Review

lncRNAs and microRNAs with a role in cancer development

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ABSTRACT

Most diseases, including human cancer, are frequently associated with an altered transcription pattern. The alteration of the transcriptome is not restricted to the production of aberrant levels of protein-coding RNAs, but also refers to the dysregulation of the expression of the multiple noncoding members that comprise the human genome. Unexpectedly, recent RNA-seq data of the human transcriptome have revealed that less than 2% of the genome encodes protein-coding transcripts, even though the vast majority of the genome is actively transcribed into non-coding RNAs (ncRNAs) under different conditions. In this review, we present an updated version of the mechanistic aspects of some long non-coding RNAs (lncRNAs) that play critical roles in human cancer. Most importantly, we focus on the interplay between lncRNAs and microRNAs, and the importance of such interactions during the tumorigenic process, providing new insight into the regulatory mechanisms underlying several ncRNA classes of importance in cancer, particularly transcribed ultraconserved regions (T-UCRs).

1. The non-coding RNA landscape

Almost the entire mammalian genome is actively transcribed under specific conditions [1]. Many of the resulting transcripts are processed to give rise ultimately to small RNAs. The range of types of small RNA includes microRNAs, which are the most extensively studied class of non-coding RNAs (ncRNAs). MicroRNAs are the best-known subtype and it is widely accepted that their function is crucial during developmental processes, apoptosis and cell proliferation [2]. MicroRNAs post-transcriptionally regulate the expression of their target genes through their complementarity with the mRNA sequence. The generation of functional microRNAs is a process that transforms the long primary transcript into a mature functional ~22 nt microRNA. MicroRNA genes are embedded within intronic genomic regions or within the exons of protein-coding genes [3]. They are initially transcribed by RNA polymerase II as long, capped and polyadenylated primary transcripts (pri-microRNAs) that may contain more than one hairpin structure, known as polycistronic microRNA clusters [4]. In the canonical processing pathway, pri-microRNAs are recognized and cleaved by the Microprocessor complex, which contains the RNase III enzyme Drosha and DGCR8 as a cofactor [5,6]. The cleaved portion of the hairpin (pre-microRNA) needs to be exported to the cytoplasm to complete its processing by the cytoplasmic RNAse III enzyme Dicer, leaving the ~22 nt duplex [7]. The guide strand is then joined to one of the Ago proteins to establish the core of the silencing complex, whereas the passenger strand is discarded [8,9]. The mature RISC complex can bind the untranslated region (UTR), generally the 3′-UTR, but some recent reports have shown that microRNA targeting is not always limited to 3′-UTR, since, with respect to the 5′-UTR of mRNAs, there are some naturally occurring examples in which the targeting microRNA downregulates the expression of the corresponding mRNA in a seed-dependent manner. This highlights the complexity of microRNA targeting in the post-transcriptional control of oncogenes or tumor suppressor genes [10]. Finally, microRNA–mRNA binding leads to translational inhibition or degradation of the target mRNA [11].

Considering that most mammalian protein-coding genes are regulated by at least one microRNA, and that a vast number of non-conserved sites exists, it is not unexpected that their function and biogenesis are firmly regulated, since their alteration is associated with a wide range of human diseases, including cancer and neurological disorders [12]. In this regard, the final amount of each microRNA can be regulated at the transcriptional or post-transcriptional level. As with protein-coding genes, most microRNAs are transcribed by RNA Pol II and can be regulated at the transcriptional level since they feature canonical promoters and enhancers in their regulatory upstream regions [13]. At this level, it is well established that their expression depends on the activity of transcription factors such as c-Myc and p53 [14,15], or on epigenetic modifications, such as DNA methylation and histone modifications, that occur at their promoter sequences [16]. Since microRNA biogenesis is a multistep process, any step could be tightly regulated. Moreover, in recent years, many models have revealed some post-transcriptional mechanisms that could account for the discrepancies between the expression levels of pri-microRNAs and the corresponding mature microRNAs [17]. Among the post-

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transcriptional mechanisms that regulate the final expression of any microRNA, the control of the Microprocessor complex stands out since the effectiveness of the catalytic process mediated by Drosha is crucial for determining microRNA abundance. Many mechanisms exist and these have been summarized in some excellent reviews [18,19]. However, to date, these have only been concerned with protein factors bound to distinct elements along the pri-microRNA or that interact with the Drosha-DGCR8 to control microRNA maturation [20,21]. Very recently, our group has identified a new mechanism for the post-transcriptional control of microRNAs at the level of Drosha processing. It is mediated by a long non-coding RNA (lncRNA) transcribed from an ultraconserved region, which highlights the great complexity of ncRNA regulatory networks between different ncRNA classes of importance in cancer [22]. This example and others are considered in detail in the lncRNA:microRNA section. Overall, it has been shown that many microRNAs are regulated during the catalytic process mediated by Drosha, and that this regulation represents a dominant mechanism that has consequences for microRNA expression during development and in cancer [23].

The lncRNA category comprises a very heterogeneous subclass that was first described by genome-wide sequencing of cDNA libraries of the mouse genome. It is becoming clear that a large number of lncRNAs are determining regulators of normal tissue physiology and the processes of diseases such as cancer and neurological disorders, despite so often being branded as transcriptional noise. As a result, much effort is currently being made to characterize those genetic, epigenetic and transcriptional changes that occur during disease. The methods used increasingly involve high-throughput RNA-seq technologies designed to enhance our knowledge of cancer pathogenesis [24,25]. In this context, very recent findings have revealed an expanding landscape of transcription via unbiased transcriptome reconstruction from thousands of tumors, normal tissues and cell lines, showing that the number of lncRNAs has been hitherto underestimated. This new assembly clearly demonstrates that the genomic heterogeneity of lncRNAs eclipses that of coding transcripts (60,000 lncRNA genes versus roughly 30,000 protein-coding genes), a discrepancy that may grow since additional diseases and cell types are currently being sequenced and more lncRNAs are being identified. Although the functions of the vast majority of lncRNAs have not been validated, it is thought that many of these transcripts could serve as future cancer biomarkers [26].

2. Long non-coding RNAs at the heart of gene regulation in health and disease

Every eukaryotic cell relies on a highly integrated gene expression program whose integrity is ensured by transcriptional and post-transcriptional processes. Some years ago, lncRNAs were first recognized as being crucial regulators of gene expression in a wide range of biological contexts, even though the mechanisms by which the vast majority exert their functions remain largely unknown. lncRNAs are a very heterogeneous group of transcripts longer than 200 nt that have no protein-coding capacity. Unlike microRNAs, the length of lncRNAs allows them to fold into intricate structures, by which they may perform their function as RNA sequences by themselves through secondary and tertiary structural determinants. In general, lncRNA transcripts exhibit low-level but tissue-specific expression [27] and, with some exceptions [22], their nucleotide sequence is poorly conserved [28]. In the last few years, several studies have shown the importance of lncRNAs to normal physiology as well as to the control of gene expression, wherein they modulate key cellular processes such as cell proliferation, senescence, migration and apoptosis [29,30].

The intrinsic ability of lncRNAs to interact with DNA, RNA and proteins by acting as guides, tethers, decoys and scaffolds offers the most compelling explanation of their ability to regulate gene expression, including epigenetic transcriptional control by their association with chromatin remodeling complexes [31], splicing [32,33], translation [34] and protein stability. Moreover, an increasing body of experimental evidence suggests connections between microRNAs and lncRNA. A new function for lncRNAs has become apparent from their ability to regulate microRNA activity by acting as either competitive endogenous RNAs for microRNAs or as microRNA sponges [35–37].

Considering that lncRNAs control key cellular processes, knowledge of the mechanisms by which lncRNA expression is regulated is the first step towards understanding the basic principles by which they exert their functions. Furthermore, many studies have shown that lncRNA expression is altered in a variety of human cancer types [38] and that their expression pattern may be associated with metastasis and disease prognosis [39,40]. The expression of specific lncRNAs with oncogenic features [41,42] is closely linked to the ability to promote matrix invasion of cancer cells and tumor growth [43].

In spite of their importance, the mechanisms that govern the control of lncRNAs are largely unknown but there is some evidence that demonstrate that they are regulated by a similar mechanism to that controlling protein-coding genes and microRNAs. However, there are some discrepancies about the transcriptional regulation of lncRNAs by epigenetic mechanisms, especially DNA methylation. In this context, genome studies involving 5Cm, H3K4me3, H3K9me3, H3K27me3 and H3K36me3 epigenetic marks associated with transcription start sites of lncRNAs common to various human cell types showed that the pattern of DNA methylation and H3K9me3 differs considerably between mRNAs and lncRNAs and that it does not seem to play a role in lncRNA expression [44].

Despite this disparity, it has been clearly demonstrated that, in addition to microRNAs, another singular class of lncRNAs transcribed from ultraconserved regions of the genome is also subject to transcriptional silencing driven by epigenetic mechanisms such as DNA methylation and histone modifications. This highlights the importance of epigenetic disruption of lncRNA in human tumorigenesis [45].

As with proteins, over the past decade many studies have shown that some lncRNAs are under the regulation of microRNAs to reduce their stability (Table 1). Given that the proper activity of these lncRNAs is essential to key cellular processes, changes in their expression can alter diverse molecular pathways altering physiological processes. In this context, the role of HOTTIP has been found to be the most significant oncogenic lncRNA in human hepatocellular carcinoma (HCC). Moreover, miR-125b binds to one putative microRNA binding site in the HOTTIP sequence by acting as post-transcriptional regulator of HOTTIP expression [46]. Another well characterized lncRNA whose activity is affected by microRNA function is linc-p21 RNA, which is transcriptionally activated by p53. MiR-let7b, in cooperation with the RNA

Table 1

Examples of cancer associated lncRNAs and T-UCRs post-transcriptionally regulated by microRNAs.

	microRNA	Disease/process	Ref
LncRNA			
HOTTIP	miR-125b	Hepatocellular carcinoma	Tsang et al. [46]
Linc-p21	Let-7b	Cervical carcinoma	Yoon et al. [47]
MALAT1	miR-9	Glioblastoma and Hodgkin's lymphoma	Leucci et al. [48]
	miR-101	Esophageal squamous carcinoma	Wang et al. [49]
loc285194	miR-211	Colorectal cancer	Liu et al. [19]
CASC2	miR-21	Endometrial cancer and gliomas	Baldinu et al. [51]
UCA1	miR-1	Bladder carcinoma	Fan et al. [55]
HOTAIR	miR-141	Renal carcinoma	Chiyomaru et al. [42]
T-UCR			
Uc.160+	miR-155	Chronic lymphocytic leukemia	Calin et al. [84]
	miR-24-1	and carcinomas	
Uc.346+A	miR-155		
Uc.78+	miR-15a		
	miR-16		

binding protein HuR contributes to reduce linc-p21 activity in human cervical carcinoma cell lines [47].

The abundant, ubiquitously expressed and highly conserved lncRNA MALAT-1 (Metastasis-Associated Lung Adenocarcinoma Transcript 1) is also targeted by miR-9 in an Ago2 complex-dependent manner in the nucleus of glioblastoma and Hodgkin cell lines. The regulation of these two ncRNA species relies on two binding sites located along MALAT-1 sequence, emphasizing the complexity of microRNA activity that is not restricted solely to the cytoplasm [48]. In addition, in spite of being deregulated in many types of cancer, its role in esophageal squamous carcinoma (ESCC) has been demonstrated very recently. Wang and co-workers found that the post-transcriptional silencing of MALAT1 by miR-101 and miR-217 leads to a significant reduction in proliferation by the arrest of the G2/M cell cycle that is explained by the deregulation of downstream effectors of MALAT1, such as MIA2, HNF4G, ROBO1, CCT4 and CTHRC1 [49].

The lncRNA loc285194 and its relationship with miR-211 gives rise to a repression feedback loop. As we describe below, loc285194 harbors two binding sites for miR-211 that can function as an endogenous sponge for miR-211. Additionally, loc285194 activity is also affected by the ectopic expression of miR-211 [50]. In this regard, it has been suggested that the lack of function of p53-inducible loc285194 in colon tumors is triggered by the upregulation of miR-211 when its expression is enhanced.

Tumor-suppressor properties for the lncRNA CASC2 (cancer susceptibility candidate 2) have recently been identified in endometrial cancer [51] and in human gliomas. In the case of gliomas, the oncogenic miR-21 binds to the CASC2 lncRNA sequence in an Ago2-dependent manner, reducing its expression and thereby the CASC2-mediated inhibition of glioma cell proliferation, migration and invasion. It has also been suggested that reciprocal regulation is also possible, by which the ectopic expression of CASC2 suppresses the malignancy of glioma cells mainly by miR-21. CASC2 lncRNA has been postulated as being one of the lncRNAs that act as endogenous microRNA sponges, as described below [44].

UCA1 lncRNA (urothelial carcinoma-associated 1) is significantly upregulated in most tumors and cancer cells, including colorectal cancer [52], tongue squamous cell carcinomas [53], breast cancer [54] and bladder cancer [55]. In bladder carcinomas, in which miR-1 is downregulated, UCA1 lncRNA is known to harbor predictable binding sites for this microRNA. In doing so, miR-1 reduces the expression of UCA1 lncRNA in an Ago2-slicer-dependent manner. The relation between the two types of non-coding RNAs has been demonstrated with luciferase-based reporter assays [56].

3. Mechanisms of gene regulation by long non-coding RNAs

By definition, the transcriptional regulation mediated by lncRNAs is the consequence of the assembly of the transcription machinery resulting from the action of transcriptional factors on enhancer and promoter regions. In this context, lncRNAs may be involved in the transcriptional regulation either as *cis*- or *trans*-acting elements, and could negatively or positively influence gene expression.

4. *Cis*-acting long non-coding RNAs

Transcriptional regulation by *cis*-acting elements implies when the regulatory lncRNA and the target gene are both transcribed from the same locus [57]. However, some recent findings suggest that *cis*-acting lncRNA also has the ability to act in *trans* [58–60], highlighting the importance of DNA loop formation in mediating the regulation of the two RNAs. One of the best examples of this first category is the human HOTTIP lncRNA, which is transcribed from the HOXA cluster and induces transcription of neighboring protein-coding genes by its interaction with WDR5 and the MLL histone modifier complex, which adds histone H3 lysine-4 trimethylation (H3K4me3) over the flanking gene

promoters [61]. Correlations between clinico-pathological parameters and levels of expression in hepatocellular carcinoma samples have demonstrated that the levels of HOTTIP transcript are associated with clinical progression of HCC patients and predict disease outcome, highlighting the role of this transcript in HCC and hence suggesting that HOTTIP is a predictive biomarker of this disease [62].

Another example of a *cis*-acting lncRNA is the non-spliced PANDA transcript, which is transcribed from a 5-kb upstream promoter region of the CDKN1A cell-cycle gene, and is activated in response to DNA damage [63]. PANDA lncRNA, by interacting with polycomb repressive complexes and the transcription factor NF-YA, endows cells with the capacity to maintain their proliferative state, and alterations in their levels can lead to entry or exit from the senescent state [64].

Global transcriptome analysis has revealed that ~70% transcripts have antisense counterparts, known as natural antisense transcripts (NATs), whose transcription can activate or inactivate the sense gene [65]. Consistent with their genomic locations, members of this group can be subclassified into *cis*-NATs and *trans*-NATs. In the first category, ANRIL lncRNA stands out because it is transcribed from the same gene-cluster locus that codes for the tumor suppressors *CDKN2A* and *CDKN2B* [66]. ANRIL transcripts are associated with CBX7, which has the ability to recognize the repressive histone mark H3K27me3 and to keep the target gene cluster silenced. With respect to functionality, it has very recently been reported that lncRNA ANRIL plays an important role in non-small cell lung cancer outcomes since its expression is closely associated with tumor-node metastasis stage and tumor size. The loss of expression of ANRIL induces apoptosis in vivo and in vitro, providing novel insights into the functionality of this lncRNA transcript in tumorigenesis [67].

5. *Trans*-acting elements

Increasing numbers of lncRNAs are known to be direct interactors with specific regions of the chromatin located across multiple different chromosomes to modulate gene expression at the genomic level. It is well known that *trans*-acting lncRNAs target proteins, distal chromatin regions of even others ncRNAs while the determining factor for these long-range interactions is not well established. Almost all classes of RNA molecules are governed by a characteristic secondary structure. Since this RNA architecture allows RNA to recruit and interact with proteins, other nucleic acids as well as with itself, deciphering the RNA structural language and understanding the structure–function relationship is critical for a deeper knowledge of *trans*-acting RNA function, especially for lncRNAs for which no common function is known. Some studies have suggested that the intrinsic architecture of lncRNAs is the key factor determining specific interactions [68,69].

In this category, two lncRNAs that are highly overexpressed in aggressive prostate cancer, PRNCR1 (also known as PCAT8) and PCGEM1, activate the transcription of ligand-dependent or ligand-independent androgen receptors by binding to androgen receptors, thereby enhancing proliferation programs in prostate cancer cells as was reported by Rosenfeld group in 2013. Binding of PRNCR1 to the acetylated androgen receptor is required for the sequential association with the H3K79 methyltransferase DOT1L that in turn, and is necessary for the recruitment of the second lncRNA involved in the present mechanism, PCGEM, which is methylated by DOT1L [70].

6. Competing endogenous RNA network: microRNA sponges and decoys

As mentioned above, lncRNAs may exert their function through transcriptional or post-transcriptional mechanisms. Some years ago, a new regulatory network, first described in plants [71], has emerged in the post-transcriptional control of gene expression by characterizing the ability of numerous lncRNA transcripts to compete for microRNA binding, thereby alleviating the negative effect of microRNAs on

their respective mRNA targets. These RNA transcripts are commonly known as competing endogenous RNAs (ceRNAs) or natural microRNA sponges. This category includes lncRNAs, circular RNAs and pseudogenes. The discovery of the ceRNA network reveals an additional layer of post-transcriptional regulation and, most importantly, reveals a complexity of ncRNA regulation of various non-coding RNA species of importance in cancer. As we mentioned before, almost all classes of RNA molecules are governed by an intrinsic secondary architecture that allows RNA to recruit and interact with proteins, other nucleic acids as well as with itself. Based on the principle of complementarity, Steitz and coworkers proposed the hypothesis, based on computational methods, that the existence of microRNA binding sites could be titrating specific microRNA and thus, affecting microRNA availability and, in turn, affecting to downstream processes [72]. Poliseno and collaborators subsequently demonstrated experimentally that the pseudogene derived from the PTEN locus sequesters microRNA due to their highly similar sequence. They showed that the expression of the ancestral protein-coding transcript was subordinated to the expression of the pseudogene transcript (PTENP1) by acting as a sponge for miR-19b and miR-20a binding sites shared between these two transcripts [73]. These *bona fide* microRNA competitors actively compete with their ancestral protein-coding genes for the same pool of microRNAs and so are able to regulate their relative abundances. This highlights how various non-coding RNA elements in a network may communicate with each other through microRNA Response Elements (MREs). Apart from the PTENP1 RNA transcript, many other transcripts, most of which are messenger RNAs, are known to be competing endogenous RNAs. Our review is concerned only with those lncRNAs that have the capacity to alter microRNA activity through the presence of MREs in their sequences. A handful of lncRNAs have so far been identified as decoys for microRNAs; some of these are illustrated in Table 2.

The H19 lncRNA is a highly conserved and imprinted transcript, 2.6 kb long, that is mainly found in the cytoplasm and is known to have an important role in growth [74]. This oncogenic lncRNA transcript harbors canonical and non-canonical binding sites for some members of the let-7 family, regulating their expression, which has crucial roles in cancer [75]. The functionality of this regulation was validated by genome-wide transcriptome analysis and by several *in vivo* experimental approaches to conclude that H19 knockdown releases let-7, leading to a stronger let-7 function [76].

Papillary thyroid carcinoma susceptibility candidate 3 (PTCSC3) is a recently discovered tumor suppressor lncRNA that is dramatically downregulated in thyroid cancers. After an *in silico* analysis, 20 putative binding sites for different microRNAs were identified along the PTCSC3 sequence. Of these, miR-574-5p was identified as a specific target for this lncRNA. The significant inverse correlation between PTCSC3 and miR-574-5p suggests that PTCSC3 acts as a molecular decoy to target the oncogenic miR-574-5p that consequently regulates cell growth

and apoptosis in thyroid cancer. The discovery that PTCSC3 regulation is part of miR-574's activity sheds some light on the molecular mechanisms that underlie thyroid cancer and provides new molecular markers for its earlier diagnosis [77].

As explained above, miR-21 post-transcriptionally regulates CASC2 function in a sequence-specific manner, but a negative feedback loop mediated by the RNA-induced silencing complex (RISC) has also been reported [51]. In this regard, CASC2 expression is dramatically reduced in glioma tissues, promoting the migration and proliferation of malignant cells. *In silico* and *in vivo* experimental validations using techniques such as luciferase assays and RNA pull-downs confirmed that CASC2 lncRNA behaved as a microRNA sponge for miR-21 in U251 and U87 glioma cell lines that overexpressed this lncRNA through one highly conserved MRE. Therefore, CASC2 could be a new candidate therapeutic target gene for treating gliomas [44].

Intriguingly, the regulation between the evolutionarily conserved lncRNA HOST2 (human ovarian cancer-specific transcript 2) and the microRNA let-7b also works in both directions. The HOST lncRNA binds to microRNA let-7b, a potent tumor suppressor microRNA, thereby mediating post-transcriptional regulation. HOST2 RNAs have only one binding site for let-7b, recruiting it and modulating its accessibility to its natural targets by acting as a molecular decoy. HOST2 can affect let-7b functions, which help to enhance the oncogenic features bestowed on HOST2 lncRNA by accentuating the endogenous expression of metastasis-promoting genes such as HMGA2, c-Myc and Imp3 [78].

LincRNA-RoR has been reported to act as a molecular decoy for miR-145, which in turn regulates the mRNA levels of core transcription factors such as Oct4, Sox2, and Nanog, to maintain cell renewal in human stem cells (hESCs) [79]. In doing so, the linc-RoR regulates miR-145 expression in a sequence-dependent manner, since only the overexpression of the wild-type form of the lncRNA had any effect on microRNA function. Moreover, the overexpression of linc-RoR affects the mature levels of miR-145, whereas the precursor levels were unaffected, confirming the action of the post-transcriptional mechanism [79]. Subsequently, the negative regulation of miR-145 by linc-RoR in endometrial cancer stem cells was demonstrated, which suggests that linc-RoR functions could be similar to the mechanism described in hESCs. This highlights the importance of this regulatory feedback loop in physiological aspects of early carcinogenesis [80].

In a similar manner, linc-ROR overexpression leads to an epithelial-to-mesenchymal transition (EMT) phenotype in mammary cells, emphasizing its role in tumorigenesis. In this study, Hou and coworkers showed that, in addition to miR-145, linc-RoR also functions as a microRNA sponge for miR-205. In doing so, linc-RoR prevents the downregulation of miR-205 target genes such as ZEB2, which is known to cause EMT and metastasis in breast cancer.

One of the most strongly upregulated genes in hepatocellular carcinoma is the lncRNA HULC, whose depletion causes some alterations in genes involved in liver cancer. In this particular case, HULC RNA has been described as a competing endogenous RNA for miR-372 that mediates the post-transcriptional repression of PRKACB genes which finally phosphorylates CREB, which in turn upregulates HULC [81].

Another good example of microRNA titration is the mutual relation between loc285194 RNA and miR-211 that involves the RISC complex. loc285194 is a p53-regulated tumor suppressor lncRNA that harbors two binding sites for miR-211 that explains its tumor suppressor features in osteosarcoma tumors [50].

The characterization of the ncNRFR lncRNA in colonic homeostasis by Franklin and coworkers has elucidated the functional role of lncRNA species. This lncRNA is transcribed from the *RAS* locus but appears to be an independent transcriptional unit. Their study found that the overexpression of this ncNRFR transcript in non-malignant cells gives rise to a highly invasive and metastatic phenotype. It also reported a functional interaction between ncNRFR transcript and let-7 family, in which the complementary sequences are outside of the seed region. Bearing in

Table 2
Examples of cancer associated lncRNAs and T-UCRs that post-transcriptionally target microRNAs.

	microRNA	Disease/process	Ref
LncRNA			
H19	Let-7	Pancreatic carcinoma	Ma et al. [75]
PTCSC3	miR-574	Thyroid cancer	Fan et al. [77]
CASC2	miR-21	Glioma	Wang et al. [44]
HOST2	Let-7b	Ovarian cancer	Gao et al. [78]
LincRNA-RoR	miR-145	Endometrial cancer stem cells	Zhou et al. [80]
	miR-205	Breast cancer	Hou et al. [97]
HULC	miR-372	Hepatocellular carcinoma	Wang et al. [81]
loc285194	miR-211	Osteosarcoma tumors	Liu et al. [50]
ncNRFR	Let-7	Colonic malignant transformation	Franklin et al. [82]
T-UCR			
Uc.283+A	miR-195	Colorectal cancer and neuroblastoma	Liz et al. [22]

mind the importance of the role of let-7 family members as tumor suppressors, any alteration would have important associations with a malignant phenotype. Using *in vitro* validation based on luciferase reporter assays upon ncNRFR overexpression in non-malignant cells, these authors showed that ncNRFR-positive cells ameliorate the repression of the let-7 reporter relative to controls, indicating that the ncNRFR transcript downregulates let-7 activity. Further analysis of let-7 targets has demonstrated that they are significantly upregulated by ncNRFR expression. Thus, the ncNRFR transcript inhibits let-7 function, disrupting the growth of normal colonic epithelial cells [82].

Last, but not least, an important mechanism in the post-transcriptional control of microRNAs by lncRNAs has been described very recently by our group. In this particular case, an lncRNA transcribed from an ultraconserved region of the genome impairs Microprocessor recognition by binding to the primary microRNA-195 transcript through sequence complementarity. A detailed explanation of the mechanism underlying this first reported case of RNA-directed regulation of microRNA biogenesis is provided in the next section [22].

7. lncRNAs harboring conserved elements in cancer: T-UCRs

Nowadays, the degree of conservation of lncRNAs has been a controversial topic. In this respect, an intriguing feature of the non-coding genome includes ultraconserved regions (UCRs), otherwise known as ultraconserved elements (UCEs). These genomic regions describe a subset of 481 conserved sequences longer than 200 bp that are fully conserved between orthologous regions of the mouse, rat and human genomes [83]. Due to their degree of conservation, it is possible that these 481 UCRs contain functional elements [83] that are essential to the physiology of the cell, although to date, their biological roles remain largely uncharacterized. Approximately 25% of them overlap with the mRNA of protein-coding genes and are known to be exonic elements. By contrast, the non-exonic elements are usually clustered within gene deserts and it has been suggested that they may be involved in the regulation of transcription at the DNA level, acting as enhancers of distal neighboring protein-coding genes [83].

A high percentage of these UCRs (~93%) are actively transcribed as RNAs products, and are known as T-UCRs, exhibiting ubiquitous and tissue-specific expression patterns that are very often altered in human carcinomas [84]. Analyzing their expression profiles makes it possible to distinguish human cancers. Among these aberrantly expressed T-UCRs, Uc.338+ is of particular note with respect to the pathobiology of hepatocellular carcinoma (HCC), since it is dramatically upregulated compared with adjacent non-cancerous tissues. A decrease in the anchorage-dependent and anchorage-independent growth of HCC cells was observed upon Uc.338+ knockdown, highlighting the role of this T-UCR in regulating cell transformation and suggesting that Uc.338+ is a genetic marker of susceptibility in HCC [85].

T-UCR expression also seems to be important in prostate cancer, such that a significant number of T-UCRs were differentially expressed between tumor and non-cancerous tissues. In this context, Uc.363+A and Uc.454+A appear to be the most significantly upregulated and downregulated T-UCRs, respectively. Uc.160+ also plays an important role since its downregulation has generated a robust gene expression profile in prostate LNCaP cells in which numerous genes that cluster in proliferation and cell movement pathways are upregulated or downregulated relative to control cells, suggesting that Uc.106+ is a functional transcript that may have an important role in prostate cancer [86].

It is well known that colorectal cancer (CRC) develops as a result of multiple genetic alterations. In the study carried out by Sana et al., Uc.73+ and Uc.388+ were identified as potential players in the diagnosis and prognosis of CRC patients, since they exhibit lower levels of expression than non-tumoral tissue samples, suggesting that both had tumor-suppressor properties [87]. However, the functional role of Uc.73+ in CRC is a controversial topic that is yet to be resolved. Uc.73+ has been described as one of the most frequently upregulated

T-UCRs in colon cancer and the effects of this downregulation have been studied by Calin and coworkers. Upon Uc.73+ downregulation, a significant decrease in COLO-320 cell growth and an increase in the sub-G1 fraction was observed, which suggests the presence of apoptosis in these cells in which this T-UCR is upregulated.

UCRs are often located at fragile sites and genomic regions affected in various cancers (cancer-associated genomic regions, CAGRs). This is the case, for instance, for Uc.349+A and Uc.352+, whose expression differs between normal and leukemic CD5-positive cells [84]. Both T-UCRs are immersed within the 13q21.33–q22.2 genomic region that has been associated with susceptibility to familial chronic lymphocytic leukemia [88].

As mentioned above, the alteration of T-UCR expression in cancer may be achieved by hypermethylation of CpG islands in their respective promoter regions [45] (Fig. 1) or by their interaction with microRNAs [84]. Concerning the epigenetic mechanisms that control T-UCR expression at the transcriptional level, the presence of CpG island promoter hypermethylation is associated with T-UCR silencing in a variety of tumor types and human cancer cell lines. As briefly described above, Uc.283+A, Uc.346+ and Uc.160+ are subject to epigenetic silencing associated with the hypermethylation of promoters. In such a way, after treatment with the DNA demethylating agent 5-Aza-2′-deoxycytidine (5-AzaC), these T-UCRs lose methylation in their promoter regions and undergo a concomitant restoration of their expression levels. Furthermore, Uc.160+, Uc.283+A and Uc.346+ hypomethylation was linked to a more accessible chromatin conformation of the RNA polymerase II enzyme and was accompanied by the presence of the active histone mark H3K4me3 [45].

Hudson and coworkers also found six T-UCRs underwent transcriptional silencing driven by epigenetic mechanisms such as DNA methylation. In particular, they found that Uc.308+A, Uc.434+A, Uc.241+A, Uc.283+A, Uc.285+ and Uc.85+ expression was recovered upon 5-AzaC and/or the histone deacetylase trichostatin A treatment in prostate cancer cell lines [86]. Of these, Uc.283+A had been previously found to be silenced by CpG hypermethylation in HCC cells [45].

Some instances of interactions between T-UCRs and microRNAs have been described and are discussed extensively in the following sections. Their intrinsic capacity to form RNA duplexes with microRNAs may represent a mechanism of mutual regulation that could work in both directions.

8. T-UCR targets for microRNAs

In 2007, Calin and collaborators demonstrated for the first time that some T-UCRs underwent altered expression patterns due to their direct regulation by highly expressed microRNAs in chronic lymphocytic leukemia (CLL). In particular, they confirmed that Uc.160+, Uc.346+A and Uc.348+ showed significant antisense complementarity with the miR-29a, miR-29b, miR-29c, miR-155, miR-146, miR-223 and miR-24 microRNAs, in which the expression of each T-UCR was negatively correlated with the corresponding microRNA levels. Each T-UCR:microRNA pair was extensively validated in vivo by measuring the expression levels of Uc.160+ and Uc.346+A after the overexpression of miR-155 in leukemia cells in which both T-UCRs were downregulated. Furthermore, Uc.348+ was cloned into luciferase reporter vectors to verify its interaction with miR-155, miR-24-1 and miR-29-b in vitro, in which a concomitant reduction in luciferase expression was observed. This confirms the association between the two RNA species and, most importantly, suggests that an ncRNA network underlies the tumoral phenotype.

This fortuitous discovery and the high degree of conservation of T-UCRs that hints at their biological function prompted researchers to investigate T-UCR functionality. They found altered patterns of T-UCR expression that are associated with specific tumor types. For instance, Scaruffi et al. showed that a negative correlation between T-UCRs and microRNAs activity may play an important role in the pathogenesis of
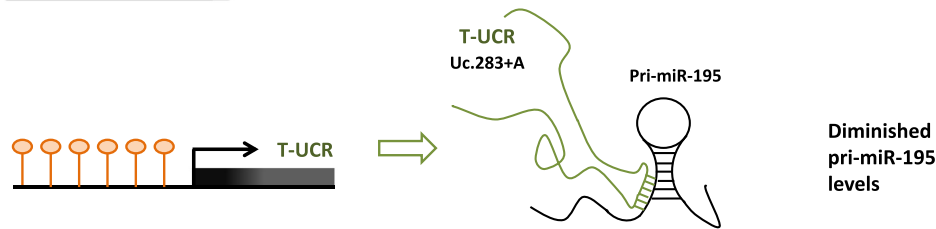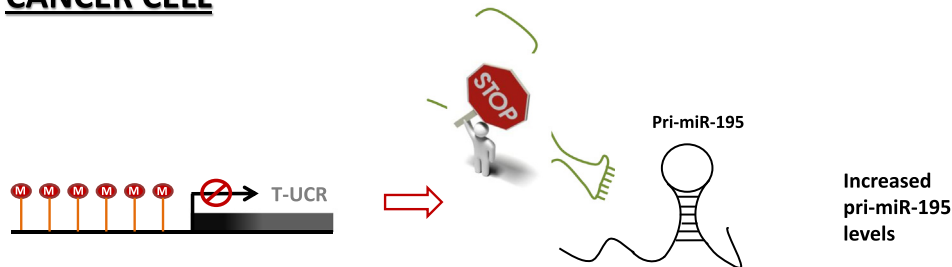
NORMAL CELL

CANCER CELL

Fig. 1. In normal cells, a T-UCR (Uc.283+A) is transcribed from an unmethylated CpG island and the complementarity between the lower stem region of a pri-miRNA (pri-miR-195) transcript and the ultraconserved sequence in the T-UCR prevents pri-miRNA cleavage by Drosha. Certain human tumors undergo T-UCR transcriptional silencing by CpG island promoter hypermethylation and the production of mature miR-195 cannot be regulated.

neuroblastoma. A thorough analysis of the expression of the 481 T-UCRs and 723 microRNAs in 34 neuroblastoma patients led them to conclude that the complementary regulatory mechanism between T-UCRs and microRNAs and the resulting T-UCR expression pattern predicted outcome in high-risk neuroblastomas [89]. In the case of pediatric neuroblastoma tumors it should be noted that T-UCRs, in addition to protein-coding genes located in CAGRs sites, may be involved in cancer susceptibility, since their expression is positively correlated with copy-number status [90].

9. MicroRNA targets for T-UCRs

The functional consequence of microRNA activity on T-UCRs (or on lncRNAs more generally) was not entirely surprising, given the high similarity of mRNA and lncRNA sequences. By contrast, the reciprocal regulation between the two classes of RNA is almost certain to yield more promising functions of lncRNAs, in addition to already known mechanisms.

Several lines of research have resulted in progress being made towards identifying functional interactions between lncRNAs as a new post-transcriptional mechanism in the control of microRNA function. Despite the importance of T-UCR expression in health and disease, the biological role and functional aspects of the vast majority of T-UCRs remain largely unknown. As described above, only one case of lncRNA derived from an ultraconserved region of the genome has so far been identified as a post-transcriptional modulator of microRNA function [22]. This new mode of regulation differs from the mode of action of other ceRNAs because the titration of microRNA from their respective targets does not necessarily reduce microRNA levels. In this particular case, the overexpression with the epigenetically disrupted Uc.283+A in HCT-116 colorectal cancer cells impairs the proper processing of miR-195 (Fig. 1). The regulation is based on the complementarity between the ~11 nucleotides upstream of the Drosha cropping site and the 11 consecutive nucleotides within the ultraconserved sequence of the Uc.283+A. Despite not being sequence-dependent, the regulation requires complementarity between molecules, which implies that disturbances in the lower stem region of pri-miR-195 underpin the negative regulation. It is well known that the lower-strand region of

pri-microRNAs is crucial to DGCR8 recognition and the subsequent catalysis mediated by the endonuclease Drosha. As a result, and as validated in vivo and in vitro, the presence of the complementarity between Uc.283+A and pri-miR-195 reduces the production of mature and functional miR-195 molecules, which in turn leads to the derepression of a functional target in vivo. It would be interesting to settle this point, since many miR-195 targets are closely related to proliferation and cell cycle progression [91,92], and Uc.283+A is highly expressed in neuroblastoma tumors [90].

Currently, much effort is being made to elucidate the well-orchestrated mechanisms that underlie the various regulatory networks controlling cell physiology in health and disease. This review summarizes some recent examples that describe the concept of lncRNA:microRNA interactions, which operate spatiotemporally during tumorigenic processes. Taken together, microRNAs and lncRNAs cooperate with each other to fine-tune gene expression at the transcriptional and post-transcriptional levels. It is clear that these non-coding RNAs species influence every aspect of cell physiology through a sophisticated and multi-layered mode of regulation, even though we currently lack information about the mechanistic aspects underlying the regulatory interactions. Recent advances have revealed fascinating new modes of interaction between non-coding RNA molecules that were once dismissed as being part of transcriptional noise. For instance, in the next few years, the analysis of RNA–RNA or RNA–protein interactions, such us those that include the Ago and RNA binding proteins, by CLASH [93], iCLIP [94], HITS-CLIP [94,95], as well as the integration of in vivo RNA structural analysis [96], seem likely to produce an expansion in our understanding of how the interaction between non-coding RNA molecules affects normophysiology and pathophysiology.

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