MicroRNAs, Cellular Behavior, and Endometrial Cancer

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Abstract

Endometrial cancer is the most common gynecological cancer and the fourth most common cancer in women worldwide. In spite of its' relative importance to overall morbidity among women, molecular research in endometrial cancers lags far behind other cancers such as breast and colorectal. Nowhere is this more true than in research in the newly emergent field of post-transcriptional gene regulation by small, regulatory RNAs, primarily microRNAs (miRNAs). Here, structure, biogenesis, and mode of action of miRNAs are presented along with a brief overview of the role of miRNAs in carcinogenesis and a review of the few miRNA studies in endometrial cancer carried out to date.

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Introduction

In a now classic review paper, Hanahan and Weinberg[1] outlined what they considered to be the hallmarks of cancer. These hallmarks, that are acquired by cancer cells, are 1. self-sufficiency in growth signals, the ability of cancer cells to promote their own growth, 2. insensitivity to anti-growth signals, the ability of cancer cells to ignore homeostatic signals, 3. evasion of apoptosis, the ability of cancer cells to ignore programmed cell death, 4. limitless replicative potential, the ability of cancer cells to grow unchecked, 5. sustained angiogenesis, the ability of cancer cells to supply themselves with resources such as oxygen and nutrients, and 6. tissue invasion and metastasis, the ability of cancer cells to colonize other regions of the body. Not all cancer cells will acquire these characteristics at the same rate nor will they acquire them in the same order nor will they acquire them by the same mechanisms thus making cancers a very heterogenous group of diseases with individual and quite variable characteristics. However, from the list of acquired

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characteristics presented above it is abundantly clear that the one thing that cancer cells do have in common from the very beginning is that they do not behave the same way that non-cancer cells do.

Normal cells, whether they be skin cells, neurons, kidney, or uterus, maintain their individual integrity, respond to their micro- and macro-environments, and interact with other cells according to a set of internally and externally driven programs designed to sustain equilibria that were established over more than three billion years of evolution. Cancer cells seem not to obey many of these rules, they create their own set of programs that ignore the established principles of cellular response and interaction. They are, in essence, an invasive species for which the equilibria of the environment in which they find themselves are irrelevant. Yet, cancer cells are not aliens that dropped in from an alternate universe. Indeed, cancer cells are ultimately all descended from perfectly normal cells that obeyed all of the rules. Thus, the rules that cancer cells follow are not written *de novo* but are, rather, *re-written* from the normal rules.

The rules of cellular behavior are enforced by a single primary agency. Most of the actions and reactions carried out in any cell are implemented by proteins. Cellular proteins are present in the cells in a bewildering array of sizes, functions, and amounts yet all of these proteins ultimately arise from the primary messages carried in the genes. The DNA sequences that encode the proteins are first transcribed into an RNA message which is, in turn, translated into the specific amino acid sequence of a protein and this is done in a carefully managed amount necessary for the cell to function. The checks and balances on the amounts of the various proteins required for a specific cell to function reside at three levels. At the DNA level there are elegant feed back systems that determine the time, the place, and the amount of transcription of genomic DNA into RNA. At the protein level there is an equally elegant system in which the primary amino acid sequences of proteins are processed into mature proteins able to carry out their specific functions. This is accompanied by cell- and tissue-specific mechanisms for protein transport as well as protein-protein and protein-RNA interactions. Until recently, it was believed that these two levels of control were all that the cell had at its disposal. In the past few years we have become aware of a level of control that lies in between the two we knew. This began in 1993 in the laboratory of Victor Ambros with the chance discovery in the tiny worm *Caenorhabtidis elegans* of a very short RNA sequence that, instead of coding for a protein, was capable of regulating the translation of the messenger RNA of another gene into its protein. This was a completely unknown level of control, now called post-transcriptional gene regulation, which has been found to be a ubiquitous and powerful mechanism throughout both the animal and plant kingdoms. In human and other animal cells, the

primary agent of this post-transcriptional gene regulation is the microRNA (miRNA). Here, we present the structure, biosynthesis, and mechanism of action of miRNAs, discuss the current level of understanding of the role of miRNA-directed gene regulation in cancer, and, finally, the status of miRNA research in endometrial cancers.

miRNA structure, biosynthesis, and mode of action

The discovery of miRNAs and the subsequent discovery of other small regulatory RNAs represent an entirely new arena in functional genomics involving the regulation of gene expression following DNA to RNA transcription. Since the first miRNA was identified in 1993[2,3], thousands of these tiny (21-24nt) regulatory elements have been identified throughout the plant and animal kingdoms as well as in a number of viruses[4]. Recognition of the role of small regulatory RNAs in post-transcriptionally regulating genes involved in a host of cellular processes, including cellular differentiation, development, and apoptosis as well as pathogenesis, continues to expand[5,6]. One important aspect of this growing recognition of the role of small regulatory RNAs in cellular processes is that they themselves are precisely regulated through as yet unknown processes. It is clear, however, that small regulatory RNA expression varies enormously by tissue type, developmental stage, and by factors such as disease and external environmental stressors. Thus, the small RNA profile of any cell in any species at any particular stage of development or circumstance cannot be predicted but, rather, must be empirically determined. Moreover, it has become apparent that one miRNA can regulate the translation of many genes, that any one gene can be post-transcriptionally regulated by more than one miRNA, and that the specific mechanisms of miRNA/target mRNA interactions are complex well beyond simple antisense base-pair binding[7-9].

MicroRNA biogenesis (figure 1) begins with the RNA polymerase II directed production of a primary RNA transcript (pri-miRNA) that can be several thousands of nucleotides in length and be either intragenic, lying in the DNA between genes, or intronic, lying in the non-coding DNA within genes. Primary miRNA transcripts are capped and polyadenylated in the same way as protein coding mRNA transcripts[10]. The pri-miRNA transcript contains within it one (usually) or more (rarely) sequences that form characteristic thermodynamically stable hairpin structures. This hairpin structure, called the precursor or pre-miRNA, typically ranges in size from 60nt to 110nt (figure 1). These hairpin structures induce the formation of an RNA-protein complex, composed of the RNA hairpin, the RNase III endonuclease DROSHA, and a double-stranded RNA binding protein partner which in mammals is called DGCR8 [11]. The latter protein binds the pre-miRNA and DROSHA excises it from the primary transcript. The remaining RNA in the pri-miRNA transcript is

simply recycled in the nucleus. Once excised, the pre-miRNA is handed off to another nuclear protein, a Ran transport receptor protein family member called Exportin 5, which binds the hairpin and transports it from the nucleus to the cytoplasm[12].

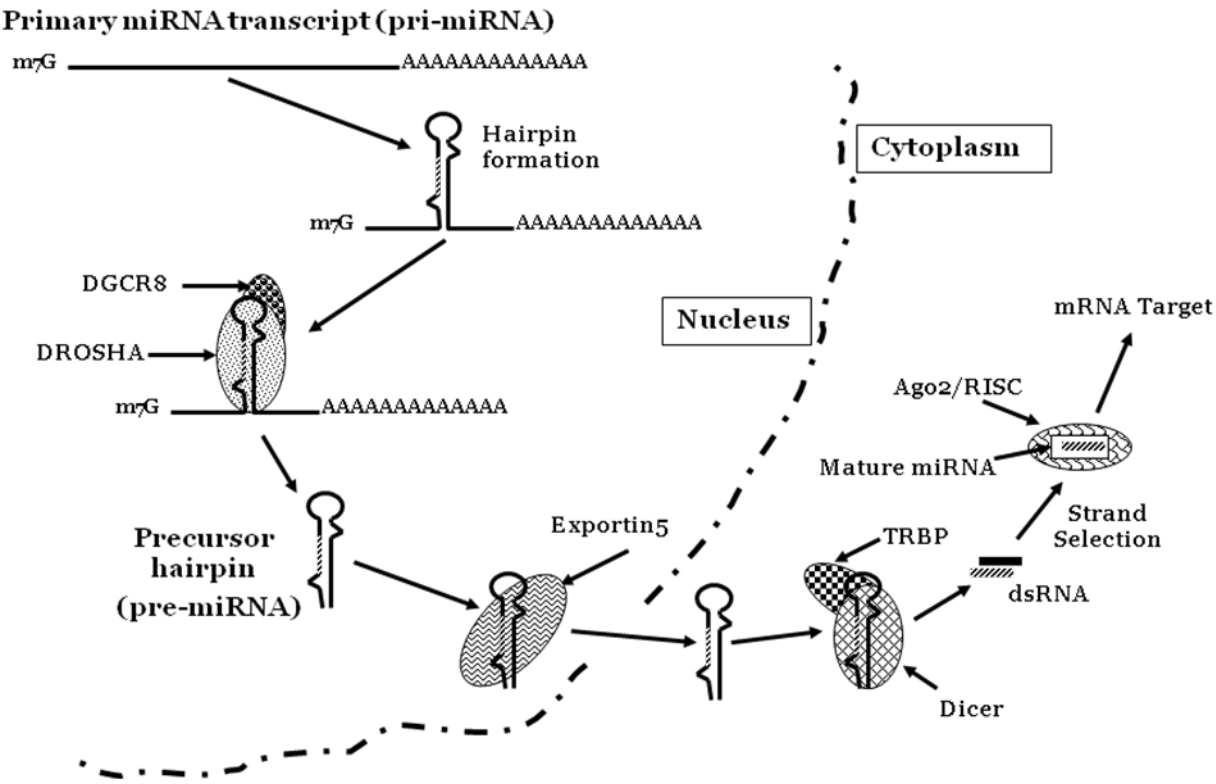

Figure 1. The pathway of microRNA biogenesis. A primary RNA transcript is generated by RNA polII which then capped and polyadenylated (pri-miRNA). Within this transcript a thermodynamically stable hairpin structure is formed (pre-miRNA). The hairpin structure is excised from the primary transcript through the combined action of the enzyme DROSHA and its partner DGCR8. This hairpin is passed off to a transport protein, Exportin 5, which ferries the hairpin from the nucleus to the cytoplasm. Once in the cytoplasm, the hairpin is bound by the enzyme Dicer and its partner TRBP which excises the small double-stranded RNA containing the mature miRNA and the star sequence. This is then handed off to an Argonaute protein, Ago2, which selects the mature miRNA strand and packages it into the RNA-induced silencing complex, RISC, for transport to its eventual mRNA target.

In the cytoplasm the pre-miRNA hairpin is recognized as a double-stranded RNA which induces a second RNase III endonuclease, called Dicer, which binds to the hairpin in association with another protein called TRBP (TAR RNA binding protein) and cleaves it in a precise manner to produce a small (21nt – 23nt) double-stranded molecule which is composed of the mature miRNA effector and its complement called the star, or miRNA*, sequence[13,14]. Conventional nomenclature refers to a microRNA as *miR*. This is preceded by the species from which it comes, for example *Hsa* for *Homo sapiens*, *Mmu* for the mouse species *Mus musculus*, and *Cfa* for the dog

Canis familiaris. It is then followed by a number that is assigned by the curatorial miRNA database, miRBase, at the University of Manchester in the United Kingdom[4]. Many miRNAs are found in multiple species and some are quite ancient so such a nomenclature avoids a great deal of confusion. In practice, however, most miRNA are simply referred to as *miR-xxx* and they will be in this paper with the understanding that, for example, the designation *miR-21* or *miR-200c* actually refers to *hsa-miR-21* or *hsa-miR-200c*.

The double-stranded RNA generated by Dicer/TRBP is nearly always imperfectly complementary, with one or more mismatched bases being the rule, and displays a 2nt 3' overhang on both strands (figure 1). This double-stranded molecule is then handed off to an Argonaute protein, called AGO2 in mammals, which is the primary component of a ribonucleoprotein complex called RISC (RNA induced silencing complex). In RISC the AGO2 protein selects the mature miRNA strand and cleaves the star strand. At this point, RISC transports the mature miRNA sequence to its target mRNA. Mature miRNAs bind in an antisense orientation to one or more locations in the 3' UTR of the mRNA where the result is either to suppress (usually) or eliminate (rarely) mRNA translation into an amino acid sequence. Complementarity of the mature miRNA sequence to its mRNA target sequence is nearly always imperfect. Indeed, it is believed that the mismatches in the miRNA/mRNA complementary sequences are necessary. There is one piece of the miRNA sequence that is quite specific, however. The 8nt RNA sequence from position two through position nine from the 5' end is called the seed and it is the seed that principally determines recognition of the miRNA and its mRNA target[5,8]. It is through this mechanism that cells can regulate protein production even if the original DNA encoding that protein is fully transcribed into RNA.

miRNAs and Cancer: Oncogenes and Suppressors

The rapidly expanding role of miRNAs in cancer has been documented in a number of excellent recent reviews[15-17]. What is clear from these and other reviews is that miRNAs can function as both tumor suppressors and as oncogenes depending upon their targets. There are currently more than 700 human microRNAs archived in miRBase. Of these, only a handful have yet to be fully evaluated for their potential involvement in cancer. However, Calin et al.[18] showed that more than half of the then known human miRNAs were located in cancer-associated genome regions or in fragile sites. Over the past few years, a number of specific human miRNAs have been shown to be differentially expressed in specific cancers. For example, Ciafre et al.[19] showed that several miRNAs (notably *miR-21*, *miR-221*, and *miR-181*) are significantly over- or under-expressed in primary glioblastoma. Other examples include differential expression of *miR-15* and *miR-16* in CLL[20], a number of miRNAs in

hepatocellular carcinoma[21], *let-7* and the *miR-17-92* cluster in lung cancer[22,23], *miR-155* and the *miR-17-92* cluster in lymphomas[24,25]; a large number of miRNAs in clear cell renal cell cancers[26] and numerous miRNAs in GI cancer[27].

A very nice compilation of the most recent findings regarding specific miRNAs was presented by Spizzo et al.[28]. They note that the functional consequences of substantial changes in the expression of miRNAs, either up or down relative to non-cancer cells, are only just starting to come to light. Overexpression of the miRNA *miR-21* can induce self-sustaining growth signals in cells, the first hallmark of cancer proposed by Hanahan and Weinberg[1] as discussed above. Insensitivity to anti-growth signals, the second hallmark of cancer, has been achieved through inhibition of E2F transcription factors in response to overexpression of the *miR-17* miRNA cluster on human chromosome 13q31.1 as well as through overexpression of the *miR-106b-25* cluster located on human chromosome 7q22.1. Spizzo et al. note that specific E2F growth factors are inhibited by specific members of these miRNA clusters, for example E2F2 and E2F3 are inhibited by *miR-20a*, a member of the *miR-17* cluster, and expression of E2F1 is inhibited by both *miR-17-5p* and *miR-20a* from the *miR-17* cluster and by *miR-106b* and *miR-92* of the *miR-106b* cluster. Alteration of apoptosis pathways potentially leading to evasion can be achieved through miRNAs targeting either anti-apoptotic proteins, such as BCL2 and MCL1 by the *miR-15/16* cluster, or pro-apoptotic proteins, such as TP53BP1 by *miR-155*. Unrestricted proliferation can be induced through down-regulation of *miR-138* which leads to repressed translation of telomerase reverse transcriptase or TERT. Sustained angiogenesis is well known to be directly caused by hypoxia and the hypoxia-inducible factor, HIF-1, which, in turn, activates several miRNAs including *miR-30b*, *miR-93*, *miR-181b*, and, in particular, *miR-210*.

As noted, whether or not a specific miRNA acts as a suppressor or as an oncogene relates to the target, or targets, of that miRNA. As a consequence of target specificity and the regulatory mechanisms involved, a miRNA can be an oncogene in one tumor and a tumor suppressor in another[28]. Two genes, HER2 and HER3, significantly associated with breast cancer survival, are suppressed by one of the human *miR-125* family[29] but both *miR-125a* and *miR-125b* target other genes where their effects are likely to be oncogenic. The previously mentioned *miR-17-92* polycistronic miRNA cluster on human chromosome 13q31.3 has been implicated as oncogenic in a number of cancers[30] but *miR-17* has an anti-tumorigenic effect in breast cancer. Other such Jekyll and Hyde miRNAs include members of the *let-7* family and the *miR-*29 family. Clearly, the role of miRNAs in cancer is and will continue to be diverse and as unpredictable as the genes they target.

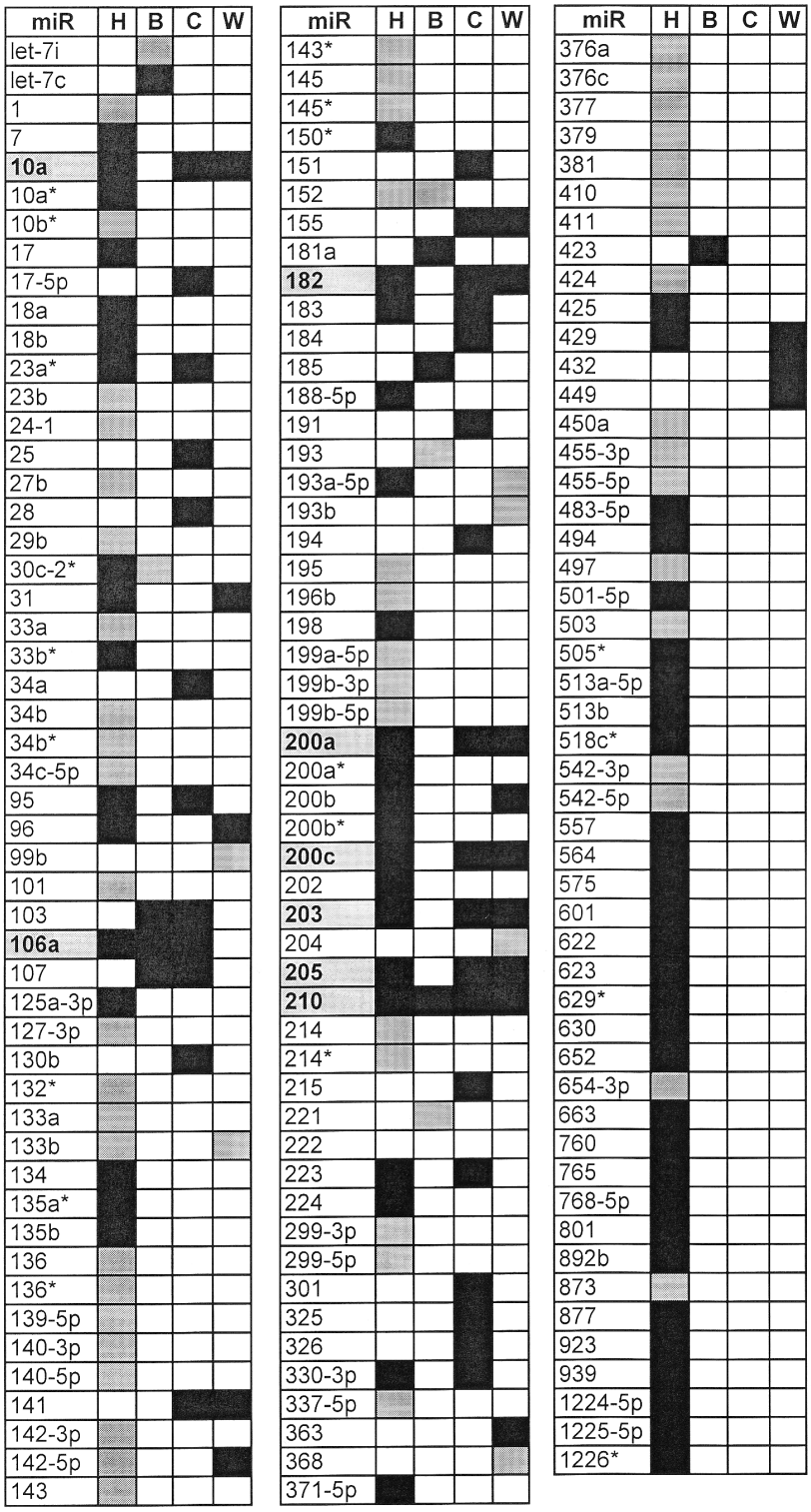

Figure 2. The miRNA landscape of endometrial cancer. microRNAs reported to be over-expressed in endometrial cancers compared to normal endometrium are shown as black squares while those reported to be under-expressed in endometrial cancers compared to normal endometrium are shown as grey squares. The eight miRNAs reported in at least three of the four surveys are bolded. The four extant miRNA surveys are; H (Hiroki et al., 2009), B (Boren et al., 2008), C (Chung et al., 2009), W (Wu et al., 2009).

MicroRNAs in Endometrial Cancer

It is not by accident or omission that the discussion of the state of knowledge of the role of miRNAs in cancer presented above did not include a single mention of endometrial cancer. Endometrial cancer is the fourth most common cancer among women worldwide[31,32] and endometrioid adenocarcinoma accounts for 75%-80% of all endometrial cancers[33] yet examination of miRNAs in these cancers is minimal in comparison to other cancers.

In the first reported survey, Boren et al.[34] examined expression of 335 human miRNAs using a hybridization array strategy followed by qPCR validation of selected candidates on the basis of expression levels. Their study sample consisted of 61fresh frozen endometrial tissues which included 37 endometrial cancers, 20 normal endometrium, and four atypical hyperplasias. They were able to identify a total of thirteen miRNAs as either significantly over-expressed or significantly under-expressed in endometrial cancers as compared to normal endometrium. Their eight over-expressed miRNAs were let-7c, *miR-103, miR-106a, miR-107, miR-181a, miR-185, miR-210* and *miR-423* and their five under-expressed miRNAs were *let-7i, miR-30c, miR-152, miR-193,* and *miR-221*. In addition to the miRNAs, Boren et al. assessed expression levels of 22,000 messenger RNAs again using a hybridization array strategy. Ninety mRNAs were seen to be differentially expressed in endometrial cancers compared with normal endometrium and, with both sets of expression levels in hand, they were able to link twenty-six differentially expressed mRNAs to their differentially expressed miRNAs by confirming that the mRNAs were predicted targets of one or more of the miRNAs. These miRNA::mRNA pairings have, as yet, to be experimentally confirmed.

Wu et al.[35] employed a similar hybridization array strategy to assess differential expression among 469 miRNAs in endometrial cancers. Their survey revealed 17 over-expressed miRNAs and six under-expressed miRNAs in a sample composed of ten endometrioid adenocarcinomas each paired with normal endometrium from the same patients. In addition, they selected six of the 23 miRNAs for subsequent qPCR validation. From the validations they observed that *miR-205* was eighteen-fold over-expressed in the cancers and that *miR-449* and *miR-429* were sixteen-fold and fifteen-fold over-expressed respectively. In the opposite direction, *miR-99b* and *miR-204* were seen to be less than 0.3-fold expressed in the cancers compared to the normal endometrium.

Using a different approach, that of direct qPCR assessments of miRNA expression levels, Chung et al.[36] surveyed 157 miRNAs in fresh frozen tissues composed of 22 normal endometrium and 30 endometrioid adenocarcinomas. They reported that thirty of the 157 miRNAs were significantly over-expressed in the cancers and they carried out subsequent validations of

fourteen (*miR-95, miR-103, miR-106a, miR-151, miR-155, miR-182, miR-183, miR-194, miR-200a, miR-200c, miR-203, miR-205, and miR-210*). As in the Wu et al. study, the most highly over-expressed microRNA was *miR-205* which, in these samples was more than 25-fold over-expressed in the adenocarcinomas than in the normal endometrium. Others included *miR-182* (>8-fold), *miR-325* (8-fold), and *miR-183* (7-fold).

The most ambitious miRNA survey in endometrial cancer to date was recently reported by Hiroki et al.[37]. Using Agilent microRNA hybridization arrays they profiled the expression levels of 470 miRNAs in a sample of seven normal endometrium and 21 endometrial serous adenocarcinomas. This survey identified a total of 66 microRNAs that were over-expressed in the cancers compared to the normal tissues and 54 that were under-expressed in the cancers compared to the normal tissues. Among these, they chose to qPCR validate eight candidate miRNAs that were among those seen to be under-expressed (*miR-10b, miR-29b, miR-34b, miR-101, miR-133a, miR-133b, miR-152, and miR-411*) and one, *miR-205*, that was very highly over-expressed (>267-fold) in the cancers compared to the normal endometrium.

Taking all four extant studies together and making allowances for differences in the methods used as well as the fact that there is no information on how much the miRNA sets overlapped, comparative miRNA expression patterns are presented in Figure 2. In spite of the limitations of such a comparison it is interesting to note that seven miRNAs (*miR-10a, miR-106a, miR-182, miR-200a, miR-200c, miR-203, and miR-205*) are significantly over-expressed in three of four surveys and an eighth, *miR-210,* is significantly over-expressed in all four. Several of these miRNAs, notably *miR-182, miR-205 and miR-210,* are so-called "cancer miRs" in that they display significant levels of over-expression in numerous cancers. In addition, the five members of the "200 family," *miR-200a, miR-200b, and miR-429* on human chromosome 1 and *miR-200c and miR-141* on human chromosome 12, are well known to be coordinately expressed in cancers[38]. Of these, the one microRNA that has been specifically studied as a validated candidate microRNA in endometrial cancer is *miR-200c*. Cochrane et al.[39] examined the potential involvement of *miR-200c* in endometrial cancer from a functional perspective. The transcription factors ZEB1 and ZEB2 (zinc-finger E-box binding homeobox 1 and 2) are known targets of *miR-200c*. ZEB1 is not normally expressed in epithelial cells but, when inappropriately expressed *in vitro*, it initiates epithelial to mesenchyme transition through repression of E-cadherin and other genes involved in polarity. *miR-200c* is highly expressed in several well-differentiated cancer cells, such as the Ishikawa-H endometrial cancer cell line, but displays very low expression in poorly differentiated cells such as the Hec50co

endometrial cancer cell line (see ref 31 for information on the characteristics of these two cell lines). This is reflected in similarly opposite levels of expression of ZEB1 (Ishikawa low / Hec50co high) and E-cadherin (Ishikawa high / Hec50co negative). Cochrane et al. showed that restoration of expression of *miR-200c* in Hec50co cells restores E-cadherin expression and decreases ZEB1 expression. This results in a concomitant decline in migration and invasiveness of Hec50co cells to levels resembling the much less aggressive Ishikawa-H cells. Thus, *miR-200c* has been confirmed to have a direct influence on the behavior of cancer cells.

Another directed miRNA study by Huang et al.[40] focused on the relationship between a specific microRNA, *miR-129-2*, and endometrial cancer. The oncogene known as the SRY-related high-mobility group box 4 gene, or SOX4, is known to be highly expressed in a number of cancers including endometrial cancer. Overexpression of this gene is related to clinical features such as increased proliferation and poor outcome. Huang et al. (2009) show that expression of *miR-129-2* is lost in endometrial cancers when compared to normal endometrium and that SOX4 is a validated *miR-129-2* target. When this microRNA was transfected into endometrial cancers cells, including the afore-mentioned Ishikawa H cells, SOX4 expression was suppressed as was proliferative behavior in these cells. Moreover, this effect was also achieved *in vitro* through demethylation reactivation of *miR-129-2* which led to SOX4 suppression. This last result suggests that hyper-methylation-mediated silencing of a microRNA leads to derepression of the SOX4 oncogene. Surprisingly, *miR-129-2* was not reported in any of the four miRNA studies discussed here. However, as these studies only listed miRNAs that displayed significant changes in expression levels, there is no way to know if *miR-129-2* was among those examined.

Unlike many other cancers, endometrial cancer, along with breast, testicular, and cervical cancers, is significantly influenced by hormones. Cellular growth in the human endometrium is controlled by the antagonistic effects of estrogen and progesterone and endometrial carcinogenesis is linked to high levels of estrogen not ameliorated by the differentiating effects of progesterone[41]. Indeed, it has been shown that expression of the two progesterone receptor isoforms, PRA and PRB, is significantly altered in more aggressive endometrial cancers and that this has profound effects on downstream cellular pathways[31,41,42]. One consequence of disrupted progesterone action in endometrial cancers is the presence of unopposed estrogen. Recently, Klinge[43] reviewed the effects of estradiol, E_2, on microRNA expression in various human cancers. Among the miRNAs whose expression was affected by E_2 are several listed in Figure 2. A few of these, *let-7c, let-7i, miR-7, miR-23b, miR-25, miR-27b, miR-151, miR-183, miR-195,* and *miR-423*, are sporadically reported but four, *miR-*

182, miR-200a, miR-200c, and *miR-203*, are among those reported in at least three of the four miRNA surveys. However, the studies cited, which focused primarily on breast cancers, showed that the effect of E_2 on miRNA expression was cell-type-dependent. Thus, it remains to be seen what effect if any both progesterone receptor changes and unopposed estrogen might have on miRNA expression in endometrial cancer.

As noted above, miRNA studies in endometrial cancer are few in number yet these few studies have already identified a number of candidate miRNAs for more detailed analysis. One of these, *miR-200c*, has proven to be very informative with regard to one of the cancer hallmarks outlined in the beginning of this review- the ability of cancer cells to colonize other regions of the body. It is this interplay between carefully designed miRNA surveys and controlled studies of candidates identified in those surveys that will lead to a much greater understanding of the role played by small regulatory RNAs in endometrial cancers.

Envoi

At the beginning of this paper it was noted that cancer cells *behave* differently than do normal cells and that these differences in behavior result from cancer cells essentially re-writing the rules that apply to normal cells existing in equilibrium. The principle means that cells use to regulate their behavior is through appropriate transcription of genes into proteins. Cancer cells have been shown to reprogram gene transcription to their advantage. The discovery of microRNAs as a complex and powerful post-transcriptional level of gene expression regulation has introduced the possibility that cancer cells alter miRNA expression to assist in carrying out that reprogramming. Indeed, it has been suggested that a global reduction of miRNA levels is emerging as an additional hallmark of cancer[44]. Such global effects can be accomplished in a number of ways including the activation of cancer-associated transcription factors like p53 and Myc which are known to have direct effects on miRNAs of the *miR-34* family (p53) and *miR-17* cluster (Myc). Chang et al.[45] have further shown that activation of Myc in cancer could directly reprogram the wider miRNA transcriptome. Other potential means of accomplishing miRNA reprogramming result from the massive genetic alterations known to occur in cancer cells[18,46] and from specific alterations of the miRNA processing machinery itself[47,48]. Thus, continued detailed studies of miRNAs in cancers will shed new light on the nature of cellular reprogramming and lead to new and more efficient ways to attack cancers in general[44] and, from our perspective, endometrial cancers in particular, in the clinic.

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