Small RNAs in development and disease

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MicroRNAs (miRNAs) and short interfering RNAs (siRNAs) are classes of regulatory small RNA molecules, ranging from 18 to 24 nucleotides in length, whose roles in development and disease are becoming increasingly recognized. They function by altering the stability or translational efficiency of messenger RNAs (mRNAs) with which they share sequence complementarity, and are predicted to affect up to one-third of all human genes. Computer algorithms and microarray data estimate the presence of nearly 1000 human miRNAs, and direct examination of candidate miRNAs has validated their involvement in various cancers, disorders of neuronal development, cardiac hypertrophy, and skin diseases such as psoriasis. This article reviews the history of miRNA and siRNA discovery, key aspects of their biogenesis and mechanism of action, and known connections to human health, with an emphasis on their roles in skin development and disease. (J Am Acad Dermatol 2008;59:725-37.)

Learning objectives: After completing this learning activity, participants should be able to summarize the relevance of microRNAs in development and disease, explain the molecular steps of how small RNAs regulate their targets within the human cell, and discuss the role of small RNAs in the diagnosis and treatment of disease.

INTRODUCTION

Regulation of gene expression: Conducting the 25,000 member orchestra

The human genome contains about 25,000 genes.[1] The expression of each of these individual genes needs to be appropriately controlled to suit the function and environment of each cell, and must change to respond to new conditions or signals. One of the general aims of biologic research is to understand how a cell's pattern of gene expression is orchestrated to promote coordinated growth and development and to understand how the inappropriate expression of genes is involved with disease.

The basic dogma of molecular biology (Fig 1, *A*) states that the template for genetic information is encoded in DNA. Genes consist of segments of DNA that are transcribed into RNA molecules, which are then transported from the nucleus to the cytoplasm, where they are translated into proteins. A detailed discussion of this process has been recently published.[2]

The activity of genes is controlled on each level of this pathway (Fig 1, *B*). At the DNA level, cells have many ways to regulate how rapidly genes are transcribed. One form of regulation is structural, in which regions of DNA are made more or less physically accessible to the cellular machinery that is required for transcription. Within the nucleus, DNA is packaged among proteins known as histones, which are responsible for organizing DNA. Histones have "tails" that extend outwards from the central part of the protein and contain amino acid sequences that can be chemically modified by the cell. These modifications modulate how tightly the histones are packaged and influence the accessibility of the DNA segments that are associated with them. When they are more accessible, genes can be more easily reached by transcription factors and can be transcribed in higher quantities. Conversely, genes can be kept inactive by being packaged more tightly, keeping them in an inaccessible state.

In addition to structural changes, another mechanism to control gene transcription is to alter the availability, quantity, or activity of transcription factors themselves. A transcription factor is a protein that recognizes a specific sequence in the regulatory region of a gene and influences the rate at which the gene is transcribed. Activity of transcription factors are regulated in many different ways: they can themselves be produced in higher or lower levels;

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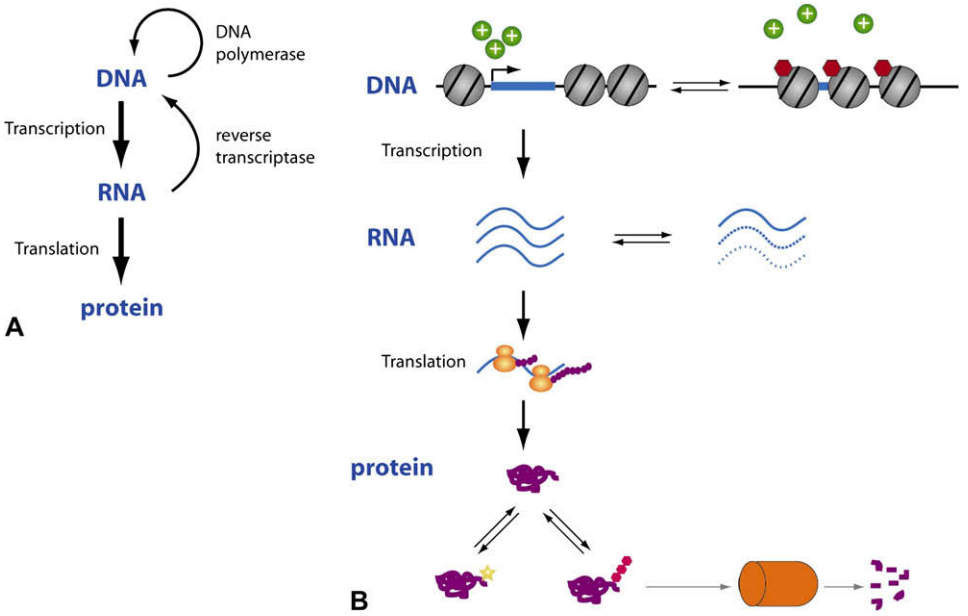

Fig 1. A, Dogma of molecular biology. Heritable genetic information is contained in DNA, which can be replicated and passed to daughter cells. DNA is transcribed to RNA, transported to the nucleus, and translated into proteins. The identification of reverse transcriptase demonstrated that RNA can also be converted back into DNA. **B**, Gene activity is regulated on many levels. Representative mechanisms of gene regulation are shown at the DNA, RNA, and protein levels. The rate of gene transcription can be affected by the quantity of transcription factors *(green circles)* that are locally available to interact with the gene. Transcription can also be structurally regulated: DNA is packaged among histone proteins *(spheres)*, which can be modified *(red octagons)* in a way to package DNA more tightly and make it less accessible to transcription factors. On the RNA level, the stability of a transcript can determine how long it persists in the cell and how much protein can be made. At the protein level, proteins can be switched to active form by chemical modifications, such as phosphorylation *(gold star)* or targeted for destruction by ubiquitination *(pink hexagons)*. Polyubiquitination causes proteins to be ferried to the proteasome, which degrades proteins into short amino acids. There are other mechanisms of gene regulation that are not shown here. MicroRNAs function at the level of altering RNA stability, as well as by affecting the rate at which RNAs are translated into proteins.

they may be activated or deactivated by chemical modifications such as phosphorylation; their localization to the nucleus can be regulated, such as by binding to a ligand; or their activity can be enhanced or inhibited by interaction with other cofactors and transcription factors. Taken together, there are numerous levels of gene regulation at the DNA level.

Gene regulation also occurs on the level of the RNA transcript. Within the cell, RNA molecules are constantly being created and broken down. Every RNA has a different longevity, depending on features such as the length of the polyadenylation tail and the presence of sequence elements in the regulatory region of the transcript. These features influence the interaction of each RNA with cellular degradation machinery and, by inference, are thought to control the amount of corresponding protein in the cell. Recent experiments indicate that the modulation of RNA stability may be a common global mechanism for cells to control gene activity. Genome-wide analyses suggest that up to 50% of all RNAs undergo significant changes to their stability in response to cellular signals.[3,4] These observations, together with the discovery that microRNAs (miRNAs) function at this level to alter target RNA stability, has sparked a growing interest to further understand the role of RNA turnover in the control of gene action.

Finally, genes are extensively regulated on the protein level. Proteins can have active or inactive conformations, or can require chemical modifications or cofactors to be functionally active. This allows a cell to control where and when a protein is active and to allow a rapid change of a protein to a functional or nonfunctional state. In addition, cellular levels of proteins can be downregulated by targeted breakdown. A well known mechanism to

selectively degrade proteins in the cell is through the ubiquitin–proteasome pathway, a process in which a small signal molecule (called "ubiquitin" because of its presence in all eukaryotes) is tagged onto designated proteins, marking them for transport to the proteasome, a barrel-shaped cellular machine that breaks ubiquitinated proteins into peptides and amino acids.

Viewed together, the activity of genes can be regulated on every level, from DNA to RNA to protein. In many instances, the control of any single gene may occur on several different levels, with adjustments being made constantly to change the levels of gene activity to an appropriate state. Despite the many varied mechanisms of gene control that are known, however, historical attention has focused on the regulatory functions of DNA and proteins. RNA has been largely envisioned as an intermediate molecule between the two, with specialized roles in splicing and translation. In this context, it was a revolutionary concept to discover the involvement of RNAs—that were of a surprisingly small size—in the extensive regulation of gene activity in humans. Our understanding of RNA-mediated regulation grows almost daily, as new studies showcase the deep and far-reaching effects of small RNAs on mammalian development and disease. Because of their small size, the very first of these regulatory small RNAs to be discovered were called miRNAs.

A brief history of microRNAs

MiRNAs were first discovered in the early 1990s in laboratories studying the genetics of development in the roundworm *Caenorhabditis elegans*. Mutations in two separate *C elegans* genes, named *lin-4* and *lin-14*, resulted in similar phenotypes in which the worms failed to mature and differentiate properly.[5] Further characterization of *lin-4* uncovered the surprising discovery that the gene itself was a 22-nucleotide (nt) RNA molecule that had multiple sites of sequence complementarity in the *lin-14* 3′-untranslated region (UTR).[6,7] A molecular model was proposed in which the translation of the *lin-14* messenger RNA (mRNA) into a protein was inhibited by the binding of the small *lin-4* RNA molecule to the *lin-14* 3′-UTR.

The discovery of the *lin-4* miRNA as a controller of developmental regulation was followed by the identification of another miRNA involved in developmental control, named *let-7*.[8] One notable feature of *let-7* was that the gene was conserved from the roundworm to other animal species, including humans. This discovery supported the idea that miRNAs might also have important functions among many different species. The use of cloning techniques and computer prediction algorithms, which identify miRNAs within the human genome based on their predicted structures, have now uncovered hundreds of miRNAs appearing in species such as the fruit fly, mouse, and human. Public databases of miRNAs are being constantly updated as investigators identify and elucidate the functions of individual miRNAs (see Britain's Sanger Institute database at http://microrna.sanger.ac.uk/).

Researchers in the field estimate that there are as many as 1000 miRNAs in the human genome,[9] and that these miRNAs may target up to one-third of all human genes.[10] Using microarray technology, which allows for the characterization of global miRNA expression patterns, the "miRNome" of normal and diseased tissue is becoming rapidly decoded. The identification and cataloguing of miRNAs has progressed much more rapidly than our detailed understanding of their individual functions. Nonetheless, the past few years have begun to reveal critical roles for miRNAs in human development and disease.

The discovery of small, interfering RNA

In the late 1990s, a landmark study demonstrated that gene expression could be inhibited by the introduction of double-stranded RNA with sequence complementarity to the gene being targeted, a mechanism that was named RNA interference (RNAi).[11] The importance of this work would be recognized by the 2006 Nobel Prize in Physiology or Medicine. Biochemical studies revealed that when long, double-stranded RNAs are introduced into a cell, they become diced into short, double-stranded, 21-nt RNAs containing 2-nt 3′ overhangs, known as short interfering RNA (siRNA). The siRNA then guide cellular machinery to target and degrade mRNAs with a similar sequence.

It was recognized that the small RNAs involved in RNAi and the ultimate effect of gene inhibition had similarities to how miRNAs inhibited mRNA protein translation. Our understanding of miRNAs has been helped in part from the parallel study of siRNAs. In particular, these two classes of small RNAs share biochemical machinery involved with their generation and processing.

Mechanism of microRNA- and short interfering RNA–mediated gene control

MicroRNAs originate from all parts of the human genome, including the introns of protein-coding genes, as a cluster with other miRNAs, or as stand-alone genes. Like protein-coding genes, they are activated by transcription factors and are transcribed by RNA polymerase II. A single miRNA has the ability to regulate multiple targets. On the other hand,

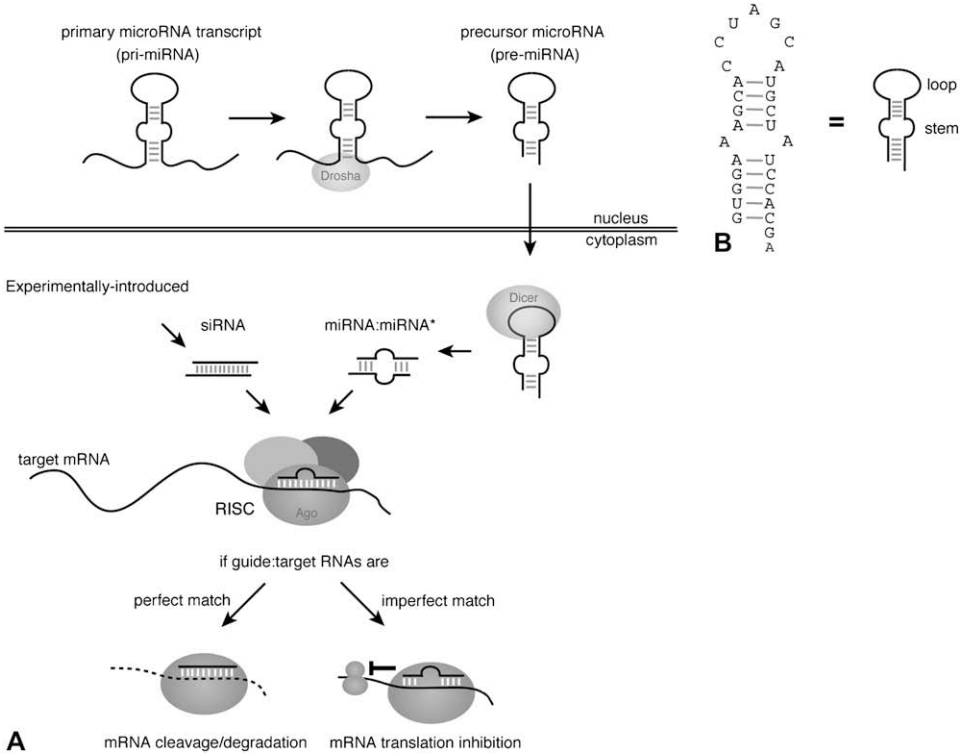

Fig 2. **A**, Schematic diagram of microRNA and small interfering RNA processing within the cell. MicroRNAs are transcribed from the genome and processed by a nuclear protein, Drosha, which excises precursor microRNA hairpins from the primary transcript. These precursor microRNAs are exported to the cytoplasm where their loop structures are removed by Dicer, forming mature microRNAs. The resulting microRNAs—or experimentally-introduced small interfering RNAs—are loaded into the RNA-induced silencing complex, which inhibits RNAs that contain sequence complementarity with the small RNA that has been loaded as a guide strand. **B**, The RNA hairpin. MicroRNAs form RNA secondary structures during processing, which are determined by intramolecular base-pairing between RNA nucleotides. Hairpins are formed by two complementary stretches that form a "stem" with an intervening set of nucleotides that do not pair, forming a "loop".

multiple different miRNAs can also converge and regulate the same target. Taken together, miRNAs form an interconnected regulatory network that does not simply turn genes on or off, but are thought to "tune" the expression level of their target genes.

MiRNA processing has been reviewed in detail,[12] and only the most critical steps and molecular players are discussed here (Fig 2, *A*). First, the miRNA is transcribed from the genome as a primary RNA transcript (pri-miRNA), which can be many kilobases long. The RNA nucleotides within the pri-miRNA transcript interact with each other to form complementary pairs, causing the RNA to form secondary structures. The most important secondary structure is known as the stem-loop—also known as the hairpin (Fig 2, *B*)—which is generated when two complementary stretches of RNA base-pair to form the "stem," with an intervening set of RNA nucleotides that do not interact, which become the circular "loop." These stretches of paired RNA can occasionally have intervening mismatches, which form a bubble (Fig 2, *B*).

The intramolecular hairpin structures are recognized by a protein complex in the nucleus that includes the enzyme Drosha, whose function is to excise the hairpin structures from the primary transcript. The excised ~60 nt hairpins are then called precursor microRNAs (pre-miRNAs), and contain a loop, a 22-nt stretch of complementary double-stranded RNA, and a 2-nt overhang at the 3′ end. Pre-miRNAs are recognized by a protein, exportin-5, that brings them from the nucleus into the cytoplasm.

In the cytoplasm, pre-miRNAs are further processed by an enzyme called Dicer, which removes the loop structure, leaving a double-stranded RNA duplex (miRNA:miRNA*) that is 22-nt in length. The processed miRNA duplex is taken up by a protein complex known as the RNA-induced silencing

complex (RISC), inside which the two RNA strands of the duplex become separated. One strand is used as a mature "guide" strand (miRNA), which will remain bound to RISC and will be the element that recognizes target RNAs. The other strand, known as miRNA*, becomes dissociated and degraded.

The inhibition of target RNAs occurs through two different mechanisms, depending on the degree with which the guide miRNA strand matches the target RNA. If the pairing between the guide miRNA and the target is imperfect—that is, if there are a significant number of mismatched pairs between the guide miRNA and the 3′-UTR of the target mRNA—then the RISC complex will inhibit the protein translation of that RNA. On the other hand, if there is a perfect match between the guide miRNA and the target RNA, then the target mRNA will be cleaved. One of the important components of RISC is Argonaute (Ago), a family of proteins which bind small RNAs. The Ago2 subtype, which is incorporated into human miRNA and siRNA RISC complexes, is responsible for cleavage of target RNAs.[13]

The components of the miRNA pathway overlap with those involved with processing siRNAs. When double-stranded RNAs are introduced experimentally into cells, Dicer cleaves them into 22-nt siRNAs. These siRNAs are then loaded into RISC in a similar manner as miRNAs (Fig 2, *A*), with the guide siRNA strand directing the inhibition of the target RNA. miRNAs in humans often do not match perfectly with their targets, and therefore lead to translational inhibition. By contrast, experimental siRNAs are often designed with perfect complementarity to their targets, leading to RNA degradation. The ability of the RISC to perform these different functions is not fully understood, but may depend in part on the subtype of Ago that is incorporated into the RISC complex. For example, although miRNAs and siRNAs in humans can associate with any of the four Ago subtypes, only Ago2 is capable of RNA cleavage.[14]

MicroRNA, small interfering RNA, and other small RNA classes

Despite the similarities between miRNAs and siRNAs, the two classes of small RNAs have several key differences, both in definition and concept. MiRNAs refer to small RNAs produced naturally from the human genome, and have diverse and widespread roles. They are generated by transcribing a single RNA that forms an intramolecular hairpin intermediate during processing. As stated before, miRNAs in humans most often have imperfect complementarity to their targets, leading to translational inhibition.

By contrast, siRNAs can be either exogenous or endogenous—that is, either naturally occurring in the genome or introduced from outside the cell. Endogenous siRNAs are created from two separate but complementary transcripts—for example, from bidirectional transcription at the same locus—resulting in a long, perfectly-matched duplex that is subsequently diced into siRNAs. The presence and roles of endogenous siRNAs in humans are still being discovered; thus far, they have been associated with providing a defense against viral infections[15,16] and in the protection against activation of mobile genomic elements.[17]

The recent attention towards small RNAs has fueled the discovery of a number of new small RNA classes, each of which are currently classified into different groups based on their different functions or mechanisms by which they are generated. These include trans-acting siRNAs (tasiRNA), a type of small RNA observed only in plants to date[18]; repeat-associated siRNAs (rasiRNA), a group of small RNAs that are produced independent of Dicer and Ago, and are thought to protect genome stability[19]; and Piwi-interacting RNAs (piRNAs), a set of small RNAs described in the mouse and rat testes that have been shown to have an essential role in the viability of germline stem cells.[20] The recent and rapid discovery of these small RNAs reflects the current excitement in the field, and suggests the possibility that small RNAs may be central to other cellular processes that are still unknown. The remainder of this review will focus only on miRNAs, whose impact is better understood at the current time, and the use of siRNAs in the context of laboratory research and disease therapy.

MicroRNAs IN DEVELOPMENT

MiRNAs play an essential role in normal development. One approach to test the global importance of miRNAs is to knock out the Dicer gene in a mouse model, which inhibits the production of all miRNAs. Developing embryos deficient for Dicer die at an early embryonic age and are depleted of pluripotent stem cells, supporting the crucial role for miRNAs in proper embryogenesis and stem cell development.[21] To evaluate the role of miRNAs in specific tissues and organs, conditional knockout alleles of Dicer have been generated, which removes Dicer only within a tissue or organ of interest. This experimental approach has demonstrated roles for Dicer in morphogenesis of several organs, including the lungs,[22] limbs,[23] and muscles,[24] and in T-cell differentiation.[25] One caveat to interpreting these lines of knockout experiments is the underlying assumption

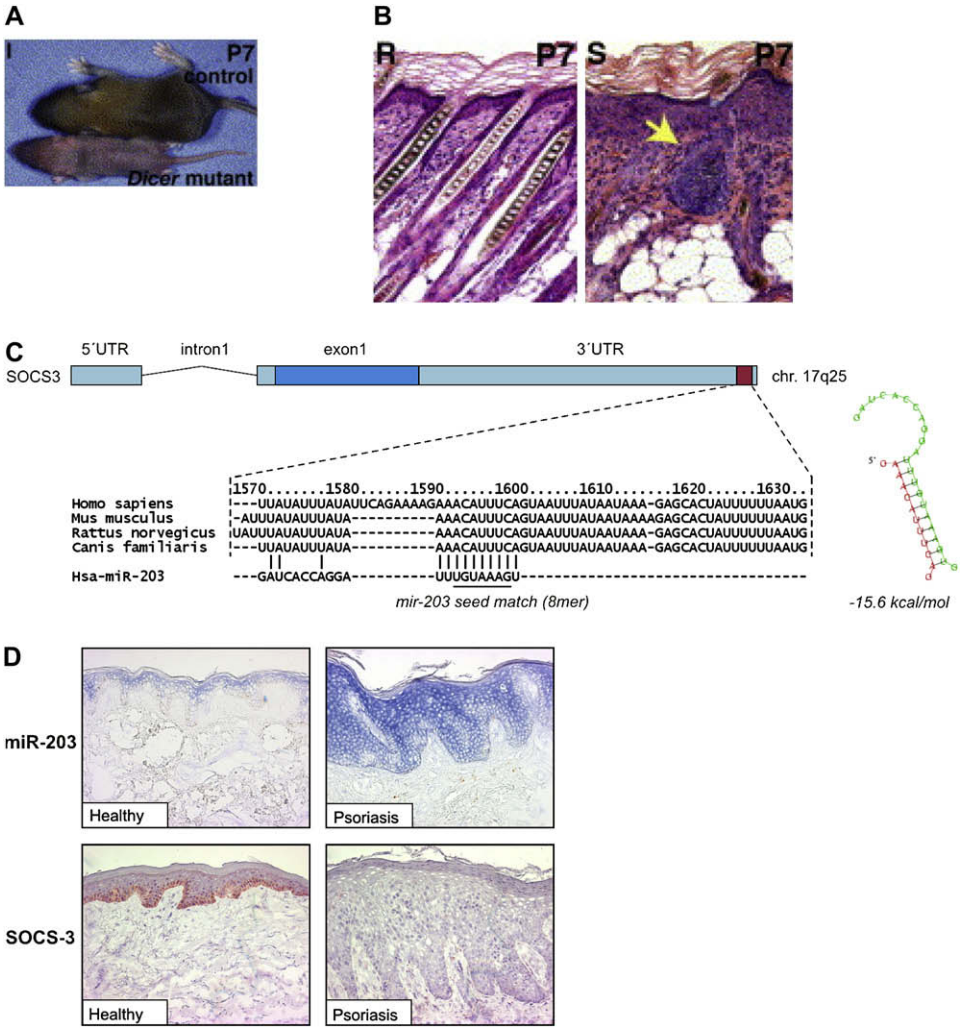

Fig 3. **A**, Effect of Dicer deficiency in the skin on mouse development. Mouse with deficient Dicer expression in the skin (mutant) on postnatal day 7 (P7) compared to its normal littermate control. **B**, Effect of Dicer deficiency in the skin on hair follicle development. Mouse with deficient Dicer expression in the skin (sample S) on postnatal day 7 (P7) compared to normal skin (sample R) shows deficient hair follicles, with cyst formation *(yellow arrow)* where follicles fail to extend into the dermis. **C**, Sequence similarity between miR-203 and the 3′-UTR of SOCS-3. miR-203 appears to be overexpressed in psoriasis, and its sequence contains conserved similarity to the 3′-UTR of the SOCS-3 protein, a known suppressor of a transcription factor known to be active in psoriatic skin. **D**, In situ hybridization demonstrates high expression of miR-203 in the epidermis of psoriatic skin with complementary loss of SOCS-3 protein in psoriatic lesions. (**A** and **B**, reprinted from Andl et al[26] with permission from Elsevier. **C** and **D**, reprinted with permission from Sonkoly et al.[42])

that Dicer does not play other roles in addition to miRNA and siRNA processing.

Using a similar approach, two recent epidermis-specific Dicer knockouts have begun to reveal the role of miRNAs in the skin.[26,27] In preliminary experiments, it was observed that Dicer is expressed at significant levels in the epidermis and that a specific subset of miRNAs appear to be enriched in the epidermal population, supporting the idea that miRNAs play a role in skin development. When Dicer was knocked out in skin epithelial progenitor cells, mature miRNAs were not produced in developing skin, as expected. Mice with deficient epidermal miRNA production were born at normal ratios, but began to lose weight shortly after birth, and only survived for a few days postnatally (Fig 3, *A*). The most striking phenotype, however, was a lack of external hair growth in these mice.

In normal skin development, the epidermis forms from the surface ectoderm of the embryo while the underlying dermis derives from the mesoderm or neural crest. Hair follicle development requires coordinated and sequential molecular signaling between the epidermis and mesenchyme.[28] First, signals from the dermis induce the formation of thickenings, or placodes, in the overlying epidermis. These epidermal placodes signal back to the dermis, causing a grouping of mesenchymal cells underneath them, known as the dermal condensate. Then, the condensate signals back to the overlying epithelium, resulting in the downward invagination of epithelial cells to surround the condensate—thereafter called the dermal papilla. The papilla continues in its role as a signaling center, directing the proliferation of local epithelial cells which eventually become the inner root sheath and hair shaft. Throughout human life, the hair follicle undergoes cycles of growth and regression. At the onset of each growth phase (anagen), the dermal papilla induces the proliferation of new cells from an epithelial stem cell population that resides permanently at the bulge region of the follicle. Coordinated, ongoing communication between epithelial and mesenchyme cells are therefore central to the life of the hair follicle. The known molecular events that dictate hair follicle development and growth have been reviewed in detail.[29]

In the epidermal Dicer knockout mice, several anatomic and molecular hair follicle defects were observed. During the first days of life, newly-forming hair germs, instead of invaginating downwards into the dermis, evaginated upwards into the epidermis (Fig 3, *B*). Several days later, these evaginating hair germs became engulfed by epithelial cells and formed cyst-like pearls in the epidermis. Interestingly enough, these epidermal pearls displayed all the components of a mature hair follicle in a concentric pattern, including the hair shaft, inner root shealth, and dermal papilla cells (in the center). This phenotype suggested that miRNAs may be important to guide proper anatomic location and orientation in hair follicle development. An additional observation in the Dicer knockout mice was the absence of hair follicle stem cells, suggesting a direct or indirect role for miRNAs in the formation and/or maintenance of the stem cell population at the bulge region. Viewed together, the phenotype of epidermal Dicer knockout mice indicate roles for miRNAs in the signaling interaction between epithelium and mesenchyme and in stem cell maintenance, and provides a fascinating model for understanding how miRNAs may influence cell-to-cell signaling.

Because the epidermal knockout of Dicer affects the production of all miRNAs, the natural next step was to detail the role of individual miRNAs in skin development. Recently, the miRNA miR-203 has been shown to be critical in mouse epithelial differentiation.[30] miR-203 levels increase during embryonic development at a time when the epidermis undergoes stratification and differentiation. Within the epidermis, it is not expressed in basal, proliferative layers, but is present in suprabasal, differentiating layers. Forced expression of miR-203 in the basal layer, where it is not usually produced, leads to the development of mice with thinned epidermis and deficient basal stem cells. These observations, together with other experiments, led the investigators to suggest that miR-203 is critical to repressing the proliferation of progenitor cells as they transition from basal to suprabasal layers, serving as a key molecular switch between proliferation and differentiation.

In support of this hypothesized function, the investigators identified p63 and Zfp281 as two downstream targets of miR-203. Both of these proteins are important to maintain proliferative potential of stem cells: p63 in the stem cells in stratified epithelia[31] and Zfp281 in embryonic stem cells.[32] The actions of the downstream targets of miR-203 are therefore consistent with a role for this miRNA as a high-level transcriptional regulator of multiple genes, and serves as an example of how a miRNA can be a critical switch that controls programs of gene expression. With the identification of the first specific miRNA with a central role in skin development, it will be interesting to see whether other important epidermal miRNAs will be uncovered in the future.

While miRNAs have been linked with functions in development and tissue identity, it is important to note they are also involved with other processes. These include the regulation of synaptic plasticity,[33] the regulation of insulin secretion in pancreatic islet cells,[34] and control of cholesterol homeostasis in the liver.[35] Even though our knowledge of miRNA function is still at an early stage, it is already clear that human miRNAs have diverse and far-reaching effects. This has fueled to the well founded suspicion that misregulation of miRNAs can lead to human disease.

MicroRNAs IN DISEASE

Given the important role that miRNAs have in the regulation of cellular differentiation and proliferation, it may not be surprising that their misregulation has been linked to cancer. One of the general strategies to understanding cancer has been to characterize the genetic and genomic changes that are associated with each type of cancer. This has led to

the identification of specific genes, such as *BRCA1* and *BRCA2* in hereditary breast cancer, and chromosomal translocations, such as BCR-ABL in chronic myelogenous leukemia, that have facilitated cancer screening, diagnosis, treatment, and assessment of prognosis.

For quite some time, certain genomic abnormalities have been repeatedly linked to cancers but have not proven to have any connection with protein-coding genes in the region. With the discovery of miRNAs, it is suspected that some of these genetic linkages may point to miRNA genes. One example involves the genetic etiology of chronic lymphocytic leukemia (CLL), the most common adult leukemia in the Western world. The overexpression of the breakpoint cluster-2 (*BCL2*) gene is common in malignant CLL cells, but in the vast majority of cases, no mechanism was known to explain how *BCL2* became misregulated. It was known for some time that a region on chromosome 13, the 13q14.3 band, was the most common chromosomal abnormality in CLL, and extensive work was performed to examine all the mapped genes in the region for mutations that correlated with CLL. No promising candidate genes were found until it was discovered that a cluster of two miRNA genes, miR-15a and miR-16-1, were located in the deleted region.[36] Subsequently, it was shown that the levels of these genes inversely correlate with *BCL2* protein expression, and that repression of *BCL2* by these miRNAs induces apoptosis in leukemia cells.[37] This study represented one of the first demonstrations of cancer associated with the misregulation of specific miRNAs, and demonstrates that in principle, miRNAs may serve as tumor suppressors or oncogenes.

Computer-based analyses suggest that a significant number of microRNAs will be linked with cancer. A genome-wide study of cancer-associated genetic regions—areas of the genome that are frequently altered in cancer cells—found that more than half of all human miRNAs fall within these cancer-associated regions.[38] In a complementary approach, a study of ovarian, breast, and melanoma cancer specimens identified significant copy-number variation in genomic regions containing miRNAs.[39] In particular, of the 283 melanoma samples that were examined in the study, 243 (85.9%) were found to contain significant copy-number aberrations in miRNA regions.

While these studies demonstrate a link between miRNAs and cancer, the exact relationship between the two may vary—that is, whether the alteration of particular miRNAs causes cancer, or whether cells become malignant by other mechanisms then subsequently acquire misregulated miRNA expression. Both relationships seem to occur. Recent evidence has demonstrated in concept that miRNAs are sufficient to trigger specific steps of tumorigenesis. In metastatic breast cancer, the miRNA miR-10b is highly expressed, and its overexpression in non-metastatic breast tumors is sufficient to cause them to invade and metastasize.[40] This finding indicates that misregulation of miRNAs may not only be associated with, but is also capable of playing a causal role in vivo to metastasis. Other studies have found that the tumorigenicity of known protein-coding oncogenes can be related to their effects on miRNAs. The oncogene c-myc is pathologically activated in many human cancers. Recently, it was shown that an important downstream result of c-myc activation involves the repression of a set of miRNAs, including the tumorigenic miR-17-92 cluster.[41] Forced expression of these repressed miRNAs in myc-activated tumor cells can significantly blunt the tumorigenicity of these cells, "rescuing" the effect of the activated oncogene. These studies highlight how closely miRNAs and protein-coding genes work together, and how the genetics of miRNAs will be critical to understanding and treating cancer.

In addition to the role of miRNAs in cancer, it is also known that they are involved with other diseases. Sonkoly et al[42] recently examined whether miRNAs may be regulators of psoriasis. In their experimental approach, they first used a microarray analysis to examine the pattern of miRNA expression in psoriatic skin, and compared this pattern of expression against an atopic dermatitis control and normal skin. They found that the psoriasis samples contained a subset of miRNAs whose expression levels was distinct from those found in the controls. They focused attention on two miRNAs, miR-203 and miR-146a, which were both overexpressed in psoriatic skin.

The two miRNA candidates displayed distinct expression patterns within different organs and cell types. miR-203 was found predominantly in organs with squamous epithelium, including the skin, esophagus, and cervix. Within the skin, miR-203 was present in keratinocytes but not melanocytes, dendritic cells, or fibroblasts. By contrast, miR-146a was found in almost all organs, although it was enriched in organs containing leukocytes, such as the spleen and liver. Consistent with this, miR-146a was enriched in T cells, dendritic cells, and mast cells within the skin, but not in keratinocytes or fibroblasts. Interestingly enough, miR-146a has been recently found to have an inhibitory effect on the tumor necrosis factor-alfa (TNF-α) pathway.[43] Blockade of this pathway by biologic drugs, such as etanercept and adalimumab, is now a therapeutic approach for treatment of psoriasis.

In contrast to miR-146a, almost nothing was known about miR-203 at the time. A computer analysis identified sequence similarity between miR-203 and the suppressor of cytokine signalling-3 (SOCS-3), a protein that is known to inhibit STAT3. STAT3 is a transcription factor that is activated during psoriatic plaque formation (Fig 3, *C*). This provided a plausible biologic connection for miR-203 among the signaling networks involved with psoriasis. Consistent with this model, in situ hybridization of normal and psoriatic skin (Fig 3, *D*) found elevated levels of miR-203 in the keratinocytes of psoriatic skin, with concomitant downregulation of SOCS-3. The investigators speculate that miR-203 might function, in part, by leading to constitutive STAT3 expression, which permits psoriatic plaque formation. This discovery also ties together the study by Yi et al[30] (described earlier) which suggests a role of miR-203 in restricting suprabasal proliferation. Future studies to further detail the downstream actions and targets of miR-203, and to find out how miR-203 itself is regulated in normal and disease states, will be areas of high interest.

One characteristic feature of psoriasis is the numerous cell types and genes that have been linked to the disease. On one hand, the complexity of cellular crosstalk and multiple genes involved may make the approach to a targeted therapy more challenging; on the other hand, it makes the potential role of miRNAs in the disease more intruiging. Because miRNAs are thought to be master gene regulators, affecting the expression of multiple targets at once, they may therefore represent a key control point for regulating genetic networks. By extension, miRNAs could prove to be good targets for therapy of complex diseases because they may have the ability to normalize a system that has gone wrong at several different levels.

miRNA DISEASE SIGNATURES

The ability to rapidly assess the cellular and genetic characteristics of a cell sample has increased the ways in which genetic information influences the diagnosis, prognosis, and treatment of diseases. Genetic medicine has reached almost all specialties of medicine, but has had one of the more visible impacts in the care of cancer patients. Breast cancer tumors, for instance, are routinely examined for the overexpression of the human epidermal growth factor receptor type 2 (Her2/neu) locus, because the amplification of this protein serves as a negative prognostic marker of disease, but also is used as an indicator of responsiveness to therapy with the monoclonal antibody against the receptor, trastuzumab (Herceptin; Genentech, South San Francisco, CA).[44] In particular subtypes of breast cancer, the expression levels of a selected set of 21 genes has been shown to provide good prognostic value for distant disease recurrence.[45] This new genetic tool is currently being validated in its ability to guide therapeutic management and serves as an example of how gene expression patterns can pave the way towards "individualized" medicine.

One general goal of analyzing genetic characteristics of disease is to be able to determine valuable clinical information that is not readily apparent from the clinical appearance. A hypothetical dermatologic application of this would be to analyze dysplastic melanocytic nevi and to have genetic markers that would predict how likely it would be for the lesion to progress to cancer. Another example would be to take a biopsy of a cancer of unknown origin, and to extract genetic information that would allow the clinician to determine the lineage or tissue from which the cancer originated, in cases where the histology was not conclusive. Broadly speaking, the genetic markers that would be useful for these scenarios would be specific to the disease type and would give some information on prognosis and response to potential treatments. The characteristic pattern of one or several genetic markers which allows the inference of a biologic state or identity is known as a genetic signature.

Because the study of many cancers has helped to delineate a series of genomic deletions, amplifications, or chromosomal translocations that correlate with characteristics of the disease, the principle of DNA-based genetic markers have already been integrated into patient care. The emergence of high-density microarrays in the past decade has allowed for the parallel analysis of expression levels of thousands of genes at once, and the subsequent analysis to identify a key set of genes whose expression best correlates with an outcome of interest. This approach has been used to develop RNA-based gene signatures that predict the diagnosis, prognosis, or treatment of leukemias,[46] lung cancer,[47] breast cancer,[48] and colon cancer,[49] among others.

The growing interest in miRNAs has sparked a natural extension of microarray technology to screen the expression level of miRNAs in parallel. In theory, miRNA gene signatures may be more useful and provide more discrimination than mRNA expression signatures, because all miRNAs presumably serve a regulatory role, whereas many mRNAs may vary in a manner that is not specific to the condition or disease in question. Preliminary studies indicate that miRNA gene signatures are promising: investigators using only miRNA data derived from marrow samples of acute lymphocytic leukemia patients were able to

distinguish between subsets of patients with different genetic characteristics—a BCR-ABL–positive group, a T-cell acute lymphocytic leukemia group, and a mixed lineage leukemia–rearranged group.[50] These miRNA signatures have also been tested against a set of poorly-differentiated tumor samples that were nondiagnostic on histologic examination.[50,51] Out of 17 test samples, miRNA classification correctly identified 12 of 17 samples compared to only 1 of 17 samples using mRNA expression. These studies suggest that miRNA signatures will have important clinical value in the future.

USES OF siRNAs IN RESEARCH AND THERAPY

The discovery that small RNAs can rapidly silence target genes has led to the development of many ideas by which these RNAs can be used as tools for biologic research and disease therapy.

In the laboratory, a prototypical genetic approach to test the function of a gene is to disable it and to determine the resulting phenotype. The "gold standard" method to accomplish this is to create a knockout animal model, in which the genomic DNA of embryonic stem cells is manipulated in culture and a gene-deficient animal generated from these cells. The development of siRNAs as a laboratory tool allows for a surrogate gene-deficiency experiment to be performed in cell culture in a matter of days. The investigator designs a siRNA sequence that is complementary to the gene of interest, introduces the siRNA into the cell, and can achieve a significant reduction in target gene activity within hours to days. This technique is often referred to as a gene "knockdown," because siRNAs often reduce gene activity but do not completely eliminate it. An additional benefit of this siRNA gene knockdown is that a combination of genes can be targeted simultaneously by introducing two (or more) different siRNAs. SiRNA knockdowns are now successfully and widely used, and are a commonplace approach to investigate the effects of genes.[52]

On a more global scale, the tool of siRNA knockdown is being used to define molecular pathways and to screen for new drug targets. A typical approach is to introduce a variety of different siRNAs onto a cell, analyze the cells for a desired phenotype, and then determine which siRNA produced the response. In one study, this approach was used to identify new molecules that affected the TNF-α pathway.[53] TNF-α signaling has been closely tied with tumor formation, and its activation upregulates the nuclear transcription factor nuclear factor kappa B (NF-$\kappa\beta$). NF-$\kappa\beta$ is broadly involved with inflammatory responses, immunity, and protection against apoptosis.

Using this siRNA screen, the cylindromatosis tumor suppressor gene, *CYLD*, was identified as a novel suppressor of NF-$\kappa\beta$ activation. *CYLD* is mutated in familial cylindromatosis, a rare autosomal dominant genetic disease with variable penetrance (OMIM 132700). Patients affected with cylindromatosis develop benign adnexal skin tumors, also known as "turban tumors," appearing principally on the head and neck. After first identifying *CYLD* as a suppressor of NF-$\kappa\beta$, the investigators next sought to understand how and where *CYLD* affected the TNF-α signaling pathway. They knew that *CYLD* functioned as a deubiquitinating enzyme, responsible for removing ubiquitin groups from specific proteins. Through further investigation, they found that the ubiquitination of TNF-receptor related factor (*TRAF*) activated its association with the inhibitor of kappa-beta kinase complex (I$\kappa\beta$), ultimately leading to upregulation of NF-$\kappa\beta$ and prevention of apoptosis (Fig 4). In a normal state, *CYLD* functioned to block *TRAF* ubiquitination, and thereby protected against NF-$\kappa\beta$ activation. In theory, therefore, deficiency in *CYLD* could lead to high levels of NF-$\kappa\beta$, aberrant cellular protection from apoptosis, and tumor formation.

Salicylates (such as aspirin) were known to inhibit NF-$\kappa\beta$ activation downstream of where *CYLD* was now known to function (Fig 4), and were seen as a reasonable candidate to treat *CYLD* deficiency. To test this hypothesis, the investigators demonstrated that in a cell culture system, the addition of aspirin restored the ability for *CYLD*-deficient cells to undergo apoptosis. Shortly thereafter, this molecular discovery was translated into a pilot clinical trial in which topical salicylic acid was used to treat patients with cylindromas.[54] Of the 12 test lesions treated, two showed complete remission and eight showed some partial response, in comparison to five control lesions which remained unchanged or grew in size. These studies in familial cylindromatosis demonstrate the potential for small RNA screens to delineate new molecular pathways, and for the new insights to pave the way for new clinical trials and therapies.

In addition to their use as experimental tools, there is significant interest in harnessing siRNAs as a form of genetic therapy. This approach applies particularly to diseases where the reduction of a specific gene target is expected to be therapeutic. Inhibition of the vascular endothelial growth factor by siRNAs is being clinically tested to prevent abnormal blood vessel growth in age-related macular degeneration and diabetic retinopathy.[55] Studies have also begun to see whether knock-down of the

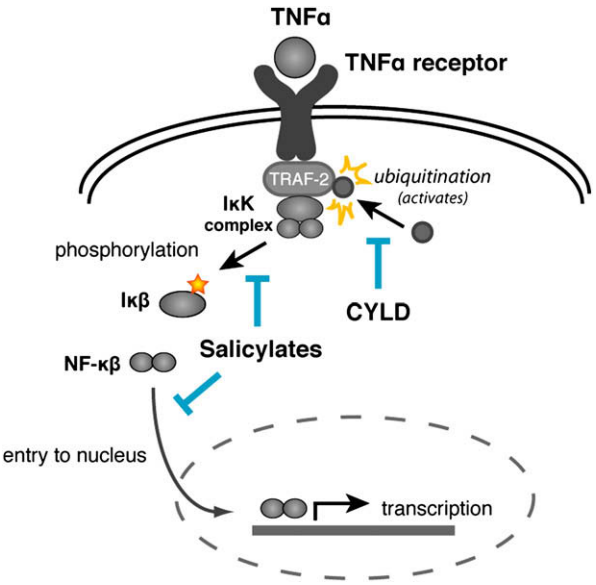

Fig 4. A small interfering RNA screen uncovers new molecular insight to tumor necrosis factor-alfa signaling and familial cylindromatosis. Using a small interfering RNA screen, the cylindromatosis (*CYLD*) gene was identified as a suppressor of nuclear factor kappa B (NF-$\kappa\beta$) activation. It functions by blocking the ubiquitination of the TNF receptor—associated factor (*TRAF*), which normally serves to activate the associated inhibitor of kappa beta complex (I$\kappa\beta$) and ultimately increase NF-$\kappa\beta$ levels, protecting the cell against apoptosis. In *CYLD* deficiency, this pathway is aberrantly activated, which is thought to lead to cylindroma formation. Salicylates inhibit I$\kappa\beta$ phosphorylation and NF-$\kappa\beta$ entry into the nucleus, and function downstream of *CYLD*. Their use has since been tested, with some encouraging preliminary results, in patients with familial cylindromas.

Huntington protein in Huntington disease can be used to reduce the amount of aberrant protein that is correlated with the progression of disease.[56] siRNAs are also being tested as antiviral therapy, by delivering targeted siRNAs against genes that are essential for viral infectivity and replication. This concept is being tested in HIV and respiratory syncytial virus.[57,58]

One of the most exciting applications of siRNA therapy is in its potential use against cancer. In theory, if the activation of an oncogene promotes tumor growth and spread, then the ability to specifically reduce oncogene expression may slow cancer growth. In cutaneous melanomas, one of the most frequently altered genes is the proto-oncogene *BRAF*, a downstream effector another key oncogene, *RAS*. A single point mutation of *BRAF* (eg, $BRAF^{T1799A}$) has been demonstrated to be strongly tumorigenic in malignant melanoma cell models, and is mutated in two-thirds of melanoma tumor specimens.[59] If an siRNA targeted selectively against $BRAF^{T1799A}$ is introduced into a malignant melanoma cell line, the growth of that line becomes inhibited, and the ability of those cells to invade in an in vitro system or to grow on xenograft models is reduced.[60,61] These early experiments in siRNA therapy represent a promising start for using RNA interference as a tool for genetic therapy against melanoma and other cancers.

The medical literature in the coming years will undoubtedly include many trials aiming to advance the delivery, safety, and efficacy of siRNA-mediated therapy. The discovery of small RNAs and their functions have revitalized the prospects of being able to control expression of specific genes in vivo, with ultimate hopes of building a new class of gene-specific medical therapies.

CONCLUSION

Small RNAs, including miRNAs and siRNAs, are a class of regulatory molecules that have diverse and important roles in human development and disease. New discoveries of their involvement in genetic and molecular pathways are being uncovered at an exciting pace. In addition to their natural roles, small RNAs are also proving to be valuable tools in research, and hold promise in helping researchers understand and treat skin disease.

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