miR-4516 is not confidently annotated in the miRNA target database TargetScan 7.2 (http://www.targetscan.org/vert_72/), elevated miR-4516 has also been implicated in perturbation of the epithelial barrier in airway epithelial cells during viral infection [58], in the proliferation and invasion of glioblastoma cells [59], and as a candidate tumour suppressor gene in pancreatic cancer [60]. Together, these suggest that miR-4516 is a biologically and clinically relevant non-coding RNA, regardless of the precise mechanisms of its biogenesis. Importantly, signal transducer and activator of transcription 3 (STAT3), an important player in psoriasis, wound healing and other aspects of epidermal biology [61] was validated as a miR-4516 target in HaCaT keratinocytes [57]. This is noteworthy because STAT3 induces the expression of miR-21-5p[62], one of the first miRNAs associated with keratinocyte migration.

Roles for miR-4516 in keratinocyte migration emerged from the observations that miR-4516 was downregulated in psoriasis lesions and that fibronectin 1 and integrin alpha 9 (ITGA9) were two direct miR-4516 targets [63]. As fibronectin is a major extracellular matrix (ECM) protein whose interactions with integrins regulate cell migration and other aspects of wound healing [64, 65], the

potential for miR-4516 to influence keratinocyte migration was explored. Indeed, migration of both HaCaT and primary human keratinocytes appeared to be impaired by a miR-4516 mimic and enhanced with an anti-miR-4516 inhibitor [63]. These effects of miR-4516 modulation were linked to its ability to dampen the STAT3:miR-21 axis of keratinocyte migration and to regulate actin cytoskeleton reorganization downstream of the small GTPases Rac1, RhoA and Cdc42 [63]. Interestingly, there was some suggestion that miR-4516 mimic triggered HaCaT differentiation, while anti-miR-4516 impaired differentiation. Hence, although inhibition of miR-4516 may promote keratinocyte migration, the impairment of keratinocyte differentiation by anti-miR-4516 may limit full re-epithelialisation in the context of wound healing. Whether miR-4516 levels are altered in wounded normal or diabetic skin is not clear.

MicroRNA inhibition to promote wound healing with evidence from diabetic mice

The above studies on HaCaT and primary keratinocyte migration are limited by the lack of data on wound closure using rodent models or *ex vivo* human skin. In contrast, several miRNAs have been shown to promote wound repair in the diabetic (*db/db*) mouse. We first examine the hypoxia-inducible miRNA miR-210 and then focus on miRNAs with clear roles in neovascularisation during wound repair given that the formation of new blood vessels is crucial for effective wound healing.[66]

Inhibiting hypoxia-induced microRNA-210-3p to promote wound healing

Hypoxia, or oxygen insufficiency, potentiates wound healing in the initial stages but is detrimental to tissue repair if sustained [67]. Early studies identified miR-210 as one of the most robustly induced miRNAs under hypoxia, with induction driven by binding of hypoxia-inducible factor (HIF-1α) to a hypoxia responsive element (HRE) in the miR-210 promoter [68, 69]. Building on these observations, Sen and colleagues found the expression of miR-210-3p was upregulated in ischemic wounds and that it was indeed dependent on the HIF-1α [70]. Expression of E2F3, a known miR-210-3p target with essential roles in cell cycle progression, was markedly lower in ischemic wounds. Inhibition of miR-210 enhanced HaCaT keratinocyte proliferation, presumably by derepression of E2F3 [70]. Recently, using layer-by-layer nanofabrication to assemble anti-miR-210-3p onto a nylon wound dressing, Johnston and co-workers found anti-miR-210-3p accelerated wound closure in *db/db*

mouse skin, with increased collagen deposition [71]. The high level of collagen deposition, along with the apparent ability of miR-210-3p inhibition to restore E2F3 in keratinocytes, appeared to supersede any impediment to tissue regeneration that might have been expected due to the loss of the pro-angiogenic function of miR-210 following inhibition [71].

Interestingly, the anti-miR used by Johnston and colleagues contained 2′-OME-substituted residues with N,N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine (ZEN)-modified ends to enhance target binding and nuclease resistance [71, 72]. This modification was reported to be particularly potent compared to LNA and phosphorothioate counterparts in luciferase reporter assays. This raises the possibility that ZEN end-modification may enhance anti-miR oligonucleotides targeting other miRNAs for wound repair. However, systematic comparisons of ZEN modified anti-miRNA and their LNA counterparts in *db/db* mouse wounds and human *ex vivo* skin wounds are required as other studies found ZEN modifications did not appear to enhance anti-miR function compared to LNA [21].

It should also be noted that beyond inhibition of miR-210 with antisense oligonucleotides, efforts have also been made to develop a small molecule inhibitor of miR-210 biosynthesis [73]. Such small molecule inhibitors have some advantages over oligonucleotide therapies, spanning ease of manufacture and purification, lower production costs, better pharmacokinetic properties and route of administration [74]. However, because the identification of selective small molecule inhibitors of miRNA biogenesis is non-trivial, the place of small molecules in the overall landscape of miRNA inhibition needs further critical consideration to maximise their translational impact.

Inhibition of microRNA-92a-3p promotes angiogenesis in diverse contexts

Early work by Dimmeler and colleagues demonstrated that miR-92a-3p, a member of the highly conserved miR-17~92a cluster, repressed migration, adhesion and sprouting angiogenesis of human umbilical vein endothelial cells (HUVECs) [75]. Focussing on muscle tissues, the authors showed that inhibition of miR-92a with an antisense oligonucleotide enhanced re-endothelialization and neovascularization in ischemic mouse limbs [75, 76] and in murine and porcine models of myocardial infarction [75, 77]. Importantly, integrin alpha 5 (ITGA5), a receptor for the extracellular

matrix protein fibronectin, was identified as a direct miR-92a target and a miR-92a inhibitor promoted sprouting angiogenesis by ECs in an ITGA5-dependent manner [75].

These observations implicating miR-92a-3p in angiogenesis and the restoration of tissue function prompted Dimmeler and colleagues to explore light-inducible controlled release of intradermally injected anti-miR-92a-3p. This approach improved tissue repair in excisional wounds in *db/db* mice and raised ITGA5 expression in dermal wound tissue, though the specific cell types with elevated ITGA5 in response to anti-miR-92a are less clear [78]. In a separate study, Jackson and colleagues explored the impact of miR-92a-3p inhibition on cutaneous wound healing using excisional wounds in the *db/db* diabetic mouse model by intradermal injection of a LNA-based miR-92a-3p inhibitor (MRG-110 from miRagen Therapeutics). The miR-92a-3p inhibitor enhanced wound re-epithelialisation, granulation tissue formation and angiogenesis [79] and this was associated with de-repression of ITGA5 in HUVECs and in human skin fibroblasts. However, whether the impact of anti-miR-92a on wound healing extends to direct effects on keratinocytes remains obscure as studies of the expression of the miR-17 cluster [80-82] have not elucidated the functional effects of miR-92a-3p on keratinocyte behaviour during wound repair.

Interestingly, lipopolysaccharide (LPS) induced miR-92a-3p expression in human pulmonary microvascular endothelial cells (HPMECs) and inhibition of miR-92a increased HPMEC migration and tube formation, improving EC cell barrier function [83]. Considering the roles of Gram-negative bacteria like *Pseudomonas aeruginosa* in chronic wounds [84], this raises the possibility that a LPS: miR-92a-3p axis may contribute to impaired angiogenesis in the dermal microvasculature. However, the ability of LPS and other ligands for LPS receptor (Toll-like receptor 4) to trigger miR-92a-3p expression in dermal ECs has not been established, though dermal ECs respond to LPS stimulation and express miR-92-3 [85, 86]. Indeed, the ability of LPS to impair sprouting angiogenesis of dermal organoid cultures [85] may be dependent on induction of miR-92a-3.

Enhanced angiogenesis and wound closure by microRNA-26a-5p inhibition

In a series of recent papers, Feinberg and colleagues identified roles for several miRNAs including miR-26a-5p, miR-615-5p, miR-135a-3p and miR-4674.[87-90] Early work by the group had shown that proangiogenic stimuli tumour necrosis factor-α (TNFα) and vascular endothelial cell growth

factor (VEGF) reduced the expression of miR-26a-5p in HUVECs [91]. Overexpression of miR-26a-5p impaired endothelial cell (EC) proliferation, migration and angiogenic sprouting, whereas inhibition of miR-26a-5p had the opposite effects on these processes [91]. Inhibition of miR-26a-5p also stimulated myocardial angiogenesis after acute myocardial infarction in a mouse model. Based on these observations, miR-26a-5p was evaluated in ECs, under high glucose conditions to reflect the diabetic microenvironment [87]. Elevated glucose (20 mM) doubled miR-26a-5p expression in EC by 24 h, implying that impaired angiogenesis in diabetic wounds may be linked to raised miR-26a-5p expression. However, given that TNFα reduced miR-26a expression, it would have been useful to assess miR-26a-5p in ECs treated concomitantly with TNFα and high glucose conditions to reflect the chronic diabetic wound more closely and reveal how ECs moderate miR-26a expression under dual signals. Nonetheless, in acute wounds in *db/db* mouse, miR-26a levels increased 3-fold by day 4 post-wounding, and inhibition of miR-26a-5p increased angiogenesis, granulation tissue and wound closure [87]. Interestingly, there was some suggestion that inhibition of miR-26a-5p could promote keratinocyte migration [87], consistent with studies showing an increase in HaCaT keratinocyte migration upon miR-26a-5p inhibition [92]. However, broadly, inhibition of miR-26-5p did not affect fibroblast or keratinocyte behaviour under basal or diabetic conditions, nor was the tissue macrophage profile altered. Thus, inhibition of miR-26a-5p in ECs alone may suffice to drive wound healing but uptake by other skin cell types may not have detrimental effects.

MicroRNA-135a-3p, MicroRNA-615-5p, MicroRNA-4674 and angiogenesis

Plasma miRNA profiling revealed elevated miR-135a-3p and miR-615-5p in patients with extensive coronary stenosis compared to those with normal coronary angiograms [88, 89]. Interestingly, both of these are minor strand miRNAs, but it is not clear whether their enhanced expression occurred at a transcriptional level or was related to enhanced stability under the disease conditions. Levels of both miR-135a-3p and miR-615-5p were elevated >2 fold in the skin of diabetic patients. Furthermore, in *db/db* mice skin, miR-135a-3p and miR-615-5p were raised ~4 and ~5 fold, respectively, compared to wild-type mice. In mouse skin sampled 7 and 9 days after wounding, levels of these miRNAs were approximately 2-fold higher than in wild type mice. Crucially, inhibition of miR-135a-3p and miR-615-5p enhanced proliferation, tube formation and migration of HUVECs while

mimics had the opposite effects. Furthermore, inhibition of these miRNAs using a LNA anti-miR induced angiogenesis, enhanced the formation of granulation tissue and promoted wound closure in diabetic mouse wounds and induced angiogenesis in human *ex vivo* skin organoids.

Mechanistically, miR-135a-3p targets mRNA encoding huntingtin-interacting protein 1 (HIP1), a co-factor in clathrin-mediated vesicle trafficking, to impair VEGF-dependent activation of p38MAPK [88]. In contrast, miR-615-5p impaired VEGF signalling through effects on two direct targets, IGF2 (insulin-like growth factor 2), and RASSF2 (Ras-associating domain family member 2) that regulate the AKT pathway [89]. Importantly, the proangiogenic agents VEGF and basic fibroblast growth factor (bFGF) both reduced miR-135a-3p and miR-615-5p expression in HUVECs, suggesting that under normal physiological conditions, VEGF dampens miR-135a-3p and miR-615-5p expression to maintain functional HIP1, IGF2 and RASSF2 functions. Conditions such as diabetes elevate miR-135a-3p and miR-615-5p through mechanisms that are not clear but the resulting repression of HIP1, IGF2 and RASSF2 dampens VEGF-dependent signalling, angiogenesis and wound healing.

In contrast to miR-135a-3p and miR-615-5p, plasma miR-4674 levels were reduced in plasma of patients with extensive coronary stenosis compared to those with normal coronary angiograms [90]. Like miR-135a-3p and miR-615-5p, however, inhibition of miR-4674 stimulated proliferation, tube formation and migration of HUVECs while a miR-4674 mimic had the opposite effects [90]. Mechanistically, miR-4674 also targeted the p38 MAPK pathway, and inhibition of miR-4674 raised *IRAK1 and BICD2* mRNA expression in ECs and human skin organoids, promoting p38MAPK activation and angiogenesis in the organoids [90]. As miR-4674 was identified as a human-specific miRNA, no studies were performed on *db/db* mice. Nonetheless, it would be interesting to determine the translational potential of miR-4674 using human skin organoids maintained with high glucose conditions that mimic the diabetic microenvironment. Further, the observation that miR-4674 was expressed at significantly higher levels in both ECs and keratinocytes compared to fibroblasts suggests inhibition of miR-4674 may mobilise axes of wound repair in both angiogenesis and re-epithelialisation, though studies of miR-4674 function in keratinocytes have not been reported yet.

Elevation of VEGF signalling by inhibition of microRNA-15b-5p and microRNA-200b-3p

One theme from the Feinberg studies is that impaired signalling downstream of VEGF and other growth factors rather than limited availability of the growth factors themselves may underpin defective angiogenesis in diabetic wounds [88]. Nonetheless, enhancement of VEGF and VEGF receptor 2 (VEGFR2) expression has been exploited for wound repair in *db/db* mice [93]. Focussing on miR-15b-5p and miR-200b-3p and their respective targets VEGF and VEGFR2 [94-96], Bitto and colleagues showed that local injection of LNA anti-miRs promoted epidermal regeneration, granulation tissue formation, neoangiogenesis and wound closure [93]. Importantly, the combination of anti-miR-15b and anti-miR-200b was more effective than either anti-miR alone.

CONCLUSION

As the pool of miRNAs whose inhibition can promote wound repair becomes clearer, questions arise about which type of anti-miRNAs are most effective in targeting these miRNAs. The successful phase 2 trial of miravirsen, an LNA-modified anti-miR targeting miR-122 in the liver to limit the replication of hepatitis C virus helped establish LNAs a leading candidate for therapeutic anti-miRNA. [97, 98]. However, an LNA anti-miR targeting miR-199b in the heart was less effective than a cholesterol-conjugated 2′OME anti-miR. Further, an anti-miR combining 2′F- and 2′-MOE-modified oligonucleotides (see Figure 2) was ineffective for miR-199b silencing even though others have shown efficacy in mice and monkeys following *in vivo* delivery [99, 100]. Hence, head-to-head comparisons of different anti-miR chemistries against the most promising miRNA targets for inhibition are needed in relation to cutaneous wound healing, using delivery vehicles that have been reviewed elsewhere [101, 102]. Such studies will help determine which anti-miR modifications are required to establish the hierarchy of translationally viable anti-miR technologies and targets for deployment in the chronic wound environment.

It will also be important to confirm the biodistribution and safety of anti-miRs applied to the skin for wound healing. Although a phosphorothioate (PS) backbone confers nuclease resistance, we know from early work on mice that hepatic and renal accumulation and toxicity is a concern for LNA-modified oligonucleotides with PS linkages [26]. The toxicity associated with PS nucleosides appears to be related at least partly to their promiscuous binding to cell surface and intracellular

proteins [103]. Hence, strategies using oligonucleotides with a limited number of PS linkages have gained traction, presumably as such oligonucleotides have fewer/weaker protein interactions [104]. One additional concern with PS modifications is the introduction of stereoisomerism to the structure, which may also be a complicating factor in toxicity [105]. The ZEN-end modified anti-miRs with 2′-OME modifications avoid such stereoisomeric complexity, confer nuclease resistance and appear relatively safe [72],[71], and perhaps offer a more straightforward route to robust stereodefined anti-miRs over PS alternatives. However, further studies of the metabolism of 2′-OME:ZEN anti-miRs are required.

Importantly, the incorporation of 2′-OME also abrogates the proinflammatory responses that were recognised early on as a barrier to the therapeutic deployment of unmodified oligonucleotides [106, 107]. However, 2′-fluoro modification may be a better choice than 2′-OME or 2′-MOE, especially when the anti-miR function is associated with loading into RISC. This is because the small size of 2′-fluoro moiety avoids the steric hindrances associated with larger modifications [108]. The high electronegativity and hydrophobicity of 2′-fluoro also support productive interactions with RNAi proteins [108]. Importantly, studies on rats and humans suggests limited toxic effects from 2′-fluoro monomer metabolites of liver-targeted siRNA [104].

In addition, critical consideration needs to be given to the ongoing use of the *db/db* mouse model in studies of wound healing due to mechanical genetic and immunological differences in mouse wound healing compared to human skin [109]. The *ex vivo* human skin used by Landén and colleagues [110] and human skin organoids exploited by Keswani, Feinberg and co-workers [88, 111] represent alternatives that can be developed further as diabetic wound healing models. For instance, a combination of high glucose, reactive oxygen species (ROS) generators and advanced glycation end products could reproduce the diabetic microenvironment and enhance the predictive quality of such models [112, 113]. Mouse models can in turn be devoted to studies, such as safety and biodistribution, which cannot be evaluated in culture. Furthermore, pigs have been used extensively in wound healing research due to the anatomical and physiological similarities between human and pig skin [114-116]. Greater exploitation of pig models of diabetic and other chronic wounds may offer deeper biological insights than observed with rodents and accelerate the translation of anti-miR therapeutics for wound repair to the clinic.

Finally, while this review has focused on cutaneous wound healing, similar side-by-side comparisons of diverse oligonucleotide chemistries in miRNA inhibitors should be conducted in other disorders such as cancer and neurodegenerative disorders where miRNA inhibition has the potential to moderate disease processes and improve patient outcomes. Such studies will contribute insights that will help predict which mode of inhibition is most effective in a given tissue context.

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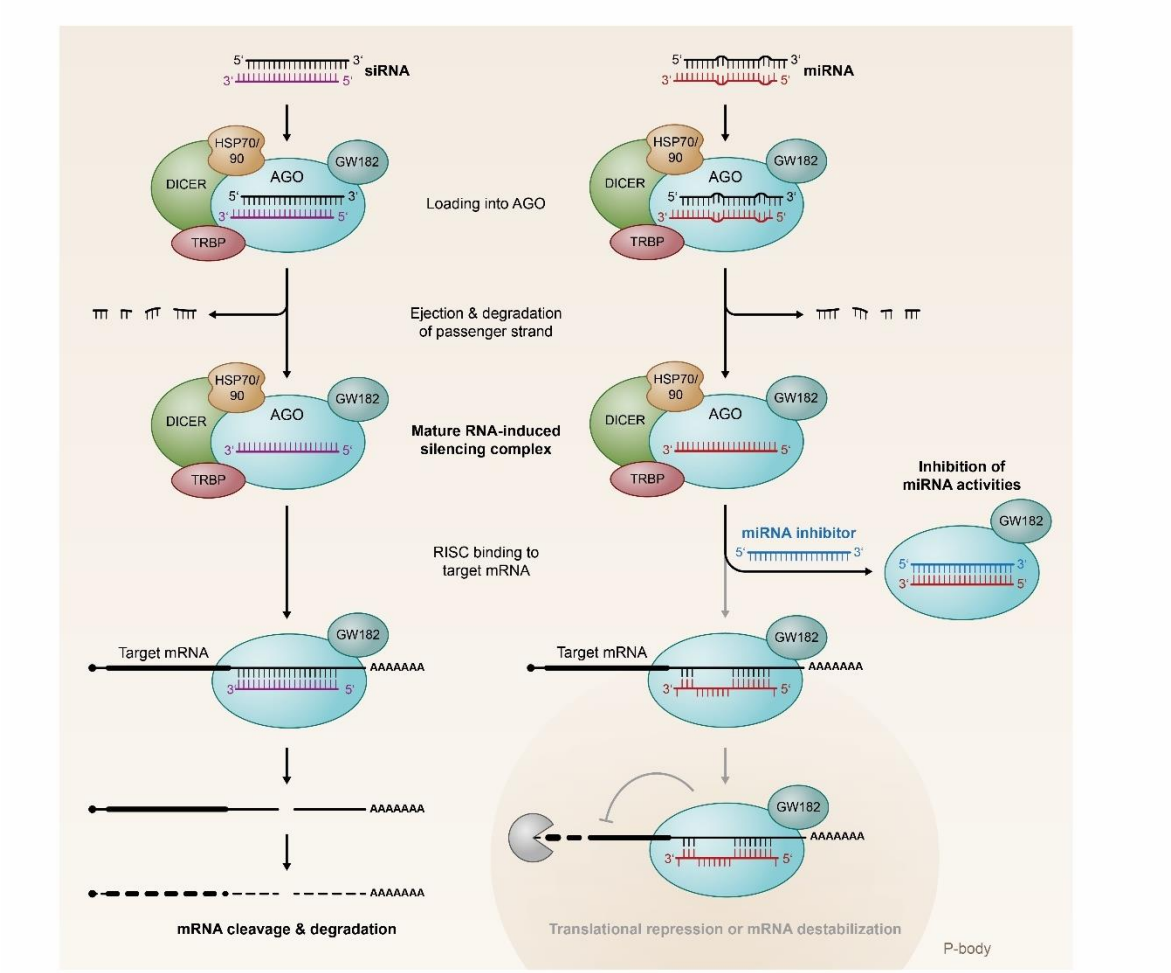
Figure 1: Comparison of siRNA and miRNA function. The co-ordinated activity of DICER, TRBP and chaperone proteins (HSP70 and HSP90) mediates the loading of siRNA or miRNA duplexes into AGO proteins. The passenger strand is displaced from the complex, leaving the guide strand to direct gene silencing by AGO2-dependent cleavage in the case of siRNA. The central mismatch between miRNA and target mRNA transcripts precludes cleavage by AGO2. Instead, gene silencing is mediated via assembly of the P body, in which protein complexes associated with translational repression and mRNA decay nucleate around the transcript-bound RISC [117]. Delivery of miRNA inhibitors into cells sequester target miRNA by direct binding, blocking the ability of the miRNA to repress target genes. AGO, Argonaute; HSP, heat shock protein; RISC, RNA-induced silencing complex; TRBP, HIV-1 transactivating response (TAR) RNA-binding protein.

Figure 2: Enhancing oligonucleotide functionality for anti-miRNA therapy. (A) Modifications to the 2′-hydroxyl ribose including 2′-O-methyl (2′OME), 2′fluoro (2′F), 2′-O-methoxyethyl (2′MOE) and locked nucleic acid (LNA). The N,N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine (ZEN) modifier original reported by Lennox and colleagues [72] is also shown. Adapted from [21]. (B, C) Structures of patisiran and givosiran, adapted from [22].

Figure 3: Enhancing wound healing in the skin through miRNA inhibition. Wound repair proceeds through a series of distinct but overlapping phases. Inhibition of specific miRNAs prevents miRNA-dependent repression of target genes, thus restoring protein expression to support proliferation and migration of endothelial cells and keratinocytes for angiogenesis and re-epithelialisation. Immune cells (*blue*) fibroblasts (*green*) which also play crucial roles in wound healing are also depicted.



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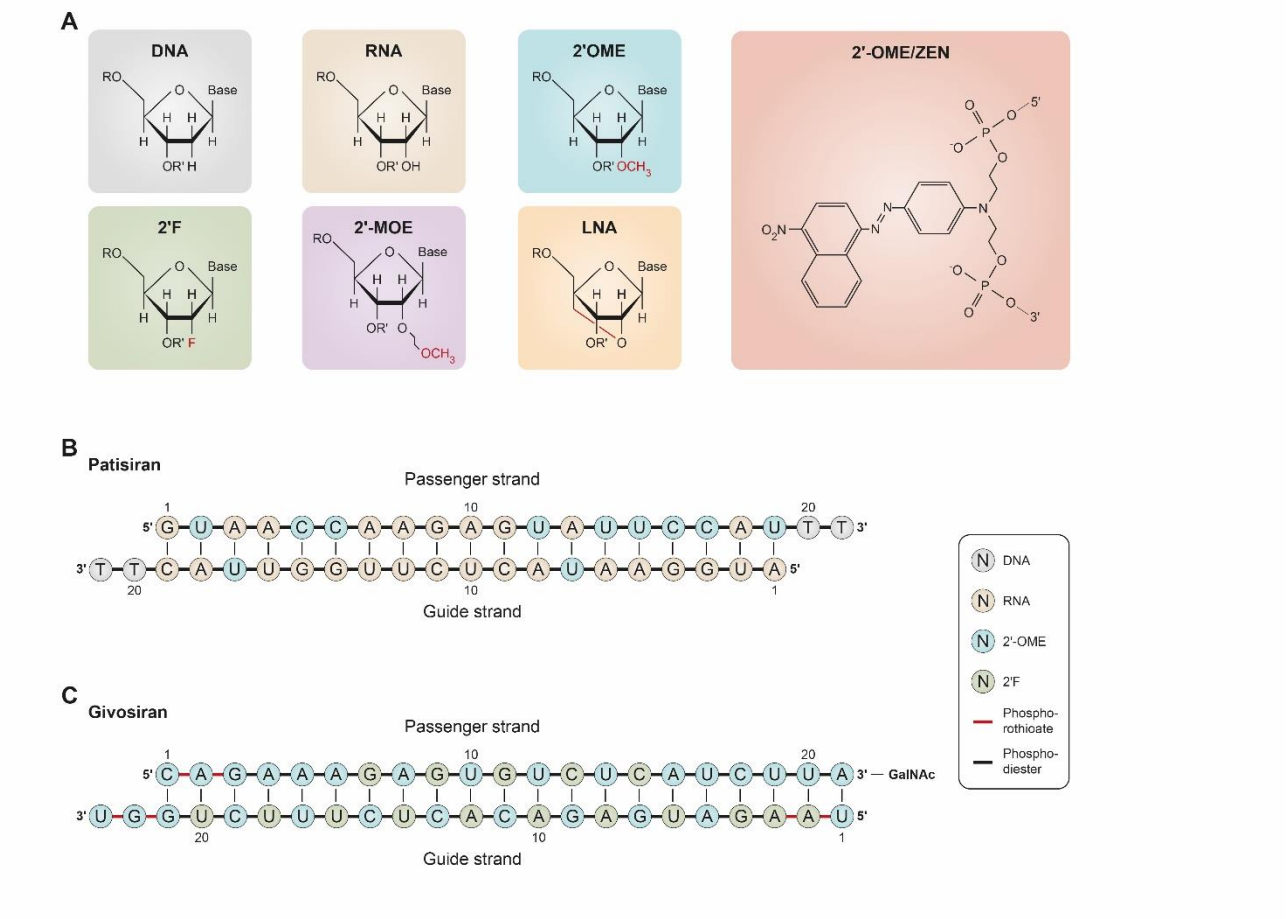

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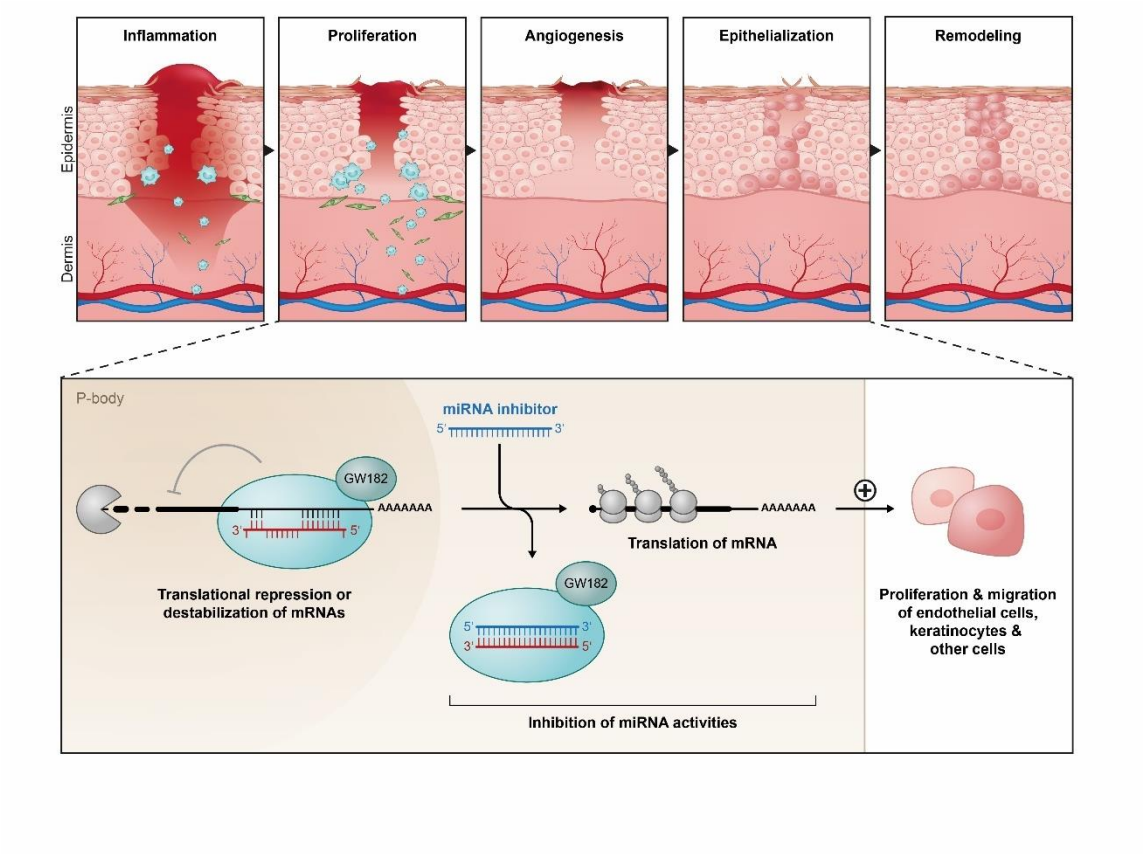

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Table 1: Promoting Wound Healing by MicroRNA Inhibition

MicroRNA	Targets	Process	Model	Ref.
miR-198	*DIAPH1* *PLAU* *LAMC2*	Keratinocyte migration	Immortalised primary keratinocytes	[53]
miR-99b-5p miR-100-5p	*IGF1R* *mTOR* *AKT1*	Keratinocyte migration	HaCaT keratinocytes	[55]
miR-4516	*STAT3* *FN1* *ITGA9*	Keratinocyte migration	Primary human keratinocytes HaCaT keratinocytes	[63]
miR-210-3p	*E2F3*	Keratinocyte proliferation Wound closure	HaCaT keratinocytes, Wounded db/db diabetic mouse skin	[70, 71]
miR-92a-3p	*ITGA5*	Angiogenesis Granulation Re-epithelialisation	HUVECs Human dermal fibroblasts Wounded db/db diabetic mouse skin	[78, 79]
miR-26a-5p	*SMAD1*	Angiogenesis Granulation Wound closure	HUVECs HMVECs Wounded db/db diabetic mouse skin	[87]
miR-135-3p	*HIP1*	Angiogenesis Granulation Wound closure	HUVEC Wounded db/db diabetic mouse skin Human skin organoids	[88]
miR-615-5p	*IGF2* *RASSF2*	Angiogenesis Granulation Wound closure	HUVEC Wounded db/db diabetic mouse skin Human skin organoids	[89]
miR-4674	*IRAK1* *BIDC2*	Angiogenesis Wound closure	HUVEC Human skin organoids	[90]
miR-15b-5p, miR-200b-3p	*VEGF* *VEGFR2*	Angiogenesis Granulation Re-epithelialisation	Wounded db/db diabetic mouse skin	[93]