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AntihypoxamiR Functionalized Gramicidin Lipid Nanoparticles Rescue Against Ischemic Memory Improving Cutaneous Wound Healing

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Abstract

Peripheral vasculopathies cause severe wound hypoxia inducing the hypoxamiR miR-210. High level of miR-210, persisting in wound-edge tissue as ischemic memory, suppresses oxidative metabolism and inhibits cell proliferation necessary for healing. In wound-edge tissue of chronic wound patients, elevated miR-210 was tightly associated with inhibition of epidermal cell proliferation as evident by lowered Ki67 immunoreactivity. To inhibit miR-210 in murine ischemic wound-edge tissue, we report the formulation of antihypoxamiR functionalized gramicidin lipid nanoparticles (AFGLN). A single intradermal delivery of AFGLN encapsulating LNA-conjugated anti-hypoximiR-210 ($AFGLN_{miR\text{-}210}$) lowered miR-210 level in the ischemic wound-edge tissue. In *rep*TOP™*mito*IRE mice, $AFGLN_{miR\text{-}210}$ rescued keratinocyte proliferation as visualized by *in vivo* imaging system (IVIS). ^{31}P NMR studies showed elevated ATP content at the ischemic wound-edge tissue following $AFGLN_{miR\text{-}210}$ treatment indicating recovering bioenergetics necessary for healing. Consistently, $AFGLN_{miR\text{-}210}$ improved ischemic wound closure. The nanoparticle based approach reported herein is effective for miR-directed wound therapeutics warranting further translational development.

Graphical abstract

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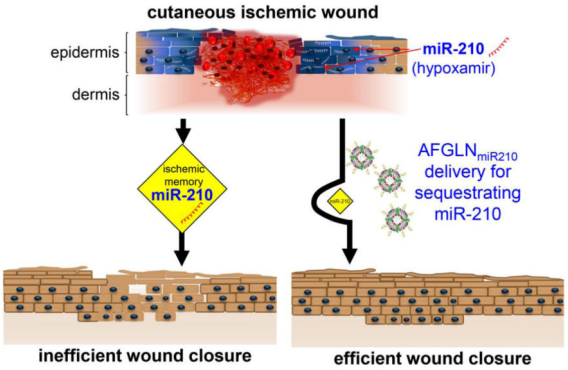

Successful intervention of non-healing ischemic wound is a major problem. Presence of hypoxia induced miR-210 limits keratinocytes proliferation and compromised wound closure. In this work, we report the formulation of antihypoxamiR functionalized gramicidin lipid nanoparticles (AFGLN). A single intradermal delivery of AFGLN encapsulating LNA-conjugated anti-hypoximiR-210 (AFGLN$_{miR-210}$) lowered miR-210 level in the ischemic wound-edge tissue. AFGLN$_{miR-210}$ delivery elevated ATP content at the ischemic wound-edge tissue recovering bioenergetics necessary for keratinocyte proliferation. Consistently, AFGLN$_{miR-210}$ improved ischemic wound closure. The nanoparticle based approach reported herein is effective for miR-directed wound therapeutics warranting further translational development.

Keywords

Ischemic wounds; tissue oxygenation; miR-210; keratinocytes proliferation; lipid nanoparticles

Peripheral vasculopathies are primarily responsible for wound ischemia and hypoxia[1]. The biological response to ischemia depends largely on the state of hypoxia. If the hypoxia is moderate, allowing for sufficient residual oxygen to support tissue survival, cells minimize oxygen cost by economizing metabolism and makes an effort to mount adaptive solutions. On the other hand, if the hypoxia is severe or near-anoxic, then adaptive rescue is no longer an option. Cells bring down oxygen cost to the threshold that separates survival and death. This is sustainable for a limited time within which the tissue may be rescued by intervention or it yields to necrotic death[1].

Our previous work has recognized the induction of master hypoxamiR miR-210 in keratinocytes of ischemic wound-edge tissue[2-4]. Elevated miR-210 lowers oxygen cost of survival by repressing mitochondrial metabolism and attenuating keratinocyte proliferation. miR-210 also silences cell cycle proteins[2, 3] (Figure S1). Such measures defend survival but oppose healing because tissue repair requires oxidative metabolism and cell proliferation. Although the state of oxygenation of the wound tissue may be corrected by intervention[1, 5, 6], growth of wound tissue is limited by an abundance of miR-210 repressing mitochondrial metabolism and cell proliferation. Thus, miR-210 may be viewed as an “ischemic memory” at the wound-edge tissue that is in direct conflict with wound healing. Healing outcomes of such ischemic wound tissue may be maximized by sequestering miR-210. In the present work we report the development of a novel antihypoxamiR

functionalized lipid nanoparticles (LNPs) that is able to rescue against the ischemic memory of miR-210 improving cutaneous wound healing.

Methods

1,2-Dioleoyl-3-dimethylammonium-propane (DODAP), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), soy phosphatidylcholine (SPC), gramicidin (GRAM), and d-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS) were dissolved in ethanol and combined at the molar ratio of 40/5/30/20/5 (DODAP/DOTAP/SPC/GRAM/TPGS). The lipid mixture was then combined with an appropriate amount of LNA based miR-210 power inhibitor in 40% ethanol followed by serial dilution (Appendix A. Supplementary data). Ischemic wound in C57BL/6 and *rep*TOP™*mito*IRE mice were induced by creating a bi-pedicle flap[2]. The *rep*TOP™*mito*IRE mice express the luciferase reporter gene under the control of an artificial minimal promoter derived from the Cyclin B2 gene, specifically induced during cell proliferation. LNA based anti-miR-210 and negative control were purchased from Exiqon. miRNA isolation, Western blot, immunohistochemistry was performed as describe previously[7]. For further details see supplementary materials.

Results

Laser capture microdissection (LCM) of human ischemic wound-edge epithelium revealed elevated miR-210 expression compared to the non-ischemic epithelium (Figure 1*A*). Expression of Ki67, a marker of cell proliferation, was also significantly lower in the epithelial tongue[8] of the human ischemic wound as compared to non-ischemic wound epithelium (Figure 1*B*). The expression of miR-210 was inversely correlated with the number of proliferating keratinocytes in patients with chronic wounds (Figure 1*C*). To evaluate the efficacy of anti-miR-210 in ischemic wound closure, a locked nucleic acid (LNA)-based anti-miR-210 power inhibitor (AM-210) was delivered intra-dermally to ischemic wound tissue of mice. At a relatively high dose of 500nM, administration of naked anti-miR-210 was ineffective in neutralizing hypoxia-induced elevated miR-210 in the wound tissue (Figure 1*D*).

We sought to develop LNPs optimized for the delivery of antagomir cargo to the cutaneous wound tissue. The particle composition and zeta potential were evaluated and presented in Figure 2A & B. The LNPs had an average diameter of 150 nm and a zeta potential of +10 to −10 mV at pH of 5 and 8, respectively. The encapsulation efficiency of these LNPs was found to be 80.12%. This LNP composition was selected for the subsequent experiments and is referred to as antihypoxamir (AM-210) functionalized gramicidin lipid nanoparticles (AFGLN) in this work. The size and concentration of the AFGLN were further examined (NanoSight™) using nanoparticle tracking analysis (Figure 2*C*).

Intradermal delivery of AFGLN containing LNA-conjugated AM-210 power-inhibitor ($AFGLN_{miR\text{-}210}$) at the wound-edge tissue in the murine model of ischemic wound significantly limited abundance of hypoxia-inducible miR-210 (Figure 3*A*). To evaluate the functional effect of $AFGLN_{miR\text{-}210}$ on ischemic wound closure, bi-pedicle flaps were

created on C57BL/6mice. Digital planimetry demonstrated significant improvement of closure in $AFGLN_{miR-210}$ group from day 3 post-wounding (Figure 3*B*). To study whether delivery of $AFGLN_{miR-210}$ accelerated wound re-epithelialization, frozen ischemic wound-edge tissue sections at day 7 post-wounding were stained for keratin-14. Wound re-epithelialization was severely compromised in the ischemic wounds that were treated with empty GLN (devoid of any cargo) alone or AFGLN packed with scrambled oligos ($AFGLN_{scramble}$) (Figure 3*C&D*). In contrast, $AFGLN_{miR-210}$ treated ischemic wound showed improved re-epithelialization with the presence of characteristic hyperproliferative epithelium as would be expected in healing wounds (Figure 3*D*). Because mouse is a loose skin animal and wound contraction may confound closure, we used a splinted wound model to limit contraction. $AFGLN_{miR-210}$ treatment improved re-epithelialization (Figure S2).

Consistent with the above-mentioned favorable effects of $AFGLN_{miR-210}$, bi-pedicle flaps were created with ischemic wounds on *rep*TOP™*mito*IRE mice. As shown in Figure 4*A*, in a setting where the perfusion of ischemic wound was equally limited in all groups, delivery of $AFGLN_{miR-210}$ rescued cell proliferation (Figure 4*B*). This finding was substantiated by immunohistochemical detection of Ki67 (Figure 4*C*). ^{31}P NMR studies validated the rescue by demonstrating elevated ATP content at the ischemic wound-edge tissue in $AFGLN_{miR-210}$ treated group compared to GLN alone or $AFGLN_{scramble}$ group (Figure 4*D*). These observations demonstrate that $AFGLN_{miR-210}$ is effective in restoring oxidative metabolism in ischemic wound edge tissue.

Discussion

Clinical intervention to reoxygenate chronic ischemic wounds such as oxygen therapy, debridement, or recanalization are likely to face metabolic barriers such as the miR-210 ischemic memory which when abundant in the wound tissue resists tissue growth (Figure S3). Thus, neutralizing such barriers are likely to improve the effectiveness of standard clinical interventions aimed at re-oxygenating the ischemic wound tissue. This work offers a lipid nanoparticle-based solution to sequester miR-210 in the wound edge tissue resulting in improved closure of ischemic wounds.

Although liposome-based nanoparticles have been widely used in skin health care, the efficiency of cargo delivery following topical application is questionable[9, 10]. There is no evidence of lipid nanoparticle based intervention of miR delivery or inhibition in the skin. In cutaneous injury, an additional challenge is the rapid removal of such nanoparticles by wound associated phagocytes[11]. Furthermore, the wound microenvironment features low pH which influences the dynamics of cargo release to the site of injury[12]. Thus design of lipid nanoparticles for skin health application, including tissue repair, must troubleshoot these challenges. Soy phosphatidylcholine has been utilized for the bilayer formation along with cationic lipid with tertiary (DODAP) and quaternary (DOTAP) amine headgroups. Incorporation of Gramicidin A was aimed at improving endosomal escape and facilitating ion channel formation in the lipid bilayer[13]. Intradermal injection of AFGLN was effective in cargo-delivery. Observations of this work substantiate the ischemic memory hypothesis by rescuing wound-re-epithelialization in response to inhibition of hypoxamir miR-210[4]. AFGLN was effective in rescuing the ischemia affected wound tissue and resuming re-

epithelialization. The formulation of AFGLN reported in this work would be effective to deliver any cargo of interest to the healing cutaneous wound tissue. A clear translational advantage of AFGLN is that all material used for its formulation has prior history of FDA approval for human use[14-16].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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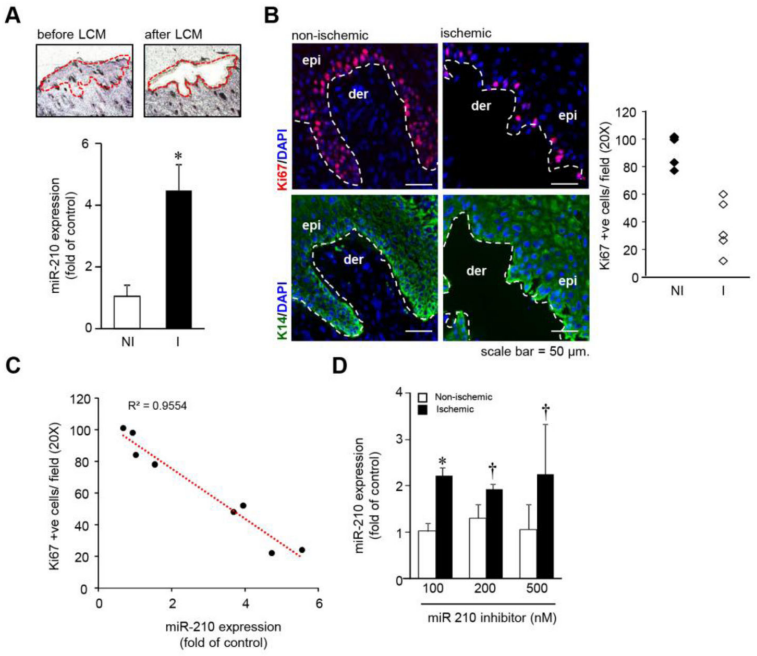

Figure 1.

(A) *miR-210* expression from laser microdissected epidermis of human wound-edge tissue. (n=7), * $p<0.001$; ANOVA. (**B**) Serial human wound cross-sections stained with anti-Ki67 and keratin-14 antibody, counter stained with DAPI. (n=4-5). The plot represents quantification of the Ki67 positive cells/ field (20X). (**C**) Regression plot of miR-210 expression from the human wound edge biopsies against number of Ki67 positive cells/ field (20X). (n=8) (**D**) *miR-210* expression from murine non-ischemic and ischemic wound-edge tissue 24h after intradermal delivery of naked LNA-anti-miR-210. (n=4). * $p<0.01$; † $p<0.05$ compared to nonischemic wound, ANOVA.

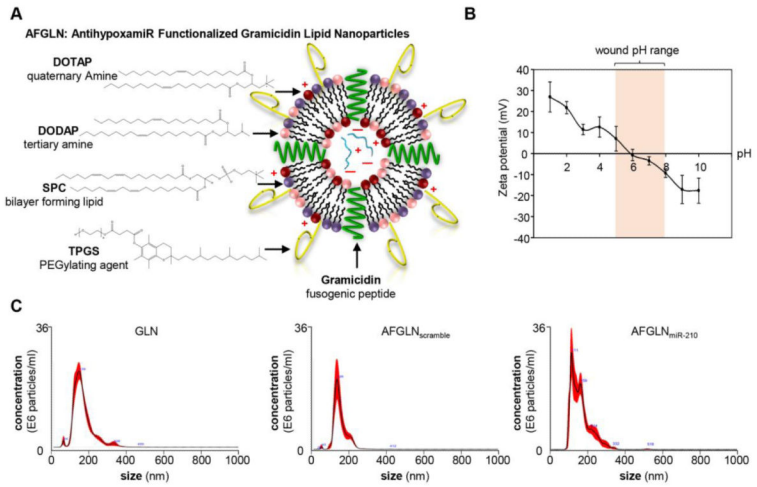

Figure 2.

(A) Schematic representation of the AntihypoxamiR Functionalized Gramicidin Lipid Nanoparticles (AFGLN). (**B**) The zeta potential of the AFGLN at different pH. Shaded region represents the pH range in chronic wounds. (**C**) Representative nanoparticle tracking analysis (NanoSight™) showing particles size and concentration of the empty GLN (devoid of any cargo), AFGLN packed with scrambled oligos ($AFGLN_{scramble}$) and AFGLN packed with anti-miR-210 ($AFGLN_{miR-210}$) (n=4).

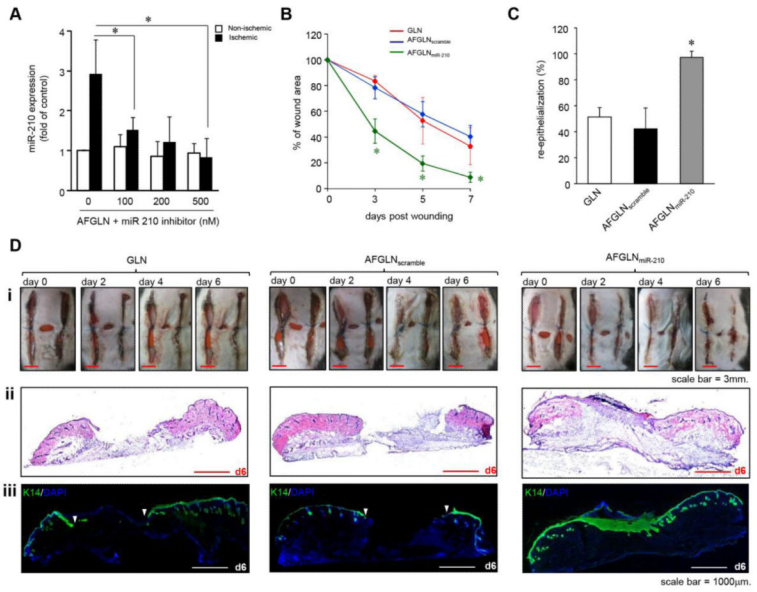

Figure 3.

(**A**) *miR-210* expression from murine non-ischemic and ischemic wound-edge tissue 24h after intradermal delivery of $AFGLN_{miR\text{-}210}$. (n=4). * $p<0.01$ compared to AFGLN, ANOVA. (**B**) Wound closure after delivery of empty GLN, $AFGLN_{scramble}$ and $AFGLN_{miR\text{-}210}$. (n=5), * $p<0.001$ compared to d0; ANOVA. (**C**) The percentage of re-epithelialization at day 6 post wounding was plotted graphically. (n=3), * $p<0.001$; ANOVA. **(D) (i)** Digital photographs of murine ischemic wound at days 0, 2, 4 and 6 after delivery of empty GLN, $AFGLN_{scramble}$ and $AFGLN_{miR\text{-}210}$. (ii) H&E images stained sections from ischemic wounds at day 6 post-wounding. (iii) Wound cross sections were stained with anti-keratin 14 antibody and counterstained with DAPI to show re-epithelialization at day 6 following treatment with GLN, $AFGLN_{scramble}$ and $AFGLN_{miR\text{-}210}$. (n=4).

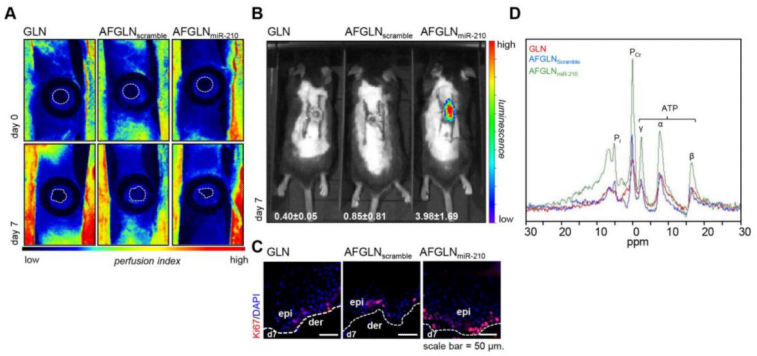

Figure 4.

(A) Laser speckle image showing perfusion level in the bi-pedicle flap at day 0 and day 7 after delivery of only GLN, $AFGLN_{scramble}$ and $AFGLN_{miR\text{-}210}$ (**B**) IVIS image from *rep*TOP™*mito*IRE showing cell proliferation in animal treated with lipid nanoparticles with $AFGLN_{miR\text{-}210}$.The images from quantification of the mean luminescence has been presented. (n=3)(**C**) Serial wound cross-sections stained with anti-Ki67 antibody counter stained with DAPI (blue). (n=3). (**D**) ^{31}P NMR spectra of the ischemic wound-edge tissue at day 7 post wounding showing iP, and the three subunits α-, β- and γ- subunit of ATP normalized with phosphocreatinine.