MicroRNAs: a complex regulatory network drives the acquisition of malignant cell phenotype

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Abstract

Several lines of evidence indicate that tumorigenesis is a complex multistep process, and that most, if not all, cancers acquire the same set of functional capabilities during development and progression, albeit through various mechanistic strategies. Increasing data show an important role of microRNAs (miRNAs or miRs) in regulating various aspects of cancer biology. This review describes the role of microRNAs during the multiple steps that drive the progressive transformation of normal cells into highly malignant derivatives, outlining the role of microRNAs in regulating the common hallmarks of tumorigenesis: self-sufficiency in growth signals, insensitivity to antigrowth signals, abnormal apoptosis, limitless replicative potential, induction and sustained angiogenesis, and tissue invasion and metastasis. Recent evidence suggests an important role of microRNAs in the regulation of the expression of most genes regulating and coordinating a wide variety of processes in endocrine glands. We will highlight microRNAs of potential relevance to endocrine tumors and hormone-dependent cancers. Through this overview of how microRNAs regulate multiple targets and entire pathways, we will provide insight into the potential to develop new molecular microRNA-targeted therapies for endocrine tumors.

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miRs and gene regulation

MicroRNAs (miRNAs or miRs) are a small (20–23 nucleotides), highly conserved noncoding class of regulatory RNA molecules expressed in a tissue- and development-specific manner (Ambros 2008). miRs are excised from a long nucleotide primary hairpin precursor RNA structure (pre-miR). The pre-miR sequence is transcribed from a larger Pol II primary transcript (pri-miR), whereas miRs are generated by a coordinated sequential processing of a long primary form into smaller miR sequences by the Drosha (in

the nucleus) and Dicer (in the cytoplasm) RNase III endonucleases and are incorporated into the RNA-induced silencing complex (Kim 2005; Fig. 1). These molecules modulate the expression of protein-coding genes by binding to complementary sequences of target mRNA transcripts. Most miRs bind to the 3' untranslated region (UTR) of the RNA transcript, but binding to coding or nontranslated 5' sequences can occur as well (Rigoutsos 2009). Perfect complementarity of miRs to target sequences leads to transcriptional repression by cleaving and degrading the mRNA transcripts. In cellular biology, miRs have the important role of reinforcing molecular networks that otherwise would be affected by random variations in gene expression. As key players in canalizing genetic programs and stabilizing developmental pathways

This paper is one of 6 papers that form part of a special Focus Section on microRNAs. The Guest Editors for this section were Professor Alfredo Fusco, Naples, Italy, and Professor Carlo M Croce, Columbus, OH, USA.

Figure 1 The pre-miR sequence is transcribed from a larger Pol II primary transcript (pri-miR), whereas miRs are generated by a coordinated sequential processing of a long primary form into smaller miR sequences by the Drosha (in the nucleus) and Dicer RNase (in the cytoplasm) III endonucleases and are incorporated into the RNA-induced silencing complex (RISC), which recognizes its multiple substrates. According to the degree of miR–mRNA complementarity, the silencing mechanism is based on the inhibition of protein translation or mRNA degradation, with a common final effect, which is a reduction of total protein expression levels.

(Hornstein & Shomron 2006), miRs minimize the noise of loose transcription regulation (Coller *et al.* 2007). This is particularly relevant in the processes of lineage commitment and cell cycle regulation, where the high extent of specific gene transcription requires fine-tuning at the translational level. Interestingly, it is now becoming clear that complex regulatory networks between miRs and their gene targets are actually common mechanisms that have evolved in mammals that enhance the robustness of gene regulation (Tsang *et al.* 2007).

Mechanisms of miR deregulation

Usually, miRs are located at cancer-associated genomic regions; however, the differential expression of miRs in malignant cells versus normal cells – and sometimes within the same tumor cell – is a complex

phenomenon, which could be explained by the complex regulation of miRs by oncogenes, tumor suppressor genes, epigenetic mechanisms, and genomic abnormalities (Calin *et al.* 2004, Calin & Croce 2006). Accordingly, studies have identified abnormalities of miRs in human cancers, revealing their transcriptional regulators (Chang *et al.* 2007, He *et al.* 2007). Cancer-associated miRs have been located downstream of major oncogenes and tumor suppressors that act as transcription factors. Similarly, transcription factors governing metastatic gene expression programs have also been found to control miRs involved in metastasis (Nicoloso *et al.* 2009). Additionally, a recent study examining epigenetic change in miR genes in tumors identified a miR hypermethylation signature characteristic of human metastasis (Lujambio *et al.* 2008). Understanding the causes of miR deregulation during the metastatic process continues to evolve; other mechanisms could be involved in this process, such as abnormal processing of precursor miRs to mature molecules and deletion or amplification because of the location of miRs in cancer-associated genomic regions.

miRs as powerful master regulators of cancer

Increasing evidence supports the supposition that miRs play an important role in different types of cancers and in various aspects of cancer biology (Nicoloso *et al.* 2009, Spizzo *et al.* 2009). Expression of miRs and the role of miRs in cancer are tissue- and tumor-specific. Abnormal miR levels in tumors have important pathogenetic consequences: miRs may act as oncogenes or suppressor genes. miRs overexpressed in tumors downregulate tumor suppressor genes, whereas miRs lost by tumors participate in oncogene overexpression. Recent evidence supports the ability of miRs to regulate several steps during neoplastic cell transformation. Many aspects of tumorigenesis, also defined as a hallmark of cancer (Hanahan & Weinberg 2000), are common to almost all, if not all, tumors: 1) self-sufficiency of tumor cells, 2) insensitivity to antigrowth signals, 3) abnormal apoptosis, 4) limitless replicative potential, 5) induction and sustained angiogenesis, and 6) invasion and metastasis. The latter is a crucial factor responsible for a worse prognosis and outcome for cancer patients. Recent data support the idea that numerous aspects of the metastatic process are also regulated by miRs. To give origin to cancer and to spread, or metastasize, a tumor cell must complete a complex set of processes, including transformation, enhanced cell motility, tissue

invasion, intravasation, dissemination, extravasation, and, finally, colonization to distant organs. However, the mechanism underlying the hallmarks of cancer and the molecular pathways underlying each of the aforementioned steps are still not well elucidated. In light of new perspectives on the role of miRs in malignant cell transformation, we will try to elucidate the significance and power of miRs in the tumorigenic process, focusing on the capabilities a cell must acquire to fulfill the malignant program with particular attention to the miRs most commonly implicated in endocrine tumors. [Figure 2](#) shows important miR functions in neoplastic cell transformation.

Self-sufficiency in growth signals

Normal cell division is regulated by stimulatory and inhibitory growth signals. These signals are transmitted to the cells by transmembrane receptors that have tyrosine kinase activity. Growth signaling involves interaction of diffusible growth factors or cytokines with transmembrane receptors as well as regulation of growth by components of the surrounding environment, extracellular matrix (ECM), and cell–cell interaction. Extracellular signals received by the tyrosine kinase receptors (TKRs) are translated into a variety of different cellular responses by a cascade of signal transductions. Malignant cells acquire the capability to escape from the tight dependency from extracellular growth signals.

The constitutive activation of TKRs, such as human epidermal growth receptor 2/3 (HER2/3), epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), and c-Met, is commonly observed in several endocrine disorders ([Heaney & Melmed 2005](#)).

Some miRs have been shown to overcome the self-sufficiency in growth signaling leading to abnormal cell proliferation. The HER members (EGFR, HER2/ERBB2, and HER3) belong to a growth factor–TKR family diffusely deregulated in tumors ([Moasser 2007](#)). A recent study demonstrated that *miR-205* is downmodulated in breast tumors, directly targets HER3 receptors, and inhibits activation of the downstream mediator Akt ([Iorio et al. 2009](#)). The reintroduction of *miR-205* in SKBr3 cells inhibits their clonogenic potential and restores a potent proapoptotic activity ([Iorio et al. 2009](#)). Another recent study showed that the cognate ERBB2 receptor can regulate the expression of *miR-21* via the mitogen-activated protein kinase and extracellular signal-regulated kinase 1/2 (ERK1/2) pathway in breast cancer cell lines,

and that *miR-21*, in turn, was able to regulate the expression of programmed cell death-4 (PDCD4), thus revealing a new mechanism for HER2/neu-induced cancer cell proliferation via miR deregulation ([Huang et al. 2009](#)). The ERBB2 TKR is also frequently overexpressed in prostate cancer, and it is associated with disease progression and poor survival. *miR-331* is decreased in prostate cancer, and it targets two specific sites within the ERBB2 mRNA 3' UTR. Transfection of multiple prostate cancer cell lines with *miR-331* showed a reduction of ERBB2 mRNA and protein expression and a block of the downstream phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway ([Epis et al. 2009](#)).

Reduction of *miR-125a* and *miR-125b* expression levels has been reported in breast, ovarian, and prostate cancers ([Iorio et al. 2005, 2007](#), [Ozen et al. 2007](#)). *miR-125a* and *miR-125b* have been proven to directly repress ERBB2/3 and to shut down the downstream phosphorylation of ERK1/2 and Akt; therefore, loss of *miR-125a* and *125b* favors the constitutive activation of the ERBB prosurvival pathway ([Scott et al. 2007](#)). Another study showed that one relevant target of *miR-210* in hypoxia was the surface receptor Ephrin-A3 ([Fasanaro et al. 2008](#)). *miR-210* was necessary and sufficient to downmodulate Ephrin-A3 expression, thereby modulating endothelial cell response to hypoxia, cell survival, migration, and differentiation ([Fasanaro et al. 2008](#)).

EGFR, also called HER1 in humans, is a surface receptor that belongs to the ErbB family of receptors and is frequently mutated or overexpressed in cancer. *miR-7* plays a critical role in the downregulation of EGFR mRNA and protein expression in different cancer cell lines (breast, lung, and glioblastoma), inducing cell cycle arrest and cell death. *miR-7* has also been shown to attenuate the activation of protein kinase B (Akt) and ERK 1/2, which are two critical effectors of the EGFR signaling ([Webster et al. 2009](#)).

A recent study on PDGFR signaling in organogenesis showed that its correct expression and function depend on *miR-140*-directed regulation ([Eberhart et al. 2008](#)). PDGFR is known to be an important tumorigenic factor in ovarian cancer ([Apte et al. 2004](#), [Iorio et al. 2007](#)), and *miR-140* was among the most significantly downregulated miRs in ovarian cancer ([Iorio et al. 2007](#)). Finally, three miRs *miR-34b*, *miR-34c*, and *miR-199a* have been shown to regulate MET receptor expression. Inhibition of these endogenous miRs resulted in increased expression of MET proteins, whereas their exogenous expression in cancer cells blocked MET-induced signal transduction ([Migliore et al. 2008](#)).

Figure 2 miRs are important regulators of gene programs involved in all the hallmarks of cancer including: (A) self-sufficiency in growth signals and insensitivity to antigrowth signals, (B) limitless replicative potential and abnormal apoptosis, (C) tissue invasion and metastasis and induction of and sustained angiogenesis.

RAS activation is a common means by which tumor cells escape growth factor dependency and become oncogene addicted. *Let-7*, one of the first miRs identified, has now become a well-documented posttranscriptional regulator of RAS (Johnson *et al.*

2005). The relevance of this mechanism has been shown by an inverse correlation between *let-7* and RAS expression levels (Johnson *et al.* 2005), and more recently, by demonstration that therapeutic approaches targeting *let-7* in lung cancer models can determine

tumor regression by affecting cell proliferation (Esquela-Kerscher *et al.* 2008, Kumar *et al.* 2008).

There are many more mitogen-kindled pathways constitutively activated by tumor cells, and for many of these, the miR regulatory functions are rapidly coming to light. *miR-143* and *miR-145* are two clustered miRs on chromosome 5q that are commonly downregulated in different tumor types, such as tumors of the colon, breast, and ovary (Michael *et al.* 2003, Iorio *et al.* 2005, 2007, Akao *et al.* 2007, Chen *et al.* 2009). Their loss has been shown to accelerate cell proliferation by deregulation of the ERK5 signaling pathway since they directly target *ERK5* mRNA (Akao *et al.* 2006). Interestingly, *miR-145*, in addition to acting on ERK5, has recently been shown to exert its antiproliferative effects on colon cancer tumor cells by suppressing insulin receptor substrate (*IRS1*) expression levels (Shi *et al.* 2007). *IRS1*, a docking protein for the insulin-like growth factor type 1 receptor (IGF1R) and insulin receptor, sends mitogenic, antiapoptotic, and antidifferentiation signals to the cell as part of a pivotal oncogenic pathway exploited by several types of tumor cells. *miR-143* has recently been shown to regulate a member of the RAS family, KRAS in the Lovo colorectal cell line; the cells that were treated with a *miR-143* inhibitor showed a stimulated cell proliferation, whereas *miR-143* overexpression had an opposite effect. Finally, inhibition of KRAS expression by *miR-143* inhibited constitutive phosphorylation of ERK1/2. Taken together, these data are evidence that *miR-143* is significant in suppressing colorectal cancer cell growth through inhibition of KRAS translation (Chen *et al.* 2009).

PI3K–Akt intracellular survival signaling is a switch diffusely turned on by tumor cells to allow malignant progression. At different levels of this pathway, miRs step in; for example, overexpression of *miR-21* and *miR-214* is responsible for PTEN repression (Yang *et al.* 2008) and consequent loss of Akt-negative regulation. Another study showed that differential expression of PTEN-targeting *miR-19a* and *miR-21* modulates PTEN protein levels and Cowden syndrome phenotypes, irrespective of the patient's mutation status, which is evidence of their roles as genetic modifiers (Pezzosoli *et al.* 2008). Another study showed a ubiquitous loss of *miR-126* expression in colon cancer cell lines (Guo *et al.* 2008a). Restoration of *miR-126* in these cancer cells induced a reduction in p85- β protein levels, which is an important regulatory subunit involved in stabilizing and propagating the PI3K signal (Guo *et al.* 2008a).

Normal cells perceive contact with the ECM component as a major prosurvival signal; therefore, modulation of malignant cell interactions with the tumor microenvironment becomes a prerequisite for tumor self-sufficiency in growth signals (Guo *et al.* 2006). *miR-124*, an epigenetically silenced miR in glioblastomas, breast cancers, and lung cancers (Lujambio & Esteller 2007, Silber *et al.* 2008), is very likely to be an important player in this process; in fact, it has been shown to regulate the integrin- β -1 subunit and laminin- γ -1 expression (Cao *et al.* 2007). Specifically in glioblastomas, *miR-124*, together with *miR-137*, was shown to contribute to tumor cell self-renewing potential by turning off the neuronal differentiation switch (Silber *et al.* 2008).

The self-sufficiency of tumor cells is also due to their ability to grow in an anchorage-independent manner, and they do not undergo apoptosis following detachment from the basal membrane (a process also termed anoikis). This quality can be achieved by down-modulation of *let-7* (Mayr *et al.* 2007), among other mechanisms, which facilitates anchorage-independent growth. This effect has been linked to de-repression of the pleiotropic architectural transcription factor HMGA2, a major *let-7* target (Lee & Dutta 2007), serving multiple differentiation programs. In addition, tumor cells support themselves by diverting bystander cells to support their proliferative needs; in line with this requirement, the oncogenic *miR-155*, overexpressed in a wide variety of tumors (Volinia *et al.* 2006), is induced in macrophages by the JNK pathway and by numerous inflammatory mediators (O'Connell *et al.* 2007), underscoring the link between inflammation and cancer.

Insensitivity to antigrowth signals

Growth inhibitory signals received by transmembrane cell surface receptors coupled to intracellular signals cause cells to enter into quiescence or a differentiated postmitotic state; both conditions require an arrest of and withdrawal from the cell cycle. Cancer cells evade these mechanisms, becoming insensitive to antigrowth signals. Specifically, this circuit, which enables cells to respond to antigrowth signals, is associated with components governing the transit of the cells through the G₁ phase of the cell cycle. Almost all antiproliferative signals are controlled by pRb and its subunits (RBb1/105, p107, and Rb2/p130); these, in turn, sequester E2F transcription factors repressing the expression of genes required for cell cycle progression (Iaquinta & Lees 2007). Disruption of the Rb pathway unlocks E2Fs and allows cell proliferation.

Although E2F transcription factors are prevalently superintended by the tumor suppressor Rb through sequestration, their expression levels are also relevant and have been illustrated to be controlled at the posttranscriptional level by a series of miRs. These belong to two highly conserved miR families, which are grouped in distinct genomic clusters – the *miR-17-92* cluster on chromosome 13q31.1 and the *miR-106b-25* cluster on chromosome 7q22.1. Specifically, *miR-20a* inhibits E2F2 and E2F3 (Sylvestre et al. 2007), whereas *miR-17-5p*, *miR-20a*, *miR-106b*, and *miR-92* inhibit E2F1 (O'Donnell et al. 2005, Petrocca et al. 2008). Furthermore, E2F-activating transcription factors have recently been shown to regulate the expression of these clustered miRs (O'Donnell et al. 2005, Woods et al. 2007, Petrocca et al. 2008). Unbound E2Fs increase *miR-17-92* and *miR-106b-25* expression, and in turn, these keep E2F levels in check and participate in the delicate equilibrium between cell proliferation and apoptosis,

which is affected by alteration of E2F levels. Additionally, the *miR-17-92* cluster preferentially shuts down the proapoptotic E2F1 in favor of the proliferative E2F3 transcriptional network. Upregulated miRs from these clusters are specifically able to target apoptotic and growth inhibitory proteins, such as p21, PTEN, and BIM (Petrocca et al. 2008). Furthermore, the so-called *oncomiR-17-92* has been shown to be directly transactivated by MYC (O'Donnell et al. 2005) and to cooperate in tumorigenesis (He et al. 2005a). Similar to MYC, the *miR-17-92* cluster appears to be essential for integrating signals from the environment into the G₁ phase of the cell cycle and determining whether a signal should be interpreted as proliferative or apoptotic (Coller et al. 2007). Under physiological conditions, the *miR-17-92* cluster avoids an excessive MYC activation, dampening the E2F positive feedback loop and ensuring the correct timing of E2F accumulation at the end of the G₁ phase of the cell cycle.

Table 1 Important deregulated *microRNAs* in endocrine tumors

Endocrine tumors ^a	Deregulated <i>microRNAs</i>	
	Downregulated	Upregulated
Breast cancers	<i>Let-7</i> family, <i>miR-9</i> family (associated with vascular invasion and lymph node metastases), <i>miR-10b</i> , <i>miR-17-92</i> ^b , <i>miR-29</i> family, <i>miR-101</i> , <i>miR-124</i> family ^c , <i>miR125a/b</i> , <i>miR-126</i> , <i>miR-143/miR-145</i> cluster <i>miR-200</i> family ^d , <i>miR-206</i> (associated to relapse), <i>miR-335</i> (during the metastatic progression)	<i>miR-10b</i> (in metastatic tumors), <i>miR-17-5p</i> (breast cancer cell lines), <i>miR-21</i> , <i>miR-27</i> (MDA-MB-231 cells), <i>miR-29b-2</i> , <i>miR-146</i> , <i>miR-155</i> , <i>miR-181</i> family, <i>miR-373</i> and <i>miR-520c</i> (in metastatic tumors)
Prostate cancers	<i>Let-7</i> family, <i>miR-15a/miR-16-1</i> cluster, <i>miR-101</i> , <i>miR-125a/b</i> , <i>miR-146a</i> , <i>miR-205</i> , <i>miR-221</i> (aggressive and metastatic tumors), <i>miR-449</i>	<i>miR-21</i> , <i>miR-106b-93-25</i> cluster, <i>miR-181</i> family
Ovarian cancers	<i>Let-7</i> family, <i>miR-9</i> , <i>miR-15-16</i> , <i>miR-17-92</i> ^b , <i>miR-29a/b</i> , <i>miR-34</i> , <i>miR-105</i> , <i>miR125a/b</i> , <i>miR-140</i> , <i>miR-145</i> , <i>miR-199a</i> , <i>miR-200</i> family ^e	<i>miR-21</i> , <i>miR-30b/d</i> , <i>miR-181b</i> , <i>miR-203</i> , <i>miR-373</i>
Liver cancers	<i>miR-1</i> , <i>miR-23b</i> , <i>miR-26a</i> , <i>miR-34a</i> , <i>miR-101</i> , <i>miR-122a</i> (in tumor with poor prognosis), <i>let-7</i> , <i>miR-141</i> , <i>miR-199a</i>	<i>miR-21</i> , <i>miR-122a</i> , <i>miR-221/222</i>
Pancreas and gastric cancers	<i>Let-7</i> family, <i>miR-15a/miR-16-1</i> cluster; <i>Let-7</i> family, <i>miR-31</i> , <i>miR-141</i> , <i>miR-143/miR145</i> , <i>miR-451</i>	<i>miR-155</i> , <i>miR-181</i> family, <i>miR196a</i> , <i>miR-217</i> , <i>miR-21</i> , <i>miR-29b-2</i> , <i>miR-106b-93-25</i> cluster, <i>miR-128b</i> <i>miR-191</i> , <i>miR-223</i>
Thyroid cancers	<i>pre-miR-146a</i> (SNP-rs2910164), <i>miR-26a</i> , <i>miR-30a-5p</i> , <i>miR-30d</i> , <i>miR-125b</i> , <i>miR-138</i>	<i>miR-17-92</i> , <i>miR-21</i> , <i>miR-106a/b</i> , <i>miR-146b</i> , <i>miR-181b</i> , <i>mR-192</i> , <i>miR-197</i> , <i>miR-221</i> , <i>miR-222</i> , <i>miR-328</i> , <i>miR-346</i>
Adrenal cancers	<i>miR-9</i> , <i>miR-34a</i> , <i>miR-125a/b</i> , <i>miR-214</i> , <i>miR-511</i>	<i>miR-17-92</i> cluster, <i>miR-184</i> , <i>miR-221</i> , <i>miR-449</i> (hyperplasia), <i>miR-503</i>
Pituitary tumors	<i>Let-7</i> family, <i>miR-15a/miR-16-1</i> cluster, <i>miR-21</i> , <i>miR-141</i> , <i>mR-143</i> , <i>miR-145</i> , <i>mR-150</i>	

^aAll tumor histotypes.
^bLoss of heterozygosity.
^cHypermethylation.
^dDownregulated in metastatic breast cancers.
^eDifferent studies reported dissimilar miRs expression levels (see text for further details).

In tumors with MYC activation, *miR-17-92* cluster upregulation overcomes protective MYC-induced apoptotic E2F responses, leading to uncontrolled cellular proliferation. The *oncomiR-17-92* is upregulated in several tumors (Table 1). Deregulated expression of MYC, which renders tumor cells nonresponsive to external antigrowth signals, is among the most common abnormalities in human malignancies, and miRs have been proven to contribute directly to MYC deregulation. Through the transcriptional repression of *let-7a*, MYC initiates a positive feedback loop that sustains high MYC expression; in fact, *let-7a* is competent to repress MYC (Sampson *et al.* 2007, Kim *et al.* 2009). Additionally, *miR-34a*, a commonly downregulated miR in cancers, also targets MYC and participates in its abnormal expression in tumors (Wei *et al.* 2008).

Several epithelial malignancies are characterized by insensitivity of cells to the homeostatic effect of transforming growth factor- β (TGF- β ; Heldin *et al.* 2009, Wu & Hill 2009). The TGF- β pathway can be altered in tumor cells by downregulation or mutations in the TGF- β receptor, loss of Smad signaling flow from the cytoplasm to the nucleus, or mutations in growth arrest effector genes (e.g. p27^{KIP1}, p21^{CIP1}, p16^{INK4a}, and p15^{INK4b}). Impaired TGF- β signaling occurs in several neoplasms where cell cycle checkpoints activated following growth arrest stimuli may be suppressed by activation of MDM2 and cyclin-dependent protein kinase-4 (CDK4) or by overexpression of cyclin D1, a G₁ phase sensor for growth factors that promotes entry into the cell cycle. It has been recently shown that cyclin D1 upregulation may be due to truncation of its 3' UTR, which renders it insensitive to *miR-16* downmodulation (Chen *et al.* 2008), which is well known to regulate transcripts involved in cell cycle progression (Linsley *et al.* 2007). Another crucial cell cycle gatekeeper to which tumor cells can become insensitive is the cyclin-dependent kinase inhibitor (CKI) p27^{KIP1}, the expression of which is frequently lost in cancers (Chu *et al.* 2008). *miR-221* and *miR-222*, two miRs found to be upregulated in many tumors such as papillary thyroid carcinomas (PTCs), hepatocarcinomas, and glioblastomas, directly target p27^{KIP1}, providing cells an additional tool to avoid cell cycle arrest (le Sage *et al.* 2007, Visone *et al.* 2007a, Fornari *et al.* 2008). *miR-221* and *miR-222* are also able to downregulate the related CKI p57^{KIP2}, preventing quiescence and favoring S-phase entry (Fornari *et al.* 2008).

Similarly, the CKI p21^{CIP1}, which is downstream of both TGF- β and DNA damage-induced growth arrest pathways, is repressed by miRs upregulated in tumors

from the *miR-106b-25* cluster (Petrocca *et al.* 2008). Cell cycle arrest in response to DNA damage is an important antitumorigenic mechanism.

The *miR-15a/miR-16*, located at chromosome 13q14, are frequently deleted or downregulated in different types of tumors, in particular, squamous cell carcinomas and adenocarcinomas of the lung where the expression of *miR-15a/miR-16* is inversely correlated with the expression of cyclin D1. In nonsmall cell lung cancer (NSCLC) cell lines, cyclins D1, D2, and E1 are physiologically regulated by *miR-15a/miR-16*. Consistent with these results, overexpression of these miRs induces cell cycle arrest in G(1)–G(0). Interestingly, H2009 cells lacking Rb are resistant to *miR-15a/miR-16*-induced cell cycle arrest, whereas reintroduction of functional Rb resensitizes these cells to miR activity. In contrast, downregulation of Rb in A549 cells by RNA interference confers resistance to these miRs. Thus, these miRs play an important role in inducing cell cycle arrest in an Rb-dependent manner. These data confirm that G(1) cyclins are major targets of *miR-15a/miR-16* in NSCLC, contributing to cell cycle control and partially implicated in the tumorigenesis of NSCLC (Bandi *et al.* 2009).

A few miRs, such as *miR-34a*, were recently shown to play key regulatory roles in cell cycle progression. *miR-34a* is induced in response to p53 activation and mediates G₁ arrest by downregulating multiple cell cycle-related transcripts. *miR-34a* directly targets *CDK6*, *CCND1*, *CDK4*, *CCNE2*, and *MET* (He *et al.* 2007, Sun *et al.* 2008). Therefore, *miR-34a* deficiency increases CDK activity with sequential Rb hyperphosphorylation in G₁, G₁–S transition, and initiation of DNA synthesis. Cyclin G₁, an atypical cyclin upregulated by Arf-dependent p53 induction, is also a mediator of Rb-dependent G₁ growth arrest (Zhao *et al.* 2003).

Also, genotoxic stress promotes the p53-dependent upregulation of the homologous miRs *miR-192* and *miR-215*. Like *miR-34a*, activation of *miR-192/215* induces cell cycle arrest, suggesting that multiple miR families operate in the p53 network (Braun *et al.* 2008, Georges *et al.* 2008).

This protective mechanism against uncontrolled cellular proliferation is lost in hepatocellular carcinomas by upregulation of *miR-122a* (Gramantieri *et al.* 2007). *miR-122* is a liver-specific miR (Chang *et al.* 2004) that is exploited by hepatitis C virus infection (Jopling *et al.* 2005) and can specifically suppress the negative *CCNG1* (Gramantieri *et al.* 2007), thereby favoring an unscheduled cell cycle progression.

Once cells have the ability to sustain their own survival and are insensitive to external inhibitory

signals, tumorigenesis as an uncontrolled cell proliferation process is still kept in check by the apoptotic and senescent programs. Normal cells use these defense mechanisms in response to insults such as environmental stress (e.g. DNA-damaging agents, reactive oxygen species (ROS), and hypoxia) and oncogenic stress, activating them mostly in a p53-dependent manner. When these hedges are halted, cells can grow beyond their normal thresholds. The most common breach in these tumor suppressor barriers is represented by p53 abnormalities.

Abnormal apoptosis

Tissue homeostasis in normal cells is maintained by a perfect equilibrium of cell division and cell loss. Acquired resistance to PDCD (apoptosis) is a hallmark of all types of cancers. Apoptosis is a complex multistep process, which requires the involvement of numerous molecules. The apoptotic machinery consists of two families of components: sensors and effectors. The first class is composed of cell surface receptors that bind to survival or death factors: IGF1R, tumor necrosis factor- α (TNF- α), TNF-related apoptosis-inducing ligand (TRAIL), and TNF-receptor superfamily member 6 (FAS) ligands. These signals usually regulate the second class of components, which function as effectors of cell death (members of the Bcl-2 family). Both families of apoptotic signals convey on the caspase cascade (i.e. caspases 8 and 9), which activates several additional effector caspases that execute the cell destruction program. Tumor cells are able to escape from the correct and complete execution of the apoptotic program despite being continuously exposed to proapoptotic stimuli (Letai 2008).

A regulatory path between miRs and apoptosis has been demonstrated at different levels in several cancer cell systems. Specifically, both the proapoptotic tumor suppressor p53–TP53 (Chang et al. 2007, Raver-Shapira et al. 2007, Welch et al. 2007) and antiapoptotic IL6/STAT3 (Löffler et al. 2007) pathways may regulate miR expression at the transcriptional level. Additionally, miRs may be able to induce (Cimmino et al. 2005) or repress (He et al. 2005a) apoptosis directly and autonomously by targeting antiapoptotic or proapoptotic proteins respectively.

Genomic regions frequently altered in cancers (e.g. fragile sites, minimal regions of loss of heterozygosity, minimal regions of amplification, and common breakpoint regions) have been characterized for their nonrandom association with miR genomic locations (Calin et al. 2004). For example, the cancer-associated genomic region 1p36, frequently lost or rearranged in

many tumor types, contains candidate tumor suppressors (i.e. *CDH5*) as well as important miRs, such as *miR-34a* (Cole et al. 2008, Hermeking 2009). The tumor-suppressive functions of *miR-34a* have been extensively studied in many tumors, especially in human neuroblastomas (Welch et al. 2007, Cole et al. 2008, Wei et al. 2008) where its loss synergizes with MYCN amplification, which is in line with the fact that *miR-34a* is a MYCN-negative regulator (Wei et al. 2008). *miR-34a* is also able to induce cell cycle arrest and subsequent caspase-dependent apoptosis through Bcl-2 (Cole et al. 2008, Wei et al. 2008) and E2F3 repression (Welch et al. 2007). In addition, analysis of the transcriptome induced by *miR-34* overexpression exhibits high similarity to that observed with p53 induction, being highly enriched for genes regulating cell cycle progression, apoptosis, DNA repair, and angiogenesis (Bommer et al. 2007, Chang et al. 2007, He et al. 2007). Accordingly, *miR-34* family members, as direct transcriptional targets of p53 (He et al. 2007), are essential for the correct execution of p53-dependent cellular responses. Although *miR-34a* proapoptotic effects seem to be cell type-dependent, *miR-34a* was proven to increase in a stress-induced renal carcinogenesis rat model, and its inhibition was shown to affect tumor cell proliferation (Dutta et al. 2007). miRs can also act upstream of p53. For example, *miR-127*, another epigenetically silenced miR in tumors, seems to repress the protooncogene *BCL6*, a p53-negative regulator (Saito et al. 2006, Lujambio & Esteller 2007).

A recently reported study showed that *miR-330* expression was significantly lower, and that this induced apoptosis in human prostate cancer cell lines (Lee et al. 2009). Bioinformatics analyses revealed a conserved target site for *miR-330* in the 3' UTR of E2F1. The same study reported that the expression level of *miR-330* and E2F1 was inversely correlated in cell lines and prostate cancer specimens, and that E2F1 was negatively regulated by *miR-330*. Additionally, *miR-330* was able to induce apoptosis in prostate cancer cells through E2F1-mediated suppression of Akt phosphorylation (Lee et al. 2009).

miR-21 overexpression is a trait common to several malignancies (Volinia et al. 2006, Hiyoshi et al. 2009, Yamamichi et al. 2009). *miR-21* acts predominantly as an antiapoptotic protein by blocking the expression of critical apoptosis-related genes (Chan et al. 2005). Among *miR-21* apoptotic targets are the tumor suppressor PDCD4 in breast cancer cells (Frankel et al. 2008, Lu et al. 2008) and the tumor suppressor PTEN in hepatocellular cancer (HCC) cells (Meng et al. 2006). *miR-21* favors the transmission of

antiapoptotic survival signals through the PI3K–Akt pathway. Additionally, *miR-21* indirectly supports Bcl-2 antiapoptotic activities (Si *et al.* 2007). Therefore, repression of *miR-21* should sensitize cells to therapeutic strategies inducing apoptosis, as demonstrated in a glioma model, by combining neural precursor cells expressing secreted TRAIL with *antimiR-21* oligonucleotides (Corsten *et al.* 2007).

The miR relevance to the TRAIL apoptotic pathway has been dissected with an RNA interference screening strategy, and many miRs (*let-7c*, *miR-10a*, *miR-144*, *miR-150*, *miR-155*, and *miR-193*) were found to affect the activation of the caspase cascade. All of the identified miRs displayed as putative target genes encoding death receptors, caspases, and other apoptosis-related genes (Ovcharenko *et al.* 2007). For *miR-155*, among others, the stress-induced proapoptotic protein TP53BP1 has been shown to be a target in pancreatic tumors and to be responsible for part of *miR-155* protumorigenic effects (Gironella *et al.* 2007).

Limitless replicative potential of tumors

Normal cells have the capacity for a finite number of cell divisions, also called limited replicative potential; normally, the cells stop proliferating and enter into a state of cellular senescence, which is a physiological withdrawal from the cell cycle in response to numerous stimuli. To ensure expansive tumor growth, a tumor cell needs to acquire additional programs independently from the cell–cell signaling described above. Disruption of the limited replicative potential leads tumor cells to expand to a macroscopic size. Senescent cells present different morphology, express senescence-associated β -galactosidase, and are insensitive to proliferative stimuli (Dimri *et al.* 1995). In normal cells, the most important players of the senescent program are p53, Rb, and p16^{INK4a} (Campisi 2005). Cells reach senescence when they complete the allowed number of doubling, entering a state of permanent proliferative arrest. This is known as ‘replicative senescence’ and is due to the progressive erosion of telomeres, which is sensed by the cell as DNA damage.

Tumor cells may circumvent replicative senescence and acquire a limitless replicative potential either by abnormal telomerase enzyme activation, alternative lengthening of telomeres, or by homologous recombination events at telomeres (Bailey *et al.* 2004). The senescent program is kept in check in tumor cells by expression of the Polycomb repressor B lymphoma Mo-MLV insertion region 1 homolog

(BMI; Grinstein & Wernet 2007). Telomerase maintenance is present in virtually all types of malignant cells, with different neoplasms presenting heterogeneous cell populations differing in their replicative potential. While most cancer cells have limited replicative potential, small subpopulations (i.e. cancer stem cells) have an infinite replicative capacity to reconstitute the tumor. Alterations of telomere dynamics and telomerase are also described in the progression of many tumors (Stewart & Weinberg 2000).

Replicative senescence can be accelerated by DNA-damaging agents, ROS, and uncontrolled oncogene activation. Hyperactive oncogenes are especially responsible for a high degree of stalling and collapsing of replication forks, causing DNA double-strand breaks and facilitating a DNA damage response (Bartek & Lukas 2006, Bartek *et al.* 2007). The molecular mechanisms underlying the escape from senescence include loss of the TP53 and telomerase maintenance.

miRs have been shown to be relevant in diverting oncogene-induced premature senescence. *miR-373* and *miR-372* were identified as capable of allowing transformation of primary cells harboring oncogenic RAS and wild-type p53 by neutralizing p53-mediated CDK inhibition through suppression of *LATS2* (Voorhoeve *et al.* 2006).

Not surprisingly, telomerase deregulation can be achieved by tumors through a reduction in normal expression levels of *miR-138*, which can repress *TERT* mRNA translation, as shown in undifferentiated and differentiated thyroid cancer cell lines (Mitomo *et al.* 2008).

Epigenetic mechanisms are also involved in telomere homeostasis, and recently, they have been shown to be controlled by *miR-290* cluster. The *miR-290* family is able to target the *RBL2* transcription factor, which in turn controls expression of DNMT3A and DNMT3B methylating enzymes that maintain telomere length (Benetti *et al.* 2008). To date, the relevance of this mechanism has not been directly assessed in cancer models.

Concerning senescence, p53-activated miRs are also important. The *miR-34* family of all transcriptional targets participates in the senescent program (Kumamoto *et al.* 2008) through modulation, at least for *miR-34a*, of the E2F signaling pathway (Tazawa *et al.* 2007). Of note, recently *RGM249* (RNA gene for miRs) was discovered, which could be among the first miR precursor genes that are involved in the differentiation and function upstream of hTERT in tumor cells (Miura *et al.* 2009).

Induction and sustained angiogenesis

In normal tissues, growth of new vessels (angiogenesis) is transitory and finely regulated, whereas tumor cells – in order to proliferate and induce and sustain neovascularization – need to turn on the ‘angiogenic switch’, thereby producing high amounts of proangiogenic factors (Semenza 2000, Tanaka et al. 2003). The rapid expansion of tumor cells creates a hypoxic environment, leading to tumor necrosis and inducing cell adaptation responses, such as hypoxia-induced factor (HIF)-dependent survival pathways and angiogenesis. Quiescent endothelial cells are thus activated and recruited by angiogenic factors, and stimulated to proliferate and form new blood vessels. Tumors count on their angiogenic potential to outgrow locally and spread to distant sites, and increased angiogenesis has been correlated with a poor prognostic outcome for patients with many types of tumors (Semenza 2003).

Recent reports have established a link between hypoxia, a key feature of the tumor microenvironment, and a group of miRs, in part explained by direct HIF1 transcriptional activation of specific miRs (Kulshreshtha et al. 2007). Microarray-based expression profiles found that a specific spectrum of miRs, including *miR-23*, *miR-24*, *miR-26*, *miR-27*, *miR-103*, *miR-107*, *miR-181*, *miR-210*, and *miR-213*, is induced in response to low oxygen. These miRs may act by decreasing proapoptotic signal-sustaining cell survival (*miR-26* family, *miR-107*, and *miR-210*) (Kulshreshtha et al. 2007) or by participating in the angiogenic process. miRs may participate in the angiogenic process like *miR-210* through direct repression of the endothelial ligand Ephrin A, which partially regulates endothelial cell chemotaxis and tubulogenesis (Fasanaro et al. 2008), or like *miR-27a*, which represses *ZBTB10* (zinc finger gene), thereby inducing specific protein-dependent transcription of both survival and angiogenic genes, such as survivin, VEGF, and VEGFRs (Mertens-Talcott et al. 2007).

A link between miRs and angiogenesis was initially investigated in Dicer knockout mice, which display angiogenic defects during embryogenesis (Yang et al. 2005). Further studies based on genetic silencing of *DROSHA* and *DICER1* in human endothelial cells confirmed the involvement of miRs in angiogenesis (Kuehbach et al. 2007, Súrez et al. 2007). In particular, *lef-7f* and *mir-27b* expression was strongly reduced, and the *let-7* family was proven to down-regulate the antiangiogenic thrombospondin-1 (TSP1).

The most important angiogenic factor is the VEGF, which is highly expressed in several tumors. VEGF is

regulated at multiple levels (both transcriptional and translational) downstream of a wide range of stimuli, such as hypoxia, HIF1, growth factors, cytokines, and hormones (Loureiro & D’Amore 2005). Recently, it has been shown that VEGF is also restrained at the posttranscriptional level by miRs. For example, miRs downregulated in hypoxic conditions, such as *miR-16*, *miR-15a*, *miR-20a*, and *miR-20b*, modulate VEGF expression levels, thereby supporting the angiogenic process (Hua et al. 2006). This creates a positive regulation loop in which hypoxia-repressed miRs reinforce the expression levels of a potent proangiogenic hypoxia-induced growth factor such as VEGF (Hua et al. 2006).

MYC is also a VEGF transcription activator promoting neovascularization in tumor cells. Colonocytes expressing c-Myc showed upregulation of *miR-17-92*, repressing TSP1 and related proteins such as connective tissue growth factor (CTGF), whereas *miR-17-92* knockdown cells restored TSP1 and CTGF expression and formed larger and better perfused tumors. These findings established a role for miRs in noncell-autonomous MYC-induced tumor phenotypes (Dews et al. 2006). Two additional miRs, *miR-18* and *miR-19*, are responsible for CTGF and TSP1 repression respectively (Dews et al. 2006), both of which are important for angiogenesis and ECM remodeling.

Another important mechanism in angiogenesis is the upregulation of growth factor receptors on endothelial cells. *miR-296* has been shown to play a major role in this process. Growth factor-induced *miR-296* contributes significantly to angiogenesis by directly targeting the hepatocyte growth factor-regulated tyrosine kinase substrate (HGS) mRNA, leading to decreased levels of HGS and thereby reducing HGS-mediated degradation of the growth factor receptors VEGFR2 and PDGFR- β (Würdinger et al. 2008). However, miRs may have different expressions and functions in different tissues (tissue-specific functions). For example, loss of *miR-221* and *miR-222* in endothelial cells sustains the proliferative and angiogenic properties of KIT, whereas upregulation of *miR-221* and *miR-222*, which is commonly observed in tumor cells, regulates the CKI p27 (le Sage et al. 2007), increasing cell proliferation and enhancing their metastatic potential (Baldassarre et al. 2005, Chu et al. 2008). Similarly, *miR-126* has an increased expression in endothelial cells, where it sustains proangiogenic factor signals through SPRED1 and PI3K regulatory subunit 2 (PI3KR2) repression (Fish et al. 2008), and its downregulation in tumor cells contributes to abnormal proliferation.

The angiogenic switch is controlled at the transcriptional level by the developmental homeobox genes, which can be grouped as pro- or antiangiogenic (Gorski & Walsh 2003). The antiangiogenic *Gax* homeobox genes are highly expressed in quiescent endothelial cells and are rapidly downregulated after mitogenic and proangiogenic signals. *miR-130a*, a miR involved in megakaryocytic differentiation and expressed by endothelial cells, was observed to be triggered by angiogenic stimuli in a reciprocal way compared to *GAX*. Accordingly, it was shown that *miR-130a* directly represses (at the posttranscriptional level) the expression of *GAX* and (to a lesser extent) the expression of a similar antiangiogenic factor, such as *HoxA5*, thereby allowing the angiogenic process (Chen & Gorski 2008).

Tumor invasion and metastasis

The spread of tumor cells to locoregional and distant sites requires that the cells detach from the primary tumor, enter the blood or lymphatic system (Chambers *et al.* 2002, Sahai 2007), and infiltrate and disseminate in the ECM as single cells or expand in solid cell strands, sheets, files, or clusters (Friedl 2004). This metastatic capability required by the cells is associated with genetic–biochemical alterations of cell–cell and cell–matrix interactions. Some tumors (e.g. sarcoma) disseminate via single cells, whereas epithelial tumors commonly use a collective migration mechanism (Friedl 2004). In particular, epithelial cells preferentially use a protease-dependent mesenchymal strategy that allows degrading ECM barriers and cells to move (Wolf *et al.* 2003, Friedl 2004).

Tissue invasion and metastases often require different cell capabilities (e.g. detachment from the primary tumor and adhesion to distant metastatic sites), suggesting a fast cellular adaptation and clonal selection that only a small subset of cancer cells will overcome (Wong *et al.* 2001). Overall, the efficiency of the metastatic process is correlated with increased cell survival (Kim *et al.* 2004); in fact, oncogenes that promote cell proliferation and survival in the primary tumor, such as *BCL2* or active *RAS*, are also involved in promoting malignant cell survival at metastatic sites (Wong *et al.* 2001). Additionally, cells at metastatic sites must display high replicative potential, as proposed by the model in which macroscopic metastases are formed only when the self-renewing ‘cancer stem cell’ arrives at a secondary site (Bjerkvig *et al.* 2005).

An increasing number of studies have revealed miR signatures of metastasis and have shown that the targets

of most of these metastasis-associated miRs are proteins involved in the regulation of cell motility, cell–cell adhesion, and cell–matrix interactions.

miR-29c, which is significantly reduced in highly invasive and metastatic nasopharyngeal carcinoma, has as preferential targets the genes encoding for ECM proteins, such as collagen or laminin- γ (Sengupta *et al.* 2008), further supporting the model in which the composition of the local microenvironment is an important variable in the ability of cancer cells to properly move and/or survive. Similarly, downmodulation of the adhesion molecule CD44, induced by *miR-373* and *miR-520c* in a breast cancer model, could explain their effect as metastasis-promoting miRs. In fact, CD44 is frequently upregulated in a wide variety of malignancies (Ponta *et al.* 2003, Marhaba *et al.* 2008), and CD44 modulates cell adhesiveness, motility, matrix degradation, proliferation, and survival – all traits that allow a tumor cell to progress through the metastatic cascade (Marhaba & Zoller 2004, Asangani *et al.* 2008, Zhu *et al.* 2008). Alterations in cytoskeletal protein expression levels by miRs can affect cell morphology and motility, as has been proven for *miR-21*, which can reduce the levels of tropomyosin 1 and affect cell invasion (Marhaba & Zoller 2004, Asangani *et al.* 2008, Zhu *et al.* 2008). In addition, *miR-21*, by acting on PTEN, potentiates tumor cell metastatic abilities by favoring the Akt survival pathway and the Akt-dependent modulation of focal adhesion kinase phosphorylation and matrix metalloprotease expression levels (Meng *et al.* 2007). *miR-21* has an important role in the tumor progression of several neoplasms, including directly targeting MARCKS and promoting apoptosis resistance and invasion in prostate cancer cells (Li *et al.* 2009b); regulating proliferation and invasion in esophageal squamous cell carcinoma (Hiyoshi *et al.* 2009); posttranscriptionally down-regulating the tumor suppressor PDCD4, stimulating invasion, intravasation, and metastasis in colorectal cancer (Asangani *et al.* 2008); and regulating tumor progression in several additional tumors. Another study reported that *miR-122*, a marker of hepatocyte-specific differentiation, is specifically repressed in a subset of primary tumors characterized by poor prognosis, and that loss of *miR-122* expression in tumor cells segregates with specific gene expression profiles linked to cancer progression and invasion. Loss of *miR-122* resulted in an increase in cell migration and invasion (Coulouarn *et al.* 2009). Downregulation of *miR-221* has been shown to be negatively correlated with the expression of the protooncogene *c-kit* in primary prostate carcinoma. In a large study cohort, *miR-221* was progressively

downregulated in aggressive forms of prostate carcinoma. Downregulation of *miR-221* was associated with clinicopathological parameters, including the Gleason score and clinical recurrence during follow-up. Statistical models showed that *miR-221* downregulation was linked to tumor progression and recurrence in a high-risk prostate cancer cohort, making this miR an important prognostic factor in high-risk prostate carcinoma (Spahn et al. 2009).

More interesting is the role of the *miR-200* family, which has been recently associated with the epithelial–mesenchymal transition (EMT) process (Gregory et al. 2008, Korpál et al. 2008, Park et al. 2008). An EMT-like process occurs as part of embryonic development and during carcinogenesis when tumor cells undergo a change from a differentiated to a dedifferentiated form, leading to a more invasive cell phenotype. This is an adaptation response, a migratory escape strategy, occurring during the course of tumor progression (Friedl 2004) where adherens junctions and desmosomes become partially dissociated and epithelial cells switch from a collective invasion pattern to a detached and disseminated cell migration method (Friedl & Wolf 2003). All members of the *miR-200* family, usually together with *miR-205*, are significantly downmodulated during the EMT process (Gregory et al. 2008). These miRs are able to regulate the expression of key transcription factors belonging to the E-box-binding family (such as Snail, Slug, ZEB1, and ZEB2/SIP-1) involved in the EMT and in tumor invasion and metastasis, which in turn regulate several EMT-related genes, such as epithelial (E)-cadherin, mucin, tight junction protein ZO3, connexin 26, and plakophilin 2 (Peinado et al. 2007). In particular, one of the ZEB1 targets is represented by a typical epithelial marker (*CDH1*) that is lost during the first step of EMT. Recent observations strengthen the role of the *miR-200–ZEB1* axis in EMT, demonstrating that on the one hand *miR-200* regulates ZEB1 expression and on the other hand ZEB1 regulates *miR-200* transcription (Burk et al. 2008), thus establishing a complex regulatory loop that may ensure the tight superintendence of the EMT process.

Given the fact that *miR-373* can act as a direct transcriptional activator of *CDH1*, it is possible that its loss during the metastatic process might also contribute to the EMT process (Place et al. 2008).

The reverse of EMT, MET, also occurs in carcinogenesis and certainly is regulated by this miR family. However, whereas EMT during cancer progression is well characterized, MET is more difficult to observe *in vivo*.

An important study found an intriguing loss of expression of *miR-200* in advanced breast cancer, where these miRs seem to regulate several oncogenic functions (Gregory et al. 2008). A dissimilar pattern of expression of this miR family has been found in different studies on ovarian cancer, most likely reflecting differences in the selection, amount, and composition of tumoral and nontumoral cells analyzed (see the review by Peter (2009)).

Crosstalk occurs between *miR-200* and *let-7* at different levels during carcinogenesis. *Let-7* is an important regulator of cell differentiation. Although *let-7* is unlikely to be a direct EMT regulator (Park et al. 2007, Shell et al. 2007), many factors are regulated by both miR families; for example, HMGA2 is directly regulated by *let-7*, and HMGA2 is often required for TGF- β -induced EMT of mouse mammary epithelial cells regulating Snail, Slug, Id2, and Twist. More recently, it was also demonstrated that ZEB1 and ZEB2 could be regulated by HMGA2, and that the regulation of Snail promoter by HMGA2 involves binding of this factor to Smad3 and Smad4 (Thiault et al. 2008). In addition, RAS, which is another target of *let-7* and a driving force for EMT, has been reported to induce HMGA2 expression (Tchernitsa et al. 2004). These two miR families could interact and be considered part of a system loop regulating dedifferentiation processes, such as EMT, and stem cells and stem cell-like properties.

miR-155 is another important factor contributing to TGF-induced epithelial cell plasticity and therefore to cell migration and invasiveness. It has been found to be a downstream effector of TGF and Smad4 and to partially account for TGF-induced RhoA suppression and therefore responsible for the dissolution of cell tight junctions (Kong et al. 2008). Consistently, *miR-155* has been reported to be upregulated in invasive breast cancer, supporting its involvement in breast cancer metastasis (Iorio et al. 2005, Volinia et al. 2006).

Epigenetic alterations with upregulated EP300-targeting miRs *miR-194*, *miR-200b*, *miR-200c*, and *miR-429* were correlated with reduced EP300 mRNA and protein in highly metastatic pancreatic ductal adenocarcinoma (PDAC) cells (Mees et al. 2009a). Another study by Mees et al. (2009b) showed that upregulated CD40-targeting *miR-224* and *miR-486* are related to downregulated CD40 protein expression at cell surfaces in highly invasive and metastatic PDAC. These results demonstrated that miRs might be able to modulate the expression of metastasis-specific suppressor genes and metastatic behavior.

Cancer cells have the potential to adopt different types of motility to successfully spread to distant organs. The faster one (amoeboid motility) is accompanied by increased activation of Rho pathways. Interestingly, two different works on breast cancer metastasis formation independently identified miRs that were able to trigger Rho pathways (Ma *et al.* 2007, Bhaumik *et al.* 2008). *miR-10b* overexpression indirectly increases, by repression of its negative transcriptional regulator, RhoC protein levels, leading to increased cell motility and metastasis formation in breast tumors (Ma *et al.* 2007). *miR-146a* expression is lost in metastatic breast cancer, and this loss has been documented as directly inhibiting the expression of the Rho target ROCK1 affecting cell movements (Bhaumik *et al.* 2008). In a similar fashion, *miR-31* mediates the inhibition of several steps during breast cancer metastasis, including local invasion, extravasation or initial survival at a distant site, and metastatic colonization. Such pleiotropy is achieved via coordinate repression of a cohort of metastasis-promoting genes, including RhoA (Valastyan *et al.* 2009). Indeed, RhoA re-expression partially reverses *miR-31*-imposed metastasis suppression. These findings indicate that *miR-31* uses multiple mechanisms to oppose metastasis. Thus, it seems likely that the proposed mesenchymal to amoeboid transition, first identified only *in vitro* (Wang *et al.* 2006), can actually play an important role in metastasis formation, and that metastatic deregulated miRs may impinge on this pathway to promote *in vivo* cell dissemination in different types of tumors. A recent study reported how metastatic cells not only down-regulate *miR-335*, inhibiting cell motility, but also downregulate *miR-126*, which inhibits cell proliferation (Tavazoie *et al.* 2008). As mentioned above, both proliferation and migration advantages are important steps of the metastatic process; therefore, ablation of both *miR-126* and *miR-335* is necessary for tumor metastasis *in vivo* (Tavazoie *et al.* 2008). *miR-335* acts, as does *miR-29c*, by modulating the ECM composition targeting the tenascin and also by halting gene differentiation programs, acting on the transcription factor *SOX4*.

miR-34b, *miR-34c*, and *miR-199a* are able to regulate the MET receptor and the invasive growth program, both in cells expressing normal levels of MET and in cancer cells overexpressing a constitutively active MET. The same miRs play a role in regulating the MET-induced migratory ability of melanoma-derived primary cells (Migliore *et al.* 2008).

Expression profile of miR in endocrine tumors

The miR profiles are surprisingly informative, reflecting the developmental lineage and differentiation and successfully classifying the tumors (Lu *et al.* 2005). Usually, we observe a general deregulation (up- or downregulation) of miRs in tumors compared with normal tissue. The deregulated miR expression profile in human cancer may prove a powerful tool for cancer detection, diagnosis, classification, and prognosis. Endocrine tumors represent a wide and heterogeneous family of tumors originating in endocrine glands. In Table 1, we have summarized all important deregulated miRs found to be associated with the pathogenesis of endocrine tumors. For each of these tumors, we provide a brief description of known important deregulated miRs discovered by miR expression profiling.

Breast cancer

Breast cancer is one of the most interesting cancers to investigate because of the different components in the mammary gland during embryogenesis, the wide variety of different tumor histotypes, and the high number of heterogeneous cell subpopulations. miR profiles in breast tumor samples have identified a variety of deregulated miRs (Iorio *et al.* 2005, Volinia *et al.* 2006, Ma *et al.* 2007, Huang *et al.* 2008, Tavazoie *et al.* 2008). Iorio *et al.* (2008) and Shi & Guo (2009) nicely summarized the most common deregulated miRs in breast cancers, including the upregulation of *miR-21* (targeting Bcl-2, TPM1, and PDCD4), *miR-155*, *miR-206* (ER- α), *miR-373*, and *miR-520*, and the downregulation of *miR-125a/b* (ERRB2–ERRB3), *miR-145*, *miR-10b* (HOXD10), *miR-9-1*, *let-7*, *miR-27a*, and *miR-17-5p*; the latter two miRs play an oncogenic role in breast cancer cell lines. Some of these miRs, such as *miR-10b*, *miR-373*, and *miR-520*, have also been shown to play a role in tumor invasion and breast cancer metastasis; others, such as *miR-335*, *miR-126*, and *miR-206*, act as metastasis suppressors (Tavazoie *et al.* 2008). Four additional miRs (*miR-7*, *miR-128a*, *miR-210*, and *miR-516-3p*) have been associated with breast cancer progression and distinct biological pathways (Foekens *et al.* 2008). Additional miRs from the *miR-200* family, in conjunction with *miR-205*, have been shown to regulate the epithelial and mesenchymal status of breast cancer cells and progression of tumors (Hurteau *et al.* 2007, Sempere *et al.* 2007, Burk *et al.* 2008, Gregory *et al.* 2008). The role played by *miR-200* in stemness regulation of breast cancer cells is interesting. A recent study showed that *miR-200c* is able to regulate the expression

of BMI1, a known regulator of stem cell self-renewal. *miR-200c* inhibited the clonal expansion of breast cancer cells and suppressed the growth of embryonal carcinoma cells *in vitro*. Most importantly, *miR-200c* strongly suppressed the ability of normal mammary stem cells to form mammary ducts and tumor formation driven by human breast cancer stem cells *in vivo* (Shimono et al. 2009).

Prostate tumors

Prostate cancer typically is composed of very small, multiple primary tumors within the prostate gland. At an early stage, the disease is often curable, although the cancer produces few or no symptoms and can be difficult to be detected. In most cases, prostate cancer is a relatively slow-growing cancer; however, a small percentage of patients experience more rapidly growing, aggressive forms of prostate cancer. miR expression profiles have identified important miRs in prostate cancer. Among these, *miR-15a* and *miR-16* act as tumor suppressor genes through the control of cell survival, proliferation, and invasion (Bonci et al. 2008). *miR-146a* is downregulated in hormone-refractory prostate cancers (Lin et al. 2008), and *miR-21* is overexpressed and functions as an oncogene by a mechanism that involves translational repression of the tumor suppressor PDCD4 (Lu et al. 2008). Among the downregulated miRs are the *miR-106b-25* cluster targeting E2F1 and p21/WAF1 (Ambs et al. 2008); *miR-221* (targeting c-kit) and *miR-101* (targeting the histone methyltransferase EZH2), which are progressively reduced in aggressive prostate cancer and metastasis (Varambally et al. 2008, Spahn et al. 2009); *miR-449*, which regulates cell growth and viability in part by repressing the expression of histone deacetylase 1 in prostate cancer cells (Noonan et al. 2009); *miR-331-3p*, which can regulate signaling pathways critical to the development and progression of prostate cancer cells (Epis et al. 2009); *miR-205* and *miR-200* (PC3 cells), which exert a tumor-suppressive effect in the human prostate by counteracting EMT and reducing cell migration and invasion (Gandellini et al. 2009, Kong et al. 2009). Other deregulated miRs include *miR-222*, *miR-373*, *miR-125a/b*, *miR-181*, and *let-7* families (Volinia et al. 2006, Galardi et al. 2007, Porkka et al. 2007).

Ovarian cancers

Ovarian cancer remains a leading cause of morbidity and mortality, with little change in survival rates over the past 30 years. Deregulation of miRs has been found to be associated with ovarian cancer. Two different

studies showed that the most deregulated miRs in ovarian cancers were *miR-15-16*, *miR-29a/b*, *miR-30b/d*, *miR-181b* (Zhang et al. 2006), *miR-105*, *miR-143*, *miR-203*, and *miR-373* (Iorio et al. 2007), with *miR-9*, *miR-34*, *miR-140*, and *let-7* commonly altered in both studies. Discordance in the expression of *miR-200* members has been reported in different studies. When ovaries from patients with ovarian cancer were compared with ovaries or ovarian surface epithelial cells from healthy patients, *miR-200* was found to be upregulated in tumors (Iorio et al. 2007, Nam et al. 2008, Yang et al. 2008); however, a recent study reported no change in *miR-200* expression (Dahiya et al. 2008), and another reported the downregulation of this family of miRs (Zhang et al. 2008) (for further details, see section titled ‘Tumor Invasion and Metastasis’).

Liver cancers

The majority of primary liver cancers (over 95%) arise from liver cells and are called HCCs or carcinomas. Downregulation of *miR-34a* expression has been reported in human HCCs and was associated with metastasis and invasion of tumors. In HepG2 cells, ectopic expression of *miR-34a* potentially inhibited tumor cell migration and invasion in a c-Met-dependent manner, in turn decreasing c-Met-induced phosphorylation of ERKs 1/2 (Li et al. 2009a). In other HCC cells (SKHep1C3), overexpression of *miR-23b* led to urokinase-type plasminogen activator and c-Met downregulation and to decreased migration and proliferation abilities (Salvi et al. 2009). *miR-26a* has been shown to be an important miR in MYC-induced hepatocarcinoma, inducing G₁ arrest in human liver cancer cells (Kota et al. 2009). *miR-101* is downregulated in hepatocellular carcinoma, promotes apoptosis, and suppresses tumorigenicity (Su et al. 2009). Additional miRs have been shown to play different but important roles in hepatocarcinoma (see the review by Gramantieri et al. (2008)), such as downregulation of *miR-1* and *miR-199a* regulating HGF/MET, downregulation of *let-7* and upregulation of *miR-21* regulating the RAS and PI3K pathways respectively, and upregulation of *miR-221* and *miR-222* implicated in the regulation of cell cycle progression through repression of p27 and p57. The role of the tumor suppressor *miR-122* in this type of cancer is noteworthy. Loss of *miR-122* expression in tumor cells has been linked to cancer progression, where transcription factors HNF1A, HNF3A, and HNF3B are able to regulate the expression of *miR-122* (Coulouarn et al. 2009). *miR-122* regulates

the expression of cyclin G₁, and the interaction *miR-122*–cyclin G₁ modulated the activity of the tumor suppressor p53 (Fornari *et al.* 2009).

Pancreatic cancers

Pancreatic cancer is a lethal disease characterized by multiple genetic alterations. The strategies currently available for diagnosis and treatment of pancreatic cancer are not satisfactory, and specific markers to identify this tumor are very scarce. Four studies have identified a pancreatic miR signature, and *miR-216* emerged as the only pancreas-specific miR (see review by Seux *et al.* (2009)). A recent study reported a differential expression that was significantly dissimilar between tumors and chronic pancreatitis, a normal pancreas, pancreatic cellular lines, and adjacent normal samples. A list of 20 miRs as signatures of PDAC was reported, with most (*miR-155*, *miR-221*, *miR-100*, *miR-125b-1*, *miR-21*, *miR-181a/c*, *miR-107*, *miR-424*, *miR-301*, *miR-212*, *miR-92-1*, *miR-16-1*, *let-7d/f1*, *let-15b*, *let-24-1/2*, and *let-376a*) being found to be upregulated and only a few downregulated (*miR-139*, *miR-345*, and *miR-142p*) (Jiang *et al.* 2005). In a second study, Szafranska *et al.* (2007) used an array containing a limited number of miRs and identified differences between pancreatic cancer, normal pancreas, chronic pancreatitis, and PDAC-derived cell lines. They selected a set of 26 miRs deregulated in PDAC as signature of disease. In a third study, Bloomston *et al.* (2007) compared miR expression in 65 microdissected PDAC cells in the adjacent noncancerous pancreatic tissue, and in a set of chronic pancreatitis specimens. Cluster analysis demonstrated that 30 miRs were upregulated and three were downregulated in pancreatic cancers compared with normal pancreatic tissue. Similar to the other two studies, they found that chronic pancreatitis and normal pancreatic specimens showed similar expression patterns, whereas patterns of normal tissue and pancreatic cancer were clearly distinct. All three studies showed a preponderant number of upregulated miRs. *miR-155* and *miR-221* were commonly found to be deregulated in all three studies. Furthermore, some miRs and genes have been shown to have mechanisms of cross-regulation. For example, loss of TP53INP1 was due to the activity of the oncogenic *miR-155*, which is overexpressed in pancreatic cancer cells. Moreover, p53 inactivation partially contributed to the reduction in *miR-34a* levels, which raises the possibility that *miR-34a* loss of function contributes to pancreatic cancer pathogenesis (see review by Seux *et al.* (2009)).

Gastric cancers

Gastric cancer is the fourth most common cancer worldwide. Metastasis occurs in 80–90% of individuals, and this disease has a high death rate, making it the second most common cause of cancer death worldwide. Only a few miRs have been confirmed to be relevant in the tumorigenesis of gastric cancer. *miR-451* was detected by *in situ* hybridization in epithelial gastric cells; however, decreased expression in gastric cancer samples was associated with a worse prognosis (Bandres *et al.* 2009). The expression levels of *miR-31*, *miR-141*, *miR-143*, and *miR-145* have been shown to be significantly downregulated in gastric cancer tissue and to be involved in the pathogenesis of this type of tumor (Du *et al.* 2009, Takagi *et al.* 2009, Zhang *et al.* 2009), whereas *miR-21* has been reported to be upregulated and involved in tumor progression (Volinia *et al.* 2006, Zhang *et al.* 2008). A study showed that HMGA2, which correlates with tumor invasiveness in gastric cancer, is negatively regulated by the *let-7* miR family (Motoyama *et al.* 2008). Further investigations are needed to identify specific miRs involved in the pathobiology of these aggressive tumors.

Thyroid tumors

Thyroid cancer may occur either in the follicular cells or in parafollicular C-cells, basically giving origin to four main types of thyroid cancers: papillary, follicular, anaplastic, and medullary. He *et al.* (2005b) were the first to report the upregulation of *miR-221*, *miR-222*, *miR-146*, *miR-21*, *miR-220*, *miR-181*, and *miR-155*, and the downregulation of *miR-138*, *miR-219*, *miR-26a*, and *miR-345* in PTCs. Additional studies confirmed *miR-221*, *miR-222*, and *miR-181b* as potential signatures for PTC (Pallante *et al.* 2006), and found that *miR-221* and *miR-222* were able to reduce p27kipl protein expression (Visone *et al.* 2007a). Another study confirmed the abnormal regulation of *miR-221* and *miR-222* in thyroid tumors (Nikiforova *et al.* 2008). *miR-30d*, *miR-125b*, *miR-26a*, *miR-138*, and *miR-30a-5p* were found to be downregulated and *miR-222* was found to be upregulated in anaplastic thyroid carcinoma (ATC; Visone *et al.* 2007b). An additional ATC study showed an upregulation of *miR-221*, *miR-222*, *miR-21*, and *miR-146b* in ATC and PTC with downregulation of *miR-26a*, *miR-219*, *miR-345*, and *miR-138* only in ATC. *miR-138* was shown to regulate hTERT (Mitomo *et al.* 2008). Overexpression of *miR-17-92* cluster, *miR-106a/b*, and *miR-17-3p/5p* was also reported in ATC cell lines (Takakura *et al.* 2008). The finding of a common single nucleotide

polymorphism (SNP: rs2910164) in pre-*miR-146a*, affecting the miR expression itself, is noteworthy. This SNP has been shown to profoundly affect downstream transcripts because of the production of different mature miRs, thus contributing to the genetic predisposition of PTC (Jazdzewski et al. 2008, 2009). Additional downregulated miRs, such as *miR-197*, *miR-346*, *miR-192*, and *miR-328*, have been described in follicular thyroid carcinoma (Weber et al. 2006). Despite *BRAF V600E* mutation, which can affect the transcriptome levels in PTC, an interesting study reported that this particular genetic alteration was not able to significantly affect miR expression (Sheu et al. 2009).

Adrenal tumors

Each adrenal gland has two main parts that function separately – the adrenal cortex and medulla. A recent study reported the involvement of miRs in the tumors originating in this gland. Tumors may originate in both components, giving rise to three main tumor groups: adrenocortical carcinoma, pheochromocytoma, and neuroblastoma. The latter cancer accounts for 15% of all childhood cancer deaths, and dysregulation of some miRs, including the *miR-17-5p-92* cluster and *miR-34a*, has been implicated in the pathobiology of this type of devastating cancer. *miR-17-5p-92* family members act in an oncogenic manner, whereas *miR-34a* has tumor suppressor functions (O'Donnell et al. 2005, Welch et al. 2007, Chayka et al. 2009). In neuroblastomas, the oncogene *MYCN* regulates the *miR-17-5p-92* cluster, which in turn inhibits p21 and BIM translation by interaction with their mRNA 3' UTRs (Fontana et al. 2008), therefore identifying a new mechanism of tumor progression in these types of tumors. Additionally, three neuronal miRs (*miR-9*, *miR-125a*, and *miR-125b*) have been shown to control human neuroblastoma cell proliferation (Laneve et al. 2007) by repressing a common target, the truncated isoform of the neurotrophin receptor tropomyosin-related kinase C, a critical isoform for regulating neuroblastoma cell growth. *miR-221* is another important overexpressed miR that has been shown to induce *MYCN* in neuroblastoma (Schulte et al. 2008).

miR-21 is an endogenously expressed miR in human adrenal cells; however, *miR-21* expression is upregulated by angiotensin II, and its overexpression causes an increase in aldosterone secretion and cell proliferation (Romero et al. 2008). Deregulation of specific miRs has been reported in adrenocortical tumors, with higher expression of *miR-184* and *miR-503* and lower expression of *miR-511* and *miR-214* in ACCs than in

adenomas and normal adrenal glands, although expression of *miR-210* was significantly lower in cortisol-secreting adenomas than in ACCs (Tömböl et al. 2009). Upregulation of *miR-449* has been reported to be associated with a bilateral form of adrenal hyperplasia often associated with Carney complex (Iliopoulos et al. 2009).

Pituitary tumors

The pituitary gland is linked in function to the hypothalamus. It is composed of two lobes – the anterior pituitary (adenohypophysis) and the posterior pituitary (neurohypophysis). It secretes hormones regulating homeostasis, including tropic hormones that stimulate other endocrine glands. Symptoms of pituitary adenomas can vary considerably, depending on whether the tumor is secreting one or more of a variety of hormones. Dysregulation of miRs has been recently associated with pituitary adenomas. *miR-15* and *miR-16* have been shown to be strongly downregulated in these types of tumors. Their expression correlates with a greater tumor diameter and a lower p43 secretion, suggesting that these genes partially influence tumor growth (Bottoni et al. 2005). A recent study showed underexpression of *miR-145*, *miR-21*, *miR-141*, *let-7a*, *miR-150*, *miR-15a*, *miR-16*, and *miR-143* in ACTH-secreting pituitary tumors (Amaral et al. 2009). A decreased expression of *let-7* was found in 42% of adenomas and was correlated with high-grade tumors. This miR inversely correlated with the high-mobility group A2 protein expression, a factor most likely involved in the tumorigenesis of the pituitary gland (Qian et al. 2009).

miRs and future treatment applications

All cancers acquire the same set of functional capabilities during their development and progression, albeit through various mechanisms. To acquire a malignant phenotype, cells require a combination of diverse genetic abnormalities. Given the aforementioned associations of miRs with cancer development and progression, the peculiar and transversal appearance of miRs in these circuits, the multiplicity of their targets, and ability to control multiple pathways in parallel, it is now held that these small regulatory RNAs could serve as targets of new anticancer gene therapies. Inhibition of overexpressed miRs can be efficiently done with the use of sequence-specific antisense oligonucleotides, chemically modified (named antagomirs when cholesterol conjugated) to enhance stability and *in vivo* delivery

(Meister *et al.* 2004, Krützfeldt *et al.* 2005). The rationale for using miRs as potential therapeutic targets comes from the many studies that have shown that specific miR deregulations (overexpression and downregulation) in cancer cells have a pathogenic effect (Tili *et al.* 2007).

For example, targeting the *oncomiR-17-92* might be an effective tool for different neoplastic conditions (e.g. breast, adrenal, and thyroid cancers) that display overexpression of the cluster. Targeting miRs from the *oncomiR-17-92* should affect more than one malignant capability, both inducing cell cycle arrest and apoptosis and suppressing the tumor's ability to sustain angiogenesis. The same should be obtained by re-expression of lost miRs, such as *miR-15a* and *miR-16* (ovarian, prostate, pancreatic, and pituitary tumors), which are known to act at the cell cycle arrest level, to induce apoptosis and block angiogenesis. The tumor xenograft model has proven to be successful with an overall reduction of tumor burden (Calin *et al.* 2008).

miR-21 and *miR-221/222* clusters are often deregulated in several endocrine tumors (including breast, prostate, thyroid, liver, and pancreatic cancers), and they could represent other good target candidates for interference with survival signals, apoptosis, and metastasis.

miR-200 and *let-7* families (breast, ovarian, gastric, and prostate cancers) are certainly among the most attractive targets for several cancer therapies because of their complex regulatory network of cell program differentiation and their involvement in tumor progression. Importantly, due to its role in 'stemness' regulation, modulation of *miR-200* might significantly improve and sensitize cancer cells to treatments with existing chemotherapeutic agents. These classes of miRs undoubtedly deserve further studies.

Possible treatments for metastatic tumors could include reducing expression levels for *miR-10b*, *miR-21*, *miR-146* family, *miR-155*, *miR-373*, and *miR-520c* in solid cancers by locked nucleic acid anti-miRs or antagomirs or re-expressing *miR-126*, *miR-148a*, *miR-206*, *miR-335*, and the *miR-200* family by mimic miRs. After preclinical studies (*in vitro* and *in vivo*), modulated miR expression could be applicable to Phase I clinical trials for cancer patients with advanced disease in combination with existing regimens. The appropriate and correct selection of patients based on miR profiling of the tumors becomes crucial in the context of a clinical trial using miR-targeted therapies. Only selected patients with specific ranges of expressions of miRs may exhibit optimal response.

Taking into account the multiple roles of miRs in cancer biology, disrupted miRs in cancer are dominant unifying players and not just an additional

layer of complexity. There are clear opportunities for miR-based anticancer therapeutics. Diagnostic potentials for miRs have been identified, and we expect that clinical diagnostic trials will test their efficacy soon.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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