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REVIEW

DNA methylation-associated silencing of tumor-suppressor microRNAs in cancer

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MicroRNAs (miRNAs) are recognized as being central players in many biological processes and cellular pathways. Their roles in disease have been highlighted first by observation of their aberrant expression profiles in human tumors, and then by *in vitro* and *in vivo* functional studies in transformed cells and model organisms. One of the most commonly observed features of miRNAs in malignancies is a defect in their production. Although several causes may be associated with this phenomenon, such as upstream oncogenic/tumor-suppressor defects and alterations in the miRNA-processing machinery, epigenetic inactivation is the prime suspect. The number of miRNAs with putative growth-inhibitory functions undergoing promoter CpG island hypermethylation in human cancer is growing fast and more detailed biological studies are necessary. The recognition of miR-124a and miR-34b/c as bona fide tumor-suppressor miRNAs undergoing DNA methylation-associated silencing in a wide spectrum of human neoplasms is a good starting point to be followed by other candidate miRNAs. Most importantly, even at this early stage, the transcriptional repression of miRNAs by hypermethylation of their corresponding promoter loci seems to be a common feature of all human tumors. This will have translational consequences for the management of the disease.
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miRNAs: Processing, targets and transport

Non-coding RNAs (ncRNAs) were formerly considered to be transcriptional noise, a byproduct of RNA polymerase during the synthesis of functional RNA. However, more than 90% of the human genome is transcribed in a network of overlapping transcripts without protein-coding capacity (Birney *et al.*, 2007). Moreover, ncRNA sequences and their secondary structures are known to be subject to purifying selection; the evolution of ncRNAs is not consistent with neutralist explanations, thereby indicating that there is selective pressure on them (Ponjavic *et al.*, 2007). ncRNAs are involved in different processes, including not only those involved in protein synthesis (transfer and ribosomal RNA), but also gene regulation, X-chromosome inactivation (Xu *et al.*, 2007) and telomerase maintenance (Azzalin *et al.*, 2007).

The most widely studied class of ncRNAs are microRNAs (miRNAs), which are small ncRNAs of ~22-nt and are involved in post-translational gene silencing by controlling mRNA translation into proteins. It has been predicted that miRNAs regulate the translation rate of more than 60% of protein-coding genes (Friedman *et al.*, 2009), and so participate in the regulation of all cellular processes. Like mRNAs, miRNAs are mainly transcribed by RNA polymerase-II (Lee *et al.*, 2004), although miRNA synthesis is known to occur by RNA polymerase-III in those miRNAs that reside near tRNA, Alu and mammalian-wide interspersed sequences (Borchert *et al.*, 2006). RNA polymerase-II synthesizes a primary transcript called pri-miRNA. The primary transcripts are long, poly-(A)-tailed and capped species measuring several kilobases. This transcript is processed by Drosha, a ribonuclease-III protein (Lee *et al.*, 2003), in collaboration with DGCR8 (DiGeorge Syndrome Critical Region Gene-8), the microprocessor protein (Han *et al.*, 2006) responsible for anchoring the pri-miRNA in the complex. The processing of the pri-miRNA produces a 70-nt-long molecule (Gregory *et al.*, 2004), named pre-miRNA, which migrates to the cytoplasm by using the nuclear export protein Exportin-5 (XPO5) (Yi *et al.*, 2003). In the cytoplasm, the pre-miRNA is released in a GTP-dependent manner and processed by Dicer (a ribonuclease-III protein) in a complex with TRBP, a double-stranded RNA-binding protein, to produce the functional 22-nt-long miRNA (Hutvágner *et al.*, 2001), which targets the mRNA. The ~22-nt molecules are loaded by the Dicer–TRPB complex into a member of the Argonaute (Ago) Protein Subfamily to form the RNA-induced silencing complex (RISC). These Ago proteins are the catalytic components of the RISC (Liu *et al.*, 2004). The miRNA biosynthesis pathway is summarized in the Figure 1.

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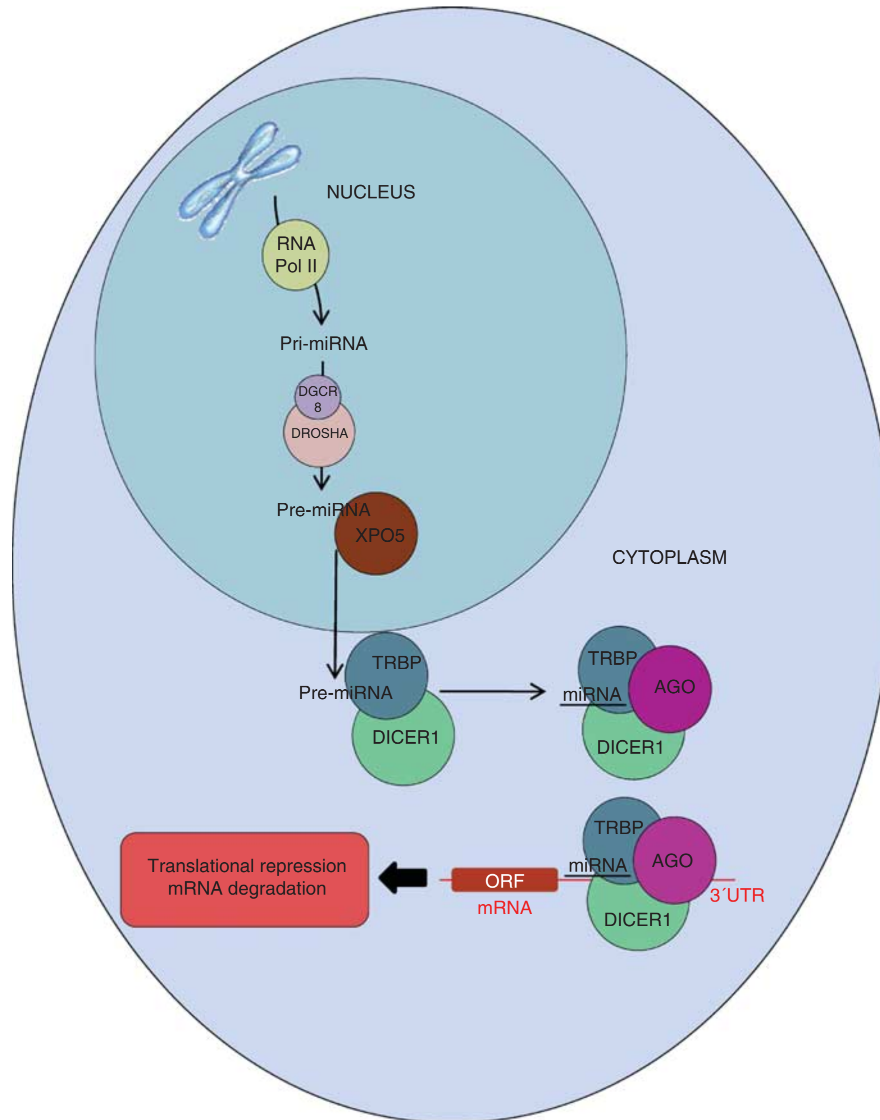

Figure 1 The miRNA biosynthesis pathway from the nucleus to the cytosol. Ago, Argonaute protein; DGCR8, DiGeorge Syndrome Critical Region Gene-8; miRNA, microRNA; ORF, open reading frame; TAR-binding protein; UTR, untranslated region; XPO5, exportin-5; TRBP.

The genes targeted by the miRNAs are defined by complementary base pairs between the miRNA loaded into the complex and, more often, the 3′-untranslated (UTR) region of the mRNA. The complementarity between the miRNA and the targeted mRNA is imperfect, the seed region of the miRNA (nucleotides 2–8) being essential to associate the miRNA with its target mRNA (Lai, 2002; Lewis *et al*., 2003). Usually, the 3′UTR region of the mRNA is bound to multiple miRNAs, improving the repressive effect of the miRNA machinery (Grimson *et al*., 2007). mRNA cleavage is one of the mechanisms by which miRNA regulates gene expression. In this mechanism, the miRNAs act as small interference RNAs (siRNAs), another type of ncRNA produced by DICER from double-stranded RNA sequences, mostly of exogenous origin. siRNAs direct RNA cleavage through the RNA interference pathway in extensive base pairing that is complementary between the siRNA and the targeted mRNA (Plasterk, 2002). The entrance of the miRNA in the RNA interference pathway depends on the degree of complementarity between the miRNA and the targeted mRNA (Hutvágner and Zamore, 2002). However, almost none of the miRNA described was highly complementary to its targeted sequence in the mRNA, preventing its entrance into the RNA interference pathway (Elbashir *et al*., 2001). In fact, the disruption of protein synthesis in the ribosomes through inhibition of translational initiation is the predominant mechanism of gene silencing mediated by miRNAs. The RISC-bound mRNAs localize in the processing bodies (P bodies) in the cytoplasm, where they accumulate or are degraded (Pillai *et al*., 2005). AU-rich elements (AREs) in the 3′UTR of unstable mRNAs dictate their degradation

(Shaw and Kamen, 1986). miRNAs and Dicer are involved in this mechanism of mRNA turnover. Many miRNAs present a complementary sequence to the ARE sequence characteristic of unstable mRNAs, the co-operation between miRNA, Dicer and Ago proteins, and ARE-binding proteins being essential for mRNA decay (Jing *et al*., 2005).

Although gene silencing is the most thoroughly studied role of miRNAs, the capability to upregulate gene transcription during cell-cycle arrest has been described; miRNA369-3 and let-7 oscillate between the ability to repress and activate, depending on the cell cycle. In proliferating cells, they repress the translation of their target genes, whereas in cell arrest, which is often the step preceding cell differentiation, they promote activation (Vasudevan *et al*., 2007). Another example of miRNA-mediated activation of mRNA translation is the case of miR-10a (Ørom *et al*., 2008). This miRNA enhances ribosomal protein synthesis by binding to the 5′UTR of the target mRNAs. The major tumor-suppressor genes, p53 and RB, negatively regulate ribosome synthesis (Ruggero and Pandolfi, 2003), and a very well known oncogene, MYC, positively regulates protein production (Boon *et al*., 2001). As for the described genes, miR-10a is involved in tumor progression (Ørom *et al*., 2008). miRNAs are known to target proteins directly. miRNAs can act as direct inhibitors of ribonucleoprotein activity in an miRNA-mediated silencing process called decoy. miR-328 interacts directly, independently of the Ago protein, with the heterogeneous ribonucleoprotein (hnRNP) E2. This interaction prevents the translational repression by hnRNP E2 of CEBPA mRNA, a key regulator of myeloid differentiation to granulocytes. Downregulation of miR-328 allows the hnRNP E2 inhibition of myeloid differentiation and thus, tumor progression (Eiring *et al*., 2010). siRNAs have the ability to direct gene silencing by heterochromatin formation. RNA-induced transcription silencing is a mechanism described in yeast (Verdel *et al*., 2004). The RNA-induced transcription silencing complex recruits histone methyltransferases after siRNA loading, directing heterochromatin formation in the DNA locus.

miRNAs circulate in the bloodstream in a very stable form; different mechanisms have been proposed to explain the high stability of the circulating RNA in an RNase-rich environment. Under normal conditions, many cell types, such as B- and T-cells, mast cells and reticulocytes, have the capacity to produce exosomes, which are small membrane vesicles that are released into the extracellular matrix with the plasma membrane. Tumoral cells in melanoma are also known to produce exosomes (Mears *et al*., 2004). The exosomes carry proteins, mRNA and miRNA. The mRNA present in the exosomes can be translated in the recipient cells, suggesting a functional role for the miRNA. This mechanism of RNA delivery is called ‘exosomal shuttle RNA’ and may constitute a way by which the donor cell regulates protein production in the recipient cell (Valadi *et al*., 2007). In this paper, the authors found 121 miRNAs in the exosomes produced by mast cells. Taking into account that every miRNA has the potential to interfere with up to 200 mRNAs (Krek *et al*., 2005), the regulatory capacity of the miRNAs present in the exosomes may modulate up to 24 000 mRNAs. More recently, it has been found that most of the circulating miRNA in plasma is confined to protein complexes rather than membrane vesicles. These proteins, like exosomes, protect miRNA from degradation, there being at least two different populations of circulating RNA. The ribonucleoprotein complex contains Ago-2, the protein that mediates RNA silencing in the RISC, suggesting that cells release functional silencing complexes into the circulation to modulate gene expression in the recipient cells (Arroyo *et al*., 2011). Cell–cell communication can be mediated by direct contact between the donor and the recipient cells by gap junction intercellular connections. miRNAs can pass from one cell to the other by these junctions and modulate the gene expression of the targeted genes in the receptor cell (Lim *et al*., 2011). These authors showed the exchange of miRNAs between bone marrow stromal cells and metastatic breast cancer cells by this mechanism; specific miRNAs target CXCL12, a chemokine involved in cell communication and signal transduction, cell proliferation is reduced and cells enter quiescence, suggesting a role for miRNAs in the dormancy of breast cancer cells in the bone marrow and explaining the recurrence of metastatic breast cancer from the bone marrow many years after primary tumor elimination (Willis *et al*., 2010). Figure 2 summarizes the different mechanisms by which miRNAs regulate gene expression.

The regulation of gene expression by miRNAs, their role in targeting different genes and their capacity to move between cells illustrate the complexity of transcriptome regulation in the cells. In addition, RNA editing increases this complexity by generating new molecules from existing ones (reviewed by Bass, 2002). RNA editing occurs by the deamination of adenosines to inosines, performed by adenosine deaminase acting on RNA enzymes (ADAR), mostly within the non-coding regions of the transcripts. The newly generated nucleotide base pairs have cytosine instead of thymidine, promoting the synthesis of new proteins from a single open reading frame (Burns *et al*., 1997) and altering miRNA targeting by editing of the miRNAs or the mRNAs (Borchert *et al*., 2009). Moreover, the role of pseudogenes in miRNA homeostasis has been described. Pseudogenes are commonly considered to be non-functional genomic loci and to have no biological relevance, although preservation of the nucleotide sequences suggests that they are under selective pressure. Pseudogenes can be transcribed or not, and those that are transcribed show a high complementarity with the protein-coding gene from the 5′ to the 3′UTR. Taking into account that miRNAs commonly regulate gene silencing by 3′UTR sequence complementarity, pseudogenes may act to regulate miRNA activity on the coding gene by competing for the miRNA molecules present in the cell. This mechanism of miRNA regulation has been modeled by studying PTEN (tumor-suppressor gene)/

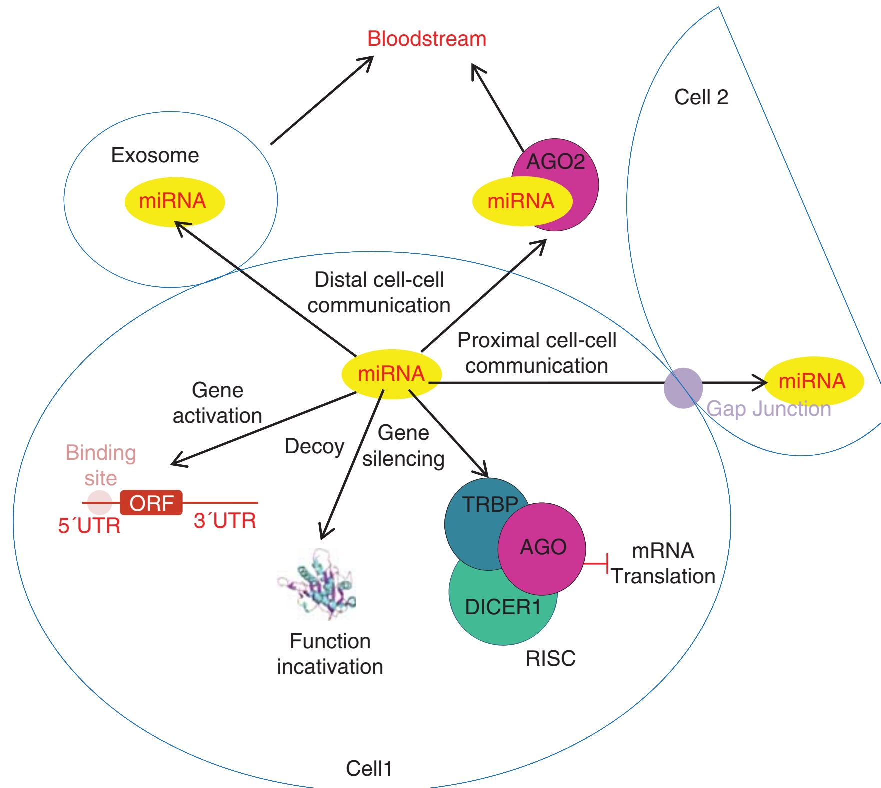

Figure 2 Mechanisms by which miRNAs regulate gene expression within a cell and in neighboring cells. Ago, Argonaute protein; ORF, open reading frame; RISC, RNA-induced silencing complex; TRBP, TAR-binding protein; UTR, untranslated region.

PTENP1 (PTEN pseudogene) expression levels in tumoral cell lines (Poliseno *et al*., 2010). The authors demonstrated the role of PTEN transcripts in modulating miRNA targeting by examining PTENP1 levels. This study provides a new perspective on transcriptional regulation. Considering the genomic rearrangements, copy-number alterations and translocations that many cancer cells suffer and that give rise to altered levels of the transcripts, changes in the tumoral cell phenotype can be due not only to the protein alteration itself, but also to global RNA deregulation by altered competition for miRNAs (Poliseno *et al*., 2010).

miRNAs: Cancer involvement and deregulation mechanisms

Cancer development involves genetic and epigenetic alterations that give rise to an uncontrolled overgrowth of cells (Jones and Baylin, 2007; Berdasco and Esteller, 2010). Research has tended to focus on coding genes and the proteins they produce, classifying them as oncogenes, if they promote cell growth, or tumor-suppressor genes, if they prevent overgrowth. With the discovery of miRNAs in *Caenorhabditis elegans* in the late 1990s (Fire *et al*., 1998) and their regulatory role in gene expression, a new front in cancer research was initiated.

As with classical genes, the non-coding loci in the DNA (ncRNAs) can be subjected to genetic and epigenetic alterations. These alterations modify the expression profile of the ncRNAs and thus alter the mechanism they regulate. Chromosome abnormalities (deletions, translocations, copy-number alterations), DNA mutations and epigenetic deregulation of the ncRNAs or the genes involved in their biogenesis have been described in tumor progression. The first association between miRNA and cancer development was described in chronic lymphocytic leukemia with chromosome 13q14 deletion. This deletion deregulates miR-15 and miR-16 (Calin *et al*., 2002). Most of the targets of these two miRNAs are involved in cell growth and cell cycle (for example, Anxa2 and Cdc2), apoptosis (Grp78 and Bcl2), and oncogenes and tumor-suppressor genes (Wt1 and Rab8B) (Calin *et al*., 2008). Surprisingly, miRNAs are frequently located in fragile regions of the chromosomes as has been shown in ovarian and breast carcinomas and melanomas (Calin *et al*., 2004; Zhang *et al*., 2006). miR-16 is also involved in the mRNA decay of tumor necrosis factor-α (Jing *et al*., 2005) by ARE sequence binding in the 3′UTR of the mRNA. This suggests that a single miRNA involved in the regulation of multiple mRNAs can use different mechanisms to achieve gene silencing: not only basepair complementary to specific sequences in the targeted genes, but also recognition of the ARE instability signal. Mutations of the miRNA sequences can also disrupt their regulatory role. Mutations in the primary transcripts of miR-15a and miR-16-1 are responsible for their reduced expression levels in chronic lymphocytic leukemias (Calin *et al*., 2005). Polymorphisms have been

described in a set of miRNAs in non-small cell lung cancer and a single single-nucleotide polymorphism in has-mir-196a2-3p is associated with survival in patients with non-small cell lung cancer, probably owing to a defect in binding to the target gene (Hu *et al*., 2008).

let-7 is one of the most widely studied miRNA families in cancer. Alterations of let-7 function have been described in several human cancer types, such as lung cancer (Takamizawa *et al*., 2004), colorectal cancer (Akao *et al*., 2006), head and neck cancer (Yu *et al*., 2011), and ovarian cancer (Wendler *et al*., 2011). It acts mainly as a tumor-suppressor miRNA. On the one hand, the members of this family negatively regulate cell-cycle-related genes such as RAS or cyclins (Johnson *et al*., 2005). Mutations or alterations of the expression profile of these oncogenes are involved in cancer development. On the other hand, the let-7 miRNA family influences apoptosis owing to its negative regulation of the Fas gene (Wang *et al*., 2011). Fas is a death receptor in peripheral T-cells, which is involved in activation-induced cell death (AICD) in the presence of the Fas ligand, a member of the tumor necrosis factor cytokine family (Krueger *et al*., 2003). In cervical carcinomas as compared with normal tissue, let-7 is overexpressed and, as a consequence, the level of Fas gene expression is reduced (Das *et al*., 2000; Lee *et al*., 2008). In this case, the let-7 miRNA family acts as an oncomiRNA (tumor-promoting miRNA).

miRNA biosynthesis is also subjected to extracellular signaling. Under mitogenic signals, the mitogen-activated protein kinase (MAPK) Erk mediates the phosphorylation of TRBP, one of the components of the DICER1 complex. TRBP phosphorylation enhances miRNA expression by increasing the stability of the biosynthesis complex (Paroo *et al*., 2009). Surprisingly, under tumorigenic signals, only miRNAs involved in cell growth promotion are upregulated when the MAPK/Erk pathway is activated, and let-7 miRNA expression levels, which are mainly involved in cell growth suppression, are reduced. let-7 downregulation has been shown under type-I collagen activation of the transforming growth factor-β1-mediated expression of MT1–MMP (membrane type-1-matrix metalloproteinase) and Erk pathway activation in pancreatic ductal adenocarcinoma (Dangi-Garimella *et al*., 2011). This mechanism modulates the metastatic capacity of the tumoral cells by enhancing the expression of metalloproteinases, which are proteins involved in collagen degradation, a key process for promoting cell invasion through the extracellular matrix (Ottaviano *et al*., 2006), and let-7 downregulation (Dangi-Garimella *et al*., 2011).

Alterations in the genes involved in miRNA expression can disrupt the correct function of these miRNAs. Overexpression of the oncogenic miRNA miR-17-92 cluster has been reported in human cancer (Volinia *et al*., 2006). This miRNA family is regulated by c-Myc, a transcriptional factor overexpressed in multiple human cancers (Chang *et al*., 2008). Overexpression of c-Myc activates miR-17-92 cluster expression. This family regulates the gene silencing of many genes involved in cell cycle and proliferation. The miR-17-92 cluster confers resistance to cell senescence and apoptosis by silencing several tumor-suppressor genes such as PTEN (Olive *et al*., 2009) and p21 (Hong *et al*., 2010). Angiogenesis is a key step in tumor development and metastasis, providing nutrients and oxygen to overgrowing cells. The miR-17-92 cluster confers a metastatic capacity on tumoral cells by inhibiting Tsp1 (anti-angiogenic thrombospondin-1) and CTGF (connective tissue growth factor), which are genes involved in angiogenesis (Dews *et al*., 2006).

Once transcribed, miRNAs are processed and exported from the nucleus to the cytoplasm. Alterations in the processing machinery can also lead to deregulation of functional miRNAs. Reduction of the expression levels of Drosha and Dicer has been described in ovarian cancer patients, leading to a downregulation of mature miRNAs (Merritt *et al*., 2008). Germline mutations in the DICER1 gene have been described in familial ovarian and pulmonary neoplasm (Rio Frio *et al*., 2011), and kidney neoplasm (Bahubeshi *et al*., 2010). An miRNA family, miR-103/107, targets Dicer to downregulate its translation into the protein and attenuate global miRNA synthesis. This mechanism creates a feedback relationship taking into account that miR-103/107 are both generated by and regulators of Dicer, producing a downscaling of Dicer levels but not their complete depletion, maintaining a basal level to regulate the miRNA pathway (Martello *et al*., 2010). Overexpression of the miR-130/107 family in breast cancer is associated with epithelial–mesenchymal transition and metastasis due to the downregulation of miR-200 levels. The miR-200 family regulates the silencing of ZEB1/ZEB2, which are genes involved in the epithelial–mesenchymal transition (Burk *et al*., 2008).

Latent infection by Epstein–Barr virus is associated with the development of human cancers such as Burkitt lymphoma, Hodgkin lymphoma and pharyngeal carcinomas (Pagano, 2002). The viral genome encodes its own miRNAs, identified in human B-cells infected by Epstein–Barr virus (Pfeffer *et al*., 2004). miR-BART6-5p, one of the miRNAs encoded by the virus, targets Dicer and suppresses its expression, altering global miRNA patterns in the infected cells (Iizasa *et al*., 2010).

TARBP2, one of the components of the DICER1 complex, presents frameshift mutations that cause diminished protein expression levels in sporadic and hereditary colorectal carcinomas. This reduced expression causes a defect in pre-miRNA processing and promotes tumor progression (Melo *et al*., 2009). XPO5 is the protein in charge of miRNA export from the nucleus to the cytoplasm. Inactivating mutations in the XPO5 gene appear in human carcinomas with satellite instability; these mutations trap pre-miRNA in the nucleus and deregulate the mature miRNA pattern in cancer cells (Melo *et al*., 2010).

miRNAs: The impact of aberrant DNA methylation profiles

One of the most common causes of the loss of tumor-suppressor miRNAs in human cancer is the silencing of

their primary transcripts by CpG island promoter hypermethylation (Saito *et al.*, 2006; Lujambio *et al.*, 2007, 2008; Toyota *et al.*, 2008; Huang *et al.*, 2009). In cancer, cells undergo global hypomethylation of the DNA (Feinberg and Vogelstein, 1983). This contributes to genome instability and the transcription of silenced transposable sequences, promoting chromosomal rearrangements and genome disruption, a characteristic feature of tumoral cells. However, the CpG islands of the promoter regions of the tumor-suppressor genes undergo DNA hypermethylation, leading to gene silencing and promotion of cancer development (Jones and Baylin, 2007; Berdasco and Esteller, 2010). The DNA methylation profile of tumors can be used as a signature to define tumor type, clinical prognosis and treatment response (Esteller, 2008; Rodríguez-Paredes and Esteller, 2011). miRNAs transcribed from CpG islands undergo DNA methylation-associated repression with a similar chromatin context to coding genes, such as binding of the transcriptional repressor methyl-CpG-binding domain proteins (Lujambio *et al.*, 2007; Urdinguio *et al.*, 2010), and a histone modification profile associated with silencing, such as in the case of loss of acetylation of histones H3 and H4 (Lujambio *et al.*, 2007; Toyota *et al.*, 2008). A similar chromatin-repressive environment has been described for other ncRNAs, such as transcribed-ultraconserved regions, which also undergo promoter CpG island hypermethylation in cancer cells (Lujambio *et al.*, 2010). Figure 3 illustrates aberrant DNA methylation patterns that occur in tumoral cells.

Epigenetic silencing of the tumor-suppressor miR-124a by aberrant DNA hypermethylation of the colorectal cancer cell line HCT-116 leads to cyclin-D kinase-6 (CDK6) overexpression, its target. Transfection of the tumoral cell line with miR-124a diminishes CDK6 protein levels and retinoblastoma (Rb) phosphorylation, one of the targets of the kinase (Lujambio *et al.*, 2007). Both proteins are involved in cell-cycle progression. Rb is an antiproliferative tumor-suppressor gene that, when phosphorylated by CDK6, diminishes its antiproliferative activity (Grossel and Hinds, 2006). Hypermethylation of this miRNA has also been observed in glioblastoma multiforme (Silber *et al.*, 2008), gastric cancer (Ando *et al.*, 2009), hematopoietic malignancies (Agirre *et al.*, 2009; Roman-Gomez *et al.*, 2009), cervical cancer (Wilting *et al.*, 2010) and hepatocellular carcinoma (Furuta *et al.*, 2010).

miR-145 is a well-known tumor-suppressor miRNA that is downregulated in many human cancers owing to aberrant DNA methylation of its promoter and/or p53 mutations (Suh *et al.*, 2011). This miRNA is a pluripotency repressor; it regulates OCT, SOX2 and KLF4 gene silencing in human embryonic stem cells, genes required for cell self-renewal and pluripotency maintenance (Xu *et al.*, 2009). In fact, they are three of the four factors known to acquire *de novo* pluripotency (Takahashi and Yamanaka, 2006). Reprogramming to pluripotency resembles the de-differentiation process that cancer cells undergo during tumor formation. In acute myeloid leukemia, the oncogene c-kit is overexpressed in 60–80% of patients (Kindler *et al.*, 2004). miR-193a has been described as a key tumor-suppressor miRNA that targets c-kit and, thus, prevents cell overgrowth and promotes cell differentiation. This miRNA is regulated by DNA methylation and its aberrant silencing by DNA hypermethylation has been described in acute myeloid leukemia cell lines (Gao *et al.*, 2011).

miR-335 was identified in a set of miRNAs involved in the suppression of breast cancer metastasis to the lungs and bone (Tavazoie *et al.*, 2008). It was found to inhibit metastasis by targeting the genes involved in cell invasion and migration such as the transcription factor SOX4 and the extracellular matrix protein tenascin-C. This miRNA undergoes genetic deletion and promoter hypermethylation silencing in metastatic cell populations derived from primary breast tumors. Cells that

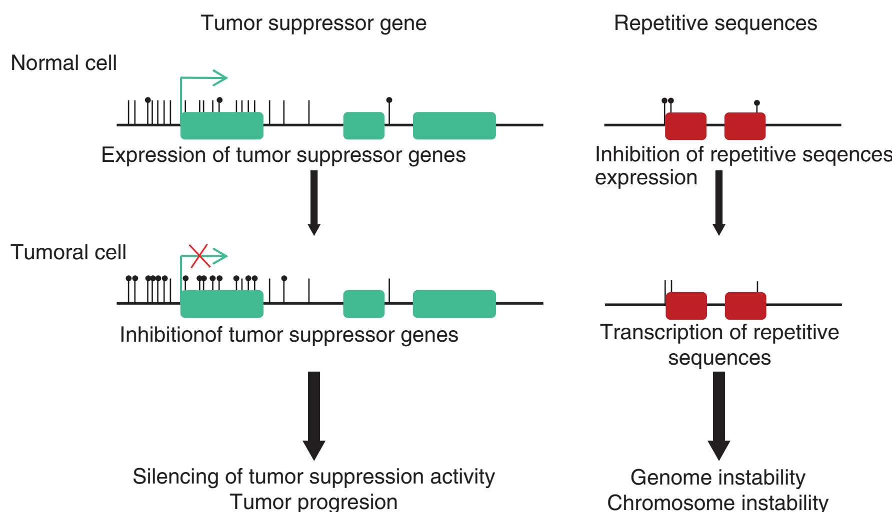

Figure 3 Aberrant DNA methylation patterns in human cancer. Heavily methylated repetitive sequences undergo hypomethylation events in transformed cells that might cause genome instability (right panel), whereas unmethylated promoter CpG islands of coding genes and miRNAs with tumor-suppressor features become hypermethylated and silenced in cancer cells (left panel).

undergo miR-335 silencing in the population have a selective advantage over the others, acquiring metastatic capacity and colonizing new tissues (Png *et al.*, 2011). The genetic and epigenetic silencing of this miRNA have been observed in ovarian cancer samples, and its deletion is correlated with the malignant progression of breast and ovarian tumors. miR-129-2 is another miRNA involved in SOX4 silencing and it is also regulated by promoter methylation. Aberrant miR-192-2 DNA hypermethylation has been found in endometrial (Huang *et al.*, 2009), colorectal (Bandres *et al.*, 2009) and gastric cancer (Shen *et al.*, 2010).

miR-199a expression is regulated by DNA methylation of the locus from which it is transcribed (Cheung *et al.*, 2011). Downregulation of this miRNA owing to aberrant DNA hypermethylation of its promoter and its association with invasive properties of the tumoral cells have been described in gastric (Ueda *et al.*, 2010), ovarian (Yin *et al.*, 2010) and testicular (Cheung *et al.*, 2011) tumors. This miRNA regulates the silencing of many genes involved in tumor progression. In testicular cancer, it targets PODXL, an anti-adhesive protein that inhibits cell–cell interaction, a key feature of the cells that acquire an invasive phenotype in the primary tumor (Cheung *et al.*, 2011).

Nuclear factor-κB is a transcription factor that regulates the proinflammatory response. Its activation is linked to cancer development by its role in the regulation of the secretion of cytokines and chemokines by the immune cells in the tumoral microenvironment. Its activation is regulated by the IκB kinases (IKKs) by phosphorylation and further degradation of the nuclear factor-κB inhibitor, IκBα. This cascade promotes the translocation of nuclear factor-κB to the nucleus, where it promotes the gene expression of the inflammatory cytokines involved in cell proliferation. In most cancer cells, the IKKs and, for instance, nuclear factor-κB are constitutively active (Chen *et al.*, 2007). miR-199a regulates the post-transcriptional silencing of IKKβ, one of the members of the IKK complex. Aberrant downregulation of the miRNA activates the IKK pathway by aberrant translation of IKKβ. This promotes the secretion of proinflammatory molecules characteristic of tumor development (Chen *et al.*, 2008).

miR-34 is a tumor-suppressor miRNA family regulated by the transcriptional factor p53 (Tarasov *et al.*, 2007) and DNA methylation (Lujambio *et al.*, 2008; Toyota *et al.*, 2008; Tsai *et al.*, 2011). p53 is one of the best studied tumor-suppressor genes; it is called the ‘guardian of the genome’ because it promotes apoptosis under DNA damage. It regulates the expression of many oncogenes such as c-Myc, Bcl2, and cell cytokines and miRNAs involved in cell proliferation. Inactivation of miR-34 members is a common feature of many tumor types. Downregulation of miR-34 owing to p53 inactivation or DNA hypermethylation of its promoter alters the Notch pathway (Pang *et al.*, 2010), which is involved in the promotion of cell invasion (Bin Hafeez *et al.*, 2009) and apoptosis of cancer cells. One of the targets of miR-34 is SIRT1, an oncogene that decreases the ability of p53 to promote cell-cycle arrest by deacetylating the protein. In this way, cells create a positive feedback loop between p53 and miR-34 for tumor prevention mediated by SIRT1 silencing (Yamakuchi *et al.*, 2008). Altered expression profile of this miRNA family has been described in gastric (Tsai *et al.*, 2011), ovarian (Corney *et al.*, 2010), colorectal (Lujambio *et al.*, 2008) and non-small cell lung cancer (Gallardo *et al.*, 2009). Promoter CpG island hypermethylation for both miR-34b/c (Lujambio *et al.*, 2008; Toyota *et al.*, 2008; Roman-Gomez *et al.*, 2009; Tsai *et al.*, 2011) and miR-34a (Lodygin *et al.*, 2008; Chim *et al.*, 2010; Corney *et al.*, 2010) represents a major mechanisms accounting for the loss of these transcripts in human cancer.

Epigenetic silencing of miRNAs is also involved in the acquisition of an invasive phenotype and the development of metastasis (Lujambio and Esteller, 2009). Treatment with the DNA methylation inhibitor 5-aza-2′-deoxycytidine and further analysis of miRNA expression arrays of metastatic cells from lymph node metastasis of different human cancers showed cancer-specific DNA hypermethylation of the CpG islands in the promoter of three miRNAs, miR-148a, miR-9 family and miR-34b/c. Epigenetic silencing of these miRNAs mediates the activation of metastatic genes such as c-Myc, CDK6 and E2F3 (E2F transcription factor-3, involved in the activation of the expression of cyclins and other proliferative genes), targeted by miR-34b/c downregulation, and TGIF2 (transforming growth factor-β-induced factor-2), targeted by miR-148a (Lujambio *et al.*, 2008). Alterations in these miRNAs have been described in many cancer types such as breast (Lehmann *et al.*, 2008), ovarian (Laios *et al.*, 2008) and pancreatic cancer (Omura *et al.*, 2008). Surprisingly, miR-9 has been described as an oncogene owing to its role in E-cadherin silencing in hepatocellular carcinomas promoting cell invasion (Tan *et al.*, 2010). This suggests the potential dual role of miRNA in promoting or inhibiting tumoral progression, depending on the tissue where they are expressed. Hypermethylation of the miR-9 family has also been reported in hematopoietic malignancies (Roman-Gomez *et al.*, 2009) and renal cell carcinoma (Hildebrandt *et al.*, 2010). Recent reports have also indicated the presence of hypermethylation-associated silencing of some miR-200 family members in cancer cells (Ceppi *et al.*, 2010; Neves *et al.*, 2010; Vrba *et al.*, 2010; Wiklund *et al.*, 2010; Chen *et al.*, 2011). The miR-200 family has an important epithelial–mesenchymal transition-regulatory activity in which it directly targets and inhibits ZEB1 and ZEB2 (Hurteau *et al.*, 2006; Park *et al.*, 2008), transcriptional repressors of E-cadherin, a cell adhesion protein crucial for maintaining epithelial structure (Peinado *et al.*, 2007). Most importantly, the DNA methylation-associated silencing of the miR-200 family is a dynamic process that determines the evolving epithelial–mesenchymal transition phenotypes of colorectal and breast tumors (Davalos *et al.*, 2011).

Other important miRNAs disrupted by promoter CpG island hypermethylation in human cancer include miR-203, which targets ABL1 and is epigenetically inactivated in hematopoietic malignancies (Bueno *et al.*,

2008) and hepatocellular carcinoma (Furuta *et al.*, 2010); miR-181c, which targets the oncogenes NOTCH4 and K-RAS, and undergoes hypermethylation in gastric tumors (Hashimoto *et al.*, 2010); and let-7a-3 in ovarian cancer with an inverse correlation with insulin-like growth factor-II expression (Lu *et al.*, 2007). The list of miRNAs undergoing promoter CpG island hypermethylation in human cancer is rapidly expanding and a recent epigenomic survey of 450 000 CpG sites revealed that among the 616 miRNAs printed in the microarray, 30–40 miRNAs underwent hypermethylation in colorectal cancer cells (Sandoval *et al.*, 2011):

As mentioned above, cell treatment with the DNA-demethylating agent 5-aza-2′-deoxycytidine and the histone deacetylase inhibitor 4-phenylbutyric acid followed by analysis of transcript expression profiles is a common approach used to identify sequences controlled by the epigenetic marks. Using this approach, the epigenetic silencing of miRNA-127 in prostate and bladder cancer described originally (Saito *et al.*, 2006). This miRNA belongs to an miRNA cluster but only miR-127 presents a CpG island, being the only one regulated by DNA methylation. Silencing of this miRNA by DNA hypermethylation leads to cancer progression owing to overexpression of its target, BCL6, a key regulator of p53 expression.

miRNAs can be transcribed from intronic regions in coding genes. Sometimes, the tumoral phenotype is thought to be the cause of the aberrant hypermethylation of a given gene, but it is also due to the downregulation of the miRNA it hosts. This is the case of miR-342, which is transcribed from the EVL gene (Grady *et al.*, 2008). The EVL promoter is hypermethylated in the early stages of colorectal adenocarcinomas and as a consequence, the gene and the miRNA are both downregulated. EVL is a gene involved in cell polarity and cytoskeleton remodeling, and can affect cell motility. The targets of miR-342 have not been described. Another miRNA aberrantly methylated in the early stages of colorectal cancer development is miR-137 (Balaguer *et al.*, 2010), which is also methylated in oral cancer (Kozaki *et al.*, 2008) and glioblastoma multiforme (Silber *et al.*, 2008). Among the targets of this miRNA, we can highlight LSD-1 (lysine-specific histone demethylase-1), a histone demethylase that has a key role in the epigenetic regulation of gene expression; CDK6 and E2F6, a transcription factor involved in cell-cycle progression. In primary hepatocellular tumors, aberrant hypermethylation of miR-1, a tumor-suppressor miRNA targeting FoxP1, a ubiquitously expressed transcription factor; MET (hepatocyte growth factor receptor) and histone deacetylase-4 has been described. Overexpression of these genes owing to miRNA silencing promotes hepatocellular carcinogenesis (Datta *et al.*, 2008).

Considered from the opposite point of view, aberrant DNA hypermethylation of tumor-suppressor genes can be promoted by an altered miRNA expression profile. In hepatitis B virus-related hepatocellular carcinomas, the levels of miRNA-152 are downregulated relative to those in non-cancerous tissues. miR-152 downregulation promotes the overexpression of DNA methyltransferase-1 (DNMT1), its target, and, as a consequence, aberrant DNA hypermethylation. The relation of the viral infection and the hypermethylation of the DNA is the hepatitis B virus X protein (HBx), the inhibitor of miR-152. This mechanism may also be involved in the methylation of the hepatitis B virus genome, promoting the survival of the virus in the host cells (Huang *et al.*, 2010). The miRNA-29 family is downregulated in lung cancer. This family of miRNAs has sequence complementarity with the 3′UTR of DNMTs 3a and 3b. These two enzymes are frequently upregulated in lung cancer and are associated with poor prognosis in patients. An inverse correlation between DNMT-3a and -3b, and the members of the miRNA-29 family has also been noted. Enforced expression of these miRNAs restores the normal DNA methylation profile by silencing the DNMTs, inducing the expression of tumor-suppressor genes that are aberrantly methylated, and inhibiting tumor growth in lung cancer cell lines and mice (Fabbri *et al.*, 2007).

Table 1 summarizes the best characterized miRNAs dysregulated by DNA hypermethylation and the functional consequences in tumoral cells.

miRNAs: Translational applications

From a clinical point of view, miRNAs have great potential at the diagnostic and therapeutic levels. Microarray technology offers the opportunity to determine the expression of thousands of miRNAs at the same time, generating a very useful miRNA profile to determine the tumor type, patient prognosis and treatment response. Microarray analysis highlights a general downregulation of miRNAs in tumors when compared with normal tissues (Lu *et al.*, 2005); furthermore, owing to the remarkable tissue specificity of the miRNAs, they become a very useful tool for defining the origin of the tumor in poorly differentiated cancers (Rosenfeld *et al.*, 2008). In fact, a specific miRNA signature is being used for cancer screening.

The prognosis and survival of patients depend on the stage of the cancer when they are diagnosed. For this reason, one of the most important issues in clinical cancer research is to find early biomarkers of the tumorigenic process. In clear cell renal carcinoma, the miRNA signature distinguishes between metastatic and non-metastatic tumors. The 5-year survival rate of patients with primary metastasis is 10% as compared with 70–90% in non-metastatic patients (Heinzelmann *et al.*, 2011), the miRNA signature being a powerful tool for early diagnosis. Moreover, analysis of circulating tumor-derived miRNAs represents a non-invasive means of diagnosing cancer (Kosaka *et al.*, 2010). The utility of altered miRNA levels in serum has been noted in many cancer types. For example, serum levels of miRNA-141 can distinguish people with prostate cancer from healthy individuals (Mitchell *et al.*, 2008). From an epigenetic point of view, there is a DNA methylation signature for human cancer metastasis (Lujambio *et al.*,

Table 1 Hypermethylated miRNAs in human cancer and their functional consequences

miRNA hypermethylated	*Chromosome*	*Target*	*Functional consequences*	*Cancer type*
miR-1	20	FoxP1 MET HDAC4	Transcriptional activation Cell proliferation, angiogenesis, tumor cell invasion Gene transcription alteration	Hepatocarcinoma
miR-9-1	1	FGF family Claudin family	Cell proliferation Cell communication and growth	Breast, ovarian, pancreas, hematological, renal
miR-9-3	15	E-cadherin	Cell migration	Colorectal, melanoma, head and neck
miR-34a	1	CD44 Notch1	Cell communication and signal transduction Transcriptional activation	Hematological, prostate, breast, renal, colorectal, ovarian
miR-34b/c	11	Notch1 c-Myc Bcl2 SIRT1	Transcriptional activation Transcriptional activation Cell proliferation Epigenetic gene silencing	Gastric, ovarian, lung, colon, melanoma, head and neck
miRNA-124a	20	CDK6	Cell-cycle deregulation	Colon, gastric, hematological, cervical, liver, glioblastoma
miR-127	14	BCL6	p53 downregulation and cell proliferation	Prostate, bladder
miR-129-2	11	SOX4	Cell dedifferentiation and proliferation	Gastric, endometrial, colorectal
miR-137	1	CDK6 E2F6 LSD-1	Cell-cycle dysregulation Cell-cycle dysregulation Gene transcription alteration	Oral, colorectal, glioblastoma,
miR-145	5	OCT/SOX2/KLF4	Cell dedifferentiation	Prostate
miR-148a	7	TGIF2	Invasive capacity	Colorectal, melanoma, head and neck, breast
miR-129-2	11	SOX4	Cell dedifferentiation and proliferation	Gastric, endometrial
miR-181c	19	Notch4 K-Ras	Transcriptional activation Signal transduction	Gastric
miRNA-199a	19	PODXL IKKβ	Invasive capacity Proinflammatory molecules secretion	Testicular, ovarian
miR-200c	12	ZEB1/ZEB2	Epithelial–mesenchymal transition	Colorectal, breast, lung
miR-141	12	ZEB1/ZEB2	Epithelial–mesenchymal transition	Colorectal, breast, lung
miR-429	1	ZEB1/ZEB2	Epithelial–mesenchymal transition	Colorectal, breast, lung
miR-203	14	ABL1	Cell communication and signal transduction	Hematological, liver
miR-205	1	ZEB1/ZEB2	Epithelial–mesenchymal transition	Bladder
miRNA-335	7	SOX4/TNC	Cell dedifferentiation and proliferation	Breast
miR-342	14	PDGFRA RASA1	Cell proliferation Cell proliferation	Colorectal
let-7a-3	22	IGF-II	Cell communication and signal transduction	Ovarian, breast

Abbreviation: miRNA, microRNA.

2008). The authors analyzed the methylation profile of lymph node metastatic cancer cells and tumors, and found a relationship between specific miRNA hypermethylation patterns and the metastatic behavior of tumors. The use of miRNA gene hypermethylation to improve cancer diagnosis has been extended recently to many types of biological fluids, such as oral rinses (Langevin *et al*., 2010), feces (Kalimutho *et al*., 2011) and colorectal mucosal wash fluids (Kamimae *et al*., 2011).

miRNAs not only have diagnostic but also therapeutic potential. Inactivation of tumor-promoting miRNAs (Medina *et al*., 2010; Obad *et al*., 2011) or restoration of tumor-suppressor miRNAs (Saito *et al*., 2006; Lujambio *et al*., 2007, 2008) offers great prospects for cancer treatment. Activation of hypermethylated tumor-suppressor miRNAs can be accomplished by using chromatin-modifying drugs such as DNA methylation inhibitors. To date, two DNMT inhibitors, 5-azacytidine and 5-aza-2′-deoxycytidine, have been approved by the US Food and Drug Administration (FDA) for the treatment of myelodysplastic syndromes and acute myeloid leukemia (Rodríguez-Paredes and Esteller, 2011). Thus, part of the clinical benefit of these drugs might relate to their capacity to hypomethylate and reactivate previously silenced tumor-suppressor miRNAs. Finally, it is worth mentioning the novel area of reactivating miRNAs with low levels of expression in human tumors (Melo *et al*., 2011). In this approach, a small drug acts as a coactivator of the miRNA-processing machinery, particularly the aforementioned TRBP protein encoded by the TARBP2 gene, to enhance the production of mature miRNAs (Melo

et al., 2011). Although it is not known whether this compound, or similar ones, can override the silencing belts imposed on the hypermethylated miRNAs, it is a pharmacological strategy that merits further attention.

Final perspective

From the candidate miRNA approach to whole-epigenome strategies, an increasing number of miRNAs undergoing DNA methylation-associated silencing in cancer cells have been described since the first reported instance in this area (Saito *et al.*, 2006). Although the functional tumor-suppressor roles and a rigorous analysis of the promoter CpG island hypermethylation-related transcriptional repression have been accomplished for a few of them, such as miR-124a and miR-34b/c, from initial observations (Lujambio *et al.*, 2007, 2008; Toyota *et al.*, 2008) to subsequent independent studies (that is, Ando *et al.*, 2009; Furuta *et al.*, 2010; Vázquez *et al.*, 2010; Wilting *et al.*, 2010), much effort lies ahead of us. A good definition of the exact transcription start sites for many of the new candidates and a detailed characterization of their mRNA targets are needed. Most importantly, further cell and mouse models will be required to test their growth-inhibitory function. However, even now, the epigenetic inactivation of miRNAs with tumor-suppressor activities is recognized as a major hallmark of human tumors, probably at the same level as the promoter CpG island hypermethylation of classical coding tumor-suppressor genes (Jones and Baylin, 2007; Berdasco and Esteller, 2010). Their use as biomarkers of the disease and as potential therapy targets will develop and exciting developments in this area are expected in the near future.

Conflict of interest

The authors declare no conflict of interest.

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