Epigenetic architecture and microRNAs: Reciprocal regulators

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Summary

Deregulation of epigenetic and microRNA (miRNA) pathways are emerging as key events in carcinogenesis. miRNA genes can be epigenetically regulated in cancer and miRNAs can themselves repress key enzymes that drive epigenetic remodeling. Epigenetic and miRNA functions are thus tightly interconnected and crucial for maintaining correct local and global genomic architecture and gene expression patterns, yet the underlying molecular mechanisms and their widespread effects remain poorly understood. Due to the tissue specificity, versatility and relative stability of miRNAs, these small non-coding RNAs are considered especially promising in clinical applications, and their biogenesis and function is a subject of active research. In this review, the current status of epigenetic miRNA regulation is summarized and future therapeutic prospects in the field are discussed with a focus on cancer.

Introduction

Epigenetics is defined as the processes that give rise to heritable changes in gene regulation not involving changes in DNA sequence. In mammals this includes methylation of CpG dinucleotides in DNA, modifications of histone tails, incorporation of alternative histone variants, changes in nucleosome positioning and expression of non-coding RNA (ncRNA). The aggregate of these processes can be referred to as the epigenetic architecture of the nucleus, which plays a fundamental role in the control of important biological processes such as proliferation, imprinting, X-chromosome inactivation and maintenance of cell identity [1, 2]. Epigenetic deregulation, often involving hypermethylation of promoter-associated CpG-islands and acquisition of repressive histone marks, can lead to silencing of tumour suppressor genes, and is a frequent event in carcinogenesis [2, 3]. Thus, epigenetic architecture adds an intricate new dimension to the dynamism of the genome, which was largely unanticipated before the era of the *Human Genome Project*. Decoding the human DNA sequence was expected to revolutionize medicine by unraveling disease causes, biomarkers and therapeutic targets. However, it is now clear that the complexity and regulation of the genome stretches far beyond the four-letter code, and the focus has therefore shifted to incorporate a comprehensive study of the human epigenome. A worldwide effort, *The International Human Epigenome Consortium* was recently initiated to make reference epigenetic maps in a variety of normal and cancer cell types to enhance our understanding of the alterations that commonly occur in disease states [4].

DNA methylation, in particular, has emerged as a powerful marker of disease states as it correlates commonly with gene expression. Many tumor suppressor genes are silenced by aberrant hypermethylation of promoter associated CpG islands in cancer [3], and some oncogenes may also be activated by CpG demethylation [5]. The cancer genome is

characterized by an overall decrease in the level of methylated cytosine, but this hypomethylation largely encompasses intergenic and intronic regions of the DNA and not typically promoter CpG islands. Repeat sequences and transposable elements in particular are associated with a loss in CpG methylation, and this is believed to contribute to the chromosomal instability and increased mutation events observed in cancer [5, 6].

CpG methylation can be analyzed with relatively good sensitivity by a variety of techniques, making it possible to identify aberrant methylation patterns in primary samples of limited quantity and quality [7]. Chromatin modifications, on the other hand, remain challenging to assess in clinical tissue samples due to the requirement for large amounts of pure cell populations. As a result, the role of nucleosome and histone architecture in disease is more obscure, although chromatin integrity appears to be broadly compromised [3]. Nonetheless, the question of whether alterations in the epigenetic landscape are a cause or consequence of gene deregulation still remains a matter of debate [8, 9], and decoding the underlying molecular mechanisms is the subject of on-going research.

Regulatory ncRNAs

A variety of ncRNAs are involved in heritable regulation of gene expression that occurs with somatic cell division and therefore included in the broad concept of epigenetics [3]. One class of growing importance is microRNA (miRNA). These are small ~22nt ncRNAs that in association with the RNA induced silencing complex (RISC) post-transcriptionally repress genes with complementary target sequences [10]. Only in recent years has the widespread biological significance of miRNAs been fully appreciated, and it seems that miRNAs are at some level involved in most cellular processes [11]. Due to the imperfect pairing between miRNAs and their target sequences, any miRNA may regulate multiple mRNAs, and thus

mediate broad cellular effects. In fact, some reports suggest that any individual miRNA can target as many as 200 transcripts simultaneously [12], hence aberrant miRNA expression can have major biological consequences and is a key effector in disease development [13].

Moreover, the expression of many miRNAs is highly tissue and cell type specific and plays important roles during development and differentiation by promoting and/or inhibiting particular lineage phenotypes [14, 15]. Therefore, deregulation of miRNA expression often induces cellular de-differentiation and lineage transitions, promoting carcinogenesis, tumor invasion and metastasis. The tissue and cell type specificity of miRNAs, apparent stability, and low number of miRNA genes compared to coding genes (ca. 940 vs. 20,000 [16]), give them good prediction value and make them promising as biomarkers and therapeutic targets in cancer and other diseases [14, 17-21].

miRNA biogenesis and regulation

The tissue specificity of miRNAs and their key role in gene regulation, suggest that miRNAs themselves must be tightly regulated. While it is well established that miRNA levels are broadly altered in cancer, the fundamental mechanisms regulating miRNA expression remain poorly defined [11, 22]. Most miRNA genes are transcribed by RNA polymerase II (polII) into primary transcripts (pri-miRNA), which are subsequently cleaved by Drosha into pre-miRNAs in the nucleus and exported by Exportin 5 to the cytoplasm where they are processed to mature miRNA duplexes by Dicer (Fig. 1; reviewed in [10]). The levels of mature miRNAs can be regulated at all these levels (transcription, processing, nuclear export, degradation), and, like traditional coding genes, they are affected by epigenetic modifications, mutations and genomic instability [18, 21, 23]. MiRNA loci can be both intergenic and embedded within coding genes, usually in introns. Intergenic miRNA genes are presumably transcribed

from their own promoters, which can be several kb away [24], and intragenic miRNA genes can either be regulated with their host gene, or expressed as individual transcriptional units [25, 26]. Decoding the network regulating miRNA biogenesis and activity is crucial to our understanding of disease and development of novel therapeutic strategies.

miRNA deregulation in cancer

The implication of miRNA deregulation in cancer was initially predicted from the observation that about 50% of miRNA genes are located in unstable chromosomal regions associated with deletions, duplications, or translocations in cancer [27]. As for coding genes, miRNA biogenesis and activity is also affected by the presence single nucleotide polymorphisms (SNPs) in miRNA loci [28]. Moreover, the expression of certain miRNA genes is directed by cancer associated transcription factors such as Myc [29], *ZEB1* (also known as *TCF8*) [30], Twist [31, 32] and p53 [33] (Table 1), which are master regulators of cell cycle, epithelial to mesenchymal transition (EMT), proliferation and stress response. Thus, miRNA expression is controlled at multiple levels, and most mechanisms traditionally associated with the deregulation of tumor suppressor and oncogenes in cancer also apply to miRNA expression.

Carcinogenesis has been associated with both an overall loss and gain of mature miRNA expression [14, 18, 20, 28, 34]. In part, this can be attributed to transcriptional and epigenetic mechanisms, but it seems the main cause of a general loss of mature miRNAs is impaired pri- and pre-miRNA processing due to Drosha and Dicer repression, which is frequently observed in poor prognosis cancer [23, 34]. Interestingly, p53 has been shown to directly enhance miRNA processing under DNA damage induced conditions by boosting the Drosha activity, which consequently leads to increased levels of certain mature miRNAs during stress response, further linking miRNA processing to cancer [35] (for a recent review of

posttranscriptional regulation of miRNA biogenesis see [23]). Although a general decrease in mature miRNAs is attributable to defects in miRNA processing, individual miRNA genes are clearly specifically deregulated, and multiple mechanisms must therefore be involved in the overall perturbation of miRNA expression in cancer.

Despite extensive research, the transcriptional regulation of miRNA genes has proven difficult to dissect [24]. MiRNA promoters and transcription start sites can be problematic to identify and validate as they may be distal to the mature miRNA sequence, and appear to be associated with lower polII activity and less prominent active histone hallmarks such as H3K4me3, H3K9ac and H3K36me3 compared to traditional active genes [24, 36, 37]. In addition, primary miRNA transcripts are processed and degraded co-transcriptionally, making them notoriously difficult to detect [38]. As such, expressed miRNA loci may have a lower propensity for active epigenetic marks and less polII accumulation compared to transcribed coding genes, and high mature miRNA levels could be a consequence of the relative stability of miRNAs when protected in the RISC complex rather than high transcription levels [23]. As a result, mapping promoters, transcription start sites (TSS) and other genetic regulatory elements controlling miRNA expression remain major challenges.

Epigenetically regulated miRNAs

A comprehensive bioinformatic analysis found that as many as 50% of miRNA genes are associated with CpG-islands [22], and a number of epigenetically regulated miRNAs have been identified (Table 1) [11, 34]. In 2006, Saito *et al.* reported that 17 out of 313 human miRNAs were upregulated, by as much as 49-fold for the top candidate miR-127, in T24 bladder cells treated with a combination of the DNA demethylating agent 5-Aza-2'-deoxycytidine (5-Aza-dC) and the chromatin-modifying drug 4-phenylbutyric acid (PBA)

[39]. Similarly, a study in colon cancer cells also identified a small number of epigenetically regulated miRNAs, with the strongest effect observed for miR-1 [40]. A more recent report demonstrated that 5-Aza-dC and PBA treatment of gastric cancer cells activated most members of a large miRNA cluster on chromosome 19q with concurrent chromatin remodeling and demethylation of local Alu-repeats [41]. Hence, a number of miRNAs are responsive to epigenetic drugs in a variety of cell types.

A different strategy for depleting DNA methylation was used by Lujambio *et al.*, which showed that 6% of 320 miRNAs analyzed were upregulated more than 3-fold in a DNA methyltransferase *DNMT1* and *DNMT3B* knock out colon cancer cell line model [42]. Notably, only one of the upregulated miRNA genes, miR-124a, is embedded in a CpG-island, thereby indicating that miRNA expression may be directed by distal CpG-elements. Neuronal miRNA expression in mice deficient for the DNA methyl-CpG binding protein *MeCP2* was also assessed in a recent study, and the expression of 7 of 218 miRNAs were significantly altered in this set-up [43]. Others have reported similar findings, suggesting CpG methylation dependent *MeCP2* regulation of specific miRNA genes, including miR-132, miR-137 and miR-184 [43-45]. However, most of the above studies have relied on global disruption of parts of the epigenetic machinery, and therefore some of the observed effects may be a result of indirect effects.

Several miRNAs that are aberrantly silenced in combination with hypermethylation of local CpG-islands have been identified in hematological, oral, lung, breast, ovarian, bladder, prostate and colon cancers. Some of these, including let-7a, miR-9, miR-34b-c, miR-124a, miR-127, miR-129, miR-137, miR-148a, miR-203, miR-205 and the miR-200 family, have been reported at least twice (Table 1) [22, 39, 42, 46-62]. In one of the more comprehensive

analyses to date, Lujambio *et al.* showed that miR-9, miR-34b-c and miR-148a are all frequently silenced and hypermethylated in aggressive cancers, and this was suggested as a possible DNA methylation signature for primary malignancy metastasis [59]. Conversely, cancer activated microRNAs (oncomiRs) could possibly be upregulated by aberrant CpG hypomethylation, as has been a suggested mechanism of oncogene activation [5], but a clear example of this has yet to be identified.

Our group recently demonstrated concerted silencing of the miR-200 family and miR-205 with concordant accumulation of repressive epigenetic marks in invasive bladder tumors and undifferentiated bladder cell lines [47]. Interestingly, the miR-200s and miR-205 have been implicated in cellular development and stemness, and epigenetic silencing of these miRNAs may be a key event during the formation of cancer stem cells that drive tumor invasion and metastasis [63, 64]. The apparent coordinated epigenetic repression of groups of miRNA loci, like for the miR-200 family and miR-205, suggests a non-random underlying mechanism specifically targeting certain combinations of miRNA genes for epigenetic remodeling. How this occurs remains an unanswered question, but it is possible that common transcriptional promoter elements may be involved in seeding of epigenetic marks, thus leading to subsequent silencing or activation of one or more loci. Examples of differential binding of transcriptional regulators triggering CpG island hypermethylation of specific genes include the cancer related transcription factors Myc, Pml-Rar and *STAT3*, which have been shown to directly interact with and guide *DNMTs* and possibly *HDACs* to target promoters [65-67]. Similarly, Fazi *et al.* demonstrated that the acute myeloid leukemia associated fusion protein Aml1/Eto directs heterochromatin formation and CpG hypermethylation of the miR-223 gene, probably by directly binding to and recruiting *DNMTs*, *MeCP2* and *HDAC1* to an Aml1 site in the miR-223 promoter [68]. Thus, putative transcriptional regulators of miR-200/miR-205

such as *ZEB1* and Twist, which are also involved in development and differentiation, could conceivably be early determinants of the coordinated epigenetic fate of these miRNA loci [30, 63, 64].

Technical hurdles in epigenetic miRNA profiling

In some cases, concomitant chromatin marks have been associated with miRNA genes silenced by CpG hypermethylation [42, 58, 68], but the general interplay between chromatin remodeling, DNA methylation and miRNA expression has not been well studied. A recent extensive screen of miRNA expression and epigenetic remodeling during lymphopoiesis in mouse concluded that lymphocyte specific miRNAs tend to be tightly controlled by polycomb silencing through H3K27me3, maintaining miRNA loci in either repressed, active or semi-active states poised for expression [36]. However, the relatively large amounts of starting material and purity of cell type currently required for the analysis of chromatin modifications by ChIP based methods, makes the histone code still difficult to study in primary human and other low quantity samples. Efforts to generate broad miRNA methylation signatures for diagnostic and prognostic purposes are underway, and some progress has been made [57, 59]. However, this has also been handicapped by technical shortcomings, and more data needs to be accumulated before clinically useful tests of miRNA CpG methylation signatures can be developed.

A major drawback of screens for aberrantly DNA methylated miRNA genes to date is the reliance on globally disruptive epigenetic drugs or knockout models with defunct epigenetic machinery [42, 43, 57, 69]. Not only does this increase the risk of detecting false positives and negatives due to widespread secondary effects, it also requires tedious validation experiments to rule out these possibilities. This in turn means that only a limited number of

bona fide candidates can realistically be verified in any one study. CpG methylation levels are typically assessed by laborious clonal bisulphite sequencing techniques, which are excellent for studying DNA methylation of individual molecules in specific regions of interest, but not ideal for screening through numerous sequences in multiple samples. This is especially problematic for miRNA genes where promoters are difficult to identify, thus often requiring analysis of more and larger regions.

The MassArray® platform, a MALDI-TOF mass spectrometry based system for detecting CpG methylation, allows for higher throughput screening of candidate regions [70]. However, this method still requires that the miRNA gene promoter region has been characterized, and has so far only been used in two published reports on epigenetic regulation of miRNAs [47, 71]. There has been some success with custom array based analysis of methyl-DNA immunoprecipitation (meDIP) and capture of methylated DNA using methylated binding domain (MBD) proteins, but this may be insufficiently sensitive for profiling elusive miRNA promoters.

Genome-wide methylation mapping using MeDIP.seq and MBD.seq, as well as pre-treating genomic DNA with methylation specific restriction enzymes prior to next generation sequencing, are currently being employed to map the epigenome, including miRNA genes. However, genome coverage and CpG sequence bias are challenges that still need to be addressed (Robinson *et al*, unpublished), and what is the most efficient, unbiased and reliable high throughput method for genome-wide DNA methylation mapping is a matter of debate (for a recent review on DNA methylation next generation sequencing strategies see [72]). Whole genome sequencing of bisulphite DNA has also recently been reported [73, 74], and this technique promises to be the most informative approach of all, but at this stage it is

expensive and still suffers from intrinsic mappability issues. Consequently, there are no comprehensive studies of the human miRNA DNA methylome available to date, but with the advent of more sophisticated second and third generation sequencing approaches this is likely to rapidly change.

Epigenetic regulation by miRNAs

miRNAs can also directly modulate the epigenetic machinery, with important downstream implications. Several miRNAs have been shown to target and destabilize the mRNAs of genes directly involved in the deposition of epigenetic marks via the canonical miRNA pathway (Table 1) [10]. In the first conclusive report on this topic, Fabbri *et al.* demonstrated that the miR-29 family targets *DNMT3A* and *DNMT3B*, thereby indirectly guiding genome wide *de novo* DNA methylation [75]. Later, it was confirmed that miR-29b induces global DNA hypomethylation in acute myeloid leukemia by directly downregulating *DNMT3A* and *DNMT3B* and indirectly repressing *DNMT1* [76]. *DNMT3A* is also a target of miR-143 [77], and, interestingly, the epigenetically regulated miR-148a represses specific isoforms of *DNMT3B* via a coding sequence target [78]. In addition, *DNMT1* is directly targeted by miR-148a and miR-152 [79]. As such, epigenetically regulated miRNAs can directly affect the DNA methylation machinery, in what may provide an important feedback mechanism (Table 1 & Fig. 1).

Indeed, *DNMT3A* and *DNMT3B* are frequently upregulated in cancers with poor prognosis and, consistently, silencing of the miR-29 family, miR-143, mir-148a and miR-152 is often reported as repressed in miRNA profiling data sets from various forms of cancer [20, 75, 77, 79-82]. Thus, aberrant miRNA expression may be an underlying cause of the extensive and often conflicting changes in DNA methylation patterns observed in cancer, resulting in both

hyper- and hypo-methylation of specific genes and/or regions of the genome. Interestingly, miRNA regulation of *DNMTs* is also required for *de novo* DNA methylation in mouse embryonic stem cells, with the miR-290 cluster being reported to indirectly activate *DNMT3A*, *DNMT3B* and *DNMT3L*, probably via silencing of the transcriptional repressor Rbl2 [83]. Hence, it seems that all *DNMTs* in mammals are directly or indirectly targeted by a subset of miRNAs all playing individual roles in fine tuning the expression levels of these important epigenetic regulators.

Another miRNA with direct effects on the epigenetic machinery is miR-101. Two independent articles have demonstrated that miR-101 targets Enhancer of Zeste homologue 2 (*EZH2*), the catalytic subunit of the polycomb repression complex 2 (PRC2), which deposits the repressive H3K27me3 mark [84, 85]. There is also evidence that miR-26a and miR-137 targets *EZH2* [43, 86]. Other direct epigenetic miRNA targets include *HDAC1* and *HDAC4* [87, 88], and the list is likely to increase dramatically as methods for determining bona fide miRNA targets improve (for a recent review of the current status of experimental approaches to identifying miRNA targets see [89]). Nonetheless, it is evident that a growing subset of miRNAs are involved in the regulation of an increasing number of DNA and histone modifying enzymes, thus underscoring the importance of these small RNAs in establishing and maintaining epigenetic architecture.

miRNAs and transcriptional gene silencing (TGS)

Perhaps the most intriguing development in the field is the potential of miRNAs to direct transcriptional gene silencing (TGS) through RNA-RNA interactions in the nucleus. It is well established that endogenous double stranded RNA (dsRNA) directly mediates TGS and heterochromatin assembly in yeast and plants [90, 91], and accumulating evidence supports

the presence of similar mechanisms in several other eukaryotic species, including mammals (for a recent review see [92]). In 1994, Wassenegger *et al.* were the first to report that viral RNA could induce DNA methylation and subsequent transcriptional silencing of viroid sequences in the *Arabidopsis thaliana* genome [93]. RNA-directed DNA methylation has since been widely studied in plants, and it is clear that both exogenous and endogenous small interfering RNAs (siRNAs) targeted to genomic promoter regions triggers DNA methylation and TGS in *A. thaliana* [94-99]. Recently, endogenous miRNAs have also been directly implicated in seeding CpG methylation both in *cis* and in *trans* in moss and rice, indicating that there might be a role for the miRNA in pathway nuclear TGS [100, 101].

In 2004, it was discovered that small ncRNAs were essential for heterochromatin and centromere assembly in the fission yeast *Schizosaccharomyces pombe* via its *AGO1* homologue [102]. The inheritance of chromatin structure through the cell cycle in *S. pombe* has since been an important focus area in epigenetic research, and it is now known to rely on centromeric transcripts that are processed into siRNAs, which in turn promote methylation of H3K9 and heterochromatin formation [91, 103, 104]. Although *S. pombe* and *A. thaliana* have emerged as the model organisms for studying RNAi directed TGS and chromatin assembly, similar pathways have been identified in other organisms, and all appear to require the general features of the RNAi machinery, including small dsRNAs, Dicer, and Argonaute proteins [91, 92, 99].

RNAi directed TGS in mammalian cells

Many molecular components involved in TGS in yeast and plants lack mammalian homologues, and whether small ncRNA mediated TGS also occurs endogenously in mammalian systems has been controversial [92, 105]. However, it now seems beyond doubt

that exogenous siRNAs targeted to promoter regions can induce TGS in cultured cells by promoting heterochromatin formation independently of DNA methylation (Fig. 2) [91, 105-110]. One study reported that siRNAs targeted to the *EF1A* promoter region triggered TGS via CpG methylation in human cells, but this was later a subject of some debate and no subsequent reports have replicated this finding [111]. Mammalian dsRNA directed TGS has, on the other hand, consistently been associated with increased localization of Argonaute proteins to chromosomal DNA, but the underlying mechanisms are poorly understood [108, 109, 112, 113]. Repression appears to be dependent on a putative transcriptional silencing complex composed of *AGO1*/*AGO2*, *HDAC1*, *HMT*, *DNMT3A*, and possibly *EZH2* and *SUV39H1*, which is guided to the to the targeted promoter by the siRNA and mediates the deposition of the silent-state histone modifications H3K9me2 and H3K27me3 (Fig. 2) [108, 112, 114, 115].

TGS directed heterochromatin formation in human cells can be triggered by both perfectly- and imperfectly-matched small dsRNAs. Gonzalez *et al.* were the first to show that both siRNAs and bulged miRNA mimics targeted to sense sites in the two promoters of the divergently transcribed *INK4b-ARF-INK4a* locus could induce epigenetic remodeling and gene silencing [108]. Interestingly, they also showed that siRNA directed TGS was associated with chromatin bound *AGO2*, whereas the miRNA mimics appeared to recruit *AGO1*. This observation suggests that there are distinct differences in TGS mediated by different types of small dsRNAs and firmly points towards miRNAs being involved in nuclear gene silencing events [108, 113]. An endogenous role for miRNAs in TGS was further underpinned by observations that miR-17-5p and miR-20a cause H3K9me3 accumulation and transcriptional repression of both mRNA and sense promoter-associated RNAs (pRNA) of genes with miRNA seed-sequence matches within 1.5kb upstream of the transcription start site (TSS)

[108]. Although speculative, another study reported that miR-320, which is encoded proximal to the *POLR3D* TSS in the antisense direction, represses mRNA transcription through targeting its own complementary sequence in *cis* on a sense *POLR3D* pRNA, thereby inducing H3K27me3 accumulation and polycomb silencing [116].

Whereas it seems evident that RNA directed TGS is mediated via interactions with local sense or antisense transcripts, the nature and regulation of these pRNAs remain elusive [92, 108, 117, 118]. A multitude of divergent transcription occurs at all active promoters, which may be required to maintain a euchromatic state and keep promoters accessible for mRNA expression [119, 120]. Two of the examples discussed above also reported a requirement for extensive overlapping transcription at promoters to induce RNA-mediated chromatin remodeling [108, 116]. One plausible scenario is that regulatory RNAs destabilize or degrade these divergent pRNA transcripts, which compromises the open euchromatic state and leads to recruitment of the putative TGS complex, heterochromatin formation and polII depletion.

Other RNAi directed effects on gene expression

In a surprising development, it was recently demonstrated that small dsRNAs targeted to promoter regions under certain circumstances also may activate gene expression [121-123]. Janowski *et al.* showed that siRNAs targeted to the *PGR* promoter can increase gene expression by apparently promoting an open chromatin conformation [123], and similar observations have been reported for p21 [122]. This phenomenon is referred to as RNA activation (RNAa), and has been further supported by findings that both exogenous siRNAs and endogenous miR-373, which has a predicted target site with high sequence complementarity in the *CDH1* promoter, increase E-cadherin levels in human cells in culture [124].

As for TGS, the currently favored hypothesis is that RNAa is mediated via interactions between the small dsRNA and local pRNAs, but the molecular mechanisms are largely unknown [117, 125]. Remarkably, shifting the target site or siRNA sequence slightly, or merely altering the cell culture conditions, can completely sequester the effect or, in some cases, even substitute RNAa with TGS of the respective gene [117, 123, 125]. Subtle changes in circumstances can therefore completely alter the outcome from activation to repression or *vice versa*. This could be a consequence of instability in local bidirectional transcription, interference with transcription factors, RNA binding proteins or secondary RNA or DNA structures, and demonstrates that RNAi directed transcriptional gene regulation is highly complex and remains unpredictable with our current understanding. It could also explain how isomiRs (variant mature miRNAs arising from alternative 5'- and 3'-end processing [126]) may have different functions, thereby adding a whole new layer to the regulation and downstream effects of miRNA biogenesis.

To complicate things further, several other types of RNA have also been linked to chromatin remodeling and gene silencing. A long endogenous antisense RNA was shown to mediate TGS of the tumor suppressor p15 in human cancer cells independently of the RNAi machinery [127], and circumstantial evidence has associated piRNAs (piwi associated small RNAs) with hypermethylation and silencing of transposable elements in developing mouse testes [128]. Notably, it has also been suggested that siRNAs targeted to introns and intron/exon junctions may favor alternative splicing by modulating local chromatin structure and polII elongation [129]. Consequently, the only thing that can be said with certainty is that the molecular machinery controlling epigenetic architecture and transcriptional gene

regulation is astonishingly complex, and we are currently only beginning to unravel this complex puzzle.

Therapeutic prospects of epigenetic miRNA regulation

Frequent deregulation and the reversible nature of epigenetic and miRNA pathways in diseases such as cancer make them attractive novel biomarkers and therapeutic targets. Moreover, the versatility of small regulatory ncRNAs and their interplay with the epigenetic machinery opens up a wide range of potential clinical approaches. Sequence based nucleic acid therapeutics offer an attractive alternative to more traditional small molecule inhibitors by providing additional flexibility for drug design and specificity. As such, miRNA gene regulation is becoming an increasingly important focus area in clinical and pharmaceutical research. Recent advances include miRNA expression signatures that can discriminate acute lymphoblastic leukemia (ALL) from acute myeloid leukemia (AML), and predict outcome in hematological, colon, lung, breast, kidney, and bladder cancers [130-135]. Applications in treatment decisions, such as response to chemo- and radio-therapy, are also being actively pursued [136]. At the current pace, the comprehensiveness and precision of cancer miRNA profiles will rapidly increase, and will likely become important diagnostic and prognostic tools in the clinic over the next decade.

Epigenetic miRNA cancer biomarkers

As discussed, repression of several miRNAs has been associated with DNA hypermethylation in a variety of cancer types (e.g. let-7, miR-9, -34bc, -137, -148a, -200, -203, -205) (Table 1), but the present list of epigenetically regulated miRNAs is too sporadic to be clinically relevant. However, with scientific and technical progress more comprehensive maps of aberrantly methylated miRNAs in cancer will soon emerge. Unlike unstable mRNA, studies

have shown that miRNAs and DNA methylation signatures are present and stable in human plasma, serum and oral fluids, making them especially suitable for the development of clinical tests [17, 48, 137-140]. OncomiRs that can be detected in this way include miR-21, miR-17-3p, miR-92 and miR-141 [17, 141]. Notably, Mitchell *et al.* showed that miRNAs from a human prostate cancer xenograft entered circulation in mouse, and could be readily detected in plasma and used to successfully discriminate xenograft mice from controls. This was extended to human samples as well, where high miR-141 plasma levels correctly differentiated between prostate cancer patients and healthy controls [17]. Furthermore, Lodes *et al*. were able to distinguish miRNA expression profiles of normal and cancer patient samples using less than 1ml of serum without any form of amplification [139], and circulating miRNAs may also be useful in tests for detecting prostate tumor progression [137]. Similarly, blood or sputum DNA methylation tests of epigenetically silenced tumor suppressor miRNAs, such as the miR-34 and miR-200 families, could provide plausible diagnostic and prognostic tools.

miRNAs as direct therapeutic targets

miRNAs are also promising as direct therapeutic targets. One approach is to use artificial miRNA mimics to replace aberrantly repressed tumor suppressor miRNAs [20, 34]. Conversely, efforts to sequester oncomiRs by using sequence specific miRNA inhibitors are widespread. This strategy has already been tested in nonhuman primates, where it was demonstrated that a systemically administrated LNA-based anti-miR-122 was functional and non-toxic in monkeys [142, 143]. However, as miRNAs tend to act in groups and networks, silencing or activating individual miRNAs may be insufficient. To overcome this problem, it has been attempted to simultaneously inhibit subsets of oncomiRs, and miR-21, miR-155 and

miR-17-5p have been successfully silenced in cell lines transfected with a multi-target anti-miR [144].

Due to the reversible nature of DNA methylation and chromatin aberrations, RNAi therapies aimed at restoring epigenetic architecture are an attractive prospect. Certain compounds based on DNA demethylating agents or *HDAC* inhibitors, such as 5-Aza-C, 5-Aza-dC and suberoylanilide hydroxamic acid (SAHA), are FDA approved as chemotherapeutics and have shown some clinical success, but as these drugs alter genome wide epigenetic architecture there is a concern of inducing broad unintended side effects [3, 145]. To attain more specific and local epigenetic remodeling, individual or pools of siRNA or miRNA could be designed to trigger TGS or RNAa of subsets of oncogenes or tumor suppressors. Indeed, siRNAs targeting the HIV-1 promoter have already been successfully employed to induce TGS and suppress viral replication in a cultured T cell line [146]. In theory, such an approach to direct local epigenetic modulations and TGS could easily be tailored to suit individual patients, thereby achieving a therapeutic precision unmatched by traditional drugs, yet the molecular mechanisms in play must be better resolved before such treatments will become reality [147].

Future perspectives

Without doubt, epigenetic and RNA-directed gene regulation provides some of the most exciting and promising current prospects for therapeutic research. This is highlighted by a strong increase in research output and also by the growing interest among life scientists in both academia and the biotech and pharmaceutical industries. Despite impressive advances in this young field, major hurdles in specificity and off-target effects remain before any miRNA-based therapies will reach the clinic. The currently most formidable challenge is siRNA/miRNA delivery [147]. However, progress is being made in this area, and data from

the first successful phase I trials demonstrating RNAi in solid tumors using a nanoparticle siRNA delivery system was recently published, a remarkable feat given that RNAi was discovered hardly a decade ago [148].

Apart from issues in therapeutic development, certain technical shortcomings are also holding back progress in epigenetic miRNA regulation research. Improved high throughput DNA methylation detection methods would facilitate the discovery of aberrantly methylated regions in disease. Similarly, more sensitive ChIP techniques from small amounts of starting material, preferably without the need for amplification steps, would allow detailed epigenetic architecture to be studied in more detail in primary samples, thereby improving our understanding of diseased epigenetic states. Technical solutions to overcome such problems will be pivotal to the progress of the field. Advances in second and third generation sequencing are especially promising and are rapidly getting better and more cost-effective, with current efforts directly aimed at solving issues relating to epigenetic profiling.

Conclusion

In summary, the tissue specificity, robust detection and impressive versatility of small RNAs make them ideal as therapeutics and drug targets and they will likely play a key role in the advent of personalized medicine. Indeed, miRNA signatures are already rapidly emerging as a reliable tool in cancer diagnosis and prognosis, and the epigenetic status of miRNA genes may soon follow. We furthermore anticipate that antimiRs and other sequence specific small RNA inhibitors will provide powerful tools once stability and delivery issues are resolved. The prospect of specifically directing TGS and RNAa of cancer related genes with siRNAs and miRNAs is also an intriguing concept, but with our current understanding of the underlying mechanisms the biological effects remain unpredictable, and clinical relevance is a

future promise that requires intense basic research support. As it stands, the relationship between epigenetic architecture and RNA in mammals remains largely unexplored, and we predict that ncRNA directed epigenetic remodeling will revolutionize our understanding of the dynamic genome and has the potential to shape the future of therapeutic research.

Executive summary

- Epigenetic architecture is crucial for maintaining local and global genomic structure, as well as gene expression.
- Widespread epigenetic alterations occur in disease and are a hallmark of cancer.
- microRNAs (miRNA) are highly tissue and cell lineage specific gene regulatory ncRNAs that are frequently deregulated in cancer.
- The tissue specificity, stability in blood and oral fluids and relatively low number of miRNA genes compared to coding genes make them promising as biomarkers and therapeutic targets.
- miRNA expression can be regulated by epigenetic mechanisms, and, *vice versa*, miRNAs can directly target DNA methylation and histone modifying genes, thereby regulating epigenetic architecture.
- An increasing number of miRNAs are confirmed as DNA hypermethylated and silenced in cancer.
- DNA methylation and chromatin remodeling of specific miRNA genes may be directed by transcriptional regulators guiding *DNMTs*, *HDACs* and other epigenetic modifiers to miRNA promoters.
- *MeCP2*, *DNMTs*, *HDACs*, *HMTs* and polycomb repression complex (PRC) components are all known direct targets of miRNAs.
- Designed exogenous promoter targeting siRNAs can induce transcriptional gene silencing (TGS) by directing local chromatin remodeling in human cells, and there are indications that endogenous miRNAs are involved in similar mechanisms.
- RNA directed TGS probably occurs via interactions between the siRNA, the putative TGS complex and bidirectional transcripts in the target promoter region.

- Promoter targeting siRNAs, and possibly endogenous miRNAs, can under certain circumstances also induce transcriptional activation (RNAa), but the mechanisms regulating these apparently opposing responses are not well understood.
- Promoter targeting siRNAs could conceivably be designed as therapeutics to direct TGS and epigenetic remodeling of specific disease genes.
- Therapeutics based on artificial miRNA mimics and inhibitory antimiRs are currently being developed.
- Cancer specific miRNAs and associated CpG methylated DNA sequences can be detected in blood and sputum, which could be exploited to develop future diagnostic and prognostic tests.
- Our current understanding of epigenetic miRNA regulation is limited, and the assembly of broad epigenetic miRNA maps is handicapped by technical limitations.
- Epigenetic miRNA regulation is a highly promising field likely to dominate future clinical research, but therapeutic success will depend on strong basic research to overcome current technical hurdles to detection, large scale profiling, bioinformatics and drug delivery.

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Conflict of interest

The authors declare no conflict of interest

Figure legends

Figure 1. The epigenetic miRNA regulation cycle. (A) Enzymes with epigenetic functions, such as *DNMT*s, *HDAC*s, *HMT*s and polycomb repression complex (PRC) components, modulate local epigenetic architecture. **(B)** DNA methylation and chromatin modifications affect polII transcription of miRNA genes. White and black lollipops indicate unmethylated and methylated CpG dinucleuotides, respectively. Colored flags represent histone modifications. **(C)** Primary miRNA transcripts (pri-miRNA) are processed into pre-miRNA short hairpins by Drosha and exported to the cytoplasm by Exportin 5 (*XPO5*), where the pre-miRNAs are subsequently processed into mature miRNA duplexes by Dicer. Mature miRNA expression is regulated at all of these levels: transcription, pri-miRNA processing, nuclear export and cytoplasmic pre-miRNA processing. **(D)** Single stranded mature miRNAs associate with an Argonaute (*AGO1-4*), forming the core of the RISC complex. **(E)** The miRNA-loaded RISC binds to mRNAs harboring complementary miRNA target sites (usually in the 3'UTR) leading to mRNA destabilization and/or translational repression. **(F)** miRNA targets may be mRNAs of DNA methylation and chromatin modifying enzymes, as depicted in **(A)**. Repression of such genes in turn directly affects epigenetic architecture, thereby completing the circle of epigenetic miRNA regulation.

Figure 2. Putative model of transcriptional gene silencing (TGS) in mammalian cells. (A) Example of an actively transcribed gene. An open chromatin conformation with H3K9Ac and H3K4me3 marks enriched around the TSS. PolII is transcribing mRNA and is also active upstream of the TSS producing promoter associated RNAs (pRNA). **(B)** TGS is induced by a designed exogenous siRNA with a target site in the promoter. The Argonaute loaded siRNA (*AGO1* or *AGO2*) binds its complementary target sequence in a pRNA, *AGO1/2* associates

either with polII or directly with the local chromatin and *HDAC1*, *DNMT3A*, *HMT*, and probably *EZH2* and *SUV39H*, are recruited. This putative TGS complex depletes H3K9Ac and catalyzes deposition of H3K9me2 and the H3K27me3 polycomb mark, leading to heterochromatin assembly and transcriptional silencing. Recent findings suggest that certain miRNAs may act in a similar endogenous mechanism.

Table 1. miRNAs that are epigenetically regulated and/or have epigenetic targets

miRNA	*Epigenetic regulation*	*Transcription factor regulation*	*Activation/repression in cancer*	*Target genes*	*References*
miR-1	CpG hypermethylation		repression	*HDAC4*	[40, 87]
let-7 family	CpG hypermethylation	NF-κβ, *LIN28*, *LIN28B*	activation/repression	Myc, *HMGA2*	[51, 52, 149, 150]
miR-9 family	CpG hypermethylation		repression	NF-κβ	[50, 53, 56, 59, 151]
miR-26a			activation	*EZH2*, *PTEN*, *SMAD1*	[86, 152]
miR-29 family		Myc	repression	*DNMT3A*/*B*, *MCL1*, *TCL1*	[75, 76]
miR-34 family	CpG hypermethylation (34bc)	p53	repression	Myc, *CDK6*, *MET*, *E2F3*	[33, 49, 57, 59, 62]
miR-101			repression	*EZH2*	[84, 85]
miR-124a	CpG hypermethylation heterochromatin		repression	*CDK6*, *FOXA2*	[42, 56, 58, 61, 69]
miR-127	CpG hypermethylation		repression	*BCL6*	[39, 69]
miR-129	CpG hypermethylation		repression		[53, 54]
miR-132		*CREB*, *MeCP2*	activation	p300, *MeCP2*?	[44]
miR-137	CpG hypermethylation	*MeCP2*	repression		[43, 53, 57]
miR-143-145 cluster	not CpG methylation	NF-κB1	repression	*DNMT3A*, *CBFB*, *PPP3CA*, *CLINT1*	[77, 80, 153]
miR-148a	CpG hypermethylation	TGFβ	repression	*DNMT1*, *DNMT3B*, *TGIF2*	[55, 56, 79, 154]
miR-152			repression	*DNMT1*	[79] [56]
miR-181c	CpG hypermethylation		repression	*NOTCH4*, *KRAS*	[155]
miR-184		*MeCP2*	activation	*AKT2*, *MeCP2*?	[45]
miR-200 family	CpG hypermethylation heterochromatin	*ZEB1*, Twist?	activation/repression	TGFβ, *ZEB1*, *ZEB2*, *BMI1*	[30, 47, 71, 156, 157]
miR-203	CpG hypermethylation		repression		[46, 57, 60]
miR-205	CpG hypermethylation heterochromatin	*ZEB1*? Twist?	repression	*MED1*, *HER3*, *LRP1*, *VEGF-A*	[47, 60, 158-160]
miR-223	CpG hypermethylation heterochromatin	Aml1/Eto, *E2F1*	repression	RhoB, *E2F1*	[68, 161, 162]
miR-342	CpG hypermethylation	expressed with host gene *EVL*	repression		[163]
miR-449a			repression	*HDAC1*	[88]
miR-512	CpG hypermethylation		repression	*MCL-1*	[41]

Key references

3. Sharma S, Kelly Tk, Jones Pa: Epigenetics in cancer. *Carcinogenesis* 31(1), 27-36 (2010).

- A comprehensive recent review on epigenetic changes in cancer, including aberrations in DNA methylation, histone modifications and variants, nucleosome positioning and deregulation of miRNA expression. The current status and future prospects of epigenetic therapies is also discussed.

17. Mitchell Ps, Parkin Rk, Kroh Em *et al.*: Circulating micrornas as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A* 105(30), 10513-10518 (2008)

- This study demonstrates that miRNAs are present in human plasma, where they appear to be stable and protected from endogenous RNase activity. It is confirmed that miRNAs from a human prostate cancer xenograft enter the circulation in mouse and can be detected in plasma, allowing discrimination between xenograft mice and controls.

59. Lujambio A, Calin Ga, Villanueva A *et al.*: A microrna DNA methylation signature for human cancer metastasis. *Proc Natl Acad Sci U S A* 105(36), 13556-13561 (2008)

- One of the first conclusive reports on aberrant silencing and DNA hypermethylation of miRNA genes in cancer compared to normal tissue. CpG hypermethylation of miR-9, miR-34bc and miR-148a is suggested as a signature of lymph node metastasis.

68. Fazi F, Racanicchi S, Zardo G *et al.*: Epigenetic silencing of the myelopoiesis regulator microrna-223 by the aml1/eto oncoprotein. *Cancer Cell* 12(5), 457-466 (2007).

- The only report to date demonstrating a mechanistic link between a transcriptional regulator and epigenetic silencing of a miRNA gene. The leukemia associated fusion transcription factor Aml1/Eto is shown to recruit *DNMTs*, *MeCP2* and *HDAC1* to an Aml1 site in the miR-223 promoter and direct epigenetic silencing of the miR-223 gene, leading to cellular dedifferentiation.

75. Fabbri M, Garzon R, Cimmino A *et al.*: Microrna-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3a and 3b. *Proc Natl Acad Sci U S A* 104(40), 15805-15810 (2007)

- The miR-29 family is shown to directly target *DNMT3A* and *DNMT3B* both *in vitro* and *in vivo* tumor models, thus repressing global *de novo* DNA methylation. Hence, loss of miR-29 expression can partly explain the global gain of DNA methylation typically observed in cancer.

108. Gonzalez S, Pisano Dg, Serrano M: Mechanistic principles of chromatin remodeling guided by sirnas and mirnas. *Cell Cycle* 7(16), 2601-2608 (2008).

- This report demonstrates that both fully complementary siRNA duplexes and bulged miRNA mimics can induce epigenetic remodeling and TGS of the *INK4b-ARF-INK4a* locus. It is shown that siRNAs mediate this function via *AGO2*, whereas the miRNA mimics appear to recruit *AGO1* to the targeted promoter.

117. Morris Kv, Santoso S, Turner Am, Pastori C, Hawkins Pg: Bidirectional transcription directs both transcriptional gene activation and suppression in human cells. *PLoS Genet* 4(11), e1000258 (2008)

- Evidence for a mechnaism of siRNA directed TGS and RNAa mediated by RNA-RNA interactions is presented. RNAa of p21 by exogenous siRNAs is shown to occur via *AGO2* dependent post-transcriptional repression of a p21 antisense transcript.

124. Place Rf, Li Lc, Pookot D, Noonan Ej, Dahiya R: Microrna-373 induces expression of genes with complementary promoter sequences. *Proc Natl Acad Sci U S A* 105(5), 1608-1613 (2008)

- The first report providing evidence for an endogenous role for a miRNA in transcriptional gene regulation in the nucleus. MiR-373 is shown to activate expression of the *CDH1* and *CSDC2* genes by targeting complementary sequences in the respective promoters. Mismatch mutations confirmed that the observed effect was dependent on the mature miR-373 sequence.

References

1. Jones Pa, Laird Pw: Cancer epigenetics comes of age. *Nat Genet* 21(2), 163-167 (1999).
2. Gronbaek K, Hother C, Jones Pa: Epigenetic changes in cancer. *APMIS* 115(10), 1039-1059 (2007).
3. Sharma S, Kelly Tk, Jones Pa: Epigenetics in cancer. *Carcinogenesis* 31(1), 27-36 (2010).
4. Time for the epigenome. *Nature* 463(7281), 587 (2010).
5. Wilson As, Power Be, Molloy Pl: DNA hypomethylation and human diseases. *Biochim Biophys Acta* 1775(1), 138-162 (2007).
6. Ross Jp, Rand Kn, Molloy Pl: Hypomethylation of repeated DNA sequences in cancer. *Epigenomics* 2(2), 245-269 (2010).

7. Clark Sj, Statham A, Stirzaker C, Molloy Pl, Frommer M: DNA methylation: Bisulphite modification and analysis. *Nat Protoc* 1(5), 2353-2364 (2006).
8. Baylin S, Bestor Th: Altered methylation patterns in cancer cell genomes: Cause or consequence? *Cancer Cell* 1(4), 299-305 (2002).
9. Clark Sj, Melki J: DNA methylation and gene silencing in cancer: Which is the guilty party? *Oncogene* 21(35), 5380-5387 (2002).
10. Bartel Dp: Microrna: Genomics, biogenesis, mechanism, and function. *Cell* 116(2), 281-297 (2004).
11. Guil S, Esteller M: DNA methylomes, histone codes and mirnas: Tying it all together. *Int J Biochem Cell Biol* 41(1), 87-95 (2009).
12. Brennecke J, Stark A, Russell Rb, Cohen Sm: Principles of microrna-target recognition. *PLoS Biol* 3(3), e85 (2005).
13. Asli Ns, Pitulescu Me, Kessel M: Micrornas in organogenesis and disease. *Curr Mol Med* 8(8), 698-710 (2008).
14. Kanwar Jr, Mahidhara G, Kanwar Rk: Microrna in human cancer and chronic inflammatory diseases. *Front Biosci (Schol Ed)* 2, 1113-1126 (2010).
15. Wang Y, Keys Dn, Au-Young Jk, Chen C: Micrornas in embryonic stem cells. *J Cell Physiol* 218(2), 251-255 (2009).
16. Griffiths-Jones S, Saini Hk, Van Dongen S, Enright Aj: Mirbase: Tools for microrna genomics. *Nucleic Acids Res* 36(Database issue), D154-158 (2008).
17. Mitchell Ps, Parkin Rk, Kroh Em *et al.*: Circulating micrornas as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A* 105(30), 10513-10518 (2008).
18. Newman Ma, Hammond Sm: Emerging paradigms of regulated microrna processing. *Genes Dev* 24(11), 1086-1092 (2010).
19. Slack Fj, Weidhaas Jb: Micrornas as a potential magic bullet in cancer. *Future Oncol* 2(1), 73-82 (2006).
20. Esquela-Kerscher A, Slack Fj: Oncomirs - micrornas with a role in cancer. *Nat Rev Cancer* 6(4), 259-269 (2006).
21. Yang N, Coukos G, Zhang L: Microrna epigenetic alterations in human cancer: One step forward in diagnosis and treatment. *Int J Cancer* 122(5), 963-968 (2008).
22. Weber B, Stresemann C, Brueckner B, Lyko F: Methylation of human microrna genes in normal and neoplastic cells. *Cell Cycle* 6(9), 1001-1005 (2007).
23. Siomi H, Siomi Mc: Posttranscriptional regulation of microrna biogenesis in animals. *Mol Cell* 38(3), 323-332 (2010).
24. Barski A, Jothi R, Cuddapah S *et al.*: Chromatin poises mirna- and protein-coding genes for expression. *Genome Res* 19(10), 1742-1751 (2009).
25. Ozsolak F, Poling Ll, Wang Z *et al.*: Chromatin structure analyses identify mirna promoters. *Genes Dev* 22(22), 3172-3183 (2008).
26. Corcoran Dl, Pandit Kv, Gordon B, Bhattacharjee A, Kaminski N, Benos Pv: Features of mammalian microrna promoters emerge from polymerase ii chromatin immunoprecipitation data. *PLoS One* 4(4), e5279 (2009).
27. Calin Ga, Sevignani C, Dumitru Cd *et al.*: Human microrna genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci U S A* 101(9), 2999-3004 (2004).
28. Ryan Bm, Robles Ai, Harris Cc: Genetic variation in microrna networks: The implications for cancer research. *Nat Rev Cancer* 10(6), 389-402 (2010).
29. Chang Tc, Yu D, Lee Ys *et al.*: Widespread microrna repression by myc contributes to tumorigenesis. *Nat Genet* 40(1), 43-50 (2008).
30. Burk U, Schubert J, Wellner U *et al.*: A reciprocal repression between zeb1 and members of the mir-200 family promotes emt and invasion in cancer cells. *EMBO Rep* 9(6), 582-589 (2008).
31. Ma L, Teruya-Feldstein J, Weinberg Ra: Tumour invasion and metastasis initiated by microrna-10b in breast cancer. *Nature* 449(7163), 682-688 (2007).
32. Lee Yb, Bantounas I, Lee Dy, Phylactou L, Caldwell Ma, Uney Jb: Twist-1 regulates the mir-199a/214 cluster during development. *Nucleic Acids Res* 37(1), 123-128 (2009).
33. He L, He X, Lim Lp *et al.*: A microrna component of the p53 tumour suppressor network. *Nature* 447(7148), 1130-1134 (2007).
34. Davalos V, Esteller M: Micrornas and cancer epigenetics: A macrorevolution. *Curr Opin Oncol* 22(1), 35-45 (2010).
35. Suzuki Hi, Yamagata K, Sugimoto K, Iwamoto T, Kato S, Miyazono K: Modulation of microrna processing by p53. *Nature* 460(7254), 529-533 (2009).
36. Kuchen S, Resch W, Yamane A *et al.*: Regulation of microrna expression and abundance during lymphopoiesis. *Immunity* 32, 1-12 (2010).

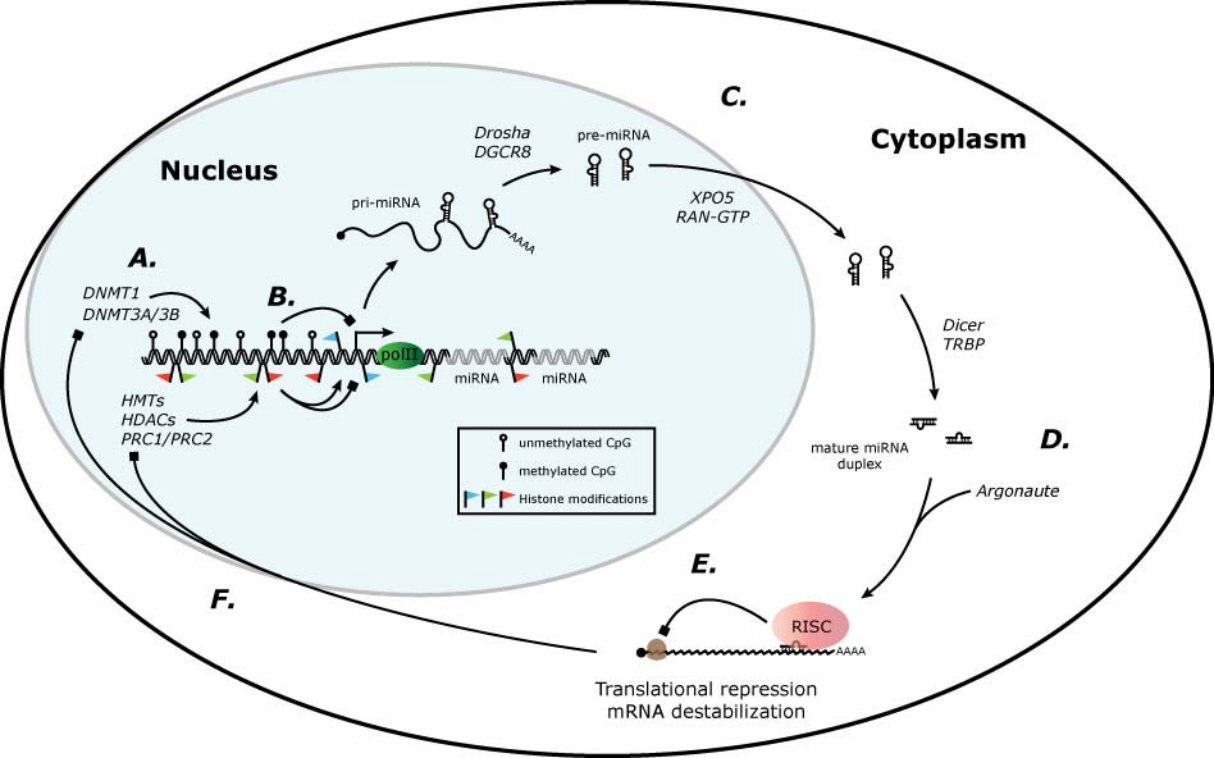
37. Ku M, Koche Rp, Rheinbay E *et al.*: Genomewide analysis of prc1 and prc2 occupancy identifies two classes of bivalent domains. *PLoS Genet* 4(10), e1000242 (2008).
38. Morlando M, Ballarino M, Gromak N, Pagano F, Bozzoni I, Proudfoot Nj: Primary microrna transcripts are processed co-transcriptionally. *Nat Struct Mol Biol* 15(9), 902-909 (2008).
39. Saito Y, Liang G, Egger G *et al.*: Specific activation of microrna-127 with downregulation of the proto-oncogene bcl6 by chromatin-modifying drugs in human cancer cells. *Cancer Cell* 9(6), 435-443 (2006).
40. Datta J, Kutay H, Nasser Mw *et al.*: Methylation mediated silencing of microrna-1 gene and its role in hepatocellular carcinogenesis. *Cancer Res* 68(13), 5049-5058 (2008).
41. Saito Y, Suzuki H, Tsugawa H *et al.*: Chromatin remodeling at alu repeats by epigenetic treatment activates silenced microrna-512-5p with downregulation of mcl-1 in human gastric cancer cells. *Oncogene* 28(30), 2738-2744 (2009).
42. Lujambio A, Ropero S, Ballestar E *et al.*: Genetic unmasking of an epigenetically silenced microrna in human cancer cells. *Cancer Res* 67(4), 1424-1429 (2007).
43. Szulwach Ke, Li X, Smrt Rd *et al.*: Cross talk between microrna and epigenetic regulation in adult neurogenesis. *J Cell Biol* 189(1), 127-141 (2010).
44. Klein Me, Lioy Dt, Ma L, Impey S, Mandel G, Goodman Rh: Homeostatic regulation of mecp2 expression by a creb-induced microrna. *Nat Neurosci* 10(12), 1513-1514 (2007).
45. Nomura T, Kimura M, Horii T *et al.*: Mecp2-dependent repression of an imprinted mir-184 released by depolarization. *Hum Mol Genet* 17(8), 1192-1199 (2008).
46. Bueno Mj, Perez De Castro I, Gomez De Cedron M *et al.*: Genetic and epigenetic silencing of microrna-203 enhances abl1 and bcr-abl1 oncogene expression. *Cancer Cell* 13(6), 496-506 (2008).
47. Wiklund Ed, Bramsen Jb, Hulf T *et al.*: Coordinated epigenetic repression of the mir-200 family and mir-205 in invasive bladder cancer. *Int J Cancer*, (2010).
48. Langevin Sm, Stone Ra, Bunker Ch, Grandis Jr, Sobol Rw, Taioli E: Microrna-137 promoter methylation in oral rinses from patients with squamous cell carcinoma of the head and neck is associated with gender and body mass index. *Carcinogenesis* 31(5), 864-870
49. Corney Dc, Hwang Ci, Matoso A *et al.*: Frequent downregulation of mir-34 family in human ovarian cancers. *Clin Cancer Res* 16(4), 1119-1128 (2010).
50. Hsu Py, Deatherage De, Rodriguez Ba *et al.*: Xenoestrogen-induced epigenetic repression of microrna-9-3 in breast epithelial cells. *Cancer Res* 69(14), 5936-5945 (2009).
51. Brueckner B, Stresemann C, Kuner R *et al.*: The human let-7a-3 locus contains an epigenetically regulated microrna gene with oncogenic function. *Cancer Res* 67(4), 1419-1423 (2007).
52. Lu L, Katsaros D, De La Longrais Ia, Sochirca O, Yu H: Hypermethylation of let-7a-3 in epithelial ovarian cancer is associated with low insulin-like growth factor-ii expression and favorable prognosis. *Cancer Res* 67(21), 10117-10122 (2007).
53. Bandres E, Agirre X, Bitarte N *et al.*: Epigenetic regulation of microrna expression in colorectal cancer. *Int J Cancer* 125(11), 2737-2743 (2009).
54. Huang Yw, Liu Jc, Deatherage De *et al.*: Epigenetic repression of microrna-129-2 leads to overexpression of sox4 oncogene in endometrial cancer. *Cancer Res* 69(23), 9038-9046 (2009).
55. Hanoun N, Delpu Y, Suriawinata Aa *et al.*: The silencing of microrna 148a production by DNA hypermethylation is an early event in pancreatic carcinogenesis. *Clin Chem*, (2010).
56. Lehmann U, Hasemeier B, Christgen M *et al.*: Epigenetic inactivation of microrna gene hsa-mir-9-1 in human breast cancer. *J Pathol* 214(1), 17-24 (2008).
57. Kozaki K, Imoto I, Mogi S, Omura K, Inazawa J: Exploration of tumor-suppressive micrornas silenced by DNA hypermethylation in oral cancer. *Cancer Res* 68(7), 2094-2105 (2008).
58. Roman-Gomez J, Agirre X, Jimenez-Velasco A *et al.*: Epigenetic regulation of micrornas in acute lymphoblastic leukemia. *J Clin Oncol*, (2009).
59. Lujambio A, Calin Ga, Villanueva A *et al.*: A microrna DNA methylation signature for human cancer metastasis. *Proc Natl Acad Sci U S A* 105(36), 13556-13561 (2008).
60. Iorio Mv, Visone R, Di Leva G *et al.*: Microrna signatures in human ovarian cancer. *Cancer Res* 67(18), 8699-8707 (2007).
61. Agirre X, Vilas-Zornoza A, Jimenez-Velasco A *et al.*: Epigenetic silencing of the tumor suppressor microrna hsa-mir-124a regulates cdk6 expression and confers a poor prognosis in acute lymphoblastic leukemia. *Cancer Res* 69(10), 4443-4453 (2009).

62. Toyota M, Suzuki H, Sasaki Y *et al.*: Epigenetic silencing of microrna-34b/c and b-cell translocation gene 4 is associated with cpg island methylation in colorectal cancer. *Cancer Res* 68(11), 4123-4132 (2008).
63. Wellner U, Schubert J, Burk Uc *et al.*: The emt-activator zeb1 promotes tumorigenicity by repressing stemness-inhibiting micrornas. *Nat Cell Biol* 11(12), 1487-1495 (2009).
64. Peter Me: Let-7 and mir-200 micrornas: Guardians against pluripotency and cancer progression. *Cell Cycle* 8(6), 843-852 (2009).
65. Zhang Q, Wang Hy, Marzec M, Raghunath Pn, Nagasawa T, Wasik Ma: Stat3- and DNA methyltransferase 1-mediated epigenetic silencing of shp-1 tyrosine phosphatase tumor suppressor gene in malignant t lymphocytes. *Proc Natl Acad Sci U S A* 102(19), 6948-6953 (2005).
66. Brenner C, Deplus R, Didelot C *et al.*: Myc represses transcription through recruitment of DNA methyltransferase corepressor. *EMBO J* 24(2), 336-346 (2005).
67. Di Croce L, Raker Va, Corsaro M *et al.*: Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. *Science* 295(5557), 1079-1082 (2002).
68. Fazi F, Racanicchi S, Zardo G *et al.*: Epigenetic silencing of the myelopoiesis regulator microrna-223 by the aml1/eto oncoprotein. *Cancer Cell* 12(5), 457-466 (2007).
69. Lujambio A, Esteller M: Cpg island hypermethylation of tumor suppressor micrornas in human cancer. *Cell Cycle* 6(12), 1455-1459 (2007).
70. Coolen Mw, Statham Al, Gardiner-Garden M, Clark Sj: Genomic profiling of cpg methylation and allelic specificity using quantitative high-throughput mass spectrometry: Critical evaluation and improvements. *Nucleic Acids Res* 35(18), e119 (2007).
71. Vrba L, Jensen Tj, Garbe Jc *et al.*: Role for DNA methylation in the regulation of mir-200c and mir-141 expression in normal and cancer cells. *PLoS One* 5(1), e8697 (2010).
72. Robinson Md, Statham Al, Speed Tp, Clark Sj: Protocol matters: Which methylome are you actually studying? *Epigenomics* In press, (2010).
73. Laurent L, Wong E, Li G *et al.*: Dynamic changes in the human methylome during differentiation. *Genome Res* 20(3), 320-331 (2010).
74. Lister R, Pelizzola M, Dowen Rh *et al.*: Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature* 462(7271), 315-322 (2009).
75. Fabbri M, Garzon R, Cimmino A *et al.*: Microrna-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3a and 3b. *Proc Natl Acad Sci U S A* 104(40), 15805-15810 (2007).
76. Garzon R, Liu S, Fabbri M *et al.*: Microrna-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly dnmt3a and 3b and indirectly dnmt1. *Blood* 113(25), 6411-6418 (2009).
77. Ng Ek, Tsang Wp, Ng Ss *et al.*: Microrna-143 targets DNA methyltransferases 3a in colorectal cancer. *Br J Cancer* 101(4), 699-706 (2009).
78. Duursma Am, Kedde M, Schrier M, Le Sage C, Agami R: Mir-148 targets human dnmt3b protein coding region. *RNA* 14(5), 872-877 (2008).
79. Braconi C, Huang N, Patel T: Microrna-dependent regulation of DNA methyltransferase-1 and tumor suppressor gene expression by interleukin-6 in human malignant cholangiocytes. *Hepatology* 51(3), 881-890 (2010).
80. Ostenfeld Ms, Bramsen Jb, Lamy P *et al.*: Mir-145 induces caspase-dependent and -independent cell death in urothelial cancer cell lines with targeting of an expression signature present in ta bladder tumors. *Oncogene* 29(7), 1073-1084 (2010).
81. Akao Y, Nakagawa Y, Kitade Y, Kinoshita T, Naoe T: Downregulation of micrornas-143 and -145 in b-cell malignancies. *Cancer Sci* 98(12), 1914-1920 (2007).
82. Girault I, Tozlu S, Lidereau R, Bieche I: Expression analysis of DNA methyltransferases 1, 3a, and 3b in sporadic breast carcinomas. *Clin Cancer Res* 9(12), 4415-4422 (2003).
83. Sinkkonen L, Hugenschmidt T, Berninger P *et al.*: Micrornas control de novo DNA methylation through regulation of transcriptional repressors in mouse embryonic stem cells. *Nat Struct Mol Biol* 15(3), 259-267 (2008).
84. Varambally S, Cao Q, Mani Rs *et al.*: Genomic loss of microrna-101 leads to overexpression of histone methyltransferase ezh2 in cancer. *Science* 322(5908), 1695-1699 (2008).
85. Friedman Jm, Liang G, Liu Cc *et al.*: The putative tumor suppressor microrna-101 modulates the cancer epigenome by repressing the polycomb group protein ezh2. *Cancer Res* 69(6), 2623-2629 (2009).
86. Wong Cf, Tellam Rl: Microrna-26a targets the histone methyltransferase enhancer of zeste homolog 2 during myogenesis. *J Biol Chem* 283(15), 9836-9843 (2008).

87. Chen Jf, Mandel Em, Thomson Jm *et al.*: The role of microrna-1 and microrna-133 in skeletal muscle proliferation and differentiation. *Nat Genet* 38(2), 228-233 (2006).
88. Noonan Ej, Place Rf, Pookot D *et al.*: Mir-449a targets hdac-1 and induces growth arrest in prostate cancer. *Oncogene* 28(14), 1714-1724 (2009).
89. Orom Ua, Lund Ah: Experimental identification of microrna targets. *Gene* 451(1-2), 1-5 (2010).
90. Matzke Ma, Birchler Ja: Rnai-mediated pathways in the nucleus. *Nat Rev Genet* 6(1), 24-35 (2005).
91. Grewal Si, Jia S: Heterochromatin revisited. *Nat Rev Genet* 8(1), 35-46 (2007).
92. Suzuki K, Kelleher Ad: Transcriptional regulation by promoter targeted rnas. *Curr Top Med Chem* 9(12), 1079-1087 (2009).
93. Wassenegger M, Heimes S, Riedel L, Sanger Hl: Rna-directed de novo methylation of genomic sequences in plants. *Cell* 76(3), 567-576 (1994).
94. Molnar A, Melnyk Cw, Bassett A, Hardcastle Tj, Dunn R, Baulcombe Dc: Small silencing rnas in plants are mobile and direct epigenetic modification in recipient cells. *Science*, (2010).
95. Wassenegger M: Rna-directed DNA methylation. *Plant Mol Biol* 43(2-3), 203-220 (2000).
96. Aufsatz W, Mette Mf, Van Der Winden J, Matzke Aj, Matzke M: Rna-directed DNA methylation in arabidopsis. *Proc Natl Acad Sci U S A* 99 Suppl 4, 16499-16506 (2002).
97. Jones L, Hamilton Aj, Voinnet O, Thomas Cl, Maule Aj, Baulcombe Dc: Rna-DNA interactions and DNA methylation in post-transcriptional gene silencing. *Plant Cell* 11(12), 2291-2301 (1999).
98. Mette Mf, Aufsatz W, Van Der Winden J, Matzke Ma, Matzke Aj: Transcriptional silencing and promoter methylation triggered by double-stranded rna. *EMBO J* 19(19), 5194-5201 (2000).
99. Huettel B, Kanno T, Daxinger L *et al.*: Rna-directed DNA methylation mediated by drd1 and pol ivb: A versatile pathway for transcriptional gene silencing in plants. *Biochim Biophys Acta* 1769(5-6), 358-374 (2007).
100. Wu L, Zhou H, Zhang Q *et al.*: DNA methylation mediated by a microrna pathway. *Mol Cell* 38(3), 465-475 (2010).
101. Khraiwesh B, Arif Ma, Seumel Gi *et al.*: Transcriptional control of gene expression by micrornas. *Cell* 140(1), 111-122 (2010).
102. Verdel A, Jia S, Gerber S *et al.*: Rnai-mediated targeting of heterochromatin by the rits complex. *Science* 303(5658), 672-676 (2004).
103. Halic M, Moazed D: Dicer-independent primal rnas trigger rnai and heterochromatin formation. *Cell* 140(4), 504-516 (2010).
104. Chen Es, Zhang K, Nicolas E, Cam Hp, Zofall M, Grewal Si: Cell cycle control of centromeric repeat transcription and heterochromatin assembly. *Nature* 451(7179), 734-737 (2008).
105. Malecova B, Morris Kv: Transcriptional gene silencing through epigenetic changes mediated by non-coding rnas. *Curr Opin Mol Ther* 12(2), 214-222 (2010).
106. Ting Ah, Schuebel Ke, Herman Jg, Baylin Sb: Short double-stranded rna induces transcriptional gene silencing in human cancer cells in the absence of DNA methylation. *Nat Genet* 37(8), 906-910 (2005).
107. Weinberg Ms, Villeneuve Lm, Ehsani A *et al.*: The antisense strand of small interfering rnas directs histone methylation and transcriptional gene silencing in human cells. *RNA* 12(2), 256-262 (2006).
108. Gonzalez S, Pisano Dg, Serrano M: Mechanistic principles of chromatin remodeling guided by sirnas and mirnas. *Cell Cycle* 7(16), 2601-2608 (2008).
109. Kim Dh, Villeneuve Lm, Morris Kv, Rossi Jj: Argonaute-1 directs sirna-mediated transcriptional gene silencing in human cells. *Nat Struct Mol Biol* 13(9), 793-797 (2006).
110. Janowski Ba, Huffman Ke, Schwartz Jc *et al.*: Inhibiting gene expression at transcription start sites in chromosomal DNA with antigene rnas. *Nat Chem Biol* 1(4), 216-222 (2005).
111. Morris Kv, Chan Sw, Jacobsen Se, Looney Dj: Small interfering rna-induced transcriptional gene silencing in human cells. *Science* 305(5688), 1289-1292 (2004).
112. Hawkins Pg, Santoso S, Adams C, Anest V, Morris Kv: Promoter targeted small rnas induce long-term transcriptional gene silencing in human cells. *Nucleic Acids Res* 37(9), 2984-2995 (2009).
113. Janowski Ba, Huffman Ke, Schwartz Jc *et al.*: Involvement of ago1 and ago2 in mammalian transcriptional silencing. *Nat Struct Mol Biol* 13(9), 787-792 (2006).
114. Fuks F, Burgers Wa, Godin N, Kasai M, Kouzarides T: Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription. *EMBO J* 20(10), 2536-2544 (2001).
115. Vire E, Brenner C, Deplus R *et al.*: The polycomb group protein ezh2 directly controls DNA methylation. *Nature* 439(7078), 871-874 (2006).

116. Kim Dh, Saetrom P, Snove O, Jr., Rossi Jj: Microrna-directed transcriptional gene silencing in mammalian cells. *Proc Natl Acad Sci U S A* 105(42), 16230-16235 (2008).
117. Morris Kv, Santoso S, Turner Am, Pastori C, Hawkins Pg: Bidirectional transcription directs both transcriptional gene activation and suppression in human cells. *PLoS Genet* 4(11), e1000258 (2008).
118. Han J, Kim D, Morris Kv: Promoter-associated rna is required for rna-directed transcriptional gene silencing in human cells. *Proc Natl Acad Sci U S A* 104(30), 12422-12427 (2007).
119. Preker P, Nielsen J, Kammler S *et al.*: Rna exosome depletion reveals transcription upstream of active human promoters. *Science* 322(5909), 1851-1854 (2008).
120. Seila Ac, Calabrese Jm, Levine Ss *et al.*: Divergent transcription from active promoters. *Science* 322(5909), 1849-1851 (2008).
121. Vasudevan S, Tong Y, Steitz Ja: Switching from repression to activation: Microrna can up-regulate translation. *Science* 318(5858), 1931-1934 (2007).
122. Li Lc, Okino St, Zhao H *et al.*: Small dsrnas induce transcriptional activation in human cells. *Proc Natl Acad Sci U S A* 103(46), 17337-17342 (2006).
123. Janowski Ba, Younger St, Hardy Db, Ram R, Huffman Ke, Corey Dr: Activating gene expression in mammalian cells with promoter-targeted duplex rnas. *Nat Chem Biol* 3(3), 166-173 (2007).
124. Place Rf, Li Lc, Pookot D, Noonan Ej, Dahiya R: Microrna-373 induces expression of genes with complementary promoter sequences. *Proc Natl Acad Sci U S A* 105(5), 1608-1613 (2008).
125. Schwartz Jc, Younger St, Nguyen Nb *et al.*: Antisense transcripts are targets for activating small rnas. *Nat Struct Mol Biol* 15(8), 842-848 (2008).
126. Morin Rd, O'connor Md, Griffith M *et al.*: Application of massively parallel sequencing to microrna profiling and discovery in human embryonic stem cells. *Genome Res* 18(4), 610-621 (2008).
127. Yu W, Gius D, Onyango P *et al.*: Epigenetic silencing of tumour suppressor gene p15 by its antisense rna. *Nature* 451(7175), 202-206 (2008).
128. Kuramochi-Miyagawa S, Watanabe T, Gotoh K *et al.*: DNA methylation of retrotransposon genes is regulated by piwi family members mili and miwi2 in murine fetal testes. *Genes Dev* 22(7), 908-917 (2008).
129. Allo M, Buggiano V, Fededa Jp *et al.*: Control of alternative splicing through sirna-mediated transcriptional gene silencing. *Nat Struct Mol Biol* 16(7), 717-724 (2009).
130. Budhu A, Jia Hl, Forgues M *et al.*: Identification of metastasis-related micrornas in hepatocellular carcinoma. *Hepatology* 47(3), 897-907 (2008).
131. Gottardo F, Liu Cg, Ferracin M *et al.*: Micro-rna profiling in kidney and bladder cancers. *Urol Oncol* 25(5), 387-392 (2007).
132. Baffa R, Fassan M, Volinia S *et al.*: Microrna expression profiling of human metastatic cancers identifies cancer gene targets. *J Pathol* 219(2), 214-221 (2009).
133. Schetter Aj, Leung Sy, Sohn Jj *et al.*: Microrna expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA* 299(4), 425-436 (2008).
134. Raponi M, Dossey L, Jatkoe T *et al.*: Microrna classifiers for predicting prognosis of squamous cell lung cancer. *Cancer Res* 69(14), 5776-5783 (2009).
135. Mi S, Lu J, Sun M *et al.*: Microrna expression signatures accurately discriminate acute lymphoblastic leukemia from acute myeloid leukemia. *Proc Natl Acad Sci U S A* 104(50), 19971-19976 (2007).
136. Hummel R, Hussey Dj, Haier J: Micrornas: Predictors and modifiers of chemo- and radiotherapy in different tumour types. *Eur J Cancer* 46(2), 298-311 (2010).
137. Brase Jc, Johannes M, Schlomm T *et al.*: Circulating mirnas are correlated with tumor progression in prostate cancer. *Int J Cancer*, (2010).
138. Ferracin M, Veronese A, Negrini M: Micromarkers: Mirnas in cancer diagnosis and prognosis. *Expert Rev Mol Diagn* 10(3), 297-308 (2010).
139. Lodes Mj, Caraballo M, Suciu D, Munro S, Kumar A, Anderson B: Detection of cancer with serum mirnas on an oligonucleotide microarray. *PLoS One* 4(7), e6229 (2009).
140. Xie Y, Todd Nw, Liu Z *et al.*: Altered mirna expression in sputum for diagnosis of non-small cell lung cancer. *Lung Cancer* 67(2), 170-176 (2010).
141. Tanaka M, Oikawa K, Takanashi M *et al.*: Down-regulation of mir-92 in human plasma is a novel marker for acute leukemia patients. *PLoS One* 4(5), e5532 (2009).

142. Elmen J, Lindow M, Schutz S *et al.*: Lna-mediated microrna silencing in non-human primates. *Nature* 452(7189), 896-899 (2008).
143. Lanford Re, Hildebrandt-Eriksen Es, Petri A *et al.*: Therapeutic silencing of microrna-122 in primates with chronic hepatitis c virus infection. *Science* 327(5962), 198-201 (2010).
144. Lu Y, Xiao J, Lin H *et al.*: A single anti-microrna antisense oligodeoxyribonucleotide (amo) targeting multiple micrornas offers an improved approach for microrna interference. *Nucleic Acids Res* 37(3), e24 (2009).
145. Cortez Cc, Jones Pa: Chromatin, cancer and drug therapies. *Mutat Res* 647(1-2), 44-51 (2008).
146. Yamagishi M, Ishida T, Miyake A *et al.*: Retroviral delivery of promoter-targeted shrna induces long-term silencing of hiv-1 transcription. *Microbes Infect* 11(4), 500-508 (2009).
147. Castanotto D, Rossi Jj: The promises and pitfalls of rna-interference-based therapeutics. *Nature* 457(7228), 426-433 (2009).
148. Davis Me, Zuckerman Je, Choi Ch *et al.*: Evidence of rnai in humans from systemically administered sirna via targeted nanoparticles. *Nature* 464(7291), 1067-1070 (2010).
149. Viswanathan Sr, Daley Gq, Gregory Ri: Selective blockade of microrna processing by lin28. *Science* 320(5872), 97-100 (2008).
150. Lee Ys, Dutta A: The tumor suppressor microrna let-7 represses the hmga2 oncogene. *Genes Dev* 21(9), 1025-1030 (2007).
151. Guo Lm, Pu Y, Han Z *et al.*: Microrna-9 inhibits ovarian cancer cell growth through regulation of nf-kappab1. *FEBS J* 276(19), 5537-5546 (2009).
152. Huse Jt, Brennan C, Hambardzumyan D *et al.*: The pten-regulating microrna mir-26a is amplified in high-grade glioma and facilitates gliomagenesis in vivo. *Genes Dev* 23(11), 1327-1337 (2009).
153. Zhang X, Liu S, Hu T, He Y, Sun S: Up-regulated microrna-143 transcribed by nuclear factor kappa b enhances hepatocarcinoma metastasis by repressing fibronectin expression. *Hepatology* 50(2), 490-499 (2009).
154. Pan W, Zhu S, Yuan M *et al.*: Microrna-21 and microrna-148a contribute to DNA hypomethylation in lupus cd4+ t cells by directly and indirectly targeting DNA methyltransferase 1. *J Immunol* 184(12), 6773-6781 (2010).
155. Hashimoto Y, Akiyama Y, Otsubo T, Shimada S, Yuasa Y: Involvement of epigenetically silenced microrna-181c in gastric carcinogenesis. *Carcinogenesis* 31(5), 777-784 (2010).
156. Gregory Pa, Bert Ag, Paterson El *et al.*: The mir-200 family and mir-205 regulate epithelial to mesenchymal transition by targeting zeb1 and sip1. *Nat Cell Biol* 10(5), 593-601 (2008).
157. Park Sm, Gaur Ab, Lengyel E, Peter Me: The mir-200 family determines the epithelial phenotype of cancer cells by targeting the e-cadherin repressors zeb1 and zeb2. *Genes Dev* 22(7), 894-907 (2008).
158. Song H, Bu G: Microrna-205 inhibits tumor cell migration through down-regulating the expression of the ldl receptor-related protein 1. *Biochem Biophys Res Commun* 388(2), 400-405 (2009).
159. Iorio Mv, Casalini P, Piovan C *et al.*: Microrna-205 regulates her3 in human breast cancer. *Cancer Res* 69(6), 2195-2200 (2009).
160. Mouillet Jf, Chu T, Nelson Dm, Mishima T, Sadovsky Y: Mir-205 silences med1 in hypoxic primary human trophoblasts. *FASEB J*, (2010).
161. Pulikkan Ja, Dengler V, Peramangalam Ps *et al.*: Cell-cycle regulator e2f1 and microrna-223 comprise an autoregulatory negative feedback loop in acute myeloid leukemia. *Blood* 115(9), 1768-1778 (2010).
162. Sun G, Li H, Rossi Jj: Sequence context outside the target region influences the effectiveness of mir-223 target sites in the rhob 3'utr. *Nucleic Acids Res* 38(1), 239-252 (2010).
163. Grady Wm, Parkin Rk, Mitchell Ps *et al.*: Epigenetic silencing of the intronic microrna hsa-mir-342 and its host gene evl in colorectal cancer. *Oncogene* 27(27), 3880-3888 (2008).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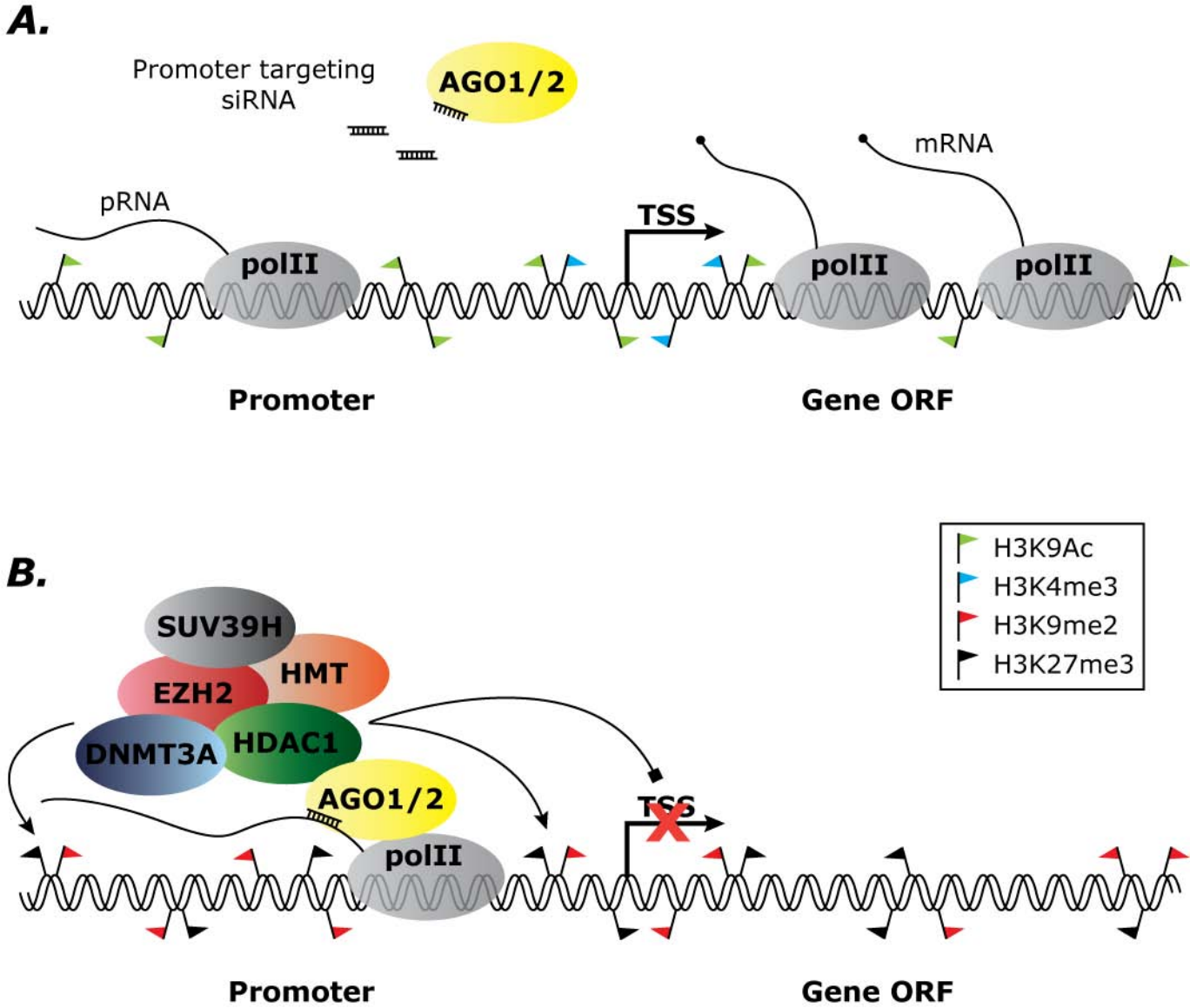
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polII
Promoter
Gene ORF