Targeting Small Non-coding RNAs to Diminish the Epigenetic Ravages of Aged Skin

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ABSTRACT

Background: Through application of epigenetic modulators, some natural aging processes can be reversed, restoring youthful skin features. Non-coding RNAs (ncRNAs) called microRNAs (miRNAs) intercept messenger RNAs (mRNAs), preventing interaction with ribosomes, and thus halting protein synthesis.

The relationship between miRNAs and skin health has been corroborated via miRNA profiling studies. Skin cells and tissues treated with two different skin care products experienced alterations in miRNA homeostatic levels as shown by qPCR.

Methods: "Mimics" of natural miRNAs augment down-regulation in expression of certain genes. In contrast, application of "antagomiRs" that irreversibly bind and sequester miRNAs promote up-regulation in expression of genes the miRNA would have targeted. The remaining hurdle for deployment of this technology remains development of suitable delivery vehicles for ncRNAs.

Results: A proprietary formulation was examined as a delivery vehicle for mimics and antagomiRs. Modulation of tyrosinase levels, the key enzyme in melanin synthesis, was tackled employing miR-218-5p mimics, which sequester tyrosinase mRNAs. Mimics complexed with the test formulation delivered to normal melanocytes, melanocyte-derived cell lines, and a melanocyte-infused skin model induced skin brightening as confirmed by visual examination and Western blot.

Alternatively, antagomiRs to miR-29a-3p were explored as this miRNA increases with age, reducing important fiber proteins. miR-29a-3p antagomiRs mixed with the test formulation were incubated with fibroblasts, resulting in increased fiber protein production visualized by immunofluorescence microscopy and Western blot.

Conclusion: These studies highlight the utility of miRNAs for improved skin health. The results confirm a novel formulation transported mimics and antagomiRs into skin cell populations.

Keywords: RNA interference (RNAi); microRNA (miRNA); mimics; antagomiRs; post-transcriptional gene silencing (PTGS); epigenetics.

INTRODUCTION

In recent years, there has been an explosion of research in the study of epigenetics – the capacity to elevate or diminish the expression of specific genes in the absence of any alterations to the deoxyribonucleic acid (DNA) sequences of the host genome. Given that one aspect of chronological aging is the gradual reduction in the activity of critical genes in skin cells, it is understandable that skin care scientists would seek to apply these scientific advancements to stimulate beneficial genes while abrogating disadvantageous ones (1). An example of such a gene is COL1A1 that produces the collagen fibers of the extracellular matrix (ECM), where decreased production with age leads to atrophied resilience, elasticity, and firmness. Imagine a topical solution infused with an epigenetic modulator that restores COL1A1 expression, thereby promoting youthful features and improved skin health.

Three mechanisms have been delineated by which epigenetic effects are promulgated: methylation of genomic DNA, alternating acetylation and deacetylation of histone proteins (histone acetyltransferase/histone deacetylase "switch"), and post-transcriptional gene silencing (PTGS) via small non-coding RNA molecules (ncRNAs) that mediate a biological process known as RNA interference (RNAi).

RNAi was first described in the late 1990s by Craig Mello and Andrew Fire in the context of gene regulation in *Caenorhabditis elegans* (2-4). For many of the years after its discovery, it was regarded as an ancient form of epigenetic control and it was not anticipated that many human genes would be subject to this form of coordination with estimates of less than 20-30%. In modern times, it is now understood that RNAi is not ancient at all, but ongoing and contemporary, exerting control over 65% or more of the human genome. As such, a multitude of skin relevant genes can have their activity modulated via this pathway (5).

The two effector arms of the RNAi regulatory pathway are short-interfering RNAs (siRNAs) and microRNAs (miRNAs or miRs)(6). One of the general distinctions between the two sets of ncRNAs is that miRNAs are produced endogenously from genes found in the genomic DNA of the organism and siRNAs are generated from exogenous sources of RNA. Another aspect that separates the two branches is the level of recognition necessary between the

ncRNA and its cognate mRNA. For siRNAs to ablate the interaction of a mRNA with ribosomes, perfect sequence complementarity is required between the siRNA molecule and a recognition sequence in the 3' end of the mRNA. In contrast, miRNAs only require partial complementarity, which allows individual miRNAs the opportunity to diminish the expression of multiple mRNAs with related sequences, potentially exerting a greater impact than their siRNA counterparts (7).

As described above, miRNAs are produced endogenously from genes found in the cell nucleus. While there are several miRNA biogenesis pathways, many of them proceed in analogous fashion to the so-called "canonical pathway". Briefly, the transcription of miRNA genes results in the a long ncRNA molecule known as a primary miRNA (or pri-miRNA) that is enzymatically processed into a smaller stem loop structure called a pre-miRNA. This RNA is subsequently exported to the cytoplasm, where the hairpin structure is cleaved creating a mature duplex miRNA. Eventually, one strand of the duplex will engage with the RNA induced silencing complex (or RISC) and seek out its cognate mRNA for sequestration or degradation (5).

The designation for the majority of miRNAs is based upon the species from which they originate (example: "hsa" for *Homo sapiens*), followed by "miR" (microRNA) and a number defining the order in which they were discovered in the relevant organism, and whether the mature miRNA molecule is derived from the 5' (5p) or 3' (3p) side of the stem loop in the pre-miRNA molecule. Therefore, hsa-miR-218-5p is the 218th miRNA identified in humans that originates from the 5' half of its progenitor pre-miRNA stem loop. As of the most recent update of the curated website miRBase – version 22.1, there are 38,589 different miRNA entries (8-13). Given this publicly available wealth of information regarding naturally occurring miRNAs, various institutions have constructed arrays of miRNA specific primers to be used to profile via reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) the relative levels of certain miRNAs in RNA samples purified from diverse biological specimens (14). So-called "miRNA profiling" efforts have become widespread and have been utilized by the skin care industry to characterize skin diseases and disorders

as well as the downstream impact on miRNA expression levels after specific topical treatments (5).

Multiple miRNAs have been identified as contributing factors in chronological aging as well as certain skin diseases and have therefore become the targets of innovative ameliorative platforms. For example, hsa-miR-146a suppresses severe inflammation associated with cases of atopic dermatitis and psoriasis (15, 16). Therefore, a topical skin care intervention that augmented miR-146a levels in the skin would be instrumental in treating patients with these skin afflictions. This is just one instance of many that illustrate the power of this approach to improve skin health.

While some cosmetic care regimens serendipitously trigger beneficial changes in impactful cellular miRNA levels, a long-term goal of the scientific community has been the targeted modulation of specific miRNAs. To that end, the development of controlled interventions using specialized ncRNAs that bolster or diminish the endogenous concentrations of individual miRNAs has been extensively investigated with sporadic claims of success. The major hurdle that must be surmounted for topical solutions containing such ncRNAs to be efficacious is the formulation of a delivery vehicle that can protect the ncRNAs from thermolytic and enzymatic degradation on the skin as well as facilitating their penetration through the stratum corneum. Herein, we reinforce the utility of miRNA profiling efforts and demonstrate the penetration and protection afforded by a candidate formulation for ncRNA delivery.

MATERIALS AND METHODS

Materials. Mimics of endogenous human miRNAs, negative control mimics, antagomiRs to endogenous human miRNAs, Qubit™ miRNA assay kits, Purelink™ RNAse A, and RNAiMAX™ positive control transfection reagent were purchased from Thermo Fisher Scientific (Waltham, MA, USA). RNEasy® purification kit, RT2 First Strand kit, miRCURY™ SYBR® green reagent, and miRCURY™ LNA focus panel array were purchased from Qiagen (Germantown, MD, USA). MIRNAC™ transfection/delivery reagent (patent pending) was produced by Biocogent, LLC (Stony Brook, NY, USA).

Cell Culture. The S91 Cloudman cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and was cultured in Advanced RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) at 37 ˚C in 5% CO_2. Normal human dermal fibroblasts (NHDF) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1% P/S, and 1% HEPES at 37 ˚C in 5% CO_2.

MicroRNA Profiling. The relative change in endogenous miRNA levels was detected via RT-qPCR (QuantStudio3, ABI) using the miRCURY™ LNA focus panel arrays. Briefly, cellular RNA was harvested and purified using the RNEasy® kit, followed by reverse transcriptase mediated conversion of the purified miRNAs into cDNA counterparts (RT2 First Strand kit). The resulting solutions were then mixed with SYBR® green based miRCURY™ reagent and applied to the PCR array for RT-qPCR analysis. The detected miRNAs were normalized against internal housekeeping control genes.

MicroRNA Target Prediction. Three different online algorithms were employed to predict the downstream gene targets of the miRNAs detected in the profiling experiments. These algorithms included: miRDB, miRmap, and miRTarBase (17-19). The online predictions were performed following user instructions on the respective websites.

Transfection Protocol. For cell plating and upon commencement of the transfection investigation, the media for the S91 cells was switched to DMEM containing 10% FBS, 1% P/S, and 1% HEPES. Lyophilized miRNA mimics were resuspended in RNAse-free water to a concentration of 20 µM and were diluted to a working concentration of 50 nM in Opti-MEM media mixed with 3% MIRNAC™.

Western Blot Analysis. Cell pellets collected from transfection experiments were lysed in a commercially available version of Laemmli lysis buffer and separated by molecular weight via sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). The gel was then electro-transferred to a nitrocellulose membrane that was blocked and probed with antibodies to specific skin proteins depending on the experiment, including but not limited to: anti-tyrosinase, anti-collagen-1, anti-fibrillin-1, and anti-elastin-1. The protein gel was stained with Coomassie blue post-transfer to normalize relative protein levels between lanes when blots were subsequently evaluated by densitometry scanning using ImageJ software.

Immunofluorescent Microscopy Analysis. Cells were cultured on glass coverslips and subject to indicated treatments. Afterwards, the coverslips were rinsed with phosphate-buffered saline (PBS), fixed with 1% paraformaldehyde, permeabilized with cold 0.1% Triton-X100 in PBS, and blocked with 3% bovine serum albumin (BSA). Subsequently, the cells were probed with rabbit anti-collagen-1 diluted in 1% BSA followed by goat-anti-rabbit secondary antibodies conjugated to AlexaFluor™-555 (red fluorophore). Finally, the coverslips were mounted onto glass microscope slides using anti-fade mounting medium infused with 4',6-diamidino-2-phenylindole (DAPI) that fluorescently stains nuclei (blue).

RNAse A Protection Assay. MiRNA mimics were mixed with MIRNAC™ at various percentages (1, 5, 10, 25, 50, and 100%) and initial concentrations were determined using the Qubit™ microRNA assay following manufacturer's protocol. The samples were subsequently incubated with RNAse A at 25 and 37 ˚C in parallel for 0, 60, 120, and 180 min. Afterwards, the relative miRNA concentration in each sample was evaluated using the Qubit™ microRNA assay.

MelanoDerm™ Assay. Three-dimensional melanocyte-infused skin tissue (MelanoDerm™ #MEL-300-B) was purchased from MatTek Corporation (Ashland, MA, USA). Briefly, upon arrival, the tissue sections were removed from the agar transport containers and cultured in Maintenance Media (EPI-100-NMM-113; MatTek) at 37 ˚C in 5% CO_2. The MelanoDerm™ tissue was treated with 100 µL of MIRNAC™ with miR-218-5p miRNA mimic or a control (deionized water or 2% Kojic acid), every other day for a total of 4 weeks. Prior to each new treatment, the tops of the tissues were rinsed with 200 µL of PBS a total of three times each, to remove any old treatment sample. The spent maintenance media which the tissue inserts sit on top of was replaced with fresh maintenance media each treatment day as well. At the end of four weeks, the tops of the tissues were rinsed a final three times with PBS, images were taken of each tissue, and then the tissues were stored dry at -20˚C.

Transmission Electron Microscopy (TEM). The MIRNAC™ reagent was outsourced for TEM imaging to the Thermomechanical and Imaging Nanoscale Characterization (ThINC) facility at the Advanced Energy Research and Technology Center (AERTC) at Stony Brook University (Stony Brook, NY, USA).

RESULTS

***Endogenous miRNA levels are changed after application of skin care ingredients*.** In an effort to demonstrate that endogenous miRNA expression in skin cells is indeed malleable to skin care interventions, normal human keratinocytes (nHEKs) were evaluated for alterations in miRNA expression levels after parallel treatment with two (2) different bio-active ingredients employed in skin care regimens: one a blue-green algae extract and the other an oligopeptide. Monolayers of nHEKs were exposed to either material and allowed to incubate for 72 h. The cells were harvested and their RNA purified, which was then analyzed by RT-qPCR on a PCR array of various skin-relevant miRNAs. As shown in Table 1, three (3) miRNAs were down-regulated from their normal endogenous levels in response to the blue-green algae extract: let-7c-5p, miR-92a-3p, and miR-150-5p. This indicates that the genes subject to PTGS by these miRNAs will experience increased expression as a result of this perturbation. Using three (3) different online miRNA target algorithms, the predicted downstream gene targets for the miRNAs was compiled and only gene targets detected in two or more of the algorithms were reported (Table 1). Many of the genes identified that would substantially benefit skin health, including various collagens, hyaluronic acid synthase, molecules promoting intercellular adhesion such as integrins and cadherins, and anti-inflammatory factors like interleukin-10 (IL-10). Intriguingly, all three miRNAs appeared to silence the expression of a similar cluster of genes.

TABLE 1

MicroRNA profile generated from normal human keratinocytes treated with a blue-green algae extract (Bio-material #1).

BIO-MATERIAL #1									
Algorithm:	**miRDB**			**miRmap**			**miRTarBase**		
microRNA:	**150-5p**	**92a-3p**	**let-7c-5p**	**150-5p**	**92a-3p**	**let-7c-5p**	**150-5p**	**92a-3p**	**let-7c-5p**
	-3.03	-1.77	-1.53	-3.03	-1.77	-1.53	-3.03	-1.77	-1.53
	p<0.05	p<0.03	p<0.03	p<0.05	p<0.03	p<0.03	p<0.05	p<0.03	p<0.03
Target	ADIPOR2	CDH10	ADIPOR2	ADIPOR2	CDH10	CLDN1	ADIPOR2	CDH10	ADIPOR2
Genes:	CERS3	COL1A2	CLDN1	CERS3	COL1A2	CLDN16	HAS2	ITGA5	CDH10
	COL19A1	COL11A1	CLDN16	COL19A1	COL11A1	COL1A2		LPIN1	ITGB3
	FNDC5	COL12A1	COL1A2	FNDC5	COL12A1	COL3A1			SIRT1
	GJC1	COL19A1	COL3A1	GJC1	COL27A1	COL4A6			
	GJD4	COL27A1	COL4A6	GJD4	DSC2	COL14A1			
	ITGAX	DSC2	COL14A1	ITGAX	FBN1	FNDC3A			
	ITGB3	FBN2	FNDC3A	ITGB3	HAS2	GJC1			
	RAD23B	HAS2	GJC1	RAD23B	HAS3	IL10			
		HAS3	HAS2	SIRT1	ITGA5	ITGB3			
		ITGA5	IL10		LPIN1	KRT5			
		LPIN1	ITGB3		SIRT6	PPARA			
		SIRT6	KRT5			SERPINB9			
			PPARA						
			SERPINB9						

The oligopeptide treatment only dysregulated two (2) miRNAs where one was up-regulated and the other was diminished: miR-320a and miR-150-5p, respectively (Table 2). While the decreased expression of miR-150-5p was shared between the blue-green algae ferment and the oligopeptide, the enhanced expression of miR-320a was different and indicative that a series of downstream genes would experience reduced gene activation. The potential genes affected by increased miR-320a were explored using the aforementioned online algorithms and candidates with skin relevance were populated in Table 2. Notably, many of the genes identified did not appear to have direct relationships with the skin, with some exceptions. One such gene was alpha-catulin (CTNNAL1) that has been described to down-regulate expression of e-cadherin, thus negatively impacting cell-to-cell contacts (20). Another was natriuretic peptide receptor 1 (NPR1) whose inhibition leads to a reduction in skin itchiness (21). And lastly, junction mediating and regulatory protein (JMY) that reportedly also impedes cell-to-cell adhesion in response to DNA damage (22). CTNNAL1 and JMY are interesting given that oligopeptide treatment was designed to promote maintenance of cell-

to-cell adhesion and interactions with the ECM. Finally, a scientific paper was found that purported that miR-320a counters fibrotic conditions by diminishing collagen production, perhaps as a feedback mechanism (23).

TABLE 2

MicroRNA profile generated from human keratinocytes treated with a proprietary short oligopeptide (Bio-material #2).

BIO-MATERIAL #2						
Algorithm:	**miRDB**		**miRmap**		**miRTarBase**	
microRNA:	**150-5p** -5.60 p<0.02	**320a** +2.01 p<0.01	**150-5p** -5.60 p<0.02	**320a** +2.01 p<0.01	**150-5p** -5.60 p<0.02	**320a** +2.01 p<0.01
Target Genes:	ADIPOR2	ADAM28	ADIPOR2	ADAM28	ADIPOR2	CD2
	CERS3	CEMIP2	CERS3	CD2	HAS2	CTNNAL1
	COL19A1	IGF1R	COL19A1	JMY		IGF1R
	FNDC5	JMY	FNDC5	MCM4		MED23
	GJC1	MCM4	GJC1	NPR1		NPR1
	GJD4	MED21	GJD4			
	ITGAX	MED23	ITGAX			
	ITGB3		IOTGB3			
	RAD23B		RAD23B			
			SIRT1			

Mimics of natural miRNAs diminish gene expression when introduced to monolayers of skin cells using an experimental formulation. Having established that normal skin care routines can serendipitously trigger potentially beneficial alterations in the miRNA levels within skin cells, a more targeted approach attempting to modulate the RNAi effectors themselves was investigated. Small ncRNA constructs that exquisitely mimic naturally occurring endogenous miRNAs (miRNA "mimics") have been tested in previous studies that demonstrated their capacity to down-regulate gene expression by augmenting the normal intracellular levels of specific miRNAs. As these are the effectors of PTGS, the resulting impact is an enhancement in the down-regulation of the corresponding gene expression, an approach termed "miRNA replacement therapy" (24-26).

To evaluate the efficacy of this method, multiple experiments were conducted using miRNA mimics of miR-218-5p which has been reported to diminish the expression of tyrosinase, the key enzyme in the melanin synthesis pathway (27). First, monolayers of a melanocyte-derived cell line (S91 Cloudman cells) were incubated for 72 h with miR-218-5p mimics complexed with a commercially available transfection reagent called RNAiMAX™. Afterwards, the cells were harvested and analyzed by Western blot probing with HRP-conjugated anti-tyrosinase. As shown in Figure 1A, there was a demonstrable decrease in tyrosinase protein levels in S91 cells following the mimic treatment in a dose-dependent manner. The Western blot data was quantified by densitometry scanning which showed that as little as 50 nM of miR-218-5p mimics was sufficient to decrease endogenous tyrosinase levels by approximately 50% (Figure 1B). Lastly, the cell pellets from the mimic transfection experiment were photographed for evidence of pigmentation change, where a decrease in melanin content was observable only when miR-218-5p mimics were compared to untransfected cells and cells transfected with a non-sense negative control miRNA mimic (miR-NC) (Figure 1C). This confirmed that the mimic approach was effective, though an alternative transfection reagent that could also function as penetrating agent on skin was sought.

FIGURE 1

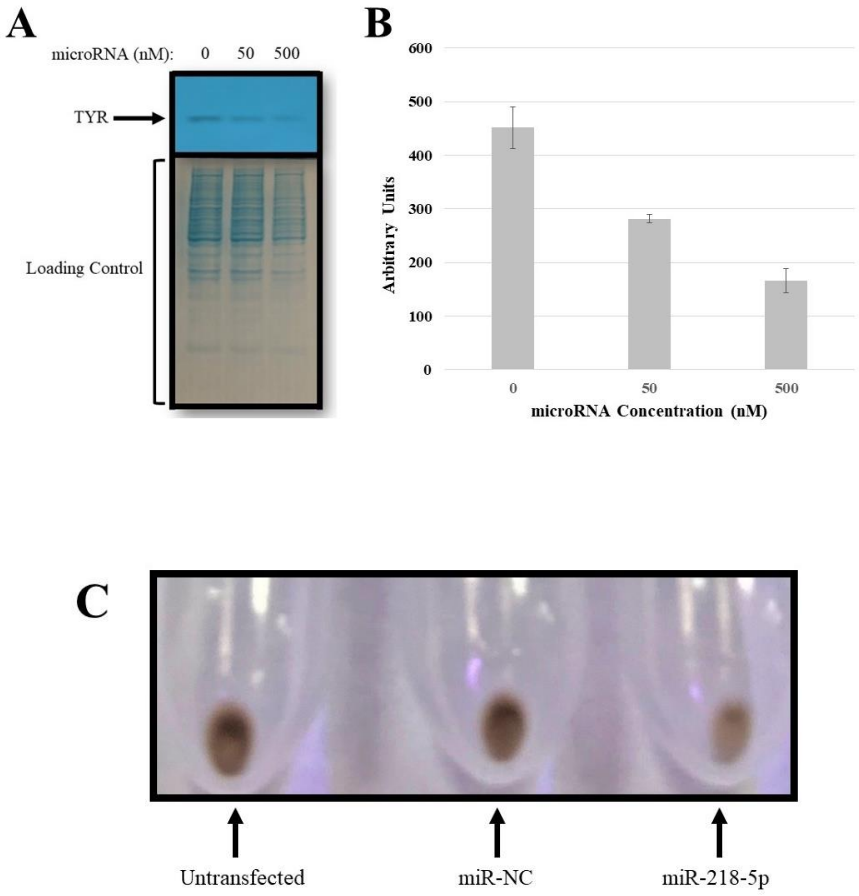

Figure 1. Mimics of miR-218-5p were examined for their ability to diminish tyrosinase expression as well as impact pigmentation in the S91 Cloudman melanocyte-derived cell line. **A.** Western blot analysis of S91 cells treated with 0, 50, 500 nM miR-218-5p mimics complexed with a commercially available transfection reagent for 72 h. The blot was probed with anti-tyrosinase-HRP. The protein gel was stained with Coomassie blue post-electro-transfer to evaluate relative protein loading between lanes. **B.** Densitometry scanning quantification of the tyrosinase-specific bands from (**A**) normalized against the protein loading detected in the Coomassie blue stained gel. **C.** The S91 cell pellets were photographed post-mimic treatment to observe any changes in pigmentation.

Finding a formulation or reagent capable of conveying miRNA-based ingredients into the skin has been a significant hurdle in this field of research given the size and electrostatic repulsion of the ncRNA molecules. Many groups have described some success with poly-arginine solutions (5), and as such a liposome-based poly-arginine formulation was generated referred to as MIRNAC™, which was visualized via TEM showing the multiple sized vesicular structures (Figure 2B). The previous experiment with S91 monolayers was

repeated, this time complexing the miR-218-5p mimics with MIRNAC™ and incubating them in cell culture for 72 h. This was followed again by Western blot analysis probing with anti-tyrosinase, which confirmed that MIRNAC™ transfected the mimics into the cells, although the relative reduction in tyrosinase was not as profound as was observed with the commercial reagent (Figure 2A). Additionally, the resulting cell pellets were resuspended in PBS and were inspected for a change in pigmentation (Figure 2C).

FIGURE 2

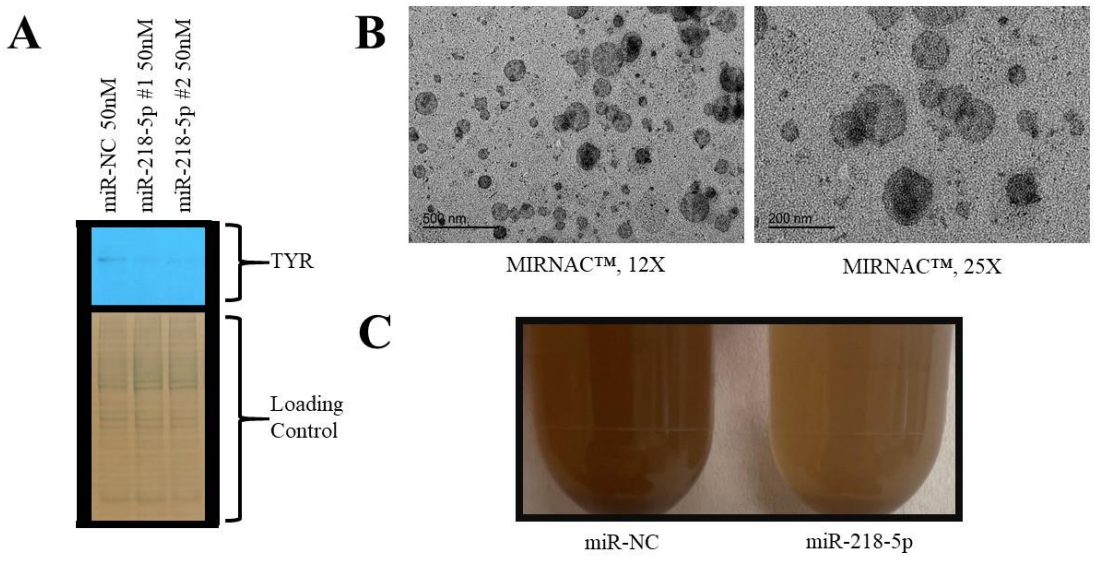

Figure 2. Mimics of miR-218-5p were complexed with an experimental reagent, MIRNAC™, and were examined for their ability to diminish tyrosinase expression as well as pigmentation in S91 cells. **A.** Western blot analysis of S91 cells treated with miR-218-5p mimics complexed with MIRNAC™ for 72 h. The blot was probed with anti-tyrosinase-HRP. **B.** Transmission electron microscopic (TEM) images of the MIRNAC™ reagent at 12X and 25X (see reference bars for size estimation). **C.** The S91 cells pellets were resuspended in PBS and photographed post-mimic treatment using MIRNAC™ (scrambled negative control miRNA mimic used as negative control, miR-NC).

Finally, a three-dimensional skin model infused with melanocytes, designed to test skin brightening agents (MelanoDerm™, Fitzpatrick Level 6), was employed to determine if MIRNAC™ also functioned as a skin penetrating solution. In preliminary experiments, MIRNAC™ was complexed with miR-218-5p mimics at a variety of concentrations (0.5, 1, 5, 10, 25, 50, and 100%) to determine an optimal usage level (data not shown). Unexpectedly,

at certain high doses, MIRNAC™ alone caused a degree of skin brightening. This effect was observed early post-application of MIRNAC™ on the tissue. Increased brightening was visualized after 4-weeks of incubation in the samples treated with MIRNAC™ complexed with miR-218-5p mimics, not MIRNAC™ alone (Figure 3A). Densitometry scanning of the tissue post-treatment showed that MIRNAC™ complexed with miR-218-5p mimics produced an approximate 50% reduction in pigmentation (Figure 3B). These results confirmed that this novel formulation was capable of delivering miR-218-5p mimics into skin and affecting a brightening of the tissue. Given the long half-life of tyrosinase, it takes approximately 4-weeks for the mimics to elicit an impact on the tissue pigmentation, thus it is fortunate that some early brightening is achieved through the MIRNAC™ reagent itself.

FIGURE 3

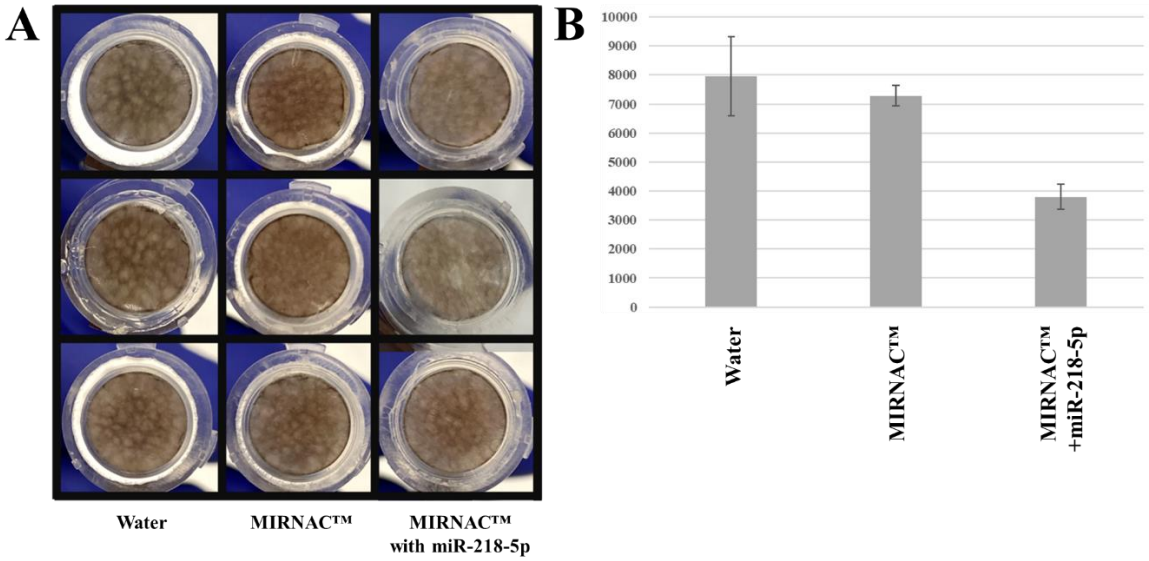

Figure 3. MIRNAC™ was evaluated for its capacity to function as a skin penetration agent for miRNA mimics. MIRNAC™ was topically applied to a melanocyte-infused three-dimensional skin model (MelanoDerm™) with and without miR-218-5p mimics. **A.** Photographed images of the treated MelanoDerm™ tissue after 4 weeks (specific experimental conditions indicated). **B.** The relative pigmentation changes were quantified via densitometry scanning in triplicate and the values plotted in a bar graph.

AntagomiRs counter the effect of endogenous miRNAs, promoting increased gene expression. As described in the Introduction, miRNA mimics are contrasted by antagomiRs, which are artificial constructs that bind to complementary sequences of natural endogenous miRNAs in an irreversibly manner, such that the cellular miRNA is prevented from

interacting with the RISC complex (28, 29). This in turn has the opposite effect of miRNA mimics resulting in increased downstream gene target expression. Here, the activity of an antagomiR to miR-29a-3p was explored in fibroblast cell culture. This particular miRNA is referred to as the "wrinkle miRNA" as it abrogates the synthesis of key protein constituents in the ECM, including collagen, fibrillin, and elastin (30). Fibroblasts (NHDF cells) were treated with anti-miR-29a-3p complexed with MIRNAC™ over a period of 72 h. Subsequently, the cells were harvested and examined by Western blot probing with antibodies directed against elastin-1 (EL1) and fibrillin (FBN1), and any alteration in protein levels was quantified by densitometry scanning of the resulting blot. As shown in Figure 4A, a detectable dose-dependent increase in protein levels was observed for both EL1 (left panel) and FBN1 (right panel) in response to the antagomiR treatment.

This above-described experiment was corroborated via immunofluorescence microscopy, where the fibroblasts were incubated for 72 h with the anti-miR-29a-3p/MIRNAC™ complexes (or controls), but with the cells cultured on glass coverslips. Post-treatment, the cells were fixed, permeabilized, and probed with collagen-1 primary antibodies followed by secondary antibodies conjugated with a red fluorophore (AF-555). The coverslips were mounted with a medium containing the DAPI counterstain allowing for the counting of cells irrespective of collagen detection. As the results illustrate in Figure 4B, there is a visually apparent increase in collagen-1 associated fluorescence in fibroblasts treated with the anti-miR-29a-3p/MIRNAC™ solution relative to control samples treated with miR-29a-3p mimics (miR-29) that saw diminished collagen-1 fluorescence and cells left untreated (untransfected) or treated with non-sense negative control constructs (miR-NC) that experienced no perturbations in homeostatic protein levels. These findings combined with the prior results with the miR-218-5p mimic study support the contention that topical formulations can be devised to epigenetically modulate specific cellular protein levels up or down in an effort to improve skin health.

FIGURE 4

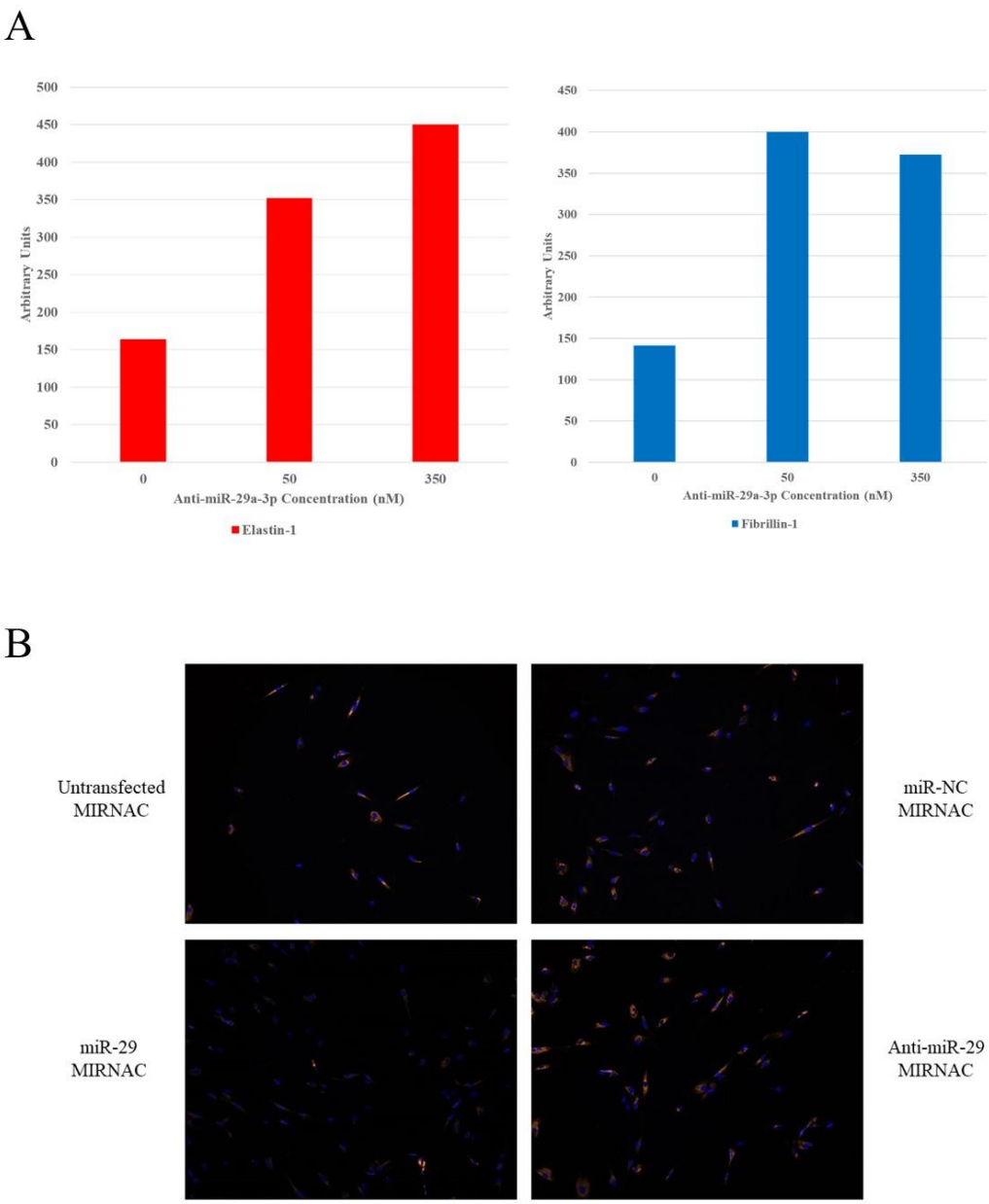

Figure 4. MIRNAC™ was tested for its ability to transfect antagomiRs of natural miRNAs into normal human dermal fibroblasts. Mimics and antagomiRs of miR-29a-3p were separately complexed with MIRNAC and applied to NHDF cells for 72 h. **A.** In the first evaluation, NHDF cell pellets that were collected 72 h after treatment with MIRNAC™ complexed with miR-29a-3p antagomiRs were analyzed by Western blot probing with either anti-elastin (left panel) and anti-fibrillin (right panel). The changes in protein levels were quantified by densitometry scanning and were graphed as shown. **B.** The Western blot experiment was corroborated, but this time investigating collagen levels, where the NHDF cells were cultured on glass coverslips and examined for changes in collagen levels by immunofluorescence microscopy utilizing an anti-collagen primary antibody followed by a secondary antibody conjugated to AF-555 (red fluorescence). The cells were counterstained with DAPI (blue fluorescence).

DISCUSSION

Epigenetics has come to the forefront in modern cosmetic care science as a means to ameliorate a variety of skin and hair conditions through thoughtful modulations of critical gene products in specific skin cell populations. Although there are multiple mechanisms by which epigenetic effects can be promulgated, PTGS via RNAi has seen considerable interest through a number of scientific investigations.

The majority of the research to date has elucidated that multiple skin diseases and disorders have hallmark signatures of dysregulation in normal homeostatic levels of endogenous miRNAs. This wealth of data has accumulated from a vast number of miRNA profiling studies that are detailed elsewhere. More recent miRNA profile examinations have looked to determine if selected skin care and hair care products can restore the dysregulated miRNAs to their original levels and thus potentially reverse certain skin afflictions. In this study, two such bio-active ingredients were tested and confirmed for their capacity to modulate endogenous miRNA levels, adding to the growing body of literature on the topic. Unknown until more investigations like this take place, many existing skin and hair care regimens may promote such impacts on skin cell gene expression.

A more novel approach would be to design, develop, and implement a new class of bio-active cosmetic care ingredients that take aim at specific miRNAs and their downstream gene targets. Such approaches are indeed feasible through employing miRNA mimics that bolster specific miRNA levels in cells, leading to augmented repression of certain gene products. In contrast, antagomiRs can toggle gene expression in the alternate direction, promoting the synthesis of certain proteins by sequestering their cognate endogenous miRNA suppressors. Here, both approaches were shown to be effective for both skin brightening and anti-wrinkle goals. The key to unlocking more widespread application of this methodology has been finding a suitable delivery platform that can deliver these powerful ncRNA molecules into the skin. And here, a novel candidate vehicle called MIRNAC™ repeatedly was shown to exhibit this type of functionality.

CONCLUSION

The field of epigenetic research continues to accrue new data at an astonishing rate. Understandably, the cosmetic care industry has investigated the utility of this technology as a means to improve skin health by driving increases in the expression of beneficial gene products and down-modulating deleterious ones. The experiments presented here and elsewhere reinforce the argument that current skin care regimens stimulate naturally occurring PTGS mechanisms. Furthermore, the effectors of these pathways can be carefully targeted and harnessed to promote a therapeutic balance in critical skin proteins. However, the success of such efforts hinges on finding qualified delivery vehicles that will allow these molecules to safely penetrate the skin barrier.

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