Opinion

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Repression of transposable-elements – a microRNA anti-cancer defense mechanism?

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MicroRNAs (miRNAs) appear to be key players in the maintenance of genomic integrity. Recent evidence implies that cancers often avoid miRNA-mediated regulation, and global repression of miRNAs is associated with increased tumorigenicity. Here we suggest that miRNAs are directly involved in the maintenance of genomic integrity through global repression of transposable elements (TEs), whose expression and transposition are well-documented causes of genomic instability in mammalian somatic tissues. Hence, one outcome of the tumor's ability to avoid miRNA-mediated regulation might be the enhancement of genomic instability and mutability due to derepression of TEs. We outline possible mechanisms underlying TE repression by miRNAs, including post-transcriptional silencing and transcriptional silencing through DNA and histone methylation. This hypothesis calls into consideration the need to study the role of miRNAs and the RNAi machinery in the nucleus, and specifically their impact on the maintenance of genomic integrity in the context of cancer.

miRNAs, cancer and genomic integrity – a question revisited

miRNAs (Box 1) have emerged in the past decade as important players in numerous cellular and organismal processes in animals and plants [1]. Their involvement in disease progression, particularly cancer, has been extensively studied [2–8], and they hold great promise for both therapeutic and diagnostic potential [9]. Whereas multiple studies addressing the connection between miRNAs and cancer have underscored the roles of individual miRNAs as oncogenes or tumor suppressors [2–4], the majority of those studies mainly consider the well-known function of miRNAs as post-transcriptional silencers of gene expression. However, there are growing indications that miRNAs might possess an additional, potentially significant role in the maintenance of genomic integrity on a global level. Here we outline recently published evidence indicating that cancers often globally repress miRNA expression, and supporting a direct involvement of miRNAs in the maintenance of genomic integrity. We propose miRNA-mediated repression of TEs as a potential mechanism, and their elimination during tumorigenesis might increase genomic instability and thereby further promote cancer progression. Hence, greater attention should be given to elucidating the function of the RNAi machinery in general, and of miRNAs in particular, in the nucleus of somatic cells during tumor formation.

Cancers selectively avoid miRNA-mediated regulation

One of the first studies that performed large-scale expression profiling of miRNAs in tumors made an intriguing observation: overall, tumors tend to express lower levels of miRNAs than normal tissues [10]. The authors suggested that this phenomenon might reflect the de-differentiated state of cancers compared to fully differentiated normal tissues.

We would like to propose an alternative explanation, namely that global avoidance of miRNA-mediated regulation might be one of the many ways that cancer cells enhance their tumorigenic potential. Consequently, we suggest that during the course of cancer progression tumors undergo positive selection to silence miRNA-mediated regulation.

Low miRNA expression might be a general common feature of relatively undifferentiated early progenitor cells, nevertheless, mouse embryonic stem cells (ES cells) express a substantial amount of miRNAs [11]. Yet, whereas undifferentiated cells progress rapidly into a differentiated state that is characterized by higher miRNA levels, cancer cells continue to replicate practically indefinitely in the presence of limiting amounts of miRNAs. Tumorigenesis and genomic instability of tumors clearly depends on other factors such as epigenetic alterations. Thus, although low miRNA levels might not suffice to drive transformation, the fact that miRNA are so commonly expressed at low levels in tumors indicates that tumors evolve to globally repress them to enhance tumorigenesis.

Avoidance of miRNA-mediated regulation by tumors is supported by several lines of evidence. For instance, widespread repression of a large set of miRNAs, mediated by the oncogene *MYC,* was reported to occur in lymphoma [12]. Additional avoidance mechanisms include genomic events such as mutations and deletions, or epigenetic silencing, of genomic loci encoding miRNAs [13–18]. Several recent publications have highlighted a more global phenomenon wherein disruption of the miRNA biogenesis

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Box 1. The biology of small RNAs

Small regulatory RNAs is a comprehensive name for a class of RNAs ~20–30 nucleotides in length that are single stranded in their mature form, and that generally act in silencing of gene expression through the RNAi (RNA interference) pathway. The RNAi pathway is comprised of small RNA biogenesis proteins, as well as the effector complex that binds the small RNAs, and is directed by them to target nucleic acid molecules via base-pairing interactions. The different classes of small RNAs differ in their origin, biogenesis, expression pattern, and utilization of the different types of RNAi effector proteins [65,68,76].

Types of small RNAs in mammals

MicroRNAs (miRNA): single-stranded RNA molecules of ~21–23 nucleotides in length that regulate gene expression through silencing. They are transcribed from distinct gene loci into long primary transcripts (pri-miRNAs), then cleaved in the nucleus by **Drosha** (see below) into a ~70 nucleotide hairpin precursor (pre-miRNA), which is exported to the cytoplasm and cleaved again by **Dicer** (see below) to its mature form.

Piwi-interacting RNA (piRNA): the largest class of small RNA molecules expressed in animal germline cells. Their expression and maturation is not dependant on Drosha and Dicer.

Small interfering RNA (siRNA): typically ~19–25 nucleotides long double-stranded RNAs that were shown to be expressed in mouse oocytes and ES cells, and are originated from pseudogenes or TEs [11,50–52]. Their maturation is independent of Drosha, whereas in oocytes they are dicer dependant, while in ES cells their Dicer dependence is minor. In the fly they were shown to be expressed in somatic tissues as well, whereas in mammals their somatic expression remains to be determined.

Protein components of the RNAi pathway

Drosha: a nuclear RNase III protein essential for the early steps of miRNA biogenesis. Following transcription of the long miRNA primary transcript (pri-miRNA) by either Pol-II or Pol-III, Drosha recognizes the ~70 nucleotide hairpin miRNA precursor termed pre-miRNA embedded in the pri-miRNA and excises it. Drosha requires its cofactor DGCR8 (encoded by the DiGeorge syndrome critical region gene 8 in mammals, also known as Pasha in other species) to form the active complex [76].

Dicer: an RNase III protein that is crucial for the biogenesis of both exogenous and endogenous siRNAs (endo-siRNAs)] and miRNAs. Different species have different numbers of Dicer homologs, and these sometimes differ in their specificities towards the various small RNAs, whereas mammals have only a single gene encoding Dicer (*DICER1* in human). Dicer cleaves the pre-miRNA hairpin, or double-stranded RNAs (dsRNA) which are the precursors of siRNAs, to generate the small ~21 nucleotide dsRNA. The mature, single-stranded small RNA is then separated and loaded into the effector complex, termed the RNAi silencing complex, or RISC [76].

Argonaute: a general name of a class of proteins that form the core of the RISC. The Argonaute family is comprised of two subfamilies: Piwi subfamily members (encoded by three distinct genes in mammals: *PIWIL1–3* in human) are specific to the germline and bind piRNAs [47], and Ago subfamily members (four genes in mammals: *EIF2C1–4* in human) bind miRNAs and siRNAs [76]. The Argonaute protein interacts with the mature small RNA and exerts silencing of gene expression either by post-transcriptional regulation (PTGS – post-transcriptional gene silencing), that includes cleavage of the target RNA and inhibition of its translation, or by transcriptional gene silencing (TGS), that includes histone and DNA methylation. Whereas the function of Argonaute proteins in PTGS in mammals and other species is well established, their role in TGS in mammalian cells has only emerged in recent years [65].

pathway itself takes place in cancer. Examples include nuclear retention of pre-miRNAs [19] and blockage of pri- or pre-miRNA biogenesis and processing [20–22], that were in some cases directly implicated in cancer promotion [23]. A recent study also demonstrated that the p53 tumor-suppressor is involved in the enhancement of miRNA maturation following DNA damage [24], further supporting the notion that global upregulation of miRNA levels is associated with cancer prevention.

miRNAs – guardians of genomic integrity

What might be the selective advantage that miRNA avoidance provides to cancer cells? Some insight into the significance of this phenomenon can be gained from a closer comparison between two studies where expression of Dicer, a key enzyme in the miRNA biogenesis pathway (Box 1), was ablated in cancer cells [25] and normal cells [26]. In the first study, Kumar *et al.* showed that depletion of Dicer from various cancer cell lines increased colony formation efficiency *in vitro* and augmented tumor burden and aggressiveness *in vivo* [25]. In the second study, Mudhasani *et al.* demonstrated that conditional *Dicer1* knock-out in normal adult mouse skin and normal mouse embryonic fibroblasts (MEFs) led to premature senescence [26]. Hence, Dicer elimination can elicit two opposite cell fates, senescence or enhanced proliferation, in normal or cancer cell backgrounds, respectively. This is reminiscent of the situation where activation of oncogenes, such as *RAS*, augments the malignant properties of cancer cells, whereas in normal cells it will often lead to activation of DNA damage checkpoints (where sensor proteins sense physical DNA damage and activate a cascade of regulators leading to DNA repair, cell-cycle arrest, apoptosis or senescence depending on the cellular context and the extent of damage [27]) with subsequent p53- and ARF-dependent senescence, a phenomenon known as 'oncogene-induced senescence' (OIS) [28]. Importantly, the premature senescence described by Mudhasani *et al.* [26] was not accompanied by the upregulation of oncogenes such as *MYC* or *RAS*, and therefore does not represent a classical case of OIS. Interestingly, however, the study demonstrated that Dicer knockout induced DNA damage (evident by γH2AX staining), and this consequently activated a p53- and ARF-dependent DNA damage checkpoint. Thus in normal cells possessing intact DNA damage checkpoints, premature senescence is enforced in order to avoid propagation of cells whose genomic integrity has been compromised by Dicer depletion. In cancer cells, these checkpoints are sub-optimal or non-functional, and thus genomic instability introduced by Dicer elimination can promote tumorigenesis further.

We therefore suggest that global miRNA avoidance contributes to cancer formation not only by enhancing proliferation, but also by directly leading to genomic instability, causing increased DNA damage and thus potentially giving rise to enhanced mutation rates. miRNAs therefore constitute an essential component in the maintenance of genomic stability throughout the lifespan of normal somatic cells, and thus can be thought of as one of the 'guardians' of genome integrity – an additional regulatory barrier whose elimination might be part of a series of events that ultimately lead to cancer.

miRNAs protect genomic integrity by global repression of transposable elements

How do miRNAs serve as guardians of genomic integrity? One immediate explanation would be that a handful of

miRNAs target genes whose excessive activity might promote genomic instability. Indeed particular miRNAs were implicated as tumor-suppressors: for example, let-7, that targets *RAS* [29] and *MYC* [30]. However, the global repression of miRNAs in cancer cells is much broader and is not restricted to tumor-suppressor miRNAs, and therefore requires a more comprehensive explanation. A related possibility might reside at the level of miRNAs network wiring within the broader regulatory network of the cell [31]. The coupling of miRNA regulation with transcriptional regulation and their tendency to influence multiple genes in the same pathway [32–34] might contribute to maintenance of proper cell fate and would thus prevent the accumulation of genomic instability [31].

An alternative explanation might be based on the conjecture that in mammalian somatic cells miRNAs are involved not only in post-transcriptional silencing, but also in repression at the DNA level. We hereby suggest that miRNAs contribute to maintenance of genomic stability by global repression of TEs in somatic cells. Therefore, the global downregulation of miRNAs observed in cancers would promote TE expression and subsequent genomic instability that benefit the evolving tumor (Figure 1). We will now provide a series of evidence to support this notion. Indeed, the expression of TEs, that are considered silent in normal cells, is widely associated with DNA damage in many species [35] (reviewed in [36]). For example, Soper *et al.* have recently demonstrated that derepression of TE transcription in mouse spermatocytes resulted in massive DNA damage [37].

The hypothesis that TE targeting by miRNA helps maintain genomic integrity and is eliminated by evolving tumors to promote tumorigenicity is an appealing one, especially in light of the observations made by Mudhasani *et al.* that ruled out two of the main hypotheses that could explain the phenomenon: Dicer elimination leads to direct physical damage to the DNA whereas cell fate was properly maintained, and expression of the oncogenic proteins MYC and RAS remained unchanged [26]. This further hints at a direct involvement (rather than through their protein targets) of the miRNA elimination in maintaining genomic instability.

The role of the RNAi pathway as protector against TE expression and expansion is well known and evolutionarily conserved in many animal and plant species, from the yeast *Schizosaccharomyces pombe* [38] to nematodes [39] and mammals (reviewed in [36,40]). For example, Dicer knockout experiments in mouse ES cells and preimplantation embryos show defective silencing of various types of TEs [41,42]. TE-defense seems to reflect the ancestral role of RNAi [40,43,44]. Accordingly, these capabilities of the RNAi machinery are likely to facilitate its contribution to the protection against potential DNA damage induced by TEs.

Mammalian cells express three classes of small RNAs that function in the RNAi pathway: piRNAs, siRNAs and miRNAs (Box 1). piRNAs are highly expressed in the animal germline, where they mediate TE silencing [44–49]. Consequently, Malone and Hannon suggested a role for the RNAi machinery, and particularly the germline-expressed piRNAs, in guarding the genome against the potentially harmful effects of TEs on long-term organismal fitness [40]. However, piRNAs are not expressed in somatic cells, and their biogenesis is Dicer-independent [44] and therefore they are less relevant to the discussion of cancer. Endogenous siRNAs (endo-siRNAs) are highly expressed in mouse oocytes, where they originate from TEs, and function in TE suppression in a Dicer-dependent manner

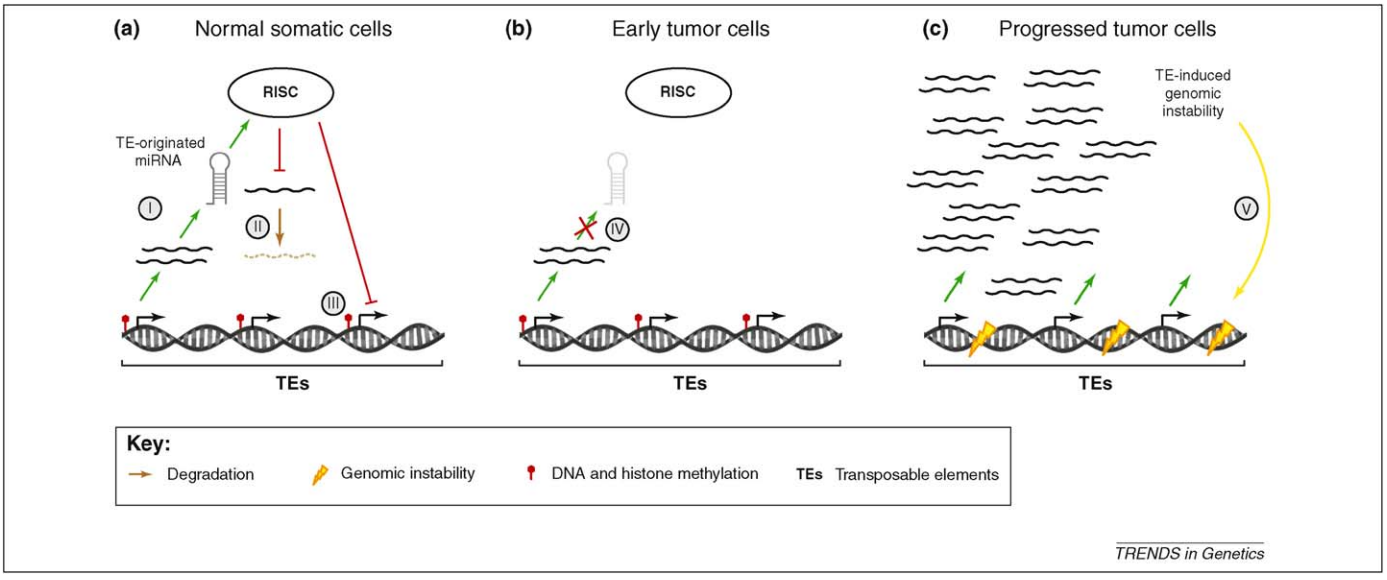

Figure 1. A proposed role for miRNAs in maintaining genomic integrity via global repression of TEs. In the proposed model, TE-derived miRNAs are incorporated into the RNAi machinery, and guide the repression of TEs, both by transcriptional and post-transcriptional silencing involving DNA and histone methylation. Thus, hundreds of mammalian and primate-specific TE-derived miRNAs serve to protect somatic cells from the DNA damage induced by TE expression. **(a)** In normal somatic cells, TE-derived and other miRNAs maintain a steady state of efficient repression of TEs. **(i)** Hundreds of mammalian and primate-specific miRNAs in the human genome originate from TEs [57]. **(ii)** TE-derived miRNAs act to silence expression and transposition of TEs in the human genome by post-transcriptional gene silencing, as suggested by others [63,64], and potentially by transcriptional silencing **(iii)**. Evidence for the involvement of RNAi machinery in transcriptional silencing via DNA and histone methylation is accumulating (Refs [74,80,81] and others). **(b)** During the early stages of tumor initiation, cancer cells are selected to avoid miRNA-mediated regulation **(iv)**. **(c)** This leads to derepression of miRNAs during tumor development. TE derepression, expression and transposition of TEs, leads to DNA damage [35–37] **(v)**, that would enhance genomic instability and contribute to cancer development.

[50,51]. They are also found in mouse ES cells [11,52], where their Dicer dependence is still unclear. Endo-siRNAs were not yet shown to be globally expressed in somatic tissues of mammals, whereas in *Drosophila melanogaster* they were shown to be expressed in both the germline and somatic tissues (reviewed in [53]). Yang *et al.* showed that an endo-siRNA derived from the L1 long interspersed nuclear element (LINE) is capable of repressing the expression and transposition of L1 in human cell lines [54]. Since the extent of endo-siRNA expression in somatic tissues of mammals, and their Dicer dependence remain to be determined, miRNAs presently appear to constitute the main class of small RNAs expressed in mammalian somatic tissues. Recent reports reveal that elimination of *Dgcr8* (Box 1), and subsequent miRNA depletion, have very little phenotypic effect in mouse oocytes [55], that express piRNAs and endo-siRNAs, whereas Dicer knockout in oocytes has a severe phenotype [56]. This is in sharp contrast to different adult tissues and cell types, where Drosha and *Dgcr8* elimination results in phenotypic defects similar to those seen upon elimination of Dicer (discussed in [55]). Hence, miRNAs are indeed the major class of small RNAs whose function is crucial in somatic cells.

Interestingly, miRNAs were shown to originate from TEs [11,57–61]. These overall constitute over 20% of the human miRNA collection (Dahary et al. personal communications), and they are mammalian and primate-specific. Moreover, mammalian and primate-specific miRNAs were previously suggested to target TEs. Expression analysis in mammalian tissues showed that TE-originated miRNAs comprise about 23% of the catalog of expressed miRNAs, and they do not tend to preserve the strand asymmetry that is typical of other miRNAs [62]. Calabrese *et al.* suggested that TE-associated miRNAs expressed in mouse ES cells serve as host defenses against TEs [11]. In further support of this notion, the degree of evolutionary expansion of different TEs in the human genome inversely correlates with the number of miRNAs predicted to target those TEs [63]. Other miRNAs were also reported to target TEs. For example, *Alu* repeats were shown to be targeted by several miRNAs, including a large cluster on chromosome 19 consisting of 46 miRNAs flanked by *Alu* TEs [60,64]. As evidence for miRNA targeting of TEs continues to accumulate there is a growing need to decipher the biological relevance of such regulation.

We now propose that, through direct global repression of TEs, miRNAs maintain genomic integrity in somatic cells and provide an important layer of anti-cancer protection (Figure 1a). There seems to be an evolutionary arms race [44] between TE expression and expansion and small RNAs of the host genomes. Given the large number of miRNAs that physically originate from TEs, especially in the mammalian and primate lineages, global avoidance of miRNA expression in cancer would promote global TE upregulation and subsequent genomic instability (Figure 1b,c). It is tempting to speculate that cells have evolved to use the RNAi pathway to turn TE-originated miRNAs against TEs in somatic tissues, where miRNAs might serve a parallel role to that of piRNAs and endo-siRNAs in the germline [11,40] as 'guardians' of genomic integrity.

Possible mechanisms for TE repression by miRNAs: post-transcriptional silencing and epigenetic silencing via DNA and histone methylation

Since the RNAi machinery is known to function both through transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS), it is conceivable that miRNAs also employ both mechanisms towards achieving global repression of TEs, as part of their role in maintaining genomic integrity. As discussed above, miRNAs were previously suggested to target TEs through their well-established function as post-transcriptional regulators.

The RNAi machinery's involvement in transcriptional silencing of DNA and heterochromatin formation is well established throughout the evolutionary tree, primarily through histone post-translational modification, as well as via DNA methylation (reviewed in [43,65,66]). siRNAs were shown to be involved in TGS in many species [67,68]. There is accumulating evidence for RNAi involvement in heterochromatin formation and DNA silencing in mammalian systems as well (reviewed in [69]). piRNA-mediated silencing of retrotransposon genes was shown to be mediated through *de novo* DNA methylation in the mouse germline [70,71]. Mammalian Ago1 (Box 1) was further implicated in TGS through histone 3 Lysine 9 (H3K9) methylation in human cells [72], and Dicer involvement in heterochromatin formation was shown in chicken [73], and in mouse ES cells [41]. Evidence linking siRNAs to DNA silencing showed that promoter DNA methylation, resulting in TGS, could be induced by a complementary siRNA that directed methylation towards the target promoter sequence in human cells [74]. Subsequently, this phenomenon was shown to cause long term DNA methylation and silencing, and to require protein components of the RNAi machinery [75]. Thus the connection between siRNAs and formation of heterochromatin is fairly well established [68,69,76].

However, whether miRNAs can exert TGS similarly to siRNAs is still very much an open question in the field. As guides of the RNAi effector complex to target genes, miRNAs should be indistinguishable from siRNAs, provided that they are also localized to the nucleus in their mature form, evidence for which has been reported [77,78], and that they are complementary to target DNA regions, a requirement obviously fulfilled in the case of TE-derived miRNAs. Recent further evidence support a direct involvement of miRNA in TGS [76]. Khraiwesh *et al.* demonstrated an involvement of miRNAs in regulating DNA methylation and gene silencing in the moss *Physcomitrella patens* [79]. Gonzalez *et al.* further implicated mammalian miRNAs complementary to promoters in transcriptional silencing via H3K9 methylation [80]. And finally, Kim *et al.* showed that mammalian miR-320, transcribed from the *POLR3D* [polymerase (RNA) III (DNA-directed) polypeptide D] promoter region, is involved in transcriptional silencing of *POLR3D* mediated by H3K27 methylation and Ago1 [81]. These examples, as well as others, demonstrate that principles which had been established for one type of small RNA were often later found to be applicable to others, and argue in favor of a renewed evaluation of the miRNA-mediated TGS.

Concluding remarks

We suggest here that miRNAs in general, and particularly TE-originated miRNAs, mediate global repression of TEs in somatic tissues, through both PTGS, and TGS (Figure 1a). We propose that repression of TEs will secure genomic stability in adult cells, whereas global suppression of miRNAs in emerging tumor cells might de-repress TE expression and transposition, and increase genomic instability, that in turn promotes tumorigenicity (Figure 1b and c)

Two complementary lines of evidence lead to the conclusion that cancers are often selected for avoidance of miRNA-mediated regulation. The first includes a variety of genetic studies showing that manipulation of the RNAi pathway, including mainly Dicer knockout, cause genomic instability, as discussed above. The second, complementary line is the observation that tumors have global lower levels of miRNAs than their normal counterparts [10]. Thus, not only that external manipulation leading to global miRNA downregulation results in genomic instability, but such downregulation is indeed observed in real tumors. Bridging between these two observations is a series of studies portraying different underlying mechanisms for global repression of miRNAs in cancer, from widespread methylation to defects in the miRNA biogenesis pathway, further strengthening the notion of global miRNA avoidance in cancer.

miRNAs are the major class of small RNAs expressed in somatic tissues of mammals. The mechanistic evidence for miRNA avoidance in cancer focuses on miRNAs genes, and components related to the miRNA pathway such as Drosha [22,23]. In addition, more than 20% of human miRNAs physically originate from TEs, making them ideal candidates for TE targeting in somatic tissues, in a similar way to the function of piRNAs and endo-siRNAs in the germline. This large amount of TE-originated miRNAs explains the tendency of tumors for global downregulation of miRNAs. Moreover, other miRNAs were also suggested to target TEs, and might also play a role in the defense of genomic stability. It is plausible that miRNAs act as the somatic parallels of piRNAs and endo-siRNAs, serving as 'guardians of the genome' in somatic tissues. Nevertheless, because endo-siRNAs, that are known to originate from TEs in mammalian oocytes, were shown to be expressed in somatic tissues in the fly, and their biogenesis is, at least in part, Dicer-dependent, it is possible that they too participate in the task of maintaining genomic stability in mammalian somatic tissues. Further studies focusing on expression of other forms of small RNAs in the nucleus of mammalian somatic cells will shed light on this question.

The involvement of miRNAs and the RNAi pathway in TE silencing in human somatic cells and its impact on genomic stability and cancer remain to be tested. Nonetheless, this is an appealing hypothesis, especially in light of the involvement of piRNAs and the RNAi pathway in repression of TEs that has been established in germline cells [51]. Mammalian and primate DNA repeats have expanded rapidly in evolution, and it seems that the RNAi pathway is a major part of the host genome's fight to defend itself, as these TEs have served as a cradle for more than a hundred miRNAs in the human genome. This hypothesis postulates a link between tumor evolution and enhanced expression of TEs, which is nowadays testable with the increasing use of advanced high-throughput sequencing methodologies. The extent of TE expression in human tumors versus normal tissues can be tested using high-throughput RNA sequencing approaches. In addition, the state of chromatin near TEs, that is predicted here to be more open in cancer compared to normal cells, can be assayed using high-throughput bisulfite sequencing to examine the DNA methylation patterns near regions of TEs in the genome, and chromatin immunoprecipitation followed by sequencing (ChIP-seq) methods can be used to look at histone modification marks in these regions. In addition, close examination of TE expression and transposition could be performed in cancer models in conjunction with Dicer, Drosha and Ago elimination to determine a causative link between miRNAs, TE derepression, genomic instability, and tumorigenesis.

Finally, the evidence for RNAi involvement in silencing of DNA in other species, and in the mammalian germline, has been reported in the literature for several years. The RNAi machinery plays an important role in the nucleus, regulating processes that have long-term effects on the DNA epigenetic state. Nevertheless, the majority of the studies on miRNA involvement in cancer have considered mainly their activity as post-transcriptional inhibitors. Integrating the proposed involvement of miRNAs in regulation of genomic stability, is essential for a more complete understanding of the roles of miRNAs in the regulation of complex phenotypes, especially in the study of human cancer.

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