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Piwis and Piwi-Interacting RNAs in the Epigenetics of Cancer

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ABSTRACT

An increasing body of evidence suggests that cancer cells acquire "stem-like" epigenetic and signaling characteristics during the tumorigenic process, including global DNA hypo-methylation, gene-specific DNA hyper-methylation, and small RNA deregulation. RNAs have been known to be epigenetic regulators, both in stem cells and in differentiated cells. A novel class of small RNAs, called piwi-interacting RNAs (piRNAs), maintains genome integrity by epigenetically silencing transposons via DNA methylation, especially in germline stem cells. piRNAs interact exclusively with the Piwi family of proteins. The human Piwi ortholog, Hiwi, has been found to be aberrantly expressed in a variety of human cancers and in some, its expression correlates with poor clinical prognosis. However, there has been little investigation into the potential role that Piwi and piRNAs might play in contributing to the "stem-like" epigenetic state of a cancer. This review will highlight the current evidence supporting the importance of Piwi and piRNAs in the epigenetics of cancer and provide a potential model for the role of Piwi and piRNAs in tumorigenesis. J. Cell. Biochem. 113: 373–380, 2012.

KEY WORDS: CANCER; PIRNA; PIWI; STEM CELL; DNA METHYLATION; EPIGENETIC REGULATION

Cancer is often classified as a disease with multiple hallmarks, including mutational genetic alterations, aberrant epigenetic modifications, angiogenesis, and evasion of endogenous surveillance mechanisms [Macaluso et al., 2003]. The epigenetic alterations found in cancer, such as global DNA hypomethylation and gene-specific DNA hypermethylation, result in both expression of oncogenes and repression of tumor suppressor genes. Recent work from many groups suggests cancer cells show epigenetic and signaling pathway similarities with stem cells [Hendrix et al., 2003], allowing cancer cells to share functional similarities with a stem cell, including self-renewal capacity and the ability to give rise to diverse progeny. In this way, cancer cells are able to aberrantly recapitulate a "stem-like" state.

In addition to DNA methylation, both histone modifications and small RNA regulation make up the epigenetic landscape of a cell. It is has been well known for a long time that in plants, yeast, mice, and other model organisms, RNAs are important epigenetic regulators. For a decade, small non-coding RNAs have been implicated as critical mediators of gene expression. Both microRNAs (miRNAs) and short interfering RNA (siRNAs) are thought to exert their silencing effects via post-transcriptional target gene silencing, though there are emerging models of siRNA-based epigenetic chromatin silencing [Verdel et al., 2009]. The ubiquitous presence of both miRNAs and siRNAs in model organisms—from plants to humans—underlies their important role in small RNA-based regulation of gene expression.

Recently a distinct class of small RNAs, called piwi-interacting RNAs (piRNAs), was discovered. piRNAs were first described as Piwi-dependent small RNAs mapping to the Drosophila flamenco locus [Sarot et al., 2004], though at the time they were termed repeat-associated small interfering RNAs (rasiRNAs) [Aravin et al., 2003]. These novel small RNAs were then found to (1) associate with various rodent Piwi orthologs, (2) be longer than either miRNAs or siRNAs, (3) cluster at repeat regions and transposons in the genome, and (4) be testes-specific, all of which suggest that piRNAs are a distinct class of small non-coding RNAs. Because piRNAs have thus far been found to cluster at transposon loci in the genome (in association with Piwi family members), they are thought to be critical for silencing these mobile genetic elements, via DNA methylation, in order to maintain genomic integrity in germline stem cells. However, only ~20% of known mammalian piRNAs map to transposon and other repeat genomic regions [Kim, 2006],

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suggesting that there may be a diverse set of functions for piRNAs. Further supporting that hypothesis, piRNAs have recently been identified outside the germline and in human cancer cells [Lu et al., 2010; Wu et al., 2010; Cheng et al., 2011; Cui et al., 2011].

While piRNAs have been implicated in the epigenetic regulation of germline stem cells, there has been little insight into the role that piRNAs—and Piwi proteins—may play in contributing to the aberrantly "stem-like" state found in cancer. Preliminary work from our group [Siddiqi et al., 2011, unpublished data] finds that the human Piwi ortholog—Hiwi—is expressed in sarcomas and drives tumorigenesis via increased global DNA methylation. This review will focus on the potential role of piRNAs in cancer, provide a mechanistic model for their function in cancer, and offer novel therapeutic avenues to treat cancers with altered piRNA/Piwi expression.

CANCER AS AN ABERRANT STEM CELL-LIKE STATE

The epigenetic landscape is widely distorted in cancer cells, both at specific genes and globally. The epigenetic global alterations found in many cancers include hypomethylation of DNA [Feinberg and Vogelstein, 1983] and hypoacetylation of histones. Along with gene-specific hypermethylation of DNA, these global alterations lead to oncogene activation (R-ras, cyclin D2) [Wilson et al., 2007] and tumor suppressor silencing (RB1, p16) [Baylin, 2005]. Further details of the epigenetic aberrancies in cancer have been reviewed by us [Siddiqi et al., 2010] and others [Tsai and Baylin, 2011]. In addition to changes in DNA methylation and histone marks, many cancers have deregulated expression of small RNAs [Lu et al., 2005].

The exact reasons for the epigenetic aberrancies that are characteristic of cancer cells are still under investigation. However, one theory gaining supportive evidence from studies of both liquid and solid tumors is that cancers reflect the epigenetic state of an adult progenitor cell [Sell, 2006]. Many types of stem cells are present in an organism: embryonic, germline, and adult tissue stem cells, the latter of which are able to mature into various cellular lineages. The epigenetic signature of cancer cells, including the repressive hyper-methylation of DNA, is very similar to that of progenitor cells. DNA methylation in progenitors cells silences differentiation genes, but in cancer, hyper-methylation is found at promoter regions of tumor suppressor genes [Esteller, 2007]. The repressive histone 3 lysine 27 tri-methylation mark, mediated by the EZH2 methyltransferase, is found in stem cells to silence differentiation genes [Simon and Lange, 2008]. However, EZH2 is aberrantly highly expressed in many cancers, including breast and prostate, and EZH2 expression correlates with tumor aggressiveness [Tsang and Cheng, 2011]. This suggests that cancer cells may take on an aberrantly "stem-like" epigenetic state.

Adult tissue stem cells or their immature progenitors can give rise to cancers, upon arrest of their normal maturation program [Sell and Pierce, 1994], or when pathways controlling a "stem-like" state are altered. For example, Oct-4, whose expression is normally restricted to early progenitor cells to prevent cellular differentiation [Pesce and Scholer, 2001], is also highly expressed in germ cell tumors [Cheng, 2004]. Moreover, ectopic over-expression of Oct-4 in adult somatic tissues results in epithelial dysplasia [Hochedlinger et al., 2005]. Additionally, the Wnt signaling pathway, which regulates the ability of B-catenin to drive expression of certain target genes, is critical for self-renewal of progenitor cells. Wnt signaling is known to be deregulated in many cancers including liver hepatocellular carcinomas [Colnot et al., 2004], colorectal carcinomas [Powell et al., 1992], and sarcomas [Matushansky et al., 2007], likely due to epigenetic aberrations rather than genetic mutations [Tsang and Cheng, 2011]. Another pathway important in progenitor cells and, aberrantly, in cancers is Notch signaling. In both embryonic and adult tissue stem cells, Notch regulates proliferation and differentiation [Katoh, 2007]. However, in a cancer context, Notch has been implicated in both growth arrest and apoptosis induction [Wang et al., 2009]. In this way, cancers can be described as having an aberrantly "stem-like" state, based on their transcriptional, epigenetic and signaling pathway alterations.

Much of the work suggesting that cancer cells recapitulate the epigenetic and signaling pathways states of progenitor cells that experience maturation arrest was first established in the hematopoietic system [reviewed in Rattis et al., 2004]. There is considerable evidence that blood cancers such as leukemia and lymphomas are very similar to HSCs that undergo aberrant maturation [reviewed in Dick, 2008], based on phenotypic similarities (i.e., self-renewal capacity) and functional similarities (i.e., clonogenic potential of serially transplanted cancer cells) [Bonnet and Dick, 1997]. Additionally, gene expression profiles of specific liposarcomas subtypes correlate closely with specific points in adipocytic differentiation of mesenchymal stem cells (MSCs) [Matushansky et al., 2008], suggesting a direct relationship between stage of MSC maturation arrest and sarcoma tumor subtype. Taken together, these data suggest that cancer cells have recapitulated a "stem-like" state, likely due to aberrant epigenetic signatures, maturation arrest of the normal progenitor cells and deregulation of signaling pathways that control a cell's "stem-like" state.

STEM CELL EPIGENETICS VERSUS SOMATIC EPIGENETICS

During development, germline, embryonic and adult tissue stem cells/early progenitors differentiate into all the mature cell types of an organism. These differentiation processes are carefully regulated at the epigenetic level, resulting in a stem cell epigenetic landscape that is very different from that of non-stem cells.

In stem cells, most CpG islands in the genome are methylated but remain unmethylated during development and in differentiated cells. However, specific CpG sites acquire methylation only during development—often associated with long-term epigenetic silencing. For example, X-chromosome inactivation and methylation of other imprinted genes is critical for epigenetic repression during development [Ng and Gurdon, 2008]. Importantly, non-CpG sites cover about 40% of the genome, but the role of DNA methylation in regulating expression at these promoters is still poorly understood. For example, the Oct-4 promoter does not contain a CpG island, but

its methylation still regulates Oct-4 expression in stem cells [Feldman et al., 2006]. DNA methylation patterns in stem cells are distinct from those in differentiated cells.

DNA methylation patterns along with histone acetylation and methylation marks provide more comprehensive insight into the diverging epigenetic states of stem cells and somatic cells. Embryonic stem cells are thought to have "bivalent domains" which contain both active (H3K4 tri-methylation) and repressive (H3K27 tri-methylation) histone marks [Collas, 2009]. The presence of both active and repressive marks allows the stem cell to tightly regulate differentiation programs. Differentiated cells lose the bivalency and consequently, lose the increased plasticity that bivalency affords. Differentiated cells have a more rigid chromatin structure—one that is either repressive or active at a given locus—which is critical for faithful replication during cellular expansion. The unique epigenetic state of both stem and somatic cells are critical for the careful regulation of differentiation.

It has long been known that RNAs are also important epigenetic regulators, controlling both long-term cell fate decisions and also short-term gene expression decisions in stem cells and differentiated cells. There are many types of regulatory RNAs, including long non-coding RNA (lncRNA), antisense RNA, siRNA, and piRNA. In plants, fission yeast and worms and vertebrates, siRNAs in the nucleus form an RNAi-induced transcriptional silencing (RITS) complex which binds target chromatin, resulting in repressive chromatin modifications. Also, in plants, a process called RNA-directed DNA methylation uses siRNAs to increase site-specific de novo DNA methylation during differentiation [Verdel et al., 2009].

The role of the lncRNA, Xist in X chromosome inactivation is a classical example of the importance of RNAs in epigenetic regulation in mammals. Xist is expressed weakly in embryonic stem cells and then is highly up-regulated during differentiation. This results in the Xist RNA coating one of the X chromosomes, inactivating it. In an additional layer of epigenetic regulation, Xist's antisense RNA, Tsix, negatively regulates Xist by repressive marking of the Xist promoter [reviewed in Navarro and Avner, 2010]. While many RNAs have been implicated as epigenetic regulators across a variety of model organisms, piRNAs have remained generally understudied. Even in male germline stem cells, where piRNAs are best understood, the picture is only emerging of piRNAs (and their associated Piwi proteins) in mediating transposon silencing via DNA methylation.

piRNAs AND STEM CELL EPIGENETICS

A large focus of piRNA research has been dedicated to elucidating their role in germline stem cells. piRNAs were isolated from rodent testes by four different groups simultaneously [Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Watanabe et al., 2006], suggesting that this novel class of small RNAs were specific to male germline stem cells. Additionally, the zebrafish Piwi ortholog, Ziwi, is expressed exclusively in the gonad [Tan et al., 2002], further suggesting that the piRNA-Piwi pathway is specific to the germline. Analysis of mouse oocytes revealed no small RNAs that correspond to piRNAs, further strengthening the assertion that piRNAs played a specific role in the male germline.

The questions of piRNA function in all tissues in which they have been identified remain open, although work from germline stem cells in both Drosophila and mouse models partially answer these questions. Most piRNAs are not complementary to the mRNA of potential target genes (unlike miRNAs), further supporting the hypothesis that piRNAs are more likely to be involved in epigenetic regulation rather than post-transcriptional regulation [Kim, 2006]. While many mechanistic details remain unclear, there is mounting evidence that piRNAs (and Piwi family proteins) mediate transposon silencing via epigenetic, rather than post-transcriptional mechanisms.

Work in Drosophila implicates piRNAs in using epigenetic mechanisms to exert their gene expression effects. The piRNA-Piwi pathway is epigenetically regulated in Drosophila [Yin and Lin, 2007]: piRNAs have been identified at heterochromatic regions and heterochromatin protein 1a (HP1a) was found to bind to piRNA and Piwi. This work implies that piRNA-Piwi pathway components may form a piRNA complex on chromatin that includes other chromatin modifying agents such as HP1a.

Though mechanistic insights into the function of piRNAs in the germline are limited, work from mouse models of Miwi and Mili (mouse Piwi orthologs) deficiency find that expression of these Piwi orthologs is necessary for spermatogenesis [Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004]. Upon knock out of Mili or Miwi, transposon expression is activated [Kuramochi-Miyagawa et al., 2008]. Moreover, de novo methylation of IAP and Line1 transposon region is reduced. Additionally, Mili and Miwi null testes were sequenced to examine changes in piRNA populations. Indeed, piRNAs were dramatically reduced upon Mili knock out, while the miRNA population remained unchanged. These data implicate de novo methylation as an important epigenetic mechanism of the piRNA-Piwi pathway to maintain transposon silencing.

In a complementary study, loss of murine Piwi ortholog Miwi2 in germ stem cells results in increased transposon expression due to a loss of DNA methylation at transposon sites [Carmell et al., 2007]. This epigenetic methylation mark has been further implicated as a mechanism for piRNA-mediated transposon silencing in order to maintain genome integrity: Mili and Miwi2 are required for de novo methylation of transposons in the murine testis [Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008], thus showing their importance in establishing rather than maintaining DNA methylation patterns. It is possible that piRNAs might play a role as guide for DNA methylation machinery to transposons. Testes from mice mutant for DNMT3L, a DNA methyltransferase enzyme which regulates de novo methylation, still have a piRNA population. This suggests that not only are Mili and Miwi2 required for de novo methylation of transposon sites, but they also act upstream of DNMT3L [Aravin et al., 2008]. These data are highly suggestive of the importance of epigenetic regulation—rather than post-transcriptional regulation—of transposon silencing in order to maintain genomic integrity of the germline.

Interestingly, piRNAs identified in the male germline seem to fall into two categories: those that associate with Mili during early spermatogenesis, and those that associate with Miwi during late

spermatogenesis. Mili-associated piRNAs are typically 3–6 nucleotides shorter than those that associate with Miwi. These data suggest that these two groups of piRNAs might play distinct roles in the germline, although more detailed aspects of these roles are still being investigated.

Given the evidence that Piwis mediate transposon silencing via DNA methylation, it is reasonable to expect Piwi localization to the nucleus. However, many groups have identified cytoplasmic Hiwi expression [Liu et al., 2006; Wilczynska et al., 2009; Beyret and Lin, 2011]. This incongruity suggests that Piwis (and their corresponding piRNAs) might be shuttled into the nucleus in a carefully regulated manner. A recent review by Siomi and coworkers [Ishizu et al., 2011] suggest a model for Drosophila in which mature piRNAs complex with Piwis in the cytoplasm and this Piwi-piRNA complex is transported into the nucleus to carry out its epigenetic silencing functions. Because there is a high degree of homology of Piwis from different organisms, it's likely that this model might be more widely applicable.

The germline restriction of piRNAs has recently been called into question by a growing body of evidence examining piRNAs in various cell types. piRNAs have been identified outside germ stem cells, for example, in somatic cells of the Drosophila ovary [Haase et al., 2010]. A recent study also found piRNAs in neurons of the mouse central nervous system [Lee et al., 2011]. The authors found that piRNA inhibition results in a decrease in dendritic spine formation, a key measure of synaptic function. A wide-ranging study by Yan et al. [2011] identified piRNA-like molecules in various fly, mouse and macaque somatic tissues, including pancreas and cortex. While these piRNA-like molecules were comparable to piRNAs in most criteria (length, distribution) and Piwi family proteins were identified in the examined tissues, a direct association of these small RNAs with Piwi family proteins was not shown. Taken together, these data suggest that piRNA populations may exist in a variety of somatic tissues.

A piRNA population has also been identified in HeLa cells [Lu et al., 2010], an ovarian cancer-derived cell line. Although the piRNA population in HeLa cell is much smaller compared to that found in murine germline stem cells, it is still localized to the periphery of the nucleus, consistent with previous studies [Klattenhoff and Theurkauf, 2008]. Moreover, Wu et al. [2010] recently identified a piRNA population in murine MSCs. These piRNAs associate with Mili in the cytoplasm of MSCs and are thought to be important for keeping cellular proliferation in check. Importantly, Mili expression was examined by immunoprecipitation and Western blot using the same Mili antibody, suggesting that actual Mili expression is much lower than the enriched band shown. There was no evidence that other murine Piwi orthologs are present in MSCs. Additionally work from our group has shown that Mili, Miwi, and Miwi2 (all three murine Piwi orthologs) are not readily detectable by RT-PCR from murine MSCs (unpublished data). Together, these data suggest that, while piRNAs may be present in a variety of stem cell and somatic cell types, Piwi protein expression is much more limited, as it is found only in the germline during normal development.

Furthermore, piRNAs have recently been found in adult human brain and testes samples [Esposito et al., 2011]. Intriguingly, a specific piRNA, piR_015520 has been identified in the human melatonin receptor 1A gene and exogenous expression of this piRNA is able to downregulate melatonin receptor 1A gene transcript levels. This is the first report of piRNA-mediated regulation of a protein-coding gene, rather than a transposon repeat element. Much more work must be done to determine additional piRNAs which correspond to gene sites and regulate gene expression, but these data suggest that piRNAs may be regulating gene expression (not only transposon expression) outside the germline and in human cells, suggesting that piRNAs regulate gene expression more broadly that previously thought.

Despite initial evidence that piRNA expression was restricted to the male germline, piRNAs continue to be identified outside of germline stem cells, including in mesenchymal stem cells. These data open the door for additional studies which identify piRNAs in a variety of cell types and would suggest roles for piRNAs that are more universal than previously expected.

Importantly, the potential epigenetic DNA methylation marks that have been the crux of piRNA mechanistic function in germ stem cells have not yet been explored in non-germline stem cells or other somatic cell types. Work from our group [Siddiqi et al., 2011, submitted] finds that Hiwi (human Piwi ortholog) expression in sarcoma progenitor cells (MSCs) results in increased global DNA methylation levels and decreased transposon expression. Interestingly, this DNA methylation increase occurs at non-CpG sites. Further studies must be done to further characterize the exact epigenetic mechanisms underlying piRNA-Piwi pathway mediated transposon silencing. piRNA-Piwi pathway components play a crucial role in the development of germline stem cells by epigenetically silencing transposons in order to maintain genome integrity; whether piRNA-Piwi pathway components play similar roles outside the germline remain unexplored.

The presence of piRNAs has been well-established in germline stem cells. Although they have only recently been identified in cancers, it is possible that the piRNAs that mediate transposon silencing during normal germline differentiation are hijacked in cancer cells to silence other parts of the genome, resulting in a tumorigenic state.

A ROLE FOR piRNAs AND PIWIS IN THE EPIGENETICS OF CANCER

Much work has been undertaken to elucidate the role of small RNAs in cancer. miRNAs and their gene targets have been characterized in a variety of cancers, including breast [Corcoran et al., 2011], prostate [Coppola et al., 2010], and liver [Huang and He, 2011] cancers. However, piRNAs are understudied in cancers and until recently were thought to be entirely absent from cancer cells. Determining the roles of piRNAs in cancers is becoming increasingly important to fully understand the cellular changes that occur during cancer.

While Piwi family members are normally expressed in germline stem cells, their expression is lost during normal development. A growing number studies have found human Piwi orthologs—called Hiwi and Hili—to be over-expressed in a variety of human cancers [Liu et al., 2006; Grochola et al., 2008; He et al., 2009; Jiang et al.,

2011; Sun et al., 2011], including cancers originating from the germline [testicular seminomas; Qiao et al., 2002] and those originating from MSCs [sarcomas; Taubert et al., 2007]. These reports suggest that in a tumorigenic state, a cell's piRNA-Piwi pathway components are altered.

piRNAs have not been thoroughly studied in cancers, however a few preliminary studies suggest that piRNAs are altered in cancers. A specific piRNA, piRNA 651, has been shown to be aberrantly over-expressed in multiple cancers, including gastric, colon, lung, and breast cancer tissues, when compared with normal tissues [Cheng et al., 2011]. Furthermore, inhibition of this piRNA in gastric cancer cell lines (another cancer in which Hiwi is over-expressed [Liu et al., 2006]) decreased cellular growth. While the growth inhibition effect of the piRNA antagonist was modest, these data are suggestive of an important potential role for aberrant piRNA expression in contributing to over-proliferation in cancer cells.

piRNAs were further investigated in gastric cancer by their detection in peripheral blood from patients [Cui et al., 2011]. Two specific piRNAs, one of which was piRNA 651, were chosen for evaluation and both were significantly lower in peripheral blood from gastric cancer patients, compared to healthy controls. This report suggests that piRNA detection might be a valid biomarker for identifying circulating gastric cancer cells in the blood. While these data may seem contradictory to the Liu et al. [2006], report which finds high piRNA 651 levels in cancer tissues, it is important to note that these are the first studies to examine piRNA expression levels in cancer and much more investigation is necessary, both to validate these studies and to identify piRNA expression in additional cancer types. Importantly, these studies did not provide evidence for a general alteration of the piRNA population in cancer versus normal cells; rather they focused on one of two specific piRNAs which may or may not be indicative of the entire piRNA population. Further, because no mechanistic insight into the role of piRNAs in cancer has been explored, it is not known if piRNAs might be driving tumorigenesis or if their alteration is merely a bystander effect that is part of the cell's stem cell recapitulation process.

The discovery of piRNAs in HeLa cells [Lu et al., 2010] is an important contribution to the body of work implicating piRNAs in cancer cells because many groups use HeLa cells as a tool to study their field of interest. Piwi-piRNA pathway components have been further implicated in determining the makeup of the piRNA population in HeLa cells: Hili over-expression results in decreased Line1 transposon expression and decreased expression of piRNAs that are derived from Line1 repeat sequences. Importantly, the overall number of piRNAs did not change significantly when Hili was expressed. These data suggest that piRNA populations may be modulated in response to expression of Piwi family proteins. However, the exact role of piRNAs in cancer, and the mechanism by which they may be performing that role, remains largely unexplored both in cancer cell lines and in cancer tissues.

While the role of piRNAs in cancer is only just beginning to be investigated, it is possible to develop a model for Piwis and piRNAs outside the germline, in a cancer context. Based on data from a variety of model organisms where piRNA populations have been identified outside the germline, there may be a much wider expanse of piRNAs across a variety of cell types, including both normal and cancer cells. A well-defined aspect of piRNAs is their association with Piwi family members, which have been found at high levels both in normal germline stem cells and in all cancers examined so far. This opens the possibility that piRNA populations are present in many cell types, but are only able to carry out their epigenetic silencing effects when there is also high expression of Piwi orthologs, such as in normal germline stem cells and in cancer cells. In this way, piRNAs, along with abundant Piwi expression, in germline stem cells result in normal transposon silencing during spermatogenesis via DNA methylation (Fig. 1A) and develop normally into somatic tissues in which Piwis are absent (Fig. 1B). However, in a cancer context—where Piwi orthologs are highly expressed—the presence of both Piwi and piRNAs results in aberrant DNA methylation and over-silencing of genomic regions (perhaps including regions of tumor suppressor genes), which results in an aberrantly "stem-like" state and consequent tumorigenesis (Fig. 1C).

In support of this model, direct evidence from our group [Siddiqi et al., 2011, submitted] finds that Hiwi plays an important role as a driver of tumorigenesis by increasing DNA methylation levels and consequent cyclin-dependent kinase inhibitor (CDKI) silencing. Moreover, both tumor formation and DNA methylation levels are reduced upon treatment with a DNA methyltransferase inhibitor. Our studies reveal not only a novel oncogenic role for Hiwi as a driver of tumorigenesis, but also suggest that the use of epigenetic agents may be clinically beneficial for treatment of tumors that express Hiwi. Additionally, these data show that Hiwi-mediated methylation results in DNA hyper-methylation with subsequent genetic and epigenetic changes that recapitulate an aberrant stem-like state and tumorigenesis. In this way, Hiwi may act appropriately to promote genomic integrity during early development (via transposon silencing) and inappropriately in adult tissues with subsequent tumorigenesis. There is considerably less evidence for the exact role of piRNAs in this model of tumorigenesis. However, data from many labs reveal that piRNAs are critical transposon silencing via DNA methylation in a normal developmental context and emerging evidence suggests the piRNAs may play a similar epigenetic silencing role in a cancer context as well.

In order for this model to be fully tested, many details still need to be determined. While Piwi orthologs have been found to be highly expressed in a variety of cancer types, the presence or absence of piRNAs in a variety of cancers has not been fully explored. The potential aberrant over-silencing of genomic regions (beyond transposons) which may occur in a cancer context must also be proven. Moreover, an extensive characterization of the methylation changes that occur in cancers with high piRNA-Piwi pathway components have yet to be elucidated. Work from our group [Siddiqi et al., 2011, submitted], finds that Hiwi is over-expressed in sarcomas and that Hiwi expression is a driver of tumorigenesis via aberrant DNA hyper-methylation and is not a passive bystander effect. Additional epigenetic marks, such as histone methylation and histone acetylation, which may occur aberrantly in cancers with piRNA populations, have also not been examined. Developing a complete picture of the relationship between piRNAs, Piwi orthologs, and epigenetic marks will give important mechanistic clues into the function of piRNAs in cancer.

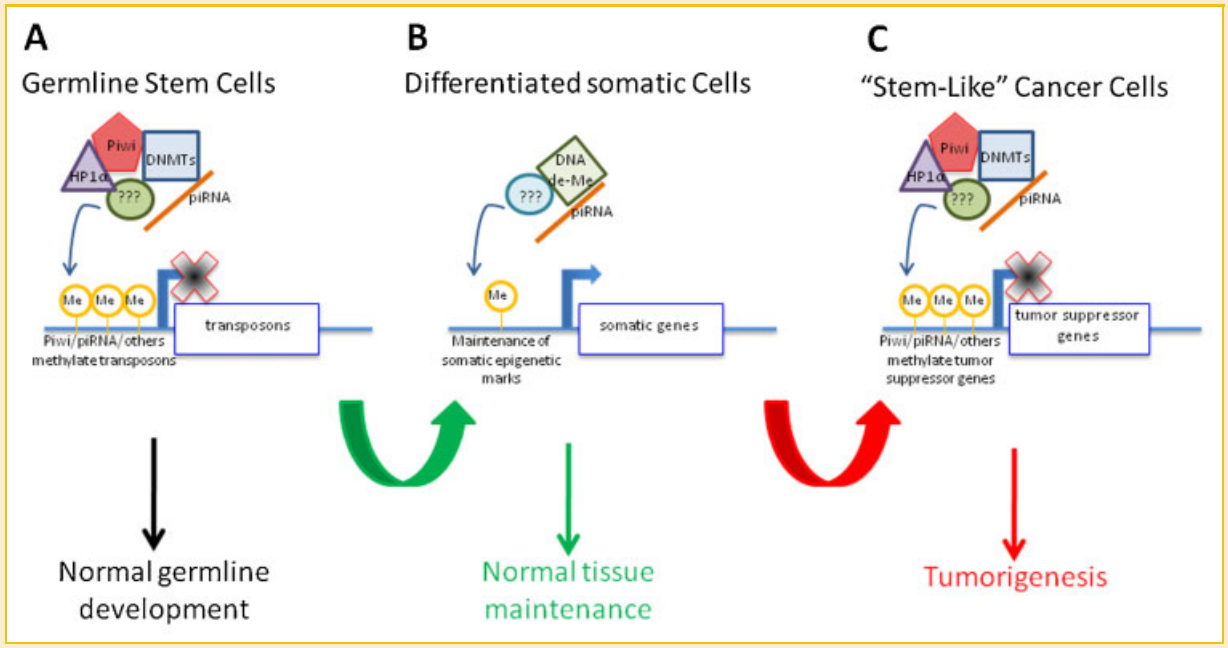

Fig. 1. Proposed model for Piwi and piRNA function in normal development and in cancer. A: In germline stem cells, the presence of piRNAs and high Piwi levels, along with other chromatin modifying agents, results in DNA methylation to silence transposons. B: During normal development (green arrow), Piwis are absent and the epigenetic marks of differentiated somatic cells allow for transcription of genes that maintain cells in a differentiated state, including reduced DNA methylation (C) piRNAs may be present in a variety of cell types. However, when Piwi orthologs are highly expressed, there is aberrant over-methylation and over-silencing of the genome, perhaps including tumor suppressor genes. This results in aberrant "stem like" epigenetic state (red arrow) and consequent tumorigenesis. For simplicity, only DNA methylation changes are shown. See text for further details.

miRNA profiles in healthy and tumor samples have been extensively characterized, resulting in increased interest in miRNAs as diagnostic tools. Additionally, miRNA inhibitor molecules and siRNA-based therapeutics are currently being developed as potential therapeutic avenues [reviewed in Heneghan et al., 2010]. However, piRNAs have not been explored as a therapeutic target. An exact elucidation of aberrant piRNA function in cancer may lead to the development of piRNA-based rational therapies against this disease. Taken together, the presence of piRNAs and Piwi orthologs in cancers suggests a potentially crucial therapeutic avenue in which the antisense piRNAs or a Piwi-inhibitor is delivered to cancer cells in order to mitigate their silencing effects. Since piRNA-Piwi pathway components are known to epigenetically silence transposons in a normal developmental context and may aberrantly over-silence the genome in a cancer context, treatment of tumors with epigenetic modifying agents might prove beneficial. For example, DNA methyltransferase inhibitors, such as 5-azacytidine, may be particularly useful in treating tumors which have high levels of piRNA-Piwi pathway components [such as Hiwi, as described in Siddiqi et al., 2011, submitted]. In a related potential therapeutic avenue, epigenetic modifying agents may also be used in conjunction with piRNA-Piwi pathway inhibitors to decrease the aberrant increased DNA methylation and over-silencing of the genome. While these are intriguing potential therapeutic applications of piRNA-Piwi pathway inhibitors, much work must still be done to elucidate the exact role of Piwis and piRNAs in cancers and the mechanisms used to play those roles.

FUTURE PERSPECTIVES

piRNAs have been discovered and characterized within the past decade, but already much work on their roles in germline stem cells and development has been undertaken. piRNAs are thought to be crucial for maintaining genomic integrity in the male germline by silencing transposons via DNA methylation. Emerging work from a variety of model organisms suggests that piRNA populations are present in many more cell types than previously thought. Moreover, piRNA populations have been identified in HeLa and gastric cancer cells and in colon, breast, and lung cancer tissues. While their exact role in cancers remains to be determined, there is evidence from a variety of human cancers that Piwi orthologs are highly expressed. These data suggest that perhaps piRNA-Piwi pathway components are aberrantly expressed in cancers and results in aberrant silencing of the genome in a cancer context.

In order to more completely understand the role of piRNAs in cancer, it is crucial to identify additional protein components of the piRNA-Piwi pathway, including any DNA methyltransferases (DNMTs) which may be recruited to methylate the genome and other chromatin modifiers. Current mechanistic insights into piRNA-mediated DNA methylation remain limited. The exact epigenetic mechanism by which piRNA-Piwi pathway components carry out their silencing effect remains to be explored, including the potential for other chromatin modifying marks, such as histone acetylation, to play a role as well. Additionally, the identification of gene targets of piRNA-mediated silencing will be important for determining the exact role of piRNAs—both in a developmental and

in a cancer context. While current evidence suggests that piRNAs target transposon silencing in normal germline development, this silencing may be expanded to additional genomic regions upon aberrant Piwi ortholog expression in a cancer context.

The discovery of piRNAs in multiple cancer tissues and cells opens up the intriguing possibility that piRNA/Piwi may be potential targets for therapeutics against the disease. Because Hiwi is not expressed normally in adult tissues, it is an attractive therapeutic target. Though the exact role of piRNAs in a cancer context must be further characterized, piRNAs may potentially be used as a viable diagnostic biomarker and their inhibitors might be effective therapies for certain cancers.

The emerging data implicating piRNA populations in a variety of cancer types, along with the discovery of high levels of Piwi orthologs in all cancers examined, suggest that piRNAs may play an important role in the epigenetics of cancer. Upon further elucidation of the exact roles and mechanisms of piRNAs in cancer, these novel small RNAs may be recognized as crucial molecules that further contribute to cancer progression.

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