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Emerging roles of microRNAs as molecular switches in the integrated circuit of the cancer cell

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ABSTRACT

Transformation of normal cells into malignant tumors requires the acquisition of six hallmark traits, e.g., self-sufficiency in growth signals, insensitivity to antigrowth signals and self-renewal, evasion of apoptosis, limitless replication potential, angiogenesis, invasion, and metastasis, which are common to all cancers (Hanahan and Weinberg 2000). These new cellular traits evolve from defects in major regulatory microcircuits that are fundamental for normal homeostasis. The discovery of microRNAs (miRNAs) as a new class of small non-protein-coding RNAs that control gene expression post-transcriptionally by binding to various mRNA targets suggests that these tiny RNA molecules likely act as molecular switches in the extensive regulatory web that involves thousands of transcripts. Most importantly, accumulating evidence suggests that numerous microRNAs are aberrantly expressed in human cancers. In this review, we discuss the emergent roles of microRNAs as switches that function to turn on/off known cellular microcircuits. We outline recent compelling evidence that deregulated microRNA-mediated control of cellular microcircuits cooperates with other well-established regulatory mechanisms to confer the hallmark traits of the cancer cell. Furthermore, these exciting insights into aberrant microRNA control in cancer-associated circuits may be exploited for cancer therapies that will target deregulated miRNA switches.

Keywords: miRNAs; oncomirs; tumor suppressor miRNAs; cancer-cell circuit; molecular switch

INTRODUCTION

MicroRNAs (miRNAs) were first described in *Caenorhabditis elegans* more than 15 years ago as part of a cascade that leads to post-transcriptional gene silencing (PTGS) (Lee et al. 1993). Today, miRNAs have been identified in mammals, including humans, as well as in fish, frogs, insects, worms, flowers, and viruses, where they act post-transcriptionally to reduce the levels of multiple target transcripts and their encoded proteins (for review, see Bartel 2004). A recent study estimates that the human genome contains an estimated number of 3441 miRNAs (Sheng et al. 2007), of which only 695 miRNAs were identified experimentally and were found scattered on all human chromosomes except for the Y chromosome (data were retrieved from miRBase: http://microrna.sanger.ac.uk/cgi-bin/sequences/mirna_summary.pl?org=hsa). Based on this estimation, ~2%–3% of human genes encode for miRNAs (Alvarez-Garcia and Miska 2005). MiRNAs regulate important cellular functions such as cell proliferation, apoptosis, differentiation, timing of developmental transitions, and organ development (Alvarez-Garcia and Miska 2005). Accumulating evidence points to important roles exerted by specific miRNAs in cancer development and progression, as well as in other common diseases.

Biosynthesis

Most miRNAs are transcribed by RNA polymerase II as primary miRNAs (pri-miRNAs) that are 5′ 7-methylguanosine-capped and polyadenylated. The length of these transcripts varies from hundreds to thousands of nucleotides (Lee et al. 2004; Du and Zamore 2005; Liu et al. 2008). About 37% of human miRNAs are found in clusters that are transcribed as polycistronic RNAs (Altuvia et al. 2005). Subsequently, pri-miRNAs are processed by a 500–600-kDa microprocessor complex that consists of the nuclear RNase III endonuclease Drosha and cofactor DiGeorge-syndrome

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critical region protein 8 (DGCR8) to generate pre-miRNAs that consist of 60–70 nucleotides (nt) with a 3′ overhang of 2 nt (Gregory et al. 2004). Pre-miRNAs are exported from the nucleus by exportin-5 (Yi et al. 2003; Lund et al. 2004). In the cytoplasm, the RNase III enzyme Dicer cleaves pre-miRNAs further to generate their mature forms, miRNA–miRNA*. Finally, one strand of the miRNA duplex is incorporated into the RNA-induced silencing complex (RISC) that mediates the gene suppression effect, while the other, miRNA*, is degraded (Gregory et al. 2005). Dicer was first recognized for its role in generating small interfering RNAs (siRNAs) that mediate RNA interference (RNAi) (Bernstein et al. 2001) and later was shown to play a role in miRNA maturation (Grishok et al. 2001; Hutvágner et al. 2001; Ketting et al. 2001). Further, Dicer, along with the trans-activating response RNA-binding protein (TRBP), PACT, and an Argonaute protein, contribute to the formation of the RISC loading complex (Kim et al. 2009). In humans, four AGO proteins (AGO1–AGO4) have been described as being associated with miRNAs (Peters and Meister 2007). AGO2 represents the catalytic component of RISC for miRNA-mediated degradation of mRNA by displaying RNase H activity for RNA–RNA hybrids (Liu et al. 2004; Meister et al. 2004; Song et al. 2004). For this reason AGO2 is also referred to as slicer. AGO1, AGO3, and AGO4 lack endonuclease activity (Peters and Meister 2007). In addition, AGO2 was shown to interact with the elF4E factor that binds to m^7G Cap sites and represses the translation of mRNAs (Kiriakidou et al. 2007). Argonautes may also function to inhibit protein production by actively translated ribosomes or ribosomes falling off during elongation (for review, see Hutvágner and Simard 2008). Finally, it has been demonstrated that RISC activity can be reconstituted after mixing small RNA with recombinant AGO2 (Rivas et al. 2005), and that fractionation of RISC activity followed by mass spectrometric analysis identifies AGO2 as the only protein present (Rand et al. 2004). Therefore, it can be concluded that AGO proteins along with miRNA form the effector RISC complex (Filipowicz et al. 2008).

A subset of miRNAs that are located inside introns is transcribed along with the corresponding genes (Ruby et al. 2007). These intronic miRNAs, also referred to as mirtrons, have been found in flies and worms (Okamura et al. 2007; Ruby et al. 2007). Recently, Berezikov et al. (2007) showed that mirtrons are also present in mammalian cells. Initial processing of mirtrons differs from that of miRNAs encoded by genes. Figure 1 illustrates known mechanisms of miRNA production in mammalian cells, including human. Irrespective of how miRNAs are produced, eventually they all display a hairpin-like structure that is further processed to yield mature miRNAs of 21–24 nt (Zamore and Haley 2005). Finally, a small percentage of miRNAs, found interspersed among repetitive elements, is transcribed by RNA polymerase III and, subsequently, processed in the same way (Borchert et al. 2006). Computational analyses showed that each miRNA recognizes hundreds of different transcript targets. Most miRNAs in animals function to inhibit effective mRNA translation through imperfect base pairing at their 3′-untranslated region (Bartel 2004). The underlying mechanism is currently not well understood, but it seems to involve the inhibition of translational initiation (Pillai et al. 2005). Using an in vitro translation system, it was shown recently that endogenous let-7 miRNAs could repress the 5′ cap recognition process while the transcript remains intact (Mathonnet et al. 2007).

miRNA editing

RNA editing is a process that results in the modification of adenosine to inosine, thus generating RNA and protein diversity in higher eukaryotes (for review, see Bass 2002). Inosine acts as guanosine during translation; therefore,

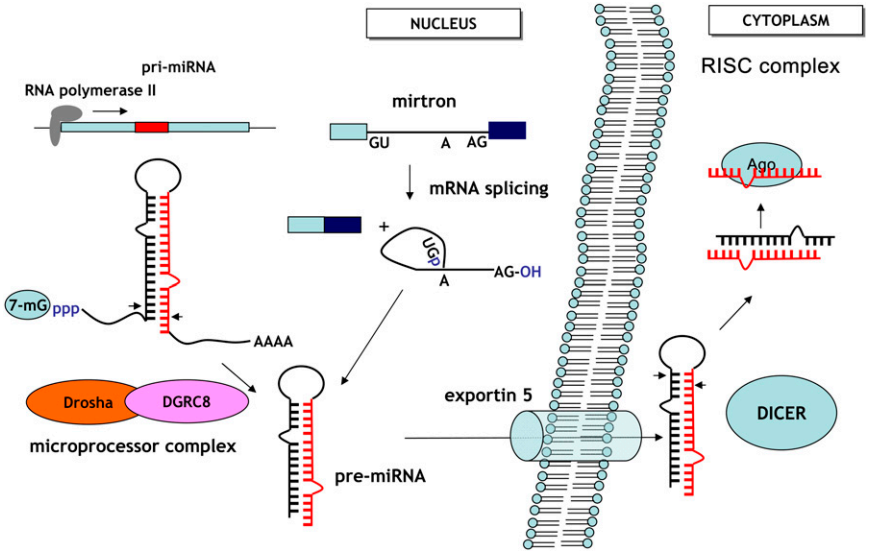

FIGURE 1. Mechanisms of microRNA production. Intracellularly, miRNAs can be generated either by transcription of a miRNA gene by RNA polymerase II (rarely by RNA polymerase III) or from introns containing miRNAs (mirtrons), as delineated in *Drosophila* (Ruby et al. 2007) and in humans (Berezikov et al. 2007). MiRNAs are derived from longer primary transcripts that contain one or more local hairpins, which are cleaved by RNase III to yield RNA duplexes of ~21–24 nt and single strands that associate with Argonaute proteins. Target selection in animals is dictated by sequences at the miRNA 5′end. Although most miRNAs end up in the cytoplasm, attachment of *cis*-acting regulatory motifs to certain miRNAs or siRNAs was shown to direct their nuclear import (Hwang et al. 2007).

editing in coding regions results in amino acid changes in the encoded protein. Adenosine deaminases (ADARs) enzymes are responsible for this modification and were found ubiquitously expressed in mammals. ADARs recognize partially double-stranded RNA structures (Bass 2002). The stem–loop structure of miRNA precursors makes them targets for ADARs in vivo. miR-22 was the first miRNA shown to be edited in various tissues and to be present in low amounts in human and mouse brains, as well as in human lung and testis (Luciano et al. 2004). Recent studies showed that editing of miRNAs represents a control mechanism for biogenesis (Kawahara et al. 2007a) or altered specificity of miRNAs (Kawahara et al. 2007b) as shown for miR-376 (Kawahara et al. 2007b). For pre-miR-151, RNA editing affects its interaction with the Dicer–TRBP complex (Kawahara et al. 2007a). TRBP is an integral component of a Dicer-containing complex that is required for recruitment of Ago2 to miRNAs bound to Dicer (Chendrimada et al. 2005).

MICRORNAS IN CANCER

Recent studies implicate miRNAs in various diseases, including fragile X syndrome (Jin et al. 2004), DiGeorge syndrome (Gregory et al. 2004), Alzheimer's disease (Wang et al. 2008b), psoriasis (Sonkoly et al. 2007), diabetes (Tang et al. 2008), and obesity (Takanabe et al. 2008). This review will focus on the role of miRNAs in cancer. The first evidence for the involvement of miRNAs in cancer came from chronic lymphocytic leukemia (CLL), which represents the most common form of adult leukemia and is caused by a deletion in chromosomal locus 13q14 observed in >50% of CLL patients. Interestingly, miR-15 and miR-16, which were mapped to this locus, were both shown to be deleted or down-regulated in CLL. Furthermore, in a subset of CLLs, a pre-miR-15 intermediate was detected by Northern blotting, indicating inefficient miR-15 processing (Calin et al. 2002). On the other hand, Dicer, which is responsible for miRNA production, is up-regulated in prostatic adenocarcinomas, leading to a global increase in miRNA levels (Chiosea et al. 2006). *Dicer* overexpression was also detected in lung adenocarcinomas (Chiosea et al. 2007), while reduced expression of *Dicer* is associated with a poor prognosis in lung cancer patients (Karube et al. 2005). Furthermore, *Dicer* gene amplifications are present in some cases of ovarian cancer (Zhang et al. 2006b), while alternative splicing of *Dicer* was observed in breast cancer (Irvin-Wilson and Chaudhuri 2005). Interestingly, an alternative promoter that modifies the length and the 5′ leader mRNA sequence is located 16 kbp upstream of the initiation site and is active in primary breast cancer, but not in normal breast cells (Irvin-Wilson and Chaudhuri 2005). Similarly to CLL, deletion of the chromosomal region 11q23-24, where miR-125b resides, is often detected in breast, lung, and ovarian cancers (Negrini et al. 1995; Rasio et al. 1995). Furthermore, miR-125b was found to be down-regulated in breast cancer (Iorio et al. 2005). MiR-140 was mapped on chromosome 6q22, again a genomic region often deleted in ovarian tumors, and miR-140 is down-regulated in ovarian cancers (Iorio et al. 2007). An extended survey of miRNA inactivation, which correlated with chromosomal deletions, was performed by Calin et al. (2004). On the other hand, global inhibition of miRNA processing was recently shown to lead to increased tumorigenicity and transformation, suggesting that the decreased expression of multiple miRNAs might be important in tumor formation and progression (Kumar et al. 2007).

The use of microarray technologies for analysis of miRNAs resulted in the rapid identification of miRNAs that are up- or down-regulated in various forms of cancer, including prostate (Porkka et al. 2007), breast (Iorio et al. 2005), ovarian (Iorio et al. 2007), colorectal (Cummins et al. 2006), kidney, and bladder cancer (Gottardo et al. 2007), as well as in cervical cancer cell lines (Lui et al. 2007). High-throughput methods for the rapid profiling of all known miRNAs were described (Nelson et al. 2004). Often, the term microRNome (in analogy to genome, transcriptome) is used to describe the set of miRNA species that are produced by a specific tissue (Cummins et al. 2006). Similarly to genes that encode for mRNAs, specific miRNAs were classified as oncogenes (oncomirs) or tumor suppressor genes based on their expression patterns in tumors and their cellular functions in the multistage process of malignant transformation and progression (Calin and Croce 2006; Esquela-Kerscher and Slack 2006; Zhang et al. 2007a). Table 1 illustrates miRNAs that are currently known to display deregulated expression in various forms of cancer based on microarray data subsequently verified by RT-PCR, qRT-PCR, or Northern blot analysis.

The "expanded" integrated circuit of the cancer cell

The model of the cellular integrated circuit proposed by Hanahan and Weinberg in their hallmark review (Hanahan and Weinberg 2000) suggests that cell signaling pathways mimic electronic integrated circuits, where transistors are replaced by proteins and electrons by phosphates and lipids. They suggested that progressive accumulation of genetic and epigenetic changes in the cancer cell genome results in deregulation of major regulatory microcircuits that proceeds in a combinatorial fashion to confer six hallmark phenotypic characteristics shared by most cancer cells, namely: sustained self-sufficiency to growth signals, insensitivity to antigrowth signals and self-renewal, evasion of apoptosis, limitless replicative potential, angiogenesis, invasion, and metastasis. It is reasonable to assume that each microcircuit is regulated by its own switch that will turn it on and off. Defects in these molecular switches result in wrong circuit activation/deactivation that results in aberrant signaling associated with transformation of a

normal cell and malignant progression. With the identification and characterization of human miRNAs it becomes evident that this class of tiny molecules can play an important role as molecular switches, in analogy to the electrical switch, that will turn the expression of specific proteins on and off. Recent studies indicate that specific miRNAs regulate the expression of major metastasis-associated genes. Interestingly, "miRNA switches" can direct an epithelial-to-mesenchymal transition (EMT), a critical change of the cell phenotype that promotes cancer metastasis. In Figure 2, we have attempted to update the schematic representation of the integrated cancer-cell circuit, originally described by Hanahan and Weinberg (2000), in view of the massive information published in recent years on the important roles played by "miRNAs switches" in the deregulation of cellular signaling and the acquisition of the above six hallmark traits of the cancer cell, as described below.

TABLE 1. miRNAs with altered expression in cancer determined by Northern blotting, quantitative RT-PCR, or semiquantitative RT-PCR

miRNA	Cancer type	Method	Up or down	Reference
let-7a	Gastric	Q	↓	Zhang et al. (2007b)
miR-9	Breast	Q	↑	Ma et al. (2007)
miR-10b	Breast (metastatic)	Q	↑	Ma et al. (2007)
miR-10b	Glioblastoma	N	↑	Ciafrè et al. (2005)
miR-15	CLL	N	↓	Calin et al. (2002)
miR-15a	Pituitary adenomas	N	↓	Bottoni et al. 2005
miR-16	CLL	N	↓	Calin et al. (2002)
miR-16-1	Pituitary adenomas	N	↓	Bottoni et al. (2005)
miR-17-5p	Breast cancer cell lines	N	↓	Hossain et al. (2006)
miR-17-92	Lymphoma	Q	↑	He et al. (2005b)
miR-17-92	Colorectal	Q	↑	He et al. (2005b)
miR-21	HCC	N	↑	Meng et al. (2007)
miR-21	Breast	N	↑	Iorio et al. (2005)
miR-21	Well-differentiated pancreatic endocrine carcinoma following liver metastasis	N	↑	Roldo et al. (2006)
miR-21	Cervical cancer cell lines	N	↑	Lui et al. (2007)
miR-21	Cholangiocarcinoma cell lines	N, Q	↑	Meng et al. (2006)
miR-29a	CLL	Q	↑	Zanette et al. (2007)
miR-29c	CLL	Q	↑	Zanette et al. (2007)
miR-34a	CLL	Q	↑	Zanette et al. (2007)
miR-34a	Neuroblastoma	Q	↓	Welch et al. (2007)
miR-34a	Colon	Q	↓	Tazawa et al. (2007)
miR-103	Well-differentiated pancreatic endocrine carcinoma	N	↑	Roldo et al. (2006)
miR-122	HCC	N	↓	Kutay et al. (2006)
miR-125b	Breast	N	↓	Iorio et al. (2005)
miR-125b1	Ovary	N	↓	Iorio et al. (2007)
miR-127	Cervical SSC	Q	↑	Lee et al. (2008)
miR-128	Glioblastoma	N	↓	Ciafrè et al. (2005)
miR-128b	ALL	Q	↑	Zanette et al. (2007)
miR-140	Ovary	Q	↓	Iorio et al. (2007)
miR-141	Ovary	N	↑	Iorio et al. (2007)
miR-141	Cholangiocarcinoma cell lines	N, Q	↑	Roldo et al. (2006)
miR-143	CLL	R, Q	↓	Akao et al. (2007)
miR-143	Colorectal neoplasia	N	↓	Michael et al. (2003)
miR-143	B-cell lymphoma	R, Q	↓	Akao et al. (2007)
miR-143	Cervical cancer cell lines	N	↓	Lui et al. (2007)
miR-145	Breast	N	↓	Iorio et al. (2005)
miR-145	Colorectal neoplasia	N	↓	Michael et al. (2003)
miR-145	Ovary	N	↓	Iorio et al. (2007)
miR-145	CLL	R, Q	↓	Akao et al. (2007)
miR-145	B-cell lymphoma	R, Q	↓	Akao et al. (2007)
miR-146b	PTC	N, R	↑	He et al. (2005a)
miR-155	Breast	Q	↑	Ma et al. (2007)
miR-155	Pancreas	N	↓	Roldo et al. (2006)
miR-155	Lymphomas	Q	↑	Eis et al. (2005)
miR-181a	Glioblastoma	N	↓	Ciafrè et al. (2005)
miR-181b	Glioblastoma	N	↓	Ciafrè et al. (2005)
miR-181b	PTC	N, Q	↑	Pallante et al. (2006)
miR-181b-1	ALL	Q	↑	Zanette et al. (2007)
miR-184	Tongue SSC	Q	↑	Wong et al. (2008)
miR-195	CLL	Q	↑	Zanette et al. (2007)
miR-199a	Ovary	N	↓	Iorio et al. (2007)
miR-199a	Cervical SSC	Q	↑	Lee et al. (2007b)
miR-200a	Ovary	N	↑	Iorio et al. (2007)
miR-204	ALL	Q	↑	Zanette et al. (2007)
miR-204	Pancreas (insulinomas)	N	↑	Roldo et al. (2006)

(*continued*)

Self-sufficiency in growth signals

Normal cells require mitogenic signals in order to leave the quiescent state and become actively proliferating cells. Many cancer cells can produce their own growth factors in order to succeed at this, a condition known as autocrine regulation (Fedi et al. 1997). RAS is a major intermediate regulator that participates in the aforementioned signaling cascade. *RAS* is mutated in many malignancies; however, miRNAs provide an alternative way to control the expression of RAS protein levels. It has been well established that let-7 binds to the 3′ UTR of *RAS* mRNA, thus inhibiting translation (Johnson et al. 2005). *H-RAS*, *N-RAS*, and *K-RAS* all have *let-7* binding sites at their 3′ UTRs. Due to frequent down-regulation of let-7 in tumors, it is suggested that this miRNA has tumor suppressor activity. In concordance with this, it was found that

TABLE 1. *Continued*

miRNA	Cancer type	Method	Up or down	Reference
miR-218	ALL	Q	↑	Zanette et al. (2007)
miR-221	PTC	N, Q	↑	Eis et al. (2005)
miR-221	PTC	N, R	↑	He et al. (2005a)
miR-221	Glioblastoma	N	↑	Ciafrè et al. (2005)
miR-222	PTC	N, Q	↑	Eis et al. (2005)
miR-222	PTC	R	↑	He et al. (2005a)
miR-331	ALL	Q	↑	Zanette et al. (2007)
miR-331	CLL	Q	↑	Zanette et al. (2007)
miR-372	Testicular	N	↑	Voorhoeve et al. (2006)
miR-373	Breast cancer metastasis	Q	↑	Huang et al. (2008)

(CLL) Chronic lymphocytic leukemia; (PTC) pappilary thyroid carcinoma; (HCC) hepatocellular carcinoma; (ALL) acute lymphoblastic leukemia; (SSC) squamous cell carcinoma; (N) Northern blotting; (Q) quantitative RT-PCR; (R) semiquantitative RT-PCR.

let-7 targets the *HMGA-2* oncogene (Lee and Dutta 2007), down-regulates *MYC*, and can revert MYC-induced growth of Burkitt lymphoma cells (Sampson et al. 2007). It should be noted, however, that one member of the let-7 family, let-7a-3, is heavily methylated in normal lung cells, while it is hypomethylated and expressed in a subset of lung adenocarcinomas. Therefore, it was concluded that this member may have a tumor-promoting role (Brueckner et al. 2007). Recently, let-7 was used as a model to investigate the mechanism of miRNA-induced inhibition of mRNA translation initiation (Kiriakidou et al. 2007). The miRNA let-7 is also considered a marker for less advanced cancer, and more importantly, a better marker than E-cadherin (Shell et al. 2007). Most importantly, let-7 targets *Dicer*, which has three highly conserved let-7 sites, pointing to a negative feedback mechanism on miRNA processing (Forman et al. 2008).

Up-regulation of cell surface receptors results in hypersensitivity of cancer cells to low concentrations of growth factors that normally do not trigger proliferation. Deregulation of miRNA expression in cancer can result in aberrant regulation of cell surface receptors. miRNA-125a and miRNA-125b target the oncogenes *ERBB2* and *ERBB3* that encode for cellular receptors (Scott et al. 2007). Estrogen Receptor alpha (ERα) represents a key regulator in cancer cell signaling. Alterations in the expression of genes that are under hormonal regulation are a very common event in cancer, especially in hormonally regulated tumors, such as breast, ovarian, and prostate. As is shown in Figure 2, miR-206 down-regulates protein and mRNA levels of *ERα* (Adams et al. 2007). Reduction of ERα levels in the cells is expected to have a great impact in the expression profiles of estrogen-responsive genes. For example, kallikrein-related peptidases, the largest group of serine proteases in the human genome that includes 15 members, are well documented to be regulated by hormonal receptors (Borgoño and Diamandis 2004). Furthermore, many members of this family are characterized by their concurrent down- or up-regulation in various forms of cancer. Some members of this family have extracellular matrix-degrading activity, while one member (KLK10) is a tumor suppressor in breast cancer. It is possible that differential expression of miRNAs that target ERα could be responsible for switching on/off the expression of *KLK* genes. ERα is also targeted by miR-221 and miR-222, two miRNAs found to be specifically elevated in ERα-negative breast cancer cell lines. Most importantly, it was demonstrated that knockdown of miR-221 and/or miR-222 expression sensitizes breast cancer cells to tamoxifen (Zhao et al. 2008), while ectopic expression confers resistance, as expected (Miller et al. 2008).

Insensitivity to antigrowth signals and increased capacity of self-renewal

As described by Hanahan and Weinberg (2000), antigrowth signals act in two different ways, either to force cells to enter a quiescent phase or to differentiate. TGF-β is a major regulator of cell signaling that transduces antigrowth signals via multiple pathways, but mainly through prevention of phosphorylation and inactivation of the tumor suppressor protein Rb, an effect mediated by p15 (Hannon and Beach 1994). Recently, miR-106 was shown to target retinoblastoma 1 and miR-20a, the receptor of TGF-β (Volinia et al. 2006). MiR-124a is epigenetically silenced in human cancer cells, and its down-regulation was correlated with Rb hypophosphorylation (Lujambio et al. 2007). As mentioned, miRNAs are important regulators of cell differentiation, as shown for members of the let-7 family in tissue regeneration studies in newts: During initiation of regeneration in newt lens and inner ear hair cells, which is characterized by dedifferentiation of terminally differentiated cells, all let-7 members were found to be down-regulated (Tsonis et al. 2007).

On the other hand, miR-17-92 was implicated in erythroleukemia. This polycistron is derived from a miRNA cluster that resides in intron 3 of the *C13orf25* gene on human chromosome 13q31.3 and contains seven miRNAs: miR-17-5p, miR-17-3p, miR-18, miR-19a, miR-20, miR-19b-1, and miR-92-1. In a mouse model infected with the Friend murine leukemia virus that causes retroviral insertional mutagenesis, induction of erythroleukemia was observed upon insertion of the viral genome 298 base pairs (bp) upstream of the miR-17-92 polycistron. This insertion results in activation of the gene cluster, enhancement of erythroblast transformation, and proliferation. Under these conditions, erythroleukemic cells became erythropoietin-dependent and altered their normal response to

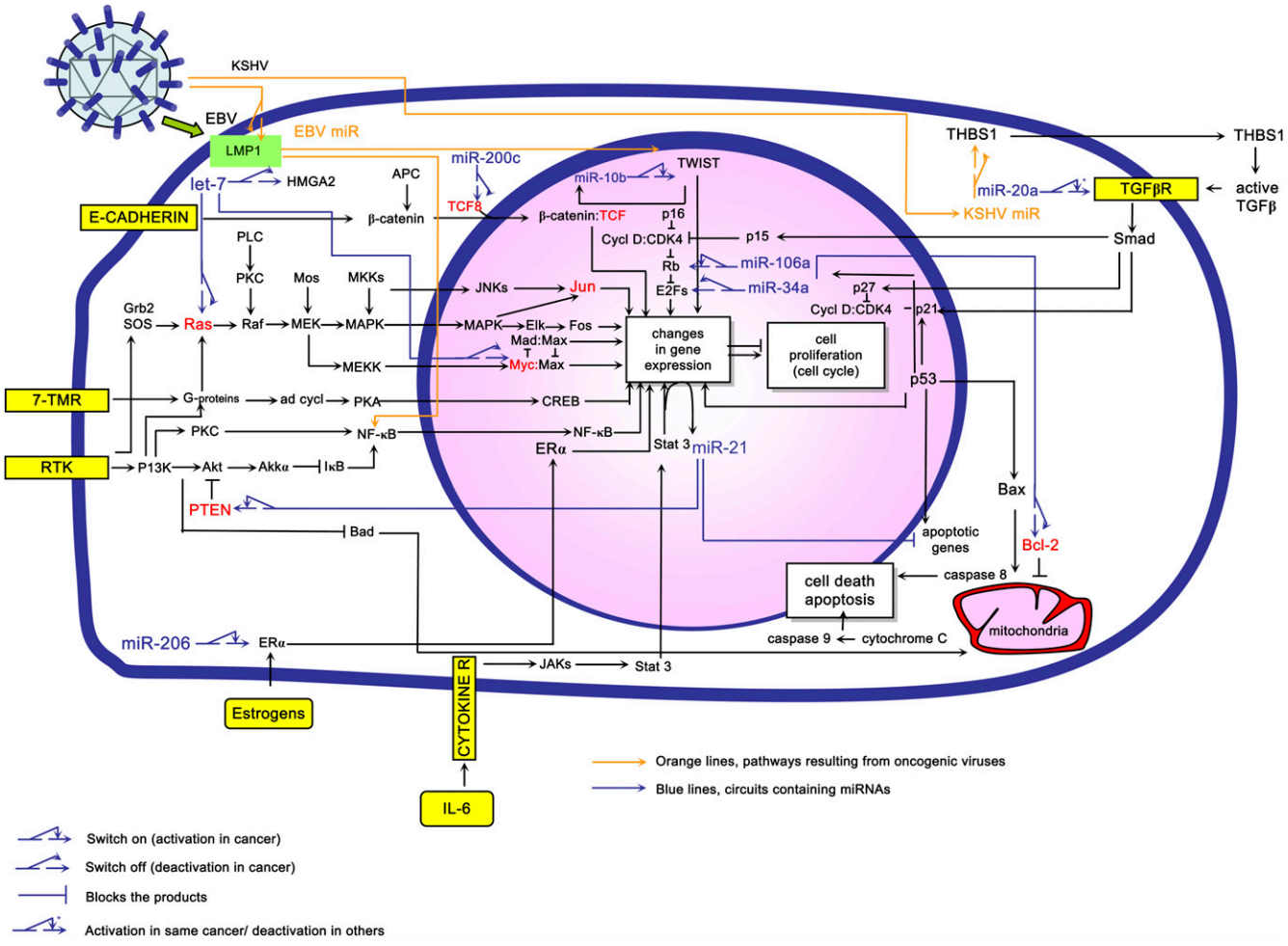

FIGURE 2. The "expanded" integrated circuit of the cancer cell. Cell signaling pathways resemble an electronic integrated circuit, where miRNAs act as switches that turn the circuits on/off. Up- or down-regulation of specific miRNAs in cancer results in turning on/off specific cellular microcircuits. In addition, these switches may be controlled by miRNAs encoded by oncogenic viruses. It should be noted that genetic reprogramming of the integrated cell circuit during malignant transformation and/or progression (e.g., mutation of p53) most likely cooperates with miRNA-mediated signaling (based on data from Fig. 2 of Hanahan and Weinberg [2000]).

erythropoietin from maturation to self-renewal (Cui et al. 2007). The same activation event was observed previously in mice infected with the SL3-3 murine leukemia virus (Wang et al. 2006). Further, two mouse models that expressed miR-17-92 were generated following its identification as an oncogene (oncomir) (He et al. 2005b; Lu et al. 2007). In the first case, expression of the miR-17-92 cluster or a truncated form of the cluster (miR-17-19b-1) in cooperation with c-myc expression resulted in significant acceleration of tumor development in a mouse model for B-cell lymphoma (He et al. 2005b). In the other case, in order to study potential developmental effects, a transgenic mouse was produced that overexpresses the mouse miR-17-92 cluster in lungs under the control of the mouse surfactant protein C promoter (Lu et al. 2007). MiR-17-92 overexpression caused major abnormalities in the lungs, while some of the animals were born dead without air in their lungs (Lu et al. 2007).

An increasing number of recent studies confirm the existence of cancer stem cells (or tumor-initiating cells), which are rare cells with indefinite proliferative potential that are believed to trigger tumor formation (Reya et al. 2001). Due to the fact that both cancer cells and normal stem cells share similar potential to indefinitely self-renew, it is logical to assume that cancer stem cells use the same machinery for self-renewing cell division as that used by normal stem cells. MiRNAs play important roles in determining the self-renewal potential of normal stem cells during development (Zhang et al. 2006a). Let-7 was found to be down-regulated in breast cancer stem cells, but its expression was induced upon induction of differentiation in breast cancer stem cells (or breast-tumor-initiating cells) (Yu et al. 2007). Lentiviral transduction of let-7a into breast-tumor-initiating cells resulted in reduction of tumor formation in SCID mice, and reduced the burden of liver and lung metastasis, as well as their self-renewal ability (Yu

et al. 2007). The inhibition of self-renewal is partly attributed to inhibition of RAS by let-7, while the induction of differentiation is partly due to inhibition of HMGA2. Also, let-7 expression was shown to deplete self-renewal of mammary progenitor cells (Ibarra et al. 2007).

Evading apoptosis: The role of oncomirs

Oncogenic miRNAs, also known as oncomirs, usually promote tumor development by inhibiting known tumor suppressor genes or genes that control cell differentiation or apoptosis (Cho 2007; Zhang et al. 2007a). As shown by several studies, expression of the miR-21 oncomir is regulated by an upstream enhancer located ~800 bp upstream of the transcriptional start site that contains two functional stat3 binding sites (Löffler et al. 2007). In myeloma cells, activation of stat3 by IL-6 activates miR-21, providing the basis for IL-6-dependent survival of multiple myeloma cells. Withdrawal of IL-6 results in rapid apoptosis of INA myeloma cells. However, ectopically expressed miR-21 inhibited significantly the number of cells undergoing apoptosis (Löffler et al. 2007). The tumor suppressor gene tropomyosin 1 (*TPM1*) is another target of the miR-21 oncomir that specifically inhibits translation of *TPM1* mRNA, while *TPM1* mRNA levels remain constant (Zhu et al. 2007). Furthermore, miR-21 targets the tumor suppressor genes *PTEN* (phosphatase and tensin homolog deleted on chromosome 10) (Meng et al. 2007), PDCD4 (programmed cell death 4) (Asangani et al. 2008; Frankel et al. 2008; Zhu et al. 2008), and maspin (Zhu et al. 2008). Additionally, miR-21 is induced by AP-1 in response to RAS activation. Because PDCD4 is a negative regulator of AP-1, down-regulation of PDCD4 by miR-21 provides an autoregulatory loop that controls RAS-mediated AP-1 activity (Talotta et al. 2009). Moreover, it was shown that inhibition of miR-21 in MCF-7 resulted in the up-regulation of various genes (*FAM3C*, *ACTA2*, *APAF1*, *BTG2*, *FAS*, *CDKN1A*, and *SESN1*) that are known to be controlled by p53. However, miR-21 does not target p53 directly; therefore, miR-21 likely antagonizes the p53 pathway via other downstream targets (Frankel et al. 2008). Interestingly, inhibition of miR-21 in MDA-MB-231 has a subtle effect on the growth of primary tumors, but it significantly reduces invasion and lung metastasis (Zhu et al. 2008).

Mutations in p53 are found nearly in all types of cancer. Importantly, p53 regulates the expression of the miR-34 family of miRNAs, which consists of three members: miR-34a, which is generated from a transcriptional unit located on chromosome 1p36, and miR-34b and miR-34c, both of which are produced by processing of a bicistronic transcript from chromosome 11q23 (Bommer et al. 2007). The miR-34 family is down-regulated in cancer cells either due to p53 inactivation by mutations or epigenetically (Hermeking 2007). In neuroblastoma, loss of miR-34a was observed, which is probably caused by a 1p36 deletion (Welch et al. 2007). It is likely that *miR-34* family members are necessary mediators of the p53 tumor suppressor activity, since the ectopic expression of miR-34a causes a cell-cycle arrest in G1 phase (Bommer et al. 2007; He et al. 2007; Tarasov et al. 2007), as did miR-34b/c (He et al. 2007). In addition, upon ectopic expression of miR-34a, human colon cancer cell lines HCT116 and RKO showed signs of senescence (Tazawa et al. 2007). Moreover, miR-34a suppressed the formation of tumors by HCT116 and RKO cells in mice. Gene-expression microarray and immunoblot analyses revealed down-regulation of the E2F pathway by miR-34a and up-regulation of the p53 pathway (Tazawa et al. 2007). In another study, it was shown that miR-34b and miR-34c cooperate in suppressing proliferation and soft-agar colony formation of neoplastic epithelial ovarian cells, while doxorubicin, which up-regulates p53, also up-regulates the expression of these two miRNAs (Corney et al. 2007). Furthermore, direct binding of p53 to a promoter element on the miR-34a promoter was demonstrated (Raver-Shapira et al. 2007; Tarasov et al. 2007). In addition, inactivation of miR-34a compromises p53-dependent apoptosis, indicating that miR-34a is a proapoptotic transcriptional target of p53 (Raver-Shapira et al. 2007). Indeed, other groups have verified that expression of miR-34a can induce apoptosis (Chang et al. 2007; Welch et al. 2007). Transfection of an episomal plasmid driving the conditional expression of miR-34a in U2OS osteosarcoma cells and conditional up-regulation of its expression resulted in a significant decrease of colony formation (Tarasov et al. 2007). Finally, miR-34a-deficient embryonic stem cells showed a slight decrease in spontaneous apoptosis after differentiation, while *BCL2* was identified as one of miR-34a targets (Bommer et al. 2007). Based on these observations, the miR-34 gene family was suggested as a new tumor suppressor miRNA family. On the other hand, miR-125b was implicated in the development of androgen-independent prostate cancer (Shi et al. 2007). Differential expression of miR-125b in androgen-dependent and -independent prostate cancer cells, as well as in benign and malignant prostate tissues, has been observed. Androgen-induced expression of miR-125b-2 is mediated by androgen receptor binding on the miR-125b-2 promoter (Shi et al. 2007). MiR-125b suppresses the expression of the proapoptotic gene *Bak1* and induces androgen-independent growth of prostate cancer cells; therefore, it was suggested that miR-125b functions as an oncomir in prostate cancer. Recently, it was shown that miRNAs can modulate TRAIL-induced apoptosis by indirectly targeting caspases (Ovcharenko et al. 2007). MiR-133 is the only miRNA that was shown to down-regulate the levels of caspase-9 by direct interaction (Xu et al. 2007). Finally, miR-15 and miR-16 induce apoptosis by targeting the antiapoptotic gene *BCL2* (Cimmino et al. 2005).

Limitless replicative potential

Limitless replicative potential is correlated with progressive shortening of telomere sequences located at the end of

chromosomes containing the motif $(TTAGGG)_n$. In every cell division, telomeres lose 50–150 bp to a certain threshold, at which an irreversible growth arrest is triggered, which is referred to as senescence. Cancer cells have adapted mechanisms to escape this limitation, acquiring a limitless replicative potential phenotype. Telomere length is maintained by the enzyme telomerase, human telomere reverse transcriptase (hTERT), which is a reverse transcriptase that synthesizes the telomeres at the ends of the chromosomes (Blackburn 2001). Another mechanism that maintains the length of telomeres is the alternative lengthening of telomeres (ALT) (Muntoni and Redder 2005), which relies on homologous recombination events at telomeres. Many miRNAs were predicted to target the mRNA that encodes for hTERT. However, only miR-138 was functionally related to the regulation of *hTERT* (Mitomo et al. 2008). miR-138 expression was found to be down-regulated in anaplastic thyroid carcinoma cell lines, leading to overexpression of *hTERT*. Interestingly, miRNAs may regulate *hTERT* expression in a subset of osteosarcomas, since in some osteosarcoma cell lines expression of the *hTERT* mRNA did not correlate with protein levels (Blackburn 2001).

Dicer1 deficiency results in decreased DNA methylation in mouse embryonic stem cells. Specifically, Dicer1-null cells displayed reduced amounts of DNMT1, DNMT3a, and DNMT3b and were characterized by hypomethylation of genomic DNA, including subtelomeric regions of chromosomes. On the other hand, retinoblastoma-like protein 2 (Rbl-2) and retinoblastoma proteins, in general, are repressors of DNMT3a and DNMT3b. The Rbl-2 transcript is a direct target of the miR-290 family (Benetti et al. 2008; Sinkkonen et al. 2008). In fact, all members of the miR-290 family except for miR-290 (i.e., miR-291-3p, miR-291-5p, miR-292-3p, miR-292-5p, miR-293, miR-294, miR-295) were shown to repress *Rbl-2* mRNA and protein levels. These results show that defects in DNA methylation lead to increased telomere recombination and aberrant telomere elongation, as demonstrated in the Dicer1-null embryonic stem cells (Benetti et al. 2008).

Angiogenesis

Angiogenesis plays an important role in cancer growth and progression, since it provides tumor cells with nutrients. Vascular endothelial cells are stimulated by proangiogenic factors secreted by tumor cells to undergo distinctive phenotypic changes that finally give rise to new mature blood vessels. The first evidence for the involvement of miRNAs in the regulation of angiogenesis came from *Dicer* knockout mice carrying a deletion by homologous recombination of the first two exons. Knockout mice died between 12.5 and 14.5 d of gestation, and it was found that their blood vessel formation was compromised (Yang et al. 2005). Recently, miR-221 and miR-222 were shown to modulate the stem cell factor (SCF) by targeting its receptor c-kit. Activation of c-kit by SCF controls tube formation, migration, and survival of HUVEC (Poliseno et al. 2006). Retroviral transduction of miR-17-92 into p53-null mouse colonocytes resulted in down-regulation of thrombospondin 1 (THBS1) and connective tissue growth factor (CTGF) and concomitant inhibition of angiogenesis (Dews et al. 2006). Knockdown of miR-17-92 resulted in partial restoration of THBS1 and CTGF protein expression (Dews et al. 2006). Under hypoxic conditions in CNE human nasopharyngeal cancer cells, VEGF is up-regulated due to down-regulation of specific miRNAs that blocked its expression. Using miRNA chip assays, it was shown that *VEGF* mRNA is targeted by miR-15b, miR-16, miR-20a, and miR-20b (Hua et al. 2006). Transfection of cells with the above miRNAs resulted in reduction of VEGF protein levels. Furthermore, miRNAs can regulate other angiogenic factors, such as cycloxygenase 2 (COX2), uPAR, and c-MET. More specifically, miR-20b, miR-15b, and miR-16 target COX2; miR-15b and miR-16 were shown to down-regulate uPAR; while c-MET is down-regulated by miR-15b, miR-16, miR-20a, and miR20b (Hua et al. 2006).

The major antiangiogenic homeobox gene *GAX* as well as *HOXA5* are direct targets of miR-130a (Chen and Gorski 2008). Quiescent endothelial cells express *GAX* but they do not express miR-130a. Induction of proliferation and differentiation by serum, proangiogenic factors (VEGF or bFGF), or proinflammatory factors (TNF-a) results in the expression of miR-130a and down-regulation of *GAX* and *HOXA5*. HOXA5 is an interesting molecule because it inhibits the expression of VEGF receptor 2 (*VEGFR2*), *COX-2*, and other important angiogenesis-associated genes. This fact probably indicates that resting cells express low levels of critical angiogenesis genes; however, induction of proliferation down-regulates the expression of *HOXA5* mediated by miR-130a, while it further enhances the angiogenesis potential by inducing the cell to express its own angiogenesis genes. In addition, miR-126 is another miRNA that regulates the response of endothelial cells to VEGF (Fish et al. 2008) and FGF (Wang et al. 2008a), and developmental angiogenesis in vivo (Wang et al. 2008a). It is located inside an intron sequence of the *Egfl7* gene and is regulated by a negative feedback loop (Fish et al. 2008). The function of miR-126 has been elucidated with the use of animal models. Specifically, it was shown that knockdown of miR-126 in zebrafish resulted in loss of vascular integrity and hemorrhage during embryonic development. However, miR-126 is not sufficient by itself to promote differentiation of pluripotent cells toward the endothelial lineage (Fish et al. 2008). A targeted deletion of miR-126 (miR-$126^{-/-}$) in mice results in similar effects, including leaky vessels, hemorrhaging, and partial (~40%) embryonic lethality. MiR-$126^{-/-}$ mice that survived to adulthood appeared normal, indicating the important effect of miR-126 in vascular integrity during embryogenesis (Wang et al.

2008a). MiR-126-targeted deletion reduces survival after myocardial infraction, since neovascularization is essential for cardiac repair (Wang et al. 2008a). Other miRNAs that were implicated in promoting angiogenesis include miR-378 (Lee et al. 2007a), miR-27b, and let-7f (Kuehbacher et al. 2007), while miRNAs may also act via interconnected complex networks. It was described that RAS modulates *myc* activity to repress the thrombospondin-1 (Watnick et al. 2003). This results in a new microcircuit within cells. Viral miRNAs also target antiangiogenic factors, as was demonstrated for KSHV-encoded miRNAs that target *THBS1* (Samols et al. 2007).

Invasion and metastasis: Epithelial-to-mesenchymal transition

Transition to the invasive/metastatic phenotype is necessary for primary tumor cells, in order for them to escape from the primary site and colonize a new tissue in the body. Recently, it was shown that miR-10b acts to promote metastasis of breast cancer (Ma et al. 2007). This study indicated that miRNAs likely play an important role in regulating the invasive and metastatic potential of the cancer cell. It was demonstrated that miR-10b expression levels in MDA-MB-231 (a metastatic breast cancer cell line) were increased by 50-fold when compared with MCF-7 (a nonmetastatic breast cancer cell line). Silencing of miR-10b resulted in a 10-fold reduction of the invasive properties of MDA-MB-231 cells assessed in vitro. Ectopic expression of miR-10b in the nonmetastatic breast cancer cell line SUM149 had no effect on the in vivo growth rate of cells implanted in SCID mice, but it increased dramatically the in vivo invasive potential of these cells. Furthermore, induction of miR-10b expression initiated the formation of lung metastasis. These results were confirmed by using another nonmetastatic breast cancer cell line, SUM159, in which ectopic expression of miR-10b resulted in the appearance of metastatic sites in the lungs and peritoneum (Ma et al. 2007). Detailed molecular analysis identified the transcription factor Twist as a key molecule for miR-10b up-regulation. Twist acts as a master regulator not only in morphogenesis but also in metastasis. Twist is responsible for the induction of epithelial-to-mesenchymal transition (EMT) (Yang et al. 2004). Furthermore, Twist is up-regulated by the LMP1 protein of Epstein-Barr virus (EBV) (Horikawa et al. 2007), a protein that is autoregulated by *BARTs* miRNAs encoded by EBV (Lo et al. 2007). Recently, it was shown that TGF-β signaling induces HMGA2, which, in turn, mediates EMT. Twist is also up-regulated by TGF-β and synergistically up-regulated with HMGA2 and TGF-β (Thuault et al. 2006). Finally, up-regulation of Twist induces tumor angiogenesis (Niu et al. 2007), thus linking the invasive potential of the cancer cell to neovascularization.

Generally, EMTs are required for the acquisition of metastatic potential by transformed cells. Recently, several miRNAs were identified that regulate the transition from the epithelial-to-mesenchymal phenotype, thereby increasing the aggressiveness of cancer cells. The miR-200 family (miR-200a, miR-200b, miR-200c, and miR-429) has recently attracted attention as a regulator of EMT. All members of the miR-200 family are down-regulated upon induction of EMT transition of mouse mammary epithelial cells induced by TGF-β (Korpal et al. 2008). Re-expression of the miR-200 family was able to reverse the mesenchymal phenotype of 4TO7 metastatic breast cancer cells (Korpal et al. 2008). Stable overexpression of miR-200c in A549 nonsmall cell lung carcinoma resulted in a loss of ZEB1 (also referred to as TCF8, δEF1, Nil-2-α) and an increase of E-cadherin expression. Also, miR-200c when expressed in MDA-MB-231 restored E-cadherin expression, which represents a major event in EMT (Thiery 2002). Furthermore, miR-200b mediates the posttransciptional repression of *ZEB2* (Christoffersen et al. 2007), which is involved in regulation of the TGF-β pathway and induction of EMT, while it induces Rb hypophosphorylation (Mejlvang et al. 2007).

Recently, it was demonstrated that miR-373 and miR-520c promote breast cancer metastasis. MCF-7, a nonmigratory, nonmetastatic breast cancer cell line, was transfected with a library of human miRNAs. Transfectants were subjected to multiple rounds of *trans*-well cell migration assay, and miR-373, miR-520c, and miR-520e were found to stimulate cell migration. Enforced expression of miR-373 or miR-520c produced a potent migratory phenotype in MCF-7 cells. In addition, injection of MCF-7 transfected with miR-373 or miR-520c in the tail vein of SCID mice resulted in extensive lung and osteolytic metastasis. This effect was attributed, at least in part, to down-regulation of CD44, a cell surface receptor of hyaluronan. Knockdown of CD44 in MCF-7 cells by shRNA had similar in vivo effects as observed when miR-373 or miR-520c became up-regulated (Huang et al. 2008). It should be noted that miR-373 was recently shown to up-regulate the expression of E-cadherin in PC-3 prostate cancer cells by targeting complementary promoter regions in the encoding gene, a phenomenon named RNA activation (RNAa). RNAa represents a mechanism that links EMT to microRNAs (Place et al. 2008). Based on the above data, miR-373 is considered a putative tumor suppressor; however, miR-372 and miR-373 act as oncogenes in testicular germ cell tumors by debilitating the p53 pathway, thus allowing tumor growth in the presence of wild-type p53 (Voorhoeve et al. 2006).

Based on the fact that miR-126, miR-206, and miR-335 are down-regulated in metastatic breast cancer cells, these miRNAs are considered metastasis suppressors. Restoration of miR-126, miR-335, or miR-206 in highly metastatic MDA-MB-231-derivatives decreased their lung colonization capacity and bone metastasis (Tavazoie et al. 2008). In particular, miR-206 also inhibited primary tumor formation in mice implanted with MDA-MB-231 in their mammary fat pad. Notably, although the metastatic potential

and motility were reduced, re-expression of these microRNAs did not alter the levels of the mesenchymal marker vimentin. Therefore, inhibition of metastasis was not due to a classical mesenchymal-to-epithelial transition (MET), despite the fact that cells changed their structure to a more rounded form (Tavazoie et al. 2008). Based on the observations outlined above, miR-126 exerts "dual" roles, since it mediates the action of VEGF, as described previously, but it plays a tumor suppressor role in breast cancer.

It is well established that proteases play significant roles in determining the invasive and metastatic potential of tumor cells. Recently, it was shown that KLK6 and KLK10 human tissue kallikrein-related peptidases are regulated by let-7f (Chow et al. 2008). Targeting of uPAR by miRNAs was also demonstrated (Hua et al. 2006). The uPAR is not only responsible for angiogenesis but also is involved in promoting metastasis of cancer cells. Furthermore, uPAR was found to induce EMT in breast cancer cells (Lester et al. 2007). Modulation of miR-21 expression was shown to correlate with the expression of MMP2 and MMP9 in human hepatocellular cancer (HCC); however, this is mediated by the PTEN tumor suppressor, which is a direct target of miR-21 (Meng et al. 2007).

DEREGULATION MECHANISMS OF MICRORNA SWITCHES IN CANCER

Deregulation of miRNA molecular switches during cancer development and metastasis is supported by an increasing number of studies. How this deregulation is initiated is currently unclear. However, it seems to involve the interplay of multiple mechanisms, such as genomic alterations, oncogenic transcription factors, epigenetic mechanisms, and hypoxia (O'Donnell et al. 2005; Kulshreshtha et al. 2007; Giannakakis et al. 2008). In some cases, protein or miRNA products of oncogenic viruses are responsible for the origin of deregulation (Pfeffer and Voinnet 2006). Genomic mutations in major "caretaker" genes, as for example the key regulator p53 tumor suppressor, deregulate multiple cellular mechanisms that can in turn deregulate the miRNA switches. Independent of the original causative effect, the endpoint is always the acquisition by the cancer cell of the six hallmark characteristics (Hanahan and Weinberg 2000). The major mechanisms underlying the deregulation of miRNA switches will be outlined in the following section, as they have emerged in numerous recent studies.

Genomic alterations

Genomic alterations often account for deregulated miRNA expression profiles in cancer. For example, down-regulation of miR-15 and miR-16 in CLL was described above. A detailed analysis of 283 different miRNA genes for gene copy number (gain or loss) in ovarian, breast, and melanoma cancer clinical specimens and/or cell lines was performed recently (Zhang et al. 2006b). Gene amplification was demonstrated for miR-17-92 (Hayashita et al. 2005). The mode of miR-223 regulation in leukemia represents an interesting example of how gross genomic alterations may be involved in the deregulation of miRNA switches. It was shown that the t(8;21) translocation associated with primary leukemia produces the oncogenic factor AML1/ETO. By recruiting chromatin remodeling enzymes at an AML1-binding site on the pre-miR-223 gene, AML1/ETO induces heterochromatic silencing of miR-223. Thereby, the miR-223 signaling pathway is turned off (Fazi et al. 2007). This example further illustrates the interconnection of multiple regulatory pathways and extends our understanding of the integrated circuit of the cancer cell in that gross genomic alterations lead to the production of a new tumor-specific oncogenic factor that induces epigenetic silencing of a miRNA by chromatin remodeling.

Transcriptional regulation of microRNAs

Changes in the expression levels of transcription factors known for their oncogenic or tumor suppressor function may have a direct effect on the expression of miRNAs. In gastric cancer, E2F1 overexpression is positively correlated with overexpression of the miR-106b-25 cluster (Petrocca et al. 2008). The oncogenic transcription factor c-MYC regulates the expression of the miR-17-92 cluster by binding to E-boxes located on the promoter of the miRNA gene (O'Donnell et al. 2005). Importantly, as discussed above, the miR-34 family is down-regulated in a panel of cancer cells due to p53 inactivation (Hermeking 2007). In addition, p53 was shown to directly bind to the miR-145 gene promoter to activate its transcription (Sachdeva et al. 2009). MiR-145 exerts an important tumor suppressor activity by silencing *c-myc* expression (Sachdeva et al. 2009).

Epigenetic regulation of microRNAs

Aberrant DNA methylation, which is a major cause of genome instability, can be triggered by miR-29 targeting of the DNA methyltransferases *DNMT3a* and *DNMT3b*. Specifically, it was shown that expression of the miR-29 cluster (29a, 29b, 29c) was down-regulated in lung cancer, while the de novo DNA methyltransferases *DNMT3A* and *DNMT3B* were up-regulated. Restoration of miR-29s expression resulted in normal patterns of DNA methylation in lung cancer cell lines, re-expression of epigenetically silenced tumor suppressor genes, such as *WWOX* and *FHIT*, and inhibition of tumorigenicity in vitro and in vivo (Fabbri et al. 2007). MiR-1 was identified by expression profiling of hepatocellular carcinoma cell lines treated with 5-aza-dC and/ or TSA. In *DNMT1*$^{-/-}$ HCT116 the miR-1 gene was found hypomethylated and activated. Re-expression of miR-1 results in inhibition of cell growth and reduction

of replication potential and clonogenic survival in HepG2 cells, indicating a putative tumor suppressor role in analogy with the classical tumor suppressor genes silenced by DNA hypermethylation (Datta et al. 2008). The tumor suppressor role of miR-1 was also demonstrated in lung cancer cell lines (Nasser et al. 2008). Other examples include miR-9-1, which was found hypermethylated in breast cancer cells (Lehmann et al. 2008) and miR-203, which is silenced either by DNA methylation or genomic deletion (Bueno et al. 2008). Identification of miRNAs that are regulated by DNA methylation was attempted by differential profiling of miRNA expression patterns in *DNMT1* and *DNMT3b* double-gene knockouts in HCT116 cells (Han et al. 2007), and in metastatic lymph node cancer cells after 5-aza-2′-dC-induced demethylation (Lujambio et al. 2008).

Polymorphisms and mutations in microRNAs

Point mutations or polymorphisms in miRNA genes may have implications in cancer, since they may abnormally turn on/off miRNAs that act as molecular switches in the integrated circuit of the cancer cell. Also, mutations in miRNA target sequences in mRNAs will have an impact on gene expression. For the human dihydrofolate reductase (*DHFR*) gene, it was found that a single nucleotide polymorphism (SNP) (C829T) at the 3′ UTR occurs inside the miR-24 recognition sequence, resulting in *DHFR* overexpression associated with methotrexate resistance (Mishra et al. 2007). Cells with a mutant *DHFR* 3′ UTR exhibited a twofold increase in mRNA half-life, expressed higher *DHFR* mRNA and protein, while they were four times more resistant to methotrexate. This SNP was named miRSNP (SNP located at or near the microRNA binding site) (Mishra et al. 2007). A similar polymorphism was also found in target mRNA encoding the angiotensin II type 1 receptor (AT1R) at the A1166C binding position recognized by miR-155 (Martin et al. 2006; Sethupathy et al. 2007). Moreover, a SNP in the complementary site of let-7 in K-RAS increases the risk for nonsmall lung cancer (Chin et al. 2008). Recently, the PolymiRTS Database (http://compbio.utmem.edu/miRSNP) was constructed to collect naturally occurring DNA variations in putative microRNA target sites (Bao et al. 2007). In a large comprehensive study published by Iwai and Naraba (2005), 96 individuals were analyzed for 173 different miRNAs. Polymorphisms were identified in various regions of 10 different miRNAs. Only one polymorphic site in miR-30c-2 was localized in the recognition sequence, which is speculated to alter the selection of mRNA targets. Recently, another study was published showing sequence variations in one precursor miRNA and 15 pri-miRNAs in various human cancer cells lines (Diederich and Haber 2006). Finally, a large study for human polymorphisms in 227 miRNAs was performed that led to the identification of 323 polymorphisms (Duan et al. 2007). One of these polymorphisms was located in the eighth nucleotide of mature miR-125a, which is expected to block processing of pri-miRNA to pre-miRNA in addition to reducing miRNA-mediated translational suppression.

MICRORNAS ENCODED BY ONCOGENIC VIRUSES: EXTERNAL SWITCHES TO THE CELLULAR CIRCUIT

Up to 15% of human cancers are associated with a single or multiple viral infections (Pfeffer and Voinnet 2006). The first evidence for the presence of miRNAs in oncogenic viruses came from the observation that the Epstein-Barr virus produces a large number of miRNAs (Pfeffer et al. 2004). The discovery of viral miRNAs indicates that miRNAs are critical modulators of virus-induced oncogenesis. Viral miRNAs can also act as molecular switches to turn on/off cellular microcircuits but may also be involved in the regulation of viral genes. EBV has been studied in detail and expresses a total of 23 miRNAs. These are arranged in two clusters. Twenty miRNAs are located in introns of the viral *BART* gene (miR-BART1–20), while three miRNAs are located adjacent to *BHRF1* (miR-BHRF1-1–3) (Grundhoff et al. 2006). All 23 miRNAs were successfully mapped in EBV-infected cells by a combination of bioinformatic approaches for viral miRNA prediction and analysis of microarray data (Grundhoff et al. 2006). EBV is also associated with ~6%–16% of gastric carcinoma cases. It was demonstrated that BART but not BHRF1 miRNAs are expressed in EBV-infected gastric carcinoma cell lines, in animal models, and in tumor samples from patients (Kim et al. 2007). Viral miRNAs were found that recognize and target viral transcripts, as demonstrated for EBV-encoded miR-BART2 that down-regulates the viral DNA polymerase BALF5, which represent a mechanism of virus self-regulation (Barth et al. 2008). Further, infection with EBV results in up-regulation of miR-155 and its precursor RNA BIC. MiR-155 modulates the NF-κB pathway, thus contributing to EBV-immortalization (Lu et al. 2008).

Kaposi's sarcoma-associated herpesvirus (KSHV) encodes for 12 different miRNAs that are detected during the latency period and are expressed from a single genetic locus (Cai et al. 2005). The KSHV miRNA (miR-K12-10) was shown to undergo RNA editing (Pfeffer et al. 2005). Since most viral miRNAs display no sequence conservation with metazoan miRNAs, they are not expected to interfere with endogenous miRNAs. Therefore, their ectopic expression allows for the identification of their targets and delineation of their functions. This approach was successfully applied to the identification of KSHV cellular targets. As mentioned, thrombospondin 1 (*THBS1*) represents a major target for KSHV miRNAs, since multiple KSHV miRNAs (miR-K12–1, miR-K12-3-3p, miR-K12-6-3p, and miR-K12–11) target *THBS1* expression (Samols et al. 2007). THBS1 was found to be down-regulated in Kaposi sarcoma lesions and has known activity as a strong tumor suppressor and antiangiogenic factor, exerting its antiangiogenic effect in part by activating

latent TGFβ (Samols et al. 2007). MiR-K12-11, a miRNA encoded by KSHV, is an exception to the rule, since it displays significant homology with cellular miR-155, including the entire seed region. It was experimentally shown that expression of physiological levels of miR-K12-11 or miR-155 results in down-regulation of an extensive set of common mRNA targets, including genes with known roles in cell growth regulation, suggesting that viral miR-K12-11 functions as an ortholog of cellular miR-155 (Gottwein et al. 2007).

It is essential to understand the role(s) that these viral miRNAs may play in oncogenesis (for review, see Pfeffer and Voinnet 2006). Based on current knowledge, they can act directly as oncogenes, as demonstrated for the KSHV miR-K12-10 and miR-K12-12. Interestingly, the p53 tumor suppressor is targeted by the EBV-encoded miR-BHRF1-1. Viral miRNAs can additionally facilitate tumor progression in tissues that are already prone to develop cancer in the absence of an infection. In a mouse B-cell lymphoma model it was found that enforced expression of the miR-17-92 cluster significantly accelerated lymphoma development (He et al. 2007). It is therefore possible that some viral miRNAs act similarly to the human oncomir miR-17-92. Finally, viral miRNAs facilitate evasion of innate and adaptive immune responses, thereby enabling long-lasting infections that are presumably required to establish a ground for cancer development. A recent computational study suggested the presence of one miRNA in hepatitis B virus (HBV) that was confirmed by Northern blot analysis. However, no cellular targets were predicted for this miRNA; therefore, it is possible that this miRNA acts as a virus self-regulatory mechanism (Jin et al. 2007). Among the many viruses that are known to infect the human liver, HBV and hepatitis C virus (HCV) are unique because of their prodigious capacity to cause persistent infection, cirrhosis, and liver cancer (for review, see Guidotti and Chisari 2006). The physiological RNAi mechanism operating in mammalian cells is thought to contribute to antiviral defense. Contrary to this, knockdown of Dicer inhibits HCV replication. Further, human miR-122 contributes to HCV replication. Depletion of human miR-122 by modified antisense oligonucleotides prevents the production of infectious viral particles (Randall et al. 2007). This is the first example of how an oncogenic virus exploits the RNAi factory of the cell for its own replication. Finally, it is interesting to note that human papilloma virus HPV31 does not seem to encode for any miRNAs, and it is possible that this is a general feature of human papilloma viruses (Cai et al. 2006).

EXPLOITATION OF MICRORNAS IN MOLECULAR DIAGNOSIS OF CANCER

MicroRNAs have tremendous potential as a new class of molecular tumor biomarker, since the extraordinary level of diversity in their expression across human cancers can provide a large amount of diagnostic, prognostic, and predictive information. Because this information is encoded by a relatively small number of miRNAs when compared with the many thousands of transcripts analyzed for gene expression profiling, analysis of miRNAs is much more effective for succesful mining of novel tumor biomarkers suitable for routine clinical applications. Numerous recent studies have shown clearly that specific miRNAs display differential expression in tumors and their adjacent normal tissues and that specific miRNA expression signatures are correlated with prognosis, indicating that miRNAs are determinants of clinical aggressiveness (Calin et al. 2004; Lu et al. 2005; Volinia et al. 2006; Zhang et al. 2007a). MiRNA signatures were used to successfully classify human cancers and to define miRNA markers for favorable prognosis (Lu et al. 2005). As shown in Table 1, miR-10b, miR-125b, and miR-145 were found to be down-regulated in human breast cancer while miR-21 and miR-155 were up-regulated (Iorio et al. 2005; Si et al. 2007). MicroRNA-21 (miR-21) has been shown to be up-regulated in breast cancer (Iorio et al. 2005), lung cancer (Volinia et al. 2006), and glioblastoma (Ciafrè et al. 2005). Interestingly, mature miR-21 has been associated with reduced overall survival of NSCLC patients (Markou et al. 2008). On the other side, miRNA-205 (miR-205) was shown to be overexpressed in head and neck cancer when compared with other cancer lines from lung, breast, colorectal, prostate, and pancreas (Jiang et al. 2005) and in bladder cancer (Gottardo et al. 2007). Yanaihara et al. (2006) have shown for the first time that miRNA expression profiles are diagnostic and prognostic markers in lung cancer. In lung cancer, reduced let-7 expression was found to be significantly associated with shortened postoperative survival, independent of disease stage (Takamizawa et al. 2004), while the expression of miRNA cluster mir-17-92 was found to have remarkably increased, especially in small cell lung cancer (Hayashita et al. 2005). A microRNA signature was found to be associated with prognosis and progression in chronic lymphocytic leukaemia (Calin et al. 2005). MicroRNA expression patterns were shown to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis, six miRNAs were proposed to be used as prognostic biomarkers, while 21 miRNAs were found to differentiate tumors from normal pancreatic tissues (Bloomston et al. 2007). Altogether, deregulated expression of miRNAs is a frequent occurrence in diverse types of cancer, highlighting the potential utility of miRNA profiling for molecular diagnosis and prognosis (Lu et al. 2005).

THERAPEUTIC IMPLICATIONS: IS PHARMACOLOGICAL MODULATION OF MICRORNA SWITCHES FEASIBLE?

As described, it is becoming increasingly evident that miRNA switches play important roles in regulating the cell circuit. Several miRNA switches are deregulated in cancer

cells, thus contributing to the acquisition and progression of malignant phenotypes. Pharmacological modulation of miRNA expression may contribute to "normalization" of the malignant phenotypes, and, therefore, it could represent a potential approach in cancer therapeutics. This will require a better understanding of miRNA functions in the different steps of tumor formation and progression. A number of recent technological advances will be helpful in this direction, for example, RNA interference (RNAi), which is used to knock down the expression of genes of interest (for review, see Martin and Caplen 2007). The best-characterized triggers of RNAi are siRNAs. In addition, systems used for in vivo delivery of siRNAs could be also applied for the delivery of miRNAs. Interestingly, artificial miRNAs, or amiRNAs, were designed recently and used for gene targeting in plants (Niu et al. 2006). Such amiRNAs could be designed to target specific oncogenes. A synthetic miRNA that targets the chemokine receptor CXCR4 was designed using the backbone of miR-155 (Liang et al. 2007), based on a previous observation that siRNA targeting of the CXCR4 inhibited metastasis of breast cancer cells in vivo (Liang et al. 2004). This miRNA, when introduced into MDA-MB-231 breast cancer cells, reduced in vitro motility and invasion, as well as in vivo lung metastatic sites (Liang et al. 2007). In a similar study, the *PRL-3* gene was targeted by a modified miR-155 (Li et al. 2006). The development of locked nucleic acids (LNAs) will aid the therapeutic applications of miRNAs. In these analogs, the ribose ring is locked by a methylene brigde connecting the 2′-O atom with the 4′-C atom (Petersen et al. 2002). LNAs were shown to exhibit no toxicity at dosages capable of producing antitumor effects in vivo. Specifically, inhibition of tumor growth in vivo by an LNA-based anti-H-RAS oligonucleotide was observed at 0.5 mg $\times$ kg^{-1} $\times$ d^{-1} with no signs of toxicity (Fluiter et al. 2005). Increasing the dose above 1 mg $\times$ kg^{-1} $\times$ d^{-1} resulted in inhibition of tumor growth that, however, was observed not only for LNAs targeting H-RAS, but also with mismatch control oligonucleotides. This indicates that miRNA-unrelated mechanism(s) may underlie the observed effect (Fluiter et al. 2005). LNAs were also used as probes for Northern blot analysis (Válóczi et al. 2004). Recently, targeting of miR-122 by LNA and peptide nucleic acids (PNAs) in human and rat liver cells was described (Fabani and Gait 2008). Efficient uptake of PNAs was achieved by conjugation of PNAs to a cell-permeable peptide (Fabani and Gait 2008). LNAs were also used to knock down the expression of miR-21 (Corsten et al. 2007). Further, LNAs were used to target miR-122 in African green monkeys and in mice, indicating that they have the potential to be used in humans (Elmén et al. 2008). Another chemical modification that has been introduced and used along with LNAs includes cholesterol-conjugated complementary miRNAs that were named antagomirs (Krützfeldt et al. 2005). In addition to chemically modified oligonucleotides, "miRNA sponges" were used recently as microRNA inhibitors that are expressed intracellularly under the control of a strong promoter (Ebert et al. 2007). These are RNA molecules with multiple miRNA binding sites that are complementary to the heptameric seeding sequence; therefore, unlike chemically modified oligonucleotides that can target only one miRNA, a single miRNA sponge can block an entire miRNA seed family. Finally, the combination of miRNA targeting with classical chemotherapy or radiation therapy may provide new tools against cancer. As mentioned, re-expression of miR-221/222 in ERα-negative breast cancer cells sensitizes cells to tamoxifen (Zhao et al. 2008). In lung cancer, the combination of let-7 reactivation (let-7a or let-7b) and radiation therapy improved efficacy (Weidhaas et al. 2007).

CONCLUSIONS

In the last two years, our knowledge of the roles of miRNAs in cancer has expanded significantly, as shown by the tremendous number of reported studies. Bioinformatic tools, e.g., search algorithms and databases, enabled the identification of a large number of novel miRNAs that will eventually allow for the construction of the miRNome of normal and cancer cells. This calls for a revision of the integrated cancer-cell circuit originally described by Hanahan and Weinberg (2000) to include not only the established signaling pathways but also the "miRNA switches" that set them on and off. In a systems biology approach, miRNAs should prospectively be included in the complete cell interactome along with protein–protein or protein–small molecule interaction networks, as recently attempted by the construction of the let-7a interactome (Sampson et al. 2007). These networks will extend our understanding of the roles of miRNAs in normal physiology and pathophysiology. Additionally, they can be exploited for informed choices in cancer chemotherapy in a way similar to pharmacogenomics. Nonetheless, important questions remain to be answered before miRNAs can be used therapeutically. For example, a high complexity of miRNA mechanisms of action was revealed by a recent study that showed dual roles of miRNAs. It was shown that miR-369-3, let-7, and the synthetic miRNA CXCR4 all could activate the translation of mRNAs during cell cycle arrest, but they inhibited translation during cell proliferation by recruiting the protein-activating complexes on AU-rich elements (AREs) of mRNAs at the 3′ end. Recruitment is mediated by binding of the miRNA to its complementary sequence on the target mRNA (Vasudevan et al. 2007). Complexity is further increased by additional mechanisms of mRNA activation that include miRNA binding sequences at gene promoter regions, as described for E-cadherin (Place et al. 2008). Finally, it remains to be answered whether we can use miRNA profiling for cancer prognosis and/or selection of chemotherapy. Mapping of all human miRNAs and the availability of complete miRNA microarrays will be required to address this question

successfully. In addition, the development of animal models will extend our understanding of the physiological functions of miRNAs and will allow testing and validation of miRNA targeting for therapeutic purposes.

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