How MicroRNAs Modify Protein Production

Jessica L. Feig[1], Keith M. Giles[2], Iman Osman[2] and Andrew G. Franks Jr.[2]

Journal of Investigative Dermatology (2015) **135**, e32. doi:10.1038/jid.2015.99

OVERVIEW

It is generally accepted that the flow of genetic information from genomic DNA to functional protein is not unidirectional and linear. Rather, numerous molecular pathways regulate the processes of replication, transcription, and translation to fine-tune the output of protein-coding genes. Specifically, RNA interference is a biological process in which specialized small RNAs—short interfering RNAs (siRNAs) or microRNAs (miRNAs)—posttranscriptionally regulate the expression of specific target genes. In the December 2013 Research Techniques Made Simple, Nambudiri and Widlund described siRNAs and applications in investigative dermatology (Nambudiri and Widlund, 2013). Here, we outline how miRNAs modulate expression of genes relevant to skin pathology by inhibiting protein translation.

INTRODUCTION

miRNAs are short (~22-nucleotide) double-stranded RNAs that mediate key biological and developmental processes by binding with imperfect complementarity to specific sites within their target genes to induce repression of gene expression. The mechanism of various miRNAs' actions is similar, irrespective of the nucleotide sequence or the gene target. miRNAs are thought to regulate more than a third of all genes (Lewis *et al.*, 2005), and they may exert their effects in a coordinate fashion; that is, multiple miRNAs may target and regulate a single, specific gene transcript (Figure 1a). Conversely, a single miRNA can bind to and repress the expression of hundreds or even thousands of target transcripts (Figure 1b), enabling miRNAs to simultaneously regulate multiple genes within a single physiological pathway (Webster *et al.*, 2009).

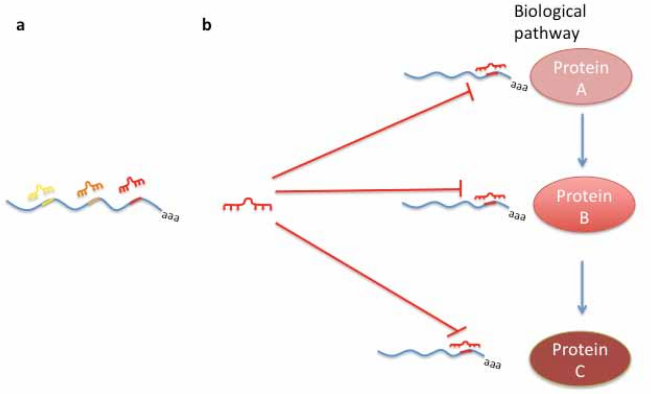

Figure 1. Mode of action of microRNAs. (**a**) Multiple miRNAs can work together to target and regulate a single, specific gene transcript in a coordinate fashion, thereby reinforcing the developmental program via redundant effectors of regulation. (**b**) A single miRNA can have multiple mRNA targets, potentially providing simultaneous and powerful control of a single physiological pathway by regulation of multiple target genes relevant to that process (e.g., proteins A, B, and C).

WHAT miRNAs DO

- miRNAs modulate transcription and repress translation, providing a level of control before the production of protein can exert biological effects.
- A single miRNA can have multiple targets, potentially providing simultaneous regulation of many genes involved in a physiological pathway.
- Multiple miRNAs with the same target reinforce the developmental program via redundant effectors of regulation.

LIMITATIONS

- Delivery: Because miRNAs do not freely diffuse into cells, special delivery approaches may be needed for both experimental and therapeutic design.
- Instability: Small RNAs are unstable and may be degraded upon entering a cell.
- Off-target effects: miRNA therapy can target an array of different gene products, with the possibility of undesired effects.

THE DISCOVERY

In a landmark discovery, Lee and co-workers identified that *lin-4*, a gene critically implicated in *Caenorhabditis elegans* larval development, does not code for a protein but rather controls the expression of RNA species, one of which is of 22-nucleotide base length. Crucially, the group found that *lin-4* RNAs had antisense complementarity to multiple sites in the 3′ untranslated region (3′ UTR) of the *lin-14* mRNA (Lee *et al.*, 1993) that had earlier been suggested to control *lin-14* expression (Ambros, 1989). Furthermore, LIN-14 protein levels were found to be inversely proportional to those of *lin-4* RNA, suggesting that as a regulatory small RNA (a miRNA), *lin-4* might regulate LIN-14 protein expression dur-

[1]*Medical Scientist Training Program, New York University School of Medicine, New York, New York, USA and* [2]*Ronald O. Perelman Department of Dermatology, New York University School of Medicine, New York, New York, USA.*

Correspondence: Jessica L. Feig, New York University School of Medicine, 550 First Avenue, New York, New York 10016, USA.
E-mail: jessicafeig@hotmail.com

ing development. Together, these discoveries suggested a model in which *lin-4*, the first characterized miRNA, binds to the *lin-14* 3′ UTR of mRNA in a complementary fashion to dictate translational repression of the *lin-14* transcript and control the basic biology of nematode development. More recently, many miRNAs have been found to be conserved across species, including humans, and there is an ever-increasing awareness of and interest in the critical roles that they play in controlling both normal and disease physiology, including many diverse areas of medicine such as cancer biology, cardiology, and dermatology.

miRNA BIOGENESIS, PROCESSING, AND TARGET RECOGNITION

miRNA biogenesis involves a sequential series of processes, each of which can be tightly regulated within a given cell. miRNA genes, contained within the introns of other genes (intragenic) or in intergenic regions, are transcribed by RNA polymerase II to produce a long primary RNA transcript (pri-miRNA). With base complementarity, the pri-miRNA transcript forms a hairpin that is then sequentially processed by Drosha/Pasha (Dgcr8) in the nucleus to produce a precursor miRNA (pre-miRNA) hairpin, which is next exported from the nucleus by the exportin 5 complex. Once in the cytoplasm, Dicer cleaves the pre-miRNA hairpin loop (Lee *et al*., 2003). One strand of the miRNA duplex (the "guide" strand) loads on the argonaute protein, forming the miRNA silencing complex, which targets specific mRNAs by two proposed mechanisms of action: mRNA destabilization and decay or translational repression (Figure 2).

One of the factors that determine whether an miRNA targets a specific mRNA is the degree of complementarity in base pairing between miRNA and mRNA. Extensive pairing between the miRNA and mRNA-3′ UTR typically induces mRNA destabilization, thereby altering the cell's transcriptional profile. mRNA destabilization occurs through recruitment of adapter proteins and deadenylase complex, which deadenylates the transcript (breaking down the poly-A tail) to inhibit translation initiation. Alternatively, less extensive base pairing can halt mRNA translation if the minimum binding requirement, known as the 7-base seed sequence, between the miRNA and the 3′ UTR of the target mRNA is fulfilled. In this manner, miRNAs may act as a "brake" to upregulated cellular responses by modulating both mRNA and protein levels of effector molecules critical to these processes.

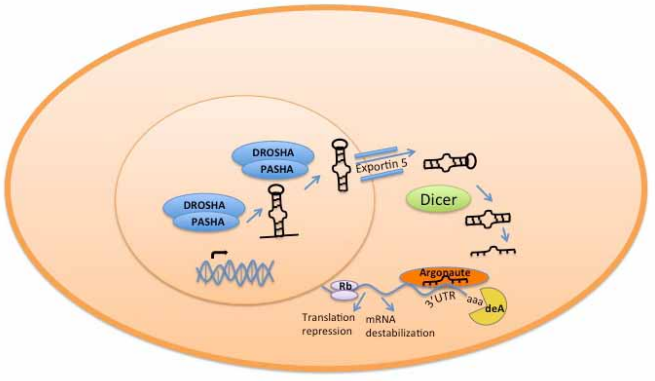

Figure 2. MicroRNA processing. MicroRNAs are sequentially processed in both the nucleus (DROSHA/PASHA) and the cytoplasm (Dicer). After the pre-miRNA hairpin is exported from the nucleus by exportin 5, Dicer cleaves the hairpin loop. The miRNA silencing complex (miRNA and argonaute protein) targets specific mRNAs at the 3′ untranslated region (3′ UTR) and modifies protein expression by two mechanisms: mRNA destabilization and decay or translational repression. The deadenylase complex (deA) deadenylates the transcript by removal of the poly-A tail. This destabilizes the mRNA and also inhibits translation initiation.

IDENTIFICATION OF miRNA TARGETS

There are four steps required for the identification of miRNAs and their subsequent physiologic characterization (Figure 3). First, expression profiling of miRNAs is accomplished with miRNA arrays or quantitative RT-PCR assays designed to detect and accurately quantify mature miRNAs in genetic material extracted from cell lines or tissue. Second, after miRNA expression profiling produces the data set of differentially regulated miRNAs (e.g., between control and diseased tissue), target prediction analysis follows to identify candidate gene targets for individual miRNAs of interest. This is accomplished using computational methods such as TargetScan (http://www.targetscan.org) or miRBase (http://www.mirbase.org). These database tools predict biological targets of miRNAs by searching for the presence of sites that match the seed region of each miRNA. Third, *in vitro* target validation, by a luciferase reporter assay, confirms the functional ability of a given miRNA to bind to the predicted 3′ UTR target sequence and repress protein production (as indicated by a decreased reporter signal) (Figure 4). Mutations in the 3′ UTR luciferase reporter would abrogate any changes in reporter gene expression induced by cotransfection of an miRNA targeting the site of interest. Fourth, phenotypic confirmation is used to functionally characterize the miRNA–mRNA interaction using methods of protein detection, such as western blotting. miRNAs are transfected into cells to turn down protein production, detected by a band of decreased intensity on a western blot. This effect can be reversed by

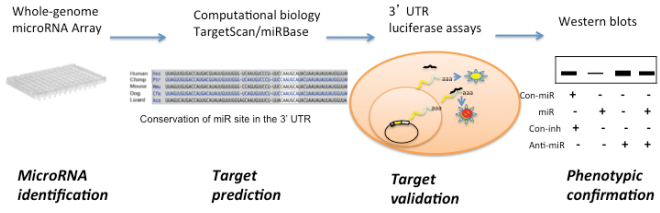

Figure 3. Four steps for miRNA identification and characterization. (1) miRNA identification is accomplished with miRNA arrays or quantitative RT-PCR assays. (2) Target prediction uses computational methods such as TargetScan or miRBase. (3) Target validation, by a luciferase reporter assay, highlights the functional ability of miRNA to bind to the predicted 3′ untranslated region (3′ UTR) target sequence and repress protein production (decreased signal). (4) Phenotypic confirmation is performed by western blots, which detect the level of proteins. miRNAs (miR), which turn down protein production, would decrease the band intensity (lane 2). This can be reversed by anti-microRNAs (anti-miR) or antagomiRs, oligonucleotides designed to antagonize the role of miRNAs through antisense complementary base pairing (lane 4).

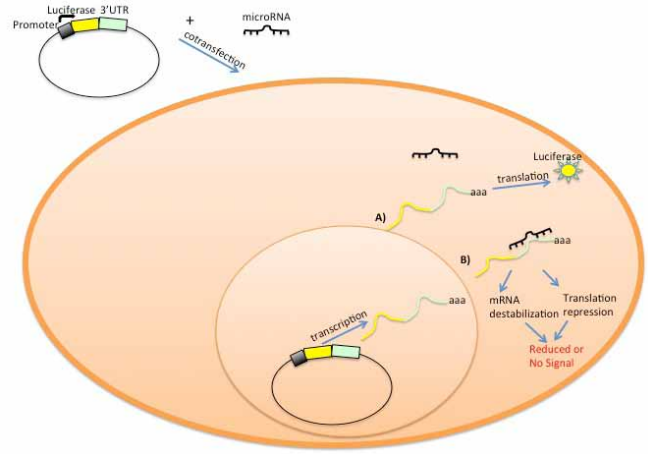

Figure 4. 3′ Untranslated region (3′ UTR) luciferase assay to validate the interaction between a microRNA and its target 3′ UTR. MicroRNA mimics and a vector containing a chimeric reporter gene consisting of luciferase fused to the 3′ UTR for a specific gene of interest are introduced into cells by transfection. (A) If the microRNA does not interact with the transcript, translation and subsequent production of the protein product occur. The luciferase product may then be detected with a luminometer. (B) If the microRNA specifically targets the transcript through complementary base pairing, then luciferase protein product is not produced. Thus, little or no luciferase signal is detected, representing an interaction between the microRNA and the 3′ UTR.

anti-microRNAs, or antagomiRs, oligonucleotides designed to antagonize the activity of a given miRNA through antisense complementary base pairing.

RELEVANCE OF miRNAS TO DERMATOLOGY

miRNAs are critical both for the normal development of the integumentary system and in diseases involving the skin. Initial investigations of the role of miRNAs in skin focused at the level of miRNA biogenesis and processing. Because the essential enzymes Dicer and Dgcr are inextricably linked to miRNA processing (Figure 2; see also "miRNA Biogenesis, Processing, and Target Recognition" above), their loss led to global depletion of miRNAs. Because mice that constitutively lacked Dicer or Dgcr8 died during early embryogenesis, before the development of the integumentary system, the Cre-loxP technology was employed to restrict the loss of these components to the skin alone at later stages. Under the control of the keratin 14 promoter, which is expressed specifically in the basal layer of the epidermis, epidermal-specific deletion of Dicer or Dgcr8 in embryonic skin progenitors in murine models perturbed epidermal organization, dermal papillae formation, and the architecture of the developing hair follicle, emphasizing the importance of miRNAs in morphogenesis (Yi *et al*., 2006, 2009). More recently, specific miRNAs were identified as playing a role in the developing skin. The epithelium is both stratified and dynamic, with deeper stem cells differentiating, becoming suprabasal, and requiring miRNA control. Specifically, miR-203, identified as a master regulator of epidermal differentiation, restricts stemness by targeting and suppressing expression of the stem cell-marker p63 (Yi *et al*., 2008).

miRNAs with roles in skin development have also been identified in disease states that involve impaired tissue homeostasis (Figure 5). Recently, a study by Noguchi and

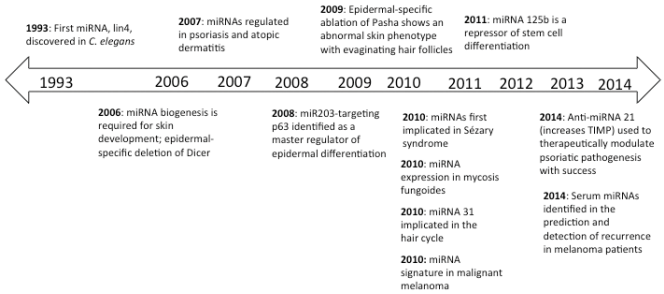

Figure 5. Breakthrough discoveries in miRNA biology. The timeline focuses on the field of dermatology.

colleagues identified and characterized the role of miR-203 in malignant melanoma. Following a miRNA profiling array that identified decreased miR-203 expression in human and canine malignant melanoma samples (Noguchi *et al*., 2013), the group defined a role for this miRNA in melanocyte biology, specifically in melanosome transport and melanogenesis (Noguchi *et al*., 2014). The study confirmed that miR-203 acts as a tumor suppressor by demonstrating its ability to negatively influence the proliferation of two human melanoma cell lines, Mewo and A2058, after the miRNA was introduced into the cell by transfection (Figure 6a). Furthermore, miR-203 transfection induced pigmentation of the cells (Figure 6b and c), which led to the hypothesis that miR-203 impacts melanogenesis via melanosome transport mechanisms. The computational miRNA target prediction algorithm TargetScan was used to predict the candidate targets of miR-203, and this analysis suggested that members of the kinesin family (kifs), responsible for intracellular transport of melanosomes, were targets of miR-203. To validate these predicted targets, luciferase assays were performed in which miR-203 significantly suppressed luciferase activities when the 3′ UTR of kif2a or kif5b was present (Figure 6e). To confirm the specificity of the interaction, mutation of the putative miRNA-binding region within the target 3′ UTR markedly abolished the effect of miR-203 (Figure 6e). These findings indicate that miR-203 modulates kinesin production, at both the mRNA and protein levels, effects that were reversed by anti-miR-203 (Figure 6c, d, and f). Results from this study suggest that miR-203 plays a pivotal role in melanoma development, and future experimental efforts will provide further insight into the functional role of this and other miRNAs in the melanoma setting.

FUTURE POTENTIAL FOR miRNAS IN DERMATOLOGY

Although miRNAs have been best characterized in melanoma (Poliseno *et al*., 2012; Fleming *et al*., 2014), their roles in the skin extend beyond the field of cancer biology. miRNAs have also been implicated in psoriasis, atopic eczema, and Sézary syndrome (Sonkoly *et al*., 2007; Ballabio *et al*., 2010; Guinea-Viniegra *et al*., 2014). With the advancement of the field, we will better understand how miRNAs regulate these processes. Increased understanding of mRNAs' contribution to disease will aid in the development of miRNA-targeted therapy in the future, as well as exploitation of miRNAs as clinical biomarkers. Yet, many therapeutic challenges remain: therapeutic delivery, instability, and non-specificity. miRNA therapy, which targets an array of

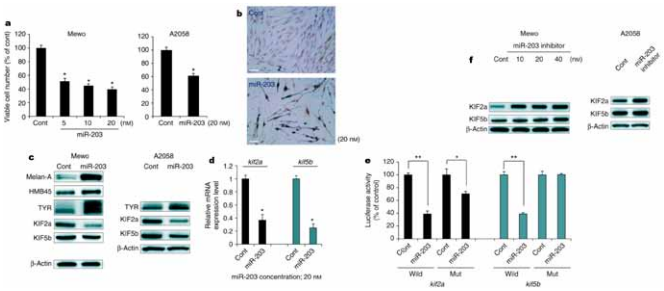

Figure 6. miR-203 regulates kinesin at the miRNA and protein levels. (**a**) Exogenous miR-203 suppressed the growth of melanoma cells. (**b**) Mewo cells stained with Masson–Fontana ammoniacal silver stain shows increased pigmentation. Bar = 20 μm. (**c**) Expression levels of various proteins by western blotting. Melan-A (Melanoma-antigen), HMB45 (melanosome marker), TYR (tyrosinase), kif (kinesins). (**d**) mRNA expression levels of *kif2a* and *kif5b* in Mewo cells were downregulated by exogenous miR-203. (**e**) Relative luciferase activities after cotransfection with control miR or miR-203 and each of the sensor vectors having the 3′-untranslated region of *kif2a* or *kif5b*. (**f**) Expression levels of various proteins by western blotting. The assays were performed at 96 (Mewo) or 72 (A2058) hours after the transfection. Values were determined for differences between the cells transfected with control miR and those transfected with miR-203 inhibitor. Data are expressed as the mean + SD ($n = 3$). Cont, control. $^{*}P < 0.05$ and $^{**}P < 0.01$. Adapted from Noguchi *et al*. (2014).

different gene products, carries with it the possibility of off-target, undesired, and nonphysiological effects. As we tackle future challenges, scientists may be able to therapeutically modulate pathogenesis by way of miRNAs or their antagomiRs. The future seems bright with the recent promising work of scientists who have successfully controlled psoriasis in murine models with such oligonucleotides (Guinea-Viniegra *et al*., 2014), and therapeutic delivery of miRNA mimics or antagomiRs has entered human clinical trials for cancer and other diseases (https://clinicaltrials.gov). miRNAs hold considerable promise as biomarkers of dermatologic diseases in addition to their potential as tiny "magic bullets" to normalize gene expression in an array of skin pathologies.

CONFLICT OF INTEREST

The authors state no conflict of interest.

CME ACCREDITATION

This activity has been planned and implemented in accordance with the Essential Areas and Policies of the Accreditation Council for Continuing Medical Education through the joint sponsorship of the Duke University School of Medicine and Society for Investigative Dermatology. The Duke University School of Medicine is accredited by the ACCME to provide continuing medical education for physicians. To participate in the CME activity, follow the link provided. Physicians should only claim credit commensurate with the extent of their participation in the activity.

To take the online quiz, follow the link below:
http://continuingeducation.dcri.duke.edu/research-techniques-made-simple-journal-based-cme-rtms

SUPPLEMENTARY MATERIAL

A PowerPoint slide presentation appropriate for journal club or other teaching exercises is available at http://dx.doi.org/10.1038/jid.2015.99.

QUESTIONS

This article has been approved for 1 hour of Category 1 CME credit. To take the quiz, with or without CME credit, follow the link under the "CME ACCREDITATION" heading.

For each question, more than one answer may be correct.

1. What is the typical length of miRNA nucleotides and its SEED sequence?

A. 22; 7.
B. 40; 22.
C. 7; 22.
D. 8; 7.

2. miRNA regulation involves which level(s) of control?

A. DNA replication.
B. mRNA destabilization.
C. Protein repression.
D. Both B and C.

3. Describe the mechanism of action of miRNAs.

A. miRNAs use adapter proteins to interfere with replication.
B. miRNAs cause destabilization of mRNA and inhibit translation initiation.
C. miRNAs usurp the polymerase II machinery to decrease its activity and lower the levels of the endogenous mRNA.
D. miRNAs rely on anti-microRNAs to potentiate their effects.

4. The best assay to identify whether a specific miRNA targets a particular transcript is which of the following?

A. PCR.
B. Western blot.
C. MicroArray.
D. Luciferase assay.

5. When a specific miRNA interacts with its target 3′ UTR in a luciferase assay, the reporter signal

A. Increases.
B. Decreases.
C. Stays the same.

REFERENCES

Ambros V (1989) A hierarchy of regulatory genes controls a larva-to-adult developmental switch in *C. elegans*. *Cell* 57: 49–57

Ballabio E, Mitchell T, van Kester MS *et al*. (2010) MicroRNA expression in Sezary syndrome: identification, function, and diagnostic potential. *Blood* 116:1105–13

Fleming NH, Zhong J, da Silva IP *et al*. (2014) Serum-based miRNAs in the prediction and detection of recurrence in melanoma patients. *Cancer* 121:51–9

Guinea-Viniegra J, Jimenez M, Schonthaler HB *et al*. (2014) Targeting miR-21 to treat psoriasis. *Sci Transl Med* 6:225re221

Lee RC, Feinbaum RL, Ambros V (1993) The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 75:843–54

Lee Y, Ahn C, Han J *et al*. (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature* 425:415–9

Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* 120:15–20

Nambudiri VE, Widlund HR (2013) Small interfering RNA. *J Invest Dermatol* 133:e15

Noguchi S, Mori T, Hoshino Y *et al*. (2013) MicroRNAs as tumour suppressors in canine and human melanoma cells and as a prognostic factor in canine melanomas. *Vet Comp Oncol* 11:113–23

Noguchi S, Kumazaki M, Yasui Y *et al*. (2014). MicroRNA-203 regulates melanosome transport and tyrosinase expression in melanoma cells by targeting kinesin superfamily protein 5b. *J Invest Dermatol* 134:461–9

Poliseno L, Haimovic A, Segura MF *et al*. (2012) Histology-specific microRNA alterations in melanoma. *J Invest Dermatol* 132:1860–8

Sonkoly E, Wei T, Janson PC *et al*. (2007) MicroRNAs: novel regulators involved in the pathogenesis of psoriasis? *PLoS One* 2:e610

Webster RJ, Giles KM, Price KJ *et al*. (2009) Regulation of epidermal growth factor receptor signaling in human cancer cells by microRNA-7. *J Biol Chem* 284:5731–41

Yi R, O'Carroll D, Pasolli HA *et al*. (2006) Morphogenesis in skin is governed by discrete sets of differentially expressed microRNAs. *Nat Genet* 38:356–62

Yi R, Poy MN, Stoffel M *et al*. (2008) A skin microRNA promotes differentiation by repressing "stemness." *Nature* 452:225–9

Yi R, Pasolli HA, Landthaler M *et al*. (2009) DGCR8-dependent microRNA biogenesis is essential for skin development. *Proc Natl Acad Sci USA* 106:498–502