Chapter 7
Unraveling the Complex Network of Interactions Between Noncoding RNAs and Epigenetics in Cancer

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Abstract Epigenetics is the study of heritable changes in gene expression that do not involve changes in the underlying DNA sequence. The most studied epigenetic modifications include DNA methylation and histone changes. These modifications are able to modulate the chromatin conformation and have a critical role in regulating gene expression. Over the last years, growing evidences have revealed the crucial role of epigenetic mechanisms controlling noncoding RNAs (ncRNAs) expression, in the same way as previously shown for protein-coding genes. Most interestingly, the link between ncRNAs and epigenetics is not limited to epigenetic regulation of ncRNAs, but also takes place in the opposite direction, meaning that these RNA molecules are able to control gene expression by regulating effectors of the epigenetic machinery. In this chapter both of these scenarios will be discussed, focusing in the cancer context. The complex network of reciprocal interactions between ncRNAs and epigenetics is just beginning to unravel and an exciting future in research about the role of ncRNAs in cancer epigenetics is guaranteed.

Keywords Epigenetics • Cancer • ncRNA • miRNA • lncRNA • DNA methylation • Histone modifications • Chromatin

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7.1 Introduction

The term Epigenetics was introduced by C.H Waddington in 1939 to name "the causal interactions between genes and their products, which bring the phenotype into being" [1]. It was later redefined as those heritable changes in gene expression that do not involve changes in the underlying DNA sequence [2]. The most studied epigenetic modifications include DNA methylation and histone changes. These modifications are able to modulate the chromatin conformation and have a critical role in regulating gene expression and the accessibility of transcription factors, co-activators, and co-repressors.

In humans, DNA methylation occurs at the carbon-5 of the cytosine in CpG dinucleotides. This reaction is catalyzed by DNA methyltransferases (DNMTs): DNMT3a and DNMT3b (de novo DNMTs) transfer a methyl group from *S-adenosyl-methionine* to previously unmethylated cytosines, while DNMT1 (*maintenance* DNMT) preserves the methylation patterns throughout each cell division. CpG sites are not randomly distributed in the genome, but instead there are CpG-rich zones known as CpG islands, located mainly at the regulatory regions in 40–60 % of all genes [3, 4]. Methylation of CpG islands is a rare event in normal cells, restricted to untranscribed genes in X chromosome, imprinted genes, germ line genes, and some tissue-specific genes. However, CpG island hypermethylation is a common hallmark in cancer cells, first associated to tumor suppressor gene silencing [4, 5].

Along with DNA methylation, histone modifications are the most studied epigenetic events related to cancer progression. Regulation of gene expression can occur through posttranslational modifications of the histone tails, including covalent changes such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation, proline isomerization, and ADP-ribosylation. Their presence on histones form the called "histone code" that dictates the chromatin structure in which DNA is packaged and can orchestrate the ordered recruitment of enzyme complexes to wrap the DNA [6, 7]. This histone code is "written" and "erased" by histone modifying enzymes. The "writer" of histone modification refers to an enzyme that catalyzes a chemical modification of histones in a residue-specific manner (i.e., histone methyltransferases HMTs or histone acetyltransferases HATs), and the "eraser" of histone marks refers to an enzyme that removes a chemical modification from histones (i.e., histone demethylases HDMs or histone deacetylases HDACs). This code is interpreted by "reader" or "effector" proteins that specifically bind to a certain type or a combination of histone modifications and translate the histone code into a meaningful biological outcome, whether it is transcriptional activation or silencing, or other cellular responses. In addition to this recruitment mechanism, histone marks per se can modulate the chromatin conformation based on steric or charge interactions (i.e., neutralization of the positive charges of histones by acetylation of lysines) [6, 8].

Another crucial player in epigenetic silencing is the Polycomb system. In mammals, two main Polycomb complexes have been identified: Polycomb repressive complex 1 (PRC1) and 2 (PRC2). PRC1 compacts chromatin and catalyzes the monoubiquitylation of histone H2A, and PRC2 also contributes to chromatin folding and catalyzes

the methylation of histone H3 at lysine 27 (H3K27). Polycomb complexes have been implicated in diverse biological processes such as epigenetic inheritance, differentiation, stem cell plasticity, proliferation, and senescence [9]. Altogether, this complex epigenetic network guarantees a dynamic and accurate control of gene expression.

Over the last years, growing evidences have revealed the crucial role of epigenetic mechanisms controlling noncoding RNAs (ncRNAs) expression, in the same way as previously shown for protein-coding genes. Most interestingly, the link between ncRNAs and epigenetics is not limited to epigenetic regulation of ncRNAs, but also takes place in the opposite direction, meaning that these RNA molecules are able to control gene expression by regulating effectors of the epigenetic machinery. In this chapter both of these scenarios will be discussed, focusing in the cancer context.

7.2 Epigenetically Regulated Noncoding RNAs in Cancer

7.2.1 Short and Midsize ncRNAs

Epigenetic silencing is considered a hallmark of cancer. The first evidences showing how this mechanism is able to control ncRNA expression were found in microRNAs (miRNAs), the most widely studied class of ncRNAs. Using different strategies, independent groups were able to demonstrate that miRNA levels are epigenetically regulated [10–12]. Treating the human bladder cell line T24 with the chromatin-modifying drugs 5-*aza*-2′-deoxycytidine (a DNA demethylating agent) and 4-phenylbutyric acid (a histone deacetylase inhibitor, HDACi), Saito et al. found that about 5 % of a set of 313 miRNAs incorporated in a microarray platform were upregulated more than threefold, including **miR-127** [10]. Using an analogous approach but another HDACi, the hydroxamic acid LAQ824, Scott et al. reported that levels of 27 miRNAs were rapidly deregulated after treatment in the breast cancer cell line SKBr3; for instance expression of **miR-27** was significantly decreased [11]. Our group also contributed with the first proofs of the role of epigenetics in ncRNAs. Thus, using as a model system a colon cancer cell line genetically deficient for the DNA methyltransferases DNMT1 and DNMT3b (HCT-116 double knockout) and comparing it with the wild-type cell line (HCT-116), we identified epigenetic silencing of **miR-124a**. Furthermore, we demonstrated that miR-124a inhibition results in an increased expression of its target CDK6, and consequent phosphorylation of the downstream CDK6-regulated Rb protein [12]. CpG hypermethylation-associated silencing of this miRNA has also been detected in other tumors, such as glioblastoma multiforme [13], gastric cancer [14], hematopoietic malignancies [15, 16], hepatocellular carcinoma [17], and cervical tumors [18]. The HCT-116 DNMT1/3b double knockout cell line has been a valuable tool in the identification not only of epigenetically regulated miRNAs with tumor suppressor capacity, but also oncogenic miRNAs, as is the case for **let-7a-3**. This member of the archetypal let-7 miRNA gene family was found heavily methylated in normal human tissues but hypomethylated in some lung adenocarcinomas

[19]. In contrast, hypermethylation of this miRNA has been detected in ovarian and breast cancer [20, 21].

During the last few years, the list of miRNAs regulated by CpG methylation and histone modifications is in accelerated expansion. Some relevant examples are included in Table 7.1 and Fig. 7.1, e.g., **miR-1** in hepatocarcinoma [22]; **miR-107** in pancreatic cancer [34]; **miR-129-2** in colorectal [25], endometrial [35] and gastric cancer [33, 36]; **miR-137** in colorectal [25, 37]; gastric [39] and oral cancer [38, 41, 42]**; miR-193a** in hematological malignances [44], oral [43] and lung cancer [27]; **miR-196b** in gastric cancer [46] and hematopoietic malignancies [45]; **miR-199a** in gastric [47], ovarian [48], and testicular tumors [49]; **miR-203** in hematological malignances [32, 55, 56] and hepatocellular carcinomas [17] or **miR-9-1** in colorectal cancer. Interestingly, in this case its methylation was associated with the presence of lymph node metastasis [25]. The relevance of epigenetic control of miRNAs in cancer progression and metastasis has also been extensively documented. In 2008, our group described a miRNA DNA methylation signature for metastasis. By means of a pharmacological and genomic approach, treating lymph node metastatic cancer cells with a DNA demethylating agent followed by hybridization to an expression microarray, we detected reexpression of **miR-9**, **miR-34b/c,** and **miR-148a** upon drug treatment and confirmed cancer-specific hypermethylation-associated silencing. Most relevantly, the reintroduction of miR-148a and miR-34b/c in cancer cells with epigenetic inactivation inhibited their motility, reduced tumor growth, and inhibited metastasis formation in xenograft models, with an associated downregulation of the miRNA oncogenic target genes, such as C-MYC, E2F3, CDK6, and TGIF2. Moreover, miR-9, miR-34b/c and miR-148a hypermethylation was significantly associated with the appearance of lymph node human metastasis [24]. Epigenetic control of these miRNAs has been also confirmed by other studies [16, 23, 26, 27, 31–33]. Recently, our group and others have also shown the epigenetic silencing of a miRNA family, the **miR-200 family**, recognized as a master regulator of the epithelial phenotype, by targeting ZEB1 and ZEB2, important repressors of E-cadherin and cell polarity genes [50–54]. During tumor progression, the loss of epithelial features and acquisition of mesenchymal attributes through epithelial–mesenchymal transition (EMT) define tumor fate and metastatic dissemination. However, once cancer cells have reached a secondary site, regain of some epithelial properties (mesenchymal to epithelial transition, MET) could be advantageous for colonizing and establishing a secondary tumor. The plasticity and reversibility make epigenetic mechanisms excellent strategies to modulate miRNAs with variable expression during tumor progression. In this regard, we described a reversible mechanism to regulate expression of both clusters of miR-200 family, **miR-200ba429** and **miR-200c141**, during EMT and cancer progression through dynamic epigenetic regulation mediated by CpG island promoter methylation [53]. Interestingly, in bladder and lung cancer a coordinated epigenetic silencing of miR-200 and **miR-205** has been reported, the last one also involved in EMT [54, 57].

Most of the aforementioned examples describe miRNAs regulated by CpG methylation. Epigenetic control is nevertheless mainly achieved not only by DNA methylation but also by histone modifications, both epigenetic marks being closely

Table 7.1 Epigenetically regulated noncoding RNAs in cancer

ncRNA	Cancer type	Target genes	References
Let-7a-3	Breast, lung, ovarian	IGF-II	[19–21]
miR-1	Hepatocellular carcinoma	HDAC4	[22]
miR-9 family	Metastatic tumors: breast, lung, melanoma; hematological malignances, renal cell carcinoma		[16, 23–27]
miR-15a/miR-16-1 cluster	Chronic lymphocytic leukemia (CLL)	BCL2, MCL1	[28]
miR-17-92 cluster	Colorectal	PTEN, BCL2L11, CDKN1A, miR-18a: NEDD9, and CDK19	[29]
miR-27	Breast	ZBTB10/RINZF, RYBP/DEDAF	[11]
miR-29a	Chronic lymphocytic leukemia (CLL)	BCL2, MCL1	[28]
miR-31	Breast	WAVE3	[30]
miR-34b/c	Metastatic tumors: breast, lung, melanoma; hematological malignances; gastric and colorectal cancer	c-Myc, CDK6, E2F3	[16, 24, 31–33]
miR-107	Pancreas	CDK6	[34]
miR-124a	Breast, colorectal, lung, gastric, cervical, HCC, glioblastoma, leukemia, lymphoma	CDK6, FOXA2	[12–18]
miR-127	Bladder	BCL6	[10]
miR-129-2	Endometrial, gastric, colorectal	SOX4	[25, 33, 35, 36]
miR-137	Colorectal, gastric, oral	Cdc42, LSD-1	([25, 37– 39]; Chen et al. [41, 42])
miR-148a	Metastatic tumors: breast, lung, melanoma; breast cancer	TGIF2	[23, 24]
miR-193a	Hematological malignances, lung, oral	c-kit, E2F6	[27, 43, 44]
miR-196b	Gastric, hematopoietic malignancies		[45, 46]
miR-199a	Gastric, ovarian, testicular	PODXL, IKKβ	[47–49]
miR-200 family	Colorectal, breast, lung, bladder	ZEB1, ZEB2, CRB3, LGL2	[50–54]
miR-203	Hematological malignances, hepatocellular carcinoma	ABL1	[17, 32, 55, 56]

(continued)

Table 7.1 (continued)

ncRNA	Cancer type	Target genes	References
miR-205	Bladder, lung	PTEN, ZEB1, ZEB2	[54, 57]
miR-224	Hepatocellular carcinoma		[58]
SNORD123	Acute lymphoblastoid leukemia, acute myelogenous leukemia, and cancer cell lines		[59]
U70C	Acute lymphoblastoid leukemia and cancer cell lines		[59]
ACA59B	Acute lymphoblastoid leukemia, primary multiple myeloma, and cancer cell lines		[59]
HBII-240	Testicular germ cell tumors		[60]
ACA8	Testicular germ cell tumors		[60]
ACA33	Testicular germ cell tumors		[60]
Uc.160+	Colon, breast, lung		[61]
Uc.283+A	Colon, breast, lung		[61]
Uc.346+	Colon, breast, lung		[61]
MEG3	Pituitary adenomas, hematological malignances		[62–65]
LOC554202	Breast		[30]

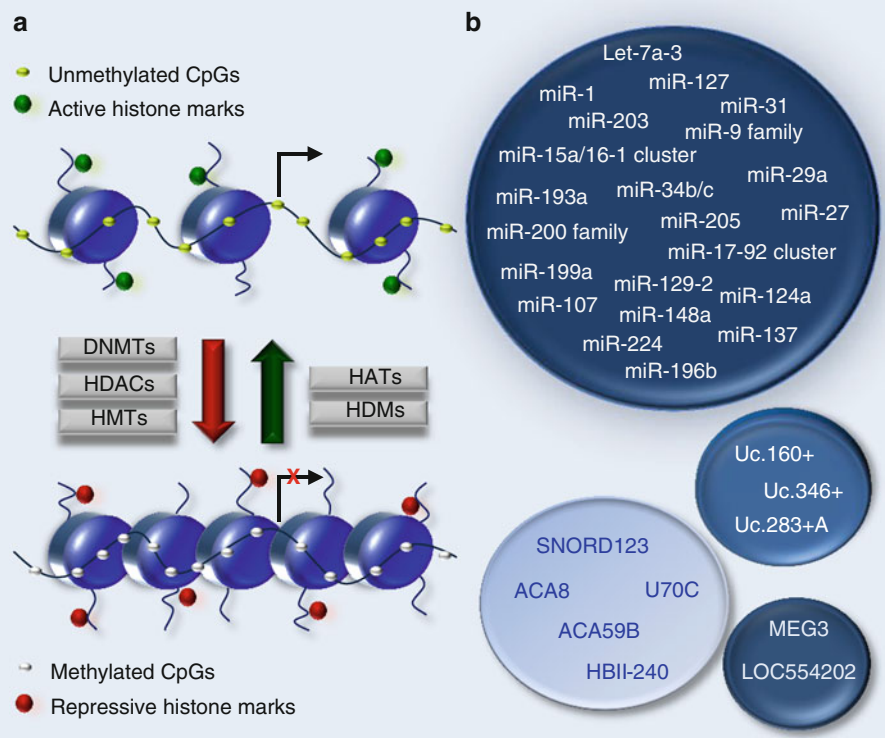

Fig. 7.1 Epigenetically regulated noncoding RNAs in cancer. (**a**). Epigenetic mechanisms involved in regulating ncRNA expression. Unmethylated CpGs and presence of active histone marks in regulatory regions of ncRNAs are related to active transcription, while CpG hypermethylation and presence of repressive histone marks are common events associated to gene silencing in cancer. The enzymes responsible of modifying DNA and histones include DNA methyltransferases (DNMTs), histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone demethylases (HDMs). (**b**). Epigenetically controlled ncRNAs, classified according to size (nt): miRNAs (*big circle*), snoRNAs (*medium circle*), and lncRNAs (*small circles*)

interconnected in order to guarantee a strict control of gene expression. However, many studies are focused on DNA methylation, perhaps largely due to technical constrains. But it is important to remark the crucial role of histone modifications controlling miRNA expression. Illustrative examples include **miR-15a**, **miR-16**, and **miR-29b** in chronic lymphocytic leukemia (CLL) [28]. miR-15a/miR-16 cluster was the first evidence linking miRNAs to human cancer, and common deletions in their genomic loci (13q14) were described as the main causal factor [66]. However, genomic deletions are not able to explain downregulation of these miRNAs in all the CLL cases. Recently, Sampath et al. have demonstrated that the common overexpression of HDACs in CLL mediates epigenetic silencing not only of the miR-15a/miR-16 cluster, but also of miR-29b [28]. HDACs are also important players in **miR-17-92** cluster regulation, as recently described in colorectal

cancer, where HDAC inhibitors decreased expression of miR-17-92 cluster with a corresponding increase in their target genes, including PTEN, BCL2L11 and CDKN1A [29]. In hepatocellular carcinoma, it has been reported that **miR-224** is reciprocally regulated by HDAC1, HDAC3, and the histone acetylase protein EP300 (E1A binding protein p300) [58]. Moreover, the recent development of genome-wide technologies, such as high-resolution ChIP-seq, has improved our knowledge about the role of histone modifications in miRNA regulation in cancer [67].

The new technical advances and vanguard strategies guarantee a booming understanding of the role of epigenetics not only in miRNA regulation, but also in other ncRNAs. It is the case of Small Nucleolar RNAs (snoRNAs), midsize ncRNAs of about 60 to 300nt located in the nucleolus whose main role is to guide chemical modifications of other RNAs. Thus, they are responsible for methylation and pseudouridylation of ribosomal RNA (rRNA) at about 50–100 sites per eukaryotic ribosome [68–70]. Our group has recently shown the existence of cancer-specific hypermethylation events in CpG islands associated with snoRNAs that lead to their transcriptional inactivation in transformed cells [59]. By data mining snoRNA databases and the scientific literature, we selected forty-nine snoRNAs that had a CpG island within ≤2 Kb or that were processed from a host gene with a 5′-CpG island. Interestingly, the host gene-associated 5′-CpG islands of the snoRNAs **SNORD123**, **U70C**, and **ACA59B** were hypermethylated in the cancer cell lines but not in the corresponding normal tissues. Most importantly, CpG island hypermethylation was associated with the transcriptional silencing of the respective snoRNAs not only in cancer cell lines, but also in a comprehensive cohort of primary tumors, demonstrating that the observed hypermethylation of snoRNAs was a common feature of various cancer types, particularly in leukemia [59]. Cheung et al. have also described epigenetic regulation of snoRNAs. They detected CpG hypomethylation in **HBII-240**, **ACA8**, and **ACA33** in testicular germ cell tumors, associated to snoRNAs upregulation in comparison with normal testis tissue [60]. Although the specific role of these snoRNAs in cancer remains to be elucidated, they are likely to contribute to tumorigenesis through an effect on ribosomes and protein translation, especially if we consider that translation is often perturbed in cancer cells [71].

7.2.2 Long ncRNAs

Regarding long ncRNAs (lncRNAs), we have found that Transcribed Ultraconserved Regions (T-UCRs) undergo epigenetic silencing in cancer [61]. T-UCRs are ncRNAs >200 nt that are absolutely conserved between orthologous regions of the human, rat and mouse genomes. Previous studies have shown that UCR expression levels are altered in cancer and that T-UCR expression signatures define different human tumor types [72]. Treating cancer cells with a DNA-demethylating agent followed by hybridization to an expression microarray containing T-UCR sequences, we detected that **Uc.160+**, **Uc283+A** and **Uc.346+** undergo specific CpG island hypermethylation-associated silencing in cancer cells compared with

normal tissues. Most importantly, this finding was not only an in vitro phenomenon but also it was confirmed in a large set of primary human tumors [61]. Other studies describe negative regulation of T-UCRs by direct interaction with miRNAs in neuroblastoma [73].

Another example of epigenetically controlled lncRNA is the Maternally Expressed Gene 3 (**MEG3**). MEG3 was the first lncRNA proposed to function as a tumor suppressor, studying human pituitary adenomas [74]. CpG hypermethylation within its regulatory region has been involved in the loss of MEG3 expression in clinically nonfunctioning pituitary adenomas [62, 64], multiple myeloma [63], acute myeloid leukemia, and myelodysplastic syndromes [65]. Other interesting examples include **miR-31** and its host gene, the lncRNA **LOC554202**. The CpG island upstream of the miR-31 locus, which also spans the first exon of LOC554202, is hypermethylated in breast cancer resulting in silencing of both miRNA and the host lncRNA [30].

Remarkably, most lincRNAs (long noncoding RNAs located in intergenic regions) were first identified using a histone mark signature previously associated to active transcription in protein-coding genes: the "K4K36 signature" that consists of a short stretch of trimethylation of lysine 4 in the histone H3 (H3K4me3), which corresponds to promoter regions, followed by a longer stretch of trimethylation of lysine 36 in the histone H3 (H3K36me3), which covers the entire transcribed region. After excluding known genes, chromatin signatures revealed approximately 1,600 regions in the mouse genome and 3,000 regions in the human genome that were actively transcribed [75, 76]. This fact highlights the key role of epigenetic marks in ncRNA regulation. Moreover, it has been reported that numerous lncRNAs that are expressed at lower levels in embryonic stem cells exhibit higher levels of H3K27me3 at their promoters. In agreement with this fact, knockdown of the H3K27me3 methyltransferase Enhancer of zeste homolog 2 (Ezh2) results in derepression of these lncRNAs [77].

7.3 Noncoding RNAs as Regulators of the Cancer Epigenome

7.3.1 Short and Midsize ncRNAs

Noncoding RNAs are not only regulated by epigenetic mechanisms but also modulate DNA methylation, histone modifications and chromatin remodeling by interfering with the effectors of the epigenetic machinery (Table 7.2). Thus, ncRNAs are able to regulate gene expression by two different ways: direct interaction with their target mRNAs and/or acting as master regulators of the epigenetic processes. Recent studies in this field have shown that DNMTs, HDACs, and HMTs are direct targets of miRNAs (Fig. 7.2). This subgroup of miRNAs that modulate the epigenetic machinery has been called "epi-miRNAs." The first evidence was reported by Fabbri et al.,

Table 7.2 Noncoding RNAs as regulators of the cancer epigenome

ncRNA	Cancer type	Target genes	References
miR-29 family	Acute myeloid leukemia, lung	DNMT1, DNMT3a, DNMT3b	[78, 79]
miR-101	Bladder, prostate	EZH2	[80, 81]
miR-140	Colon and osteosarcoma	HDAC4	[82]
miR-143	Colorectal	DNMT3a	[83]
miR-148	Cervical, cholangiocarcinoma	DNMT1, DNMT3b	[84, 85]
miR-152	Cholangiocarcinoma	DNMT1	[85]
miR-185	Glioma	DNMT1	[86]
miR-212	Gastric	MeCP2	[87]
miR-342	Colorectal	DNMT1	[88]
miR-373	Cholangiocarcinoma	MBD2	(Chen et al. [40])
miR-449a	Prostate	HDAC1	[89]
HOTAIR	Breast, colorectal, gastrointestinal stromal tumors, hepatocellular carcinoma	PRC2, LSD1	[90–95]
ANRIL	Leukemia, Prostate	PRC1 (CBX7), PRC2 (SUZ12)	[96–98]
p21 antisense	EST sequenced from neuroblastoma. Potentially oncogenic	Ago-1	[99]
lincRNA-p21	Potentially oncogenic	hnRNP-K	[100]
lncRNA-HEIH	Hepatocellular carcinoma	EZH2	[101]
ncRNAs-Cyclin D1	Potential tumor suppressor	p300/CBP	[102]

who demonstrated that **miR-29 family** induces global DNA hypomethylation by decreasing DNMTs expression, through direct targeting in the case of DNMT3a and DNMT3b or indirectly in DNMT1. Restoration of miR-29 expression in cancer cell lines resulted in demethylation and consequent reexpression of p15/INK4b and ESR1 comparable to that of using DNMTs inhibitors [78, 79]. DNMT3a is also target of **miR-143**, a frequent downregulated miRNA in CRC. More significantly, enforced expression of miR-143 in colon cancer cells decreases tumor cell growth and soft-agar colony formation [83]. In addition, **miR-148** is able to repress DNMT3b expression targeting a region in its coding sequence instead of the 3′UTR as is usual in miRNA–mRNA interactions [84]. **miR-148** and **miR-152** also target DNMT1, increasing Rassf1a and p16/INK4a expression, which in turn reduce cell proliferation in cholangiocarcinoma cells [85]. **miR-342** and **miR-185** also directly target the 3′ untranslated region of DNMT1, as has been described in colorectal cancer [88] and gliomas [86]. **miR-449a** is another miRNA targeting enzymes involved in epigenetic regulation, specifically HDAC1. Thus, tumor-related downregulation of this miRNA could be one of the mechanisms responsible for the frequent HDAC1 overexpression found in several cancer types. Introduction of miR-449a into prostate cancer cells results in cell-cycle arrest, a senescence-like phenotype and apoptosis [89]. Another HDAC, HDAC4, is targeted by **miR-140**. Remarkably, blocking endogenous miR-140 partially sensitized resistant colon

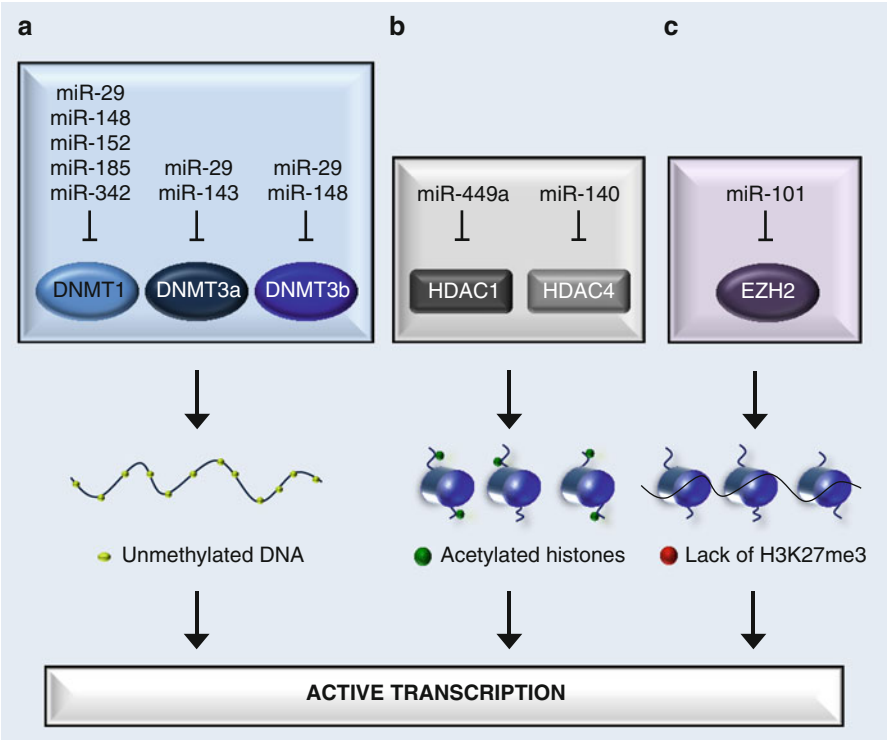

Fig. 7.2 Noncoding RNAs as regulators of the cancer epigenome. ncRNAs are able to regulate the effectors of the epigenetic machinery. (**a**) DNA methyltransferases (DNMTs), (**b**) histone deacetylases (HDACs), and (**c**) members of the Polycomb repressive complexes, such as Enhancer of zeste homolog 2 (EZH2) are direct targets of miRNAs. Posttranscriptional inhibition of these molecules by miRNAs leads to a global deregulation of gene expression in cancer

cancer cells to 5-fluorouracil chemotherapeutic treatment [82]. In addition, HMTs are also targets of miRNAs. Studies in bladder transitional cell carcinomas and prostate tumors have revealed that EZH2, the catalytic subunit of the Polycomb repressive complex 2 (PRC2), is target of **miR-101**. Hence, the miR-101 downregulation observed in cancer could be involved in the common overexpression of EZH2 in aggressive solid tumors. Significantly, restoration of miR-101 expression attenuates cancer invasion, whilst miR-101 inhibition induces an invasive phenotype [80, 81]. Another interesting example is **miR-373,** which has been recognized as a negative regulator of the Methyl-CpG-binding Domain Protein 2 (MBD2) in hilar cholangiocarcinoma. MBD proteins bind selectively to methylated DNA and serve as a molecular link to recruit histone deacetylases (HDAC) and other transcription repression factors to the chromatin. miR-373 inhibition leads to increase of MBD2, which in turn inhibits methylation-silenced genes such as RASSF1A [40]. Another MBD controlled through miRNAs is MeCP2, targeted by **miR-212**. This miRNA has been found downregulated in gastric cancer, resulting in higher MeCP2 protein levels

that could favor the epigenetic deregulation observed in these tumors [87]. These miRNA-controlled adjustments in the epigenetic machinery could in turn affect the regulation not only of protein-coding genes, but also of ncRNAs epigenetically regulated.

In addition to the role of miRNAs as regulators of the epigenome, other short ncRNAs have been proposed as components of the epigenetic regulatory networks. **piRNAs** (PIWI-interacting RNAs) are germ line-specific ncRNAs of 24–30 nt in length that bind to Piwi proteins and guide them to their targets. These ncRNAs have been implicated in DNA methylation [103]. Previously, it had been described that loss of Piwi proteins MIWI2 (mouse piwi 2) and MILI (miwi-like) in male germ cells results in defective DNA methylation of regulatory regions of retrotransposons in a similar way to that in DNMT3L-deficient mice, indicating that the piRNA pathway plays essential roles in establishing de novo DNA methylation of retrotransposons in fetal male germ cells in mice [104, 105]. Along the same lines, Watanabe et al. have recently reported that the components of the piRNA pathway are required for de novo methylation of the differentially methylated region (DMR) of the imprinted mouse Rasgrf1 locus [103]. Although their specific functions in tumorigenesis are not clearly defined, piRNAs and PIWI proteins have been implicated in cancer [106–109]. Strikingly, piRNAs have been involved not only in testicular tumors but also are aberrantly expressed in human somatic tumors, implying a role outside germ line cells that make it worth an exhaustive research. For instance, expression of pi-651 was found upregulated in several cancer types [110], and tumor suppressive properties in human gastric cancer cells have been described for pi-823 [111]. In addition, these piRNAs have been proposed as useful biomarkers for detecting circulating gastric cancer cells [111].

A role for another type of ncRNA, **pRNAs** (promoter-associated RNAs), in the regulatory networks controlling the epigenetic state of chromatin, have also been reported. Schmitz et al. have shown that pRNA interacts with the target site of the transcription factor TTF-I, forming a DNA:RNA triplex that is specifically recognized by the DNA methyltransferase DNMT3b. More relevantly, they found that elevated levels of synthetic pRNA trigger de novo DNA methylation, heterochromatin formation and transcriptional silencing. Remarkably, as commented by the authors, recruitment of DNMT3b by DNA–RNA triplexes may be a common and generally used pathway in epigenetic regulation that could act on virtually any gene that is silenced by DNA methylation [112].

7.3.2 Long ncRNAs

The abovementioned example of a triplex structure consisting of ncRNA:DNA: DNMT3b illustrates the versatility of ncRNAs, feature that makes them ideal orchestrators of epigenetic networks. In particular, long noncoding RNAs are considered flexible modular scaffolds of proteins that bring specific regulatory components into proximity with each other, which results in the formation of functional

complexes. However, as described for several ncRNAs, the model of lncRNAs as modular scaffolds should not be limited to protein interactions. Base-pairing alignment with DNA could be used to guide complexes to specific DNA sequences or bridge together sets of DNA-binding proteins. Additionally, lncRNAs could also interact with other RNAs in order to assemble complexes that can interact with proteins [113]. This attribute of lncRNAs is critical in their function as effectors of epigenetic control and gene expression regulation. The ability of lncRNAs to bind to multiple histone modifier enzymes, recruit chromatin remodeling complexes, and recruit RNA binding proteins to gene promoters is broadly recognized.

Many of the identified lncRNAs have a spatial and temporal expression patterns, indicating that lncRNA expression is strongly regulated. This fact suggests that lncRNAs have specific biological functions. In agreement with this idea, increasing evidences are demonstrating their role in a wide repertoire of biological processes, such as cell cycle control, cell differentiation, translation, splicing, etc. Considering that disruption of some of these functions has been implicated in tumorigenesis, it is expected that lncRNAs deregulation might be associated with cancer. In fact, there is growing information showing differential lncRNA expression in tumors and supporting the repercussion of lncRNA disruption in this disease. Below some examples of lncRNAs acting as epigenetic regulators with a proposed role in cancer will be discussed.

The most well-known lncRNA that acts as mediator of epigenetic modifications by recruiting chromatin remodeling complexes to specific loci is **XIST** (X-inactivation specific transcript). During early development in females, XIST transcript is expressed from the inactive X and coats the X chromosome from which it is transcribed [114]. Another lncRNA, RepA, which is transcribed from the repeat A element of the XIST locus, recruits the Polycomb repressive complex 2 (PRC2) to the XIST promoter [115]. PRC2 in turns trimethylates H3K27 creating a heterochromatic patch that leads to XIST transactivation resulting in widespread X silencing. Interestingly TSIX, another lncRNA, is transcribed from the XIST promoter in the antisense direction and regulates XIST levels during X-chromosome inactivation, at least in mice [116, 117]. Although a role for XIST in cancer has been suggested [118, 119], this issue remains controversial. While only one X chromosome is active in human cells, many tumors from both sexes have supernumerary X chromosomes and dysregulation of XIST expression has been detected. However, it remains to be determined whether the X chromosome duplications/reactivations, XIST dysregulation, and overexpression of X-linked genes are involved in cancer development or are merely a consequence of overall epigenetic instability in these cancers [120]. It is remarkable that in a mouse thymic lymphoma model, the induction of Xist results in the initiation of X inactivation and the inhibition of tumor growth, suggesting that Xist deregulation might play a significant role in tumorigenesis [121]. Moreover SATB1, a cofactor required for Xist-mediated silencing [121], is able to reprogramme chromatin organization upregulating metastasis-associated genes while downregulating tumor suppressor genes to promote tumor growth and metastasis in breast [122].

A mechanism of lncRNA-mediated epigenetic silencing similar to the aforementioned for XIST has been described for AIR and KCNQ1OT1. Both lncRNAs epigenetically silence large domains of the genome through their interaction with chromatin. In mice, **Air** is imprinted and expressed only from the paternal allele, whereas a near cluster including Igf2r, Slc22a2 and Slc22a3 protein-coding genes is expressed only from the maternal allele. Air is transcribed in antisense direction to Igf2r and Slc22a3 and is responsible for *cis*-silencing of the three paternally inherited genes, through binding to the Slc22a3 promoter and recruiting the histone lysine methyltransferase Kmt1c, which methylates H3K9 and drives the epigenetic silencing. Conversely, imprinted expression of Air itself is caused by a DNA methylation imprint acquired during oocyte development that silences the maternal Air promoter [123]. In humans, the IGF2R gene contains an intronic CpG island promoter that expresses the AIR ncRNA. The human AIR ncRNA is expressed in 16–40 % of Wilms' tumors, however the AIR-mediated silencing of IGF2R is not clear. Only in a 50 % of tumor samples, high expression of the AIR ncRNA correlated with reduced IGF2R expression [124].

A similar example is **KCNQ1OT1**, expressed from the paternal allele and responsible for the *cis*-silencing of a neighbor cluster of protein-coding genes. This lncRNA immunoprecipitates with members of the PRC2 complex (EZH2 and SUZ12) and the HMT G9, suggesting that KCNQ1OT1 is able to recruit these histone methyltransferases in *cis* to establish the repressive H3K27me3 and H3K9me3 marks, respectively [125]. A role in loss of imprinting for KCNQ1OT1 (LIT1) via epigenetic disruption has been suggested in colorectal carcinogenesis [126].

It has been proposed that as many as 20 % of lncRNAs expressed in various cell types are bound by PRC2, and that additional lincRNAs are bound by other chromatin-modifying complexes [76]. In this context, it is noteworthy that members of PRC2 complex, such as EZH2, have been associated with cancer, although contrasting evidences point to both oncogenic and tumor suppressor roles [127]. For example, Simon et al. have recently demonstrated that disruption of Ezh2 is sufficient to cause T-acute lymphoblastic leukemia (T-ALL) in mice [128]. This tumor suppressor behavior of EZH2 has been also observed in human T-ALL [129]. In contrast, EZH2 seems to have an oncogenic role in nasopharyngeal carcinoma, where it is able to form a co-repressor complex with HDAC1/HDAC2/Snail to repress E-cadherin, regulating cell invasion and metastasis [130].

One of the first lncRNAs described to have a direct implication in cancer progression by remodeling the chromatin landscape was **HOTAIR** (Hox antisense intergenic RNA) (Fig. 7.3). Among other 231 transcripts with low-coding potential, this ncRNA was identified in a comprehensive study evaluating the transcriptional activity of human HOX loci [131]. HOTAIR is a 2.2-kb spliced and polyadenylated transcript that is transcribed from the HOXC locus. This ncRNA negatively transregulates the distant HOXD locus by acting as a molecular scaffold, binding and recruiting at least two distinct histone modification complexes. The 5′ domain of the HOTAIR binds the PRC2 complex responsible for H3K27 methylation, while the 3′ region binds LSD1/CoREST/REST complex, which mediates enzymatic demethylation of H3K4me2 [90]. This lncRNA has been found highly upregulated in both primary and metastatic breast tumors. More significantly, it has been demonstrated that

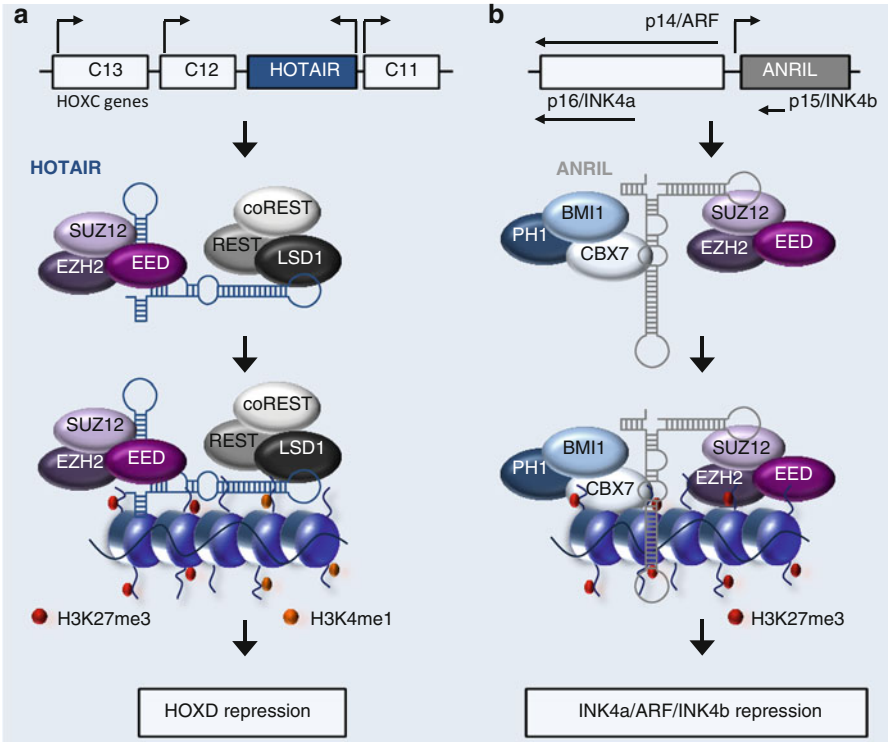

Fig. 7.3 Role of long noncoding RNAs in chromatin remodeling in cancer. lncRNAs, like HOTAIR and ANRIL, can recruit chromatin remodeling complexes to specific genomic loci to mediate tumor suppressor gene silencing. (**a**). HOTAIR is transcribed antisense to HOXC genes and its expression is correlated with the repression of genes in the HOXD locus. The 5′ domain of the HOTAIR binds the Polycomb repressive complex 2 (PRC2) responsible for H3K27 methylation, while the 3′ region binds LSD1/CoREST/REST complex, which mediates enzymatic demethylation of H3K4me2. Recruitment of PRC2 complex to specific target genes genome-wide leads to epigenetic silencing of metastasis suppressor genes. (**b**). Long ncRNA ANRIL is transcribed antisense of the INK4a/ARF/INK4b tumor suppressor genes. ANRIL mediates gene silencing of this locus by interaction and recruitment of members of the Polycomb repressive complexes PRC1 and PRC2. CBX7, a H3K27me3-recognizing component of PRC1, can bind directly to both ANRIL and H3K27me3 via its chromodomain, and both interactions are required for CBX7 to repress the INK4a and INK4b loci

HOTAIR acts as an epigenomic reprogrammer regulating metastatic progression in breast tumors. Thus, this lncRNA recruits PRC2 complex to specific target genes genome-wide, leading to H3K27 trimethylation and epigenetic silencing of metastasis suppressor genes [91]. Remarkably, their function in cancer progression and metastasis is not restricted to breast tumors, since a significant correlation between HOTAIR overexpression and metastasis has also been detected in colorectal cancer [92], gastrointestinal stromal tumors [93], and hepatocellular carcinoma (HCC) [94]. In addition, in HCC patients who have undergone liver transplant therapy, HOTAIR has been proposed as candidate biomarker for predicting tumor

recurrence. Also, its potential as therapeutic target has been suggested in view of experiments in which siRNA-mediated suppression of this lncRNA in a liver cancer cell line increased chemotherapeutic sensitivity to cisplatin and doxorubicin [95].

ANRIL (Antisense noncoding RNA in the INK4 locus) (Fig. 7.3) is another example of cancer-related lncRNA. It is transcribed from the INK4a/ARF locus that encodes the p15, p16, and ARF proteins, which regulate cell cycle progression and senescence. ANRIL is transcribed antisense to the INK4b/ARF/INK4a promoter and overlaps with two exons of p15/CDKN2B. It has been reported that ANRIL overexpression results in silencing of INK4b/ARF/INK4a and p15/CDKN2B in leukemia [96] and prostate cancer [97]. ANRIL-mediated epigenetic silencing is achieved by their interaction with members of the Polycomb repressive complexes PRC1 and PRC2. Yap and colleagues have reported that CBX7, a H3K27me3-recognizing component of PRC1, can bind directly to both ANRIL and H3K27me3 via its chromodomain, and both interactions are required for CBX7 to repress the INK4a and INK4b loci [97]. Along the same lines, studies from Kotake et al. have revealed that ANRIL binds and is required for the recruitment of SUZ12, a component of PRC2, to p15/INK4B locus [98]. p15/INK4B silencing has been correlated with an increase in the repressive mark H3K9me2 and a decrease of the active mark H3K4me2 at the promoter region, but no increase in DNA methylation has been observed [96]. These studies revealed the critical role of ANRIL-*cis*-mediated epigenetic silencing of a well-recognized cancer-related locus. It is noteworthy that specific SNPs in ANRIL have been correlated with an increased susceptibility to cancer and other diseases. For instance, SNPs in ANRIL influence the number of plexiform neurofibromas in Neurofibromatosis type 1 (NF1), a tumor predisposition syndrome [132].

LncRNA-mediated silencing of tumor suppressor genes has also been reported as a mechanism controlling the cell cycle regulator p21/CDKN1A. Morris et al. described the bidirectional transcription at the p21 genomic locus where p21 antisense (**p21AS**) acts as effector molecule driving transcriptional gene silencing of p21. p21AS maintains low-level epigenetic silencing by direct recruitment of Argonaute 1 (Ago-1) and the repressive mark H3K27me3 to the p21 sense promoter [99]. Although p21AS has not been associated to cancer thus far, the direct consequence of an imbalance in endogenous bidirectional transcription could potentially lead to the deregulation of p21 expression and contribute to tumorigenesis, specially taking into account the crucial role of p21 in cell cycle control. Additionally, another ncRNA located approximately 15 Kb upstream of the p21/CDKN1A gene has been identified. The so-called **lincRNA-p21**, (due to its proximity to p21 locus), is transcribed from an independent promoter in the opposite orientation to the p21/CDKN1A gene. This transcript was identified as a p53-activated lincRNA whose binding to hnRNP-K (heterogeneous nuclear ribonucleoprotein K) is required for the proper localization of the ribonucleoprotein [100]. hnRNP-K had been previously described as a key component of a repressor complex that acts in the p53 pathway [133] and lincRNA-p21 was found to be a global repressor of genes in the p53 pathway, playing an important role in the p53-dependent induction of apoptosis [100].

Collectively, these examples highlight the lncRNA-mediated epigenetic silencing as an important mechanism driving critical cancer-related pathways.

Recently, a specific lncRNA High Expressed In Hepatocellular Carcinoma (HCC), called **lncRNA-HEIH**, has been identified. Interestingly, the expression level of this lncRNA has been significantly associated with recurrence and has been proposed as an independent prognostic factor for survival in hepatitis B virus-related HCC. This lncRNA is able to associate with EZH2 and experimental evidences have demonstrated that this association is required for the repression of EZH2 target genes in HCC progression [101].

In addition to binding to and recruiting chromatin remodeling complexes and histone modifier enzymes, the ability of lncRNAs to regulate gene expression is also achieved through their capacity to recruit RNA binding proteins and/or regulate its accessibility to gene promoters. ncRNAs transcribed from 5′ end of the Cyclin D1 gene are significant examples. Cyclin D1/CCND1 is a cell cycle regulator frequently disrupted in several cancer types [134]. In response to DNA damage, **ncRNAs-Cyclin D1** are expressed and interact with the TLS (Translocated in Liposarcoma) protein, inducing an allosteric modification that allows its association with the CCND1 promoter. Recruitment of TLS to the promoter causes Cyclin D1 repression by inhibition of enzymatic activities of the histone acetyltransferases CREB-binding protein (CBP) and p300 [102].

Another example is an lncRNA transcribed from the DHFR (Dihydrofolate Reductase) minor promoter. This **lncRNA-DHFR** has a key function in an epigenetic mechanism of promoter-specific transcriptional repression of the gene encoding DHFR. The lncRNA forms a triplex structure with the major promoter while interacting directly with the transcription factor TFIIB, which results in the disruption of the preinitiation complex at the major promoter [135]. Considering the role of DHFR in the synthesis of DNA precursors, competitive inhibitors of DHFR (i.e., methotrexate) are used in anticancer therapy in order to limit the growth and proliferation of tumor cells [136]. Therefore, although so far a link between lncRNA-DHFR and cancer has not been identified, this represents a topic worthy of future research.

Finally, ncRNAs can also function as molecular “decoys” by preventing correct regulation through competitive binding. For instance, **GAS5** (Growth arrest-specific 5) is an ncRNA whose conformation mimics that of the glucocorticoid responsive element (GRE) DNA. Consequently, GAS5 is able to bind to the DNA-binding domain of the glucocorticoid receptor (GR) by acting as a decoy glucocorticoid response element (GRE). The competition with DNA GREs for binding to the receptor blocks the transcriptional induction by GR of the target genes. GAS5 is highly expressed in cells whose growth has been arrested because of lack of nutrients or growth factors and sensitizes cells to apoptosis by suppressing glucocorticoid-mediated induction of several responsive genes [137]. Interestingly, GAS5 has been found downregulated in breast cancer and some introns of GAS5 encode snoRNAs that sensitize mammalian cells to apoptosis, supporting its tumor suppressor role [138]. Overall, multiple examples have been discussed emphasizing the function of ncRNAs as regulators of the cancer epigenome.

7.4 Perspectives

The complex network of reciprocal interactions between ncRNAs and epigenetics is just beginning to unravel. The exponential increase in the number of studies and publications in the ncRNA field highlights the fast advance of our knowledge in this matter, directly promoted by technological and bioinformatics advances at genome-wide level. In general, it has been demonstrated that the more complex an organism, the greater the number of its ncRNAs. Moreover, gene expression regulation by ncRNAs has significant advantages, including rapid response speed (attributable to the proximity between ncRNA production and the target gene), or a lower energetic cost to the cell (due to the lack of protein synthesis), among others. Furthermore, their molecular structure consisting of primary nucleotide sequences leads to more plastic structure–function constraints than proteins. Collectively, these facts support the proposition that expansion of regulatory RNAs was fundamental in cellular reprogramming and ultimately in the evolution of eukaryotes. Although until quite recently argued to be spurious transcriptional noise, the biological significance and critical roles of ncRNA in cellular function are now supported by increasingly strong evidences. Versatility of these molecules makes them not only ideal orchestrators of biological networks, but also key components in tumorigenesis. An exciting future in research about the role of ncRNAs in cancer epigenetics is guaranteed.

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