

**COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD,  
CONSUMER PRODUCTS AND THE ENVIRONMENT**

**Role of miRNA Related Effects and Chemicals on Cancer**

At the November 2009 meeting, the Committee discussed a paper on RNA mechanisms of carcinogenicity. The paper (CC/09/13) provided a review of the role played by RNA mechanisms in cancer development. It concentrated primarily on RNA editing, alternative splicing, non-sense mediated decay (NMD), RNA binding proteins and RNA interference and the role of microRNAs (miRNAs), as mechanisms that potentially influence tumorigenesis.

One area of particular interest was the role played by miRNAs in carcinogenesis. At the time it was noted by the Committee that literature was available on the effect of therapeutic drugs on RNA mechanisms and how the manipulation of these processes could be exploited for therapeutic purposes, but similar information was not available for environmental chemicals. However, since that time a number of papers have been published on environmental chemicals influencing the RNA processes involved in carcinogenesis. The attached paper, outlines the role played by miRNAs in carcinogenesis as previously discussed in CC/09/13 and discusses the recent publications on the effect of chemicals on miRNAs involved in the carcinogenic process.

*Questions for the committee*

Would members like additional information on any of the studies summarised in this paper?

Will information such as that described here be of help in assessments by the Committee?

Do members have any comments on the possible utility of specific miRNAs as biomarkers of cancer, whether in experimental models or in humans?

Do members have any research or other recommendations with respect to miRNAs and cancer?

Do members consider that it would be appropriate to produce a committee statement on miRNAs at this stage?

Secretariat  
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## COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

### Role of miRNA Related Effects and Chemicals on Cancer

#### Introduction

1. Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) are found in many organisms including plants, animals, bacteria and some viruses. DNA and RNA differ functionally. DNA primarily serves as the storage material for genetic information. RNAs are versatile molecules capable of an array of functions in a cell. One of the primary functions of RNA is to facilitate the translation of DNA into protein. RNAs also function as information-carrying intermediaries in gene expression, and they act as key catalytic, structural, and regulatory elements in the cell.

2. In recent years many new small functional RNAs have been found. These non-coding RNA molecules are transcribed from a DNA sequence, but are not translated into proteins. The encoding DNA sequence is often referred to as an RNA gene. Functional RNA genes in the human genome include transfer RNA (tRNA), ribosomal RNA (rRNA) and various other small non-coding RNAs. Several hundred genes in our genome encode small functional RNA molecules collectively called microRNAs (miRNAs).

#### MicroRNAs (miRNAs)

3. RNAi is a process of sequence specific post-transcriptional gene silencing initiated by double stranded RNA (dsRNA). The two main types of short RNAs that target complementary messengers in animals are 1) small interfering RNA (siRNA) and 2) miRNA. RNAi regulation of endogenous genes in mammalian cells occurs via production of miRNAs. MiRNAs are a class of small non-coding RNAs whose mature products are ~22 nucleotides (nt) long. MiRNAs are pleiotropic regulators of gene expression at the post-transcriptional and/or translational level and play a role in the processes of tumour suppression and oncogenic transformation.

4. Figure 1 illustrates miRNA biogenesis (Cullen, 2005). Briefly, miRNAs are encoded in the genome and transcribed by RNA polymerase II as primary transcripts known as pri-miRNAs (primary-miRNAs). Pri-miRNAs are typically 3 to 4 kilobases long, single-stranded RNAs with a 5' cap, 3' poly (A) tail and complicated secondary structure. The pri-miRNAs are processed in the nucleus into one or more precursor-miRNA by the Drosha RNAase III endonuclease to give a 60-70 nt stem loop intermediate, having a 5' phosphate and ~2nt 3' overhang. This pre-miRNA is actively transported from the nucleus to the cytoplasm by Ran-GTP (RAS-related Nuclear protein-GTP) and the export receptor, Exportin 5. The cuts made by the RNase III enzyme Drosha in the nucleus define one end of the mature miRNA and the other is defined in the cytoplasm by DICER, which recognises the double stranded portion of the pre-miRNA and cuts the two helical turns away from the base of the

stem loop. This eliminates the loop itself and leaves the 5' phosphate and the ~2nt 3' overhang characteristic of the RNAase III products. Once incorporated into the RNA-induced silencing complex (RISC) (see paragraph 6 below), there is no substantial difference between miRNA and siRNA towards the target mRNA. The final short RNA duplex possesses a strand which is highly, but not perfectly, complementary to one or more of the mRNAs which are targeted by mRNAs. This complementarity drives the assembly of an RNA-protein complex on the target mRNAs which, in turn, prevents the latter from being translated.

5. There are estimated to be at least 700 miRNAs in the human genome (Liang and Li, 2009), comprising 1-4% of all expressed human genes, which makes miRNAs one of the largest classes of gene regulators. Most human miRNAs are found within introns of either protein-coding or non-coding mRNA transcripts (Rodriguez et al., 2004). The remaining miRNAs are either located far from the other transcripts in the genome, within the exons of noncoding mRNA genes or within 3' untranslated regions (UTRs) of mRNA genes, or they are clustered with other miRNA genes (Bartel, 2004).

6. They negatively regulate their targets in one of two ways depending on the degree of complementary between the miRNA and the target. The first mechanism involves miRNAs that bind with perfect, or nearly perfect, complementarity to protein-coding mRNA sequences inducing the RNA-mediated interference (RNAi) pathway. This involves mRNA transcript cleavage by ribonucleases in the miRNA associated, multi-protein RNA-induced silencing complex (miRISC) which results in the degradation of target mRNAs. The second mechanism does not involve cleavage of their mRNA targets. These miRNAs exert their regulatory effects by binding to imperfect complementary sites within the 3' UTR of their mRNA targets and they repress target-gene expression post-transcriptionally, apparently at the level of translation, through a RISC complex that is similar to, or possibly identical with, the one that is used for the RNAi pathway.

## **MiRNAs and Cancer**

7. A large amount of evidence has been presented in the literature indicating a role for miRNAs in many human diseases, including cancer. This evidence includes but is not limited to 1) a unique set of miRNAs exists in a specific disease; 2) unique expression of miRNAs in a certain human disease and 3) aberrant expression of miRNAs in human disease (Zhang and Farwell, 2008). According to Sassen et al. (2008), three important observations early in the history of miRNAs suggested a role in human cancer. Firstly, the earliest miRNAs discovered in the nematode worm *C. elegans* and the fruit fly *Drosophila* were shown to control cell proliferation and apoptosis by Lee et al. (1993) and Brennecke et al. (2003). Their deregulation may therefore contribute to proliferative diseases such as cancer. Secondly, when human miRNAs were discovered, it was noticed that many miRNA genes were located at fragile sites in the genome or regions that are commonly amplified or deleted in human cancer (Calin et al. 2004). Thirdly, malignant tumours and tumour cell lines were found to have widespread deregulated miRNA expression compared to normal tissues (Calin and Croce, 2006; Gaur et al., 2007; Lu et al. 2005).

8. The first evidence of a link between miRNAs and human cancer came from an observation in patients diagnosed with a common form of adult leukaemia, B cell chronic lymphocytic leukaemia (CLL). It was observed that two miRNA genes, miR-15a and miR-16-1 located in a 30kb region on chromosome 13, were deleted or reduced in CLL cases (Calin et al., 2002). Deletions within the 13q14 locus occur in more than 65% of all CLL cases, as well as in 50% of mantle cell lymphomas, 16-40% of multiple myelomas and 60% of prostate cancers. The miR-15a/16-1 cluster is also reported to be down-regulated in pituitary adenoma (Bottoni et al., 2005) and prostate carcinoma (Porkka et al., 2007). In these cancers, miR-15a/16-1 expression is preferentially down-regulated to favour cancer development by inhibiting apoptosis. Calin et al (2008), using microarray analysis, studied the down regulation of miRNA in CLL patients with high levels of miR15a/16-1 with respect to CLL patients with low levels of these two miRNAs. They identified a signature of 60 genes in common between CLLs and MEG-01 cells transfected with miR15a/16-1. Genes, such as MCL1, ETS1 and JUN, which play a role in apoptosis and cell cycle control, were found to be significantly differentially expressed in these cells (Calin et al., 2008). Cimmino et al (2005) demonstrated that miR-15a and miR-16-1 expression is inversely correlated to Bcl2 expression in CLL and that both miR-15a and miR-16-1 negatively regulate Bcl2, which is an anti-apoptotic gene that is often over-expressed in many types of human cancers, including leukaemias and lymphomas. The tumour suppressor role of miR-16-1 was further supported by the identification of a germ line mutation (C to T mutation, 7 base pairs downstream of the miR-16-1 precursor) which causes low levels of miRNA expression in vitro and in vivo and which was associated with deletions of the normal allele.

9. Michael et al. (2003) identified many miRNAs that are expressed in human colorectal epithelium. Characterisation of these miRNAs has shown an association between reduced levels of two specific miRNAs (miR-143 and miR-145) and precancerous and neoplastic colorectal tissue.

10. Iorio et al. (2005) identified miRNAs where expression is significantly down-regulated in cancer versus normal breast tissues. MiR-106, miR-125b and miR-145, and miR-21 and miR-155 were up-regulated suggesting they potentially act as tumour suppressor genes and oncogenes, respectively (Iorio et al., 2005). The expression of miRNAs was correlated with specific breast cancer pathological features such as oestrogen-receptor status, tumour stage, vascular invasion and proliferative index.

11. Glioblastoma multiforme is the most frequently occurring and malignant form of primary brain tumour. Ciafre et al. (2005) found that miR-21 was strongly upregulated and miR-181a, miR-181b and miR-181c were downregulated in glioblastoma compared to normal brain controls, using microarray analysis. It has been shown also that glioblastoma strongly over-expresses miR-21 (Chan et al., 2005). Its expression was increased 5 to 100 fold in human glioblastoma multiforme tissue compared with control non-neoplastic brain tissues. Inhibition of miR-21 expression led to caspase activation and associated apoptotic cell death in multiple glioblastoma cell lines. These findings suggest that overexpression of miR-21 may function as an oncogene in glioblastomas by blocking key apoptosis-enabling genes.

12. MiR-21 is the most consistently up-regulated miRNA across many cancer types of solid tissues (lung, breast, stomach, prostate, colon, brain, head and neck, oesophagus

and pancreas) (Chan et al., 2005; Iorio et al., 2005; Diederichs and Haber, 2006; Roldo et al., 2006). It is also overexpressed in diffuse large B-cell lymphomas (Lawrie et al., 2007), in chronic lymphocytic leukaemia (Calin et al., 2005), in uterine leiomyomas (Wang et al., 2007), in human malignant hepatocytes and in hepatocellular carcinoma (Meng et al., 2007). Volinia et al. (2006) performed a large scale miRNome analysis on 540 samples including breast, stomach, prostate, colon and pancreatic cancers and identified a solid-cancer signature comprising a large proportion of overexpressed miRNAs. These include miR-155, miR-17-5p, miR-20c, miR-92 and miR-106a as well as miR-21.

13. Primary thyroid carcinoma (PTC) is the most common malignancy in thyroid tissue, accounting for 80% of all thyroid cancers (He et al., 2005). Pairwise significance analysis of microarray data indicated that six miRNA genes were significantly overexpressed in tumour tissue from PTC with fold changes >1.5 in at least seven patients. These miRNAs were as follows: miR-146, miR-221, miR-222, miR-21, miR-155, and miR-181a. Prediction analysis using the microarray data indicated that five of the over-expressed miRNAs (miR-221, miR-222, miR-146, miR-21, and miR-181a) were sufficient to successfully predict cancer status (He et al., 2005). Three of the miRNAs (miR-146, miR-221, and miR-222) which showed substantial overexpression (11- to 19-fold higher level in PTC tumours) showed a loss of KIT transcript and Kit protein. KIT is an important tyrosine kinase receptor in cell differentiation and growth. He et al. (2005) also showed polymorphisms in 5 out of 10 primary thyroid cancers in the KIT 3'UTR that corresponds to the site of interaction of miR-221, miR-222 and miR-146, suggesting an altered miRNA-target interaction.

#### **PTEN**

14. PTEN (Phosphatase and tensin homolog) is a tumour suppressor gene encoding a phosphatase that regulates cell cycle, akt and p53 activity (Li and Ross, 2007). MiR-21 directly targets PTEN whose down regulation will release its inhibition on protein kinase B resulting in significantly reduced apoptosis in cancer cells. Meng et al. (2007) showed that inhibition of miR-21 expression increased PTEN expression in human hepatocellular carcinomas. Focal Adhesion Kinase (FAK), an established downstream target of PTEN, is a protein tyrosine kinase involved in the regulation of cell-cycle progression, cell survival and cell migration. Meng et al. (2007) demonstrated that inhibition of miR-21 in HCC cell lines significantly reduced the phosphorylation of FAK and Akt and significantly reduced the expression of matrix metalloproteases 2 and 9, downstream targets of PTEN and key mediators of tumour cell survival, cell migration and invasion. Interestingly, Frankel et al. (2008), investigating the role of PTEN-miR-21 interaction in breast cancer cells, showed that inhibition of miR-21 caused only subtle changes in PTEN protein levels, suggesting that cell and tissue specific differences may result in different functional miR-21 targets.

#### **Pdcd4**

15. Pdcd4 (programmed cell death 4) is a tumour suppressor known to be up-regulated in apoptosis (Yang et al., 2003) and down-regulated in several cancer forms (Chen et al., 2008; Gao et al., 2007; and Zhang et al., 2006). Recently, two independent reports revealed that Pdcd4 is a target of miR-21 in colon cancer (Asangani et al., 2008) and MCF-7 human breast cancer cells (Frankel et al., 2008). Asangani et al. (2008) showed that Pdcd4 is negatively regulated by miR-21 at the

post-transcriptional level, via a specific target site (nt 228-249) within the 3'UTR. They also demonstrated that miR-21 induces invasion/intra-vasation/metastasis in colorectal cancer cells. Lu et al. (2008) demonstrated that the translation of Pdc4 is negatively regulated by miR-21 in HEK-293T, MCF-7 and JB6 cell lines.

16. Si et al. (2007) found that miR-21 was highly expressed in breast tumours and found that the anti-miR-21-mediated cell growth inhibition was associated with increased apoptosis and decreased cell proliferation. In a study by the same laboratory, Zhu et al. (2007) found that the tumour suppressor gene tropomyosin 1(TPM1), which carries a putative miR-21 binding site, was down-regulated by miR-21, whereas anti-miR-21 up-regulated TPM1 expression through the miR-21 binding site at the 3'-UTR region.

### MYC

17. Pathologically activated expression of MYC is one of the most common oncogenic events in human cancers (Nesbit et al., 1999). The MYC oncogene encodes the transcription factor c-Myc that regulates cell proliferation, growth and apoptosis. There appears to be a close relationship between miRNAs and the increased expression of MYC, leading to the development of B-cell malignancies.

18. An aggressive B-cell leukaemia occurs when MYC is translocated into the miR-142 locus (Gauwerky et al., 1989). The MYC gene translocated only four nucleotides downstream of the miR-142 3'-end and alignment of mouse and human miR-142 containing EST sequences indicates a ~20 nt conserved sequence element downstream of the miR-142 hairpin, which is lost in the translocation (Lagos-Quintana et al., 2002). It was suggested that the absence of this conserved downstream sequence element in the putative miR-142/mRNA fusion prevented the recognition of the transcript as a miRNA precursor to be properly processed, and therefore may have caused accumulation of fusion transcripts and overexpression of MYC.

19. Another miRNA that has been linked with MYC overexpression and B-cell cancers is miR-155. This miRNA is encoded by nucleotides 241-262 of B-cell integration cluster (BIC). Metzler et al. (2004) found that the only phylogenetically conserved region within BIC spans 138 nucleotides and encodes the hairpin region of miR-155. High expression of miR-155 and its host gene BIC have been reported in paediatric Burkitt lymphoma (BL) (Metzler et al., 2004), human B cell lymphomas (Eis et al., 2005) and Hodgkin's lymphoma (Kluiver et al., 2005) A 100 fold upregulation of the miR-155 precursor has been reported in paediatric BL (Metzler et al., 2004). Yanaihara et al (2006) found that high expression of miR-155 has a significantly worse impact on patients with lung adenocarcinoma as an independent risk factor. MiR-155 has also been reported to be upregulated in breast cancer (Iorio et al., 2005).

20. Articles by He et al. (2005) and O'Donnell et al. (2005) have indicated a clear association between miRNAs and cancer. A polycistronic miRNA cluster known as miR-17-92 comprises seven miRNAs (miR-17-5p, miR-18a, miR-19a, miR-196-1 and miR-92-1) and resides in intron 3 of the C13orf25 gene at 13q31.3. It is markedly overexpressed in lung cancers, especially with small-cell lung cancer histology (Hayashita et al., 2005) and upregulated in 65% of B-cell lymphoma samples (He et

al., 2005). Expression profiling studies have revealed widespread overexpression of these miRNAs in diverse tumour subtypes including such as those derived from breast, colon, lung, pancreas, prostate and stomach (Petrocca et al., 2008; Volinia et al., 2006). Using the well-studied E $\mu$ -myc transgenic mouse model of B cell lymphoma, He et al. (2005) provided direct evidence that the miR-17-92 cluster has oncogenic activity. Enforced expression of the miR-17-92 miRNAs dramatically accelerated disease onset and progression. O'Donnell et al. (2005) reported that transcription of the miR-17-92 cluster is directly transactivated by c-MYC. In particular, the miR-17-92 cluster seems to be tightly linked to the functions of the E2F family of transcription factors, which are critical regulators of the cell cycle and apoptosis. O'Donnell et al. (2005) found that the expression of E2F1 was negatively regulated by miR-17-5p and miR-20a (2 miRNAs in the miR-17-92 cluster) in HeLa cells. Their findings revealed a mechanism through which the c-MYC protein simultaneously activates E2F1 transcription and limited its translation, resulting in a tightly controlled proliferative signal.

21. Moreover, Woods et al. (2007) demonstrated that both E2F1 and E2F3 can directly activate transcription of the miR-17-92 cluster. Woods et al. (2007) proposed a model whereby miR-17-92 promoted cell proliferation by shifting the E2F transcriptional balance away from the pro-apoptotic E2F1 and towards the proliferative E2F3 transcriptional network.

22. In mammals, a paralog of the miR-17-92 exists. The miR-106b-25 has been shown to be differentially expressed in a number of different cancers. Li et al. (2009) demonstrated the upregulation of the miRNAs encoded by the miR-106b-25 cluster in hepatocellular carcinoma. Other studies have also shown that miR-106b-25 is overexpressed in HCC and in human liver tumour cell lines (Meng et al. 2007; Li et al. 2009). Ambs et al. (2008) determined genome-wide expression of miRNAs and mRNAs in primary prostate tumours and non tumour prostate tissues. They reported that components of miRNA processing and several miRNA host genes (MCM7 and C9orf5) were significantly upregulated in prostate tumours and that tumours expressed the miR-106b-25 cluster at significantly higher levels than non-tumour tissues. Knockdown studies for the miR-106b-25 cluster showed that inhibition of each member of the miR-106b-25 cluster inhibited cell proliferation (Li et al. 2009). They also showed that the entire miR-106b-25 cluster resulted in more effective inhibition of cell proliferation and also suppressed anchorage-independent growth. The role of the miR-106b-25 cluster in cell growth and proliferation has also been described by Ivonovska et al. (2008) and Petrocca et al. (2008).

### Let-7

23. Another putative tumour suppressor in human cancers is the let-7 miRNA. The *C. elegans* let-7 miRNA was identified by genetic analysis of the developmental timing defects of mutants and the human let-7 is abundantly expressed in normal lungs. The let-7 family contains miRNAs that have been shown to regulate expression of a proto-oncogene, the rat sarcoma viral oncogene homolog (RAS), through post-transcriptional repression (Johnson et al., 2005). RAS proteins are membrane associated signalling proteins that regulate cell growth and differentiation. Almost 15-30% of human tumours possess mutations in RAS genes. The miRNAs that are encoded by the let-7 family were implicated as tumour suppressors because they map to fragile sites associated with lung, breast, urothelial and cervical cancers (Calin et

al., 2004). Johnson et al. (2005) showed that over-expression of RAS proteins in lung cancer tissue correlated with reduced expression of let-7 miRNA. Johnson et al. (2005) also demonstrated that 3'UTRs of the human RAS gene contain let-7 complementary sites, allowing let-7 to regulate RAS expression. Takamizawa et al. (2004) demonstrated that transcripts of certain let-7 homologues were significantly down-regulated in human lung cancer and found that this was correlated with a poor prognosis. Takamizawa et al. (2004) demonstrated that over-expression of the let-7 miRNA in an A549 adenocarcinoma cell line resulted in inhibition of lung cancer cell growth in vitro.

#### **MiRNAs and their role in metastasis**

24. Metastasis is complex, multi-step process where primary tumour cells invade adjacent tissue, enter the systemic circulation and lymphoid system. The tumour cells are then carried through the vasculature, penetrate distant tissue parenchyma and finally proliferate from microscopic growths (micro-metastases) into macro secondary tumours (Filder et al., 2003). The precise role played by expressed miRNA in specific steps of malignant progression including metastasis, are still unknown. However, a number of studies have investigated the association of specific miRNAs with specific stages of malignant progression.

#### **EMT**

25. Epithelial to mesenchymal transition (EMT) has been considered an essential early step in metastasis of epithelial-derived tumours. Gregory et al. (2008) found that all five members of the microRNA-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) and miR-205 were markedly downregulated in cells that had undergone EMT in response to transforming growth factor (TGF)-beta or to ectopic expression of the protein tyrosine phosphatase (Pez). Enforced expression of the miR-200 family alone was sufficient to prevent TGF-beta-induced EMT. Together, these miRNAs cooperatively regulate expression of the E-cadherin transcriptional repressors ZEB1 (also known as  $\delta$ -EF1) and SIP1 (also known as ZEB2), factors previously implicated in EMT and tumour metastasis. These findings were confirmed by four other independent studies (Bracken et al., 2008; Korpala et al., 2008; Park et al., 2008 and Burk et al., 2008).

#### **Breast Cancer**

26. Using the 29 identified miRNAs that were differentially expressed between primary breast carcinomas and normal mammary tissues, reported by Iorio et al. (2005), Ma et al. (2007) found that miR-106 was highly expressed only in metastatic breast cancer cells. They reported that the expression level of miR-106 was 50 fold higher in cells of the MDA-MB-231 line, which are capable of metastasizing, than in cells of the MCF-7 human breast cancer cell line, which have little if any metastatic capabilities (Ma et al. 2007). They also found that miR-106 expression levels were lower in all of the breast carcinomas from metastasis-free patients. However, they found 50% of metastatic-positive patients had elevated miR-106 levels in primary tumours. Ma et al. (2007) also demonstrated that miR-106 positively regulates cell migration and invasion in vitro and can initiate cell invasion in vivo. They also identified that the transcription factor Twist (a metastasis-promoting gene) directly regulates miR-106, which in turn inhibits synthesis of the HOXD10 protein, permitting the expression of the pro-metastatic gene product, RHOC. This gene

product favours cancer cell migration and invasion. Conversely, silencing of miR-106 inhibits Twist-mediated cell migration and invasion.

27. In a study of 219 patients, Gee et al (2008) found less miR-106 expression in patients without metastasis (n=114) than in normal breast tissue (n=10). However, unlike Ma et al. (2007) they found lower miR-106 expression in patients with distant relapse and local recurrence. Also, whereas Ma et al. (2007) found that miR-106 over-expression increases tumour size and invasiveness, Gee et al (2008) found its expression correlates inversely and significantly with tumour size and grade.

28. Zhu et al. (2008) investigated the role of miR-21 in cell invasion and tumour metastasis and found that suppression of miR-21 in metastatic breast cancer MDA-MB-231 cells significantly reduced invasion and lung metastasis. They showed that over-expression of TMP1 (tropomyosin1), which is down-regulated by miR-21, suppresses the invasiveness of MDA-MB-231 cells, and they also identified Pcdcd4 and maspin as candidate target genes for miR-21. Yan et al. (2008) investigated the global miRNA expression profile in primary breast cancer cells to evaluate their involvement in the malignant progression of the tumour. Their principle finding agrees with that of Zhu et al. (2007) that over-expression of miR-21, one of the most significantly altered miRNAs in breast cancer, is associated with progression and poor prognosis of the patient.

29. Tavazoie et al. (2008) identified a set of 6 miRNAs (miR-335, miR-126, miR-206, miR-122a, miR-199a and miR-489) for which expression is specifically lost as human breast cancer cells develop metastatic potential. Restoration of miR-126, miR-206 and miR-335 expression in malignant cells suppressed lung and bone metastasis by human cancer cells (MDA-MB-231) *in vivo* in the mouse. MiR-126 significantly suppressed overall tumour growth and proliferation, whereas miR-335 inhibited metastatic cell invasion. In clinical metastasis, Tavazoie et al. (2008) found that patients whose primary tumours displayed low expression of miR-335 and miR-126 or miR-206 had a shorter median time to metastatic relapse. It was also reported that miR-335 regulates metastatic gene SRY-box containing transcription factor (SOX4); tenascin C (TNC); c-mer tyrosine kinase (MERTIC) and receptor type tyrosine protein phosphatase (p-TPRN2), whose collective expression in a large cohort of human tumours is associated with risk of distal metastasis.

30. Huang et al. (2008) demonstrated that miR-373 and miR-520c have metastasis promoting abilities. They showed that miR-373 and miR-520c did not affect cell proliferation but promoted a migratory and invasive phenotype of MCF7 cells *in vitro*. Furthermore, MCF7 cells over-expressing miR-373 and miR-520c developed into metastatic nodules, which did not occur with control cells. Elucidating the molecular pathways involved, of nine potential targets CD44 was found to be a direct target of miR-373 and miR-520c. The migration phenotype of miR-373 and miR-520c can be explained by the suppression of CD44 expression. Huang et al (2008) also found significant up-regulation of miR-373 in clinical breast cancer metastasis samples that correlated inversely with CD44 expression.

31. Breast cancer metastasis suppressor I (BRMSI) inhibits breast melanoma, non-small cell lung and ovarian cancer metastasis in a number of experimental models. BRMSI forms complexes with SIN3, histone deacetylases and selected transcription

factors that modify metastasis-associated gene expression (EGFR, OPN, PLAU, P14PSKIA). Edmonds et al. (2009) found that BRMS1 altered miRNA expression in metastatic breast carcinoma cells, down-regulating miR-106, miR-373 and miR-520c and their downstream targets (for example RohC which is a downstream target of miR-106) and up-regulating miR-146a, miR-146b and miR-335. These results are in agreement with previous publications of Ma et al., (2007), Hurst et al., 2009, Tavazoie et al., 2007 and Huang et al., 2008.

### **Hepatocellular Carcinoma**

32. Budhu et al. (2008) identified a 20 miRNA signature that is associated with hepatocellular carcinoma (HCC) venous metastasis. The signature was capable of predicting survival and recurrence of HCC in patients with multi-nodular or solitary tumours, including those with early stage disease.

### **Colorectal cancer**

33. Asangani et al. (2008) demonstrated that miR-21 induces invasion / intravasation / metastasis in colorectal cancer cells and that the tumour suppressor Pcd4 is negatively regulated by miR-21 at the post transcriptional level, via a specific target site (nt 228- nt249) within the 3'-UTR.

### **Epigenetics and miRNAs.**

34. Cancer has been recognised as both a genetic and epigenetic disease. Genetic lesions alone cannot explain the complexity of the aberrations that arise in a cancer cell (Lujambio and Esteller, 2009). Epigenetics is defined as the inheritance of changes in gene activities that are independent of DNA sequence. The two main epigenetic mechanisms involved in gene regulation, development and carcinogenesis are DNA methylation and histone modification (Lujambio and Esteller, 2009). In cancer cells, the fine control of epigenetic mechanisms is lost and the disruption of epigenetic patterns promotes the expression of the tumoural phenotype. Three main epigenetic alterations occur in human cancer. These are 1) DNA hypermethylation of tumour suppressor genes, 2) global genomic hypomethylation and 3) aberrant histone modification signatures.

### **DNA hypermethylation of tumour suppressor genes**

35. DNA methylation is a normal process used by mammalian cells in maintaining expression patterns; it is involved in the regulation of imprinted gene expression and X-chromosome inactivation among others (Robertson and Jones, 2000). DNA methylation occurs almost exclusively on a cytosine in a CpG dinucleotide and is achieved by the addition of a methyl group to the 5 position of a cytosine ring by the action of three DNA methyltransferases namely Dnmt1, Dnmt3a and Dnmt3b. Dnmt3a and Dnmt3b are de novo methyltransferases and DNMT1 is the maintenance DNMT that ensures that methylation patterns are copied faithfully through each cell division (Klose and Bird, 2006). CpG sites are asymmetrically distributed into CpG poor regions and dense regions known as CpG islands, which are located in the promoter regions of roughly half of all protein-coding genes (Chuang and Jones, 2007). Methylation of CpG islands in promoter regions is often associated with gene silencing and aberrant DNA methylation occurs in most cancers, leading to the silencing of some tumour suppressor genes (Jones, 2002). Lehmann et al (2008) reported that 61 CpG islands associated with human miRNA genes have been identified, thus far.

36. Exploiting the pharmacologic manipulation of the epigenome, Saito et al. (2006) demonstrated that a subset of miRNAs are upregulated in bladder transitional cells (T24) by treatment with chromatin modifying drugs (5'aza-2'deoxy-cytidine; 5'aza-CdR) and 4-phenylbutyric acid, PBA, which inhibit DNA methylation and histone deacetylase, respectively). In particular, miR-127, which is located within a CpG island and silenced in human cancer cells, was highly induced after treatment with 5'aza CdR and PBA, with downregulation of its potential target the proto-oncogene BCL6.

37. A similar approach was recently taken by Toyota et al. (2008), demonstrating that aza-5CrD treatment of colon cancer cell lines upregulates over 30 miRNAs, including miR-346 and miR-34c. They also reported that downregulation of miR-346/c expression was strongly associated with hypermethylation of its neighbouring CpG island, which notably harbours bidirectional promoter activity and also regulates expression of another candidate tumour suppressor gene, B-cell translocation gene 4 (BTG4). The expression of BTG4 suppressed colony formation of CRC implying that BTG4 as well as miR-346/c are novel tumour suppressors in colon cancer (Toyota et al., 2008).

38. Lujambio et al. (2007) used a genetic model to compare the miRNA expression profile of the wild-type colon cancer cell line (HCT-116) with the same cell line after genetic disruption by homologous recombination of DNA methyltransferases 1 (DNMT1) and DNMT3b (Double knockout, DKO), using a miRNA microarray profiling method. The double knockout (DKO) cells showed a reduction of DNMT activity and 5-methylcytosine DNA content leading to a release of gene silencing associated with CpG island hypermethylation (Lujambio et al., 2007). Their results demonstrated that DNA hypermethylation contributed to the transcriptional down-regulation of miRNAs in human tumours and that the epigenetic silencing of miR-124a leads to the activation of cyclin D kinase 6 (CDK6), a bona fide oncogenic factor, and the phosphorylation of the retinoblastoma (Rb) tumour suppressor gene.

39. Aberrant hypermethylation events in the regulatory regions of miRNAs may also play a role in the establishment of human metastasis. Lujambio et al. (2008) used a pharmacological and genomic approach to unmask an aberrant epigenetic silencing program by treating lymph node metastatic cancer cells with a DNA demethylating agent followed by hybridization to an expression microarray. MiR-148a, miR-34B/C and miR-9 were found to undergo specific hypermethylation-associated silencing in cancer cells compared to normal cells. The reintroduction of miR-148a and miR-34b/c in cancer cells with epigenetic inactivation inhibited their motility, reduced tumour growth, and inhibited metastasis formation in xenograft models, with an associated down-regulation of the miRNA oncogenic target genes, such as C-MYC, E2F3, CDK6, and TGIF2. The involvement of miR-148a, miR-34b/c, and miR-9 hypermethylation in metastasis formation was also suggested in human primary malignancies (Lujambio et al., 2008).

40. In a clinical study of epithelial ovarian cancer, Lu et al. (2007) analysed the methylation of let-7a-3 in tumour tissues using real time methylation specific PCR and evaluated the effect of methylation on let-7a expression, insulin-like growth factor II (IGF-II), a potential target of let-7a and patient survival outcomes. They

showed that let-7a-3 was methylated in epithelial ovarian cancer and that low let-7a-3 methylation was associated in ovarian cancer with high IGF-II and low IGFBP-3 expression as well as poor prognosis of epithelial ovarian cancer.

### **Global Genomic Hypomethylation**

41. Global hypomethylation events can also affect miRNAs. Brueckner et al. (2007) showed that the human let-7a-3 gene on chromosome 22q13.31 is associated with a CpG island. Using bisulfite sequencing, they found that the let-7a-3 gene is heavily methylated in normal human tissues but hypomethylated in some lung adenocarcinomas. They also reported that let-7a-3 hypomethylation facilitated epigenetic reactivation of the gene and elevated expression of let-7a-3 in a human lung cancer cell line resulted in enhanced tumour phenotypes and oncogenic changes in transcriptional profiles (Brueckner et al., 2007).

42. Using miRNA microarray analysis in hepatocellular carcinoma cells (HCC) treated with 5-AzaC (5-azacytidine) alone or in combination with trichostatin A (TSA), a histone deacetylase inhibitor, Datta et al. (2007) attempted to identify candidate tumour suppressor miRNAs that are silenced by epigenetic mechanisms in HCC cells. They found that miR-1-1 is one such gene that is methylated in human HCC cells and primary HCC, and its activation by epigenetically-acting drugs suppresses tumour cell growth by down-regulating its oncogenic targets MET, FoxP1 and HDAC4.

43. Iorio et al. (2007) investigated whether aberrant DNA methylation patterns could contribute to the altered miRNA expression characterising human ovarian cancer, using miRNA profiling of the ovarian carcinoma cell line (OVCAR3) before and after treatment with the demethylating agent 5-Aza (5-aza-2'-deoxycytidine). The analysis of microarray data showed 11 human miRNAs differentially expressed, 9 upregulated and 2 down regulated, with miR-21, miR-203, miR-146b, miR-205, miR-30-5b and miR-30c, the most significantly induced on treatment. They also found that miR-21, miR-203 and miR-205 are overexpressed in ovarian carcinomas compared to normal tissue. The finding that reactivation of the miRNA genes occurs after demethylation treatment suggests that hypomethylation could be the mechanism responsible for the overexpression in vivo.

### **Chemical Induction of miRNAs and Cancer**

44. MiRNAs play an important role in cancer and the aberrant expression of miRNAs has been observed for a number of different cancers as outlined above. These findings have led a number of researchers to hypothesise that exposure to specific environmental carcinogens would induce alterations in miRNA expression and that this altered miRNA expression would contribute to carcinogenesis. This is a relatively new field of research and to date only ~25 scientific papers have been published. The role played in the induction of miRNAs by environmental carcinogens such as cigarette smoke, dioxins, conazoles, PPAR $\alpha$  agonists, vinylcarbamate, and estrogens have been investigated thus far.

### **Genotoxic Carcinogens**

45. In an oral feeding rat study, Pogribny et al. (2010) investigated the effect of the genotoxic liver carcinogen 2-aminoacetylfluorene (2-AFF, 0.02% for 12 or 24 weeks)

on miRNA and gene expression patterns associated with the p53 signalling pathway in the livers of control and 2-AAF treated rats. Of the 86 genes associated with p53 signalling investigated, 13 genes were significantly up- or down-regulated ( $p < 0.05$  and fold change  $> 2.0$ ). They reported that the number of upregulated anti-apoptotic growth related genes was greater than the number of pro-apoptotic genes. Additionally the expression of p53 did not change significantly at any time point and that the expression of p73 gene was substantially down-regulated. Using miRNA microarrays, and confirmed by qRT-PCR, they reported that 21 and 33 miRNAs were significantly up-regulated or down-regulated in the livers of 2-AAF treated rats, respectively, as compared to age matched control rats. The up-regulated miRNA genes included those involved in apoptosis /cell proliferation control (miR-34A, miR-18, miR-21, miR-96, miR-182 and members of miR-183/96/182 cluster) and cell-cell contact (miR-200s, miR-200b, miR-200c and miR-429), two pathways disrupted during the carcinogenic process. Protein levels of confirmed targets of the differentially expressed miRNAs showed decreased protein levels of E2F3, ZEB1, ZEB2 and PTEN regulated by miR-349, miR-200a, miR-200b, miR-200c, miR-429, and miR-21 in the livers of rats fed 2-AAF compared to age-matched controls and increased protein levels of c-MYC directly activated by miRNAs from the miR-17-92 cluster.

46. Pogribny et al. (2007) investigated the effect of a tamoxifen containing diet on the miRNA expression profiles in treated and age-matched control livers of F344 rats. 33 miRNAs (20 upregulated and 13 downregulated) were differentially expressed in the livers of tamoxifen treated rats ( $p < 0.05$ ) and a sustained alteration of miRNA expression occurred at both 12 and 24 weeks. No effect on miRNA expression was observed in the kidneys and mammary glands of tamoxifen treated and age-matched control rats. Analysis of the protein levels of confirmed targets of differentially regulated miRNAs induced by tamoxifen showed BCL2 (miR-16) and E2F1 (miR-20) levels in the liver of tamoxifen treated rats were decreased by 32% and 26% respectively compared to controls. They also found decreased levels of RB1 and NOTCH1 proteins by 50% and 45% respectively, in the livers of tamoxifen treated rats, which was associated with an upregulation of miR-106a and miR-34. These targets are involved in cell cycle, DNA replication, chromatin maintenance and apoptosis.

47. Yu et al. (2009) examined the relative expression of miRNAs in 7,12-dimethylbenz[a]anthracene (DMBA) induced oral squamous cell carcinoma (OSCC) in Syrian Hamsters using tri-weekly applications of a 5% solution of DMBA in acetone. They identified 5 miRNAs that were upregulated (miR-21, miR-200b, miR-221, miR-338, miR-762) and 12 miRNAs that were downregulated (miR-16, miR-26a, miR-29a, miR-124a, miR-125b, miR-126-5p, miR-145, miR-148b, miR-155, miR-199a and miR-203) in cancer tissues compared with normal tissues. Only three of the miRNAs (miR-338, miR-762 and miR-126-5p) identified by Yu et al (2009) have not previously been correlated with a cancer type.

48. Kalsheuer et al. (2008) examined the alteration in miRNA expression at the early stage of lung cancer development by obtaining miRNA expression profiles from the lungs of male F344 rats continuously fed the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) at a dose of 10ppm in drinking water for up to 20 weeks. The authors found that the majority of miRNAs maintained

their normal expression levels during NNK treatment. However, miR-101, miR-126\*, miR-199 and miR-34b were downregulated in NNK-treated rats compared to control rats. These results were confirmed by Northern Blots and qRT-PCR. To further understand the role of miRNAs in NNK-induced tumorigenesis, the authors used the miRBase computer program to predict targets of the 4 miRNAs identified. They found the human CYP2A13 gene to be predicted as a target by miR-126\*. The orthologue of CYP2A13 in rats is CYP2A3. In a reporter assay performed on transiently transfected 293T cells, miR-126\* repressed the expression of a firefly luciferase gene containing the 3'UTR of CYP2A3 mRNA. The authors identification of CYP2A3 as a potential target of miR-126\* suggest a mechanism by which NNK exerts its toxicity potential in the lungs.

49. Conzoles including triadimefon and propiconazole are antifungal agents and some are hepatotoxic and induce hepatocellular tumours in two year mice bioassays. Ross et al. (2010) investigated the effect of exposure to tumourigenic doses of triadimefon and propiconazole and exposure to the non tumourigenic myclobutanil on miRNA expression profiles in the livers of CD-1 male mice. They found that propiconazole and triadimefon induced alterations in the expression of 63 miRNAs and 28 miRNAs, respectively. Myclobutanil induced alterations in the expression of one miRNA. The expression of 19 miRNAs were altered by both propiconazole and triadimefon compared to control animals.

### **Epigenetic Carcinogens**

50. Hsu et al. (2009) investigated the effects of exposure of diethylstilbestrol (DES) (70 nmol/L) on the epigenetic alterations of miRNA in mammosphere-derived epithelial cells (MDEC) in vitro. Using microarray analysis of DES exposed MDEC cells and control cells, the authors found 9.1% of miRNAs were differentially expressed (37 upregulated and 45 downregulated). The findings were validated and confirmed for 10 randomly selected miRNAs by qRT-PCR. The authors further investigated the epigenetic characterisation of miR-9-3 as this miRNA was previously implicated in breast tumorigenesis (Iorio et al., 2005). In a series of experiments, Hsu et al. examined the role played by miR-9-3 in breast cancer. Levels of repressive chromatin makers (HBK27me3 and H3K9me2) were increased in MDECs after DES exposure, followed by increased DNA methyltransferase 1, causing an increase in DNA methylation of its promoter CpG islands. Further analysis demonstrated that 32.4% of primary breast tumours exhibited promoter hypermethylation in the upstream region of the CpG islands, whereas normal controls showed little or no methylation. This epigenetic event occurred preferentially in ER- $\alpha$  positive tumours. They also found that DES can activate an ER- $\alpha$ -mediated signalling pathway, causing epigenetic repression of miR-9-3 or, in the absence of hormone receptors, an epigenetic repression of miR-9-3 can be mediated via an ER- $\alpha$ -independent pathway. Functional analysis of miR-9-3 in a knockout study was used to determine the role of miR-9-3 silencing in breast tumorigenesis. Target genes of miR-9-3 were identified and included 70 upregulated genes and 32 downregulated protein coding genes. Further analysis identified 8 upregulated and 3 downregulated genes that were significantly associated with the p53 signalling pathway.

51. Shah et al. (2007) demonstrated that activated PPAR $\alpha$  was a major regulator of hepatic miRNA expression. Wild-type (WT) mice were fed either a control diet or a diet containing Wy-14,643, a specific PPAR $\alpha$  agonist, and microarray expression

profiling demonstrated that expression of 27 miRNAs was significantly changed following Wy-14,643 treatment. Shah et al. found that the PPAR $\alpha$  agonist WY-14,643 suppressed let-7C expression in WT mice but not in PPAR $\alpha$ -null mice, indicating that the repression was PPAR $\alpha$ -dependent. Basal levels of expression of let-7C inhibit c-MYC leading to inhibition of cell growth and cell cycle arrest. The overexpression of the c-MYC gene has been demonstrated to increase hepatic proliferation and increase the incidence of liver cancers. Let-7C was shown by Shah et al. (2007) to target c-MYC via interaction with the 3' untranslated region of c-MYC mRNA. The PPAR $\alpha$ -mediated induction of c-MYC via let-7C subsequently increased expression of the oncogenic miR-17-92 cluster. The authors suggest that collectively this pathway may lead to increased hepatic proliferation and tumourgenesis. Interestingly, these effects of Wy-14,643 were not apparent in PPAR $\alpha$ -null mice expressing the human PPAR $\alpha$  receptor transgene (knocked in).

52. Marsit et al. (2006) examined the *in vitro* effect of sodium arsenite,  $\gamma$ -irradiation and folate on the expression of miRNAs in human lymphoblast cell line TK-6. Arsenite exposure caused an increase in miRNA expression with 4 miRNAs exhibiting statistically significant upregulation. No significant alteration was observed following treatment with  $\gamma$ -irradiation. Folate deficiency caused a significant general increase in miRNA expression, with 22 miRNAs significantly upregulated. For example, miR-222 and miR-22 expression were increased in the *in vitro* study under folate deficient conditions. Returning the cells to full medium (no longer folate deficient), miRNA expression profiles did not differ from control cells. The authors also found statistically significant increased expression of hsa-miR-222 in human peripheral blood derived cells of low folate status individuals, but they did not observe a difference in the relative expression of miR-22 in the peripheral blood of subjects of differing folate status. MiR-18 and miR-210 did not show any significant alteration under folate deficient conditions in the *in vitro* study and similarly no significant alteration in relative expression of miR-18 and miR-210 was observed between subjects of differing folate status.

53. Schembri et al. (2009) determined the miRNAs expressed in a relatively pure population of bronchial airway epithelial cells from 10 current smokers and 10 never smokers and identified the miRNAs that are differentially expressed with smoking. Principle component analysis (PCA) of all 232 detected miRNAs demonstrated modest separation of subjects by smoking status and 28 miRNAs were found to be differentially expressed between current and never smokers ( $p \leq 0.05$ ). Of these 28 miRNAs, 23 were downregulated in smokers and the most significant altered miRNA was miR-218 ( $p = 5 \times 10^{-4}$ ), which was downregulated 4 fold in smokers. The other top differentially expressed miRNAs were miR-128, miR-500 and miR-181d. They found that many of the 28 differentially expressed miRNAs were highly inversely correlated with their predicted targets compared with all miRNAs and their predicted mRNA targets. Using a mRNA microarray expression dataset of normal bronchial epithelial cell line (NHBE), they found that there was significant over representation of miR-218 targets among the genes most upregulated by cigarette smoke exposure. In both NHBE and H1299 cells, increasing and decreasing miR-218 levels was sufficient to cause a corresponding increase or decrease in the expression of predicted miR-218 mRNA targets, respectively. Also, they found exposure of NHBE cells to cigarette smoke condensate (CSC) caused an almost 2-fold reduction in miR-218 expression levels. Modulation of miR-218 levels by CSC in NHBE cells contributes

to the smoking dependent regulation of MAFG (transcription factor) expression levels.

54. Using an *in vitro* system, Xi et al. (2010) examined miRNA alterations mediated by cigarette smoke condensate (CSC) in normal human respiratory epithelia (SAEC and NHBE), immortalised human bronchial epithelial cells (HBEC), lung cancer cell lines from smokers (Calu-6, H841 and H1299) and lung cancer cells from non smokers (H1650 and H1975). They reported that untreated Calu-6 and H841 cells exhibited higher endogenous levels of miR-31 relative to SAEC and NHBE cells. Treatment of SAEC and NHBE cells for 5 days with CSC upregulated miR-31 expression 5.5 fold and 3.6 fold, relative to controls, respectively. Calu-6 and H841 cells with CSC treatment increased miR-31 expression 3.1 and 6.5 fold respectively. A recent study by Corcoran et al. (2009) showed that the host gene for miR-31 is LOC554202. Using this information, Xi et al (2010) found that 5 day CSC treatment of SAEC cells induced expression of LOC554202 and this expression was maintained for 20 days following CSC removal. They authors suggest that these data all suggest that CSC exposure contributes to the upregulation of LOC554202 and miR-31.

55. Izzotti et al. (2009a) analysed miRNA alterations in the lung of Sprague Dawley rats following exposure to environmental cigarette smoke (ECS) for 28 days. The exposure was equivalent to a whole body exposure to smoke generated by 600 cigarettes/week. Using microarray analysis, exposure to ECS downregulated 126 miRNA 2 fold and 24 miRNA three fold including let-7, miR-10, miR-26, miR-30, miR-34, miR-99, miR-122, miR-1213, miR-124, miR-125, miR-140, miR-145, miR-146, miR-191, miR-192, miR-219, miR-222 and miR-223. These miRNA are known to regulate stress response, apoptosis, proliferation, angiogenesis and gene expression.

56. In a follow on study, Izzotti et al. (2010a) examined the role of orally administered chemopreventive agents (N-acetylcysteine, oltipraz, indole-3-carbinol, 5,6 benzoflavone and phenethyl isothiocyanate (singly or combined)) against miRNA alterations caused by ECS exposure in Sprague Dawley rats. Exposure to the chemopreventative agents either singly or in combination did not appreciably affect baseline miRNA profiles in the lungs. ECS exposure caused significant downregulation of miRNA as described in Izzotti et al (2009). The authors found that all the agents tended to upregulate miRNAs expression, thus counteracting the downregulation induced by ECS alone.

57. Izzotti et al. (2009b) determined the miRNA profiles in the lungs of variously aged CD-1 mice using microarray analysis. They also investigated the alterations of miRNA expression in the lung following exposure of various aged mice to ECS and/or UV-containing light. The authors found distinct age related variations in the baseline miRNA expression profiles in the lungs of the mice. UV light did not significantly affect miRNA expression in the lungs of the mice. However, exposure to ECS caused a significant reduction in 15 miRNA expression (let-7, miR-26a, miR-30b, miR-30c, miR-34b, miR-99b, miR-122a, miR-124a, miR-125a, miR-125b, miR-140, miR-192 and miR-431) compared to control mice. The microarray data was validated by analysing let-7f expression by qRT-PCR in the lungs of post-weanling males and were in agreement with the microarray analysis.

58. In a follow on study, Izzotti et al. (2010b) examined the role played by chemopreventive agents (budesonide (BUD) and phenethyl isothiocyanate (PEITC)) in both the liver and lung of control or ECS exposed neonatal CD-1 mice. The expression of 576 miRNAs were analysed by microarray in the livers and lungs of the mice. The authors found the baseline expression of miRNAs higher in the liver than the lung. Exposure of neonatal mice to ECS downregulated 43 miRNAs and upregulated 29 miRNAs in the lung and downregulated 90 miRNAs and upregulated 76 miRNAs in the liver. The effect of both chemopreventative agents on the baseline expression was negligible in the lung, whereas variations were observed in the liver. The prevailing trend by the two agents in both organs was a downregulation of miRNA expression (downregulation of 6 miRNAs in the lung and 23 in the liver). They found the agents counteracted the ECS-induced dysregulation in the mouse lung of let-7a, miR-26A, miR-31 and miR-382. BUD alone tended to restore miR-463 and PEITC alone tended to restore the expression of let-7c, miR-29b, miR-125b, miR-135b and miR-200b. However, in the liver, PEITC and in particular BUD exhibited a poor ability in counteracting the effects of ECS on miRNA expression and exhibited some adverse effects in the mouse liver.

59. All studies outlined thus far by Izzotti's group investigated miRNA expression as an early response to exposure to ECS and/or treatment with chemopreventative agents. To investigate effects at late stage carcinogenesis, Izzotti et al. (2010c) examined miRNA alterations when tumours were detectable following exposure to mainstream cigarette smoke (MCS) and chemopreventative agents by H strain mice. Analysis of 576 miRNAs showed that exposed MCS mice had extensively dysregulated miRNA expression profiles. Both chemopreventive agents (PEITC and NAC) efficiently inhibited MCS-induced miRNA alterations. The authors also reported specific miRNA signatures related to different diseases such as pneumonia, adenoma or bronchoalveolar carcinoma and how these profiles are affected by the histopathology of the disease state.

60. Izzotti et al. (2011) investigated the dose-responsiveness of miRNA dysregulation in groups of neonatal CD-1 mice exposed to increasing doses of MCS (0, 119, 292, 438 mg/m<sup>3</sup>) and evaluated the persistence of CS-induced miRNA alterations in the lung after smoking cessation. At the two lower doses (119 and 292 mg/m<sup>3</sup>), exposure had only a moderate effect on basal miRNA expression of miRNAs (ie only 2 and 12 downregulated, 2 and 3 upregulated more than 2 fold, for 119 and 292 mg/m<sup>3</sup> doses). 32 miRNAs were significantly altered by the highest dose. MiR-30 and let-7 were very sensitive to MCS downregulation. 5 miRNAs significantly upregulated at the highest dose were miR-153, miR-214, miR-301, miR-297 and miR-324. They found that let-7 expression, which was significantly downregulated by MCS, was restored one week after smoking cessation. However, a number of miRNAs that were downregulated at least 2 fold failed to recover to their basal expression levels after smoking cessation including miR-34b, miR-345, miR-421, miR-450b, miR-46b and miR-469. The authors note that on the whole, miRNAs appear to be less sensitive markers than bulky DNA adducts and 8-*oxo-dGuo* in revealing exposure at low MCS doses. Exposure at higher MSC doses resulted in alterations of DNA adducts and 8-*oxo-dGuo* levels and dysregulation of miRNAs, mainly in a downregulation trend.

61. Kasashima et al. (2004) investigated the effect of 12-o-tetradecanoylphorbol 13 acetate (TPA) induced differentiation of human leukaemia cells (HL-60) into monocyte/macrophage-like cells on miRNA expression. In the TPA-induced cells, they found increased expression of miRNAs including miR-21, Mir-23, miR-24 and miR-27a. They also reported downregulation of miR-17-5p, miR-142-5p, miR-142-3p and miR-320 following TPA treatment. They suggest the miRNA-induced control of cell differentiation in HL-60 cells may involve two mechanisms, one concerning gene silencing by upregulation of miRNA and one concerning gene activation by terminating miRNA-catalysed gene silencing by downregulation miRNAs.

62. Various studies outlined here have demonstrated that miRNAs are responsive to toxin treatment in the livers of experimental animals in vivo. In contrast, Moffat et al (2007) found limited hepatic response of miRNAs following exposure to 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD). Using microarray techniques and qRT-PCR, Moffat et al. (2007) determined miRNA expression levels in WT-type (WT) versus AhR null-mice, dioxin sensitive rats versus dioxin-resistant rats and hepatoma cells in culture. They found 45 miRNAs and 17 miRNAs were responsive to TCDD (max. 2.5 fold TCDD effect), in the mouse and rat models respectively. Only 8 miRNAs were altered by TCDD in both species (miR-203, miR-20a, miR-212, miR-373, miR-422B, miR-485-3P, miR-498, and miR-542-5p). In the mouse Hepa-1-cell line, candidate miRNAs were unresponsive to TCDD with the exception of miR-122a, which was increased in the Hepa-1 mouse cell and miR-203 which was increased in the rat 5L cells. They found that AhR genotype did not have a significant effect on hepatic miRNA levels in either TCDD treated animals or in constitutive expression (only some miRNAs differed in expression between AhR-null mice compared to WT mice or between L-E rats (WT AhR) compared to Han Wistar rats). Overall, Moffat et al. (2007) results suggest that down-regulation of mRNA levels by dioxins in adult rodent livers is unlikely to involve miRNAs.

### **Chemical Induced Carcinogenesis**

63. Shen et al. (2009) investigated the miRNA expression profile in human bronchial epithelial cells (16 HBE cells), following treatment with a metabolite of benzo[a]pyrene, anti-benzo[a]pyrene-7,8-diol-9,10 epoxide (anti-BPDE). They found that miRNA expression in 16HBE-treated cells as markedly different from the 16HBE-control cells. 54 miRNAs were differentially expressed (45 were upregulated (>2.0 fold), while 9 miRNAs were down-regulated (<0.5 fold) in 16HBE-related cells. The most highly expressed miRNAs in 16HBE-treated cells were miR-494, miR-320, miR-498, miR-129 and miR-106a (22.04, 17.78, 16.23, 12.12, and 11.20 –fold change respectively). The lowest expression of miRNAs was observed for miR-109, miR-493-5P, AND miR-363. The miRNA microarray data was validated by qRT-PCR for two miRNAs, miR-10a and miR-320 and the qRT-PCR results were consistent with the microarray data. The authors also performed a predicted target analysis for miR-10a, chosen as it has previously been associated with human cancer and one of its predicted targets, HOXA1, maybe be involved in oncogenicity. They found that HOXA1 mRNA was upregulated 3-9 fold in 16HBE-treated cells compared to control 16-HBE cells.

64. Shen et al. (2009) reported that the most highly expressed miRNAs following treatment of human bronchial epithelial cells with anti-BPDE were miR-320 and miR-494. Using this information, Duan et al. (2010) investigated the roles of miR-320 and

miR-494 in cell cycle control of primary murine bronchial epithelial cells exposed to B[a]P. They found that time dependent increases in miR-320 and miR-494 expression were observed in treated cells exposed to 1  $\mu$ M B[a]P for 24 hours. They also found that inhibitors of miR-320 and miR-494 induced a relief to G1 arrest caused by B[a]P treatment and caused upregulation of CDK6 in the B[a]P-treated primary murine bronchial epithelial cells. The authors suggest that aberrant expression levels of miR-320 and miR-494 regulating B[a]P exposed cell-cycle progression, may affect G1/S transition through CDK6.

65. Using the finding from Shen et al. (2009) that the most highly expressed miRNAs was miR-494 with a 22.04 fold increase following treatment of 16HBE cells with anti-BPDE, Liu et al. (2010) determined the expression pattern of miR-494 and PTEN in anti-BPDE induced 16HBE cells. They examined whether miR-494 plays a role in the regulation of PTEN expression. They detected both miR-494 and PTEN expression in both treated and control 16HBE cells passaged 15 and 30 times and found an inverse trend between miR-494 and PTEN protein expression with no observable change in mRNA levels. For cells passaged 15 times, miR-494 expression was lower in 16HBE treated cells than 16-HBE control cells (16HBE-15T versus 16HBE-15C), but was significantly upregulated (12.6 fold) in 16HBE treated compared to 16HBE control cells passaged 30 times (16HBE-30T versus 16HBE-30C). PTEN protein levels in 16HBE-15T cells slightly increased, while PTEN protein levels in 16HBE-30T cells were significantly reduced by  $40.0 \pm 12.5$  %. They performed transient co-transfections with an anti-miR-494 (or a pre- miR-494) with a luciferase vector containing the wild-type (WT) or mutated (MT) putative binding sites for miR-494 in 16HBE-30T cells to determine whether PTEN is a putative target for miR-494. They found that expressing WT vector in 16HBE-30T (high endogenous miR-494) generated lower luciferase activity than it did in 16HBE-30C cells (low endogenous miR-494) and in 16HBE-30T cells, the WT vector also generated a lower luciferase activity than the MT vector. They suggest that these results indicated that high endogenous miR-494 represses the expression of the WT vector, indicating that Mir-494 may regulate PTEN expression. They also examined the effect of miR-494 on cell apoptosis and found that increased miR-494 expression decreased caspase 3/7 activity and vice-versa. Also miR-494-expressing cells showed decreased motility in comparison to control cells as determined by the wound scratch healing assay. The malignancy stage of tumour development was also tested and inhibition of miR-494 decreased the malignancy of cells. The authors suggest that all their findings between the expression of miR-494 and PTEN support the computational analysis results that PTEN may be a target of miR-494.

66. Liu et al. (2010) examined the miRNA expression in anti-BPDE transformed 16HBE and report on the regulatory effect of miR-22 on PTEN expression in transformed cells. QRT-PCR analysis showed that miR-22 was significantly upregulated by 2.3 fold in 16HBE-treated cells. PTEN mRNA levels were not significantly different between control and transformed cells. PTEN protein levels in 16HBE-treated cells were significantly reduced by 40%. The protein levels of PTEN and the expression of miR-22 were inversely correlated (overexpression of miR-22 lead to a decrease in PTEN levels by 48% in 16HBE-treated cells). In dual luciferase assays, the authors report that high expression of miR-22 decreases luciferase activity of pgl3 containing the putative WT-binding site and inhibition of miR-22 show the reverse results. They also examined the effect of miR-22 on cell apoptosis and found

that increased miR-22 expression decreased caspase3/7 activity and miR-22-expressing cells showed decreased motility in comparison to control cells as determined by the wound scratch healing assay. Hoescht 33258 staining for DNA fragmentation showed that inhibition of miR-22 in 16HBE-treated cells increased the number of apoptotic cells compared to 16HBE control cells. In cell growth assays, miR-22 also decreased colony formation on soft agar. The authors suggest that the relationship between miR-22 and PTEN from their results support the computational analysis that suggests PTEN is a target of miR-22.

67. Yauk et al. (2010) exposed male mice to B[a]P at a dose of 150 mg/kg/d for 3 days by oral gavage and investigated the correlation between hepatic mRNA and miRNA response to B[a]P. They found that exposure to B[a]P caused a large response in liver gene expression (>400 differentially expressed) but no changes were observed for miRNA expression, as analysed by DNA microarray.

68. Estradiol (E2) regulates gene expression at the transcriptional level by functioning as a ligand for estrogen receptors (ER $\alpha$  and ER $\beta$ ). Bhat-Nakshatri et al. (2009) examined whether E2 regulates miRNA expression in luminal type A/ER $\alpha$ -positive breast cancer cells and whether the E2-regulated miRNAs subsequently control the expression of E2-regulated genes post-transcriptionally. They found that E2 upregulated 21 miRNAs (including Let-7 and miR-21 which can depress levels of c-MYC and E2F2 proteins) and downregulated 7 miRNAs in MCF-7 cells. They also reported that E2 induced expression of DICER, an enzyme required for miRNA processing.

69. Vinylcarbamate (VC) is a potent lung carcinogen that induces malignant lung tumours and pulmonary surface tumours. Melkamu et al. (2010) investigated the effect of vinylcarbamate on miRNA expression profile in the lungs of A/J mice and assessed whether dietary administration of the chemopreventative agent indole-3-carbinol (I3C) can modulate miRNA expression in vinylcarbamate treated mice. Microarray analysis, confirmed by qRT-PCR, found 5 miRNAs (miR-21, miR-31, miR-130a, miR-146a and miR-377) increased and 2 miRNAs (miR-1 and miR-143) decreased by  $\geq 2$  fold in lung tumour tissue of VC-treated mice compared with normal lungs from vehicle-treated mice. Dietary treatment with I3C reversed the effect of VC by downregulating miR-21, miR-31, miR-130a and miR-146b, miR-377 in I3C treated and VC treated mice compared to VC only treated mice. Prediction analysis of miR-21 targets identified 6 tumour suppressor genes, sprouty homolog 2, TGFB, PDCD4, PTEN, tropomyosin 1 and RECK. Downregulation of PTEN (not significant), PDCD4 (significant) and RECK (significant) mRNA was observed between vehicle treated mice and lung tumours from VC and VC-I3C-treated mice with treatment with I3C significantly upregulating the mRNA levels of both PDCD4 and RECK.

70. In an oral feeding study, Zhang et al. (2009) found RDX (5 mg/kg for 28 days) induced significant changes in the miRNA expression profile in the brain and livers of female B6C3F1 mice. They found 113 miRNAs significantly altered following exposure to RDX. The expression level of 10 miRNA (miR-99a, miR-30d, miR-30e, miR-22, miR-194, miR-195, miR-15a, miR-139-5p and miR-101b) were significantly increased in both mouse liver and brain ( $p < 0.01$ ). Three miRNAs (miR-762, miR-425-5p and miR-185) had expression levels significantly decreased in both brain and

liver. They confirmed the findings from the microarray analysis using qRT-PCR for 4 miRNAs (miR-206, miR-200c, miR-27a and let-7c).

## **Conclusion**

71. Studies in recent years have suggested that miRNAs play an important role in the mechanisms of tumorigenesis, although the precise contribution of individual RNA molecules and RNA regulated processes on cancer development and progression needs further investigation. Some miRNAs may be directly involved in cancer development by controlling cell differentiation and apoptosis, while others may be involved in cancers by targeting cancer oncogenes and/or tumour suppressors. It is thought that understanding the function of miRNAs will provide new insights with regard to the molecular basis of cancers, and new biomarkers for cancer diagnoses and cancer therapy.

72. This review has also highlighted that several miRNAs can regulate the same gene. The interaction between these miRNAs and what mechanisms control these miRNAs for targeting the same gene are still unclear. Evidence from the studies available in the literature indicates that miRNAs are regulated by epigenetic processes and act as regulators of the epigenome. However, further studies are needed to shed light on the full magnitude of interactions between miRNAs and the epigenetic machinery.

73. Over the past two years, there has been a number of publications indicating a role of environmental chemicals in the induction of miRNAs expression and thus affecting the mechanism by which miRNAs affect tumorigenesis. This field of research will more than likely grow and give us further insight into the mechanisms by which chemicals can alter miRNA expression.

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Figure 1. MiRNA biogenesis (taken from Cullen (2005))

