Chapter 14
RNA Interference and Cancer: Endogenous Pathways and Therapeutic Approaches

Derek M. Dykxhoorn, Dipanjan Chowdhury, and Judy Lieberman*

Abstract The endogenous RNA interference (RNAi) pathway regulates cellular differentiation and development using small noncoding hairpin RNAs, called microRNAs. This chapter will review the link between mammalian microRNAs and genes involved in cellular proliferation, differentiation, and apoptosis. Some microRNAs act as oncogenes or tumor suppressor genes, but the target gene networks they regulate are just beginning to be described. Cancer cells have altered patterns of microRNA expression, which can be used to identify the cell of origin and to subtype cancers. RNAi has also been used to identify novel genes involved in cellular transformation using forward genetic screening methods previously only possible in invertebrates. Possible strategies and obstacles to harnessing RNAi for cancer therapy will also be discussed.

Keywords RNA interference, microRNA, cancer, microarray, tumor profile, siRNA, therapy, prognosis

1 Introduction

RNA interference (RNAi) is an endogenous, ubiquitous, and evolutionarily conserved pathway for regulating gene expression. Noncoding stem-loop RNAs, encoded within exons or in intergenic regions, are processed by specialized intracellular RNase III enzymes into small RNAs, called microRNAs or miRNAs.[1–4] The microRNAs are taken up by a multiprotein cytoplasmic complex, called the RNA-induced silencing complex (RISC), which directs the posttranscriptional silencing of a partially complementary mRNA target. Silencing of highly complementary mRNAs can occur through mRNA degradation, but for less complementary targets, gene silencing occurs by inhibiting translation. Most mammalian microRNAs work by the latter

Derek M. Dykxhoorn, Dipanjan Chowdhury, and Judy Lieberman
CBR Institute for Biomedical Research and Department of Pediatrics, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115, USA

* To whom correspondence should be addressed: e-mail: lieberman@cbr.med.harvard.edu

R. Khosravi-Far and E. White (eds.), *Programmed Cell Death in Cancer Progression and Therapy*.

pathway. The rules for identifying silenced target genes are still poorly defined; therefore, only a handful of mammalian genes have been clearly shown to be regulated by the endogenous RNAi pathway. However, current bioinformatic estimates suggest that the expression of a third or more of all genes may be regulated by microRNAs. In other species, such as plants, worms, and flies, RNAi regulates critical genes involved in cellular differentiation and survival. In fact, the first identified endogenous microRNAs regulated the progression from one larval state to another in *Caenorhabditis elegans* development.

There is increasing evidence of a role for microRNAs in cancer.[5–8] This should not be surprising since malignant transformation results from abnormally regulated cell differentiation and survival – processes regulated by microRNAs in other organisms. Here, we review how microRNAs are processed within mammalian cells and then describe the evidence for microRNA regulation of genes implicated in cancer and apoptosis. Recent studies provide examples of emerging networks that regulate the expression of microRNAs and transcription factors to control terminal differentiation in a variety of cell types, a step that is aberrant in cancer. We will discuss how microRNA expression profiles are altered in cancer and might be used for diagnosis and prognosis. We will also discuss recent examples of RNAi-based screens to identify tumor-promoting and suppressor-coding genes and microRNAs. Lastly, we will discuss the therapeutic prospects for harnessing RNAi to silence oncogenes or other genes involved in cell proliferation and survival or for interfering with microRNAs that play a role in tumorigenesis.

2 microRNA Biogenesis and the Endogenous RNAi Pathway

Most microRNAs are transcribed within coding mRNAs or as independent transcripts by RNA polymerase II as long precursor primary transcripts that are capped and polyadenylated (Fig. 14.1).[4,9–11] microRNA transcripts are highly structured with an elongated hairpin that contains frequent mismatches, bulges, and non-Watson–Crick base-pairings. In some cases, several microRNAs are coordinately expressed as polycistrons from the same primary transcript.[12–16] The microRNA precursors, called pri-miRNAs, have a characteristic fold-back structure that is recognized in the nucleus by an RNase III-type enzyme, Drosha, and its binding partner, variously called DiGeorge syndrome critical region gene 8 (DGCR8) protein in mammals and partner of Drosha (Pasha) in *Drosophila* and *C. elegans*.[15,17–21] Drosha cleaves the pri-miRNA into a ~70 nt fold-back structure, termed the pre-miRNA, which is exported into the cytoplasm by exportin 5.[22–25] The pre-miRNA is then recognized by Dicer and cleaved into a small dsRNA intermediate that contains both the mature microRNA and the accompanying complementary strand.[26–31] The strand whose 5′-end is less tightly bound to its complementary strand is incorporated into the effector RISC or miRISC.[32,33] The complementary strand is rapidly lost when the microRNA is taken up into RISC.[34,35] In some cases, presumably when both ends are comparably paired, microRNAs can be found that correspond

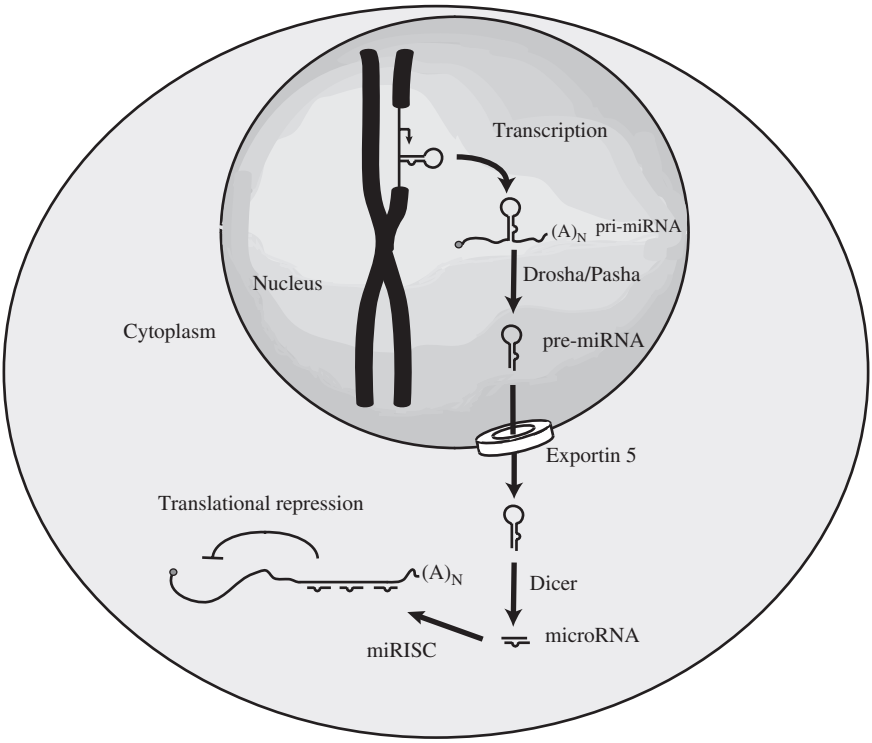

Fig. 14.1 RNA interference pathway. microRNAs that direct the posttranscriptional silencing of gene expression are derived from longer primary transcripts that are expressed from RNA polymerase II promoters.[4,9–11] These primary transcripts, termed pri-miRNAs, can range from several hundred to thousands of nucleotides long with the microRNA sequence encoded in a highly structured RNA hairpin that contains frequent bulges and mismatches.[4] These long hairpins are recognized and cleaved into shorter (~70 nt) hairpin RNAs, pre-miRNAs, in the nucleus by Drosha in conjunction with the double-stranded RNA recognition protein, termed Pasha in *Drosophila* and *Caenorhabditis elegans* and DGCR8 in mammalian cells.[15,17–21] pre-miRNAs are exported from the nucleus into the cytoplasm by Exportin 5 where they are recognized and cleaved into the ~22 nt microRNA by Dicer in conjunction with another dsRNA-binding protein, called Loquacious in *Drosophila* and TRBP in mammals.[22–25,28–30] The miRNA is taken up by the effector complex, miRISC, and the passenger strand is lost, leaving the mature microRNA to guide the recognition of the microRNA-binding sites on the target mRNA, leading to silencing of target gene expression.[34,35] Originally, mammalian microRNAs were thought to mediate target gene silencing by binding to sites on the mRNA that had incomplete complementarity with the microRNA and inducing translational repression, in contrast to small interfering (si)RNAs which have complete (or nearly complete) homology and direct mRNA cleavage.[2,4] However, microRNAs with partial complementarity can facilitate some mRNA degradation, in addition to inducing translational repression[177]

to both strands of the microRNA precursor. The exact composition of the RISC is still unknown. (In fact, this term probably refers to several complexes that may have some core components in common but have additional factors that determine their individual function.) A key component of the RISC is an Argonaute family protein, often Ago 2, which is the RISC endonuclease.[36]

Regulation of gene expression by microRNAs operates through several mechanisms including two that work posttranscriptionally – degradation of the targeted mRNA by cleavage and inhibition of translation.[2,4] The first mechanism has a more potent effect on gene expression, probably because the same RISC-incorporated small RNA can be used repeatedly to guide the degradation of multiple target mRNAs.[37] It is controversial whether the other mechanism (translational inhibition) involves blocking the initiation of translation or a more distal step in translation, and possibly multiple mechanisms may operate in different circumstances. A less well-understood mechanism of gene silencing by noncoding RNAs involves inhibition of transcription by the formation and maintenance of regions of silenced chromatin.[38] In fact, Dicer-deficient cells are impaired in heterochromatin formation.[39] The specificity of posttranscriptional silencing is determined by complementarity of the microRNA to the target mRNA, usually at sites in the 3′ untranslated region (UTR) of the message. The 5′-end of the guide strand is buried into a pocket of the Ago protein, while nucleotides 2–8 are exposed on the surface of the molecule forming a seed sequence that directs target mRNA recognition.[40,41] How strongly base-pairing of the remaining nucleotides of the typical 19–23 nucleotide sequence of the microRNA to the target sequence influences gene silencing is uncertain. Other properties of the target mRNA that might influence gene silencing (such as lack of secondary structure of flanking sequences) are not well understood, making prediction of microRNA gene targets still a challenge.

mRNAs undergoing microRNA-induced translational inhibition appear to be sequestered in distinct cytoplasmic foci.[42–47] These sites, referred to by a variety of names including processing (P)-, cytoplasmic-, GW-, Dcp-, or Lsm-bodies, serve as foci for the accumulation of mRNAs that are destined for degradation.[48–50] In addition to the mRNA, these sites contain essential components of the mRNA degradation pathway, the mRNA decapping enzymes (Dcp1/Dcp2), as well as the 5′-3′ exonuclease Xrn1, Dhh1p, and Pat1p, and in mammalian cells, GW182.[51] The first hint of the interaction of the microRNA machinery with these sites of mRNA turnover was the demonstration that the mammalian Ago proteins implicated in RNAi colocalize with components of mammalian P-bodies.[43,46] Another family of Argonaute proteins, the Piwi family, that have not been found to be associated with microRNAs, do not colocalize.[46] A direct physical interaction between Ago1 and Ago2 with Dcp1 and Dcp2 was also shown by co-immunoprecipitation, even in the absence of RNA or using Ago2 protein, mutated in the Piwi Argonaute Zwille (PAZ) domain required for small RNA binding.[46] However, Ago2 localization to P-bodies is a microRNA/siRNA-dependent process.[45] This was further confirmed by following the fate of reporter mRNAs containing multiple MS2-binding sites that can be visualized with a fluorescently tagged MS2 protein and sites for either an endogenous microRNA or an exogenously introduced siRNA that mimics microRNA function by binding to multiple imperfectly complementary sites on the mRNA.[45] The tagged mRNAs, but not reporter mRNAs that lack the microRNA-binding sites, localize in P-bodies only in the presence of their respective microRNA. A functional link between P-bodies and RNAi-mediated silencing was shown by silencing GW182, which disrupts the formation of P-bodies and

significantly impairs gene silencing by both translational repression and mRNA cleavage.[44,45]

The physical and functional link between the sites of mRNA turnover and microRNA/siRNA-mediated silencing raises questions about the potential role of the RNAi machinery in other translational regulation mechanisms. P-bodies are increasingly thought to be sites for the storage of translationally repressed mRNAs, with mRNAs being able to move between the active and inactive pool as needed. One hypothesis put forward by the Parker and Hannon groups is that microRNAs may mediate their repressive function by selectively transporting and possibly even maintaining their mRNA targets in these sites of translational repression, segregated from the translational machinery. It is possible to envision a variety of potential mechanisms by which the RISC could inhibit translation, including impairing various steps in translation (e.g., blocking the processivity or binding of ribosomes along the mRNA) or "tagging" newly formed proteins for degradation, in addition to sequestering the target mRNA from the translational apparatus. The interaction of RNAi components with other sites of mRNA storage and translational regulation, such as stress granules, which are distinct structures that interact with P-bodies, remains to be clarified.

3 Changes in microRNA Expression in Cancer

Mammalian microRNAs were first predicted using RNA-folding algorithms that identified evolutionarily conserved sequences that form into energetically favorable short hairpins that are structurally similar to microRNAs identified in other organisms.[52,53] These algorithms identified about 200 predicted microRNAs in mammalian genomes. A substantial subset of the predicted microRNAs was then verified by cloning small RNAs from a variety of cells. However, when the requirement for evolutionary conservation was relaxed, additional microRNAs were predicted (and the actual number may well exceed 1,000) and some of these have been cloned.[54] These less conserved microRNAs may regulate specialized functions (such as immune responses) that have evolved recently. The makeup and size of the universe of functional mammalian microRNAs is still uncertain, but will soon be more accurately defined using recently available methods for efficiently cloning small RNAs.

microRNAs are expressed in temporally regulated patterns during cell differentiation with distinct expression patterns in different cell types and tissues.[55–62] The total number of microRNAs in a cell can also vary during differentiation and typically constitutes about 1% of the total cellular RNA. Highly expressed microRNAs can be present at as many as 10^4 copies/cell. Highly efficient cloning has enabled researchers to identify microRNAs in rare cell types that are expressed at fewer than 100 copies/cell.[54,63] The functional significance of these rare microRNAs on gene expression is unclear. Figuring this out will be challenging because the gene targets of most microRNAs are unknown, and current target gene prediction

algorithms are poor at identifying them. Moreover, the effect on gene expression of a single microRNA binding to an mRNA may be small, particularly when silencing is via translational inhibition. In fact, when single microRNAs are genetically deleted or inhibited, it is rare to find any significant difference in cellular function or fate.[64] However, binding of multiple microRNAs to different sites in the 3′ UTR of a gene can coordinately have an impact on its expression.[65] This model of cooperative regulation is reminiscent of models of transcriptional regulation by groups of transcription factors binding to promoter sites on the DNA.

microRNAs have been associated with the regulation of a variety of biological processes from fat metabolism and insulin secretion to cell proliferation, apoptosis, and developmental timing.[66–70] Since microRNAs play such an important role in the regulation of invertebrate development and differentiation, it is not surprising that dysregulation of microRNA expression would be associated with oncogenic transformation in mammals. microRNAs might function as either tumor suppressors or oncogenes depending on their target genes and could contribute to cancer either by enhanced or reduced expression in tumor cells (Fig. 14.2). The first hint that microRNAs might be associated with the development of cancer was the identification of two microRNAs, miR15 and miR16, encoded in a small region of chromosome 13 that is frequently deleted in B-cell chronic lymphocytic leukemia (CLL).[71] These two microRNAs were later found to suppress the expression of bcl-2, an antiapoptotic protein that is frequently overexpressed in B-cell lymphomas and other malignancies. Similarly, expression of miR143 and miR145 is significantly decreased in colorectal cancer specimens compared to matched normal tissue.[72] Expression of these microRNAs is also reduced in a variety of colorectal, breast, prostate, lymphoid, and cervical cancers. In addition, miR26a and mir99a, expressed from regions associated with loss of heterozygosity in lung tumors, have reduced expressed in lung tumors and lung cancer cell lines. Bioinformatic analysis of the regions encoding microRNAs found that most microRNA genes (98 of 186 microRNAs examined) are encoded in regions of the genome associated with cancer, including regions associated with loss of heterozygosity, gene amplification, common break point regions, and fragile sites.[73] Importantly, one of these breakpoint region translocations (t(8,17)) associated with aggressive B-cell lymphoma places the MYC oncogene downstream of the miR142s gene promoter leading to MYC overexpression.[74] Although these studies correlate decreased microRNA expression with the development of cancer, they do not generally identify targets of the microRNAs that can explain their role in tumorigenesis.

Other microRNAs are overexpressed in specific malignancies. A conserved noncoding RNA termed BIC was first identified as a site of insertion of avian leukosis retroviruses, and enhanced expression in chicken B-cell lymphomas.[75] Recently, BIC was found to encode for miR155[74] and was found to be upregulated in human diffuse large B-cell lymphomas (DLBCL) with an activated B-cell phenotype. miR155 overexpressing tumors have poorer prognosis than B-cell lymphomas of the germinal center phenotype.[76–78]

Cancers result from the accumulation of multiple spontaneous and/or inherited mutations that lead to dramatic changes in the pattern of gene expression, particularly

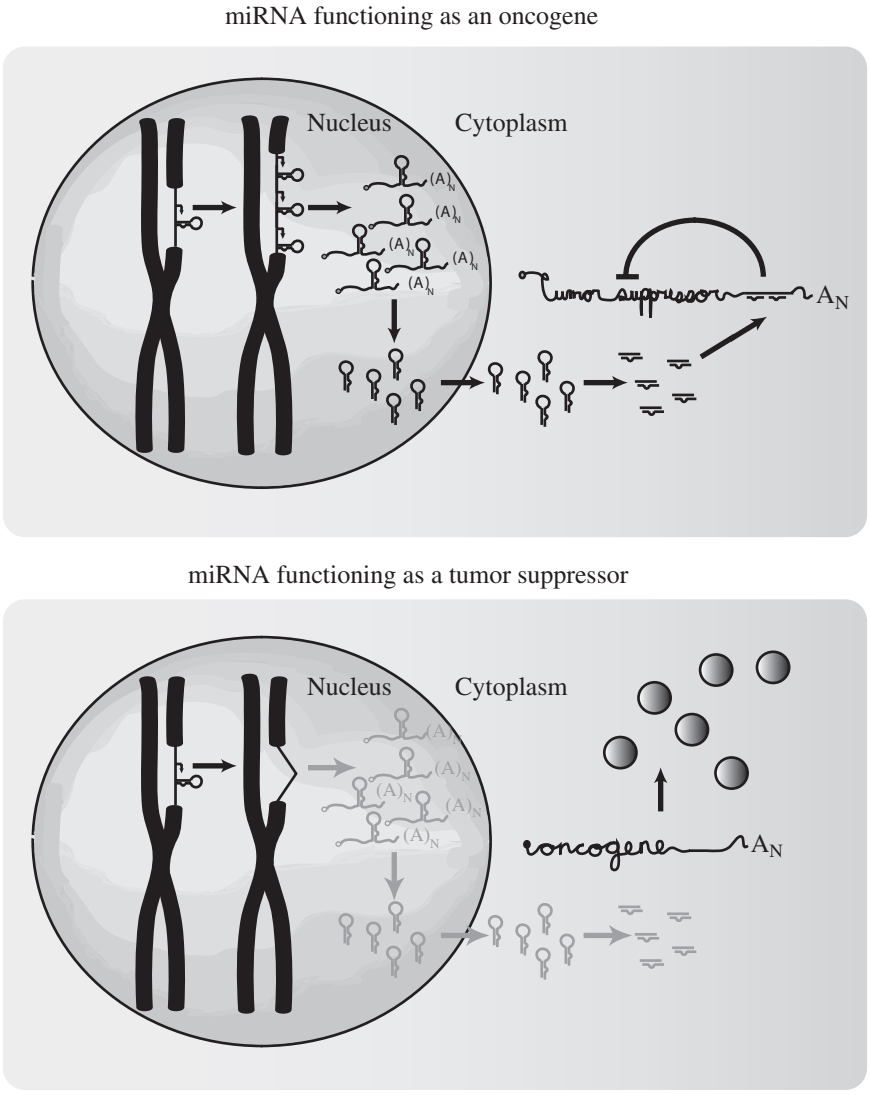

Fig. 14.2 microRNAs can act as either tumor suppressors or oncogenes depending on their targets. (A) *microRNAs as oncogenes*. microRNAs that target a tumor suppressor gene and are overexpressed because of gene amplification (e.g., the miR17–92 polycistron),[86] inappropriate expression of factors that upregulate transcription of the miRNA (e.g., c-Myc upregulation of the miR17–92 cluster)[94] or translocation into a genome locus that alters microRNA expression[73] can lead to cellular transformation, dysregulated proliferation, and tumor formation. (B) *microRNAs as tumor suppressors*. Tumor formation can be induced by the loss or decreased expression of a microRNA whose normal function would be to suppress expression of an oncogene. Inappropriate expression of the oncogene would then lead to cellular transformation. In either case, tumor formation could be a result of increased proliferation, angiogenesis or invasiveness, decreased levels of apoptosis, or alteration of the state of cellular differentiation

in pathways that control cell proliferation and cell cycle regulation, cell signaling, angiogenesis, apoptosis, protein degradation, transcriptional regulation, and the immune response. The assessment of changes in gene expression profiles in specific cancers by mRNA microarrays can be used to some extent to enhance definition of tumor subtypes and improve diagnosis and prognosis. However, a better understanding of the changes that are necessary for oncogenic transformation is seen when the mRNA microarray data are analyzed for changes in groups of related molecules, "molecular modules." This allows for the identification of specific pathways and biological processes that are disrupted in particular cancers. Changes in microRNA expression in different tissues, developmental and differentiation states were initially assessed using a cloning strategy that took advantage of the unique structure and size of Dicer cleavage products to isolate and sequence microRNAs.[13] Cloning microRNAs, however, is not suitable for high-throughput analysis and often does not provide reliable quantitative comparisons of expression. Until recently, low-abundance microRNAs were not readily detected by cloning. To address some of these concerns, microarray technologies used for profiling mRNA levels have been adapted to analyze microRNA expression[79–84] in a variety of normal and cancerous tissues, including CLL[85,86] and solid tumors, including lung, breast, stomach, prostate, colon, and pancreatic cancer.[87,88]

To begin to explore the role of microRNAs in CLL, Calin and colleagues compared the microRNA profile of CLL patient samples with that of normal CD5 + B cells.[85] The CLL samples fell into two distinct clusters of microRNA expression. Some microRNAs were upregulated in both groups compared to CD5 + B cells (e.g., miR183, miR190, and miR24–1) and some downregulated (e.g., miR213 and miR220). CLL patients can be grouped into two major subtypes according to whether their tumor cells express high levels of the signaling molecule ZAP70 and unmutated immunologlobulin heavy chain (more rapid disease progression) or low or undetectable ZAP70 and mutated immunoglobulin (slower disease progression). Expression of 13 microRNAs differed between the two groups.[89] When patients were classified by the interval between diagnosis and initiation of therapy (another indication of tumor grade), expression of 9 of the 13 microRNAs identified the slower progressing tumors. Eight of the nine differentially expressed microRNAs were overexpressed in the more rapidly progressing tumors. Some of the changes in microRNA expression could be linked to mutations within or near the microRNA sequences.

Microarray analysis also found that microRNA expression differed between normal and cancerous tissue in solid tumors, as well as between solid tumors arising from different organs.[90] microRNA expression by prostate, colon, stomach, and pancreatic adenocarcinomas tend to cluster together. On the other hand, lung and breast cancer samples have distinct patterns of microRNA expression. A few microRNAs (miR21, miR17–5p, and miR191) are overexpressed in a majority of solid tumors. One would expect that these common microRNAs might be involved in dysregulating cellular processes, such as cell proliferation, that are aberrant in all malignancies, while the tissue-specific microRNAs might be involved in oncogenic or differentiation events relevant to specific tissues.[91]

To facilitate microRNA profiling in human cancers, Lu et al. developed a highly effective and specific bead-based solution hybridization procedure.[90] This technique uses oligonucleotide-capture probes linked to polystyrene beads impregnated with a variable combination of fluorescent dyes, a specific combination for each microRNA that is being tested, to analyze rapidly the microRNA composition of large numbers of samples. By binding the oligomer-capture probes to the microRNAs in solution, as opposed to on a solid support (e.g., glass slides), microRNA family members that differ from one another by only a single nucleotide can be distinguished without much cross-reactivity. This method has a robust dynamic range with linear detection over a 100-fold range of microRNA expression. It was used to analyze the microRNA profile from 334 primary tumors representing a variety of tumor types and tissues of origin. Tumor samples showed decreased overall microRNA expression. Tumors of related lineage (i.e., epithelial, endodermal, and hematopoietic) clustered together, and expression patterns differed between tumors and their normal cellular counterparts. Moreover, tumors whose histology was not diagnostic could be assigned based on their microRNA expression profile with much more assurance than would be possible from mRNA profiling.

Early indications suggest that microRNA expression patterns will be more informative than mRNA microarrays in characterizing cancer cells.[90] It is likely that microRNA profiling will soon be used to refine diagnosis and subtype tumors to improve prognostic information and guide the choice of therapies. As the targets of the microRNAs whose expression is altered in various cancers are elucidated, this information will hopefully shed light also on the key events that contribute to the development and progression of cancers. It would not be surprising, for example, if alterations in microRNA genes might underlie poorly understood processes, such as metastasis.

4 microRNAs as Oncogenes or Tumor Suppressor Genes

Recently, a few pathways for microRNA regulation of genes implicated in cellular transformation have begun to be uncovered, but it is clear that this is just the beginning (Table 14.1). let-7, one of the first identified and most well-conserved microRNAs, regulates developmental timing in *C. elegans*. Upregulation of let-7 is necessary for the terminal differentiation of seam cells in adult animals by facilitating their exit from the cell cycle. In worms that lack let-7 expression, seam cells continue to divide and fail to differentiate, similar to cancer cells. In fact, let-7 is downregulated in lung cancer cells and cell growth of a lung cancer cell line is inhibited by overexpression of let-7.[92] These results suggest that let-7 acts as a tumor suppressor. This was demonstrated to be the case when the RAS oncogene was identified as a let-7 target in mammalian cells.[93] In *C. elegans* as well, the RAS homolog, let-60/RAS, is inhibited by the let-7 family members, let-7 and miR84, which bind to multiple target sites in the 3′ UTR of the let-60/RAS mRNA. Overexpression of miR84 in vulval cells

Table 14.1 Validated microRNA targets in mammalian cell proliferation, differentiation, and apoptosis

microRNA	Target gene	Function	Reference
let-7 family	Ras and its homologues	Cell proliferation	93
miR17–5p	E2F1	Transcription and cell proliferation	94
miR20a	E2F1	Transcription and cell proliferation	94
miR181	Hox A11	Hematopoiesis	99
		Skeletal myoblast differentiation	108
miR223	Nuclear factor I-A (NFI-A)	Granulopoiesis	100
miR221	c-kit receptor	Erythropoiesis	101
miR222	c-kit receptor	Erythropoiesis	101
miR130a	Transcription factor MAFB	Platelet physiology	102
miR10a	HoxA1	Megakaryocyte differentiation	102
miR196a	HoxB8	Limb development	106,107
miR1	Histone deacetylase 4 (HDAC 4)	Skeletal myogenesis	112
	Hand2	Cardiac development	111
miR133	Serum response factor (SRF)	Myoblast proliferation	112
miR134	Lim-domain-containing protein kinase 1 (Limk1)	Dendritic spine development	115
miR375	Myotrophin	Insulin secretion	66
miR143	ERK5/BMK1	Adipocyte differentiation	117
miR15a	bcl-2	Antiapoptosis	120
miR16–1	bcl-2	Antiapoptosis	120
miR372	LATS2	Tumor suppressor (germ cells)	135
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(vulval development being a good model for let-60/RAS function) leads to abnormal vulval development and precocious seam cell terminal differentiation. In addition, miR84 overexpression suppresses the effects of activating mutations of let-60/RAS. Similar to the *C. elegans* let-60/RAS, the human RAS homologues, HRAS, KRAS, and NRAS, contain multiple putative let-7 family member binding sites in their 3′ UTRs. The introduction of a let-7a siRNA that mimics the let-7a microRNA suppresses RAS expression in a liver cancer cell line. Reciprocally, inhibiting let-7a in HeLa cells by transfection of complementary 2′-*O*-methyl antisense oligomers increases RAS expression (Fig. 14.3). Of note, several of the human let-7 family members, let-7a, let-7c, and let-7g, are encoded in chromosomal locations that are commonly deleted in lung cancer samples,[73] and let-7 expression is reduced in lung tumor samples relative to normal adjacent tissue. In fact, the extent of let-7 reduction is an important independent prognostic indicator; patients with the most drastic reductions in let-7 have the poorest prognosis after potentially curative tumor resection. Moreover, reduced let-7 expression is inversely correlated with the level of NRAS protein. These experiments suggest that let-7 family members are tumor suppressors.

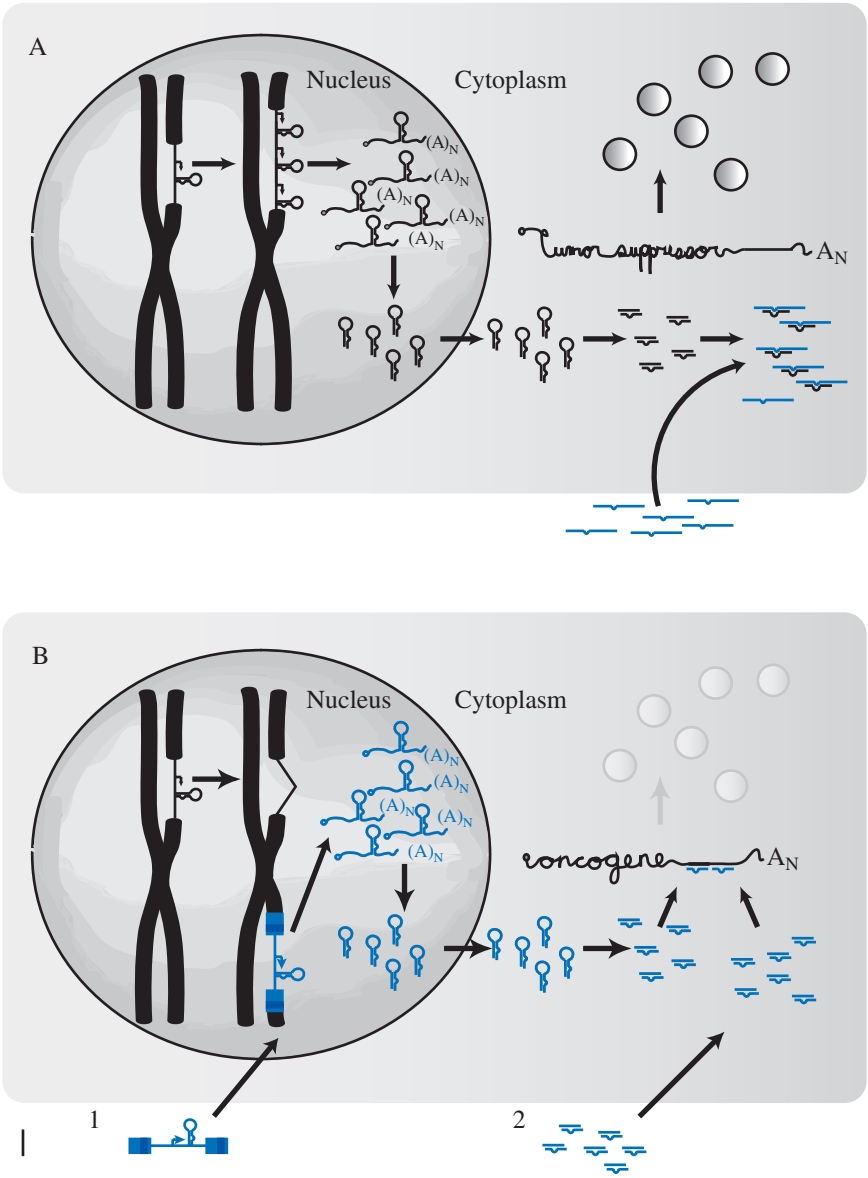

Fig. 14.3 Potential therapeutic approaches to inhibit tumorigenesis associated with altered microRNA expression patterns. (A) Tumor cells resulting from the overexpression of a microRNA that functions as an oncogene can be treated with cleavage-resistant single-stranded RNA molecules (e.g., chemically modifying the RNA by replacing the 2′-hydroxy groups on the sugar backbone with 2′-*O*-methyl groups)[178] that are complementary to the mature miRNA. These RNA molecules can effectively bind to the microRNA, preventing the association of the microRNA with its target gene(s) and thereby restore expression of the tumor suppressor gene and inhibit tumor growth. (B) Tumors that result from the loss of expression of a microRNA that acts as a tumor suppressor can be treated by reintroducing the microRNA into the cells. This can be

(continued)

In some instances, multiple microRNAs are encoded as polycistrons from a single common transcript. One of these microRNA clusters, the miR17–92 microRNA polycistron, maps to a region of chromosome 13 (13q31–q32) that is frequently amplified in B-cell lymphomas.[86] microRNA microarray analysis of several B-cell tumor lines that carry known amplifications of this region show increases in five of the six microRNAs in the miR17–92 cluster, compared to normal B cells and leukemia, and lymphoma cell lines lacking amplification of this region. In fact, expression of these microRNAs correlates with the copy number of the amplified region. Expression of the primary miR17–92 transcript is elevated in DLBCL and follicular lymphomas, suggesting that increased expression of this microRNA cluster might contribute to tumor formation. To test this hypothesis, He et al. overexpressed the first five of the microRNAs (miR17–19b), in the context of c-myc overexpression from the immunoglobin heavy chain enhancer (Eμ-myc), a well-established mouse model of B-cell lymphomas. While Eμ-myc transgenic mice typically develop B-cell lymphomas by 4–6 months of age, tumor formation was accelerated in Eμ-myc mice overexpressing miR17–19b, with a mean age of tumor formation of 51 days. Overexpression of each of the microRNAs in the miR17–19b cluster separately failed to enhance the rate of tumor formation. These tumors were particularly aggressive, invading visceral organs outside the lymphoid compartment, including liver, lung, and kidneys. One potential clue to the oncogenic nature of this microRNA cluster was reduced apoptosis in the miR17–19b/Eμ-myc tumors compared to control tumors.

The choice of the Eμ-myc transgenic mouse to test the oncogenic potential of miR17–92 may have been especially apt since O'Donnell et al. (2005) showed that the miR17–92 promoter contains c-Myc E-box binding sites and c-Myc activates the expression of this miR cluster.[94] This study also identified the transcription factor gene E2F1, which regulates progression through G1/S, as a target of two members of the miR17–92 polycistron. c-Myc is known to activate transcription of E2F1, and E2F1 activates c-Myc expression, suggesting a positive feedback loop to enhance cell proliferation. c-Myc induction of miR17–92 then serves to dampen this loop by suppressing c-Myc-induced E2F1 expression. This study illustrates the potential of microRNAs to fine-tune gene expression patterns for important genes that regulate cell cycle progression. It is likely that other genes involved in regulating cell proliferation will also be targeted by this microRNA cluster. These studies provide an example of how dysregulating microRNA expression might disrupt the

Fig. 14.3 (continued) achieved either by (1) introducing a DNA-based microRNA expression construct (e.g., using an oncoretroviral or lentiviral vector) that stably expresses the microRNA or (2) by directly introducing a chemically synthesized duplexed form of the microRNA that can enter the microRNA pathway and direct the silencing of the target oncogene. Alternatively, therapeutic benefit could be achieved by introducing siRNAs that silence expression of the dysregulated oncogene or any gene that will inhibit tumor growth (e.g., genes involved in cell cycle progression or angiogenesis) or make the tumor more sensitive to radiation or chemotherapy

fine balance that regulates cell growth, disrupted during oncogenic transformation. However, these regulatory networks may be more complicated than this story suggests; although the miR17–92 cluster is amplified in some B-cell lymphomas and clearly promotes tumor formation in the Eμ-myc mouse, the same region is associated with loss of heterozygosity in some hepatocellular carcinomas.

5 microRNAs and Differentiation in Mammalian Cells

Cancer cells are sometimes considered to be "frozen" in an undifferentiated or partially differentiated state. Until recently, differentiation research primarily focused on transcriptional regulation by regulatory DNA sequences (promoters, enhancers, and locus control regions) that are proximal to protein-coding sequences, paying little attention to the "noncoding" genomic DNA. The discovery of microRNAs has focused attention on mechanisms of posttranscriptional regulation of differentiation. As a general rule, total microRNA expression is higher in terminally differentiated cells than in less-differentiated cells and is higher in adult tissues than in embryos. Moreover, microRNAs have well-defined and distinct expression patterns in different tissues, particularly in cells of different developmental lineages. These findings suggest that microRNAs might play an important role in regulating terminal differentiation in different lineages. In fact, several recent studies provide compelling examples of regulatory networks (or the beginnings of networks) involving microRNAs, discussed below, that hint at an important role for microRNAs in controlling terminal differentiation, a step that is aberrant in cancer. These emerging regulatory circuits often involve intimate connections between transcription factors and microRNAs with, on the one hand, microRNA gene expression being regulated by transcription factors known to be important in lineage determination and, on the other, microRNAs suppressing the expression of key transcription factors.

The first evidence that microRNAs play a role in the differentiation of mammalian cells came from the conditional deletion of Dicer1. (Loss of Dicer1 is lethal early in development.[95]) Conditional deletion of Dicer1 in embryonic stem cells,[39] T cells,[96] limb mesoderm,[97] and skin[98] showed gross defects in differentiation in all these lineages. The logical inference is that impaired production of microRNAs in the absence of Dicer1 interferes with cellular differentiation.

microRNAs in hematopoiesis Much of the initial work in studying the role of microRNAs in mammalian cell differentiation has been elucidated in hematopoiesis, probably the best-studied system of mammalian cellular differentiation. The first example implicated miR181, whose expression is increased in thymus, lymphoid tissues, and bone marrow, in promoting B-cell differentiation. Ectopic expression of miR181 in mouse hematopoietic precursor cells leads to a dramatic increase in B lineage cells.[99] Another microRNA, miR223, expressed in the bone marrow, is important in granulopoiesis.[100] An elegant network involving miR223 and two competing transcription factors, C/EBPα and NFI-A, appears to control

the differentiation of promyelocytes into granulocytes. During in vitro and in vivo retinoic acid-induced differentiation of leukemic promyleocytes, miR223 expression is upregulated. The miR223 promoter contains overlapping sites for C/EBPα and NFI-A binding. C/EBPα upregulates and NFI-A inhibits miR223 expression. Upon retinoic acid treatment, NFI-A expression declines, while C/EBPα is upregulated. C/EBPα then binds and displaces NFI-A from the miR223 promoter to enhance miR223 expression. This molecular circuit is complete when miR223 binds the 3′ UTR of NFI-A transcripts, blocking further expression of NFI-A. The importance of miR223 in regulating granulocyte differentiation was shown by inducing differentiation of promyelocytes by ectopic expression of miR223 without retinoic acid and by blocking retinoic acid-induced differentiation by inhibiting miR223.

Two clustered microRNAs, miR221 and miR222, abundantly expressed in CD34 + hematopoietic precursor cells, are downregulated upon in vitro differentiation into the erythroid lineage.[101] One likely target of these microRNAs is the kit receptor, required for proliferation and erythroid differentiation in response to kit ligand. Overexpressing either of these microRNAs reduces kit expression, cell proliferation under erythroid-promoting conditions, and engraftment of CD34+ cord blood cells into immunodeficient mice. Because constitutively activated c-kit has been implicated in leukemias and gastrointestinal stromal tumors, inducing expression or transducing cells with these microRNAs (or their siRNA analogues) might have therapeutic benefit.

Another study that looked at in vitro differentiation of CD34+ progenitor cells into megakaryocytes found a group of downregulated microRNAs, one of which might be involved in targeting the transcription factor *MAFB*, upregulated during megakaryopoiesis and involved in activating transcription of the megakaryocyte-specific gene GPIIB.[102] Another downregulated microRNA miR10a is embedded in the HOX gene cluster and potentially targets HoxA1.

HOX gene microRNAs miR10 and miR196 microRNA families are embedded within the four HOX clusters of mammalian homeobox transcription factor genes,[103] which play an important conserved role in determining the identity of cells in the developing embryo. The intricate expression pattern of HOX genes persists in adult tissues, but their roles are modified according to specific cellular needs (reviewed in[104]). The embryonic expression of the HOX-embedded microRNAs closely follows that of their "host" HOX cluster genes.[105] Moreover, the HOX-embedded microRNAs have been shown in a few examples to regulate the expression of HOX genes. miR196a binds to the HOXB8 3′ UTR and inhibits HOXB8 expression by cleaving the HOXB8 transcript.[106] HOXB8 and miR196a also have complementary expression patterns during embryogenesis, supporting the idea that miR196a regulates the expression of HOXB8 during development.[103] miR196a is overexpressed in embryonic mouse hindlimbs compared to forelimbs, while the expression pattern of HOXB8 is the opposite. At least in chickens, miR196a appears to impede the retinoic acid-induced expression of HOXB8 and sonic hedgehog (Shh) in forelimb development to establish anterior–posterior patterning.[107] However, loss of microRNAs in Dicer-deficient hindlimbs does not induce

HOXB8 expression. This result suggests that the primary regulation of HOX gene expression might not be via microRNAs or that multiple microRNAs might be involved in more complicated regulatory networks.[107]

Another illustration of HOX gene regulation by microRNAs is the regulation of HOXA11 in differentiating myoblasts by miR181.[108] miR181 is upregulated in differentiating muscle cells during development or during regeneration in response to injury, but is not expressed in undifferentiated myoblasts or fully differentiated muscle cells. Its target HoxA11, which inhibits myoblast differentiation,[109,110] is reciprocally expressed in myoblasts and (at low levels) in adult muscle cells, but turned off during differentiation. Inhibiting miR181 interferes with myoblast differentiation, but does not completely restore HoxA11 expression, suggesting that multiple microRNAs or other pathways contribute to this process.[108] Moreover, the ectopic expression of miR181 does not induce myoblast differentiation, again suggesting a more complex regulatory network. Nonetheless, the involvement of miR181 in both B-cell and myoblast differentiation suggests that miR181 might be involved in regulating common pathways activated during the terminal differentiation of cells of mesodermal origin.

microRNAs and muscle development The miR1 family and miR133 genes are specifically and highly expressed in adult skeletal and cardiac muscle tissues and to a lesser extent during development of these tissues. Expression of miR1 genes is activated in the heart by the serum response factor (SRF) transcription factor and its cofactor myocardin and in skeletal muscle by the Mef2 and MyoD transcription factors. One of the targets of miR1 is the Hand2 transcription factor that promotes proliferation of cardiac muscle precursor cells. Cardiac embryonic development is activated when Hand2 begins to be expressed. Although Hand2 mRNA persists in adult cardiac tissue, Hand2 protein is downregulated coincident with miR1 expression. Precocious expression of miR1 in the developing heart leads to severe defects in heart formation because of decreased cell division.[111] Therefore, miR1 controls terminal differentiation of myocardiocytes.

Another microRNA (miR133) is clustered with miR1-1, and they are transcribed as a single transcript beginning late in embryonic development.[112] However, miR1 and miR133 have opposing effects on myoblast fate – as it does for the heart, miR1 promotes skeletal myoblast differentiation, whereas miR133 promotes myoblast proliferation and inhibits differentiation.[112] One of the targets of miR1 in skeletal muscle is HDAC4, which globally represses transcription, including transcription of the muscle-specific transcription factor MEF2C.[113] One way that miR133 inhibits differentiation is by suppressing expression of SRF, which activates myoblast differentiation.[114] Recall that SRF activates expression of miR1 (and thus miR133). This negative feedback loop indicates a complicated microRNA-transcription factor-regulated mechanism for controlling muscle cell differentiation. Likely, there will be more to this story.

microRNAs also regulate the function of terminally differentiated cells microRNAs are especially abundant in terminally differentiated cells compared to their precursors, suggesting that they may not only suppress the genes required for proliferation and progenitor cell pluripotency, but may also regulate their effector functions.

Regulating the function of terminally differentiated cells might not be directly related to cellular transformation and cancer. However, a few instructive examples of tissue-specific microRNAs and their role in differentiated cell function will be briefly described. miR375 appears to be exclusively expressed in pancreatic β cells and regulates insulin response to glucose.[66] Increasing miR375 in β cells suppresses glucose-induced insulin secretion, while inhibiting miR375 has the opposite effect. This effect is mediated by the effect of miR375 on Myotrophin (Mtpn), a protein previously not know to play a role in insulin secretion. Silencing Mtpn by siRNA reproduces the miR375-suppressive effect on insulin secretion.

Another interesting example involves the role of miR134, highly expressed in brain, in regulating dendritic spine development of neurons in response to synaptic stimulation.[115] miR134 inhibits translation of Lim-domain-containing protein kinase 1 (Limk1) which regulates dendritic spine formation.[116] miR134 is localized near synapses where Limk1 synthesis takes place. The authors speculate that miR134 might bind to Limk1 mRNA as it is being transported from the cell body to the dendrites and be responsible for suppressing Limk1 translation during transport and before synaptic stimulation. In response to activating stimuli, such as brain-derived neurotrophic factor (BDNF), the inhibitory effect of miR134 on Limk1 translation is reversed.[115] A surprising observation is that even after BDNF stimulation when Limk1 mRNA is being translated, miR134 continues to associate with the Limk1 transcript. How BDNF stimulation might interfere with miR134-mediated silencing of Limk1 translation or bypass it remains a puzzle.[117]

6 microRNAs and Apoptosis

Deregulation of cell death is an important feature of many cancers (reviewed in[118]). Highly conserved caspase-dependent pathways are often inactivated in transformed cells, principally by overexpression of inhibitors of apoptosis, including antiapoptotic bcl-2 family members, survivin, and other IAP family members. The first example of a role for microRNAs inhibiting apoptosis was in *Drosophila*, where expression of the proapoptotic factor hid is repressed by the microRNA bantam.[67] Bantam not only blocks apoptosis, but also directly increases cell proliferation.[67] In flies, miR14, the miR2 gene family and miR278 also act as potent cell death suppressors.[68,119,120]

In mammals the first evidence for a role of microRNAs in regulating apoptosis comes from conditional deletion of Dicer, which in embryonic limbs causes extensive apoptosis.[97] Deletion of Dicer in the T-cell lineage reduces the numbers of mature T cells, which both proliferate more slowly and are more prone to apoptosis in response to stimulation.[96] Expression of antiapoptotic Bcl-2 in B-cell lymphomas is a likely target of miR15a and 16-1, which are deleted in many high-grade B-cell malignancies.[121] Not only is bcl-2 expression tightly correlated with expression of these microRNAs, but transfection of bcl-2+ leukemia cells with an expression plasmid for either or for both of these microRNAs leads to downregulation of bcl-2

and induction of apoptosis. Glioblastoma cells and some other tumors strongly overexpress miR21.[122] Depletion of miR21 in cultured glioblastoma cells activates caspases and leads to increased apoptosis through an unknown mechanism. Interestingly, another study using antisense microRNA inhibitors in cervical adenocarcinoma HeLa cells identified miR21 as an inhibitor of cell growth with no direct effect on apoptosis.[123] The biological effects of any particular microRNA, including miR21, in different cells are likely to vary depending on the cell-specific repertoire of expressed target genes. Although these studies support a role for microRNA regulation of apoptosis, understanding the target genes and pathways in mammalian cells awaits further research.

7 RNA Interference-Based Screens to Identify Novel Tumor Suppressor Genes and Oncogenes

RNAi has provided new opportunities to identify novel genes implicated in a variety of diseases by forward genetic screens. Before the discovery that RNAi worked in mammalian cells, the power of unbiased screens to identify unexpected participants in biologically important pathways was only available in invertebrates. Libraries of retroviruses encoding short hairpin RNAs (shRNAs) or arrays of siRNAs mixed with a transfection reagent, designed to silence a large proportion of human-expressed genes or functionally related subsets of genes (i.e., all kinases and phosphatases, all known ubiquitin ligases), can be used to identify genes involved in cellular transformation, susceptibility to apoptosis, or drug resistance. Similarly, libraries of retroviruses encoding microRNAs can be used to identify microRNAs involved in cancer. Identifying a gene candidate in any screen is only the first step to validating its role in a biological pathway or disease. Some illustrative examples of RNAi-based cancer screens are given below.

Cancer cells are especially sensitive to apoptosis induced by the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). To identify genes that might enhance or suppress TRAIL-mediated apoptosis, TRAIL was added to HeLa cells transfected in microtiter plates with a panel of siRNAs targeting 510 genes, including 380 kinases.[124] This screen was able to identify several unknown genes whose silencing either sensitized or desensitized cells to TRAIL-induced apoptosis and to identify several signaling pathways (WNT, MYC) required for maintaining TRAIL sensitivity, since silencing multiple genes in these pathways differentially affected cell survival in response to TRAIL. Another siRNA-based loss-of-function screen surveyed hundreds of kinase and phosphatase genes to identify those that enhance or suppress apoptosis of HeLa cells either on their own or in conjunction with chemotherapeutic drugs.[125] A large proportion of these enzymes (i.e., more than one third of the phosphatases) affected cell survival by at least twofold. The large number of "hits" suggests that more refined screens or biological verification would be needed to winnow through these leads to identify attractive targets for drug development. For example, identifying kinases or phosphatases that are preferentially

needed for survival of different types of cancer cells vs normal cells or are only required for survival in the face of radiation or chemotherapy would be a first step.

Another screening approach uses libraries of DNA or viral vectors to express shRNAs, processed intracellularly into active siRNAs. Most libraries have used retroviral vectors because of the transduction efficiency and stability of gene expression afforded by these vectors. Brummelkamp et al.,[126] examining what effect silencing the expression of 50 deubiquitylating enzymes had on TNFα activation of NF-κB, singled out the cylindromatosis (CYLD) tumor suppressor gene, which is linked to a familial proliferative skin disease. With this lead they were then able to pinpoint a role for CYLD in deubiquitinating TRAF2, which activates IKK and consequently NF-κB. By interfering with NF-κB activation using sodium salicylate, they could enhance apoptosis of CYLD-silenced cells. This result was rapidly translated to show that topical aspirin derivatives could be used to treat this rare disfiguring disorder.

Although this study screened a small set of genes, large-scale plasmid and retroviral shRNA expression libraries targeting a large proportion of the human and mouse genome have been constructed and validated by several groups.[127,128–133] Some of these vectors express the shRNA within a microRNA sequence to enhance its processing and increase the efficiency of silencing.[129] In screens for tumor suppressor genes, cells at the brink of transformation because of expression of combinations of oncogenes are transduced to express shRNAs and then selected for outgrowth of transformed cells. One retroviral-based RNAi screen took advantage of a conditionally transformed cell line that expresses the catalytic subunit of telomerase (hTERT) and a temperature-sensitive allele of SV40 large T antigen (tsLT), which allows cells to proliferate at 32°C (the temperature at which tsLT is functional and can inactivate pRb and p53), but not at 39°C at which growth arrest occurs, to identify novel factors that modulate p53-dependent proliferation arrest.[133] After infection with the library, positive colonies, containing cells able to proliferate at 39°C, were selected and sequenced to identify the gene being silenced. shRNAs targeting six genes were pulled out of the screen, including the p53 gene, as well as five novel genes – RPS6KA6 (ribosomal S6 kinase 4, RSK4), Tip60 (histone acetyltransferase), HDAC4 (histone deacetylase), KIAA0828 (putatitive *S*-adenosyl-L-homocysteine hydrolase, SAH3), and CCT2 (T-complex protein 1, β-subunit). These novel genes were validated by showing that shRNAs targeting each of the genes selected in the screen were able to inhibit growth arrest induced by ionizing irradiation or $p19^{ARF}$ overexpression. In a similar manner, Westbrook et al.[134] used another shRNA expression library to look for potential tumor suppressor genes that inhibit transformation of human mammary epithelial cells (HMECs) expressing hTERT and SV40 large T antigen. Colonies of cells that demonstrated anchorage-independent growth after infection with the shRNA library were isolated. The silenced genes were identified by DNA sequencing and bar code (a sequence identifier specific for each shRNA construct) microarray analysis. This approach identified several previously known tumor suppressor genes, including TGFBR2 and PTEN, as well as a gene that had not been previously shown to have tumor suppressing properties, REST/NRSF (RE1-silencing transcription

factor/neuron-restrictive silencing factor). The role of REST as a tumor suppressor was confirmed by expressing a dominant negative REST gene. The tumor suppressor activity of REST was then found to be mediated by its ability to suppress PI(3)K-dependent signaling. REST is often deleted in colorectal cancer cell lines.

In a similar assay system, a retroviral shRNA-expressing library was used to screen for potential tumor suppressor genes whose silencing could substitute for overexpression of a constitutively active form of RAS (RAS^{V12}) and permit anchorage-independent growth in fibroblasts that overexpressed the catalytic subunit of telomerase (hTERT), SV40 small t antigen, and had silenced p53 and $p16^{INK1A130}$. The homeodomain pituitary transcription factor PITX1 was identified and confirmed as a tumor suppressor gene by showing that inhibiting PITX1 expression activates the RAS pathway by activating the promoter of RASAL1, a RAS-GTPase activating protein that connects Ca^{2+} signaling to RAS activity.

These previously described screens could identify tumor suppressor genes whose silencing promotes cellular proliferation or anchorage-independent growth, but they could not be used to identify potential oncogenes, whose silencing would cause growth arrest or cell death. To identify putative oncogenes, Staudt and colleagues[135] used an inducible shRNA retroviral library to identify by microarray analysis shRNAs that were depleted in abundance when transduced and induced DLBCL lines were cultured for 3 weeks. All of the depleted shRNAs silenced NF-κB pathway components, including IKBKB, CARD11, MALT1, and BCL10. Interestingly, these genes were required for the proliferation of only activated-type DLBCL and not germinal center-type DLBCL, suggesting that they might be good selective drug or siRNA targets.

Another type of screen was used to identify microRNAs that act as oncogenes. Using a retroviral library to express many of the known human microRNAs, Voorhoeve and colleagues[136] identified two microRNAs, miR372, and 373, which share the same seed sequence, that cooperate with a constitutively active form of RAS (RAS^{V12}) to transform primary human fibroblasts that express wild-type p53. miR372 and 373 expression was elevated in testicular germ cell tumors, which mostly contain functional p53, but not in normal testes or in samples from breast, colon, lung, and brain tumors. Expression of a putative tumor suppressor gene, large tumor suppressor homolog 2 (LATS2), predicted to contain two potential miR372/373-binding sites, is decreased in cells overexpressing these microRNAs, and its silencing may be contributing to the oncogenic effect of these microRNAs.

8 Harnessing RNA Interference for Cancer Target Validation and Therapeutics

Although we are just beginning to understand the role of microRNAs in cancer, many investigators are already exploring the possibility of exploiting the power of RNAi for cancer therapy and drug target validation in animal models. (A discussion

of this extensive body of work is beyond the scope of this review [see, e.g.,[137,138]]). RNAi has become a standard tool to identify the importance of any particular gene in diverse biological pathways, including those implicated in cellular transformation. siRNAs can be highly (but not completely) specific and can distinguish a single nucleotide polymorphism, as was first demonstrated by targeting the point mutation that constitutively activates a RAS oncogene, leaving wild-type RAS unaffected. RNAi can be used effectively to silence the expression of any gene in any cell in vitro.[139] Transgenic mice expressing shRNAs can also be used to identify the importance of particular genes or microRNAs in cancer formation in vivo.[140]

Cleavage of the target mRNA is likely the most potent RNAi mechanism to harness for therapy, because the same RISC-incorporated small RNA can direct the cleavage of many transcripts and because the transcript is eliminated, not merely repressed. (The relative effectiveness of mechanisms in which chromatin is silenced to inhibit transcription is unknown. This mechanism is too poorly understood to use as the basis for therapy at present.) The RISC-stabilized small RNA is highly stable within the cell – probably with a half-life of 1 week or more. The major determinant of durability of silencing is the rate of cell proliferation, where small RNAs are diluted with each cell division.[141] In terminally differentiated nondividing cells silencing can last for weeks, while in rapidly dividing cell lines silencing peaks 3 days after transduction and is gone by 1 week. For cancer cells, frequent and repetitive dosing will likely be required for siRNA-based drugs. However, less rapidly dividing precancerous lesions or potentially cancer stem cells might be particularly effective targets requiring infrequent treatments. In addition to silencing transcripts for oncogenes, RNAi could be used either to mimic microRNAs identified in promoting differentiation, inducing apoptosis or reducing proliferation or to inhibit cancer-promoting microRNAs. If the studies reviewed here that suggest that microRNAs may be master regulatory switches for terminal differentiation hold up, then transducing cells with siRNAs that mimic such microRNAs may be a highly attractive strategy for cancer therapy.

RNAi-based therapy for cancer could be used to target more than oncogenes. Genes implicated in cell cycle progression[142–146] and angiogenesis[147–150] would be good targets. Particularly, if siRNAs can be targeted preferentially to tumor cells, then any gene required for viability is a potential target, although genes needed only for cell division are particularly attractive since they will cause less toxicity to the majority of nondividing cells. Growth factors or their receptors required for tumor growth are also possible targets.[151–154] Targeting viral oncogenes encoded by EBV,[155–157] HPV,[158,159] and other oncogenic viruses also provides an opportunity for specificity. RNAi-based therapy could also be used in conjunction with chemotherapy or radiation to make cells more susceptible to these agents, particularly by silencing genes involved in drug resistance (i.e., transporters that efflux drugs), DNA repair, or metabolic pathways targeted by these drugs.[160–166] It may also be possible to target cells involved in either supporting the growth of the tumor or eliminating it rather than the tumor itself. For example, tumor infiltrating lymphocytes are

largely incapacitated in their ability to destroy tumor cells; by targeting inhibitory receptors or regulatory cells, these tumor-specific immune cells might be activated to eliminate residual tumor cells.[138]

Two strategies can be used to harness RNAi for therapy – one is gene therapy (transducing cells with viral vectors that encode for shRNA precursors processed intracellularly like endogenous microRNAs); the other is to develop siRNAs as small molecule drugs.[167,168] The latter is more suitable for cancer therapy and closer to clinical application. siRNAs can be chemically modified to enhance their pharmacokinetics and reduce potential off-target effects caused by binding to Toll-like receptors, immune sensors of pathogenic double-stranded RNAs. The main obstacle to using siRNAs is delivering them into the cytoplasm of cells, where they work. Cancer cells can be transfected in vitro, but except for superficial sites, this is not a viable strategy for treating most cancers, particularly micrometastases and macrometastases. Although most cells do not readily internalize siRNAs, mucosal surfaces appear to be especially susceptible to topically applied siRNAs.[168] Initial siRNA phase I and II studies targeting the eye and lung (to treat age-related macular degeneration and respiratory syncytial virus infection, respectively) have not met with any unexpected toxicity. Therefore, malignancies that are located at these sites or spread locally are good initial targets. Attractive examples for initial studies might include HPV-related cervical cancer (targeting the E6 and E7 oncogenes), EBV-related nasopharyngeal cancer, lung squamous cell carcinomas, retinoblastoma, or head and neck cancer.

However, for most cancers a method for effective systemic administration is needed and is the major obstacle to using siRNAs for cancer therapy. Recently, several systemic siRNA delivery strategies have begun to be described in animal models. These involve covalently coupling the passenger strand of the siRNA to a targeting molecule (e.g., cholesterol),[169] incorporating the siRNA into liposomes,[170,171] lipoplexes,[172–174] or nanoparticles,[149,175] or mixing the siRNA with fusion proteins, capable of specific targeting by binding to cell surface receptors.[176] This latter approach was used to target and inhibit the outgrowth of a subcutaneous mouse melanoma cell line by intravenous injection of 1 mg/kg of a cocktail of siRNAs. siRNA delivery was highly specific since adjacent normal tissues did not take up the siRNAs.

As for other cancer therapies, drug resistance caused by mutating the target site sequence is an anticipated problem. This may be more of an obstacle for siRNAs than for other types of drugs, since conservative mutations that do not alter the encoded protein may interfere with gene silencing. However, dealing with drug resistance to siRNAs is a much simpler problem than for other small-molecule drugs, which usually work by targeting a single active site on a protein. Since multiple sequences can be used to target any gene, alternate siRNAs can readily be designed. Combinations of siRNAs that target more than one sequence in a gene or multiple genes at once are likely to work synergistically to enhance tumor suppression and reduce the likelihood of emerging drug resistance.[141]

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