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Not miR-ly micromanagers: the functions and regulatory networks of miRNAs in mammalian skin

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Abstract

The microRNA (miRNA) pathway is a widespread mechanism of post-transcriptional gene regulation in eukaryotic cells. In animals, each miRNA species can regulate hundreds of protein coding genes, resulting in pervasive functions for miRNAs in numerous cellular processes. Since the identification of the first mammalian miRNA, the function of miRNAs in mammals has been a topic of great interest, both because of the versatile roles of miRNAs in biological systems, as well as the clinical potential of these regulatory RNAs. With well-defined cell lineages and the availability of versatile tools for both *in vivo* and *in vitro* studies, mammalian skin has emerged as an important system in which to examine miRNAs' functions in adult tissues. In this review, we discuss recent insights into the functions and regulatory networks of miRNAs in mammals, with a specific focus on murine skin development as a model system. We first introduce functional analyses of the miRNA biogenesis pathway in the skin, then highlight the functions of individual miRNAs in skin development, followed by an examination of miRNA roles in skin stress responses. We finish with a discussion of miRNA regulatory networks and emphasize future challenges and emerging technologies that permit the genome-wide study of miRNA functions and regulatory mechanisms in mammalian skin.

Mammalian skin as a model system to study the miRNA pathway

The first mammalian miRNA, let-7, was discovered in 2000, and thousands have subsequently been identified by computational analysis and small-RNA cloning techniques¹. Important roles for the miRNA pathway have been revealed in virtually every biological system examined². Our present-day understanding of miRNA networks indicates that miRNA activity is likely a crucial regulatory mechanism in most gene regulatory networks that operate in mammalian cells.

The skin is the largest organ in humans and fulfills a variety of essential functions. Principally, the skin serves an ancient role as the critical first barrier between the environment and the organism. In addition to mechanical functions, the skin provides

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essential roles in defense from pathogens and maintaining body temperature, as well as sociological functions such as providing organismal identity. The skin is composed of the squamous stratified interfollicular epidermis and associated appendages such as hair follicles, sweat glands, and sebaceous glands embedded within the collagenous dermis, which provides structure and subcutaneous fat deposits, and contains fibroblast and immune cell populations³.

Mammalian skin is a traditional model for studying complicated developmental and cell biological processes. Complex and integrated regulatory networks orchestrate self-renewal and differentiation of stem/progenitor cell populations as they are specified into the numerous cell types of the epidermis and hair follicle⁴. Initially, the epidermis, hair follicle, and other epidermal appendages develop from multipotent keratin-14⁺ ectodermal progenitor cells. Hair follicle morphogenesis begins during embryogenesis through reciprocal interactions of mesenchymal and epithelial progenitor cells⁵. Activation of β -catenin/Wnt signaling is one of the earliest known signals for specifying the epidermal progenitors towards a hair follicle fate and initiating hair follicle morphogenesis⁶. Subsequent to specification, the hair follicle progenitors undergo rapid proliferation, cytoskeletal reorganization, and migration into the dermis, coordinated by multiple signaling pathways⁷. During this period of downward growth and movement, hair follicle stem cells (HFSCs) are specified, and a subset then further differentiate to form the complex hair follicle structure⁸. After initial morphogenesis, the HFSCs are maintained in the specialized stem cell niche known as the bulge⁹. Throughout the adult's lifetime the hair follicle cycles between states of growth (anagen), destruction (catagen) and rest (telogen)¹⁰. In contrast, the interfollicular epidermis is a static structure that maintains homeostasis by continual self-renewal throughout the lifetime of the organism¹¹. The stem/progenitor cell populations of the basal epidermal layer maintain a constant supply of suprabasal keratinocytes, which undergo a dramatic terminal differentiation process to produce the cornified barrier of the epidermis¹². The extensive proliferative potential of epidermal stem/progenitors has been utilized in the clinic as a pioneering stem cell treatment for replacing skin lost to burn injuries. Readers interested in learning more about this system are directed to a set of thorough reviews on the topics of skin stem cells, skin barrier functions, and the clinical potential of skin stem cells^{4,13,14}.

The skin is one of the first tissues in which functions of the miRNA pathway were delineated using genetically engineered mouse models^{15,16}. These pioneering studies defined the expression pattern of and requirement for the miRNA pathway in embryonic and neonatal skin development. Mice lacking the canonical microRNA biogenesis machinery in epidermal lineages have a severe phenotype resulting in perinatal lethality. Proper hair follicle migration is disrupted, resulting in aberrant cyst-like structures residing in the interfollicular epidermis; additionally, the balance of proliferation and differentiation is improperly maintained, resulting in a hyperproliferative epidermis. Within the numerous cell types of the epidermis and hair follicle, many miRNA families are present, including stratified epithelia-enriched miRNAs such as miR-203 and miR-205, as well as more ubiquitously expressed miRNAs, such as the let-7, miR-125, and miR-17/92 families. Genetic manipulation of a small subset of individual miRNAs has demonstrated that no single miRNA examined to date is responsible for all the phenotypes that result from

compromised miRNA biogenesis in the skin. Instead, loss of individual miRNAs only contributes to a subset of the phenotypes, indicating that many miRNAs are likely working in concert with additional miRNA families and other regulatory pathways to control skin development. In the following sections we discuss insights gained into the mechanisms of miRNA functions provided by studying murine skin, followed by a discussion of progress in determining the functions of individual miRNAs in development and stress responses, and end with a discussion of new insights into the function and regulation of miRNA activity in gene regulatory networks.

Canonical and non-canonical mechanisms of miRNA biogenesis

Most mammalian miRNAs are transcribed by RNA polymerase II from diverse regions of the genome; some miRNAs are located in the introns of coding genes, and are co-transcribed with the host gene, while others are under the control of distinct promoters. Recent evidence has also shown that some miRNAs can be generated from highly-expressed non-coding RNAs, such as tRNAs or snoRNAs^{17,18}. In the canonical miRNA biogenesis pathway, mammalian miRNAs are first transcribed into long, primary miRNAs (pri-miRNAs), which can consist of one or several miRNA hairpins. These hairpins are then processed into pre-miRNAs by the microprocessor complex, composed of the RNase-III enzyme Drosha and the RNA-binding protein Dgcr8. The hairpin structure and the 2-nt 3' overhang of the pre-miRNA are recognized by Exportin-5, which exports pre-miRNAs to the cytoplasm. Once in the cytoplasm, pre-miRNA hairpins are further processed by another RNase-III enzyme, Dicer, to produce a duplex RNA. One strand of the duplex is selected and loaded into an Argonaute (Ago) protein to form a miRNA-Induced Silencing Complex (miRISC). The miRISC then utilizes the miRNA as a guide to select target mRNAs through sequence homology, particularly with the seed-sequence region of the miRNA (nucleotides 2–8 from the 5' end of the miRNA). Such miRNA target sites are generally located within the 3' UTR of the mRNA. Once an mRNA target has been selected, its expression is modulated through translational repression and/or degradation. For readers interested in more detailed information regarding the canonical miRNA biogenesis pathway, general mechanisms of miRNA activity, and the history of these discoveries, see the following reviews^{2,19,20}.

The skin is one of only a few mammalian organs in which most of the individual components of the canonical miRNA biogenesis pathway have been examined using mouse knock-out models. Mice with skin-specific, keratin-14-Cre-driven deletion of Dicer¹⁵ or Dgcr8²¹ suffer pleiotropic skin defects and die within 4 to 6 days of birth. The phenotypes of these skin-specific knock-outs are very similar, resulting in a gross disruption of proper epidermal and hair follicle development. Collectively, these mutant analyses demonstrate that Dgcr8- and Dicer-dependent miRNAs are the predominant regulatory small RNAs important for skin development.

While the canonical miRNA biogenesis pathway has been characterized for some time, studies into the nuances of the pathway continue to expand our understanding. Herbert and colleagues recently mapped phosphorylation sites of DGCR8, and showed that phosphorylation increases its intracellular stability²². DGCR8 phosphorylation by the ERK/MAPK pathway may be a response to extracellular cues that can affect global miRNA

production, revealing a further level of regulation of miRNA biogenesis than previously appreciated. Similarly, Ago proteins are also subject to post-translational modifications that regulate their stability and function²³. In addition, further genetic analysis of the functions of the Ago proteins has also yielded new insights. Mammals express four Ago proteins (Ago1-4) which are all functional for miRISC activity. Of these four, Ago2 is the only one capable of performing siRNA-like slicer activity. Additionally, of individual knock-outs of the four Ago proteins, only global Ago2 ablation results in embryonic lethality²⁴. Despite this difference in phenotype, miRNAs do not have a specific preference for loading into any of the four Ago proteins either *in vivo* or *in vitro*^{25,26}. In the skin, individual loss of either Ago1 or Ago2 leads to a mild decrease in miRNA expression, but causes no overt phenotype²⁵. This result suggests that the slicer activity of Ago2 is dispensable for the miRNA pathway in the skin. However, when both Ago1 and Ago2 are knocked out together, the overall abundance of miRNAs is reduced 80%, resulting in catastrophic effects on hair follicle morphogenesis similar to those seen with Dicer and Dgcr8 skin knock-outs. Importantly, Ago3 is still able to load all miRNA species in the absence of Agos 1 and 2, although it is unable to compensate for their loss, indicating that a high level of Ago proteins is necessary to properly sustain miRNA biogenesis²⁵. While Ago2 slicer activity is dispensable in the skin, recent studies in other systems have described a Dicer-independent biogenesis pathway for miR-451, a miRNA highly enriched in the blood, that is dependent on the slicer activity of Ago2^{27,28}. Loss of Ago2 slicer activity, and therefore of miR-451, results in neonatal lethality, revealing an essential function for the broadly-conserved but previously poorly-understood slicer activity of Ago2²⁷. However, animals with loss of only miR-451 are viable while displaying a mild anemia^{29,30}. Thus, there may be additional functions specific to the slicer activity of Ago2 that are yet to be uncovered.

In addition to the Dicer-independent biogenesis of miR-451, some other miRNAs are generated through additional non-canonical biogenesis pathways. Recently, Xie et al showed evidence for a microprocessor-independent biogenesis pathway³¹. In this pathway, a small subset of pre-miRNAs receive a 7-methylguanosine 5' cap and bypass the microprocessor. These capped pre-miRNAs are exported through the PHAX-Exportin-1 pathway, contrasting from canonical export by Exportin-5. While canonical miRNA biogenesis allows either the 3p or 5p strand of the miRNA duplex to be selected for loading into Ago, these capped miRNAs allow Dicer processing and loading of only the 3p-miRNA. Although this pathway generates only a small number of miRNAs, some of them, such as miR-484 and miR-320, are expressed in the skin, indicating that this pathway may be of importance for skin-specific processes. For further reading on alternative miRNA biogenesis pathways, see an excellent review by Yang and Lai³².

To date, a largely unexplored aspect of miRNA biology has been upstream regulators of their biogenesis. A few studies have begun to elucidate this novel aspect of the pathway. For example, Hippo signaling was recently shown to be a link between contact-inhibition in cells and the global down-regulation of miRNAs in cancer³³. Further exploration into potential upstream regulators of miRNA biogenesis will be essential for our complete understanding of miRNAs and how they impact such diverse processes as development and disease.

Diverse miRNA families control skin development

A critical first-step approach to studying miRNAs is accurately defining their spatiotemporal expression patterns. Intensive efforts have been applied to defining miRNA expression patterns in different skin cell types and stress conditions. Classic techniques such as Northern blotting, in-situ hybridization and qRT-PCR together can provide quantitative and spatial measurements of miRNA abundance. However, these techniques are generally unable to accurately quantify miRNA abundances relative to other miRNAs in a high-throughput manner. Small-RNA cloning followed by deep sequencing and miRNA microarrays have been used to provide more comprehensive miRNA expression profiles, but suffer from RNA-ligation and hybridization based biases, respectively^{34,35}. However, recent advances in small-RNA sequencing library preparation protocols have permitted the unbiased quantification of miRNA abundances as well as differential expression between samples³⁶. By incorporating absolute abundance measurements, one can more readily discern miRNAs that not only are differentially expressed, but also expressed at levels sufficient to elicit a detectable biological response. In most samples studied, a few hundred to a thousand miRNAs can be detected by deep sequencing; however only a small number of miRNAs achieve high expression levels, with fewer than fifty miRNAs accounting for greater than 90% of all miRNAs present (K.R. and R.Y., unpublished observation). Additionally, current estimates indicate that individual miRNAs can reach 10^4 - 10^5 copies per cell, whereas individual protein-coding transcript abundances are estimated to plateau at an order of magnitude lower^{37,38}. However, total miRNA abundances can vary dramatically between cell types; for example, human hematopoietic stem cells have ~11,000 total copies of miRNA, whereas liver cells contain upwards of ~115,000 copies³⁹. Since the potency of miRNA regulation is dependent upon the relative ratio between miRNAs and their targets^{40,41}, accurate measurement of individual miRNA abundances in a defined cellular context yields important insights into a given miRNA's regulatory abilities. Small-RNA sequencing measurements of miRNA abundance from diverse skin cell types will further enhance candidate miRNA selection for genetic analyses, as well as contribute to our overall understanding of genome-wide miRNA activities in different cell lineages, developmental processes, diseases and other stress conditions.

Within the skin, hundreds of miRNAs can be reliably detected by small-RNA cloning techniques, yet only a handful of these account for the majority of miRNAs detectable²¹. The spatiotemporal expression pattern and abundance of a miRNA can provide key insights into potential functions and regulatory abilities. Shown in Figure 1 are the expression patterns of well-studied skin miRNAs as defined by small-RNA cloning complemented with precise in situ hybridization studies. Given the critical importance of the miRNA pathway for regulation of hair follicle morphogenesis and epidermal homeostasis, elucidating the functions of individual miRNAs in these processes has been a key focus of investigation. Below we discuss recent progress and review the current state of knowledge in understanding the functions of individual miRNAs in skin development.

miR-205 modulates the PI(3)K/Akt pathway in epithelia

miR-205 is an intergenic miRNA that is widely conserved among vertebrates. It is strongly and specifically expressed in stratified epithelia, and is among the most highly expressed miRNAs in mouse skin according to small RNA cloning analysis (R.Y. et al., unpublished observation). In addition to the epidermis and hair follicle, miR-205 has been shown to have robust expression in the corneal epithelia, esophagus, mammary tissue, and prostate, as well as the vast majority of other organs derived from stratified epithelia, but is absent from most other critical organs and tissues^{42–44}.

The expression pattern of miR-205 was first described in corneal and retinal epithelia⁴². A specific function for this miRNA was not described until 2008, when Yu and colleagues showed that it directly targets the SH2-containing phosphoinositide 5' phosphatase 2 (SHIP2), a negative regulator of the PI(3)K/Akt pathway⁴⁵. In human epidermal keratinocytes (HEKs) and human corneal epithelial keratinocytes (HCEKs), suppression of miR-205 by antagomiRs also leads to an increase in Rho-ROCK1 activity, as well as phosphorylation of cofilin and a decrease in filamentous actin, which together impair the ability of the mentioned cell types to repair scratch wounds in culture⁴⁶. Interesting, the same group also showed that miR-184, an epithelial miRNA with a partial overlap in expression with miR-205 in the cornea, was able to repress the targeting of SHIP2 by miR-205, without causing any decrease in SHIP2 expression itself⁴⁵. These findings highlight the largely unexplored concept of crosstalk between miRNAs in regulating cellular processes.

In developing skin, miR-205 is enriched in interfollicular progenitor cells and stem cells, and is detectable as early as embryonic day 10 (E10) in Np63⁺ skin progenitors⁴³. After hair follicles have matured, miR-205 is highly enriched in HFSCs and interfollicular progenitors, and is present in the outer root sheath, but is mostly absent from the matrix⁴³. While the vast majority of individual miRNAs are dispensable for development, our lab was the first to show that miR-205 is unique in that its ablation results in lethality within two weeks of birth^{43,44}. Loss of miR-205 results in thinner epidermis, shorter and mis-angled hair follicles, and defects in proliferation. We showed that in developing hair follicles, miR-205 co-represses several negative regulators of the PI(3)K/Akt pathway (SHIP2, Frk, Inpp4b and Phlda3), sustaining a high level of pAkt and thereby promoting the proliferation and expansion of progenitors and SCs (see Figure 1). Therefore, HFSCs that have lost miR-205 prematurely exit the cell cycle to become quiescent. Many additional potential targets of miR-205 were identified but not yet validated, some of which are involved in cell migration and adhesion. Further study will be required to discern other roles for miR-205 in regulating cell migration and adhesion in the interfollicular epidermis and hair follicle.

miR-205 has attracted interest not only for its role during development, but also because it has been described as both a tumor suppressor and an oncogene in distinct situations. Recently, it was found to promote the epithelial identity of breast cancers, along with the miR-200 family, by repressing negative regulators of E-cadherin⁴¹. For further reading on the roles of miR-205 and other miRNAs in epithelial cancers, see these excellent reviews^{47,48}.

miR-203 restricts epidermal progenitor proliferative ability through diverse mechanisms

miR-203 is found expressed at high levels only within stratified squamous epithelia such as the skin, oral mucosa, esophagus, tongue, and cervix⁴⁹. miR-203 was one of the first miRNAs to be comprehensively examined in the skin and was originally identified as a miRNA upregulated in human psoriatic skin samples⁴⁵. In normal skin, miR-203 is expressed specifically in the differentiated post-mitotic keratinocytes of the suprabasal layer of the interfollicular epidermis, and the pre-cortex, inner root sheath, and companion layer of the hair follicle, as well as the differentiated sebocytes of the sebaceous gland. Manipulation of miR-203 expression by keratin-14-driven transgenic overexpression or *in vivo* ablation via antagomiR delivery demonstrates that miR-203 acts to suppress proliferation of epidermal progenitor cells during differentiation⁴⁹. In other tissue culture systems, miR-203 maintains the ability to suppress cell proliferation, even in cell-types that normally do not have high steady-state levels of miR-203^{50,51}.

Mechanistically, miR-203 controls cell-proliferation through the regulation of diverse mRNA targets (see Figure 1). By using a doxycycline-includible allele to precisely control miR-203 expression *in vivo*, it was found miR-203 targets transcripts with functions involved in cell-cycle regulation, cell-division, and response to DNA damage⁵². Np63, the p53 homologue critical for epidermal development, is targeted by miR-203 through conserved 3'UTR binding sites. Additionally, miR-203 targets Skp2, the important substrate recognition subunit of the SCF-Skp2 complex, which promotes CDK inhibitor degradation. Subsequently Np63 has been shown to regulate the transcription of Skp2, demonstrating that miR-203 regulates both the transcription factor, Np63 and its transcriptional target Skp2, further reinforcing suppression of the Np63 transcriptional network⁵³. In addition to regulating the Np63 network, miR-203 also regulates the RNA-binding protein Mushashi2 (Msi2). Msi2 is a fundamental regulator of cell-cycle progression in part through translational repression of the CDK inhibitor, p21 (CDKN1A). In normal development Msi2 is required for hematopoietic stem cell self-renewal and likely has similar functions in epidermal progenitor cells⁵⁴. Reconstituting Np63, Msi2, and Skp2 levels in miR-203-overexpressing keratinocytes robustly rescues the self-renewal capacity of keratinocytes, demonstrating that these targets contribute to miR-203's ability to suppress keratinocyte progenitor self-renewal.

Outside of its role in differentiation, miR-203 expression is often disrupted during stress conditions. In the skin, miR-203 is strongly suppressed in migrating keratinocytes during wound healing, and is thought to suppress cell migration through regulation of the target genes RAN, Np63, and LASP1⁵⁵. This speculated function is consistent with reports from human cancer cell lines that miR-203 suppresses Epithelial-to-Mesenchymal Transition through regulation of the pro-metastatic factors Snai1/2^{56–58}. In addition to wound healing, miR-203 is also commonly suppressed during cancer progression in epithelial cancers, and is directly suppressed by growth factor signaling and known oncoproteins such as HPV-E6 and Zeb1/2^{59,60}. Additionally, and surprisingly, miR-203 is commonly silenced in lymphomas, a cancer derived from a primary tissue that normally poorly expresses miR-203,

suggesting the possibility that miR-203 can be activated by stress conditions outside of the skin to contribute to cell-cycle arrest processes⁵¹. Future studies using genetic mouse models will be needed to clarify the role of miR-203 in tumorigenesis and wound healing *in vivo*.

miR-125b balances hair follicle stem cell self-renewal and differentiation

Although miR-205 and miR-203 are specifically expressed in epithelia, and have been shown to have important functions during skin morphogenesis, there are examples of more ubiquitous miRNAs that also have important functions in skin. A well-characterized example of this is miR-125b, a mammalian orthologue of *Caenorhabditis elegans* lin-4, the first miRNA to be identified. The repression of lin-28 by lin-4 (miR-125b) is a highly conserved mechanism essential for mammalian embryonic stem cell differentiation^{61,62}. Additionally, miR-125b has been shown to play a role in hematopoietic stem cells. In particular, overexpression of miR-125b stimulates proliferation and self-renewal of human and mouse megakaryocytic progenitors⁶³. As one of the most conserved miRNAs, miR-125b is also highly expressed in many tissues in both mice and humans⁶⁴.

Zhang, et. al., recently generated miRNA profiles for four populations of keratinocytes (basal epidermis, HFSCs, outer root sheath, and matrix) and observed that miR-125b is highly expressed in HFSCs and is expressed at modest levels in basal epidermal cells, but has significantly reduced expression in outer root sheath and matrix cells⁶⁵. Sustained levels of miR-125b through an inducible transgenic mouse model leads to grossly enlarged sebaceous glands at the expense of normal hair follicle growth. miR-125b was found to target Blimp1 in sebaceous gland progenitors, leading to additional rounds of proliferation when its levels are sustained (see Figure 1). When miR-125b levels are maintained in stem cell progeny, the cells preferentially retain their stemness, blocking differentiation of HFSCs into different lineages and preventing hair growth. However, this effect is completely reversible when miR-125b regulation was restored to normal, even after several months of induction. Thus, miR-125b dynamically regulates stemness rather than permanently preventing cells from differentiation; it was shown that miR-125b targets a variety of transcripts within stem cell progeny, acting as a rheostat to control the balance between self-renewal and differentiation (see Figure 1). These findings highlight the important concept of miRNAs functioning in networks, controlling such processes as cell fate decision. Because of the potent role of miR-125b in blocking HFSC differentiation, it could also play an important role during oncogenic transformation, wherein HFSCs have been identified as cells-of-origin for skin tumor initiation^{66,67}.

The miR-23/24/27 cluster regulates keratinocyte actin-cytoskeletal remodeling

The conserved vertebrate-specific miR-23/24/27 family consists of 5 miRNAs, miR-23a, 23b, 24, 27a and 27b. In most vertebrates studied to date, the family exists in two paralogous clusters with varying miRNA composition, although they are generally organized into the miR-23a/27a/24-2 cluster and the miR-23b/27b/24-1 cluster. In humans and mice the mature sequences of miR-23a and 23b differ by only a single nucleotide at the distal 3' end of the

mature mRNA; similarly miR-27a and 27b share nearly identical sequences (see excellent review)⁶⁸. However, the seed-sequences of miR-23, miR-24, and miR-27 are divergent, suggesting that these miRNAs recognize distinct target sites. Despite the differences in predicted targeting abilities, the evolutionarily conserved clustering of several members of the family on the same primary transcript indicates that they function in a coherent manner to modulate cellular phenotypes.

Within the skin, all members of the two clusters are highly expressed, as assessed by small-RNA cloning techniques²¹; despite this, miR-24 is the only member that has been extensively studied in the skin. miR-24 is preferentially expressed in the post-mitotic differentiated suprabasal keratinocytes of the epidermis and the inner root sheath of the hair follicle, exhibiting an expression pattern similar to miR-203⁶⁹. Melino and colleagues have studied the function of miR-24 in both the hair follicle and interfollicular epidermis by combining keratinocyte-specific over-expression with subcutaneous antagomiR injections. Their studies demonstrate that miR-24 is up-regulated during keratinocyte differentiation, and functions during that process to regulate actin-cytoskeletal remodeling dynamics (see Figure 1). As a consequence of precocious miR-24 expression, actin-cytoskeletal dynamics are altered and differentiation is triggered. PAK4, Tsk5, and ArhGAP19 were identified as crucial targets for this phenotype by screening for putative mRNA targets down-regulated by miR-24 over-expression in primary culture. These regulators are suppressed during high-calcium-induced differentiation, and individual abrogation of these mRNAs results in cytoskeletal rearrangements similar to those induced by high-calcium and miR-24 induction. Surprisingly, silencing a single target of miR-24 is able to partly recapitulate the phenotype of attenuated miR-24 activity, suggesting that ArhGAP19, Tsk5, and PAK4 act as critical nodes in the regulation of actin-cytoskeletal dynamics. These elegant studies also highlight the precise and complex mechanisms utilized to regulate cellular migration and cytoskeletal rearrangements during proper epidermal differentiation. Additionally, in the hair follicle, miR-24 activation via the keratin-5 promoter results in stunted cyst-like hair follicles with reduced proliferative capacity⁷⁰. miR-24 is hypothesized to modulate hair follicle development by directly suppressing the transcription factor Tcf3, which in combination with Tcf4 maintains epidermal and hair follicle progenitor self-renewal (see Figure 1)⁷¹. Although miR-24 transgenics develop severe epidermal and hair follicle phenotypes, it remains essential to perform loss-of-function experiments in a cell-type-defined genetic manner to tease apart potential complications due to the gross morphologic perturbations caused by miR-24 overexpression.

Interestingly, the miR-23/24/27 cluster has diverse and sometimes contradictory functions. Within certain types of cancer, the miR-23/24/27 cluster promotes tumorigenesis, while in other cancers it has tumor-suppressive functions^{72,73}. At the molecular level, the defined targets of the cluster are varied and sometimes contrasting in cellular function. For example, miR-24 can target c-MYC and E2F2, enforcing cell-cycle arrest, while in other systems, such as keratinocytes, miR-24 targets the cell-cycle inhibitors p16 (CDKN2A) and p27 (CDKN1B), reinforcing cell proliferation^{74,75}. Moreover, miR-23 suppresses metastasis in colorectal carcinoma and breast cancer models through targeting TGFBR2, FZD7, PAK2 and other pro-metastatic factors; in contrast, however, miR-23 promotes invasion and metastatic traits in collaboration with miR-24 and miR-27 through the targeting of Spry2 in

other breast cancer models^{72,73}. Additionally, miR-23 and presumably miR-24 and miR-27, are also repressed or activated in a c-Myc dependent fashion in different cellular contexts^{73,76}. Given the important roles of these miRNAs in both developmental and disease processes, future work will be required to address the functions and targets of these clustered miRNAs within defined cell types of the skin.

miRNA involvement in skin stress responses

It has been well-established that miRNA processing machinery must be intact for the development and survival of many organisms; however, recent studies in *C. elegans* have revealed that the majority of individual miRNAs, and even entire miRNA families, are dispensable for normal development^{77,78}. The same appears to hold true in mammalian systems, with a few notable exceptions⁷⁹. The results of these studies suggest a level of functional redundancy between miRNAs and other regulatory networks, which likely evolved to guarantee developmental robustness. To test this hypothesis in *C. elegans*, J. Brenner and colleagues employed the approach of a sensitized genetic background to analyze miRNA loss-of-function phenotypes, in which the organisms were missing one but not both of the worm Argonaute-like (alg) proteins⁸⁰. This partial Argonaute knock-out results in worms that are viable, but have lower levels of mature miRNAs compared with wild-type. When crossed with worms in which individual miRNAs were genetically deleted, mutant phenotypes were revealed for many miRNAs that had previously been shown to have no overt phenotypes when the miRNA alone was deleted; in fact, 25 of the 31 miRNAs studied were shown to have a mutant phenotype. This study has highlighted the complexities of studying individual miRNA knock-out models, which often have subtle or indiscernible developmental phenotypes.

Applying this approach to mammalian systems may provide invaluable insights into the functions of miRNAs, and should be considered for all functional studies of individual miRNAs and miRNA families. One notable example of the application of this technique in mammals, albeit not in the skin, supplied valuable insights into the role of a miRNA in skeletal muscle. Williams, et al., noted that miR-206, a skeletal muscle-specific miRNA, was among the most up-regulated miRNAs in a mouse model of amyotrophic lateral sclerosis (ALS)⁸¹. They then combined this mouse model with a loss-of-function model for miR-206, and determined that loss of miR-206 accelerated the progression of ALS symptoms. In addition, though the miR-206 knock-out alone showed no overt phenotype during normal development, when muscle was injured, reinnervation was delayed in the miR-206 loss-of-function model compared with wild-type. This work has further highlighted the importance of studying miRNAs under a variety of conditions, including not only normal homeostasis but also conditions of stress.

As the barrier between the organism and the environment, the skin provides a unique and valuable platform for examining the stress- or injury-related functions of miRNAs. The skin is constantly subjected to many stresses such as wound healing, viral infection, and UV radiation. In the following sections we highlight studies that have been conducted as well as questions that require further pursuit.

Wound healing and regeneration

Wound healing is a complex process involving not only proliferation and migration of epithelial cells, but also inflammation, immune response, and angiogenesis. This complicated process has been extensively studied in the skin, where there is high accessibility to and high visibility of wounds. Additionally, wounding creates a stress to the skin which is likely to reveal functions for miRNAs whose role cannot be uncovered during normal homeostasis. Indeed, a recent study showed that there is a change in expression of several miRNAs after dermal wounding when compared with healthy skin, indicating that miRNAs are potential regulators of wound repair⁸². The same study showed that the miR-99 family targets several genes in the AKT/mTOR pathway; thus, a decrease in their expression in keratinocytes following wounding promotes proper wound closure⁸². miR-203 was shown to be down-regulated upon wounding, suggesting that it may play a role in mediating wound repair⁸³. Many other miRNAs have been shown to be involved in keratinocyte migration and proliferation^{43,46,69}, both essential processes for wound repair; whether any of these miRNAs are involved in wound healing in the skin remains to be seen.

Though most of this review has focused on the epithelial linages of the skin, there are additional cell populations such as endothelial cells and immune cells which are key regulators of wound healing, which are also subject to miRNA regulation. In endothelial tissue, down-regulation of both miR-199a-5p⁸⁴ and miR-200b⁸⁵ has been shown to support wound healing by stimulating angiogenesis. In addition, a recent study showed that mice lacking miR-155, a miRNA known to be involved in immune response, show an increased rate of wound healing compared with wild-type⁸⁶. Thus, miRNAs are involved in regulating wound repair in a variety of cell populations within the skin. Importantly however, chronic wounds are a world-wide clinical problem that requires much more effort to be well understood.

Viral infection

Skin is the primary site of infection for many types of viruses, including cutaneous papilloma and herpes viruses. miRNAs from both the host and the virus have been shown to be important for viral infection in many epithelial tissues. In the skin, human papilloma virus (HPV) has been shown to regulate the expression of miR-203, miR-24 and miR-205^{59,87,88}. The life-cycle of HPV requires that cells continue to replicate DNA post-differentiation. The virus has been shown to down-regulate miR-203 expression, thereby promoting continued replication by increasing expression of Np63⁸⁷. In addition, HPV affects the expression of both miR-24 and miR-205 during differentiation, providing an additional mechanism by which the virus skews the balance between differentiation and proliferation⁸⁸. Though the precise mechanisms by which HPV regulates these skin-specific miRNAs have not yet been described, there are likely to be a variety of direct and indirect modulations of miRNA activity employed by various viruses. The authors of the above studies suggest that viral alteration of certain signaling pathways, such as MAPK, could affect the levels of specific miRNAs such as miR-203. There is also evidence that viruses are able to affect miRNA expression more broadly. For example, the adenovirus RNA VA1 inhibits the function of Dicer, which reduces the global expression of miRNAs⁸⁹. For further reading on the relationship between viruses and miRNAs, see excellent review by Cullen⁹⁰.

Few studies have been conducted examining the role of miRNAs in viral infection specifically in the skin; however, the above studies, as well as numerous additional studies demonstrating that the miRNA pathway interacts intimately with viruses, strongly suggest that skin miRNAs are likely important mediators of viral infection^{91–93}. In addition, the study of viral infection in the skin may provide insights into the role of miRNAs in the early stages of cancer, as many epithelial cancers originate with viral infection, such as from HPV.

Skin diseases

There are several diseases of the skin which provide models of stress, and in which miRNAs have been shown to play a role. For example, both psoriasis and atopic eczema exhibit distinct miRNA profiles from healthy skin; both exhibit down-regulation of miR-125b⁹⁴. However, in psoriasis, but not atopic eczema, miR-203 and miR-146a are up-regulated⁹⁴. The proliferation of keratinocytes is enhanced in psoriasis, which appears to contradict the finding that miR-203 represses proliferation in differentiating epidermal cells⁵². However, miR-203 was shown to target SOCS-3 only in psoriatic, but not healthy skin⁹⁴, indicating that miR-203 and other miRNAs regulate distinct targets under conditions of stress. This study highlights the importance of studying miRNAs not only in the tissues and cells where they are endogenously expressed, but also under a variety of conditions such as stress, in order to reveal their functions in specific situations. For a schematic of miRNAs involved in stress responses, see Figure 2.

Defining miRNA regulatory networks in the skin

miRNA regulatory networks are evolutionarily conserved, with key components maintained over hundreds of millions of years of evolution¹⁰³. These networks are proposed to serve a variety of roles, including buffering phenotypic and genetic variation, imparting robustness to gene regulatory networks, or acting as critical rheostats for components of regulatory networks (see excellent reviews^{104,105}). Mechanistically, miRNAs function to set thresholds for processes, suppress transcriptional noise, or reduce network activity by targeting multiple members of a pathway. miRNA networks are organized distinctly from other regulatory molecules such as transcription factors, which tend to group into key network hubs and acting as master regulators for biological processes to establish cell-state-specific gene expression programs¹⁰⁶. In contrast, miRNAs act downstream of master regulatory factors to fine-tune gene expression levels and reinforce cell-state-specific gene expression (see Figure 3). For example, in the skin, miR-203 acts to reinforce suprabasal gene expression by suppressing genes related to the basal cell fate, including the transcription factor Np63, and thereby restricting progenitor basal cell proliferation. While miRNAs do not function as master regulators, miRNA targets sites are enriched in genes that act as network hubs such as transcription factors, regulatory metabolic enzymes, or signaling cascade modifiers¹⁰⁷. For example, miR-205 targets multiple negative regulators of the PI(3)K pathway, which collectively function to modify the activity of pAkt⁴³. Whereas a single miRNA can target hundreds of protein coding genes, few studies have addressed the intrinsic relationship between the large number of targets and how to distinguish functionally important targets in a given cellular context. A key challenge for the study of

miRNA activities is moving beyond the paradigm of studying an individual miRNA and only a handful of targets. miRNAs generally function through the coordinated activity of numerous targets, and in the future defining the interactions of such targets within larger networks will be a central focus.

Recent technological developments now permit the genome-wide identification of miRNA binding sites and facilitate gene expression measurements by High-Throughput Sequencing (see sidebar). By integrating quantitative gene expression measurements with HITS-CLIP analyses (or similar derivatives), miRNA/mRNA interaction networks can be experimentally defined⁹⁹. The genetic study of candidate miRNAs in the skin has identified many miRNAs with important functions in development. However, it is essential to recognize that many mRNAs can be targeted by multiple miRNAs, complicating the interpretation of a single miRNA's regulation of given transcript (see Figure 3). For example, Np63 was originally identified as critical target of the suprabasal miRNA miR-203; however, subsequent studies also demonstrated that miR-130 regulates Np63 during keratinocyte senescence, suggesting that these microRNAs may act coordinately to regulate Np63 in keratinocytes^{49,108}. Traditional studies of individual miRNA targets fail to uncover the complex combinatorial interactions between multiple miRNAs on similar target mRNAs or biological networks. An added layer of conceptual complexity is introduced when one considers interactions between not only the individual miRNA/mRNA, but additionally, interactions with abundant mRNAs or ncRNAs. Recent studies provide evidence that the ability of a miRNA to target a set of transcripts can be controlled by modulating the abundance of available miRNA binding sites and therefore the abundance of active RISC-associated miRNA complexes (see Figure 3)¹⁰⁹. For example, abundant pseudogenes can recruit RISC-loaded miRNAs and titrate away active miRNA targeting interactions from protein-coding genes. Additional noncoding RNAs, such as long non-coding RNAs (lncRNAs) and circular RNA (circRNA) species have also been demonstrated to actively recruit miRNA complexes away from traditional protein coding target genes^{110,111}. These non-canonical mechanisms for regulating miRNA activity can determine the functional pool of miRNA-loaded RISC, and in a handful of cases are critical for proper miRNA function. RNAs that have the ability to titrate functional RISC and perturb miRNA targeting have been named competing endogenous RNAs (ceRNAs). Systematic profiling of the different cell types of the skin by high-throughput methods will be required to provide insights into these non-canonical and higher-level miRNA/mRNA target relationships, to further develop our understanding of miRNA regulation in the skin.

Perspective

Over the past decade and a half, miRNA regulation has emerged as a major mechanism for post-transcriptional gene control in mammals. Intensive efforts have been made to define the mechanisms of miRNA biogenesis, their functions in biological processes, and the regulation of miRNA activity. However, key challenges remain to be addressed, such as experimental identification of biologically relevant miRNA targets in complex gene-regulatory networks, as well as elucidation of the functions of miRNAs in stress responses relevant for human diseases.

miRNAs are a unique class of regulatory molecules that continually challenge our notions of biological significance. miRNA targeting rarely perturbs target gene output by more than 50%, a level of regulation that is likely within the levels of cell-to-cell variation in gene expression. Defining miRNA targeting networks experimentally therefore requires sophisticated and precise measurements of gene-expression. Rapid developments in high-throughput sequencing now provide genome-wide insights into gene regulatory networks that were previously unattainable, such as single-cell analysis of gene-expression, direct identification of miRNA targets from *in vivo* samples, and measurement of translational rates. The skin is an ideal system in which to apply these techniques due the numerous cell lineages amenable to isolation and genomics profiling. Additionally, these techniques will allow the systematic identification of not only mRNA targets, but will also uncover additional layers of regulation, such as combinatorial miRNA regulation of mRNAs, and non-canonical miRNA interactions with lincRNA, circRNA, and other ncRNA. By delineating the mechanisms by which miRNAs elicit biological function *in vivo* we stand to gain important insights into complex gene-regulatory network organization and regulation.

Despite the deep conservation and abundance of many miRNAs, surprisingly few miRNA knockout animals display overt phenotypes. The paucity of strong developmental phenotypes in miRNA knockout animals suggests that we need to incorporate stress paradigms into our phenotypic analysis and reconsider how we define biologically significant phenotypes in complex animal models. The skin is aptly suited for studying stress responses, being subjected to a wide variety of stresses as the primary barrier between the environment and the organism. Furthermore, classical stress protocols have been established in murine skin such as wounding-healing, chemical carcinogenesis, and infection models, that can be readily applied to the study of miRNAs involved in stress responses. Moving forward, identifying miRNA functions under stress conditions relevant to human disease processes remains perhaps the most important and difficult challenge in realizing the therapeutic potential of miRNAs.

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Genome-wide technologies for identifying miRNA regulatory networks

Advancements in high-throughput sequencing now permit genome-wide and quantitative approaches to identify miRNA targets in *in vivo* samples. The use of these methods, combined with established Flow Cytometry methods, facilitates the characterization of miRNA dynamics in different skin cell lineages.

HITS-CLIP/PAR-CLIP

High-Throughput Sequencing of Cross-Linked Immuno**P**recipitations (HITS-CLIP), identifies RNA-binding protein binding sites transcriptome-wide⁹⁵. HITS-CLIP uses UVC light to covalently crosslink RNABPs to target RNAs. Following limited RNase digestion and stringent complex purification, the isolated RNA is subject to high throughput sequencing⁹⁶. The related techniques PAR-CLIP or CLASH utilize modified ribonucleosides or direct RNA ligation, respectively, to facilitate direct AGO binding site identification^{97,98}. Using Ago HITS-Clip, combined with miRNA knockout mutants, can directly identify miRNA targets genome-wide from *in vivo* samples⁹⁹.

Ribosome profiling

miRNAs suppress target mRNAs by RNA deadenylation and through translational repression¹⁰⁰. Much focus has defined transcriptome-wide miRNA target repression, however translation dynamics and protein levels have been difficult to assess for technical reasons. Ribosome footprinting techniques measure genome-wide ribosome occupancy, allowing researchers to more accurately define miRNA regulatory effects on the proteome¹⁰¹.

Quantitative small-RNA-Seq and mRNA-Seq

Small-RNA and total RNA-Seq methods are powerful methods for defining transcript abundance, differential expression and identifying novel RNA species. These techniques can be readily adapted to profile rare cell populations¹⁰². RNA-Seq identifies steady-state RNA levels from diverse RNA species, including ncRNA such as lncRNA. Additionally, recent advances in small-RNA sequencing methods now provide quantitative measurements of miRNA abundances from *in vivo* samples³⁶.

Figure 1.

Murine skin is comprised of a variety of cell lineages within the interfollicular epidermis and hair follicle. In the interfollicular epidermis, proliferative basal cells contribute to the above layers of differentiating cells, which perform the barrier function of the skin. Within those differentiated cells, miR-203 and miR-24 regulate proliferation and actin-cytoskeletal dynamics, respectively. The hair follicle is comprised of cells at varying stages of differentiation. A subset of hair follicle stem cells (HFSCs) housed in the bulge differentiate and move downwards to become the highly proliferative, transit-amplifying cells of the matrix. Those cells then differentiate to become the inner and outer root sheaths. The balance of quiescence and differentiation in HFSCs is maintained in part by miR-205 and miR-125b. Within the inner root sheath, miR-24 ensures that self-renewal is repressed and differentiation is preferred. Finally, within the sebaceous gland, an accessory structure to the hair follicle, miR-125b contributes to the control of proliferation. In addition to the examples shown, there are likely to be additional miRNAs with important functions in the various cell types of the interfollicular epidermis and hair follicle that have yet to be uncovered.

Figure 2.

miRNAs regulate a variety of processes involved in stress responses. The skin undergoes a variety of stresses, including wound healing, viral infection, and tumorigenesis. Examples of specific miRNA regulation have been uncovered for most of the major processes which contribute to these stress responses. However, our understanding of how miRNAs are involved in regulating stress responses is incomplete, and extensive effort will be required to extend our comprehension.

Figure 3.

(A) Conceptual functions for miRNA targeting in networks. miRNAs can act to reinforce a cellular state by promoting negative regulation of factors. In this case, $miR-203$ antagonizes $Np63$ during the epidermal differentiation. We note that $miR-203$ represses many other genes in addition to $Np63$ during the transition. (B) By imparting negative regulation to transcripts, miRNAs can buffer biological noise inherent in gene expression networks. By suppressing noise in gene expression, miRNAs contribute to establishing thresholds at which target genes must cross to promote a relevant cellular outcome. (C) One of the barriers in studying miRNA function in complex biological systems is that many mRNAs are under coordinated regulation by multiple miRNAs. Shown are representative Ago2 HITS-CLIP tags mapping to $Np63$'s 3'UTR. $miR-130b$ and $miR-203$ have both independently been demonstrated to regulate $Np63$, and shown here both co-target $Np63$ in keratinocytes. (D) Further complexity in dissecting miRNAs function on a genome-wide scale is introduced by competing endogenous RNAs (ceRNAs). ceRNAs, such as pseudogenes or circular RNAs, titrate away active RISC from mRNA targets, thereby influencing miRNA repressive activities.