Local delivery of Therapeutic RNAs Accelerates Wound Healing in Diabetic Mice

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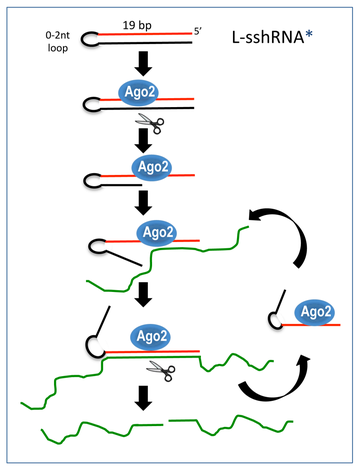
Abstract

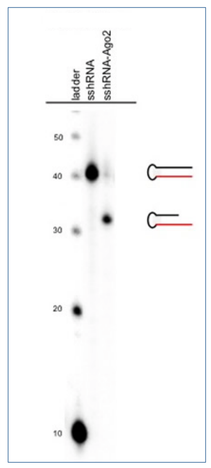
We are presenting an approach to accelerate wound healing in diabetics. In diabetes-associated chronic wounds, the normal response to hypoxia is impaired and many of the cellular processes involved in wound healing are hindered. In the wound healing pathway, HIF-1α (Hypoxia Induced Factor-1α) activates multiple factors, including VEGF and SDF-1, that enhance wound healing by promoting cellular motility and proliferation, new vessel formation, and re-epithelialization. Under normoxia, PHD2 (Prolyl Hydroxylase Domain-containing protein 2) negatively regulates HIF-1α activity by targeting it for degradation. HIF-1α also upregulates microRNA miR-210, which in turn regulates proteins involved in cell cycle control, DNA repair, and mitochondrial respiration in ways that are antagonistic to wound repair. We have identified a highly potent sshRNA (short synthetic hairpin RNA) that inhibits expression of PHD2 in cell culture and an antisense oligonucleotide (antimiR) targeting miR-210. Both oligonucleotides were chemically modified for improved biostability and to mitigate potential immunostimulation. Silencing PHD2 transcripts stabilizes HIF-1α and, in combination with the antimiR targeting miR-210, increases proliferation and migration of keratinocytes in vitro. To assess activity and delivery in a mouse model of type II diabetes, PHD2-targeting sshRNAs and miR-210 antimiRs both alone and in combination were formulated for local delivery to wounds using layer-by-layer (LbL) technology. LbL nanofabrication was applied to incorporate sshRNA into a thin polymer coating on a Tegaderm mesh. This coating gradually degrades under physiological conditions, releasing sshRNA and antimiR for sustained cellular uptake. Formulated treatments were applied directly to splinted full-thickness excisional wounds in db/db mice. Cellular uptake was confirmed using fluorescent sshRNA. Wounds treated with a single application of LbL-PHD2 sshRNA or LbL-anti-miR-210 closed 4 days faster than control group wounds, and wounds treated with both oligonucleotides closed on average 4.75 days faster. SDF-1 and VEGF levels were significantly increased along with markers for neovascularization and cell proliferation (CD31 and Ki67, respectively) in the wound area at Day 2 ($p<0.05$). These results suggest that silencing of PHD2 and miR-210 by localized delivery of sshRNAs and antimiRs is a promising approach for the treatment of chronic wounds.

Features of sshRNAs

- RNAi effectors with
  - Short base-paired stems (16-19 bp)
    - not processed by Dicer
  - Small loops (≤ 2-nt)
    - more stable & resistant to endonuclease cleavage
- Efficiently loaded into RISC by binding directly to Ago2
- Highly potent (low picomolar IC_{50})
- Loop blocks passenger strand off-targeting
- Chemically synthesized instead of vector-expressed
  - Allows for precise and flexible chemical modification patterns
  - Enhanced stability against nucleases
- Single chemical entity
  - Simplified production, purification, and formulation
- Proven *in vivo* efficacy in pre-clinical mouse models
  - Durable target knockdown
  - Well-tolerated

sshRNAs are processed by a Dicer-independent pathway





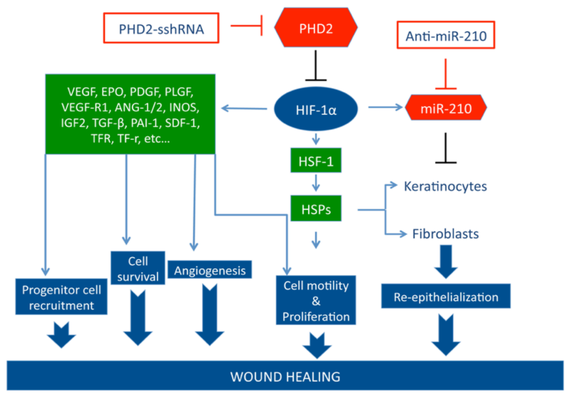
Ago2 pull-down of radiolabeled sshRNA shows full-length and processed sshRNA

1. sshRNAs are loaded intact into RISC (40 nt)
2. Passenger arm is sliced
3. Processed ¾-length molecule (30 nt) containing guide strand remains stably bound to Ago2

3 overlapping phases of wound healing

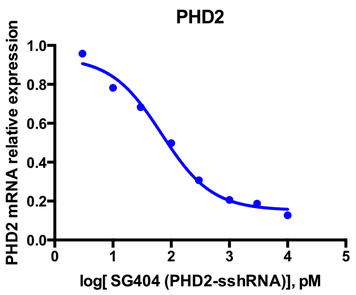
- Inflammation (2-5 days)
  - Vasoconstriction
  - Platelet aggregation
  - Clot formation
  - Phagocytosis
- Proliferation (2 days-3 weeks)
  - Granulation
  - Wound contraction
  - Epithelialization
- Remodeling (3 weeks- 2 years)
  - Collagen deposition
  - Remodeling

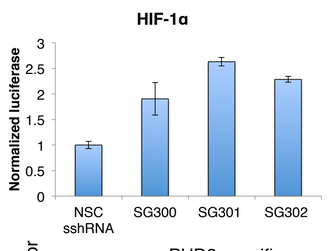
HIF-1α-regulated pathways in wound healing

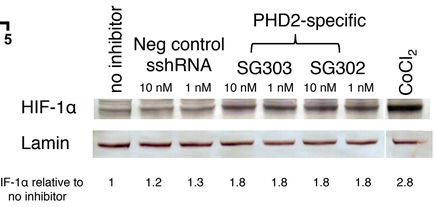


Therapeutic approach: targeting PHD2 and miR-210 to restore the normal hypoxia response

Inhibition of PHD2 mRNA and induction of HIF-1α by PHD2-sshRNA

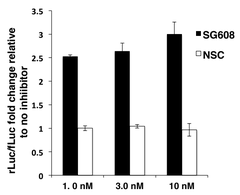






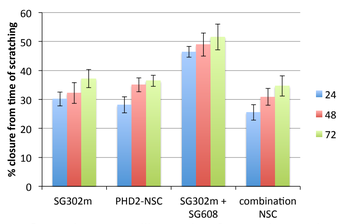
Dose response of PHD2 inhibition (left) and HIF-1α induction (right) by PHD2-targeting sshRNAs. Left panel: Total RNA was isolated 48h after transfection. PHD2 was quantified by qPCR $2^{-\Delta\Delta Ct}$ method, normalized to GAPDH. Quantification is expressed as fold-inhibition relative to cells that were not transfected with inhibitors. Right panels: Induction of HIF-1α by several PHD2-targeting sshRNAs measured by luciferase reporter assay (top) and Western blot (bottom). PHD2-sshRNAs and non-specific controls were transfected and HIF-1α levels were analyzed 48 h later. Cells treated with $CoCl_2$ were used as a positive control. Protein levels in the Western blot were quantified using ImageJ software with Lamin as a loading control.

Potent antimiR inhibitor of miR-210

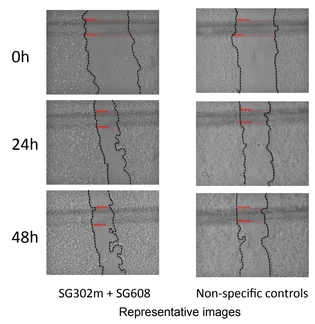


Luciferase biosensor assay to measure inhibition of miR-210 by antimiRs. De-repression of rLuc signal shows specific activity of miR-210-targeting antimiR SG608 relative to non-specific control antimiR (NSC).

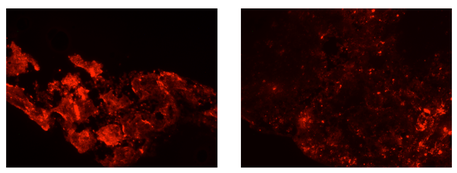
Keratinocyte growth is stimulated by therapeutic RNAs



Scratch assays illustrating faster closure in response to combination transfection with a PHD2 sshRNA and antimiR targeting miR-210.



Fluorescently-labeled LbL-formulated PHD2 sshRNA enters cells in wound area

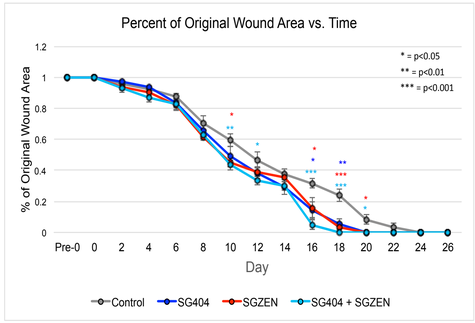


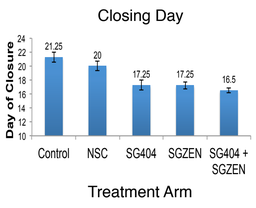
AlexaFluor594-labeled sshRNA Fluorescence

Therapeutic oligonucleotides in this study were formulated into a thin film coating onto the surface of a woven nylon wound dressing by LayerBio Inc. using its proprietary drug delivery technology. LbL formulations provide slow release of the encapsulated oligonucleotides into the wound bed over the course of 7-10 days[1].

Castleberry, S. et al. ACS Nano 7, 5251, 2013

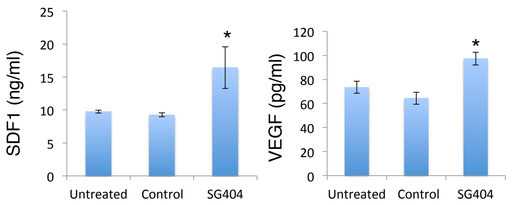
LbL-formulated PHD2-sshRNA and anti-miR210 improve diabetic wound healing both alone and in combination





LbL-formulated sshRNA (SG404) and antimiR (SGZEN) monotherapy treatments result in wound closer that is 4 days faster than the control treatment. The combination treatment leads to a 5-day faster wound closure.

Downstream factors that enhance wound healing are induced by Day 2 after treatment with LbL-SG404

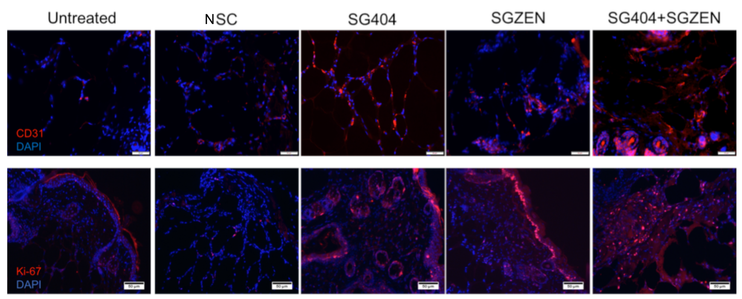


*p<0.05 vs. SG221c control

ELISA assays, n = 5 mice per treatment

- SDF-1 is thought to recruit progenitor cells to injured tissue and is associated with angiogenesis

Sequence-specific treatment increases neovascularization in all targeted treatment groups



Images are taken 4 days after treatments were applied. Top row: CD31 (red) staining shows neovascularization and Dapi (blue) stains cell nuclei. Bottom row: Ki-67(red) staining shows cell proliferation and Dapi (blue) stains cell nuclei.

Summary

- Identified effective sshRNA inhibitors of PHD2 and miR-210
- Lbl-formulated sshRNA is taken up by cells in the wound area at all time points
- PHD2 sshRNAs stabilize HIF-1α and induce downstream genes involved in wound healing
- PHD2 sshRNAs and anti-miR-210 are effective in promoting keratinocyte migration
- Therapeutically significant increase in rate of wound closure and reduction in time to closure in db/db mice
- Significant increase in neovascularization and cell proliferation in wound area

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