“Using liver miRNA profiles to predict chemical hepatocarcinogenesis”

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ABSTRACT

Industrial, agricultural, and pharmaceutical requirements drive the development of a plethora of new chemical entities each year, many of which -for example drugs, pesticides, and food additives- have to be assessed for potential human health hazard. The current benchmark for risk assessement is the lifetime rodent bioassay which is expensive, time-consuming, laborious, requires the sacrifice of numerous animals, and is often irrelevant to humans. Hence alternative strategies to the rodent lifetime bioassay for prediction of chemical carcinogens are being pursued, especially for the liver which is an organ frequently affected by exogenous chemicals due to its detoxifying and metabolic roles. Numerous studies in recent years support the important role of microRNAs in cancer development, including hepatocellular carcinoma. The principal hypothesis of this project was that hepatic microRNA signatures can contribute to the earlier prediction of chemical hepatocarcinogens. Examination of livers from male Fischer rats treated with six chemical hepatocarcinogens, with diverse mode of actions for 90 days revealed that all the tested hepatocarcinogens affected the liver miRNAome from that early stage. Interestingly, a small set of microRNAs were identified whose expression was frequently deregulated by the hepatocarcinogens. Bioinformatic analysis indicates that these microRNAs can regulate pathways which are important in hepatocellular carcinoma. A more detailed investigation of one of those hepatocarcinogens, phenobarbital, showed that its effects on liver microRNAs were both dose and time dependent, with a progressive induction of specific microRNA clusters.Thus this study was the first to investigate in depth the effects of chemical hepatocarcinogens on the liver miRNAome and supports the potential usefulness of hepatic microRNA signatures in risk assessment.

Statement of Originality

I affirm that all the work presented in this thesis is my own original work, unless otherwise stated. Where the work of others has been used, it has been dully acknowledged.

Costas Koufaris

April 2011

"Σα βγεις στον πηγαιμό για την Ιθάκη,

να εύχεσαι νάναι μακρύς ο δρόμος,

γεμάτος περιπέτειες, γεμάτος γνώσεις…"

"As you set out on the way to Ithaca,

wish that the road is a long one,

full of adventures, full of knowledge..."

Constantine P. Cavafy

Dedication

To my parents

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List of Abbreviations

Abbreviation	Meaning
2-AAF	2-Acetyl aminofluorene
AHF	Altered Hepatic Foci
ANOVA	Analysis of Variance
BP	Benzophenone
CAR	Constitutive androstane receptor
Chl. Ac	Chlorendic Acid
CTR	Control technical replicates
Cy-3	Cyanine 3-pCp
CYP	Cytochrome P450
CYP2B	cytochrome 450 type 2b
DEHP	Diethylhexylphthalate
DEN	Diethylnitrosamine
DETU	Diethylthiourea
DGCR8	DiGeorge Syndrome Critical Region 8
DNMT	DNA methyltransferase
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial to mesenchymal transition
EGF	Epidermal growth factor
FDR	False Detection Rate
FE	Feature extraction
FIG	Figurre
FOXO	Forkhead box other

List of Abbreviations

Abbreviation	Meaning
GJIC	Gap junctional intercellular communication
GNTX	Genotoxic
HCC	Hepatocellular carcinoma
HNF4α	Hepatic nuclear factor-4 alpha
HSCs	Hepatic stellate cells
KC	Kupffer cells
LI	Labelling Index
MTD	Maximum Tolerated dose
mRNA	Messenger RNA
MP HCL	Methapyrilene HCl
miRNA	microRNA
MOA	Mode of Action
MON	Monuron
NGTX	Non-genotoxic
NOAEL	No observable adverse effect level
NTP	National Toxicology Program
NK	Natural killers
PAGE	Polyacrylamide gel electrophoresis
PAM	Prediction Analysis of Microarrays
PB	Phenobarbital
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
piRNAs	Piwi-interacting RNAs

List of Abbreviations

Abbreviation	Meaning
PPARα	Peroxisome proliferator activated receptor alpha
PPC	Peroxisome proliferator compounds
ppm	Part per million
pre-miRNA	Precursor microRNA
pri-miRNA	Primary-microRNA
PCA	Principal Component Analysis
PTR	Phenobarbital technical replicates
PXR	Pregnane X receptor
QC	Quality Control
qPCR	Quantitative PCR
RIN	RNA integrity number
RLM	RNA ligation mediated
RACE	Rapid Amplification of cDNA Ends
Rb	Retinblastoma
ROS	Reactive oxygen species
RNS	Reactive nitrogen species.
RISC	RNA activated silencing complex
SAM	Significance analysis of microarrays
SDS	Sodium dodecyl sulfate
SEC	Sinusoidal endothelial cells
SiRNA	Small interfering RNA
TAE	Tris-Acetate-EDTA
TCPOBOP	1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene

List of Abbreviations

Abbreviation	**Meaning**
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
TE	Tris- EDTA
TGF	Transforming growth factor
TSS	Transcriptional start site
UTR	Untranslated region
Zeb1	Zinc finger E-box-binding homeobox 1
Zeb2	Zinc finger E-box-binding homeobox 2
Wy-14,643	4-chloro-6-(2,3-xylidino)-pyrimidynylthioaceticacid

Chapter 1: Introduction

1.1 Mechanisms of carcinogenesis

1.1.1 Carcinogenesis is a multi-step, progressive process

The term cancer describes more than 100 types of the disease with distinct cellular origins, symptoms, prognosis, and treatments. Nevertheless, all cancer cells are fundamentally different in their cellular physiology from normal cells. Hanahan and Weinberg initially identified six key hallmarks which are common to cancer cells (Hanahan & Weinberg, 2000, Hanahan & Weinberg, 2011):

(**1**) **Self-sufficiency in growth signals**. All normal cells require stimulation by extra-cellular growth factors to initiate cell division. A necessary step for carcinogenesis is the acquired ability of pre-malignant and malignant cells to undergo abnormal, excessive proliferation in the absence of the appropriate mitogenic signals.

(**2**) **Insensitivity to anti-growth signals**. Tissues abound with anti-proliferative signals which maintain tissue homeostasis by suppressing proliferation. Incipient cancer cells acquire the ability to proliferate even in the presence of such growth-suppressive signals.

(**3**) **Evasion of apoptosis**. Apoptosis is the instigation of a program of cell death in a cell, when given the appropriate internal or external stimuli. Cancer cells are characterized by a resistance to the external and internal stimuli which normally stimulate apoptosis.

(**4**) **Limitless replicative potential**. Normal cells can only replicate a limited number of times before reaching the Hayflick limit and entering a state of senescence. The

appearance of tumours requires the acquisition by cancer cells of the ability for unlimited replication.

(**5**) **Sustained angiogenesis**. As tumours grow in size existing capillaries are not sufficient to supply the oxygen and nutrients needed by the growing tumour. In response to this problem cells within lesions of abnormal growth acquire the ability to trigger angiogenesis, thus increasing the supply of required substances.

(**6**) **Tissue invasion and metastasis**. Aberrantly proliferating cells may not be harmful so long as they are contained. These are benign tumours. Eventually a fraction of the benign tumours will progress to become invasive (malignant) cancers which possess the ability to invade adjacent tissues. Invasive cancer cells can eventually become metastatic and invade other organs through the lymphatic system.

Besides these six essential hallmarks, two other acquired biological capabilities of cancer cells that may be essential for the development of tumours are the abilities to evade the immune system and to limit their metabolism to glycolysis (Hanahan & Weinberg, 2011).

Given the detrimental effects of cancers, long-lived animals such as mammals possess multiple safeguards to prevent their occurrence. Therefore no single factor can cause cancer. Rather, the development of all cancers requires multiple independent events (Hanahan & Weinberg, 2001, Vogelstein & Kinzler, 2004). Simultaneous occurrence of these multiple changes needed for the transformation of a specific cell from a normal to a transformed state is too improbable to occur. Instead cancers probably

originate in a process comparable to Darwinian evolution, whereby cancer cells gradually develop from a single original cell in a series of distinct steps (**Figure 1.1**).

Figure 1.1 *Clonal evolution of cancer cells*

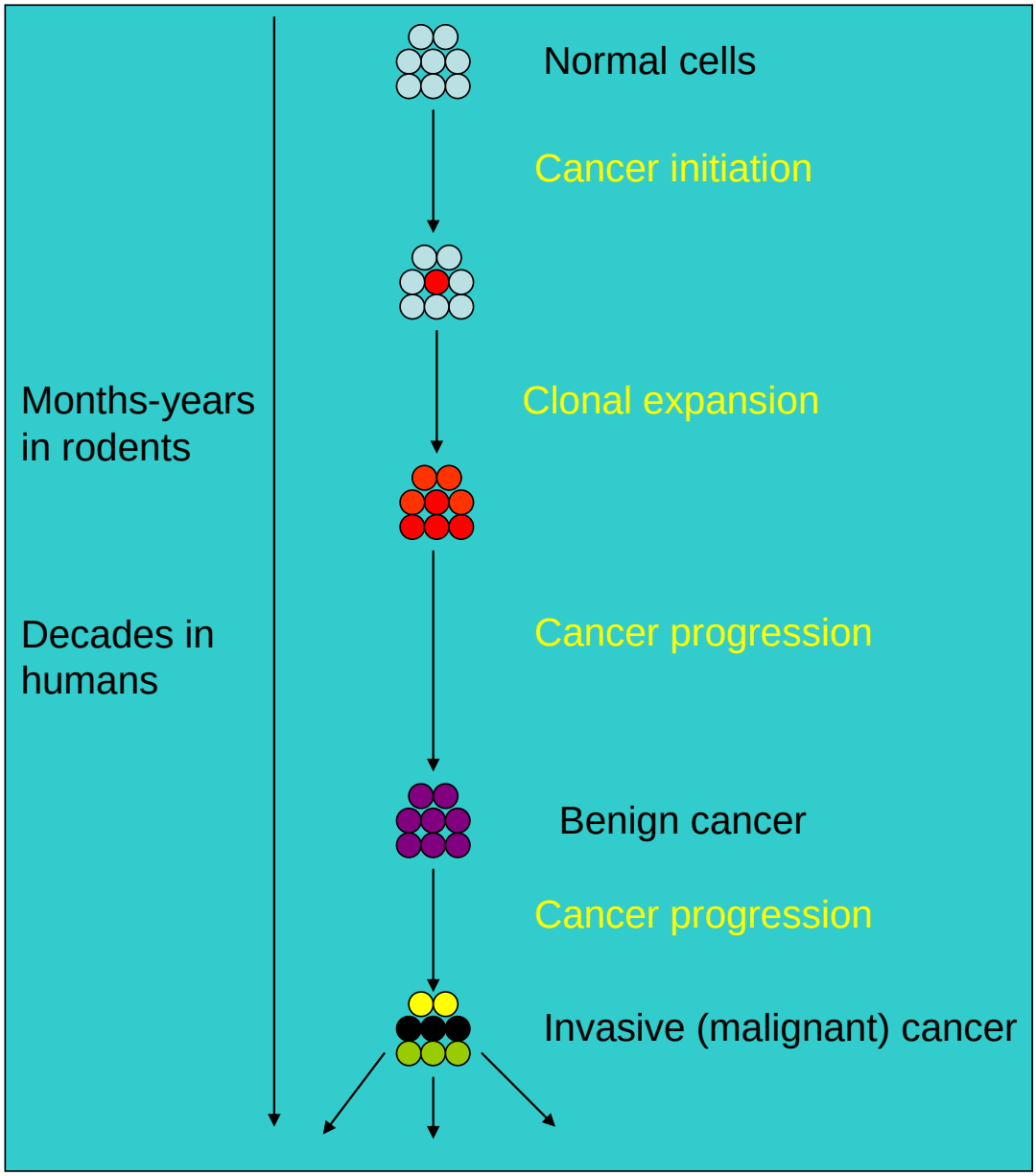


Fig. 1.1 Cancer is initiated after acquisition by a cell (usually an epithelial type) of a selective advantage (Cancer initiation). This pre-cancerous cell then undergoes clonal expansion. During cancer progression the initiated cell is transformed to a benign and then a malignant cancer cell through repeated acquisition of new traits followed by clonal expansion. The tumour is heterogeneous made up from subclones originating from the parental cancer cell. The process can take years in rodents and decades in humans.

Initiation of cancer occurs with the acquisition by a replication-competent cell of a selective advantage compared to normal cells e.g. an increased rate of cell division. Consequently clones of this altered cell will outcompete normal cells and become more abundant in their resident tissue. Progression of the initiated cell to more cancerous states occurs through repetitive rounds of this evolutionary process: acquisition by descendants of the original cell of new selective advantages followed by clonal expansion. Eventually this iterative process results in the generation of full-blown malignant cancer (Nowell, 1976). Multiple facts support this multi-step model of carcinogenesis. First, within tissues lesions represent intermediate steps from normal to pre-malignant to invasive cancers. Second, mathematical models of cancer incidence throughout a human lifetime suggest that at least 4-7 events are required for cancer development. Third, transformation of cells *in vitro* to a cancer state requires altered activity of multiple genes. Fourth, germline mutations cause a predisposition to cancer, not definite cancer appearance. Fifth, transgenic mouse models also support the requirement for multiple steps before the appearance of tumours (Hanahan & Weinberg, 2000).

Cancer initiation and progression is driven by deregulation of cancer genes. Most mammalian genes are irrelevant for cancer. Cancer genes are the small subset of animal genes which drive tumour formation and growth. So far about 400 cancer genes have been identified (Stratton *et al.*, 2009, Futreal *et al.*, 2004). In normal cells most of these cancer genes are part of the normal cellular regulatory networks controlling cell division, apoptosis, and homeostasis(Hanahan & Weinberg, 2000). Cancer genes are classified into two categories based on the functional effects of their deregulation: oncogenes and tumour-suppressor genes. Oncogenes are inappropriately activated in cancers in order to facilitate tumour growth. Usually inappropriate

activation of a single allele of an oncogene is sufficient to confer a selective advantage to a cell. For example activated Ras may stimulate cell proliferation while activated MDM2 inhibits cell death of tumour cells (Croce, 2008). Conversely for tumour-suppressors genes it is the loss of their activity which contributes to the progression of the disease. For tumour-suppressor genes it is normally required that both alleles are inactivated in a cancer cell for the effect to be observable. Two tumour suppressor genes that are frequently inactivated in tumours are *p53* and retinoblastoma (*Rb*). Loss of *P53* makes cancer cells more resistant to apoptosis while loss of *Rb* allows cells to be more resistant to anti-growth signals (Sherr, 2004).

Ultimately cancer genes contribute to tumour development *via* the cellular pathways, which they control. Inappropriate activation of oncogenes and/or loss of tumour-suppressor genes results in deregulation of key pathways and acquisition of the cellular traits essential to cancer development. Several such pathways have been demonstrated to be implicated with cancer, including the Rb, P53, PI3K, APC, HIF1, Gli, and Smad pathways (Vogelstein & Kinzler, 2004). Since cellular pathways consist of many genes, they can be disrupted by the deregulation of any one of several cancer genes. For example the Rb pathway can be blocked either by inactivation of Rb and $p16^{INK4}$ or activation of CDK4 and Cyclin D1. Indeed deregulation of all these four genes has been found in multiple cancers and appears to follow an "exclusivity principle" (Vogelstein & Kinzler, 2004).

"Gatekeeper" pathways are those that when disrupted within the appropriate cell initiate tumourigenesis. The gatekeeper pathways are different between tissues e.g. the Rb1 is a gatekeeper for retinoblastoma, but in colon cancer that role is performed by the APC pathway. For many tissues the gatekeeper pathway is not known. It is still

not clear whether some tissues may have multiple gatekeeper pathways or just one(Vogelstein & Kinzler, 2004).

Besides the different gatekeeper pathways, the process of carcinogenesis is variable between different cell types in other ways as well. For example, it has been found that mutations of the same cancer genes can occur at different stages of cancer development in different tissues. Consequently the timing of carcinogenic traits associated with those cancer genes also differs between cancers e.g. one tumour type may acquire resistance to apoptosis at a much earlier stage than other tumour types. Furthermore, cancer genes can have distinct effects in different cellular contexts. Activation of *KRAS2* initiates the neoplastic process in pancreatic duct, but not in other tissues. Hence, a given cancer gene may be implicated in some tumour types, but not others (Hanahan & Weinberg, 2000, Vogelstein & Kinzler, 2004).

The molecular processes underlying carcinogenesis also seem to be partially different between liquid tumours (e.g. leukaemia) and solid tumours (e.g. liver cancer). Solid tumours appear to require several mutations in order to develop malignant tumours, while liquid tumours perhaps only need one or two. Moreover, for liquid tumours chromosomal translocations that activate oncogenes are much more common than for solid tumours. It is probable then that the genesis of liquid tumours may follow quite different pathways from that of solid tumours(Vogelstein & Kinzler, 2004).

1.1.2 Genetic and epigenetic mechanisms of gene disruption in cancer

All normal somatic cells of an individual contain copies of the same genome. By some manner the functioning of cancer genes and of the pathways which they control must be disrupted in cancer cells for tumours to form. Two fundamentally different mechanisms exist by which this can occur: genetic mutations and epigenetic defects. Genetic mutations permanently modify genes by altering their DNA sequence. In cancer genetic mutations activate oncogenes and deactivate tumour suppressor genes. Occasionally mutated cancer genes are germ-cell transmitted and underlie the predisposition of related individuals to familiar cancers (e.g. *BRCA1* to breast cancer and *Rb1* to retinoblastomas) (Vogelstein & Kinzler, 2004). In most cases though, cancer genes are disrupted within the somatic cells of individuals. The recent deployment of the powerful DNA sequencing technologies to the sequencing of tumour genomes has confirmed the wide-spread occurrence of somatic mutations in cancer genes. One study sequenced a malignant melanoma cell and a lymphoblastoid cell line (representing germline DNA) originating form the same individual (Pleasance *et al.*, 2010a). They identified more than 30000 somatic base substitutions in the melanoma, affecting 292 coding genes and 319 non-coding genes. A second study used the same approach for a small-cell lung carcinoma cell line and a lymphoblastoid cell line derived from a different patient (Pleasance *et al.*, 2010b). They identified more than 22000 somatic mutations including 134 in coding genes and 182 in non-coding genes. It is expected that the mutated genes in these cancers represent both “passengers” as well as “drivers” i.e. mutations that are by-products of carcinogenesis, as well as those which were crucial to the formation of the tumour.

Gene mutations can be subtle or prominent. Subtle mutations include changes of a single nucleotide base (point mutations) or small insertions and deletions within a

gene. Missense mutations (change in a single nucleotide resulting in amino acid substitution) can affect normal functioning of a protein. One of the most commonly mutated genes in several cancer types is *BRAF*, resulting in stimulation of aberrant tumour growth. Oncogenic mutations for *BRAF* are missense mutations within its kinase domain that disrupt its molecular structure rendering it constitutively active (Wan *et al.*, 2004). Nonsense mutations (change in a nucleotide causing a premature end in protein chain) and frameshift mutations (changes in reading frame) alter large parts of proteins. For example *APC* is a tumour suppressor gene that is often inactivated in cancers by nonsense and frameshift mutations (Samowitz *et al.*, 2007). Alternatively, mutations in cancer can be much more prominent such as deletions of large chromosomal regions, translocations of genes to new locations, and copy number amplification. *MDM2* is an oncogene that acts to repress apoptosis by tagging P53 for degradation and blocking its activation. In several cancers the gene for *MDM2* is amplified so that several functional copies are present that can target P53(Oliner *et al.*, 1992). Moreover, gross chromosomal abnormalities are found in all solid tumours. This results in loss of heterozygocity, with a large fraction of alleles being lost in cancer cells (Duesberg, 2003).

One interesting hypothesis is that acquisition by cancer cells of genetic instability - and hence increased mutation rate- is required to allow the acquisition of sufficient number of mutations for cancer appearance within a lifetime (Loeb, 1991). In favour of this "mutator phenotype" idea some hereditary cancer genes are involved in genetic instability, e.g. *ATM* and *BRCA1* (Vogelstein & Kinzler, 2004). Many cancers also possess somatic mutations of genes that are involved in sensing DNA damage and in coordinating proper chromosomal segregation (Lengauer *et al.*, 1997). The matter of whether genetic instability is a side-effect, a contributing factor, or absolutely

essential to the process of cancer formation is still being debated. Either way, whether sufficient mutations to cause cancer arise in a cancer cell as a result of the natural mutation rate or require the prior acquisition of a mutator phenotype, genetic mutations have an essential role in cancer.

Unlike genetic mutations, epigenetic mechanisms regulate the expression of genes in a mitotically heritable manner without affecting their DNA sequence. Epigenetic regulation of genes is fundamental for cell development, differentiation, and maintenance. For example hepatocytes and nerve cells express different genes even though their genomes are identical, due to differential epigenetic regulation. The basic units of chromosomes are nucleosomes, DNA wrapped around an octomer core of histone proteins. Gene expression is epigenetically regulated through alterations of this basic unit by methylation of DNA, covalent modification of histone, and non-covalent nucleosome repositioning and utilisation of histone variants (Berger, 2007). DNA methylation is the addition of methyl groups on cytosine residues found in CpG nucleotide duplets. In mammals CpG sites are disproportionally concentrated in the genome in regions called CpG islands, usually found close to the 5' end of genes. A large fraction of human promoters contain CpG islands which can permanently silence gene transcription when methylated (Larsen *et al.*, 1992). Various forms of covalent modifications of histones exist including acetylation, methylation, SUMOylation, ubiquitylation, and phosphorylation. Modifications of histone can either activate or repress associated genes according to the modification and type of residue affected. The positioning of nucleosomes affects the ability of transcription factors to bind to DNA and activate transcription (Jiang & Pugh, 2009). The presence of alternative histone variants in the nucleosome can also affect the rate of gene transcription (Santenard & Torres-Padilla, 2009).

Just as for genetic mutations, defects in the epigenome have been implicated in cancer (Feinberg, 2007, Jones & Baylin, 2007, Sharma *et al.*, 2010). Cancer genomes are characterized by genome wide hypomethylation and hypermethylation of specific genes. Hypomethylation results in the activation of oncogenes, while hypermethylation results in silencing of tumour suppressor genes. For examples *Rb* is silenced in cancers by hypermethylation (Greger *et al.*, 1989) while *PAX2* expression is induced by tamoxifen in ovarian tissues stimulating proliferation and ovarian carcinogenesis by hypomethylation (Wu *et al.*, 2005). Loss of global methylation can also contribute to carcinogenesis by causing genomic instability. Methylation is responsible for silencing repeated DNA sequences. Global loss of methylation for example results in activation of transposable elements which cause insertional mutations. Repetitive DNA sequences also promote recombination resulting in chromosomal aberrations and translocations (Wilson *et al.*, 2007). Cancer cells also exhibit widespread changes in their histone acetylation and methylation patterns. Changes in the nucleosomal positioning in the promoters of genes have also been linked with inappropriate gene regulation in cancers. The SWI-SNF complex is an ATP-dependent chromatin remodeling complex made up of 8-11 components that regulate gene expression by modifying the nucleosome into a conformation that facilitates the initiation of transcription. This complex has an important role in the regulation of cell division and differentiation. Loss of several protein components of the SWI-SNF complex occur in cancer and are involved in the cancer process (Reisman *et al.*, 2009).

To summarise, both genetic and epigenetic mechanisms are involved in deregulation of genes in cancers. In certain instances the two mechanisms are alternative methods

of deregulating cancer genes e.g. in colon cancer the *APC* gene is the gatekeeper that can be silenced either by genetic mutations or by hypermethylation. Examination of colorectal tumours reveals that the two processes are almost mutually exclusive, presumably because only one is needed to silence the gene (Esteller *et al.*, 2000). In other cases though genetic and epigenetic processes can cooperate e.g. in some primary tumours one *p16* allele is silenced by methylation while the other is lost by chromosomal deletion (Merlo *et al.*, 1995). In certain cases epigenetic defects of a pathway in cancer predate the genetic mutations and it has been suggested that they may predispose cells to accumulate genetic mutations in the same pathways to drive tumour progression (Baylin & Ohm, 2006).

1.1.3 Mechanisms of carcinogenesis by exogenous chemicals

It has been recognised for a long time that cancers can have either endogenous or exogenous instigators. Cancers with endogenous origins are driven by the natural biochemical and metabolic cellular processes. Conversely, cancers with exogenous origins are induced by environmental agents that increase the incidence of tumours in their target organ(s). The relative contribution of endogenous and exogenous sources on the incidence of cancers in the human population has involved considerable debate (Shuker, 2002, Irigaray & Belpomme, 2010, Loeb & Harris, 2008). Ultimately, it can be deduced from the multi-stage progressive model of carcinogenesis that both endogenous and exogenous carcinogenic processes have to act by generating epigenetic and genetic defects in key pathways of incipient cancer cells. Endogenous sources of genetic mutations of cancer genes which arise spontaneously in cells are well understood and accepted. These include the depurination or deamination of DNA bases, the misincorporation of nucleotide bases during DNA replication, and damage

of DNA by metabolically generated reactive intermediates such as free radicals (Jackson & Loeb, 2001). At present the mechanisms which control and maintain the epigenetic status of cells are not well understood, hence it is not clear how they arise. Nevertheless, it is possible that spontaneous epigenetic errors may drive cancer initiation and maintenance (Wilson *et al.*, 2007).

Besides the endogenous causes of cancer, hundreds of exogenous carcinogenic agents are currently known, including ionizing radiation, biological agents, and chemical substances. Chemical carcinogens are particularly worrisome due to the increased exposure of humans to compounds through the environment, workplace, and as medications. Therefore, a considerable effort has been invested into studying the mechanisms of chemical carcinogenesis. Various different mechanisms by which chemical carcinogens may induce cancers have been proposed (Irigaray & Belpomme, 2010, Butterworth *et al.*, 1995, Williams, 2001, Silva Lima & Van der Laan, 2000, Bombail *et al.*, 2004, Hernandez *et al.*, 2009).

One commonly used classification of carcinogenic mechanisms is genotoxic (GNTX), also known as mutagenic and DNA-reactive, and the non-genotoxic (NGTX), also known as non-mutagenic and non-DNA reactive. GNTX compounds contain chemical groups, or are chemically modified to contain active chemical groups, which interact and damage DNA. The majority of GNTX carcinogens are procarcinogens which have to be converted first to DNA-reactive chemicals by metabolic processing. (Nebert & Dalton, 2006). On the other hand, NGTX mechanisms of carcinogenesis do not rely on direct damage of DNA. Instead NGTX carcinogens act by either

increasing indirectly the mutation rate in target cells or by facilitating the expansion of initiated cancer cells (**Table 1.1**).

Table 1.1 Postulated mechanisms of chemical carcinogens

Mechanism	Carcinogenic effects
Genotoxicity	Reactive chemical groups interact with DNA directly to induce DNA mutations
NON-GENOTOXIC MECHANISMS	
Stimulation of free radicals	Free radicals can damage DNA to cause mutations; perturb intracellular signaling; cause cell death and inflammation
Cytotoxicity	Death of cells in target organs results in regenerative hyperplasia; inflammation
Immunosuppression	Initiated cells can escape immune surveillance
Targeting of cell receptors	Endocrine Modification; Mitogenic; anti-apoptotic; CYP450 activation
Altered epigenome	Altered expression of oncogenes and tumour-suppressors; genetic instability
Inhibition of gap junctional intercellular communication	Loss of homeostatic communication between cells

The mechanisms of carcinogenesis described above are not mutually exclusive. Indeed most carcinogens affect more than one of these mechanisms. For example many NGTX carcinogens which are cell receptor agonists also inhibit gap junctional

intercellular communication (GJIC). GNTX mechanisms of carcinogenesis are intuitively the most straightforward to understand. An example of a GNTX carcinogen is 2-acetylaminofluorene (2-AAF). This chemical is not mutagenic *per se*, but is metabolically activated by the liver first to N-hydroxy-2-AAF and then to esterified derivatives such as acetoxy or sulfoxy moieties. These two metabolic products are strongly electrophilic and bind covalently to DNA. *In vivo* animal studies have shown 2-AAF to be a potent liver, bladder, and kidney carcinogen (Ogiso *et al.*, 1985). Hundreds of GNTX carcinogens are known, including most recognised human carcinogens (Purchase, 1994). Each cell contains intricate machinery responsible for repairing damaged DNA. However some of the DNA adducts formed following GNTX exposure are incorrectly repaired during DNA replication, resulting in mutations. The increased mutation rate in cells exposed to GNTX carcinogens increases the probability of a detrimental mutation occurring within a cancer gene.

Evidence also exists that several GNTX compounds also possess epigenetic faculties which may be important in cancer development by facilitating the expansion of the initiated cells. Tamoxifen is a hepatocarcinogenic GNTX compound (Gamboa da Costa *et al.*, 2001) which also induces global DNA hypomethylation, hypomethylation of repetitive sequences, and altered patterns of histone methylation (Tryndyak *et al.*, 2007). Similarly 2-AAF exposure causes hypermethylation of the p16(INK4) gene in the liver, as well as global and long interspersed nucleotide element-1-associated histone H4 lysine 20 trimethylation (Bagnyukovay *et al.*, 2008). 2-AAF also has anti-apoptotic propertied arising from blockage of the liver mitochondrial proapoptotic pathway from the early stages of carcinogenesis (Klohn *et al.*, 2003).

NGTX carcinogens can also indirectly damage DNA damage and subsequently cause mutations through increased production of free radicals, i.e. reactive oxygen species (ROS) and reactive nitrogen species (RNS). During inflammation both ROS and RNS are produced by macrophages and other phagocyting cells. ROS can also be byproducts of metabolism of exogenous substances by CYP450s or of normal cellular metabolism. Multiple antioxidant and scavenging systems exist to remove ROS and RNS. Oxidative and nitrate stress occurs when these systems are unable to clear an excess of free radicals. High level of ROS and RNS can then damage DNA and other macromolecules or even cause cell death. In recent years it has also been found that the ROS and RNS also have physiological cellular signaling roles within cells. They can especially affect cell proliferation and apoptosis through the MAPK and NF-kappa pathways. Therefore this is an alternative way by which free radicals can affect carcinogenesis (Roberts *et al.*, 2010).

A common mechanism of many NGTX carcinogens is the perturbation of normal cell physiology through inappropriate activation or repression of cellular receptors. These then result in the propagation of multiple cellular effects that facilitate cancer development. Several recognised human NGTX carcinogens affect cellular signalling by binding to receptors (e.g. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) binding the aryl hydrocarbon receptor; estrogenic compounds binding the estrogen receptor; progestins binding to the progesterone receptor). Several NGTX hepatocarcinogens are also known to target cellular receptors in the liver, for example 4-chloro-6-(2,3-xylidino)-pyrimidynylthioaceticacid (Wy-14,643) induced carcinogenicity requires activation of the peroxisome proliferator activated receptor alpha (PPARα) (Gonzalez *et al.*, 1998). Cellular receptors can drive carcinogenesis through stimulation of cell

proliferation, inhibition of apoptosis, changes in the epigenome, induction of CYP450 enzymes, altered metabolism etc. The increased rate of proliferation may allow the generation of new mutations as the rate of spontaneous mutation is higher in replicating than in quiescent cells. Additionally, conversion of DNA adducts to mutations occurs when cells are stimulated to enter the cell cycle. The inhibition of apoptosis may allow the survival of cells carrying mutations that would stimulate apoptosis, so that they can survive and accumulate more mutations at subsequent stages. Induction of CYP450s is a mechanism by which ROS may be generated and result in DNA damage.

Another established mechanism of NGTX carcinogens is cytotoxicity followed by regenerative proliferation. Such chemicals cause cell death in their target organs and this is followed by proliferating of the remaining cells in order to restore the damaged organ. For cytotoxic chemicals the doses which cause cytolethality are closely associated with the carcinogenic doses. It is thought that this induced hyperproliferative state can drive carcinogenesis through various ways (Butterworth *et al.*, 1995). The first is the increased rate of cell proliferation. Second, the state of inflammation causes the generation of free radicals which may damage DNA. Thirdly, the secretion of growth factors which allow tissue repair may facilitate growth of pre-neoplastic cells.

Immunosuppression, inhibition of gap junctional intercellular communication (GJIC), and disruption of the cellular epigenome have also been suggested as mechanisms of NGTX carcinogenesis. Numerous components of the immune system are thought to be involved in cancer immunosurveillance whereby nascent transformed cells are

recognised and removed. Suppression of the immune system allows a greater proportion of incipient cancer cells to escape the host immune system and initiate cancer development (Zitvogel *et al.*, 2006). An example of a chemical with such properties is cyclosporine, a compound that is used to suppress the immune system after organ transplant. Unfortunately its immunosuppressive activity also results in increased malignancies (Hojo *et al.*, 1999). Gap junctions are formed between adjacent cells whose plasma membranes link to form channels around the cells, that are thought to be important for homeostasis. Through these channels small molecular substances can travel between cells. Disruption of the GJIC between cells affects cell growth and cell death. GJIC is a common target of many NGTX carcinogens, e.g. phenobarbital, Aroclor, pregnenolone-16 alpha-carbonitrile (Kolaja *et al.*, 2000).

Exposures to many carcinogens results in an altered epigenome. The disruption of the cellular epigenome can contribute to cancer by directly deregulating critical cancer genes. For example in many cancers, tumour suppressor gene *P16* is hypermethylated and the oncogene *RAS* is hypomethylated. Alternatively, altered epigenome can promote carcinogenesis by causing genetic instability. Indeed, global hypomethylation is commonly observed in cancers. Mechanisms by which exogenous insult can disrupt the epigenome have been suggested (depletion of the S-adenosylmethionine and S-adenosylhomocysteine pools; repression of DNA methyltransferases; DNA damage; passive demethylation during cell replication; active demethylation) (Wilson *et al.*, 2007, Pogribny *et al.*, 2008). Arsenic is a NGTX carcinogen that is thought to act partly by altering the epigenome. It can induce global liver hypomethylation by depleting the S-adenosylmethionine pool (Zhao *et al.*, 1997) and inhibiting the DNA methyltransferases (Reichard *et al.*, 2007).

Several distinct mechanisms of carcinogenesis have been described here, while perhaps more remain to be identified in the future. Nevertheless, many more carcinogens exist than carcinogenic mechanisms. It is thus expected that groups of chemical carcinogens will cause tumours through similar mechanisms. Indeed, research has confirmed that groups of carcinogens act in similar fashion to cause cancer. This idea underpins the concept of a mode of action (MOA) for a carcinogen (Boobis *et al.*, 2009b). MOA in cancer is defined as a biologically plausible series of key events which are initiated with the interaction of the substance with the cell and result in cancer formation. In contrast to MOA which is a plausible hypothesis of how a carcinogen works, the mechanism of action is a more complete and detailed description of the events driving carcinogenesis. Key events are quantifiable consequences of exposure (e.g. biochemical processes or histopathological alterations) which are necessary or a biological marker of a necessary event to the eventual carcinogenesis. A key event is necessary, but often not sufficient for cancer formation. Associative events are not causal, but are reliable markers of key events (U.S. Environmental Protection Agency, 2005). The MOA therefore forms a useful framework for studying chemical carcinogens and their risk to humans. As an example, for chloroform the MOA is cytotoxicity followed by regenerative hyperplasia. Chloroform does not damage DNA, but causes cell death in the mouse liver followed by regenerative cell proliferation at the same doses that cause liver tumour formation (Larson *et al.*, 1994). Key events of chloroform carcinogenicity are (1) biotransformation of chloroform to reactive phosphogene (2) Phosphogene-induced cytotoxicity and cell death of hepatocytes (3) regenerative proliferation (4) development of tumours (Boobis *et al.*, 2009b). Another MOA is activation of PPARα by agonists (e.g. Wy-14,643; Di(2-ethylhexyl)phthalate (DEHP)) resulting in

liver hepatocarcinogenesis in rodents. The key events are altered cell proliferation, altered apoptosis, and selective clonal expansion (Klaunig *et al.*, 2003) .

1.2 MicroRNAs have important roles in cancer

1.2.1 Non-coding RNAs are conserved in eukaryotes

The classic view in molecular biology had been that RNA is a more or less passive intermediate in the process of gene expression, possessing no regulatory functions. This notion has been overturned in the past decade with the discovery in eukaryotes of endogenous small non-protein coding RNAs which have important physiological roles in gene regulation and for protecting genome stability. At present three classes of small non-coding RNA are known: the piwi-interacting RNAs (piRNAs), the short interfering RNAs (siRNAs), and microRNAs (miRNAs). These non-coding RNAs act to silence homologous sequences found within the organism by multiple mechanisms (Moazed, 2009). PiRNAs are 24-30 nucleotides long and have been found in the germline of animals. They act to silence selfish DNA such as transposons, thus protecting genetic stability (Klattenhoff & Theurkauf, 2008). Endogenous siRNAs and miRNAs differ from piRNAs in being 21-25 nucleotides long, being active in both somatic and germline cells, have been found in both plants and animals, and originate from double stranded precursors (Carthew & Sontheimer, 2009).

Animal miRNAs have been extensively studied in recent years due to their important roles in physiology and pathology, including cancer (Bartel, 2004, Bartel, 2009). Despite the importance of miRNAs, lin-4 which was the first miRNA to be identified as such, was only discovered in 1993. Lin-4 was initially identified as a heterochronic

non-coding RNA which was involved in the timing of larval development in *C.elegans* (Lee *et al.*, 1993). As lin-4 was only detected in nematodes it was thought that this gene was a peculiarity restricted to this animal. The big breakthrough came with the isolation of a second nematode miRNA, let-7, in 2000. Unlike lin-4 this miRNA had homologs in several species, including human (Pasquinelli *et al.*, 2000, Reinhart *et al.*, 2000). This finding revealed that a non-coding RNA was an evolutionary conserved mechanism of gene regulation. The field of miRNAs only really grew with the subsequent isolation of hundreds of miRNAs shortly afterwards (Lagos-Quintana *et al.*, 2001, Lau *et al.*, 2001, Lee & Ambros, 2001).

It is now known that miRNAs are evolutionarily conserved, some of them very broadly, while others are restricted to more closely related species, and have been discovered in the genome of all known plant and animal genomes (Carthew & Sontheimer, 2009, Bartel, 2004). MiRNAs are a fairly abundant class of genes in mammals, as it has been estimated that 2-3% of all genes code for miRNAs (Alvarez-Garcia & Miska, 2005). The last few years have seen a continued increase in the number of identified miRNAs in various species. The latest release of the miRNA database has 17341 mature miRNAs in 142 species (miRbase database Dec 2010). In that database there are 1048 miRNAs identified in humans, 672 in mice, and 408 in the rat.

1.2.2 Biogenesis and function of mature miRNAs

MiRNA genes are located either within protein coding genes or at their own independent intergenic loci. A large fraction (30-40%) of miRNAs is positioned within protein coding genes, usually within introns, but can be found within exons as

well. Intergenic miRNAs have their own promoters and regulatory elements. The generation of the mature, functioning miRNAs from these genes is a multi-step process requiring the activity of numerous enzymes. Most miRNAs are transcribed by the same enzyme that performs mRNA transcription, DNA polymerase II (Lee *et al.*, 2004), although some miRNAs are transcribed instead by RNA polymerase III (Borchert *et al.*, 2006). The canonical pathway of miRNA generation (See **Figure 1.2**) starts with the transcription of a long primary-miRNA (pri-miRNA) that can be hundreds to thousands of kilobases in length. A distinguishing characteristic of miRNA biogenesis is the folding of the pri-miRNA onto itself to form hairpin structures. A given pri-miRNA can contain from one to six mature miRNAs located within such intramolecular hairpins. The pri-miRNA will subsequently undergo two cleavage steps from RNAse III type enzymes in order to release the mature miRNA(s). While still in the nucleus the pri-miRNA is cleaved by the microprocessor complex made up from DiGeorge Syndrome Critical Region 8 (DGCR8) and Drosha. The former protein recognizes double-stranded RNA hairpins and positions Drosha to process the pri-miRNA and release the 60-80 nucleotide long pre-miRNAs. These molecules are then transported from the nucleus to the cytoplasm by Exportin-5 and Ran-GTP. In the cytoplasm the pre-miRNA is processed further by Dicer which cuts the pre-miRNA at 21-24 nucleotides from one end to form a miRNA-miRNA* duplex. The mature miRNA strand is then selectively incorporated onto the multi-protein RNA activated silencing complex (RISC), while the miRNA* is usually degraded and is found at much lower steady-state levels. The incorporated mature miRNA then acts as the guiding strand directing the RISC to silence its mRNA target (see later). The conserved argonaute family of proteins which are contained within the

RISC are the effectors of RISC-induced silencing of targets (Bartel, 2004, Sotiropoulou *et al.*, 2009).

An alternative process by which mature miRNAs can be generated has also been identified. These miRNAs, termed mirtrons, are localised within introns and are transcribed along with the protein-coding gene in which they reside. Pre-miRNAs are then generated directly from the introns by activity of the spliceosome and debranching enzymes. These pre-miRNAs are then exported to the cytoplasm and processed to mature miRNAs as in the canonical pathway (Berezikov *et al.*, 2007) (**Figure 1.2**).

Figure 1.2 *microRNA biogenesis in mammals*

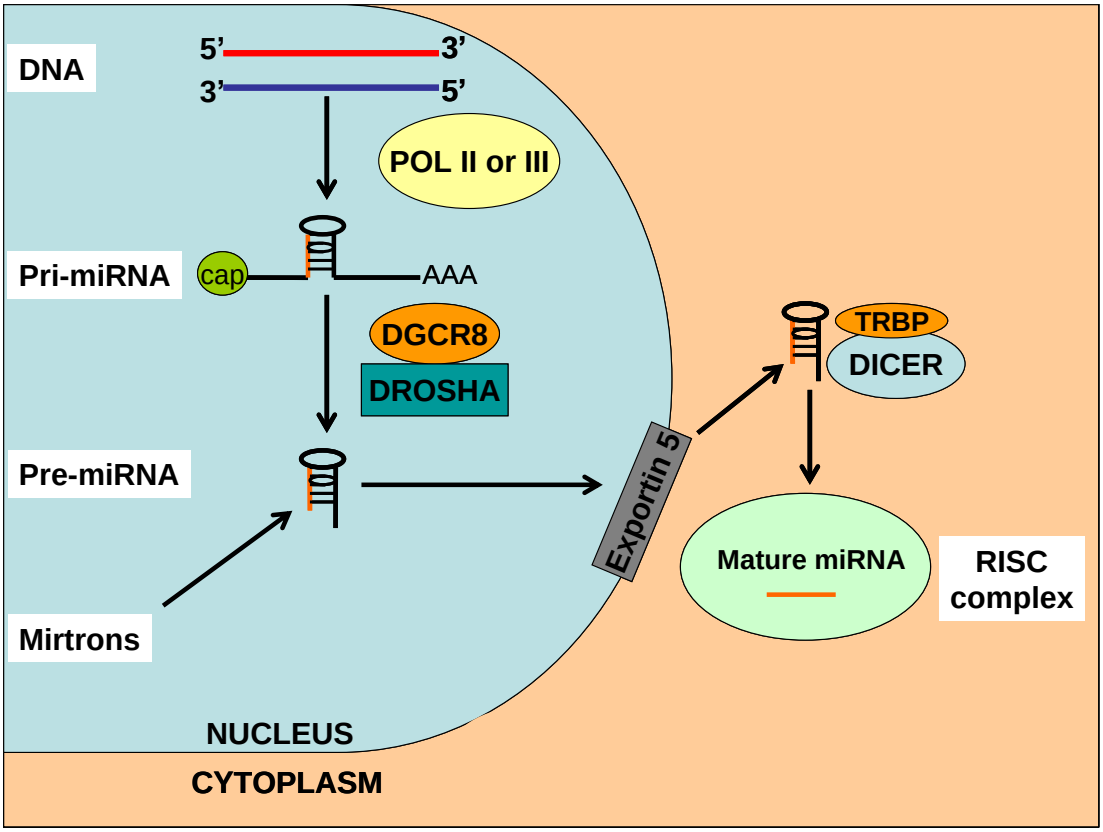


Fig.1.2 In the canonical pathway pri-miRNAs are transcribed by DNA polymerase II or III. This are capped and contain a polyA tail. The pri-miRNA folds into itself to form one to six hairpin structures containing base-pair stems (here one is shown). Within these intramolecular structures the mature miRNA sequences are contained. Drosha and DGCR8 process the pri-miRNA to release the ~70 nucleotide long pre-miRNA. In an alternative pathway for mirtrons pre-miRNAs are formed by the splicing machinery without forming pri-miRNA first. The pre-miRNA are transported to the cytoplasm by exportin 5. Within the cytoplasm the pre-miRNA is processed by dicer/TRBP in mammals and the mature miRNA strand is loaded onto the RISC complex.

Another processing mechanism during biogenesis of miRNAs is editing of their sequence. The adenosine acting on RNA (ADAR) enzyme drives the change of adenosines to inosines in RNA duplexes. This results in the change of a stable adenosine:cytosine pairing to a less stable inosine:cytosine. The miRNA editing can have profound effects on the processing and functioning of the miRNA. This editing

blocks processing of the pri-miR-151 by Drosha (Kawahara *et al.*, 2007). For at least one miRNA, miR-376, RNA editing alters the seed region of the miRNA so that the miRNA targets a different set of genes (Kawahara *et al.*, 2007).

Bioinformatic methodologies predict that approximately 30% of all mRNAs are subject to miRNA regulation(Krek *et al.*, 2005, Bartel & Chen, 2004). Additionally, a given miRNA is thought to be able to regulate the levels of hundreds or possibly thousands of genes. It has been shown experimentally that manipulation of the levels of a miRNA affected the expression of thousands of genes both at the level of mRNA (Lim *et al.*, 2005) and protein output (Selbach *et al.*, 2008, Baek *et al.*, 2008). MiRNAs and their target mRNAs display appreciable, but not complete, sequence complementarity, allowing Watson-Crick pairing of the two nucleotide strands. For animal miRNAs this is usually imperfect pairing and will result in bulges and mismatches. Strong repression efficiency depends on pairing of 7-8 nucleotide regions in the 3' UTR of mRNA to the "seed" region of the miRNAs (residues 2 to 8 on the 5' end). Repressional efficiency is also increased if the mRNA nucleotide at position one is an adenosine and at position 9 either an adenosine or uracil. Usually multiple binding sites for the regulating miRNAs can be found on the 3'UTR of mRNAs. MiRNA-mRNA pairings on 5'UTR or the coding regions of the mRNA can also induce post-translational silencing, but are less effective (Baek *et al.*, 2008, Grimson *et al.*, 2007). When miRNAs were first discovered it was thought that they acted exclusively through translational repression. It was later found though that miRNAs can silence genes by induction of mRNA degradation and/or inhibition of translation. It is still not clear what determines whether the target mRNA is cleaved or translationally repressed. The mechanisms of miRNA regulation are still being

investigated and debated (Carthew & Sontheimer, 2009, Filipowicz *et al.*, 2008, Eulalio *et al.*, 2008) (**Figure 1.3**).

Figure 1.3 *Proposed mechanisms of microRNA-induced silencing of target genes*

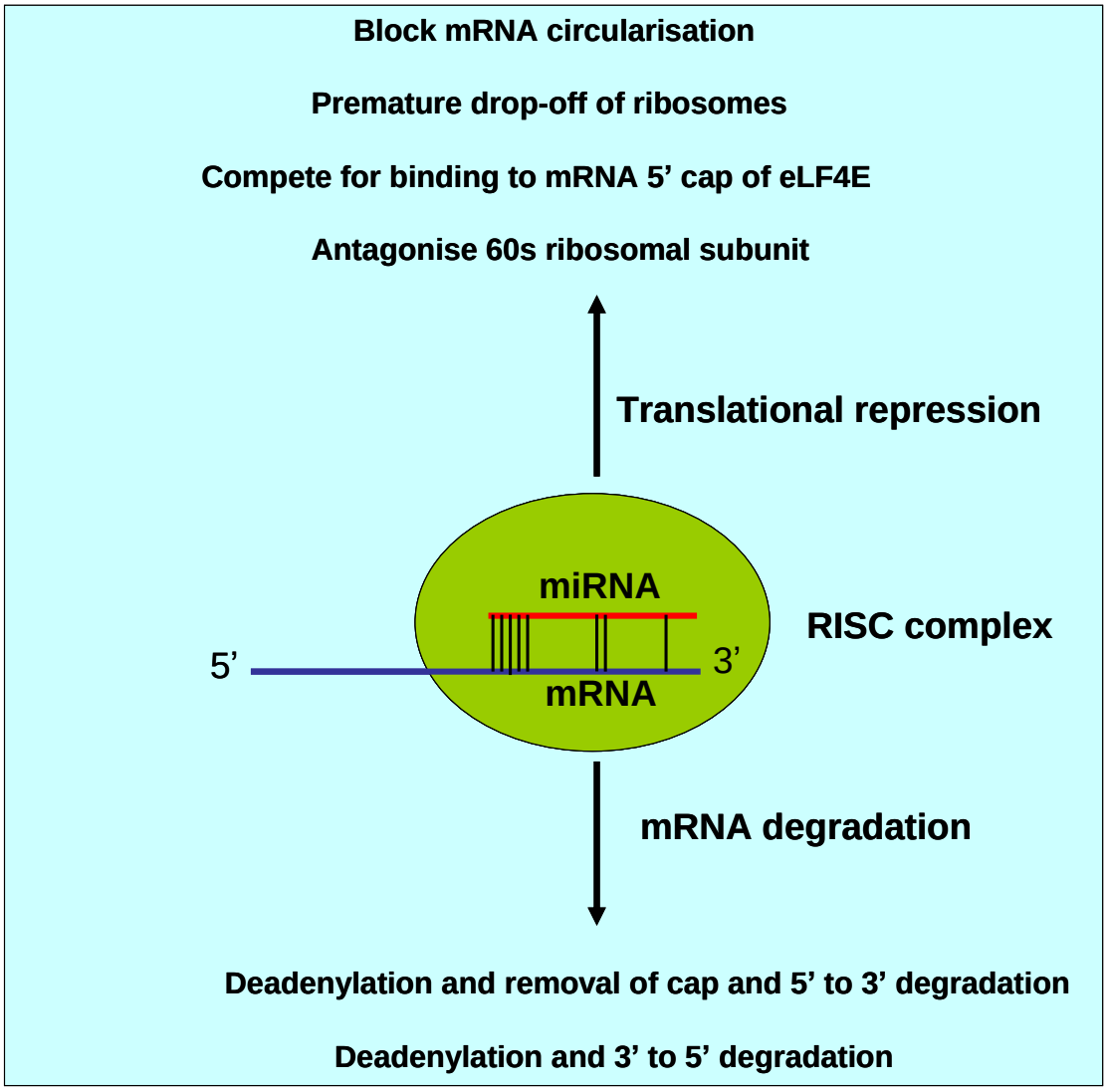


Fig.1.3 Incomplete sequence complementarity between miRNA and mRNA, especially between residues 2-8 on 5' end of miRNA to 3' UTR of mRNA, directs the RISC multi-protein complex to silence the mRNA. Silencing can occur by mRNA degradation or translational repression, and the mechanisms are still under investigation.

Degradation of mRNAs is not by direct cleavage by the RISC complex, but instead is the result of destabilization of target genes. First the poly(A) tail is shortened. Then the mRNA can have its cap removed and degraded in a 5' to 3' direction or be

degraded in a 3' to 5' direction while its cap is intact. Translation of a mRNA entails initiation, elongation, and termination. Mechanisms by which miRNA can block either the initiation or elongation of translation have been suggested: RISC complex competes with binding of the eukaryotic initiation factor (eLF) 4E onto the 5'cap to initiate translation; it may prevent the association of the 60S ribosomal subunit with the pre-initiation complex; deadenylation of the mRNA tail can block its circularization; ribosomes may be induced to drop-off from the mRNA prematurely. Which of these models of translational repression are valid is an area of active research.

1.2.3 MicroRNAs in cancer: small but deadly

Early suspicions that miRNAs may be involved in cancer were raised when it was found that miRNAs regulate cell proliferation, apoptosis, and differentiation, processes which are important in cancer (Bartel, 2004). Since then evidence has been amassed confirming the important roles of these genes in cancer development. A first observation was that more than 50% of miRNA loci are located at fragile sites or cancer-associated genomic regions – such as sites of minimal loss of heterozygocity and common breakage sites. Such areas of non-random chromosomal abnormalities are indicative that those regions harbour cancer genes (Calin *et al.*, 2004, Calin & Croce, 2006).

Second, miRNAs expression arrays have showed that in all types of tumours thus far the miRNA profile is distinct from their corresponding healthy tissue. For example deregulation of miRNA profiles has been observed in tumours of the liver (Pineau *et al.*, 2010), pancreas (Lee *et al.*, 2007), ovaries (Iorio *et al.*, 2007), lung (Yanaihara *et*

al., 2006), neuroblastoma (Chen & Stallings, 2007), breast , colorectal (Slaby *et al.*, 2007), and melanoma (Schultz *et al.*, 2008). Importantly, the miRNA signatures of tumours have been found to be informative on the diagnosis, progression, prognosis, and treatment of human cancers (Calin & Croce, 2006). Third, it was demonstrated that inhibition of the miRNA biogenesis and processing cellular machinery is sufficient to promote carcinogenesis. Blockage of Dicer, Drosha, or DGCR8- enzymes essential to the production of mature miRNAs- promoted cell transformation and tumourigenesis (Kumar *et al.*, 2007).

Finally, functional studies have linked miRNAs which are deregulated in cancer with the acquisition of cancer traits by developing cancer cells (Sotiropoulou *et al.*, 2009, Esquela-Kerscher & Slack, 2006). MiRNAs bring about their functional effects in cancer by targeting oncogenes, tumour-suppressor genes, and the epigenetic machinery. Hence miRNAs themselves act as oncogenes or tumour-suppressors. Selected examples of miRNAs involved in cancer are shown in Table 1.2 below.

Table 1.2 Examples of microRNAs implicated in cancer

MiRNA	Example of known targets	Effect of cancer deregulation
Let-7 family	Ras	cell proliferation and differentiation
miR-17-92 cluster	E2F1	cell proliferation
miR-34 family	Bcl-2; cyclin D1; SITR1; CDK1	Inhibit apoptosis, cell cycle arrest, and senescence
miR-21	PTEN	Evasion of apoptosis
miR-221/222	c-kit	Stimulation of angiogenesis
miR-15/miR-16	Bcl-2	Evasion of apoptosis
miR-29 family	DNMT3a and DNMT3b	Altered methylation
miR-200 family	Zeb1 and Zeb2	epithelial to mesenchymal transition
miR-101	EZH2; MCL-1	Altered chromatin status & anti-apoptosis
miR-106	P21/CDKN1A	Resistance of anti-growth signal
miR-122	Cyclin G1	Cell cycle
miR-96/182	FOXO	Evasion of apoptosis
miR-31	RhoA	Invasion and metastasis
miR-10b	TWIST	Invasion and metastasis

The underlying mechanisms by which the expression of key miRNAs is altered in cancer are still under investigation. So far epigenetic changes, dysregulation by transcription factors, mutations, chromosomal alterations, and defects in the miRNA biogenesis machinery have been implicated with miRNA deregulation in cancer (Deng *et al.*, 2008, Croce, 2009). From the fact that many miRNA genes map to chromosomal regions that are altered in cancers, it can be deduced that chromosomal deletions and amplifications will deregulate miRNAs (Calin *et al.*, 2004). One well-

known example is the deletion of the chromosomal region containing the tumour suppressors miR-15a and miR-16-1 in 50% of patients with chronic lymphocytic leukemia (CLL) (Calin *et al.*, 2002). Expression of oncogenic miRNAs can also be enhanced in cancers by amplification of the miRNA locus as has been found for the miR-17-92 cluster (Hayashita *et al.*, 2005). In theory point mutations of miRNAs could alter regulation of its target genes e.g. a substitution in its seed sequence. Yet, so far no miRNAs with somatic point mutations that are associated with cancer have been identified. Mutations can also affect miRNA regulation of genes if they affect the binding of miRNAs to their target genes. One identified case is for the oncogene HMAG2 which is expressed in multiple types of cancer. Chromosomal translocation of this gene results in loss of binding sites of *let-7* on its 3' UTR releasing it from miRNA regulation (Mayr *et al.*, 2007).

Altered methylation has also been shown to deregulate several miRNAs in multiple cancers. The members of the miR-34 family (miR-34a, miR-34b, and miR-34c) are silenced by methylation in several cancer types (Hermeking, 2010). MiR-203 shows altered methylation in ovarian (Iorio *et al.*, 2007), oral (Kozaki *et al.*, 2008), and liver cancers (Furuta *et al.*, 2010). In pancreatic cancers hypomethylation was correlated with increased expression of the miR-200a/200b/429 cluster (Li *et al.*, 2010). Research has identified numerous transcription factors, cytokines, and hormones that regulate expression of miRNAs. For example p53 binds to the promoter of the miR-34a and miR-34b/c miRNAs. Deregulation of p53 is common in cancers and results in reduced expression of miR-34a (Hermeking, 2010). Therefore, perturbations of upstream factors can also deregulate miRNAs in cancers.

1.3. Liver is a frequent target of chemical carcinogens

1.3.1. Liver architecture and function

The liver is a large complex organ with multiple key functions in physiology and disease. This organ is a frequent target of exogenous chemicals, including carcinogens (Hardisty & Brix, 2005). This is due to the metabolising activities of hepatocytes which activate many chemicals to a more reactive metabolite capable of attacking DNA. Additionally, the liver is exposed to high concentrations of orally ingested chemicals which pass to it directly from the gastrointestinal tract.

In mammals the liver is an abdominal organ located below the diaphragm. Mammalian livers have a lobular structure consisting of four anatomically distinct areas (right, medial, left, and caudate lobes). In humans the largest lobe is the right one, while in rats it is the left lobe. The right and left lobes are divided further by veins into smaller parts (anterior-posterior for the right lobe; medial and lateral for the left lobe). Lobes are made up of roughly hexagonal compartments called lobules which are the fundamental structural and functional units of the liver (Ishibashi *et al.*, 2009). In the centre of each lobule is a hepatic venule from which one-cell thick rows of 15-25 hepatocytes radiate outwards. Oxygen, nutrients, endogenous and exogenous substances are carried to the hepatic lobules through the hepatic portal vein and the hepatic artery. The former transports venous blood containing nutrients and other ingested substances from the gastrointestinal tract. The hepatic artery carries oxygenated blood to the liver. In resting animals 25-30% of their circulation is present at any one time within the liver. The hepatic portal vein, hepatic artery, and the bile duct are located in the portal area at the edges of the lobules (**Figure 1.4**).

Figure 1.4 *Architecture of hepatic lobules*

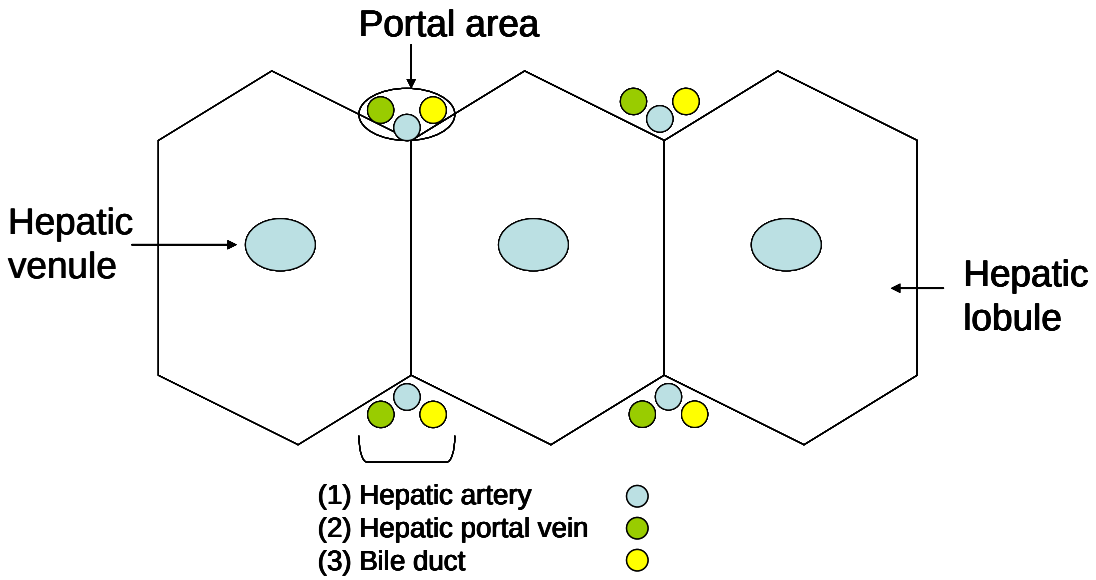


Fig. 1.4 Adapted from Ishibashi *et al.* 2009. Hepatic lobules are the main structural and functional units of the liver. In the diagram three hepatic lobules are shown with their roughly hexagonal shapes. At the centre of each lobule is a hepatic venule from which rows of hepatocytes radiate outwards. At the edges of the lobules are portal areas containing hepatic arteries, hepatic portal veins, and bile ducts.

Between the hepatocyte rows flows blood from the portal area within specialised blood vessels called sinusoids. Eventually the blood originating from the portal area drains from the sinusoids into the hepatic venule, to larger hepatic veins, and into the vena cava from where it is exported from the liver. In the opposite direction to the blood flows bile, a digestive fluid that assists fat metabolism. Bile is produced by hepatocytes and is transported to the bile ducts located in the portal area through minute channels called bile canaliculi. Bile then flows to the gall bladder where it is stored (Ishibashi *et al.*, 2009) (**Figure 1.5**).

Figure 1.5 *Flow of substances through the liver*

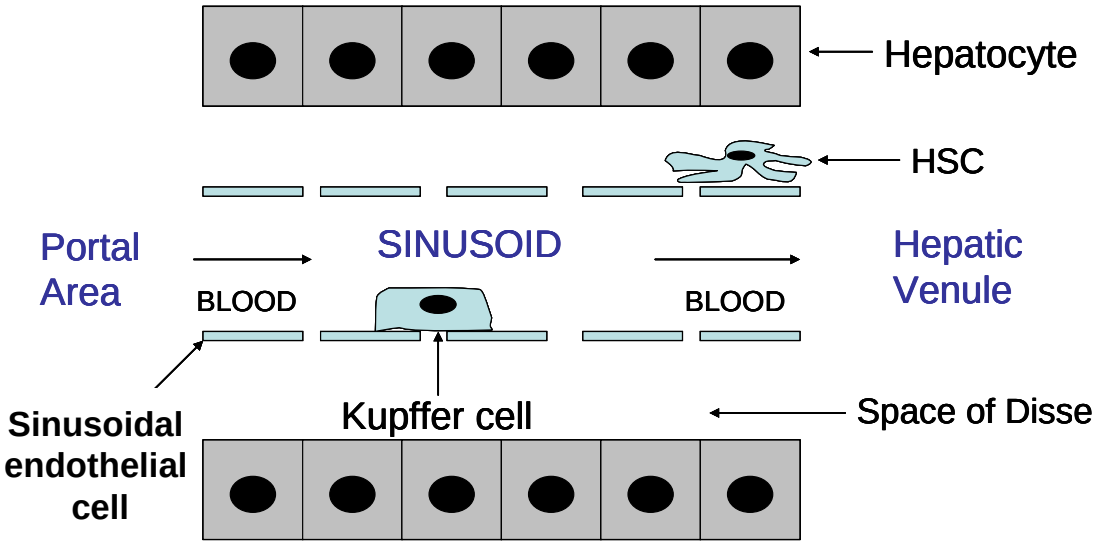


Fig.1.5 Adapted from Ishibashi *et al.* 2009. Blood flows from the portal area via the sinusoid into the hepatic venule. Bile flows in the opposite direction from hepatocytes to the bile duct through the bile canaliculi (not shown). Hepatocytes absorb, secrete, and modify substances as they flow within the blood through the sinusoid. The sinusoid is made from endothelial cells and also contains Kupffer cells (KC), Hepatic stellate cells (HSCs), and natural killer cells (NK) (not shown).

All hepatocytes appear similar when viewed microscopically. Nevertheless, there exists diversity in their functional properties according to their location within hepatic lobules. Expression of genes is different for hepatocytes along the axis from the periportal to the central venule region. The hepatic heterogeneity facilitates the proper functioning of the liver. First it allows the development of hepatocytes with distinct functional properties and therefore a liver capable of more diverse responses. Second substances that progress through the sinusoid can be sequentially metabolised (Gumucio, 1989).

The most abundant cells of the liver are the hepatocytes which constitute 60% of total cells in the liver and 80% of the liver volume. Besides hepatocytes at least fourteen

other cell types can be found in the liver (Malarkey *et al.*, 2005). The next most abundant liver cells after the hepatocytes are the sinusoidal endothelial cells (SEC). These cells constitute 20% of liver cells and make up the walls of the sinusoid. Unlike normal epithelial cells SECs are fenestrated, which facilitates the flow of solutes into the space of Disse where they can be acted upon by hepatocytes. The third most abundant cells are KC which make up 15% of liver cells. These are macrophages that are found within the sinusoidal space and remove big particulate material by phagocytosis. They also produce a number of cytokines. Hepatic stellate cells (HSC), also known as Ito cells, represent 5% of the liver population and are found in the space of Disse. Liver-specific NK cells, also known as Pit cells, can be found between SECs and fibroblasts. They constitute 1% of liver cells. Collectively the SECs, KC, HSC, and NK cells are known as the hepatic sinusoidal cells as they are associated with this structure. Also fairly abundant (4% of liver cells) are biliary epithelial cells (also called cholangiocytes) which make up the lining of the bile duct.

Hepatocytes are the parenchymal cells of the liver responsible for performing the diverse functions of the liver. About half of the volume of hepatocytes is attributable to organelles. Hepatocytes are a major centre for the production of energy (in the form of glucose) and secrete it to the blood. Hexokinase, phosphoenolpyruvate carboxykinase, and glycerol kinase, drive the production of glucose from carbohydrates, amino acids, and glycogen respectively (Williams & Iatropoulos, 2002) . Hepatocytes are also the main cells responsible for biotransformation of xenobiotic chemicals. This biochemical processing converts lipophilic xenobiotic chemicals into more water soluble compounds that can be excreted from the body. Phase I reactions oxidise, reduce, or hydrolyse the chemicals. The most common

phase I event is oxidation by P450 enzymes. In phase II metabolism conjugating enzymes attached an ionizable functional group (such as glucuronyl or sulphate) to the molecules to make them water-soluble, as well as to often deactivate them. The enzymes responsible for both phase I and phase II metabolism are abundant within the endoplasmic reticulum of hepatocytes (Williams & Iatropoulos, 2002).

1.3.2. Aetiology, types, and frequencies of liver cancers

Liver cancers originate in the hepatic tissue itself, rather than tumours which arise in different tissues and metastasise to the liver. Liver cancers are the sixth most frequent cancer worldwide with more than 600000 new cases every year. The levels of liver cancer have been increasing worldwide over the past decades. The incidence of liver cancers is 2.5 fold more frequent in males than females. For both sexes the incidence of the disease is much higher in the developing world. The most important risk factors for liver cancer are viral infections (hepatitis B or C) and dietary aflatoxins. Several other risk factors have been identified such as alcoholic liver disease, obesity, steatosis, hemochromatosis, smoking, and oral contraceptives (Kamangar *et al.*, 2006).

In adults the most common forms are hepatocellular carcinomas (HCC) which account for 85% of primary liver cancers and cholangiocarcinomas (cancers of bile ducts) which account for 10% of liver cancers. Rarer liver cancers include hepatoblastomas (liver cancers found in infants and children), hemangioendotheliomas (cancers of the blood vessels)**,** tumours displaying mixed hepatocyte/cholangiocyte phenotype**,** as well as a number of other cancer forms . In humans HCC is an aggressive disease with low survival rates. It can arise in both

normal livers, as well as from cirrhotic livers and the genetic pathways involved may differ between them (Farazi & DePinho, 2006). Development of HCC is a slow sequential process, with intervals in some cases of up to 30 years between hepatitis B and C induced cirrhosis and subsequent cancer. As in most cancers both epigenetic and genetic events are implicated with the disease. It is interesting to note that during this long preneoplastic phase most changes in gene expression are quantitatively driven by epigenetic factors (Thorgeirsson & Grisham, 2002). These epigenetic changes are not sufficient to cause carcinogenesis, as only a fraction of cirrhotic livers will eventually develop HCC. Hence, subsequent genetic mutations are required. Epigenetic alterations are likely to provide the conditions that facilitate the occurrence of the driving genetic mutations. The epigenetic alterations may also precede subsequent mutations, as for example the methylation of *c-myc* at early stages, and amplification of the gene later on.

An interesting question is which types of cells can give rise to liver cancers following carcinogen treatment. Cancers are initiated in replication-competent cells whose lineage survives long enough to accumulate carcinogenic modifications. However, many terminally differentiated cell types are short-lived or have lost their replicative potential. Recent years have seen the isolation from several types of normal tissues of adult stem cells which are long-living and can replicate. This has led to the hypothesis that in many tissues (e.g. colon and brain) cancers originate from adult stem cells (Hermann *et al.*, 2010). The liver though is a peculiar organ in that its mature cells, which are normally quiescent, possess the ability to proliferate following suitable stimulation. Hepatocytes and cholangiocytes can start proliferating rapidly after hepatectomy of 2/3 of the liver and rapidly reform the organ after two rounds of

hepatocyte replication. The regeneration is driven by many growth factors and cytokines that are released in the blood after hepatectomy (Michalopoulos & DeFrances, 1997, Michalopoulos, 2007). Serial transplantation of hepatocytes between mice showed that hepatocytes still retain their proliferative potential after at least 69 doublings (Overturf *et al.*, 1997). The fact that hepatocytes and cholangiocytes possess the properties necessary to form liver cancers suggests that they are potential targets of chemical hepatocarcinogens, cholangiocytes giving rise to cholangiocarcinomas and hepatocytes to HCC.

On the other hand, animals also host a population of hepatic progenitor cells, termed oval cells in rodents, which possess bipotential replicative potential and reside between the hepatocyte canaliculi and bile ducts. These cells can expand and differentiate into either hepatocytes or cholangiocytes if given an appropriate stimulus. It is thought that they form a reserve for liver regeneration when the mature liver cells are unable to regenerate after injury (Roskams *et al.*, 2004). Several animal models have displayed oval cell activation and differentiation. The Solt-Farber model of carcinogenesis starts with treatment of animals with an initiating carcinogen (such as diethylnitrosamine (DEN) followed by two week 2-AAF exposure and partial hepatectomy (Solt *et al.*, 1977). 2-AAF is used to block hepatocyte proliferation. Following hepatectomy oval cells are activated and proliferate to regenerate the liver. Additionally some rare forms of livers cancers show a mix of hepatocyte and cholangiocyte properties, suggesting that these cancers probably arise from the progenitor oval cells. The capacity of progenitor cells to form liver cancers was demonstrated by oval cells isolated from mice deficient in p53 which could induce HCC when transplanted to athymic nude mice(Dumble *et al.*, 2002). Oval cells

therefore can also form HCC. The liver then contains three populations of long-living, replication-competent cells that can be acted upon by chemical carcinogens to form tumours: hepatocytes, cholangiocytes, and oval cells. As of present, there exists no definite way to identify the cellular targets of chemical carcinogens, with most of the evidence being circumstantial (**Figure 1.6**).

Figure 1.6 *Cellular origins of liver cancers induced by carcinogens*

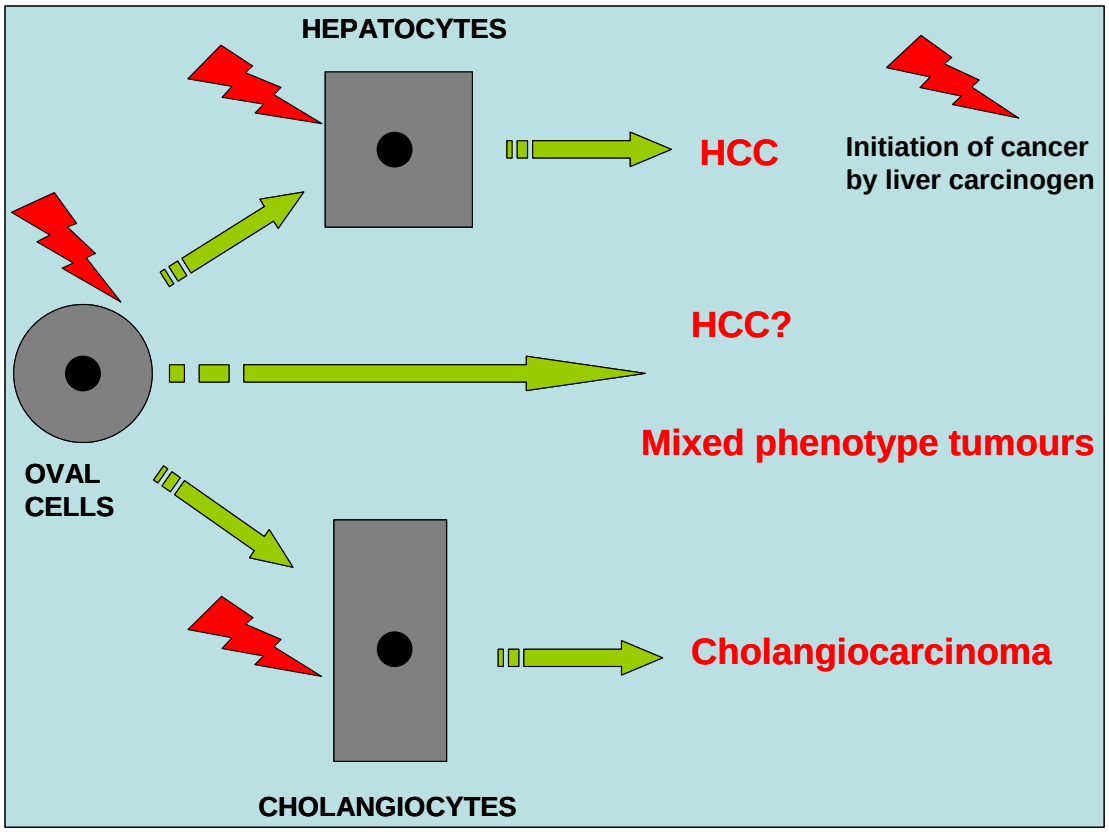


Fig. 1.6 Adapted from Roskams *et al.* 2006. Progenitor cells (oval cells in rodents) are bipotential and can give rise to either hepatocytes or cholangiocytes. The most common liver cancers in adults are HCC and cholangiocarcinomas. These can arise from hepatocytes and cholangiocytes respectively. Rare liver cancers show intermediary or mixed hepatocyte/cholangiocyte properties and are thought to arise from progenitor cells. The contribution of oval cells to HCC is still not clear.

The non-parenchymal cells of the liver are also involved in chemical carcinogenesis by affecting the initiation and progression of liver cancer. Indeed, transgenic mice where PPARα is expressed only in hepatocytes, do not develop tumours following Wy-14,643 treatment, demonstrating the essential role of non-parenchymal cells (Yang *et al.*, 2007). Activation of KC results in the generation of ROS as well as the secretion of growth factors and inflammation molecules such as TNF-α, IL-1, and IL-6, which can drive hepatic carcinogenesis (Roberts *et al.*, 2007). HSCs are important for the development of fibrosis and cirrhosis (Krizhanovsky *et al.*, 2008).

1.3.3 Phenobarbital is a prototype of non-genotoxic hepatocarcinogens

Phenobarbital (PB) has been under continual usage by humans since 1912 for its strong sedative and anticonvulsant properties. Even though its usage has declined in recent decades, it still remains the most widely-used antiepileptic drug worldwide (Kwan & Brodie, 2004). PB is also a prototype of rodent liver NGTX carcinogens that cause transient hyperplasia and liver enlargement. Numerous *in vivo* and *in vitro* genotoxicity tests have been performed for PB (DNA damage *in vivo*, assays for bacterial gene mutations, mammalian gene mutations, chromosomal aberrations etc.), which concluded that PB is a NGTX compound. The carcinogenic potency of PB is species and strain specific. The C3H and $B6C3F_1$ mice strains are much more susceptible to liver tumour induction by PB than the C57BL mouse strain, developing adenomas and carcinomas in multiple studies (Whysner *et al.*, 1996). Rats are also more resistant to PB-induced tumours than the susceptible mouse strains, developing only altered hepatic foci and/or adenomas. PB is also a powerful promoter of HCC in livers of mice and rats initiated with several genotoxic agents, such as 2-AAF (Whysner *et al.*, 1996). For humans large epidemiological studies examining cohorts

of thousand of patients concluded that PB is not carcinogenic at doses which cause liver tumours in rodents (Olsen *et al.,* 1995);(Lamminpaa *et al.,* 2002). Based on these observations the latest IARC evaluation has concluded that "inadequate evidence" exists for carcinogenicity of PB in humans, while "sufficient evidence" exists that it is carcinogenic in experimental animals (International Agency for Research on Cancer 2001). A defining characteristic of PB is the strong induction in the liver of the cytochrome 450 type 2b (CYP2B) family. Members of this gene family are found in humans, mouse, and rat (Cyp2B9 and Cyp2B10 in mouse, Cyp2B1 and Cyp2B2 in rat, and CYP2B6 in human). PB also induces other enzymes (e.g. Cyp2C and Cyp3A), but not as potently. PB-type inducers are a set of structurally diverse chemicals which nevertheless display similar strong inductions of Cyp2B. Species differences exist as well, with some compounds inducing Cyp2B in one animal, but not in others. PB-type compounds include PB, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), methoxychlor, dieldrin, acetone, o,p'-DDT, oxazepam, and methoxychlor (Sueyoshi & Negishi, 2001).

PB exposure of hepatocytes results in the activation of two members of the nuclear receptor family, the constitutive androstane receptor (CAR) and the pregnane X receptor (PXR). Nuclear receptors are transcription factors with characteristic modular structures that are activated by extracellular or intracellular ligands, bind to DNA, and affect gene expression. Due to the fact that CAR and PXR lack an identified endogenous ligand, they have been classified as orphan nuclear receptors. CAR and PXR have also been classified as xenobiotic receptors, receptors which show promiscuous binding to toxic byproducts of metabolism or exogenous chemicals. CAR is responsible for generating most of the pleiotropic effects of PB in

the liver, including cancer ((Timsit & Negishi, 2007); (Li & Wang, 2010). Inactive CAR is located in the cytoplasm as part of a multi-protein complex. PB does not bind to CAR, but instead activates it indirectly by causing its nuclear translocation through PP2A phosphorylation. Within the nucleus of hepatocytes the active CAR propagates multiple cellular effects by directly regulating genes or by affecting other transcription factors (**Figure 1.7**).

Figure 1.7 *Phenobarbital drives the CAR to the nucleus*

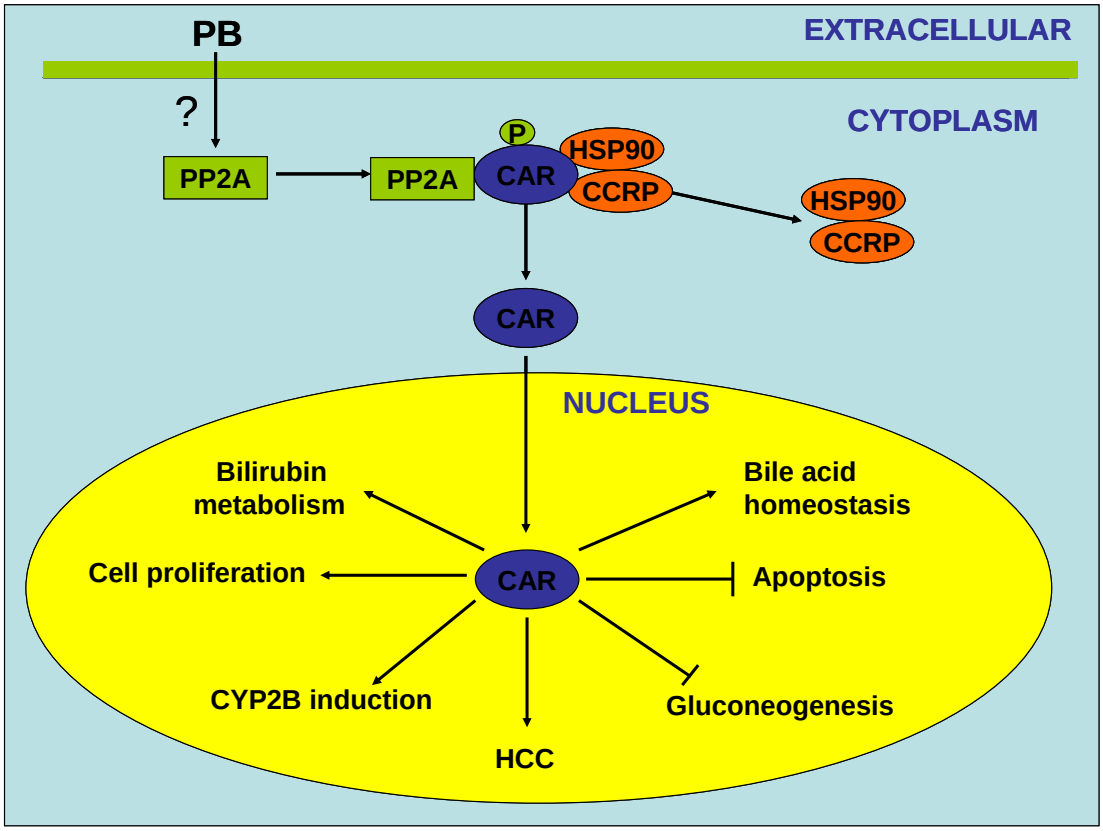


Fig. 1.7 Inactive CAR is located in the cytoplasm as part of a multi-protein complex including CCRP and HSP90. PB activates CAR by stimulating its translocation into the nucleus where it propagates multiple short and long term effects (detoxification response, suppression of gluconeogenesis, etc.). PB does not bind directly to CAR, but instead activates it indirectly through PP2A. PP2A dephosphorylates CAR at serine 202, releasing it from the complex and allowing it to translocate into the nucleus. The mechanism by which PB activates PP2A in order to stimulate CAR activation is currently not known.

Induction of the phase I and phase II metabolizing genes by CAR is thought to be an adaptive response aiming to protect the liver from toxic damage. In the nucleus CAR forms heterodimers with the retinoid X receptor alpha (RXRα) and activates CYP2B by binding onto a PB responsive element found in the promoter region of the genes (Wei *et al.*, 2000). CAR is also responsible for the induction of several other phase I and II metabolizing genes after PB exposure, such as P450s, drug transporters such as Mrp2 and Mrp4, sulfotransferases, glutathionine-S-transferase, and organic ion transporters (Ueda *et al.*, 2002);(Assem *et al.*, 2004). CAR can also regulate genes indirectly by acting as a co-regulator of gene transcription. Within the nucleus CAR binds to and represses Forkhead box other (FOXO) 1, which antagonises gluconeogenesis (Kodama *et al.*, 2004). CAR also antagonises transcriptional activity of the Hepatic nuclear factor-4 (HNF4) which is involved in lipid and glucose metabolism. This is achieved by competition for binding motifs of genes and coactivator PGC-1α (Miao *et al.*, 2006).

Some PB effects are CAR-independent. Ueda *et al.* 2002 compared transcript profiles of CAR wildtype and knockout mice 12 hours after exposure to PB dose. They found that a significant proportion of genes affected by PB were independent of CAR (60 out of 144). PB can also induce the PPARα receptor in CAR and PXR knockout hepatocytes, but this effect is repressed in wildtype animals (Tamasi *et al.*, 2009). Importantly though, experiments with CAR knockout animals demonstrated that activity of the receptor is essential for the development of HCC (Wei *et al.*, 2000) (Yamamoto *et al.*, 2004); (Huang *et al.*, 2005). In the CAR knockout mice there is no transient increase in hepatocyte proliferation or increase in liver size following acute PB or TCPOBOP exposure. CAR knockout mice which were initiated with DEN and

promoted with PB or TCPOBOP had more apoptotic bodies, fewer altered hepatic foci (AHF), and developed no adenomas or carcinomas. In contrast all wildtype animals developed liver cancers.

Activation of CAR is therefore essential to the hepatocarcinogenesis of PB-type compounds. It is thought that the CAR-dependent increases in proliferation and suppression of apoptosis drive the progression of spontaneous or chemically initiated preneoplastic cells (Holsapple *et al.*, 2006). In accordance with this PB induces more AHF and cancers in aged rats which contain more spontaneous preneoplastic cells (Ward, 1983). The induction of hepatocyte proliferation by PB is transient as judged by numerous studies utilizing hepatic labeling index and BRDU staining. However, PB treatment also results in increased number and size of AHF. These are distinct regions made up of cells with altered morphology and histology. These foci develop in the liver prior to the appearance of tumours and may develop to adenomas and carcinomas. There is experimental evidence that PB treatment increases proliferation and suppresses apoptosis of preneoplastic cells within these AHF. ((Kolaja *et al.*, 1996)) examined male mice and rats which were fed 10, 100, and 500 mg/kg PB after initiation with DEN. The 100 and 500 doses resulted in the development of hepatic foci, within which there was increased DNA synthesis and decreased apoptosis. (Schultehermann *et al.*, 1990) reported on N-nitrosomorpholine initiation in female rats followed by PB promotion. In the PB promoted animals there was increased number of foci, faster foci enlargement, and finally cancers. Within the foci, proliferation was 10-fold higher than in normal tissue. Apoptosis was lower in PB-promoter foci than in controls, and was increased if PB treatment was removed.

The MOA of PB-type inducers is then considered to be the activation of CAR which results in multiple cellular effects. The key events consist of activation of CAR, increased proliferation, suppression of apoptosis, hypertrophy, and development of altered hepatic foci (Holsapple *et al.*, 2006). Besides the key events identified above, PB also causes other events which have been suggested to be involved in its hepatocarcinogenesis. These include oxidative stress, inhibition of GJIC, and alterations in DNA methylation. It has been suggested that oxidative stress resulting from CYP2B induction contributes to hepatocarcinogenesis (Imaoka *et al.*, 2004). However, it is also possible that induction of CYP2B is a side-effect of CAR induction that is irrelevant to the process of carcinogenesis. Just like many other NGTX carcinogens, PB also causes a disruption of the GJIC in hepatocytes. Treatment with tumour-promoting dose of PB of rats for seven days blocked GJIC by 50% (Kolaja *et al.*, 2000). Also, connexin-32, the major gap junction protein, was reduced following PB exposure in male Fischer rats (Neveu *et al.*, 1994). In mice PB doses affected GJIC of the B6CF31 mice but not of the C57BL/6 mice (Warner *et al.*, 2003). Interestingly, species differences seem to exist in the role of GJIC in PB-induced carcinogenesis. Connexin 32 mutant mice develop more tumours after DEN initiation and PB promotion than wildtype (Dagli *et al.*, 2004). However, rats with similar experimental treatement had increased number of lesions, but not an increased incidence of tumours (Hokaiwado *et al.*, 2005). Studies have also determined that PB affects both global and gene-specific DNA methylation. A 20% decrease in global DNA methylation was reported for sensitive B6CF31 mice strain at 1,2, and 4 weeks, but not for the more resistant C57BL/6 strain (Counts *et al.*, 1996) . The methylation of multiple genes was altered at two and four weeks after PB exposure in the B6CF31 mice compared to C57BL/6 mice (Phillips *et al.*, 2009a). Methylation patterns were

also distinct between CAR wildtype and CAR knockout mice at 23 weeks (when foci appear) and 32 weeks (when tumours appear) (Phillips *et al.*, 2009b). Analysis of the genes altered by methylation in these studies in the B6CF31 and CAR wildtype mice, but not in the C57BL/6 or the CAR KO animals, identified a number of genes involved in cancer pathways.

At the molecular level PB has to induce cancer by altering critical cell cycle and apoptotic pathways. Various genes involved in these cellular responses have been so far linked to PB-induced carcinogenesis. A recent interesting study has revealed a pathway where CAR activates *c-myc*, which subsequently activates *FoxM1*. This cascade then drives hepatic proliferation after CAR activation in mice (Blanco-Bose *et al.*, 2008). Also, unlike tumours caused by DEN treatment, 80% of tumours generated by DEN initiation followed by PB promotion contained *b-catenin* mutations (Aydinlik *et al.*, 2001). Anti-apoptotic effects of CAR have been associated with the induction of *mdm2* (Huang *et al.*, 2005) and of *gadd45b* (Columbano *et al.*, 2005).

As has been mentioned above, extensive epidemiological evidence supports that PB is not carcinogenic in humans. The molecular underpinnings of the different response of human and rodents to PB are still not clear. Comparisons of rat and human primary hepatocytes reveal that although PB induces CYP2B in both, only in rats is DNA synthesis stimulated (Hirose *et al.*, 2009). This and other studies indicate that PB may affect proliferation and apoptosis of rodent hepatocytes, but not of humans. There exist obvious practical difficulties of studying the effects of PB treatment on the human liver *in vivo*. One interesting model for studying species-differences of PB effects are transgenic mice which lack the murine CAR and PXR receptors, but

instead contained the human CAR and PXR homologs. Treating these mice resulted in induction of CYP2B10 in wildtype and humanised mice, but not in knockout. However, cell proliferation and HCC were only observed in wildtype mice. These data suggest that human and mouse CAR may affect different genes involved in cell cycle (Ross *et al.*, 2010).

1.3.4 Involvement of miRNAs in hepatocellular carcinoma

MiRNAs are abundant in liver and play important physiological and pathological roles in this organ (Bala *et al.*, 2009). Proteomics have also demonstrated that a single miRNA can have profound effects on protein expression. MiR-34a transfection into the human liver cell line HepG2 resulted in thirty four proteins being downregulated, with 15 probably being downstream targets of the miRNA. Interestingly, they found that only 11% of proteins that were downregulated by miR-34a transfection also displayed a decrease in transcript levels (Cheng *et al.*, 2010).

Evidence has been accumulating in recent years that, just as for other cancers, miRNAs are involved in both the initiation and maintenance of liver cancers. Numerous studies have shown altered expression in liver tumours (Pineau *et al.*, 2010, Gramantieri *et al.*, 2007, Jiang *et al.*, 2008, Kutay *et al.*, 2006, Ladeiro *et al.*, 2008, Meng *et al.*, 2007, Ura *et al.*, 2009, Hou *et al.*, 2011). Profiling studies have also shown that the miRNA profile of tumours is connected with the aetiology, malignancy, and the underlying gene mutations. For example Ladeiro *et al.* 2008 associated deregulations of specific miRNAs with hepatitis B infection, alcohol consumption, and HNF1alpha and B-catenin mutations. Ura *et al.* 2009 reported that hepatitis B and hepatitis C infected tissue affected a distinct group of miRNAs which

were involved in the progression to hepatocellular carcinoma (HCC). In another study expression of specific miRNAs was associated with a subclass of HCC which displayed overactive IGF signalling (Tovar *et al.*, 2010).

The requirement of normal miRNA signalling to prevent hepatic tumours was demonstrated in a conditional knockout mouse model in which dicer1 was deleted specifically in hepatocytes three weeks after birth. Hepatocytes in these animals lacked mature miRNAs. At one year of age two-thirds of these mice developed HCC, clonally derived from dicer1 knockout hepatocytes (Sekine *et al.*, 2009). It has also been shown *in vivo* that a single miRNA can affect the progression of liver cancers. Mir-26a is a cell-cycle regulating miRNA that is lost in liver cancer cells. Introduction of this miRNA into mouse models of HCC using virus carriers protects from progression of the disease (Kota *et al.*, 2009). Several specific miRNAs have been linked with HCC. MiR-21 and miR-221 are oncogenes. MiR-21 is highly overexpressed in HCC and liver cancer lines and promotes hepatocyte growth by targeting PTEN (Meng *et al.*, 2007). MiR-221 is upegulated in 71% of HCC and controls cell cycle regulators p27 and p57 (Fornari *et al.*, 2008). MiR-122 is a tumour-suppressor downregulated in 50-70% of HCC and correlates with loss of hepatocyte phenotype and increased invasiveness (Kutay *et al.*, 2006, Coulouarn *et al.*, 2009). Mir-199a/b-3p is a miRNA with a high expression, the frequent repression of which in HCC is associated with aberrant activations of the PAK4/Raf/MEK/ERK pathway frequently repressed in HCC (Hyun *et al.*, 2009) Other miRNAs that have been implicated in HCC include let-7 family, miR-18, miR-301, miR-373, and miR-224 (Bala *et al.*, 2009). More functional studies are needed to clarify the functional significance of the deregulation of these miRNAs in HCC.

1.4. Cancer risk assessment strategies

1.4.1. Rodent lifetime bioassays are the benchmark for risk assessment

An essential requirement for regulated chemicals (such as drugs, pesticides, industrial chemicals, and food additives) is to evaluate their carcinogenic potencies to humans. This is a necessary step to prevent unnecessary exposure of the public to chemicals which will lead to the development of cancers. Unfortunately, risk assessment for carcinogenicity of chemicals is currently a laborious, complicated, time-consuming, and costly process (Butterworth *et al.*, 1995). Risk assessment currently entails tests for genotoxicity, two-year cancer bioassay in rodents, MOA considerations, and extrapolations of animal data to humans (Boobis *et al.*, 2009a).

The rodent lifetime bioassay has remained a central requirement for the evaluation of chemicals for several decades. It involves testing both sexes of a rat and mouse strain to the maximum tolerated dose (MTD) for a period of two years, which represents the lifetime of these animals. Typically 50 animals are used per sex per dose group. MTD is the highest dose that is not sufficiently toxic to result in significant effects on animal health. The value of this assay rests on the assumption that rodents and humans are comparable in respect to carcinogen hazard; therefore in the absence of extenuating information animal tumours are relevant to humans (Butterworth *et al.*, 1995).

GNTX carcinogens can be identified reliably and quickly using a weight of evidence approach which takes into account structural information, *in vitro* assays, and *in vivo* DNA damage assays (Weisburger & Williams, 2000, Kirkland *et al.*, 2005). Development of chemicals with GNTX properties can then be terminated at an early

stage. On the other hand, currently no short-term tests that can predict NGTX carcinogenicity exist. This is due to the fact that these carcinogens do not damage DNA and have multiple MOA. Consequently, most of the tested chemicals that result in the induction of tumours in the two-year bioassay will act through NGTX mechanisms. (VanOosterhout *et al.*, 1997) examined the databases of all pharmaceutical that were tested for carcinogenicity which applied for a marketing authorisation in Germany and Netherlands. Of the 221 tested compounds only six had evidence of genotoxicity. A second study by NTP looked at 301 chemicals which were tested for rodent carcinogenicity. Of those 40% were NGTX carcinogens, with only 13% being GNTX carcinogens (Ashby & Tennant, 1994).

The MOA of a carcinogen is also increasingly accepted as an important aspect in assessing human relevance of rodent carcinogens (Boobis *et al.*, 2009b, Holsapple *et al.*, 2006, Boobis, 2010). In certain cases the MOA of rodent carcinogens has been found to be irrelevant for humans. Additionally, the MOA of a rodent carcinogen may be specific to a high dose used in a bioassay, and irrelevant to the lower doses to which humans are exposed. Certain chemicals induce a2u-globulin nephropathy in male rats (e.g. d-limonene and unleaded gasoline). For these chemicals the MOA is accumulation of α2u-globulin in the kidney causing cell death, hyperplasia, and renal tumours. This MOA is irrelevant for female rats, mice, and humans as α2u-globulin does not accumulate in the kidney of these animals. Hence, regulatory agencies accept that chemicals with this MOA do not pose a human cancer risk (Whysner & Williams, 1996). As noted above epidemiological studies indicate that PB is probably not carcinogenic in humans. Therefore, chemicals with a PB-type MOA are probably not a human cancer hazard.

An important aspect of risk assessment is the extrapolation of rodent tumour data to humans. Both toxicokinetic and toxicodynamic parameters of chemicals are important for this evaluation. Toxicokinetic parameters include the uptake, processing, exposure of tissues, metabolism and excretion of the chemical from the body and relates exposure of the chemical to its toxic effects. Toxicodynamics is the mechanisms by which chemicals produce their biological effects. If it is established that the MOA of a rodent carcinogen is not plausible in humans, taking into account kinetic and dynamic factors, then rodent tumour data can be ignored. If the MOA of a rodent carcinogen is not identified or is considered plausible in humans then the human cancer risk is determined. For risk assessment purposes a clear distinction is made between GNTX and NGTX carcinogens. It is currently considered that for GNTX carcinogens their risk is non-thresholded and linear, so that there is no safe exposure level (U.S. EPA 2005). Unlike GNTX carcinogens, NGTX carcinogens are considered to be reversible and to have a dose threshold. The reversibility is due to the fact that, at least initially, they do not induce DNA damage and mutations. The threshold exists because sufficient dose is necessary to cause a biological effect e.g. proliferation, necrosis etc. The existence of a threshold means that for chemicals with NGTX carcinogenic properties a safe level of exposure can be determined. The no observable adverse effect level (NOAEL), the highest dose of exposure that has been determined experimentally or by observation to have no adverse effects, of the most sensitive species/stain/gender is then extrapolated to humans with the addition of a safety factor. Commonly this is set at a maximum human exposure of a concentration that is 100x less than the NOAEL (factor of 10 for interspecies differences and a further factor of 10 for sensitive individuals) (Butterworth *et al.*, 1995).

1.4.2 Alternative strategies to the rodent lifetime bioassays

The rodent bioassay is very expensive, costing million of pounds for each compound tested and requiring up to two years to be completed. Hence, the bioassay adds a considerable cost to the development costs of new chemicals. Moreover, a positive finding in the bioassay can delay significantly or halt the development of new compounds, which have been developed at a considerable cost. The expense of the assay also prevents the testing of many compounds such as environmental pollutants to which humans are exposed. Therefore, various alternatives to the rodent bioassay have been pursued with some positive results, but so far none has been sufficiently validated to replace the two-year animal bioassay (**Table 1.3**).

Table 1.3 Alternative methods to the two-year rodent assay

Method	Concept	Potential Advantages
In silico	Prediction of carcinogenicity using structural information of compound	Fast and cheap
In vitro	Model the liver responses *in vitro*	No need for animal testing; cheaper and faster than animal models
Human stem cells	Test compounds in human stem cell derived models of the liver	More relevant to humans than rodent models
Transgenic mouse models	Use transgenic mice which are more sensitive than wildtype animals	Tumours will appear faster in transgenic than in wildtype animals
"-omics" technologies	Examine expression profiles after short-term chemical exposure	Acquire mechanistic information ; earlier prediction of carcinogenicity

Each of these alternative strategies has certain advantages and disadvantages. The use of the "-omics" technologies is at present one of the better prospects. They rely on the use of global profiling platforms which allow the quantification of selected parameters

e.g. transcripts (transcriptomics), proteins (proteomics), or metabolites (metabonomics). The central concept behind the use of "omics" for toxicology is that chemicals with similar mechanisms will generate characteristic profiles after short-term treatment. These "signatures" can then be used to both analyse the MOA of a given chemical and to predict its carcinogenic potency after short-term exposure (Blomme *et al.*, 2009).

So far most effort has gone into examination of mRNA expression following carcinogen treatment. (Hamadeh *et al.*, 2002)reported that structurally diverse compounds produced gene expression profiles according to class of the tested compound. (Ellinger-Ziegelbauer *et al.*, 2005) examined liver mRNA expression after treatment of rats with four GNTX carcinogens and four NGTX carcinogens for up to 14 days and reported distinct cellular effects of the two classes. (Ellinger-Ziegelbauer *et al.*, 2008)used a training set of five GNTX carcinogens, five NGTX carcinogens, and three non-hepatocarcinogens to identify biomarkers. The generated classifier was then used to classify a set of 16 other carcinogens (5 GNTX hepatocarcinogens, 6 NGTX hepatocarcinogens, and 6 non-hepatocarcinogens), which it did with an 88% accuracy. In principle then it appears that transcriptomic signatures derived after short-term *in vivo* exposure have potential for assessing the carcinogenic potential of chemicals.

1.5 Justification, hypothesis, and objectives

Justification of project

The possibility of utilising rodent hepatic miRNA signatures following short-term chemical treatment for assessing the chemical risk hazard has remained so far largely unexplored. It is a fact that many fewer miRNAs exist than mRNAs (hundreds compared to thousands). Nevertheless, miRNA signatures have proved better at classifying tissue origin and development stage of cancer than mRNA signatures (Lu *et al.*, 2005, Rosenfeld *et al.*, 2008). The classification and predictive content of miRNA profiles may be even more pronounced during the early stages of hepatocarcinogenesis when epigenetic events are more important. Moreover, miRNAs can also regulate cellular responses (e.g. cell proliferation, differentiation, and apoptosis) that are important drivers of chemical hepatocarcinogenesis. It is possible then that deregulation of miRNAs by chemical carcinogens may be an important event in the process of carcinogenesis induced by many chemicals. Therefore, there is a valid and timely requirement to examine more thoroughly the usefulness of miRNAs in cancer risk assessments.

Few studies so far have examined the effects of chemical treatments on the expression of liver miRNAs. The results of such studies have produced evidence that miRNA profiles are deregulated by chemical carcinogens long before the appearance of tumours. One study examined the effect of continual treatment with a carcinogenic dose of tamoxifen for 24 weeks in female Fischer rat on the expression of liver miRNAs (Pogribny *et al.*, 2007). This study found 33 miRNAs with significant changes in their expression in tamoxifen-treated animals and confirmed the

downregulation of selected protein targets in the pre-cancerous liver tissue. Many of these protein targets were involved in carcinogenesis e.g. Rb1, E2F1, BCL2 etc. A second study examined the effect of exposure to a carcinogenic dose of Wy-14,643 for two weeks (Shah *et al.*, 2007). At this point 27 miRNAs were differentially expressed. Among them was let-7c, which was found to be consistently downregulated from 4 hours up to 11 months when tumours appear. The let-7c repression initiated a signaling cascade that drives hepatocyte proliferation. It was also shown that let-7c was not repressed following Wy-14,643 treatment in a humanised PPARα model which is much less susceptible to liver tumours induced by this chemical (Shah *et al.*, 2007). The complete carcinogen 2-acetylaminofluorene (2-AAF) has also been examined and found to affect expression of liver miRNAs (Pogribny *et al.*, 2009a). The miRNA profiles of animals treated with carcinogenic dose of 2-AAF were examined at 12 and 24 weeks. They found deregulation of miRNAs and their protein targets at both timepoints, with some miRNAs showing a temporally progressive increase in their deregulation.

On the other hand two studies found little or no effects of carcinogenic treatments on liver miRNAs. In the first study mice and rats were treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) for up to 3 days (Moffat *et al.*, 2007). Dioxin toxicity is mediated through the AH receptor. Their analysis revealed that AH signalling affected very few miRNAs, and the levels of change in expression were minimal in magnitude. A second study examined mice fed doses of benzo[a]pyrene (BaP) for 4 and 24 hours. As expected the treatment resulted in considerable changes in the expression of mRNAs. However, no miRNAs were significantly altered by this treatment (Yauk *et al.*, 2010).

Hypothesis

That the hepatic miRNA profiles of rodents following short-term chemical treatment will facilitate the mechanistic understanding and the earlier identification of carcinogens, especially those with NGTX MOA

Objectives of project

Microarrays were used to extract the hepatic miRNA profiles of animals treated with NGTX and GNTC carcinogens in their diet for periods up to 90 days. The extracted miRNA profiles were then examined for their potential to:

(a) Classify chemicals according to their MOA

(b) Predict their carcinogenicity in two-year rat bioassay

(c) Elucidate the role of miRNAs in chemical hepatocarcinogenesis

Chapter 2:
Materials and Methods

2.1 Chemicals and reagents used

For RNA extraction the Trizol reagent was purchased from Invitrogen. RNase Zap and DNA/RNA/nuclease free microcentrifuge tubes were purchased from Applied Biosystems. The integrity of the extracted RNA was assessed using the RNA Nano kit which was obtained from Agilent. The reagents for performing miRNA microarrays (microarray slides, microRNA complete labelling and hybridisation kit, gene expression washing buffer, gasket backing slides) were purchased from Agilent.

Reagents, probes, and primers for real-time quantitative polymerase chain reaction (qPCR) were purchased from Applied Biosystems. For semi-quantitative PCR the Tfi polymerase and the PCR primers were purchased from Invitrogen. Reverse transcription was performed using M-MLV enzyme (Promega). Random hexamers were purchased from Qiagen and the dNTP mix from Invitrogen. A 5x loading buffer from Invitrogen was used to load PCR products into gels. For RNA ligase-mediated rapid amplification of 5´ cDNA ends (RLM-RACE) the Generacer kit was purchased from Invitrogen.

For DNA extraction the DNeasy Blood & Tissue kit of Qiagen was used. Levels of global DNA methylation were assessed using the Methylamp Global DNA Methylation Quantification Ultra Kit of Epigentek

The 100X Halt Protease inhibitor from Pierce was used during protein extraction. The BCA (bicinchoninic acid) protein assay from Pierce was used to quantify the protein extracts. Primary and secondary antibodies were purchased from Santa Cruz

biotechnologies, except for the β-actin primary antibody which was purchased from Sigma-Aldrich. The Novex Sharp pre-stained protein standard was obtained from Invitrogen. Protein bands were visualised using the SuperSignal West Dura Extended Duration Substrate (Pierce) and Hyperfilm which was purchased from GE Healthcare. All other buffers and reagents used were obtained from Sigma-Aldrich.

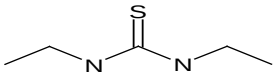
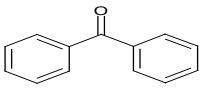
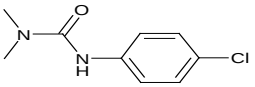
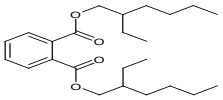
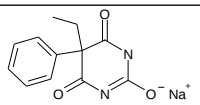
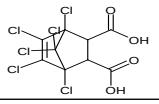
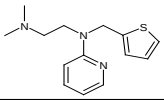
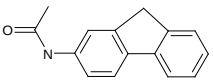
2.2 Animal studies

2.2.1 Tested compounds

This project used liver samples generated from two animal studies which were conducted in 2007 (Dow, 2007a, Dow, 2007b) by industrial collaborators at the Syngenta Central Toxicology Laboratories. **Details of the experimental design of the two studies which are reported here are taken from the research report summary documents produced for these two studies.** They are included here to facilitate the understanding of the generated data from this project. All the compounds used were purchased from Sigma-Aldrich.

The eight tested chemicals in these two studies are shown in **Figure 2.1.**

Figure 2.1 *Chemicals used in animal studies.*

COMPOUND	Chemical structure
Diethylthiourea (DETU)	
Benzophenone (BP)	
Monuron (MON)	
Diethylhexylphthalate (DEHP)	
Phenobarbital Sodium salt (PB)	
Chlorendic Acid (Chl. Ac)	
Methapyrilene HCl (MP HCL)	
2-Acetyl aminofluorene (2-AAF)	

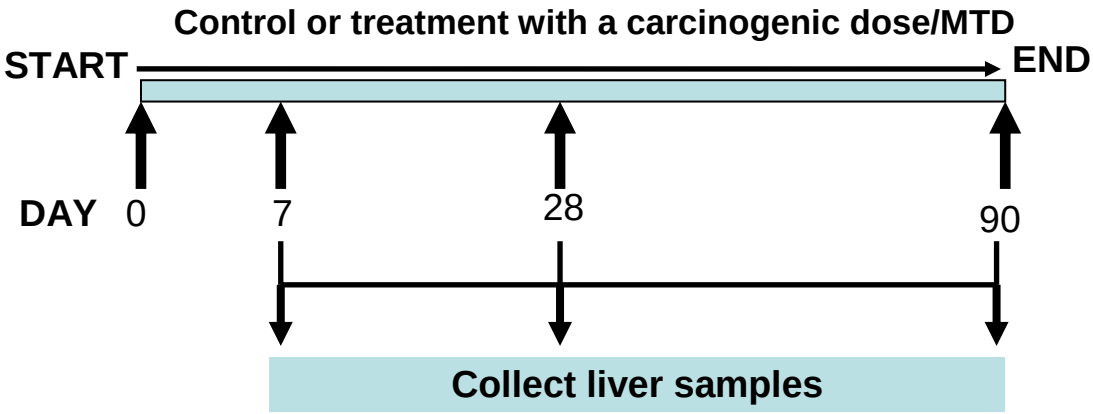
2.2.2 Design of animal studies

Nine-week old male Fischer rats were used in both animal studies, as this strain is commonly used in lifetime bioassays. Rats were given access to mains water and powdered diet *ad libitum.* The tested compounds were fed to the animals at the desired dose through their diet. Liver tissue was obtained from animals at the designated times immediately upon sacrifice, snap frozen, and stored at -80 C^{o}.

The first study was a 90 day dietary study with various known hepatocarcinogens and non-hepatocarcinogens ((Dow, 2007b)). The animals were fed a carcinogenic dose of

carcinogens (as determined by reported two-year assays in Fischer rat) or the maximum tolerated dose (MTD) for the non-carcinogens. The MTD was the maximum dose used in the literature that did not result in unacceptable toxicity in a 90 day study. The design of the 90 day study can be seen in **Figure 2.2**.

Figure 2.2 *Design of 90 day multicompound study*

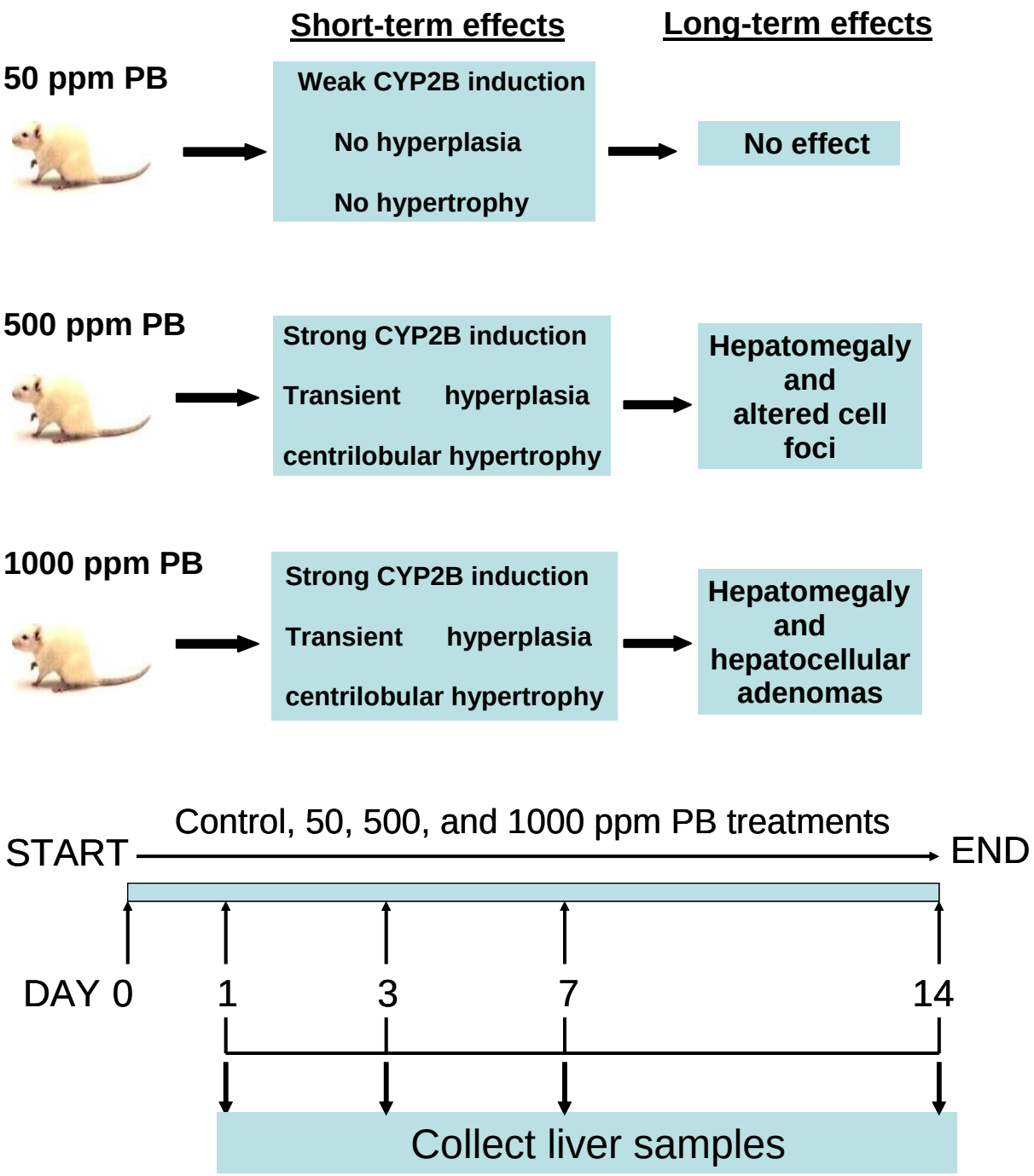


The dose, genotoxicity, and carcinogenicity in 2-year male Fischer rat bioassays of the eight tested chemicals

NGTX & non-carcinogens	NGTX carcinogens	GNTX carcinogens
BP (1250 ppm)	Chl. Ac (1250 ppm)	2-AAF (40 ppm)
DETU (250 ppm)	DEHP (12000 ppm)	
	MP HCL (250 ppm)	
	MON (1500 ppm)	
	PB (1000 ppm)	

The histopatholigical effects of these chemicals on the liver are described in sections **3.1-3.2**. For the second study (Dow, 2007a) animals were exposed to three PB doses, one carcinogenic (Butler *et al.* 1978) and two non-carcinogenic (Hagiwara *et al.*, 1996) for a period up to 14 days (**Figure 2.3**).

Figure 2.3 *Design of 14 day PB dietary study*



Throughout the two studies, treated animals were examined for evidence of overt toxicity. Besides the collection of liver samples data were also collected on important

parameters such as adjusted liver weight, hepatic Ki67 labelling index (LI), microscopic histopathology, and pathology in treated and control animals.

2.3 MicroRNA microarrays

2.3.1 RNA extraction

Total RNA was isolated from the liver tissue using Trizol (Invitrogen) following the manufacturer's instructions. RNase Zap (Applied Biosystems) was used to clean the benches and pipettes used, to remove potential RNase enzymes. To minimise RNA degradation frozen liver sections were thawed in Trizol and processed as quickly as possible. From each liver sample a 50-100mg section of left lateral lobe was pulverised with a polytron homogenizer and total RNA was then extracted using 1ml of Trizol. Following homogenisation at room temperature the insoluble material was removed by centrifugation at 12000g for 10 minutes. Chloroform was added to each sample at 200µl and the samples were then shaken vigorously for 15 seconds, incubated at room temperature for two minutes, and centrifuged at 12000g for 15 minutes to encourage phase separation. The top aqueous phase that contains RNA, but not DNA or protein, was then collected. To increase the purity of the extracted RNA chloroform extraction was performed twice. Total RNA (i.e. ribosomal RNA, mRNA, transfer RNA, miRNA etc.) was then precipitated from the aqueous solution. In order to remove proteoglycan and polysaccharide contaminants, which are widespread in the liver, the RNA was precipitated with a mixture of isopropanol and a high salt precipitation solution (0.8M sodium citrate and 1.2M sodium chloride) at equal volumes. The extracted RNA was then washed with 75% ethanol, air-dried, and dissolved in Tris- **e**thylenediaminetetraacetic acid (EDTA) buffer (TE buffer) at pH

8.0 or RNA-free water. The solution containing pure RNA was then aliquoted, and stored at -80C.

2.3.2 Assessment of RNA quantity, purity and integrity

The suitability of the extracted RNA samples for further usage was determined based on their quantification, purity, and integrity. The RNA quantity and purity of samples was assessed using a Nanodrop-ND1000 spectrophotometer (Nanodrop Technologies). Scanning spectrophotometry analysis is based on the observation that RNA has its maximum absorbance at 260nm, with pure RNA having a 260:280 ratio of 2.0-2.1. Hence low 260:280 and 260:230 absorbance ratios indicate the presence of impurities in the samples. The quantity and purity of all RNA samples used in miRNA microarrays was assessed by recording the absorbance at 260nm, the 260:280 ratios, 260: 230 ratios, and the absorbance spectrum across the spectrophotometric range (220-350 nm). To assess the integrity of the RNA an RNA Nano 6000 kit was used, which runs on the Agilent 2100 bioanalyser. This technology is based on the movement of labelled-RNA at different speeds through micro-channels, depending on the size of the molecules. The bioanalyser software then automatically generates an RNA Integrity Number (RIN), a numerical assessment of RNA integrity, based on the electrophoretic profile of the sample i.e. degree of RNA degradation. A characteristic Agilent bioanalyser analysis of the RNA extracted from the liver samples here is shown in **Figure 2.4**.

Figure 2.4 *Example of evaluation of sample RNA integrity*

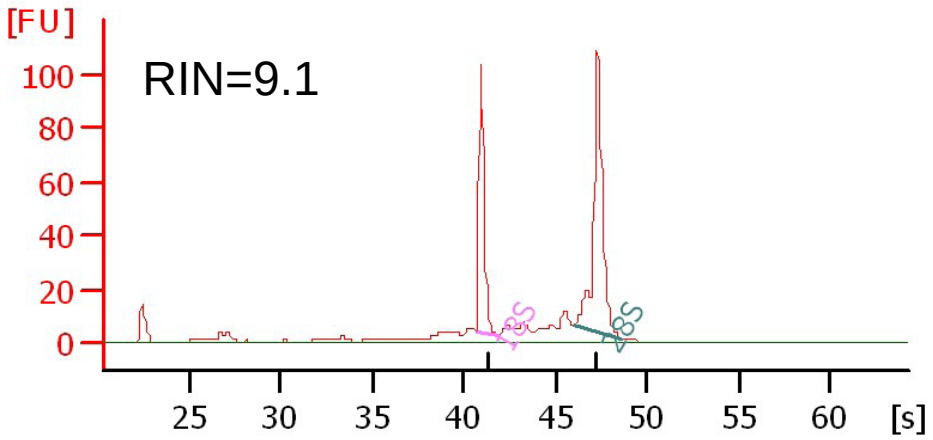


Fig. 2.4 The integrity of RNA extracted from liver samples was evaluated using the Agilent 2100 bioanalyser and RNA Nano 6000 kit. In this figure an example is shown of the evaluation of an RNA sample by this method, with the automatically-generated high RIN value demonstrating the high integrity of the RNA sample.

For miRNA profiling RNA samples were only used if they had an A260:280 ratio above 2.0 and an RIN larger than 7.

2.3.3 RNA labelling, hybridisation, and scanning

The miRNA hepatic expression was profiled using the Agilent miRNA microarray platform and the Agilent Rat miRNA microarray kit. This is a single-colour multiplexed microarray-platform with 8 identical microarrays printed on each slide. This microarray platform has been reported to produce accurate measurements spanning the range of 0.2amol to 2fmol of input RNA (Wang *et al.*, 2007). The platform had also been used to profile tissue miRNAome in published studies previously (Worley *et al.*, 2008, Sato *et al.*, 2009) The microarray comprises 350 *in situ*-synthesized rat miRNA 60mer oligonucleotides probes, based on the Sanger database v10.1 which contained all current identified rat miRNAs at January 2010.

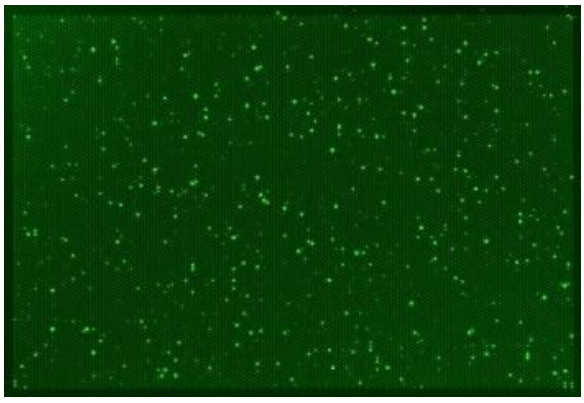
Also included on the microarrays were 23 QC and control probes. Each miRNA is represented by 2-4 probe sequences and 20-40 replicate features on each microarray.

Samples were randomly assigned positions on the microarray slides to reduce non-biological variation. For the 14 day PB dietary study (See **chapter 4**) RNA samples from three animals per day/dose group were examined. As there were 16 day/dose groups (e.g. Day 1 control, Day 1-treated with 50 ppm PB, Day 1-treated with 500 ppm PB, and Day 1- treated with1000 ppm PB), RNA was profiled from 48 animals in total. For the multicompound dietary study (See **chapter 3**) it was decided to focus on liver samples from animals treated with the tested compounds for 90 days rather than the two earlier timepoints. RNA was extracted and profiled for all 42 liver samples available from control and treated animals at 90 days (control n=5, DETU n=5, BP n=5, MON n=5, DEHP n=5, MP HCl n=5, 2-AAF n=5, Chl. Ac n=4, PB n=3). Also examined were six technical replicates (three control samples and three PB treated samples) for which RNA was re-extracted and processed together with the normal samples.

RNA was labelled and hybridised using the miRNA complete labelling and hybridisation kit (Agilent). Total RNA (100ng) was single-colour labelled by enzymatic addition of Cyanine 3-pCp (Cy-3) at the 3' end. Briefly, RNA was first dephosphorylated by incubating with calf intestinal phosphatase included in the kit for 30 minutes at 37^{o}C. The dephosphorylated RNA was denatured by addition of 100% DMSO and heating at 100^{o}C for 10 minutes. The RNA was then transferred immediately to a water-ice bath to prevent reannealing. Ligation of Cy-3 to RNA was performed by incubating at 16^{o}C for 2 hours with T4 RNA ligase that was included in

the kit. The RNA samples were then vacuum dried using a Speedvac (Savant). The dried and labelled RNA samples were reconstituted in hybridisation mix solutions (18µl labelled RNA, 22.5µl 2x hybridisation buffer, and 10x gene expression blocking agent; included in miRNA complete labelling and hybridisation kit and gene expression washing kits respectively), incubated at 100^{o}C for five minutes, and placed immediately on ice. Hybridisation of the labelled RNA to the microarray slides occurred within the Agilent Microarray hybridisation kit (Agilent). Briefly, hybridisation solutions were loaded onto a gasket which was then overlaid with a microarray slide. The sandwiched slides were then clamped and placed in a hybridisation oven set at 55^{o}C and 20 rpm for 20 hours. After 20 hours of hybridisation the microarray slide was removed and washed with two washing buffers before being scanned using an Agilent scanner (G2505B) at the MRC microarray centre at Hammersmith Hospital. In **Figure 2.5** a typical example of a scanned miRNA microarray image is shown.

Figure 2.5 *typical image of a microRNA microarray*



Data were extracted from the scanned microarray images using the Agilent Feature Extraction (FE) software v10.1. Also automatically generated by the Agilent FE

software is a number of quality control metrics that are useful for the evaluation of sample quality

2.3.4 Pre-processing and normalisation of miRNA microarray data

The expression value for each miRNA (the "gTotalGeneSignal") was automatically generated by the Agilent FE software. This value is calculated as the sum of the signal of all features (spots) corresponding to a given miRNA on that microarray, after removal of outliers. Each miRNA is represented by 2-4 distinct probe sequences and 20-40 replicate features on each microarray. The Agilent FE also automatically flagged miRNAs as being detected or not detected on each microarray. MiRNAs were determined as present if their signal magnitude was more than three times the signal error for any of its probes present on the microarrays. The miRNA signal threshold was set to 1 and then $\log_2$ transformed, which adjusts the variance so that it is the same for all intensities.

Normalisation was performed to reduce non-biological variability (e.g. hybridisation efficiencies, washing etc.) and to allow between-array comparisons. The manufacturer's advice is normalisation to the 75^{th} percentile; i.e. for each microarray the value of the 75% percentile miRNA is subtracted from the value of each miRNA in that microarray. The use of the 75% percentile instead of the more normally used median normalisation is performed because a large fraction of miRNAs will not be detected in any given experiment. Two alternative methods to the normalisation to the 75^{th} percentile were also considered (normalisation to the 90% percentile and normalisation to the 50% percentile after filtering of miRNAs which were flagged as absent). To test these alternative normalisation methods the coefficient of variation

was calculated across all samples from the 14 day PB study for four internal control spots (whose levels should be constant in the microarrays) which cover a range of intensity signals, for the three alternative normalisation methods. This analysis found the lowest coefficient of variation for all four control probes after normalisation to the 75th percentile, supporting that it is the most suitable normalisation for these samples (**Figure 2.6**)

Figure 2.6 *Comparison of normalisation methods of microRNA microarrays*

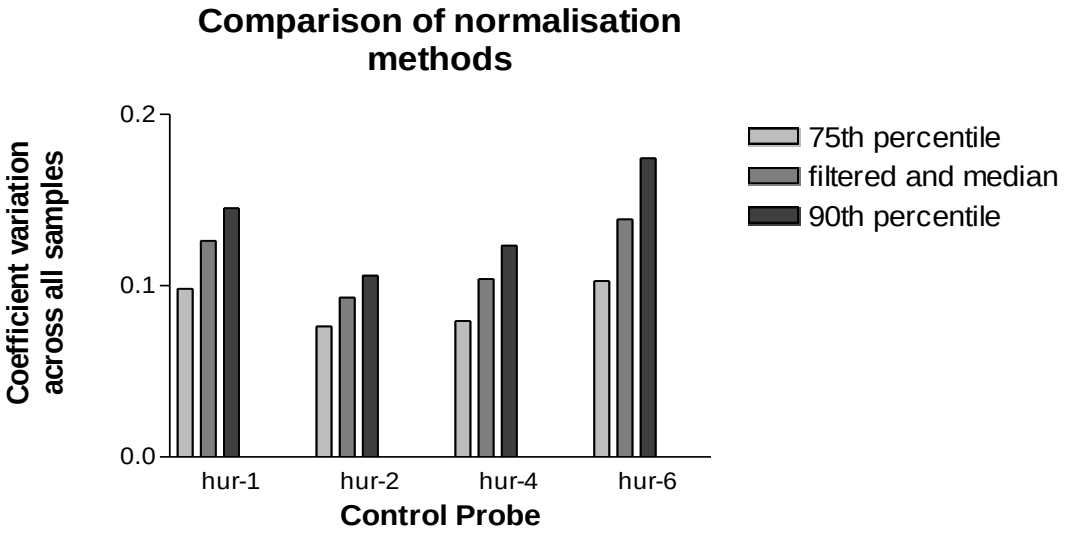


Fig. 2.6 Coefficient of variation across all samples from 14 day PB study for four internal positive control spots present in the microarrays with three different normalisation methods**.** The Y-axis shows the coefficient of variation, while on the x-axis the names of the positive control probes are shown.

Normalisation to the 75% percentile was performed in Genespring GX 10.0 (Agilent). Next, filtering of miRNAs with very low or undetected expression was required to reduce noise in the microarray data. Too stringent filtering could remove potentially interesting miRNAs from further analysis, while too relaxed filtering could result in high background noise in the data. Here it was decided to filter out from further

analysis all miRNAs which were not flagged as present in at least 50% of the samples in any one group.

2.4 Polymerase chain reaction (PCR)

2.4.1 Real-time Quantitative PCR (qPCR)

In qPCR the generation and accumulation of PCR products occurs in real-time, with genes being quantified during the exponential phase of PCR, when the enzymatic process is first order and therefore at its most reliable and precise. To detect and quantify expression levels of selected miRNAs the widely used and reliable quantitative PCR (qPCR) pre-designed Taqman MicroRNA assays (Applied Biosystems) were used. This method relies on the use of stem-loop RT primers followed by Taqman PCR analysis (Chen *et al.*, 2005). This methodology is specific for mature miRNAs (i.e. do not bind to pri-miRNA, pre-miRNA, or genomic DNA), can discriminate miRNAs that differ by a single base, and amplify them with high efficiencies. Taqman probes are oligonucleotides probes that anneal to sequences between the PCR primer sets. Attached to these probes are FAM dyes that only fluoresce- and hence can be measured- during PCR amplification, due to the 5' nuclease activity of DNA polymerase; hence no signal is generated from non-specific PCR products.

In the first step 10ng of total RNA was reverse transcribed using the reagents in the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). A MJ Research Peltier PTC-200 thermocycler (BioRad) was used to perform the reverse transcription. In the second step the generated cDNA was amplified using the Taqman MicroRNA

assays and the Taqman 2X Universal PCR master mix, No AmpErase UNG (Applied Biosystems). The PCR master mix also contained the passive reference dye ROX which was used to automatically correct for differences in reaction concentrations and volumes. The PCR was performed in 96-well plates in an ABI 7500 Fast Real-Time PCR machine (Applied Biosystems). The solutions were first heated at 95^{o}C for 10 minutes to activate the DNA polymerase. Then 40 cycles were performed of heating to 95^{o}C for 15 seconds and 60^{o}C for 1 minute. Each PCR reaction was performed in triplicate. Also included in triplicate in each run were no template control (i.e. PCR reactions in which DNA template is replaced with water) and no reverse transcription - controls (samples for which reverse transcription was performed in the absence of reverse transcriptase). PCR products in the no template control samples indicate the presence of nucleic acid contamination of the PCR reagents. PCR products in the no reverse transcriptase- samples indicate the amplification of genomic DNA instead of RNA.

QPCR data were analysed in the ABI 7500 Sequence Detection System (Applied Biosystems). The comparative Ct Method ($\Delta\Delta C_T$ Method) was used to quantify miRNA expression. Calibration was based on the expression of the 18s rRNA Taqman or snoRNA gene assay (Applied Biosystems). The 18S rRNA gene was reverse transcribed using the same reverse transcriptase used in the TaqMan MicroRNA Reverse Transcription Kit and random hexamers were used as primers. The snoRNA was processed with the same protocol as the miRNA assays. The baseline and threshold were manually chosen for each PCR plate run. Baseline was the initial phase of PCR reaction for which fluorescence is not detected. Threshold was a point above the baseline, but within the exponential phase of PCR

amplification. The threshold cycle (C_T) was the fractional cycle number at which the fluorescence of a sample passed the set threshold value. ΔC_T for a miRNA was the difference in C_T between the miRNA and the calibrator (in this case 18s rRNA). $\Delta\Delta C_T$ was the difference in average value between the ΔC_T of control and treated samples. The fold change in the expression of the examined miRNA between control and treated samples was $2^{\Delta\Delta C_T}$.

2.4.2 Semi-quantitative PCR

In traditional semi-quantitative PCR the process is performed for an appropriate number of cycles and then the PCR product is run and detected on an agarose gel. Semi-quantitative PCR offers a cheaper, but less precise method of quantifying changes in gene expression. A two-step RT-PCR protocol was used here to perform semi-quantitative PCR, with both reverse transcription and PCR performed with the MJ Research Peltier PTC-200 thermocycler. In the first step total RNA was reverse-transcribed using M-MLV (Promega) reverse transcriptase to form cDNA. Two μg of total RNA was mixed with 0.5μg random hexamers (Qiagen) and heated at 70^oC for five minutes to melt secondary structures and then placed immediately on ice. Next were added 200 units of M-MLV, 1.25μl of 10mM dNTP mixture (Invitrogen), 25 units of RNase inhibitor (Applied Biosystems), 5μl of M-MLV reaction buffer, and made up to 25μl with nuclease-free water. The mixture was heated at 37^0C for one hour and then to 75^oC for 15 minutes to denature the enzyme.

In the second step 1-2μl of the RT mix were amplified by PCR using the Tfi polymerase (Invitrogen) and custom PCR primers. First a 50μl mixture was made

containing 5x PCR reaction buffer, 200µM of each nucleotide, 1.5mM $MgCl_2$, 0.2µm of each primer set, 1-2µl cDNA, and made up with nuclease-free water to 49µl. The solution was then heated at 94^{o}C for two minutes. After the heating step 1µl of Tfi polymerase was added to the mixture. The late addition of the polymerase is termed "Hot start" PCR and it reduces the amplification of non-specific PCR products. Then 30-35 cycles of PCR were performed at 94^{o}C for 30 seconds, annealing temperature for 30 seconds, and extension for one minute. After the completion of the designated number of cycles the protocol was completed by ten minutes of extension at 70^{o}C. The PCR primers used were either custom-designed using primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) or were obtained from previously published papers (**Table 2.1)**.

Table 2.1 Primer sequences used for semi-quantitative PCR

Primer	Forward sequence (5'-3')	Reverse sequence (5'-3')	Annealing temperature	Product size	Reference
cdh1	ACAGCAAGCATGCC AGTGAA	GCACCAACACACCCA GCATA	62^{o} C	326 bp	Barth *et al.* 2005
cyp2b1/2	GGA GAG CGC TTT GAC TAC	CTC GTG GAT AAC TGC ATC	59^{o} C	514 bp	Pustylnyak et. al. 2007
gapdh	CAT GGA CTG TGG TCA TGA G	TTC AAC GGC ACA GTC AAG G	59^{o} C	373 bp	Pustylnyak et. al. 2007
Primer A	AGCGCCCCAAATCTC CAGGATC	AACACGGTCGTCACA CCTGGTG	55^{0}C	249 bp	Custom made
Primer B	ACCATCCCACCTCCC TTTAAACCC	TAGGCAGAGGTGGGT TAGCACG	55^{o}C	260 bp	Custom made

PCR products were examined by electrophoresis through 1% agarose gels. Gels were made by adding agarose powder to 1xTAE buffer (40mM Tris-acetate, 1mM EDTA), made up from 10x TAE stock (Sigma-Aldrich), and heating it to dissolve. Ethidium

bromide (0.5μg/ml) was added to the gels to allow visualisation of PCR products and the gels were then poured into agarose gel tanks and allowed to set. A 20μl volume of PCR products was mixed with 5x loading buffer (Invitrogen) and loaded into the gel A DNA ladder (Sigma-Aldrich) was used to allow determination of the molecular sizes of the observed PCR products. Electrophoresis was setup at 100V for 30-60 minutes and PCR products were visualised by exposure to UV light. Detected bands were quantified in the Aida image analyser v 3.52 (Raytest) and the expression of genes of interest were normalised to the expression of the *gapdh* gene.

2.5 Immunoblotting

2.5.1 Protein extraction from liver samples

Protein was extracted from 50 mg of liver tissue. Livers were homogenised with a polytron machine in 500 μl of lysis buffer (50 mM Tris–HCl, pH 7.4; 1% IGEPAL (Sigma-Aldrich); 0.25% sodium deoxycholate (Sigma-Aldrich); 150 mM NaCl (Sigma-Aldrich); 1 mM EDTA (SIGMA-Aldrich); 100 X Halt Protease inhibitor (Pierce)). Homogenisation was followed by sonication and incubation at 4 °C for 30 min. The solutions were then centrifuged at maximum speed for 20 minutes to remove insoluble debris. The protein extracts were quantified using the BCA (bicinchoninic acid) protein assay (Pierce). This assay is based on the reduction of Cu^{2+} ions to Cu^{1+} by proteins. The BCA then reacts with the reduced Cu^{1+} ion to form a purple coloured complex. Absorbance at 562nm is proportional to the amount of protein present in the solution.

2.5.2 Protein detection and quantification

Protein mixtures were separated according to size by electrophoresis through sodium dedocyl sulphate (SDS) 7.5-15% polyacrylamide gels at 100V for 1-2 hours. Equal amounts of protein (50μg), as determined by BCA quantification, were loaded into each lane mixed with 5x Laemmli loading buffer (Sigma-Aldrich) and made up to 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue, 60 mM Tris-Cl pH 6.8 equal volumes with water. Also loaded onto the gel was Novex Sharp pre-stained protein standard (Invitrogen) to allow estimation of protein size. The proteins were then electrophoretically transferred from the gel onto a nitrocellulose membrane (400 Amps and 200V for 90 minutes) and the efficiency of the transfer was assessed by staining the membrane with Ponceau S (Sigma-Aldrich), a reversible non-specific protein stain. The nitrocellulose membrane was blocked for one hour at room temperature with 10% fat-free milk made up in PBS-Tween 0.1% to reduce non-specific binding and then incubated with the commercially available monoclonal primary antibodies, made up to the appropriate concentration in PBS-Tween 0.1%, overnight at 4^{o}C. The next morning the blots were washed (PBS-Tween 0.1%) and incubated at room temperature for one hour with horseradish peroxidise (HRP)-conjugated secondary antibodies which bound the primary antibody. The concentrations of antibodies used are shown in **Table 2.2**.

Table 2.2 Concentrations of primary/secondary antibodies used for immunoblotting

Antibody	Primary Ab concentration	Secondary Ab concentration
Zeb1 (Santa Cruz)	1:200	1:2000
Zeb2 (Santa Cruz)	1:200	1:2000
β-actin (Sigma-Aldrich)	1:10000	1:10000

After a final thorough washing of the membrane (with PBS-Tween 0.01%), bound antibodies were detected using SuperSignal West Dura Extended Duration Substrate (Pierce). Hyperfilm ECL (GE Healthcare) was overlaid for appropriate time over the blot and then exposed. Protein bands of the protein of interest were quantified in Aida image analyser v.3.52 and normalised to that of β-actin.

2.6 Methylation analysis

2.6.1 DNA extraction and quantification

DNA was extracted from liver samples using the DNeasy Blood & Tissue kit (Qiagen) following the manufacturer's protocol, and using the reagents and buffers included in the kit. Briefly, approximately 25mg of left lateral lobe liver tissue were lysed overnight using proteinase K at 56^{o}C in ATL tissue lysis buffer. RNase A (Sigma-Aldrich) was added at 100mg/ml and left at room temperature for two minutes to digest RNAs present in the lysate. Equal amounts of buffer AW1 and ethanol (96-100%) were added to the mix and vortexed. The mixture was centrifuged in mini-spin columns containing membranes which selectively bind DNA. The mini-spin column membrane was washed with buffer AW2 to remove contaminants and the sample

DNA was eluted from the membrane with water. Extracted DNA was quantified using the Nanodrop ND-1000 and used for subsequent experiments if its 260:280 ratio was higher than 1.6.

2.6.2 Global DNA methylation analysis

The levels of global DNA methylation for extracted DNA were determined using the Methylamp Global DNA Methylation Quantification Ultra Kit (Epigentek) following the manufacturer's instructions. First 150ng of genomic DNA extracted from liver samples were immobilised onto a strip. The immobilised DNA was subsequently bound by an antibody that specifically recognises 5-methylcytosine, the methylated form of cytosine. This antibody includes a chromophore that has its maximum absorbance at 450nm, with the amount of methylated cytosine in the sample being proportional to the absorbance of the solution at that wavelength.

2.7 RLM-RACE

RNA ligase-mediated rapid amplification of 5´ cDNA ends (RLM-RACE) is a protocol that allows the identification of the 5' end and the transcriptional start site (TSS) for genes which have part of their sequence known. In this method an oligo with a known sequence is attached to the 5' end of the gene. Then PCR is performed using one primer that anneals to the attached oligo and a second primer that is designed to anneal to a known part of the gene that is downstream. The PCR product can then be sequenced to identify the 5' end of the gene (**Figure 2.7**).

Figure 2.7 *Overview of the methodology of Generacer kit*

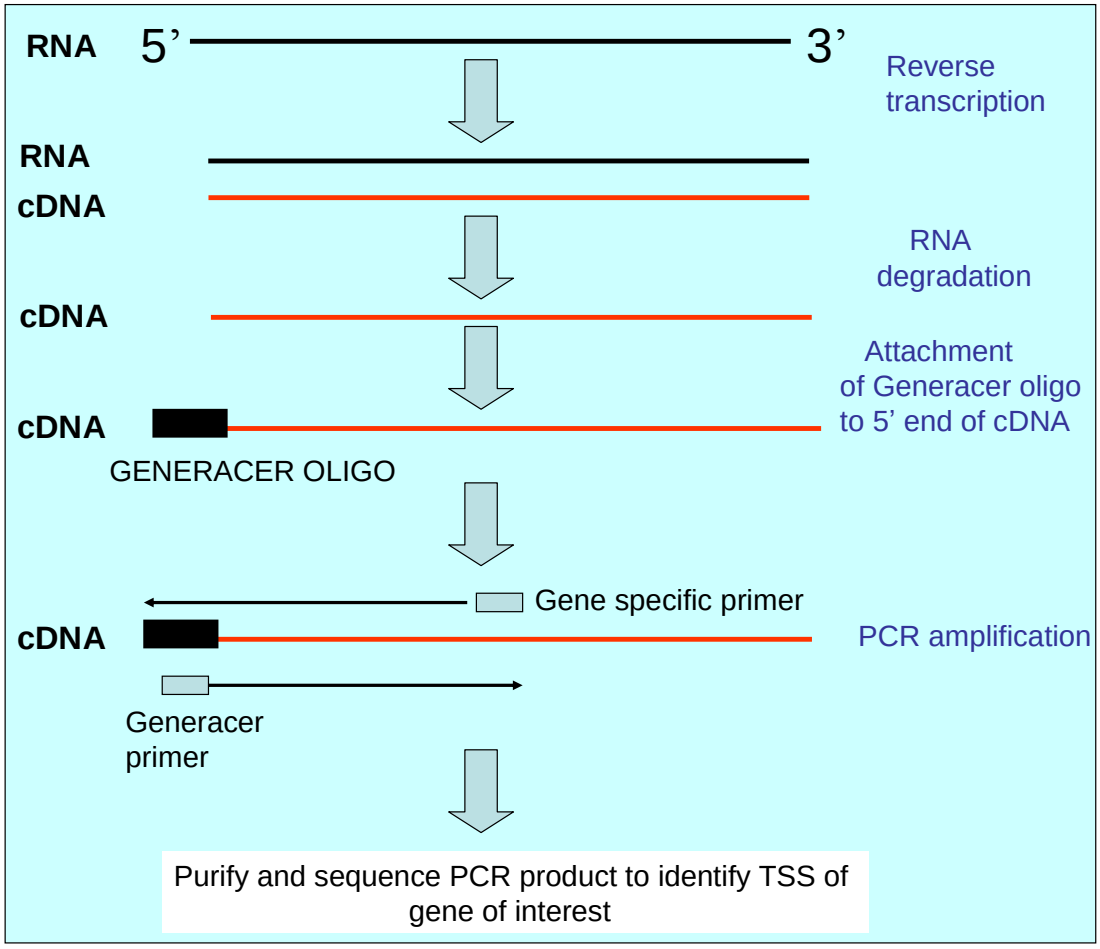


Fig. 2.7 RNA from sample cell/tissue is reverse transcribed to form cDNA. RNA is enzymatically degraded and then the Generacer oligo is attached to the 5' end of cDNAs. PCR amplification is performed using one primer specific for the Generacer primer and one primer that is specific for a region within the gene of interest. The PCR product is then purified and sequenced to identify the 5' end of the cDNA.

The Generacer kit (Invitrogen) was used here to perform RLM-RACE for miRNAs. Attachment of the Generacer RNA oligo to the 5' end of the gene requires a prior modification of the RNA. First 5µg of total RNA was treated with calf intestinal phosphatase to remove 5' terminal phosphate from non-capped RNAs and then treatment with tobacco acid pyrophosphatase removes the 5'-cap structure from capped RNAs. This allows the ligation of Generacer RNA oligo to the 5' end of RNAs. The ligated RNA was then reverse transcribed using Superscript III reverse transcriptase using random hexamers as primers. Modified RNA was then amplified

using a Generacer RNA oligo-specific primer and a custom-primer. The whole protocol was also performed on RNA from Hela cells provided by manufacturer. The *Beta-Actin* gene was then amplified as a positive control of the whole process.

2.8 *In silico analysis*

Genomic DNA sequences were obtained from the University of California Santa Cruz (UCSC) genome browser (Rhead *et al.*, 2010) from the latest available rat genome assembly (Nov 2004 Baylor 3.4/rn4). The NUBISCAN (Podvinec *et al.*, 2002) and the PROMO 3.0 software (Messeguer *et al.*, 2002) was used to predict putative transcription factor binding sites within examined DNA sequences. The NUBISCAN software identifies nuclear receptor response elements in genomic sequences. The PROMO software uses known transcription factor binding sites deposited in TRANSFAC database to generate binding site weight matrixes. The CpG island searcher software (Takai & Jones, 2002) was used to scan selected genomic sequences for the presence of putative CpG islands. CpG islands were defined as those regions which were larger than 200 bases, with a greater than 55% percentage of cytosines and guanines, and a 0.65 ratio of observed to expected cytosine-guanine dimmers. The TargetScan v5.1 software (http://www.targetscan.org) was used to predict mRNA targets of specific miRNAs. TargetScan predicts targets by scanning for conserved 7mer and 8mer sequences in the 3' UTR of mRNAs that complement the seed region of miRNAs. The algorithm also allows for some mismatch of the seed region if extra binding exists at 3' end of miRNA. The IDs of predicted miRNA targets were uploaded to the PANTHER database (Mi *et al.*, 2003, Thomas *et al.*, 2006) and allocated to pathways. Comparisons of the distribution of the genes within the rat genome into pathways with the distribution of predicted targets of miRNAs allowed

the identification of enriched pathways. The FirstEF software was used to predict promoter regions (Davuluri *et al.*, 2001)

2.9 Clustering and classification of samples

2.9.1 Hierarchical clustering

Hierarchical clustering of the generated miRNA data was used to identify the treatments which had the most similar effects on the hepatic miRNA profiles at the tested times. Additionally this method allowed the identification of co-regulated miRNAs. Clustering of the miRNA data was performed in Gene Cluster 3.0 (Eisen *et al.*, 1998) and the generated tree was viewed in Gene Treeview (Saldanha, 2004). Hierarchical clustering is unsupervised in that no information is supplied by the user besides the normalised expression data. The hierarchical clustering algorithm is termed “agglomerative” because it sequentially joins small clusters to bigger ones and “binary” due to the fact that it joins together two-clusters at a time. The hierarchical clustering algorithm initially considers each sample as an isolated cluster. The similarity between all clusters is then calculated using the chosen similarity metric (e.g. correlation centred correlation uncentred, Euclidian, etc.) and performing all possible pair-wise comparisons. The two most similar samples are then joined with the length of the branch proportional to their distance as calculated by the similarity metric (more similar samples are connected by shorter branches, less similar with longer branches). The two samples are then merged to a single cluster and the process is repeated until all samples are incorporated into the generated tree. To calculate the distance metric of clusters formed when two or more samples are joined together different options exist (simple linkage, complete linkage, average linkage, etc.). The

average linkage used here is the most robust to outliers. With this linkage the distance metric between two metrics is the average value of their members. The most commonly used distance metric is correlation. Correlation metrics group samples and genes together according to similar patterns of gene expression.

2.9.2 Sample classification

Prediction analysis of microarrays (PAM) is a class prediction tool that has been specifically developed for analysis of microarray data (Tibshirani *et al.*, 2002). Given a predefined class it can identify the smallest group of genes that characterise that group of samples. The identification of this signature is achieved using the expression of genes and the nearest shrunken centroid method. This signature can subsequently be used to predict the class of new sets of samples, if there are any available. PAM was performed as a function tool within the BRB Array tools v4.2 (BRB Array tools).

2.10 Statistical analysis

Student's t-test and ANOVA were used to test for significance of PCR, immunoblotting, and methylation data. One-way ANOVA with multiple testing correction were used to identify differentially expressed miRNAs after 90 days of chemical treatments. Parametric (Pearson's) and non-parametric (Spearman's) correlations analysis were performed in Graphpad Prism v5.0. Significance analysis of microarrays (SAM) (Tusher *et al.*, 2001) was used to identify deregulated miRNAs from the 14 day PB dietary study. This statistical method was specifically developed for microarrays and determines which genes are statistically differentially expressed. This method has the advantage of being non-parametric and that it allows control over the false detection rate (FDR).

Chapter 3:

Effects of 90 day treatment with various hepatocarcinogenic and non - hepatocarcinogenic compounds on the liver microRNAome

3.1. Introduction

The Agilent miRNA microarray platform was used to profile the expression of miRNAs in control and treated animal's livers generated by the Syngenta multiple compounds study (Dow, 2007b) as previously described (**section 2.3**) . Samples from animals treated for 90 days were chosen for microarray examination rather than earlier time-points, i.e. 28 or 7 days, as it was surmised that changes in miRNA expression relating to carcinogenesis would be more established at this later stage. Moreover, most acute toxicological responses irrelevant to carcinogenesis would have attenuated by that stage. The eight chemicals examined here represented a diversity of MOA, carcinogenic potentials, and effects on the liver:

- **2-AAF** is an aromatic amide that is metabolised into electrophilic derivatives, which react with DNA. 2-AAF was the only confirmed GNTX compound included in this study. Besides being mutagenic 2-AAF is also a powerful complete carcinogen that can cause liver tumours by itself (Heflich & Neft, 1994).

- **MP HCl** is a NGTX hepatocarcinogen. The MOA of this compound is considered to be its cytotoxicity, coupled with increased hepatic cell proliferation (Lijinsky, 1984).

- **PB** and **BP** are two "PB-type chemicals", a structurally diverse family of chemicals characterised by their ability to pronouncedly induce cyp2b enzymes in the liver. Of the two chemicals only PB can cause hepatocellular adenomas in the male rat, even though both chemicals are mice

hepatocarcinogens (Whysner *et al.*, 1996, Butler, 1978); (NTP TR-533, 2006). The MOA of PB hepatocarcinogenesis requires CAR activity, as the chemical does not cause tumours in CAR KO mice (Yamamoto *et al.*, 2004). PB also acts as a strong promoter following initiation (Whysner *et al.*, 1996).

- **DEHP** is a phthalate widely used as a plasticiser. It is a rodent hepatocarcinogen and a peroxisome proliferator chemical (PPC) (NTP TR-217, 1982). These structurally diverse chemicals (PPCs) are characterised by their induction of peroxisomal and fatty acid-oxidising enzymes, increase in both the size and number of peroxisomes, and hepatic hypertrophy and hyperplasia (Moody *et al.*, 1991). DEHP brings about its PPC activities *via* its monoester metabolite mono-ethylhexyphthalate (MEHP) which activates PPARα (Lapinskas *et al.*, 2005). DEHP does not have peroxisome proliferation effects in PPARα knockout mice (Ward *et al.*, 1998), demonstrating the important role of the receptor in driving its liver effects.

- **Chl.Ac**. and **MON** are two male rat NGTX hepatocarcinogens with unknown MOA. MON is a photosynthesis-inhibiting herbicide. It is weakly carcinogenic in male Fischer rats, but is not carcinogenic in mice or female Fischer rats (NTP TR-266, 1988). Chl.Ac. is a chlorinated hydrocarbon that is used for some chemical synthetic processes. It induces tumours in both mice and rats in oral studies (NTP TR-304, 1987).

- **DETU** is a NGTX compound which was chosen due to its lack of effects in the liver and because it is not hepatocarcinogenic in either mice or rat (NTP TR-149, 1979).

The liver samples generated from this study therefore were a good starting point to examine the hypothesis that miRNAs are useful biomarkers of cancer-inducing chemicals. In total 48 samples were examined. Of these 42 were liver samples from the control and treated animals at 90 days. The other six samples examined were technical replicates- three control technical replicates (CTR) and three PB-treated technical replicates (PTR).

Also available for these samples were data on the effects of each chemical on relevant parameters such as overall animal toxicity, liver size, hepatic proliferation, liver histopathology, etc. (Dow, 2007b), as well as transcriptomic profiling data (Richard A. Currie personal communication). These two datasets could then be compared to the generated hepatic miRNA profiles.

3.2. Effects of chemical treatments on animals

Data relevant to liver carcinogenesis are presented in **Tables 3.1-3.4** (Dow, 2007b). **These data were not collected by myself and are shown here to facilitate the miRNA data obtained in my experiments**.

Table 3.1 Frequency of liver tumours in two-year bioassay in male Fischer rat

Compound	Dose (ppm)	Number of neoplasias/carcinomas	Reference
DETU	250	0/50 (0%)	(NTP TR-149, 1979)
BP	1250	0/50 (0%)	(NTP TR-533, 2006)
MON	1500	9/50 (18%)	(NTP TR-266, 1988)
DEHP	12000	12/49 (24%)	(NTP TR-217, 1982)
PB	1000	11/33 (33%)	(Butler, 1978)
Chl.Ac.	1250	23/50 (46%)	(NTP TR-304, 1987)
MP HCL	250	18/20 (90%)	(Lijinsky, 1984)
2-AAF	40	23/23 (100%)	(Ogiso *et al.*, 1985)

Table 3.2 Liver weight adjusted to bodyweight for each treatment

Compound	Day 7	Day 28	Day 90
CON	9.4	9.5	11.6
DETU	10.3*	10.2	11.7
BP	11.2*	9.5	14.7*
MON	10.3*	10.7*	13.1*
DEHP	14.6*	18.9*	19.9*
PB	11.7*	13.7*	14.7*
Chl.Ac.	8.7*	8.9	11.5
MP HCL	9.5	9.7	11.9
2-AAF	9.4	10.5*	12.2

Values are the average ratio of liver weight to bodyweight; Analysis of covariance of organ weight to bodyweight * $p<0.05$

Table 3.3 Percentage of Ki67-labelled hepatic cells for each treatment group

Compound	Day 7	Day 28	Day 90
CON	5.68	2.47	0.81
DETU	2.71*	1.69	2.71*
BP	6.59	2.77	1.69*
MON	5.68	2.49	2.24*
DEHP	7.87	2.30	1.77*
PB	6.32	3.26	2.23*
Chl.Ac.	2.15*	3.95*	3.48*
MP HCL	4.83	7.76*	7.66*
2-AAF	5.37	3.32	4.94*

ANOVA with double arcsine transformation * $p<0.05$

Table 3.4 Liver micropathology

Treatment	Hypertrophy			Necrosis and/or apoptosis			Vacuolation			Biliary hyperplasia +/- inflammation		
DAY	**7**	**28**	**90**	**7**	**28**	**90**	**7**	**28**	**90**	**7**	**28**	**90**
CON	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
DETU	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
BP	5/5	5/5	5/5	0/5	0/5	0/5	0/5	4/5	4/5	0/5	0/5	0/5
MON	0/5	0/5	0/4	0/5	0/5	0/4	0/5	0/5	4/4	0/5	0/5	0/4
DEHP	5/5	5/5	5/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
PB	5/5	5/5	4/4	0/5	0/5	0/4	0/5	4/5	4/4	0/5	0/5	0/4
Chl. Ac.	0/5	0/5	0/4	0/5	0/5	0/4	0/5	0/5	0/4	0/5	0/5	0/4
MP HCL	0/5	0/5	0/5	1/5	3/5	4/5	0/5	0/5	0/5	0/5	5/5	5/5
2-AAF	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	5/5	5/5

At the tested doses the chemicals causing the highest frequency of tumour formation in the two year bioassay were 2-AAF, MP HCl, and Chl. Ac. These were also the chemicals with the highest Ki67 LI at 90 days. Besides the induction of proliferation 2-AAF, the most potent carcinogen of this study, also caused a transient increase in adjusted liver size at 28 days and biliary hyperplasia +/- inflammation at days 28 and 90. MP HCl caused the biggest increase in hepatic proliferation at days 28 and days 90. It was also the only compound for which minimal necrosis/apoptosis were detected. These two observations are in accord with the MOA of MP HCL being hepatotoxicity followed by regenerative hyperplasia. MP HCL was also the only other compound besides 2-AAF to cause biliary hyperplasia +/- inflammation at days 28 and 90. Chl. Ac caused a small transient decrease in liver weight at day 7.

The nuclear receptor agonists PB, BP, and DEHP were the only ones to cause hypertrophy from day 7 onwards, centrilobular for the PB-type compounds and diffuse for the PPARα activator. The three compounds also caused the biggest sustained increase in adjusted liver weight. DEHP caused the biggest increase (day 7- 155%; day 28 198%; day 90 171%). PB and BP caused similar increases except for day 28 when no change was observed for BP-treated animals (Day 7- 124% and 119%; day 28 144% and 100%; day 90 127% for both). These three compounds also caused minimal vacuolation from day 7 onwards.

Besides the nuclear receptor activators, MON was the only compound to cause a sustained statistically significant increase in adjusted liver weight (110% day 7; 113% day 28; 113% day 90) and to cause minimal vacuolation on day 90. DETU had minimal effects on the liver, with a transient increase in liver weight being observed on day 7.

3.3. Evaluation of quality and processing of microarray data

3.3.1 PCA and Quality Control metrics of samples

The first step after data extraction was to confirm that the biochemical and analytical manipulations had worked as expected and to identify any potential problematic samples. First a PCA was performed (**Figure 3.1**). Outlier samples can be indentified as those which are clearly separated from the other samples of their group.

Figure 3.1 *PCA of pre-filtered normalised samples from 90 day dietary study*

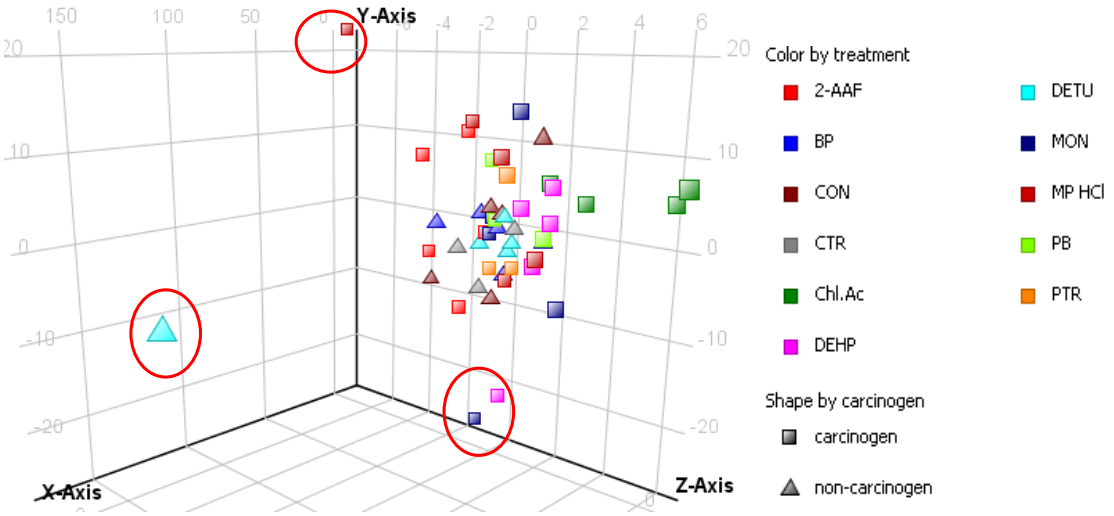


Fig. 3.1 Each symbol represents an array, with its colour indicating the group to which it belongs and its shape indicating if the treatment is carcinogenic. The red circles highlight the four samples (DETU 5, DEHP 5, MON 4, and MP HCL 3) which are clearly outliers from their groups. X-axis= principal component (PC) 1, Y-axis= PC2, Z-axis= PC3.

The PCA analysis revealed four samples (DEHP 5, DETU 4, MON 4, and MP HCL 3) as being clear outliers. Besides PCA an additional method to evaluate sample quality was based on the quality control (QC) metrics which were automatically generated for each sample by the Agilent FE software. These metrics were (**1**) "AnyColorPrcntFeatPopnOL": this is the percentage of features on the array which are population outliers. A high value can indicate that regions on the array were problematic (**2**) "gTotalSignal75pctile": the sum of the signal for all miRNAs at the 75th percentile. A very low value indicates that some part of the procedure was sub-optimal resulting in low signal. A very high signal can indicate insufficient washing of the microarray (**3**) "AddErrorEstimateGreen" is an estimate of the total error on the microarray. Higher values indicate higher error on the arrays (**4**)

"gNonCtrlMedPrcntCVBGSubSig" which is the median covariance of inlier probes minus the background. The values of these four QC metrics for each of the samples are tabulated in **Table 3.5-3.6**.

Table 3.5 QC metrics for controls and non-carcinogen treated samples

Sample	Metric 1	Metric 2	Metric 3	Metric 4
CON 1	2.4	22.4	3.3	43.8
CON 2	4.4	30.4	3.2	37.4
CON 3	2.1	39.5	3.5	6.7
CON 4	2.9	49.4	3.9	6.9
CON 5	3.2	42.7	3.1	6.1
CTR 1	3.5	35.3	3.1	16.0
CTR 2	2.2	37.2	2.9	6.7
CTR 3	2.5	30.6	6.2	7.4
BP 1	3.2	42.8	3.9	6.5
BP 2	3.2	41.8	2.7	9.4
BP 3	2.9	31.1	3.7	26.3
BP 4	3.0	40.6	3.3	22.1
BP 5	2.8	30.0	2.8	6.6
DETU 1	2.4	42.0	3.0	7.1
DETU 2	3.2	32.0	2.8	10.4
DETU 3	6.4	37.3	4.6	7.1
DETU 4	2.1	35.5	3.3	6.9
DETU 5	**2.7**	**1369.8**	**526.5**	**6.1**

Metric 1= AnyColorPrcntFeatPopnOL; Metric 2= gTotalSignal75pctile
Metric 3= AddErrorEstimateGreen; Metric 4=gNonCtrlMedPrcntCVBGSubSig
Marked in bold are outlier samples as identified by PCA. Coloured red are QC metric values that are outside normal range.

Table 3.6: QC metrics for carcinogen-treated samples

Sample	Metric 1	Metric 2	Metric 3	Metric 4
2-AAF 1	3.9	40.7	3.3	7.1
2-AAF 2	2.6	54.4	3.2	5.7
2-AAF 3	3.3	38.0	3.0	25.8
2-AAF 4	3.3	57.6	3.0	6.7
2-AAF 5	2.6	29.4	4.1	6.7
Chl. Ac. 1	2.0	51.1	3.8	6.2
Chl. Ac. 2	3.1	44.3	2.6	6.6
Chl. Ac. 3	2.7	45.0	3.0	6.7
Chl. Ac. 4	3.0	49.8	3.3	11.3
DEHP 1	3.0	41.4	3.0	5.3
DEHP 2	2.8	32.8	4.2	6.2
DEHP 3	2.6	45.5	3.2	9.0
DEHP 4	2.2	40.0	3.4	8.6
DEHP 5	**5.0**	**9.3**	**5.0**	**90.5**
MON 1	2.8	59.6	3.3	7.1
MON 2	4.0	44.3	3.1	6.3
MON 3	5.9	21.4	4.4	19.2
MON 4	**2.5**	**4.9**	**3.9**	**9.1**
MON 5	4.0	38.9	2.7	9.3
MP HCL 1	2.9	60.8	3.2	7.1
MP HCL 2	3.6	32.9	3.4	48.0
MP HCL 3	**2.2**	**78.2**	**3.0**	**6.6**
MP HCL 4	3.5	62.6	3.2	8.4
MP HCL 5	2.8	34.7	2.9	6.9
PB 1	2.5	54.2	3.1	6.7
PB 2	2.7	40.2	3.5	7.1
PB 3	3.2	40.9	4.0	9.8
PTR 1	3.7	33.1	3.4	16.6
PTR 2	2.9	30.7	3.4	31.3
PTR 3	2.5	49.6	3.3	7.5

Metric 1= AnyColorPrcntFeatPopnOL; Metric 2= gTotalSignal75pctile

Metric 3= AddErrorEstimateGreen; Metric 4= gNonCtrlMedPrcntCVBGSubSig

Marked in bold are outlier samples as identified by PCA. Coloured red are QC metric values that are outside normal range

Samples marked with bold in tables 3.1-3.2 are the four samples that were identified as outliers by PCA. Three of the four sample outliers identified by PCA had clearly unusual QC metrics. DETU 5 had a metric 2 value more than 20 times the next

sample, indicating that the sample was not washed sufficiently. Conversely, samples MON 4 and DEHP 5 had very low metric 2 values, indicating that for these samples RNA labelling or hybridisation failed. Based on the PCA and QC metric examination four samples (DETU 5, DEHP 5, MON 4, and MP HCL 3) were removed from further analysis as problematic outlier samples.

3.3.2 Filtering of miRNA data

The next step was to filter out miRNAs which were not expressed, or were expressed at very low levels in the samples. Of the 350 miRNAs represented in the microarrays 192 were detected in at least one sample, 167 were detected in 50% of samples of control or any treatment, and 90 miRNAs were detected in all samples. The fact that so many of the miRNAs were not detected could be due to the fact that (a) the expression of many miRNAs is tissue-cell type dependent as well as varying according to developmental stage (b) The sensitivity of microarrays is lower than other techniques (e.g. PCR or deep-sequencing) so that miRNAs with very low expression may not be reliably detected. Further analysis was performed on the 167 miRNAs that were detected in 50% of samples in any condition.

Box plots allow a quick visual summarisation of median, distribution, and most extreme values of a given sample. Box plots for the normalised, filtered samples showed that the microarray samples had comparable distributions and intensities, supporting their reproducibility (**Figure 3.2**).

Figure 3.2 *Box plots of normalised and filtered microarray samples*

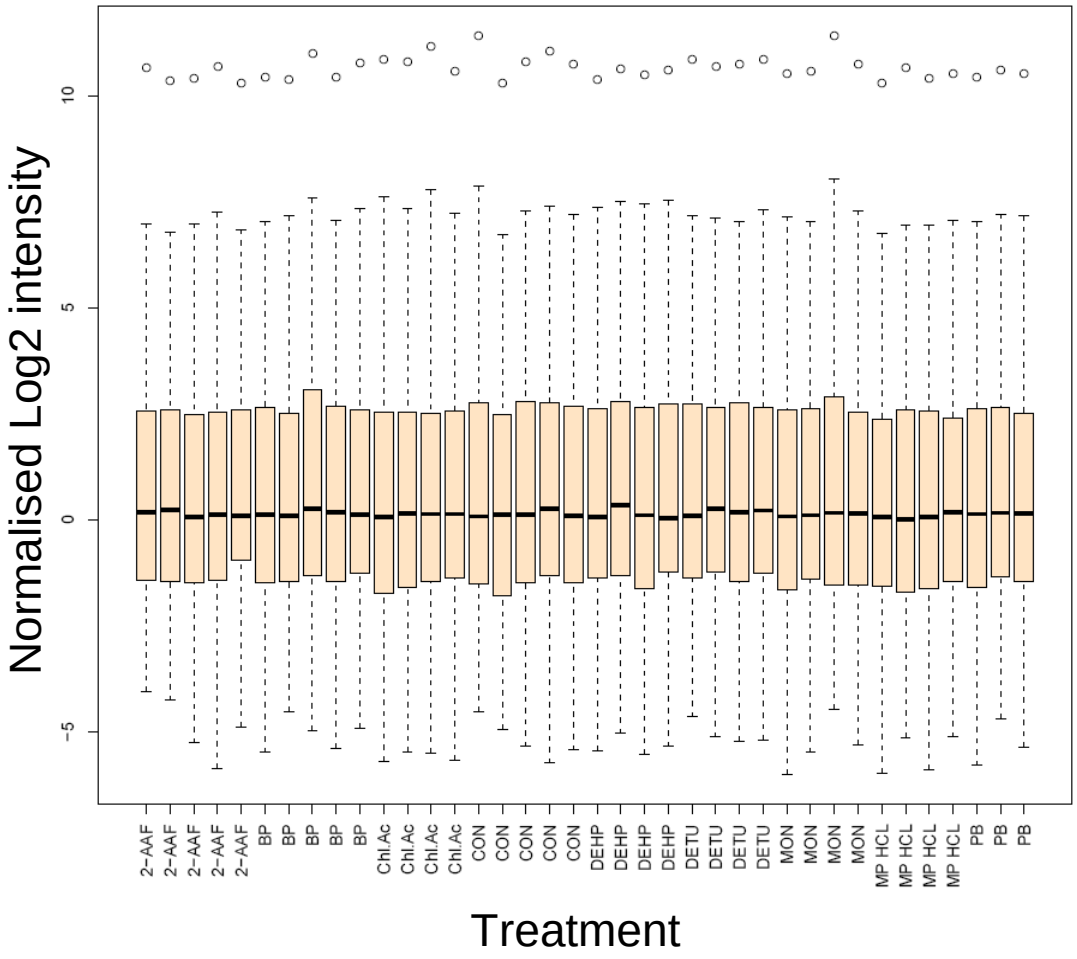


Fig. 3.2 Each box-plot on the x-axis represents a microarray sample. The lower end of the box is the 25th percentile, while the highest end is the 75th percentile. On the Y-axis is plotted the normalised log2 intensity from the microarrays. The black line in the middle of each box-plot is the median value for that sample. The open circle signifies the log2 value of the miRNA with the highest intensity in each sample. End of the whiskers contain 1.5 interquartile range.

After filtering and normalisation the most widely expressed miRNAs in the untreated rat liver were determined. In **Table 3.7** the twenty miRNAs with the highest expression in the control liver, as determined by microarrays, are shown.

Table 3.7: Top 20 abundantly expressed miRNAs in untreated rat liver

miRNA	Expression ($Log_{2)}$	Standard Deviation
rno-miR-122	10.9	0.4
rno-let-7f	7.3	0.4
rno-miR-21	7.0	0.3
rno-let-7a	6.9	0.4
rno-miR-192	6.5	0.4
rno-miR-29a	6.5	0.1
rno-miR-22	6.5	0.2
rno-miR-26a	6.3	0.1
rno-let-7c	5.9	0.4
rno-miR-26b	5.7	0.4
rno-miR-23b	5.3	0.2
rno-miR-125b-5p	5.3	0.4
rno-miR-29c	5.1	0.2
rno-let-7b	5.1	0.3
rno-miR-126	5.0	0.2
rno-miR-99a	5.0	0.1
rno-miR-194	4.9	0.1
rno-let-7d	4.9	0.3
rno-miR-16	4.8	0.3

- Expression shown is the average log_2 hybridisation signal for the five control samples

As can be seen there is a wide spread in the expressions of miRNAs. Especially high was the expression of miR-122 (~12 times higher than the next miRNA). The expression levels of all the 167 detected miRNAs can be seen in **Appendix I**.

3.3.3. Comparison of microarray technical replicates

The process of miRNA profiling is a complicated protocol involving multiple intricate steps- labelling RNA, hybridising it onto arrays, washing and scanning the arrays, and finally normalising the extracted data. Each one of these steps can introduce error into the data. Moreover, the fact that for this study RNA was extracted from whole liver samples, a tissue composed of multiple cell types, added a further source of variability. Therefore, in order to assess the robustness of the acquired microarray data of this study, miRNA profiles were compared between two sets of technical replicates. For six liver samples RNA was extracted twice and the whole process of miRNA profiling was performed twice. Comparison of the miRNA profiles of these two sets of technical replicates would then allow an estimation of the total technical error in the microarray data. As can be seen the two sets of technical replicates showed high correlation (r=0.97 Pearson's correlation), supporting the reproducibility of the miRNA data (**Figure 3.3**). A further observation was that technical variability started to increase for miRNAs with low intensity, corroborating the necessity of filtering out miRNAs with very low expression.

Figure 3.3 *Comparison of technical replicates for microarrays.*

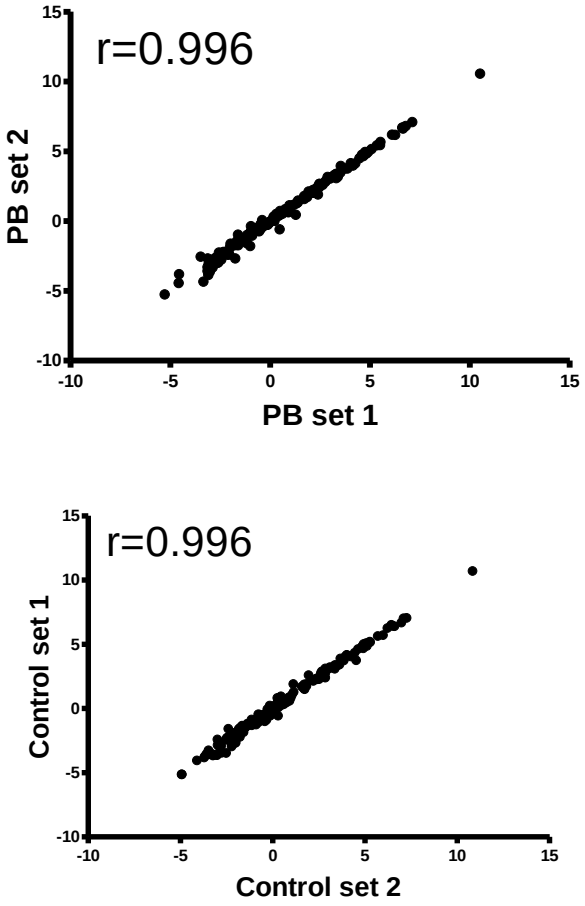


Fig. 3.3 On each axis is plotted the mean log2 intensity of a given miRNA in one of the replicate sets. Each circle represents one of the 167 miRNAs detected in at least 50% of samples in any condition. On the top panel the technical replicates for the three control samples are compared and on the bottom panel the replicates for the three PB samples are compared.

Evaluation of data quality performed here (PCA, quality control metrics, technical replicates) therefore supports the reliability and reproducibility of the acquired microarray data for 44/48 microarray samples. A few other observations also back this conclusion. First, the miRNA signals covered a wide range from negative to very high values. This observation shows that the microarrays had a large dynamic range. Second, the five positive control probes present in the arrays (hur 1,2,4,6 and mir-1) were detected on all arrays, while a number of negative controls were not detected in

any of the samples. Thirdly, the individual miRNAs that were flagged as being present or absent were consistent across the microarrays. Finally, for all samples the most abundant signal was miR-122, a hepatocyte-specific miRNA that makes up 70% of the liver miRNAome (Hou *et al.*, 2011, Jopling *et al.*, 2005).

3.4 Deregulation of hepatic miRNAs by hepatocarcinogens

3.4.1 MiRNA profiles are distinct for carcinogen-treated livers

As a first step an unpaired t-test was performed to determine the hepatic miRNAs with statistically significant differential expression between animals treated with carcinogenic treatments (PB-DEHP-MON-MP HCL- Chl.Ac- and 2-AAF) and untreated animals at 90 days. Two miRNAs were identified as being statistically differentially expressed between the two groups, with the difference in expression being relatively modest in magnitude (**Figure 3.4** and **Table 3.8**)

Figure 3.4 *Volcano plot of liver microRNAs from carcinogen vs. untreated animals*

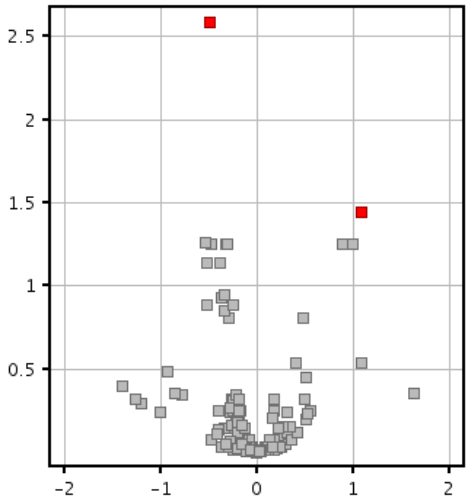


Fig. 3.4 Unpaired t-test was performed with Benjamin-Hochberg multiple testing. On Coloured red are miRNAs with p<0.05. On the Y-axis is plotted the corrected p value for each miRNA and on the x-axis the log2 fold change.

Table 3.8 Differentially expressed liver microRNA between carcinogen-treated and untreated animals

miRNA	Corrected p value	Fold Change in carcinogen treated samples compared to untreated animals
rno-miR-99a	0.002	-1.4
rno-miR-429	0.03	2.1

Unpaired T-Test with Benjamin-Hochberg multiple testing correction between untreated animals and animals exposed to carcinogenic treatments (2-AAF, MP HCL, Chl. Ac, PB, and DEHP) for 90 days. The miRNAs shown had corrected $p<0.05$

Next the PAM class prediction software (Tibshirani *et al.*, 2002) was used to identify a signature entailing the smallest possible number of miRNAs that could predict whether a chemical treatment was hepatocarcinogenic (2-AAF, MP-HCl, Chl.Ac, PB, MON, or DEHP), or non-hepatocarcinogenic (DETU or BP), in this rat strain. **Figure 3.5** shows the misclassification error of the method as the number of miRNAs used for classification decreases. The success of the resultant miRNA classifier for each liver sample can be seen in **Table 3.9**.

Figure 3.5 *PAM misclassification with decreasing number of microRNAs*

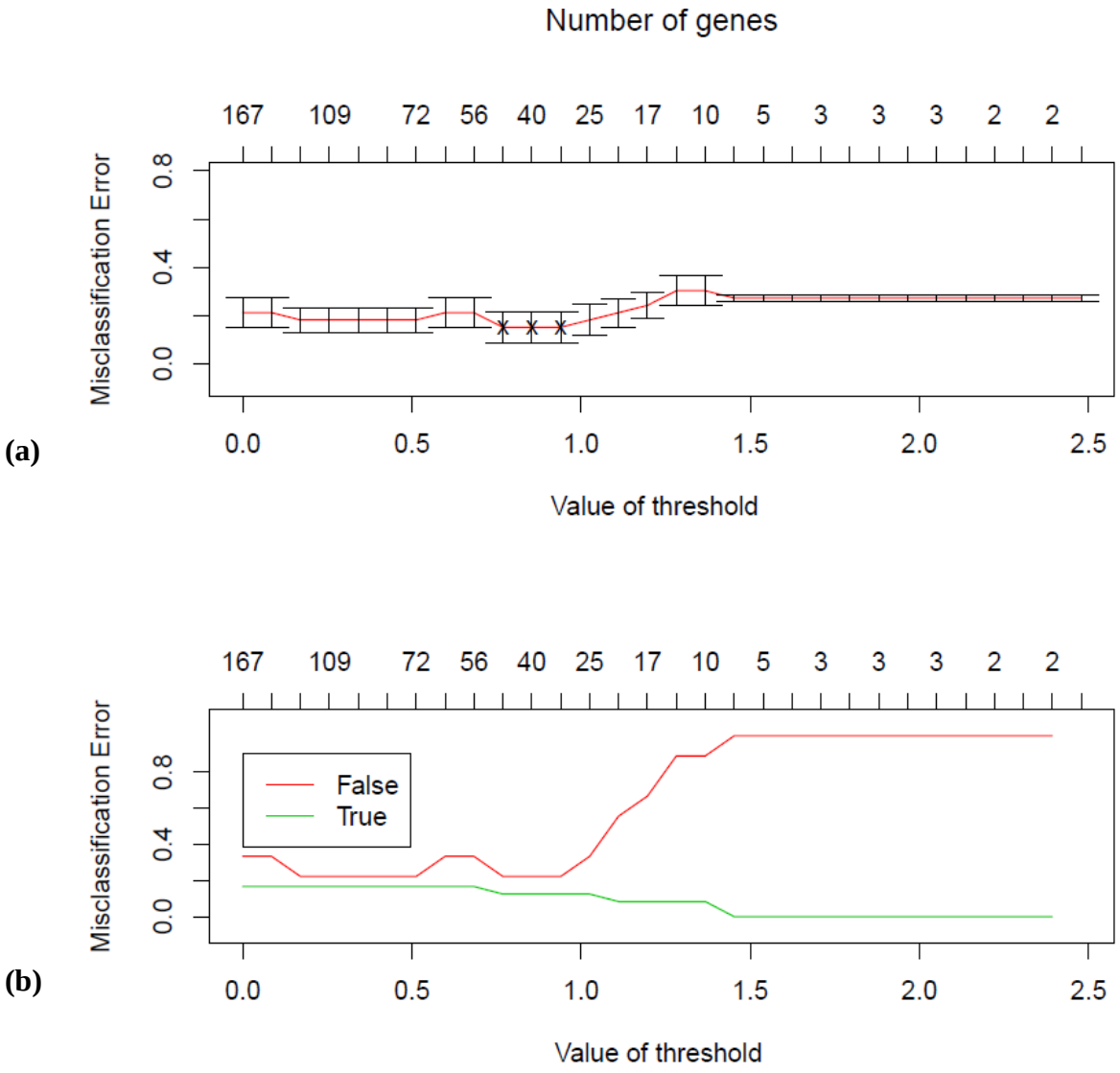


Fig. 3.5 PAM analysis was performed within BRB-array tools software. Y-axis is the misclassification error; on the x-axis is the number of miRNAs used (a) Total misclassification error (b) Misclassification error for a sample as carcinogen (True) or non-carcinogen (False) separately. The threshold was automatically set as 0.94.

Table 3.9: Performance of PAM classifier during cross-validation

Sample	Classified correctly	Classified incorrectly
DETU	**5/5 samples**	**0/5 samples**
BP	**3/5 samples**	**2/5 samples**
PB	**1/3 samples**	**2/3 samples**
DEHP	**4/4 samples**	**0/4 samples**
MON	**4/4 samples**	**0/4 samples**
Chl. Ac	**4/4 samples**	**0/4 samples**
MP HCL	**5/5 samples**	**0/5 samples**
2-AAF	**4/5 samples**	**1/5 samples**

Number of examined liver samples that were correctly or incorrectly classified as being exposed to hepatocarcinogenic treatment using PAM derived miRNA signature

For 85% of the samples the PAM classifier correctly predicted whether the liver was treated with a carcinogen. Two out of the three PB-treated samples was falsely classified as non-carcinogenic, while two out of five BP-treated samples were falsely classified as carcinogenic. The miRNA profiles could therefore correctly identify 2-AAF, MP HCL, Chl. Ac, MON, DEHP, and DETU. BP and PB are harder to discriminate due to their similar miRNA profiles. All the other carcinogenic treatments were correctly classified. The classifier consisted of 33 miRNAs, as shown in **Table 3.10**.

Table 3.10 PAM identified microRNA signature

	Array id	Class label	Prediction	Prediction Correct?
1	DETU1	False	False	YES
2	DETU2	False	False	YES
3	DETU3	False	False	YES
4	DETU4	False	False	YES
5	BP1	False	False	YES
6	BP2	False	False	YES
7	BP3	False	True	NO
8	BP4	False	False	YES
9	BP5	False	True	NO
10	PB1	True	False	NO
11	PB2	True	False	NO
12	PB3	True	True	YES
13	DEHP1	True	True	YES
14	DEHP2	True	True	YES
15	DEHP3	True	True	YES
16	DEHP4	True	True	YES
17	MON1	True	True	YES
18	MON2	True	True	YES
19	MON3	True	True	YES
20	MON4	True	True	YES
21	Chl Ac1	True	True	YES
22	Chl Ac2	True	True	YES
23	Chl Ac3	True	True	YES
24	Chl Ac4	True	True	YES
25	MP HCL1	True	True	YES
26	MP HCL2	True	True	YES
27	MP HCL3	True	True	YES
28	MP HCL4	True	True	YES
29	2-AAF1	True	True	YES
30	2-AAF2	True	True	YES
31	2-AAF3	True	True	YES
32	2-AAF4	True	True	YES
33	2-AAF5	True	False	NO

-threshold for PAM equal to 0.94

Previously, studies have reported that preneoplastic biochemical/morphological changes induced in the liver following chemical treatment can be early markers of carcinogenicity (Elcombe *et al.*, 2002, Allen *et al.*, 2004, Cohen, 2010). These markers were increased relative liver weight, liver hypertrophy, and hepatic labelling index (LI). Importantly, for some of the chemicals examined here, their miRNA profiles were better at predicting carcinogenicity than the biochemical/morphological changes that they induced. MON and Chl.Ac are particularly hard to identify as carcinogens using biochemical/morphological changes. MON only caused an equivocal increase in relative liver weight as defined by Elcombe *et al.* 2002, whilst Chl. Ac only an increase in LI at 90 days. Hence their miRNA profiles could be a better marker of their eventual carcinogenicity in the rat.

3.4.2 Identification of possible miRNA biomarkers of carcinogenicity

MiRNA biomarkers of carcinogenicity are those whose expression is a good early indicator of the carcinogenicity of a chemical. Direct comparison of carcinogenic treatments Vs non-carcinogenic treatments overlooks the fact that the effects of carcinogens on liver miRNAs may be specific to a given MOA. Therefore, as an alternative method miRNA biomarkers of carcinogenicity were identified here as those which display a significant change in their expression from untreated animals by two or more carcinogens, in the same direction (i.e. induction or repression) for each treatment. One-way ANOVA was used to identify differentially expressed miRNAs. Coupled with the Benjamini-Hochberg multiple testing correction 70 of 167 miRNAs as being differentially expressed at $p<0.05$. This statistical test identifies a high proportion of miRNAs as being differentially expressed at 90 days (~40%). These

differentiated miRNAs can be seen in **Appendix II**. In order to generate a more manageable list one-way ANOVA was performed coupled with Bonferonni-Holm multiple testing correction, which is a more stringent statistical test. This test identified 21miRNAs as being significantly deregulated at 90 days (**Table 3.11)**

Table 3.11: Differentially expressed microRNAs after 90 days of chemical treatment

microRNA	Corrected p.value	DETU	BP	PB	DEHP	MON	Chl. Ac	MP HCL	2-AAF
miR-34a	>0.01	-1.1	-1.3	1	-1.2	3.9	2.3	1.7	27.9
miR-96	>0.01	1.3	2	3.6	1.6	1.6	-1.5	1	-1.3
miR-221	>0.01	-1.5	-1.8	-1.1	3	-1.2	1.5	1	-1.1
miR-200a	>0.01	-1.2	2	3.7	1.7	1.1	2	1.7	2
miR-107	>0.01	-1.1	1.1	-1.1	2.0	1	1.1	-1.2	-1.3
miR-200b	>0.01	-1	2.5	4.5	2.0	1.2	2.3	1.6	2
miR-429	>0.01	-1.1	2.4	3.8	1.9	1.2	2.3	1.8	2.7
miR-182	>0.01	2.4	4.3	6.3	3.1	3.3	1.1	1.3	1.4
miR-375	>0.01	-2	-2.3	-2	1.8	2.2	1.7	-2.1	-1.5
miR-193	>0.01	-1.3	-1.1	1.2	-1.7	-2.4	-4.9	-1.9	-1.3
miR-17	>0.01	-1.1	1.1	-1.1	1.1	1	-1.6	-1.2	-1.2
miR-497	>0.01	1	-1.1	-1.1	-1.2	-1.3	-1.7	-1.3	-1
miR-203	0.01	-1.2	-1.1	-1.4	-1.4	-1.2	-2	-1.2	-1.6
miR-31	0.01	1.1	1.1	-1	1.3	-1	1.3	-1.4	-1.5
miR-29a	0.01	-1.1	-1	1.1	1.3	1	-1.2	-1.2	-1.2
miR-29c	0.01	-1.1	1	1.2	1.3	-1	-1.3	-1.2	-1.2
miR-99a	0.02	-1	-1.3	-1.3	-1.3	-1.5	-1.3	-1.5	-1.6
miR-20a	0.03	-1	1	-1.1	1.1	-1	-1.6	-1.2	-1.3
miR-139-5p	0.03	1.3	-1.1	1.1	1.3	1.3	3.2	1.3	-1.3
miR-28	0.04	1.1	1	1.1	-1.1	1	1.4	1	-1.3
miR-505	0.05	-1.1	1.1	1.5	1.5	1.1	2.8	1.2	-1.6

Differentially regulated miRNAs were identified using one-way ANOVA with Bonferonni-Holm multiple testing correction and $p<0.05$

Values shown are average absolute fold change from control in that treatment

The altered miRNAs included both downregulated and upregulated ones, indicating that both forms of deregulation can occur. In terms of magnitude, most altered miRNAs were changed between 1.5 to 5 fold from control, which is similar level to the changes observed between HCC and benign liver tissue (Jiang *et al.*, 2008). The miRNAs deregulated in each treatment compared to control at 90 days can be seen in **Table 3.12**.

Table 3.12 MicroRNAs differentially expressed from control for each chemical treatment at 90 days

Compound	miRNAs significantly changed from control
DETU	None
BP	mir-200b(↑) +miR-200a(↑)+ miR-429 (↑) + miR-182(↑↑) + miR-96 (↑) + mir-221 (down)
PB	mir-200b(↑↑)+miR-200a(↑) + miR-429 (↑) + miR-182(↑↑) + miR-96 (↑)
DEHP	miR-200b (↑)+ miR-200a + miR-429 + miR-182(↑)+ miR-96 + mir-221(↑) + miR-107(↑) + miR-29a
MON	miR-182(↑) + miR-96 + miR-34a(↑) +miR-193(↓) + miR-99a
Chl. Ac	miR-200b(↑) + miR-200a(↑) + miR-429(↑) + miR-34a(↑) + miR-203(↓) + miR-193(↓↓) + miR-505(↑)+ miR-139-5p(↑) + miR-17 + miR-20a + miR-96 (down) + miR-28 + miR-497
MP HCL	miR-200a + miR-99a + miR-34a
2-AAF	mir-200b(↑) + miR-200a(↑) + miR-429 (↑) + miR-34a(↑↑↑) + miR-203 + miR-99a

MiRNAs were identified as differentially expressed using one-way ANOVA with Bonferonni-Holm multiple test correction and Tukey HSD post test $p<0.05$

Underlined are miRNAs that fulfil biomarker criteria

↑Upregulated >2-fold; ↑↑↑ Upregulated > 10-fold ↓ Downregulated >2-fold; ↓↓ Downregulated >4-fold ; Coloured are miRNAs that belong to same family

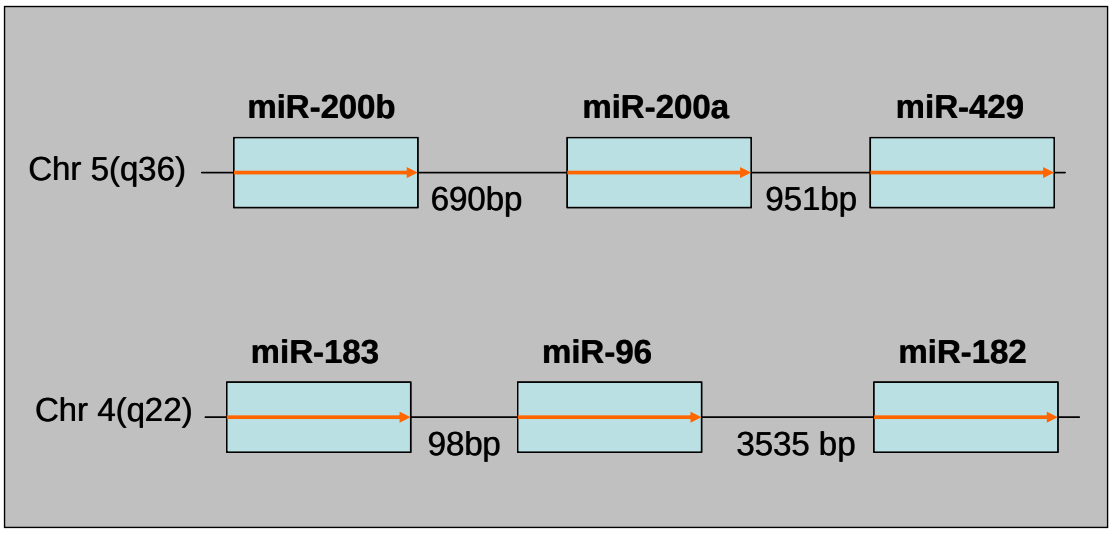
It is important to note that all carcinogens tested here resulted in altered miRNA expression at 90 days, long before the appearance of liver tumours. **Moreover, there is a small set of miRNAs that are frequently affected by carcinogens, even though they have distinct MOA**. Nine miRNAs fulfil the requirements set for biomarkers as defined previously (**Table 3.13)**

Table 3.13 Set of potential biomarker microRNAs

miRNA	Locus in rat	DNA strand	Intragenic or intergenic
miR-200a/200b/429	Chr. 5 (q36)	-	Intergenic
miR-96/182	Chr. 4 (q22)	-	Intergenic
miR-34a	Chr. 5 (q36)	+	Intergenic
miR-99a	Chr. 11 (q11)	+	Intergenic
miR-203	Chr. 6 (q32)	+	Intergenic
miR-193	Chr. 10 (q25)	+	Intergenic

Large fractions (30-40%) of miRNAs are found within protein-coding genes and are transcribed together with their host genes (Chuang & Jones, 2007). Interestingly, all of the identified biomarker miRNAs were intergenic, being transcribed from independent promoters, suggesting that these miRNAs are more receptive to chemical treatments. Of the 9 identified miRNAs five belonged into one of two separate clusters (**Figure 3.6)**

Figure 3.6 *Chromosomal organisation of the miR-200a/200b/429 and miR-96/182/183 microRNAs in the rat.*



MiR-200a, miR-200b and miR-429 are clustered together and transcribed from the same promoter as part of one long pre-miRNA (Gregory *et al.*, 2008a, Burk *et al.*, 2008). In rats this cluster is located on chromosome 5. The miR-200a/200b/429 cluster was induced at 90 days by 5/6 carcinogens, as well as non-carcinogenic BP. Similarly, miR-182 and miR-96 are transcribed from a common promoter together with another miRNA, miR-183 (Xu *et al.*, 2007). The expression of miR-183 was too low to pass the filtering criteria (i.e. be detected in at least 50% of samples in any group), so was filtered out from further analysis. MiR-96 and miR-182 are located on rat chromosome 4 and were induced by three carcinogens, as well as the non-carcinogenic BP. It is expected that the expressions of the miRNAs belonging to the same clusters (i.e. miR-200a with miR-200b/miR-429; and miR-96 with miR-182) should be correlated across treatments. Indeed, that was what was observed. The highly correlated expression of the clustered miRNAs suggests that their induction following chemical treatment is the result of altered transcriptional regulation of their promoter (**Fig. 3.7**).

Figure 3.7 *Correlated expression of clustered microRNAs miR-200a/200b/429 and miR-96/182*

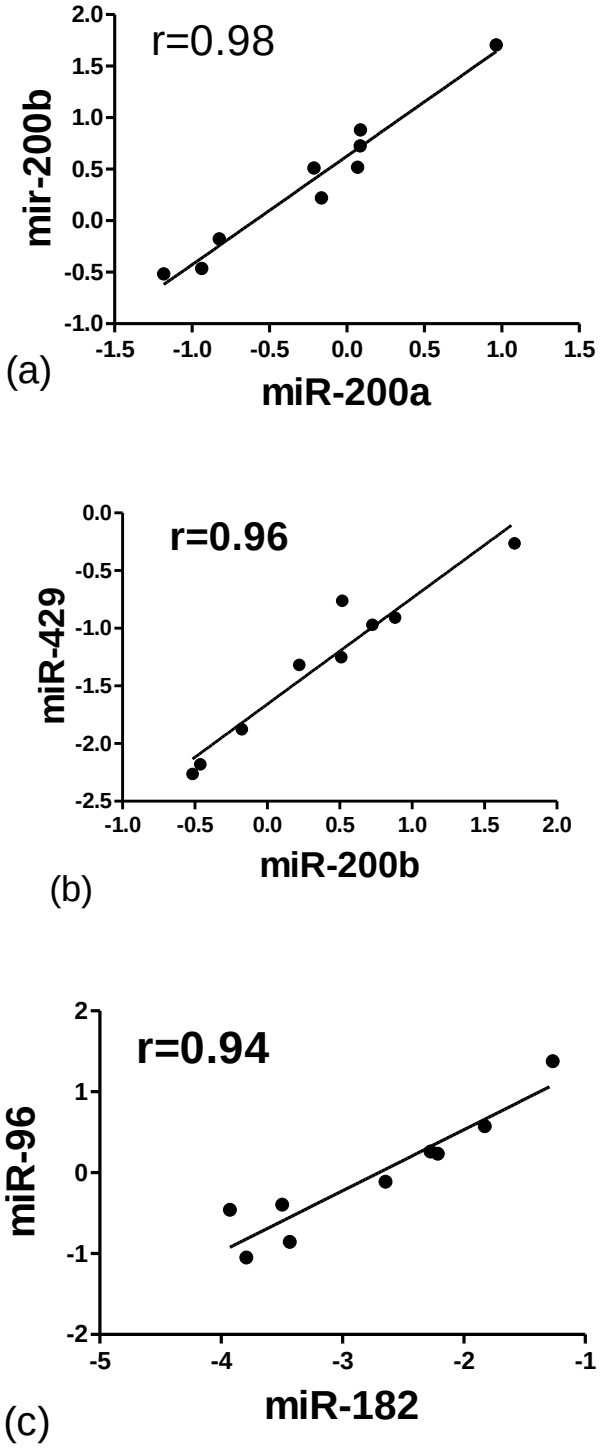


Fig. 3.7 Pearson's correlation was used for calculation of correlation strength. Values plotted are log2 intensities. Each circle is the averaged expression of the miRNA in one treatment group.

The sensitivity and specificity of expressional changes of the identified miRNA biomarkers for chemical hepatocarcinogens are shown in **Table 3.14** below.

Table 3.14 Sensitivity and specificity of "biomarker" microRNAs

miRNA	Sensitivity	Specificity
miR-200a/200b/429	5/6 (83%)	1/2 (50%)
miR-96/182	4/6 (67%)	1/2 (50%)
miR-34a	4/6 (67%)	0/2 (0%)
miR-99a	3/6 (50%)	0/2 (0%)
miR-203	2/6 (33%)	0/2 (0%)
miR-193	2/6 (33%)	0/2 (0%)

Sensitivity= Percentage of carcinogenic-treatments in which the miRNA was deregulated from control by one-way ANOVA and Dunnett's post-test
Specificity= Percentage of non-carcinogenic treatments in which miRNA was deregulated from control by one-way ANOVA and Dunnett's post-test

The highest sensitivity was observed for the miR-200a/200b/429 cluster which was induced by 5/6 carcinogenic treatments (exception was MON). Among other miRNAs identified as commonly deregulated by carcinogen treatment, induction of miR-34a was observed specifically for 4/6 tested carcinogens. Significant downregulation of miR-99a was observed only for three carcinogens, while miR-203 and miR-193 were significantly deregulated by two carcinogenic treatments and were unaffected by the non-carcinogens. Importantly, all of the indentified miRNAs have been previously reported as being deregulated in various types of cancer, including HCC (**Table 3.15**).

Table 3.15 Reported deregulation of identified microRNA biomarkers in cancers

miRNA	Compound	Cancers in which the miRNA is deregulated	Known target genes
miR-200a	PB, BP, DEHP, 2-AAF, Chl.Ac, MP HCL	HCC (Murakami *et al.*, 2006) breast (Shimono *et al.*, 2009), ovarian (Bendoraite *et al.*, 2010)	b-catenin (Xia *et al.*, 2010); Zeb1 and Zeb2 (Gregory *et al.*, 2008a); Fog2 (Hyun *et al.*, 2009)
miR-200b	PB, BP, DEHP, 2-AAF, Chl.Ac	HCC (Gramantieri *et al.*, 2007) , breast (Shimono *et al.*, 2009) , ovarian (Bendoraite *et al.*, 2010)	Zeb1 and Zeb2 (Gregory *et al.*, 2008a); Fog2 (Hyun *et al.*, 2009)
miR-429	PB, BP, DEHP, 2-AAF, Chl.Ac	Breast (Shimono *et al.*, 2009), ovarian (Bendoraite *et al.*, 2010)	Zeb1 and Zeb2 (Gregory *et al.*, 2008a); Fog2 (Hyun *et al.*, 2009)
miR-96	PB, BP, DEHP, MON, Chl.Ac. (down)	HCC (Pineau *et al.*, 2010); breast (Shimono *et al.*, 2009) Endometrial	FOXO1 (Myatt *et al.*, 2010)
miR-182	PB, BP, DEHP, MON	breast (Shimono *et al.*, 2009) Endometrial (Myatt *et al.*, 2010)	FOXO1 (Myatt *et al.*, 2010)
miR-34a	2-AAF, MP HCL, MON, Chl.Ac.	Neuroblastoma (Welch *et al.*, 2007) (Welsch *et al* 2007); Colon (Tazawa *et al.*, 2007); HCC (Pineau *et al.*, 2010)	- SIRT1, NOTCH1, E2F3, MET, Bcl-2 (Chang *et al.*, 2007)
miR-99a	2-AAF, MP HCL, MON	HCC (Hou *et al.*, 2011) Children Adenocarcinoma (Doghman *et al.*, 2010);	- IGF-IR - mTOR (Doghman *et al.*, 2010);
miR-203	2-AAF & Chl.Ac	HCC (Furuta *et al.*, 2010); Ovarian (Iorio *et al.*, 2007)	- ABCE1 - CDK6 (Furuta *et al.*, 2010)
miR-193	Chl.Ac. & MON	Oral cancer (Kozaki *et al.*, 2008); Squamous cell carcinoma (Dry *et al.*, 2011)	- E2F6 (Kozaki *et al.*, 2008)

The literature was then scrutinised to determine whether these 10 miRNAs have been reported to be deregulated in the liver after sub-chronic chemical treatments. Indeed, other studies have reported changes in some of these miRNAs after sub-chronic chemical treatments. For example miR-34a and miR-203 have also been reported to be deregulated in rodent livers treated with Wy-14,643 and tamoxifen (**Table 3.16**).

Table 3.16 Reported hepatocarcinogenic treatments affecting identified biomarker microRNAs

Reference	Treatment	GNTX?	Animal	miRNAs affected
Shah *et al.*, 2007	0.1 %Wy,14,643 two weeks	NO	mice	miR-34a; miR-203; miR-99a; miR-182
Pogribny *et al.*, 2007	420 ppm Tamoxifen 24 weeks	YES	Female F344	miR-34a; miR-193; miR-203
Pogribny *et al.*, 2009	0.02% 2-AAF 12 and 24 weeks	YES	Male Sprague-Dawley	miR-34a at 12 and 24 weeks; miR-200a/200b/429 (progressive increase from 12 to 24 weeks); miR-99a , miR-182, and miR-96 (at 24 weeks)
Wang *et al.*, 2009	choline-deficient and amino acid–defined (CDAA) diet 6,18,32, and 65 weeks	NO	C57BL/6 mice	miR-200b
Li *et al.*, 2010b	N-ethyl-N-nitrosourea (ENU) 120 mg/Kg for 1,7,15,30, and 120 days	YES	Big-blue female mice	miR-34a from day 1 onwards

3.4.3 MiRNA profiles are associated with the MOA of chemicals

It has been reported that the mRNA profile of livers reflects the MOA of chemicals with which they are treated (Ellinger-Ziegelbauer *et al.*, 2008, Nie *et al.*, 2006). Clustering analysis could clarify whether the same is true for miRNA hepatic profiles as well. The clustering tree was constructed using the 21 miRNAs identified as differentially regulated. For easier interpretation of the resulting tree the miRNA expression was averaged for the samples in each group of treatments. Besides the information regarding the grouping of chemical treatments, the clustering analysis also allowed the identification of miRNAs which undergo coordinated regulation (**Figure 3.8**).

Figure 3.8 *Clustering analysis of control and treated liver samples at 90 days*

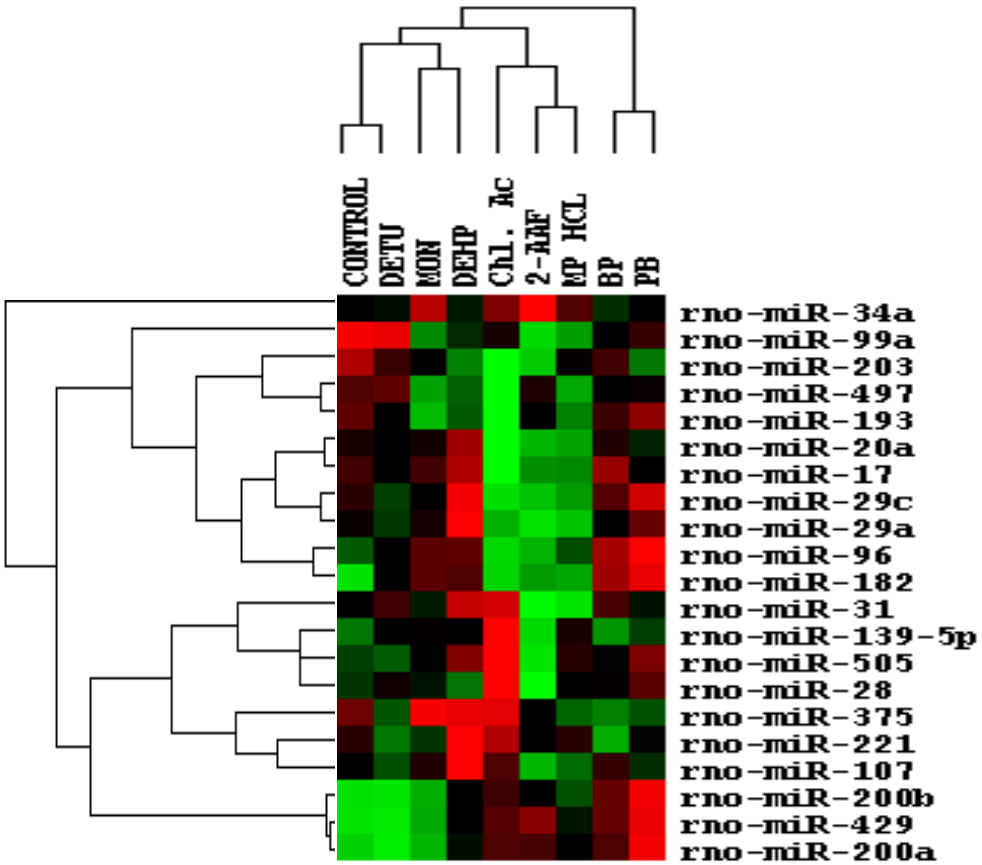


Fig. 3.8 The tree was constructed using Average linkage with correlation uncentred as the similarity metric using the 21 deregulated miRNAs (Table 3.11). MiRNAs were median centred and normalised in Cluster 3.0. Red colour indicates higher expression than the median; the green colour indicated lower expression, and the black colour similar expression.

Importantly, the clustering of the samples based on the differentially expressed miRNAs appears to follow a logical order. DETU, the chemical with the least effects on the liver, associated with the control animals. In a second node the two PB-type compounds, PB and BP, clustered together. On a third node the three most potent carcinogens (2-AAF, MP HCL, and Chl.Ac) were also clustered together. **Therefore, the clustering analysis performed here indicates that liver miRNAs profiles reflect the MOA of a chemical.**

Clustering of miRNAs demonstrated that several of them exhibited correlated changes in expression as a result of the chemical treatments. MiRNA members of the miR-200a/200b/429 and miR-96/182 clusters, which were shown previously to be highly correlated (**Figure 3.7**), grouped together. MiR-17 and miR-20a which are part of the miR-17-92 oncogenic cluster also showed very high correlation (r=0.98, Pearson's correlation). Grouped together were miRNAs miR-29c and miR-29a which belong to the same family but are found in different chromosomes (r=0.95 Pearson's correlation).

3.4.4 Comparison of chemical effects on mRNAs and miRNAs

Deregulated mRNAs for the same liver samples at 90 days were previously identified (Richard Currie, personal communication). The number of deregulated mRNAs and miRNAs per treatment at 90 days were compared to determine how the two profiles relate to each other (**Figure 3.9**).

Figure 3.9 *Comparison of chemical effects on mRNAs and miRNAs after 90 days of treatment*

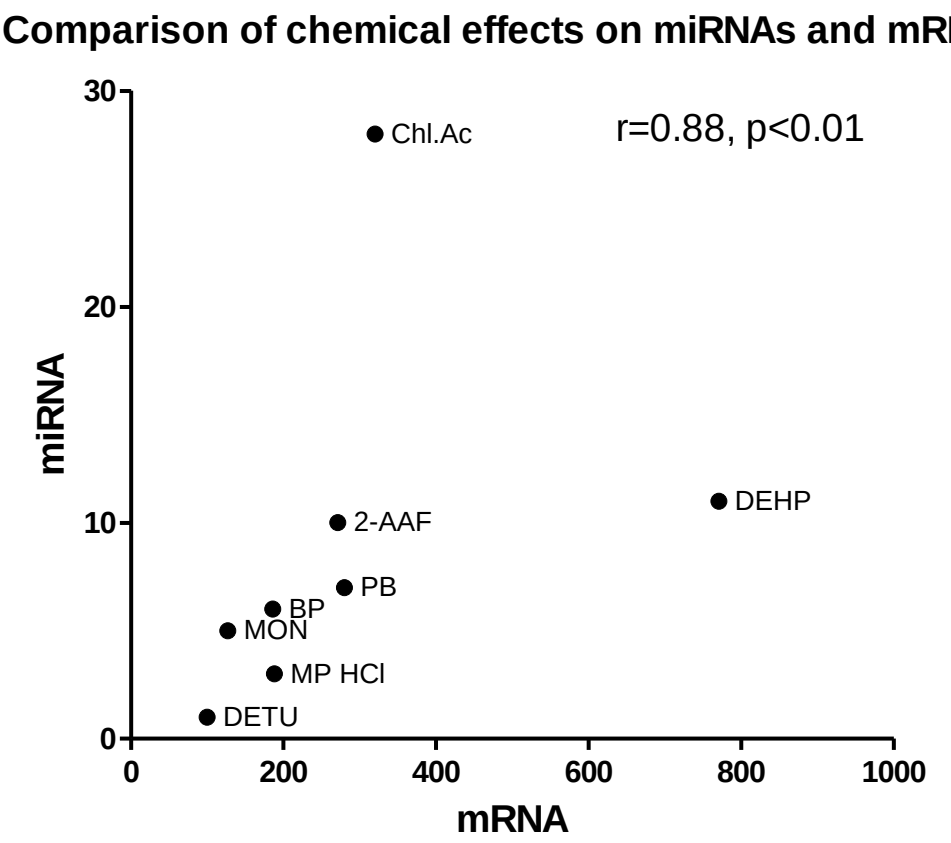


Fig. 3.9 Deregulated mRNAs were identified as those with p<0.05 (t-test from control) and more than 1.5-fold change from control. Deregulate miRNAs were those with p<0.05 by one-way ANOVA with Benjamini-Hochberg and Dunnett's HSD post-test. Correlation shown is Spearman's correlation.

For both mRNAs and miRNAs the chemical with the least effects on their expression at 90 days compared to control was DETU. The biggest divergence between the two datasets could be seen for DEHP (which had a much more pronounced effects relative to the other compounds on mRNAs compared to miRNAs) and Chl. Ac (with a much more pronounced effect relative to other compounds on miRNAs than mRNAs). The correlation between the two categories of genes was significant with Spearman's correlation.

3.4.5 Correlation of "biomarker" miRNAs with hepatic parameters

The correlation between the expression of the identified "biomarker" miRNAs and selected hepatic parameters was then examined (**Table 3.17**).

Table 3.17 Correlation of biomarker miRNAs with hepatic parameters

microRNA	% liver tumours	Adjusted liver weight	Ki67 LI
miR-200a	r=0.41	r=0.29	r=0.03
miR-200b	r=0.25	r=0.38	r=0.18
miR-429	r=0.47	r=0.29	r=0.21
miR-96	r=-0.43	r=0.54	r=-0.42
miR-182	r=-0.38	r=0.58	r=-0.39
miR-193	r=-0.25	r=0.18	r=-0.30
miR-203	r=-0.51	r=-0.06	r=-027
miR-99a	r=-0.74 * p<0.05	r=-0.17	r=-0.60
miR-34a	r=0.71 * p<0.05	r=-0.34	r=0.45

Correlation value shown is for the average expression of each miRNA in control and treated samples as determined by miRNA microarrays with the average frequency of liver tumours, adjusted liver weight, and Ki67 LI for the same liver samples (Table 3.1-3.3). Correlation used was Pearson's correlation. * $p<0.05$ correlation was significant

A significant correlation was only observed between the expression of miR-34a and miR-99a with the frequency of liver tumours. MiR-34a and miR-99a also showed the strongest correlation with the Ki67 LI even though it was not significant. MiR-96 and miR-182 showed the strongest correlation with adjusted body weight.

3.5 Chemical-specific miRNA profiles

3.5.1 PB-type compounds have characteristic miRNA profiles

This was the first study to examine the effect of PB-like compounds on the liver miRNAome. The main effect of both compounds at 90 days was the induction of two miRNA clusters, the miR-200a/200b/429 **(Figure 3.10**) and miR-182/96 (**Figure 3.11**).

Figure 3.10 *Effects of chemical treatments on the expression of miR-200a/200b/429 at 90 days*

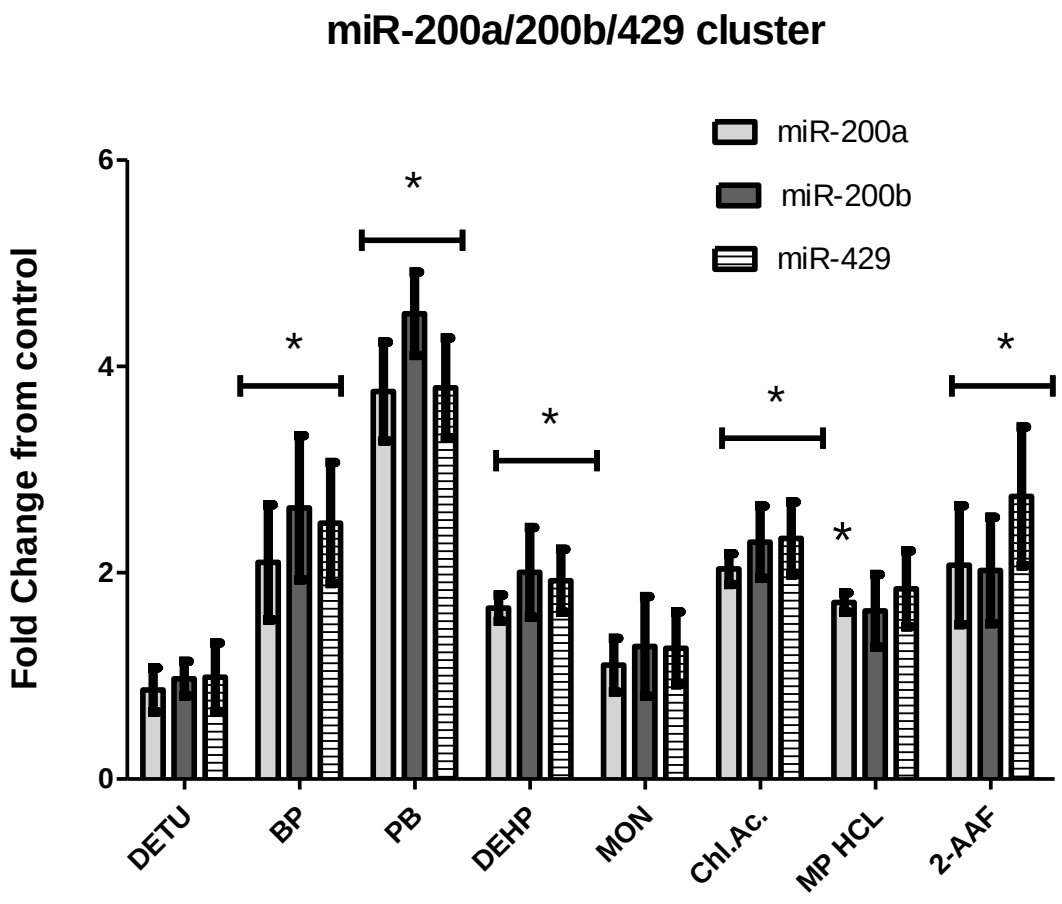


Fig. 3.10 On the Y-axis is displayed the averaged hybridisation signal normalised to control for each of the three miRNAs. On the x-axis are plotted the eight chemical treatments. Error bars are the S.D. and * signifies statistically significant expression as determined by one-way ANOVA with Tukey HSD test and corrected $p<0.05$.

Figure 3.11 *Effects of chemical treatments on the expression of miR-96/182 at 90 days*

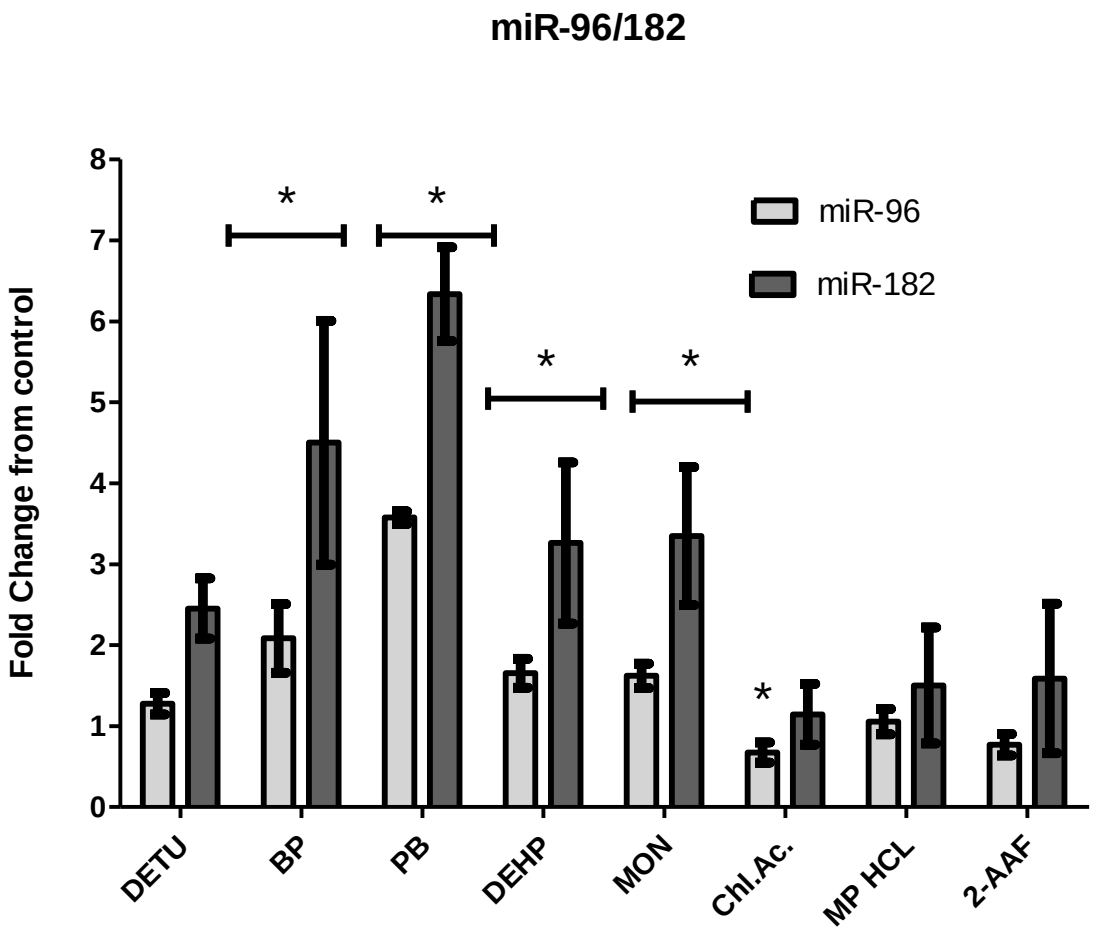


Fig. 3.11 On the Y-axis is displayed the averaged hybridisation signal normalised to control for the two miRNAs. On the x-axis are plotted the eight chemical treatments. Error bars are the S.D. and * signifies statistically significant expression as determined by one-way ANOVA with Tukey HSD test and corrected $p<0.05$.

Both of these clusters were induced by other compounds as well, although PB had the biggest effect. The level of expression of these five miRNAs were also compared for the six PB and the six control technical replicates In support of the veracity of the induction of these two clusters, the expressions of these miRNAs were very similar between the technical replicates (**Figure 3.12**).

Figure 3.12 *Expression of selected microRNAs in two sets of technical replicates*

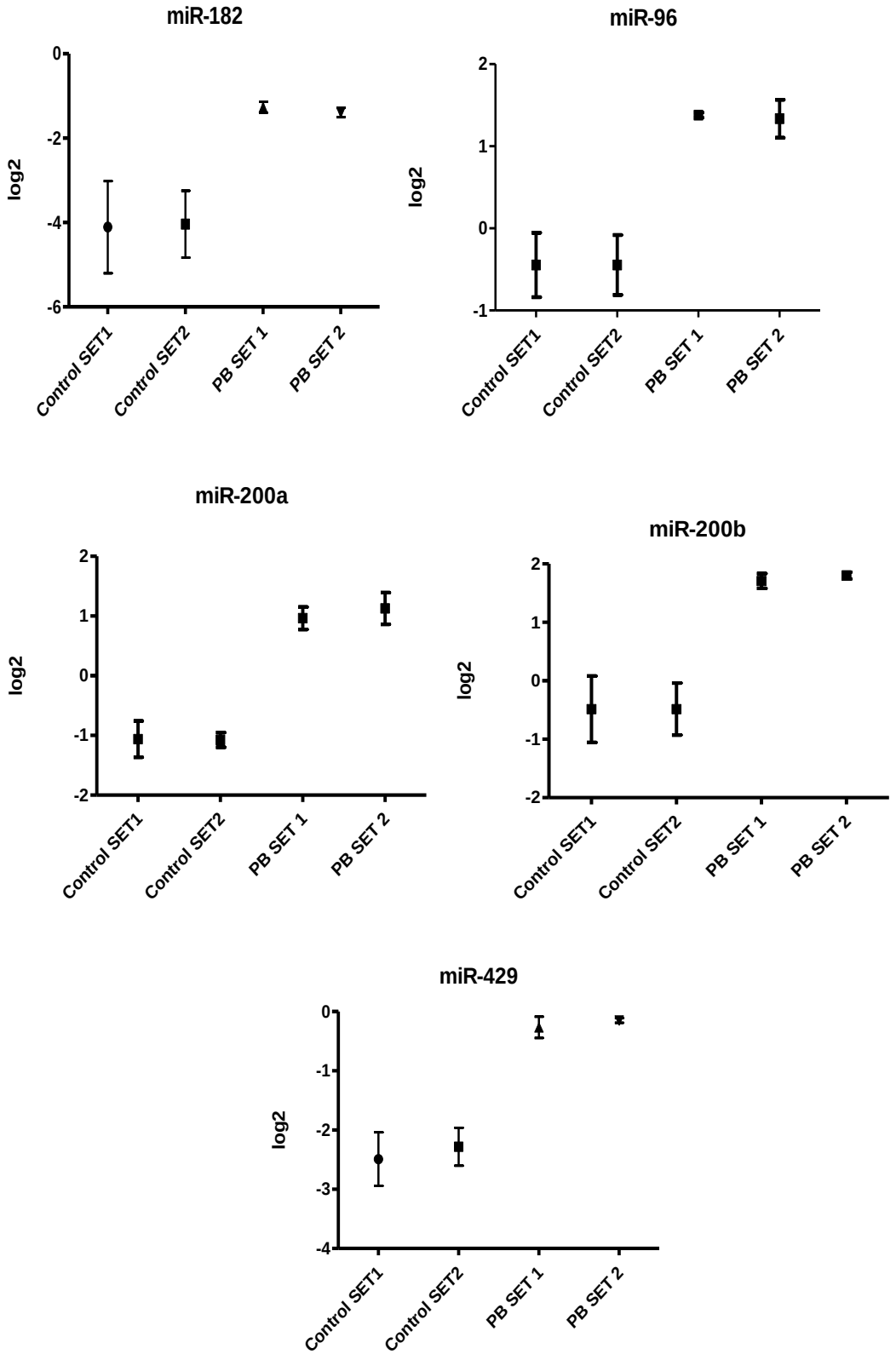


Fig. 3.12 .Y-axis is the normalised log2 hybridisation intensity, n=3 for each group. Error bars are S.D.

3.5.2. Effects of genotoxic 2-AAF on microRNAs

A previously reported study looked at the effect of 2-AAF treatment on liver miRNAs (Pogribny *et al* 2009). 2-AAF was the only compound used in the present study for which published data existed on its effects on the liver miRNAome. Albeit the Pogribny *et al* study used a lower dose of 2-AAF (20 vs. 40 ppm in the present study) and was performed in a different rat strain (Sprague Dawley vs. Fischer). An additional source of variability between this study and the (Pogribny *et al.*, 2009a) study was the use of different microarray platforms (Agilent Vs. mirVana miRNA Bioarrays (Ambion)). Nevertheless, for both studies the main effect of 2-AAF was the induction of miR-34a and the miR-200b/200a/429 cluster (**Table 3.18**).

Table 3.18 Comparison of reported effects of 2-AAF on liver microRNAs

miRNA	**Pogribny *et al 2009***	**This study**
miR-34a	3.0	27.9
miR-200a	2.8	2.0
miR-200b	2.7	2.0
miR-429	2.0	2.7

Values are average fold change from control at 12 weeks (Pogribny et al 2009) and 90 days (this study) as determined using microarrays. N=4 for Pogribny et al 2009 and n=5 for this study.

The main effect of GNTX 2-AAF treatment on hepatic miRNA expression that differentiated it from the NGTX carcinogens was the much more pronounced induction of miR-34a (**Figure 3.13**).

Figure 3.13 *Effects of chemical treatments on the expression of miR-34a at 90 days*

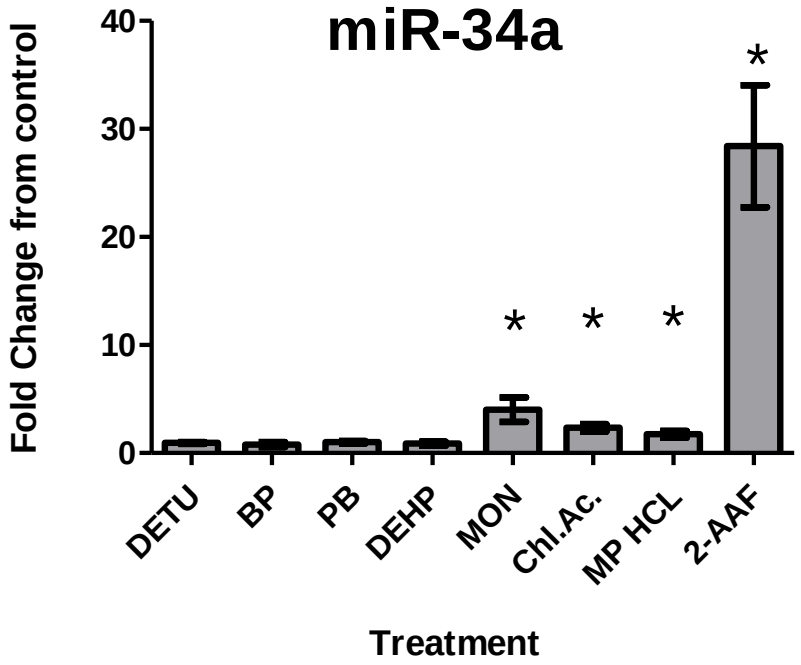


Fig. 3.13 Values are average fold change in expression from control as determined by microarrays. * Statistically significant one-way ANOVA Tukey HSD test $p<0.05$. n=3-5 animals per group. Error bars are S.D.

On the other hand 2-AAF resulted in some similar deregulations of miRNAs as NGTX carcinogens such as the induction of the miR-200a/200b/429 cluster (**Figure 3.10**) and the downregulation of miR-99a and miR-203 (**Figure 3.14**).

Figure 3.14 *Effects of chemical treatments on the expression of miR-99a and miR-203 at 90 days*

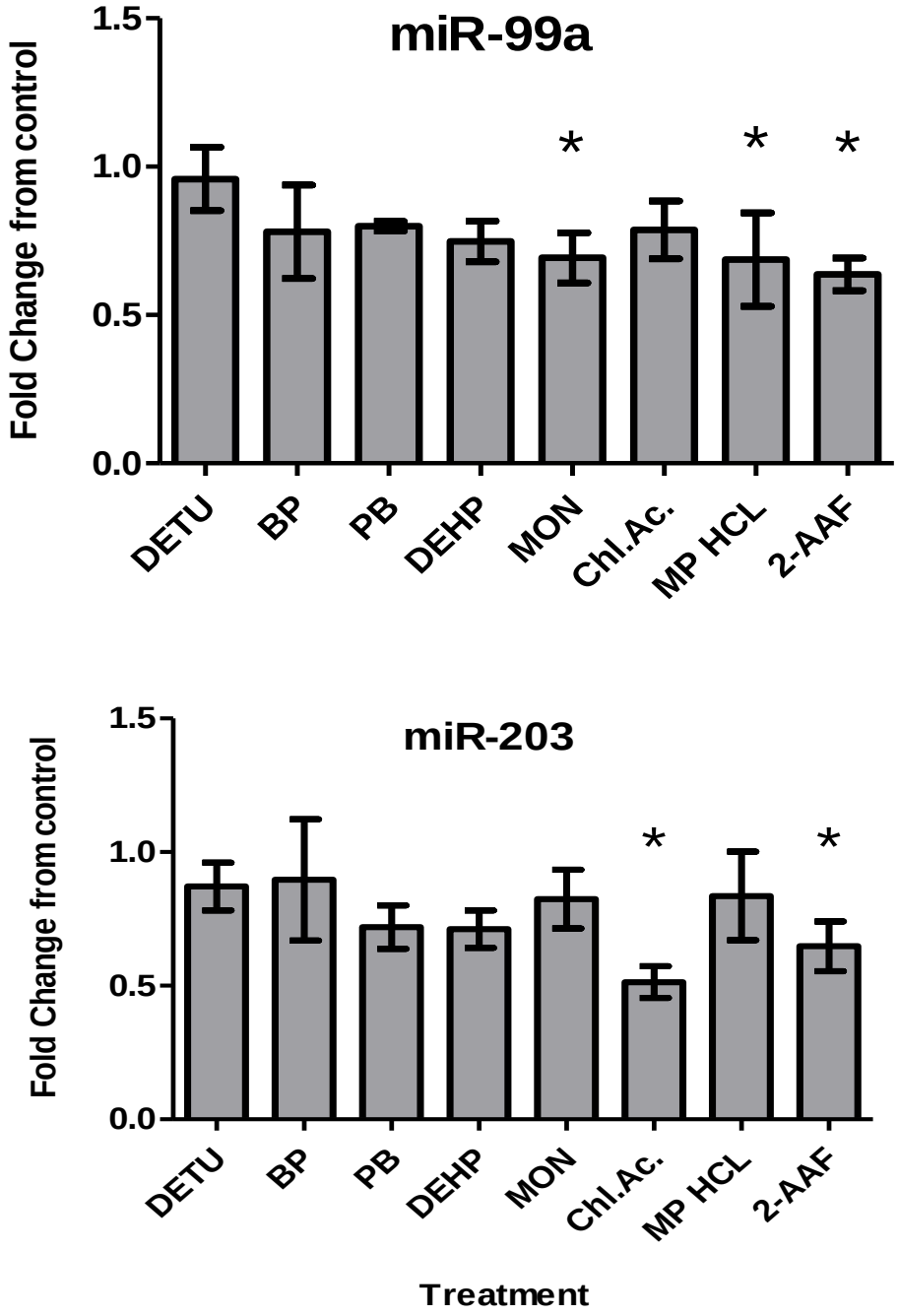


Fig. 3.14 Y-axis is fold change from control as determined by microarrays, bars are S.D. * significantly different one-way ANOVA Tukey HSD test $p<0.05$. n=3-5 animals per group.

3.5.3 DEHP shows some similarities to PB-type compounds

The effect of a PPC, Wy-14,643, on hepatic miRNAs has been examined and reported (Shah *et al.*, 2007). Their study showed that Wy-14,643 treatment, just like DEHP (another PPC) here, causes deregulation of miRNA expression. When comparing the results of the Wy-14,643 study with the DEHP data generated here it is important to note that, besides the difference in chemicals, they were performed in different species and for different durations. Nevertheless, two miRNAs that were deregulated in both studies were miR-182 and miR-107 (for miR-182 18.2-fold for Shah *et al.* 2007 vs. 3.1 for this study; for miR-107 2.3 Vs 2.0). Induction of miR-107 was similar for both compounds, but miR-182 was much more pronounced for the Wy-14,643 study.

One important difference between the effects of Wy-14,643 and DEHP was the lack of repression of let-7c by DEHP. The downregulation of let-7c by Wy-14,643 was observed from 4 hours through to two weeks, and up to 11 months when liver tumours are prominent.

Another interesting observation was a high correlation between two miRNA clusters miR-200a/200b/429 with miR-182/96 specifically for the livers treated with DEHP, BP, and PB (**Figure 3.15**).

Figure 3.15 *Correlation of miR-200b and miR-96 at 90 days.*

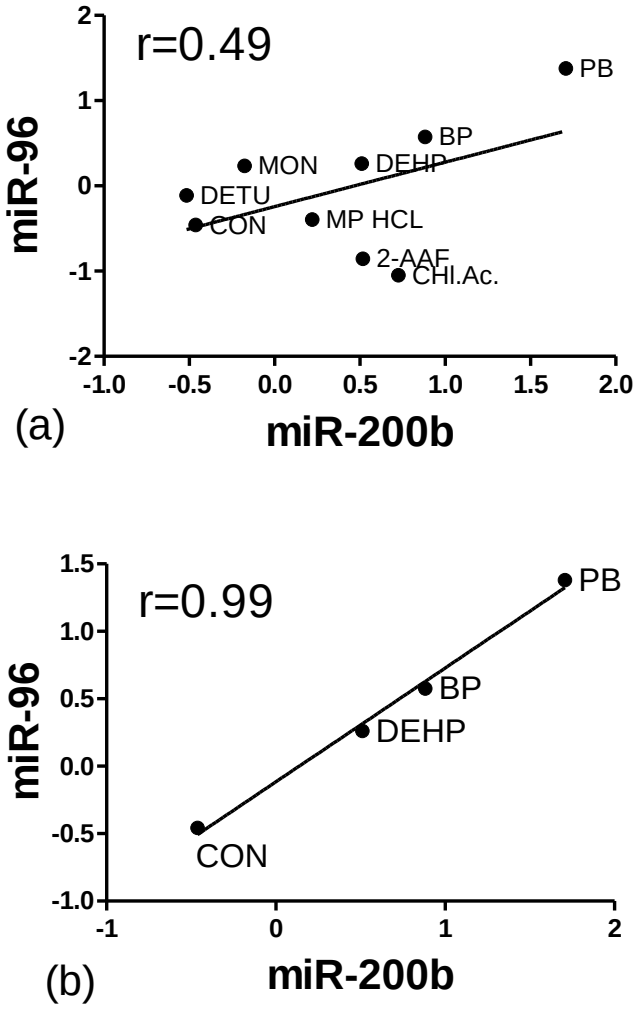


Fig. 3.15 (**a**) the correlation of the two miRNAs across all 90 day treatments can be seen (**b**) the correlation of the two miRNAs in PB, BP, and DEHP samples only can be seen. Pearson's correlation is used

This high correlation between the two miRNA clusters for DEHP, BP, and PB suggests that these two clusters are very likely under common regulation after treatment with these three compounds. It is important to note that DEHP also activates the CAR receptor (Ren *et al.*, 2010, Eveillard *et al.*, 2009a, Eveillard *et al.*, 2009b). Therefore, it is possible that a common CAR-dependent factor is involved in the correlated induction of these two miRNA clusters by DEHP, BP, and PB.

3.5.4. MiRNA profiles of MP HCL, MON, and Chl.Ac.

MP HCl had unexpectedly few effects on the liver miRNAs, given its carcinogenic potency (90% liver tumour formation (Lijinsky, 1984). The three miRNAs with significantly altered expression due to MP HCl treatment were changed less than 2-fold from control. These miRNAs were miR-99a, miR-34a, and miR-200a; all three are potential biomarkers of carcinogenicity. MON and Chl.Ac were the two carcinogenic chemicals used in this study which had an unknown MOA. Both treatments caused downregulation of miR-193 (**Figure 3.16**) as well as other of the biomarker miRNAs.

Figure 3.16 *Effects of chemical treatments on the expression of miR-193 at 90 days*

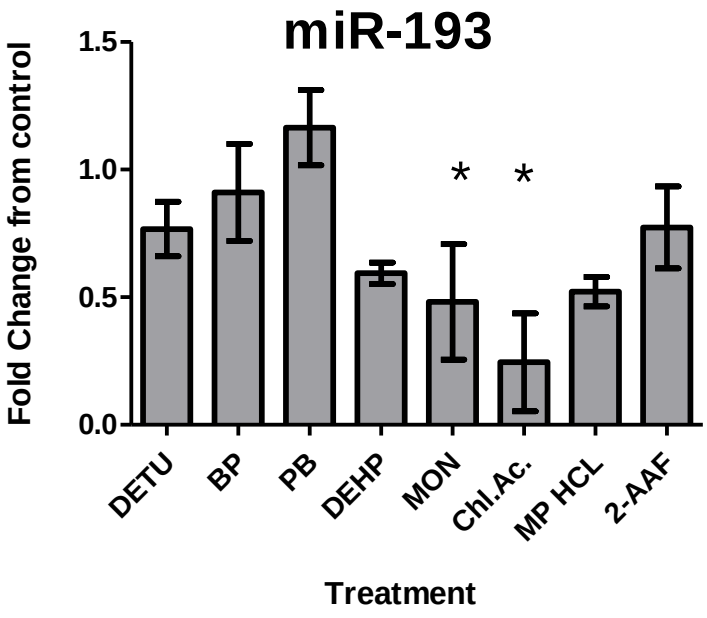


Fig. 3.16 Y-axis is fold change from control, bars are S.E. * significantly different from control one-way ANOVA with Tukey HSD $p<0.05$. n=3-5 animals per group.

MON induced the miR-96/182 cluster, miR-34a, and miR-99a. Chl.Ac induced miR-200a/200b/429, miR-34a, and repressed miR-203. Chl.Ac also had several chemical-specific effects on miRNAs (e.g. miR-497, miR-505, and miR-139-3p).

3.6 qPCR validation of miRNA changes

The change in expression for miR-200b was also quantified in samples from the 90 day dietary study by qPCR and supported the veracity of the 90 day study microarray data (**Figure 3.17**).

Figure 3.17 *Correlation of microRNA fold changes calculated by qPCR and microarrays.*

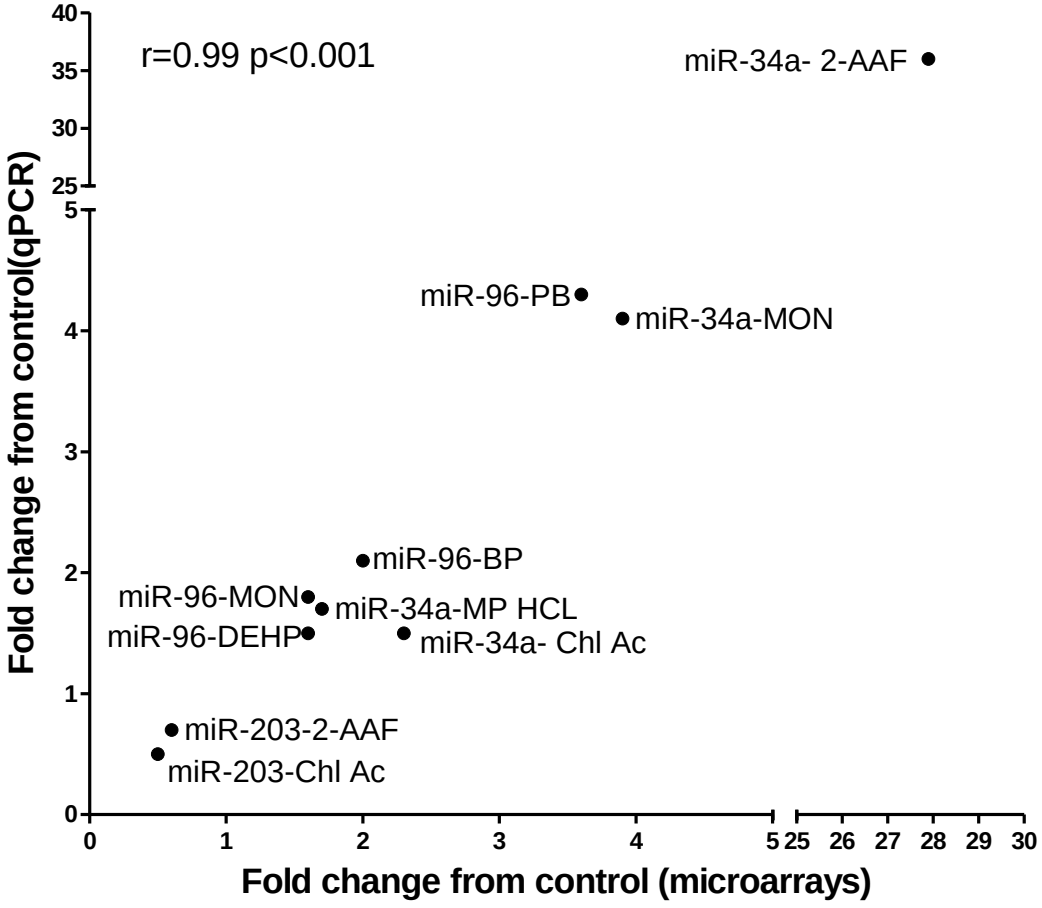


Fig. 3.17 The same liver samples were examined by qPCR and microarrays; n=3-5 animals per group. On the Y-axis the mean fold change for a miRNA calculated by the $\Delta\Delta C_T$ method is shown. On the x-axis the fold change as calculated from the hybridisation data. The correlation shown is Pearson's correlation.

3.7 Discussion

MiRNA expression profiles were determined for the liver samples from the Syngenta multiple compound study using the Agilent miRNA microarray platform. A number of observations concurred on the reliability and reproducibility of the generated microarray data (e.g. reproducibility of the technical replicates). MiRNA profiling using microarrays has become more reliable and reproducible in recent years as the involved technologies have been further developed and optimised. Nevertheless, the confirmation of miRNA deregulations detected by microarrays is still a required step. Microarrays and qPCR rely on alternative methodologies to quantify gene expression (Hybridisation of extracted RNA onto predesigned probes vs. PCR amplification of specific sequences respectively). Therefore, qPCR is often used as an independent method of validating the gene deregulations identified by microarrays. The quantifications of selected miRNAs by qPCR performed here were in agreement with the microarray data. These data hence were a further confirmation that the observed changes in the expression of miRNAs were real events.

A first conclusion from this study was that all the carcinogens tested affected hepatic miRNAs at 90 days. Other studies have previously reported perturbation in liver miRNA profiles after sub-chronic treatment with chemical carcinogens (e.g. (Pogribny *et al.*, 2007, Shah *et al.*, 2007, Pogribny *et al.*, 2009a)). However this study (a) examined a wider variety of chemicals with both known and unknown MOA (b) also examined chemicals which were less powerful carcinogens (PB, DEHP, and MON), while previous studies focused on highly potent carcinogens. Therefore, this

study has verified that alterations in the expression of hepatic miRNAs from the early stages of tumourgenesis are a common effect of chemical carcinogens.

A second key finding was that the miRNA profile of a given compound can give insights into its MOA. This was most clearly demonstrated by the two PB-type compounds, PB and BP. These two structurally diverse chemicals resulted in a characteristic "miRNA signature". Hence chemicals of this class of compounds appear to have distinguishing miRNA profiles.

Interestingly, the miRNA profile of 2-AAF suggested that besides its genotoxic activities it also potentially has epigenetic activities as well. Even though DNA damage and adduct formation is an important step in 2-AAF induced carcinogenicity, evidence suggests the requirement of subsequent epigenetic events to drive tumour formation following initiation (Williams *et al.*, 2000, Williams *et al.*, 1998). For example two chemicals closely related to 2-AAF, 2-acetylaminophenanthrene and trans-4-acetylaminostilbene, have similar genotoxic potencies but are unable to cause tumours without the action of a promoter such as PB (Bitsch *et al.*, 1993). This fact suggests that 2-AAF has properties similar to promoters. The similarities of the miRNA profile of 2-AAF to that of NGTX carcinogens used in the present study supports the epigenetic MOA of this chemical.

Additionally, the miRNA profile of DEHP shows some differences to that reported for Wy-14,643 and similarities to that of the PB-type compounds. Both Wy-14,643 and DEHP are PPC and activators of PPARα. However, unlike Wy-14,643 treatment in mice, DEHP did not downregulate let-7c in rats. Importantly, repression of let-7c by

Wy-14,643 was PPARa dependent as it did not occur in PPARα-null mice (Shah *et al.*, 2007). One difference between the two chemicals is that Wy-14,643 is a much more potent carcinogen, resulting in 100% hepatic tumours at a dose of 0.1%. Since let-7c is a suppressor of hepatic proliferation (Shah *et al.*, 2007), increased hepatic proliferation induced by let-7c repression may be part of the reason why Wy-14,643 is a much more potent rat hepatocarcinogen than DEHP.

Recent studies have demonstrated that besides activating PPARα, DEHP can also induce activity of the CAR receptor (Ren *et al.*, 2010, Eveillard *et al.*, 2009a, Eveillard *et al.*, 2009b). Nevertheless, transcriptomic analysis has shown that most genes affected by DEHP in the mouse liver are PPARα-dependent (94%), with only a small group regulated via CAR instead (Ren *et al.*, 2010). Until recently it was considered that the carcinogenicity of DEHP is wholly dependent on PPARα activity (Klaunig *et al.*, 2003). Unlike Wy-14,643 though, DEHP can also induce low levels of tumours in PPARα-null mice (Ito *et al.*, 2007). This has led to suggestions that DEHP can cause tumour formation through different pathways compared to Wy-14,643 (Guyton *et al.*, 2009). The precise nature of the MOA of phthalates is an important factor in the risk assessment of these common environmental contaminants. The highly correlated effect of PB, BP, and DEHP on the miR-200a/200b/429 and miR-96/182 clusters adds further support to the DEHP-mediated activation of CAR. It seems a possibility that miRNAs regulated by DEHP *via* CAR may be an alternative method by which phthalates may drive carcinogenesis in PPARα-null mice.

A final key finding was the identification of a set of potential miRNA biomarkers of carcinogenesis. In order for any identified miRNA signatures to be useful, they must

possess both high sensitivities (ability to detect carcinogens) and specificities (ability to discriminate carcinogens from non-carcinogens). Indeed, for the chemical treatments examined in this study, candidate miRNA signatures did show good specificities and sensitivities (**Section 3.4**). It is important to note however that a relatively small set of chemicals –six carcinogens and two non-carcinogens- were examined in this study. The determination of the specificity of the identified miRNA signatures was particularly worrisome, due to the fact that only two non-carcinogens were available for this study. Whilst the set of chemicals that were tested here were deliberately chosen to represent diverse MOA, the calculated sensitivities and specificities determined in this study must be confirmed in a larger study.

It is noteworthy that not included in this list are miRNAs which have been previously strongly associated with HCC (such as miR-122, miR-21, let-7a, miR-221, and 17-92 cluster). For example the hepatocyte specific miRNA miR-122 is downregulated in 50-70% of HCC (Gramantieri *et al.*, 2007, Kutay *et al.*, 2006, Hou *et al.*, 2011), while miR-21 is frequently upregulated in HCC and is a regulator of PTEN (Meng *et al.*, 2007).

Since carcinogenesis is a multistep process one possibility is that changes in the expression of these miRNAs do not occur until the later stages of tumour formation. Indeed, evidence supporting the gradual deregulation of miRNAs has been collected from rodent models of carcinogenesis. Unlike in humans were examination can occur only after the appearance of disease, in rodents the livers can be examined from the earliest stages of carcinogenesis. Kutay *et al.* 2006 examined male Fischer rat fed a folic acid, methionine and choline-deficient diet FMD. These animals develop

preneoplastic nodules at 36 weeks and tumours at 54 weeks. Downregulation of miR-122 was observed in the tumours of these animals at 54 weeks, but not in the preneoplastic tissue. Wang *et al* 2009 studied mice fed CDAA diet for 6, 18, 32, and 65 weeks. By 65 weeks mice livers will show signs of steatosis and preneoplastic nodules, while tumours appear at 85 weeks. MiR-122 levels were not altered in the livers of these mice until 65 weeks of treatment. **Thus miRNAs that have been found to be deregulated in HCC may not be useful for predicting carcinogenicity of chemicals following short-term exposures**.

In conclusion the study here has allowed a more thorough examination of the hypothesis that miRNAs can contribute to toxicological assessments than has been previously possible. Overall, the findings of this study support the potential usefulness of miRNAs as biomarkers of hepatocarcinogenicity. It has been shown that by 90 days all carcinogens affect expression of hepatic miRNAs. Additionally, it seems possible that information regarding the MOA of carcinogens can be extracted from their miRNA profiles. Finally a set of frequently deregulated miRNAs has been identified which could potentially assist toxicological assessments.

Chapter 4:

Hepatic microRNA profiling of rats treated with dietary phenobarbital

4.1 Introduction

The doses used for the PB dietary study (Dow, 2007a) were chosen from the literature, based on their reported short-term and long-term effects on the liver. (Kolaja *et al.*, 1996) examined the effect of treatment with 10, 50, 100, and 500 ppm PB on male B6C3F1 mice and male F344 rats over 90 days. In the rat only the 100 and the 500 ppm doses caused an increased liver weight and DNA synthesis. (Hagiwara *et al.*, 1999) tested the administration of 8, 30, 125, and 500 ppm PB in a two year animal bioassay of male Fischer rats. None of the doses caused adenomas or carcinomas of the liver, with only the 125 and 500 ppm doses causing liver enlargement and appearance of GST-P^+ foci. Uniquely for the 500 ppm dose, nodules of regenerative hyperplasia were detected. (Butler, 1978) tested a 1000 ppm dose in a two-year bioassay on male Fischer rats. It was found that 11 out of 33 animals surviving to that point had developed adenomas. The reported data therefore support a threshold in the ability of PB doses to induce hepatic tumours in the two-year bioassay with the male Fischer rat, as well as for the induction of hyperplasia and hepatomegaly. Research on PB promotion also supports the presence of thresholds for tumour promotion following initiation, although at lower levels. Following DEN initiation, 300 and 1200 ppm PB could promote liver cancers in male Fischer rats, but not 75 ppm or lower doses of PB (Maekawa *et al.*, 1992).

For the conducted animal study, nine-week old male Fischer rats were treated with 50, 500 and 1000 ppm doses of PB through their diet. By 14 days the transient induction of hepatocyte proliferation and the maximum increase in liver weight will have occurred (Kolaja *et al.*, 1996). Hence, the tested doses included one dose which has been reported to be hepatocarcinogenic in two-year bioassay (1000 ppm), one dose

which is non-hepatocarcinogenic, but is mitogenic and causes liver enlargement (500 ppm), and one dose which is not hepatocarcinogenic and does not affect liver weight or cell proliferation (50 ppm). Throughout the conduction of the animal study parameters such as overt toxicity, P450 activity, liver weight, cytotoxicity, hypertrophy, hepatic cell proliferation, and apoptosis were measured in order to confirm that the PB doses caused the expected changes on the liver.

Transcriptomic and metabonomic studies on the livers of this animal study have been already published (Waterman *et al.*, 2010). Here the hepatic miRNA profiles of control and PB-treated animals were determined at 1, 3, 7, and 14 days using three animals per group, by the same methodology as that used in chapter 3. It is well known that miRNAs regulate cell proliferation and apoptosis.

The aims of this study were: (1) to examine whether changes in miRNA expression are involved in PB-induced proliferation (2) determine whether the carcinogenic PB dose had different effects on the hepatic miRNAome from these early stages than the non-carcinogenic PB dose.

4.2 Effects of chemical treatments on animals

The effects of the three PB doses on the whole animal and in the liver in particular over the 14 days of the study have been reported previously (Waterman *et al.*, 2010; Dow, 2007a). **The data shown in tables 4.1-4.5 summarise the previously published findings with these rat samples. These data were not collected by myself and are shown here to facilitate the interpretation of the miRNA data obtained in my experiments.**

Table 4.1 Microsomal PROD activity

	Control(n=5)	50 ppm(n=5)	500 ppm (n=5)	1000 ppm (n=5)
Day 1	4.45±0.30	7.33±1.48**	179.72**	193.33**
Day 3	2.88±0.82	100.24±30.69**	569.07±97.83**	753.99±157.45**
Day 7	3.42±0.33	192.52±56.85**	1370.84±359.09**	1757.24±243.79**
Day 14	4.70±2.37	210.58±16.29**	861.36±140.50**	958.37±148.91**

PROD activity (pmol/min/mg protein); Error shown is SD; $p<0.01$

Table 4.2 Effect of phenobarbital on relative liver weight

	Control(n=5)	50 ppm(n=5)	500 ppm (n=5)	1000 ppm (n=5)
Day 1	4.6±0.2	4.7±0.1	4.9±0.1**	4.6±0.1
Day 3	4.7±0.3	5.0±0.2	5.2±0.3*	5.6±0.2**
Day 7	4.3±0.1	4.7±0.1**	5.4±0.2**	5.7±0.1**
Day 14	4.2±0.2	4.4±0.2	5.4±0.2**	5.7±0.2**

Liver to bodyweight ratio (%); Error shown is SD; * $p<0.05$ ** $p<0.01$

Table 4.3 Incidence of centrilobular hypertrophy

	Control(n=5)	50 ppm(n=5)	500 ppm (n=5)	1000 ppm (n=5)
Day 1	0	0	3	1
Day 3	0	1	3	4
Day 7	0	3	4	5
Day 14	0	3	5	5

Animals displaying centrilobular hypertrophy (%); Error shown is SD

Table 4.4 Ki67 hepatic labelling index

	Control(n=5)	50 ppm(n=5)	500 ppm (n=5)	1000 ppm (n=5)
Day 1	6.77 ±2.55	8.63 ±2.55	7.94 ±2.41	9.88 ±2.87
Day 3	6.92 ±0.73	6.14 ±0.74	9.65 ±0.58**	11.13 ±1.73**
Day 7	5.08 ±0.33	4.32 ±1.12	4.63 ±1.13	4.35 ±1.21
Day 14	2.03 ±1.04	1.10 ±0.26	1.38 ±0.49	1.83 ±0.63

Percentage of nuclei staining positive for Ki67; Error shown is SD ** $p<0.01$

Table 4.5 Incidence of apoptotic bodies

	Control(n=5)	50 ppm(n=5)	500 ppm (n=5)	1000 ppm (n=5)
Day 1	0.05±0.04	0.06±0.03	0.06±0.06	0.05±0.05
Day 3	0.06±0.04	0.03±0.03	0.07±0.04	0.04±0.03
Day 7	0.05±0.06	0.06±0.07	0.05±0.03	0.03±0.03
Day 14	0.08±0.06	0.07±0.07	0.11±0.04	0.08±0.10

Number of apoptotic bodies/mm^2; Error shown is SD

CYP2B1 is the main enzyme that mediates the dealkylation of 7-pentoxyresorufin (PROD) into resorufin, a process that can be measured fluorometrically. CYP2B1 is the major cyp enzyme induced by PB *via* CAR activation. The microsomal PROD levels (Table 4.1) hence support a consistent moderate induction of CYP2B1/2 by the 50 ppm dose and a much more pronounced induction by the 500 and the 1000 ppm doses. The adjusted liver weights of animals were consistently increased by the 500 and the 1000 ppm doses (Table 4.2). The maximum weight was reached by 7 or 14 days. Centrilobular hypertrophy was detected in the liver samples microscopically. By the 14^{th} day all animals treated with 500 and 1000 ppm PB displayed centrilobular hypertrophy, as well as 3/5 treated with 50 ppm PB. Ki67 hepatic labelling index (LI) showed a transient statistically significant increase in the livers of animals at 3 days (increase 1.4-fold in rats treated with 500 ppm and 1.6-fold for animals treated with 1000 ppm). By the 7^{th} day the Ki67 Li returned to normal. The terminal deoxynucleotidyl transferase mediated dUTP nick end labelling (TUNEL) method was used to stain apoptotic cells. The incidence of apoptotic bodies per unit area was unaffected across the study.

The above data collected and reported by others (Waterman *et al.*, 2010) and Syngenta project report, personal communication) demonstrate that overall, the effects of PB on the liver during this 14 day period were as expected i.e. induction of CYP2B, transient induction of proliferation, and sustained increase in liver size. Based on this information, the liver samples harvested in the study were used for my experiments. Microarrays were used to profile liver samples of controls and animals treated with three PB doses after 1, 3, 7, and 14 days.

4.3. Quality control of microarray data

The quality control metrics of the microarray samples were examined, as described in chapter 3, in order to evaluate the quality of the microarray samples (**Tables 4.6-4.9**).

Table 4.6 quality control metrics of day 1 microarray samples

Sample	Metric 1	Metric 2	Metric 3	Metric 4
CONTROL- 1	4.7	0.5	13.1	5.3
CONTROL- 2	5.0	0.0	8.9	6.8
CONTROL- 3	8.6	0.0	11.6	9.5
50 ppm- 1	3.3	4.2	8.8	6.2
50 ppm- 2	5.7	2.1	12.2	6.8
50 ppm- 3	4.2	2.1	10.9	7.2
500 ppm- 1	2.0	2.4	7.5	6.1
500 ppm- 2	1.9	1.1	9.3	6.6
500 ppm- 3	2.7	0.0	10.3	7.1
1000 ppm- 1	3.9	1.2	11.8	6.4
1000 ppm- 2	5.2	3.8	10.8	6.7
1000 ppm- 3	4.8	1.0	8.9	27.6

Metric 1= AnyColorPrcntFeatPopnOL; Metric 2= gTotalSignal75pctile

Metric 3= AddErrorEstimateGreen; Metric 4= gNonCtrlMedPrcntCVBGSubSig

(See Section 3.3.1 for explanation of the metrics)

Samples refer to individual animals and values are arbitrary units

Table 4.7 quality control metrics of day 3 microarray samples

Sample	Metric 1	Metric 2	Metric 3	Metric 4
CONTROL- 1	3.33	4.37	12.61	7.40
CONTROL- 2	4.06	2.98	9.49	7.17
CONTROL- 3	2.88	1.27	8.01	5.19
50 ppm- 1	3.65	5.20	11.57	7.72
50 ppm- 2	3.46	2.33	10.00	7.54
50 ppm- 3	4.72	2.25	13.23	6.25
500 ppm- 1	6.25	6.80	11.50	17.35
500 ppm- 2	5.73	3.84	9.43	7.39
500 ppm- 3	3.08	4.16	9.67	5.64
1000 ppm- 1	3.07	2.49	6.53	6.47
1000 ppm- 2	2.45	4.33	8.62	6.86
1000 ppm- 3	3.53	3.59	11.45	6.35

Metric 1= AnyColorPrcntFeatPopnOL; Metric 2= gTotalSignal75pctile

Metric 3= AddErrorEstimateGreen; Metric 4= gNonCtrlMedPrcntCVBGSubSig

(See Section 3.3.1 for explanation of the metrics)

Samples refer to individual animals and values are arbitrary units

Table 4.8 quality control metrics of day 7 microarray samples

Sample	Metric 1	Metric 2	Metric 3	Metric 4
CONTROL- 1	3.07	0.34	9.33	6.03
CONTROL- 2	3.43	7.11	10.40	6.26
CONTROL- 3	1.84	5.10	7.11	5.87
50 ppm- 1	2.65	3.70	9.33	6.80
50 ppm- 2	4.17	1.25	9.82	5.95
50 ppm- 3	4.60	1.42	9.76	5.82
500 ppm- 1	6.39	11.45	15.83	10.55
500 ppm- 2	2.90	2.98	9.18	7.00
500 ppm- 3	3.51	0.13	7.51	31.53
1000 ppm- 1	3.55	1.35	7.91	44.06
1000 ppm- 2	4.72	3.15	9.78	6.03
1000 ppm- 3	5.72	1.78	16.59	6.88

Metric 1= AnyColorPrcntFeatPopnOL; Metric 2= gTotalSignal75pctile

Metric 3= AddErrorEstimateGreen; Metric 4= gNonCtrlMedPrcntCVBGSubSig

(See Section 3.3.1 for explanation of the metrics)

Samples refer to individual animals and values are arbitrary units

Table 4.9 quality control metrics of day 14 microarray

Sample	Metric 1	Metric 2	Metric 3	Metric 4
CONTROL- 1	3.33	2.98	7.14	6.66
CONTROL- 2	2.40	2.68	14.66	6.55
CONTROL- 3	1.77	3.37	7.42	6.02
50 ppm- 1	3.61	4.51	10.06	6.08
50 ppm- 2	1.42	0.00	12.32	6.46
50 ppm- 3	8.00	3.35	13.89	8.23
500 ppm- 1	4.31	0.00	10.21	9.08
500 ppm- 2	5.17	3.13	10.59	5.80
500 ppm- 3	1.35	5.66	8.28	5.87
1000 ppm- 1	3.53	2.76	10.81	6.11
1000 ppm- 2	4.24	0.40	8.03	6.35
1000 ppm- 3	1.58	1.89	10.11	5.26

Metric 1= AnyColorPrcntFeatPopnOL; Metric 2= gTotalSignal75pctile

Metric 3= AddErrorEstimateGreen; Metric 4= gNonCtrlMedPrcntCVBGSubSig

(See Section 3.3.1 for explanation of the metrics)**;** Samples refer to individual animals and values are arbitrary units

As can be seen the values of the four metrics were consistent for the 48 microarray samples, with no obvious sample outliers. Another observation was the much lower values of metric 2, the gTotalgenesignal75pctilem, in these microarray samples compared to that for the samples from the multicompound study of chapter 3. This fact indicates that the hybridisation signal was generally lower for this set of microarrays. In accordance to this, 95 miRNAs were detected in the 14 day study

compared to 167 miRNAs in the 90 day multicompound study, using the same filtering criteria. The 72 miRNAs not detected in the present 14 day PB study were those with the lowest expression which were detected in the 90 day multicompound study. Consequently, some miRNAs which were found to be significant in the 90 day study were not detected in the 14 day study e.g. miR-182, miR-429, miR-203, etc. The difference between the hybridisation intensity of the two microarray studies was probably the result of variability in the efficiencies of hybridisation, ligation etc.

Boxplots were plotted for the 12 samples at each of the four timepoints after normalisation and filtration of the miRNAs that were not detected. The boxplots showed that the distribution of miRNAs were comparable for all the microarray samples of a given day (**Figure 4.1**).

Figure 4.1 *Boxplots for normalised, filtered microarray samples for 14 day phenobarbital study*

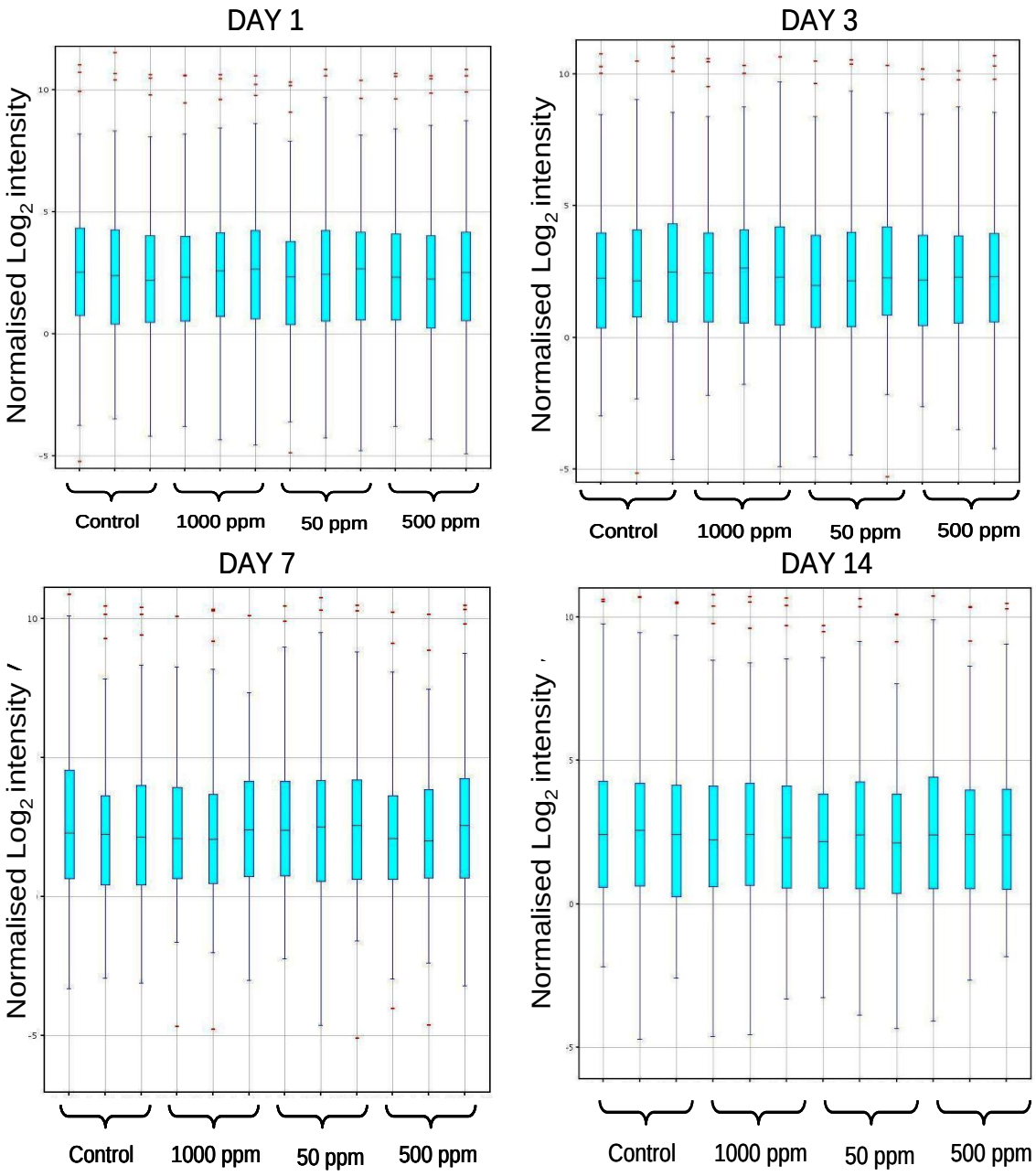


Fig. 4.1 Plot displays the 25th percentile (bottom end of box), 75th percentile (top of box), and median (line in the middle of box). Whiskers contain the 1.5 interquartile. Plotted as red circles are miRNAs with expression outside the 1.5 interquartile.

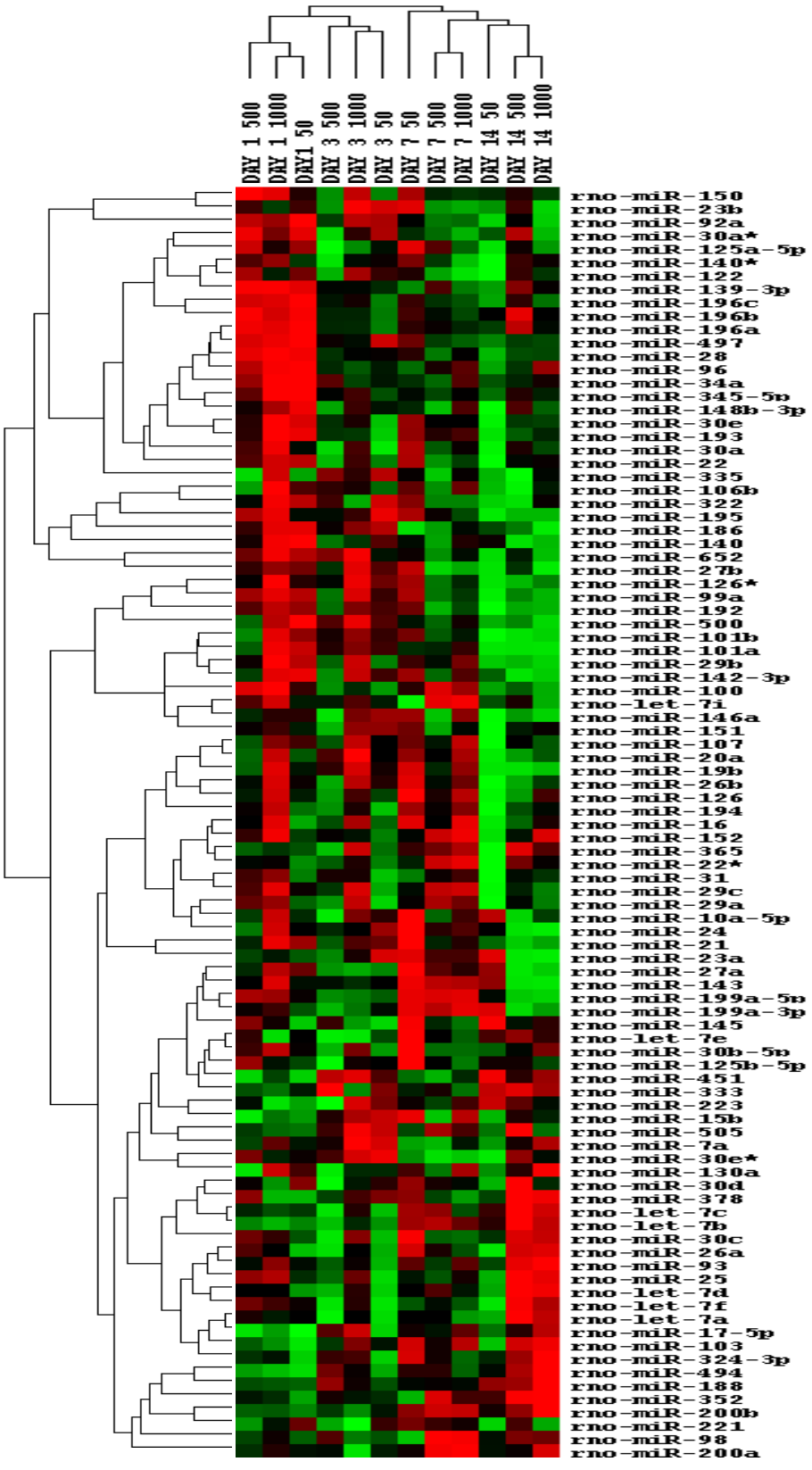
Besides the quality control metrics and the boxplots, other factors supported the reliability of the microarray data (a high miR-122 expression, expression of positive

and negative control probes of samples, the dynamic range of the microarrays, and the consistency of miRNA expression across replicates). In conclusion, the quality control analysis supports the reliability of the microarray data acquired for the 14 day PB study liver samples.

4.4 Clustering Analysis of samples

Hierarchical clustering analysis was used to study the dose and temporal patterns of PB effects on the liver miRNAome. Clustering was performed using the 95 miRNAs that were detected in at least 50% of liver samples in any condition. For each treatment the expression of miRNAs was averaged and its fold change from its day control samples was determined. It would be expected that if the dose effects of PB were consistent through time, the averaged samples would cluster according to their dose treatments e.g. the 1000 ppm doses from the different days clustering together. Instead, treatments clustered according to length of exposure, suggesting that the temporal aspects of PB effects on hepatic miRNAs are prominent. A second observation was the clustering of the treatments into early (day 1 and day 3) and late (day 7 and day 14) stages of PB treatment, indicating a distinction between the earlier and later responses (**Figure 4.2**).

Figure 4.2 *Clustering analysis of samples from 14 day phenobarbital study.*



(**Figure** legend in the next page)

Fig. 4.2 For each treatment group the fold change of each miRNA from the day control were calculated. The calculated values were then median centred and normalised in Cluster 3.0. Samples and miRNAs were clustered using Correlation centred similarity metric and average linkage. Red colour indicates high expression and green indicated low expression

From **Figure** 4.2 it can be seen that miRNAs did not show consistent repression or induction as a result of PB treatments, but instead varied over time. To examine further the temporal aspects of PB on hepatic miRNAs clustering was then performed for the control and treated samples at each of the four timepoints of the study (**Figure 4.3**).

Figure 4.3 *Clustering of samples at individual timepoints of 14 day phenobarbital study.*

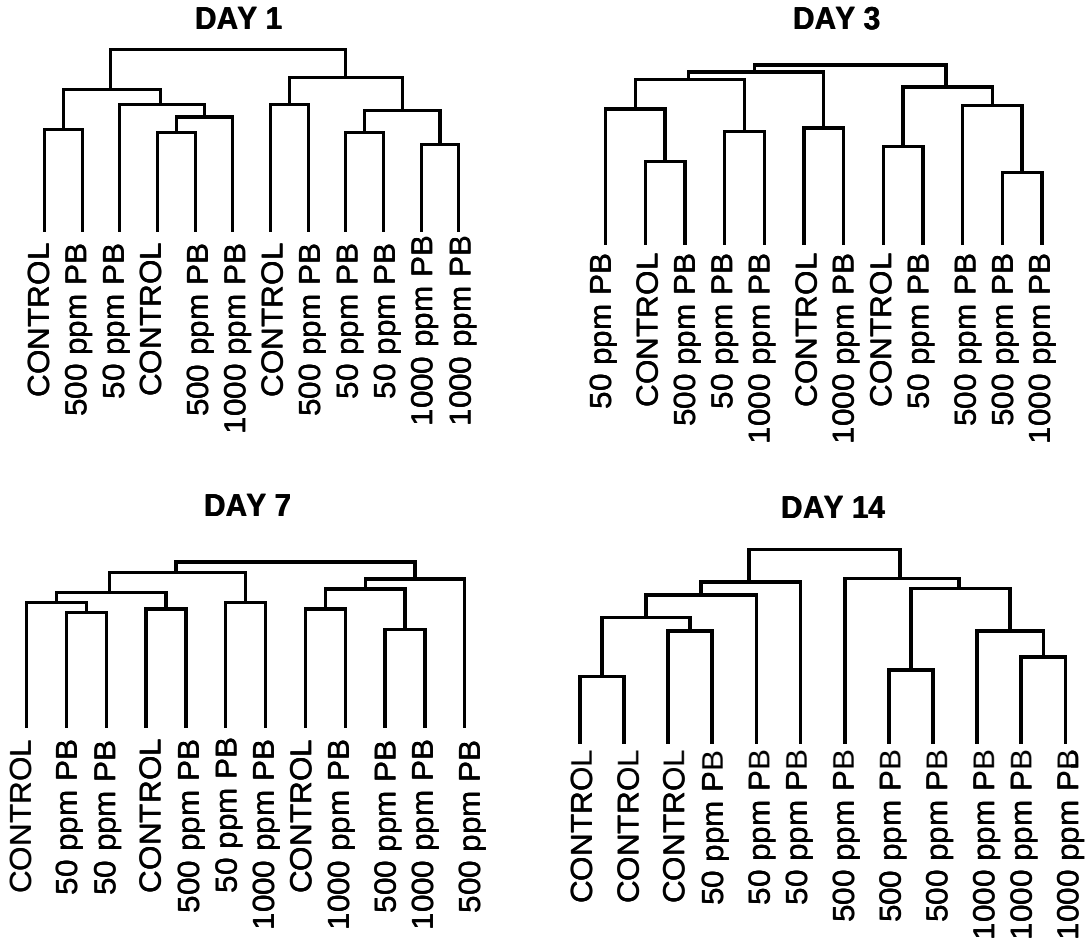


Fig. 4.3 Trees were constructed using the 95 miRNAs which were expressed in at least 50% of samples in any condition. Expressions of miRNAs were median centred and normalised in Cluster 3.0. Correlation centred was used as the similarity metric and clusters were linked by average linkage. For clarity heat maps of miRNA expression for each sample are not shown.

Interestingly, the control and PB-treated liver samples clustered at a more or less random fashion after 1, 3, and 7 days. In contrast, a clearly non-random association of samples could be seen for samples after 14 days. One cluster contained the control and 50 ppm PB dosed samples, while the high 500 and 1000 ppm PB doses were part of a distinct branch. Moreover, at 14 days of PB exposure liver samples from animals exposed to the carcinogenic 1000 ppm dose of PB clustered separately from those treated with the 50 and 500 ppm dose. This is despite the fact that up to 14 days the 500 and the 1000 ppm doses had similar effects on hepatic proliferation, CYP450 induction, and liver weight. **Therefore, hierarchical clustering analysis supports the effects of PB on the hepatic miRNAome are time and dose dependent**.

The expression and clustering of miRNAs for the day 14 samples can be seen below (**Figure 4.4**).

Figure 4.4 ***Clustering analysis after 14 days of phenobarbital treatment.***

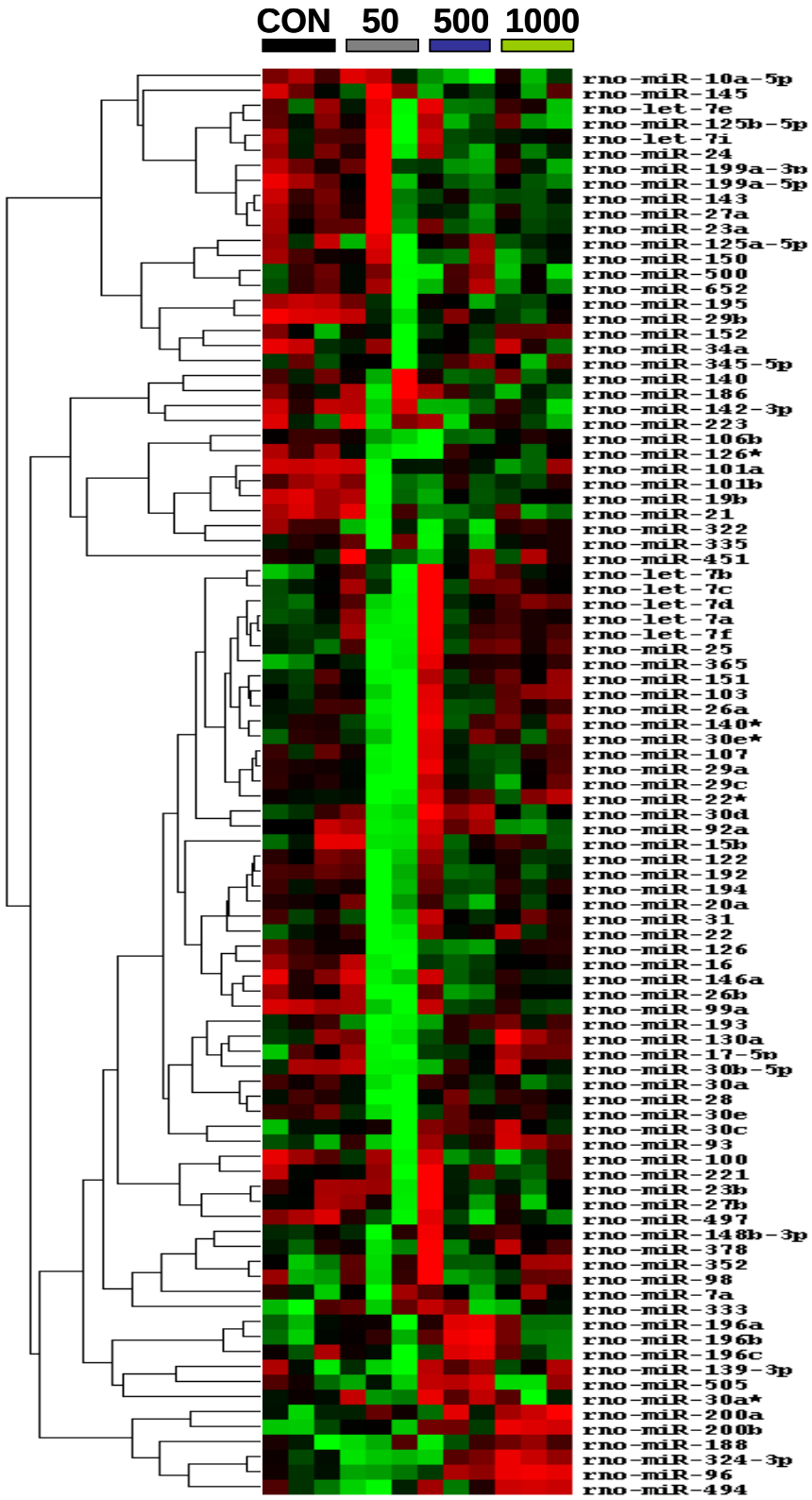


Fig. 4.4 Tree was constructed using 95 miRNAs that were detected in at least 50% of samples in any group. MiRNAs were median centred and normalised. Tree was constructed using Correlation metric and average linkage. Red indicates high expression, green low expression, and black no difference compared to median.

It can be seen that the expression of miRNAs after 14 days was more similar for the two higher PB doses. Additionally, clustering revealed one group of miRNAs which were strongly induced by the 1000 ppm PB dose at 14 days (miR-200a, miR-200b, miR-324-3p, miR-96, and miR-494). Importantly, miR-200a, miR-200b, and miR-96 were also found to be induced by 1000 ppm PB after 90 days of treatment (section 3.5.1). Just as in the 90 day samples, a significant correlation was observed for the clustered miRNAs miR-200a and miR-200b (**Figure 4.5**).

Figure 4.5 *Correlation of miR-200a with miR-200b across 14 day phenobarbital study.*

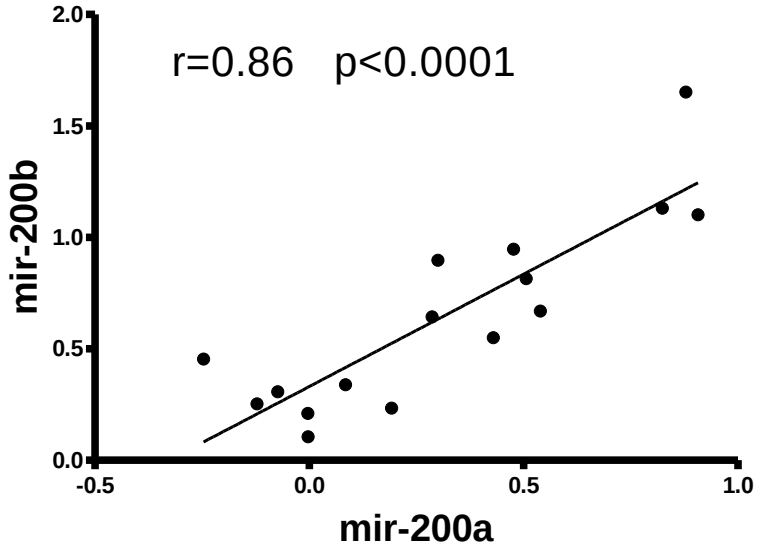


Fig. 4.5 Mean log2 hybridisation signal for miR-200b and miR-200a for each of the 16 treatment groups of 14 day PB study is plotted on each axis. Pearson's correlation analysis was performed.

In the 90 day samples a very strong correlation was also observed between the expression of miR-200b and miR-96 for the PB, BP, and DEHP treated samples. To determine whether that relationship holds true from early on, the correlation of miR-

200b with miR-96 was also determined for the 14 day PB study samples and again found to be significant (**Figure 4.6**).

Figure 4.6 *Correlation of miR-96 with miR-200b across 14 day phenobarbital study.*

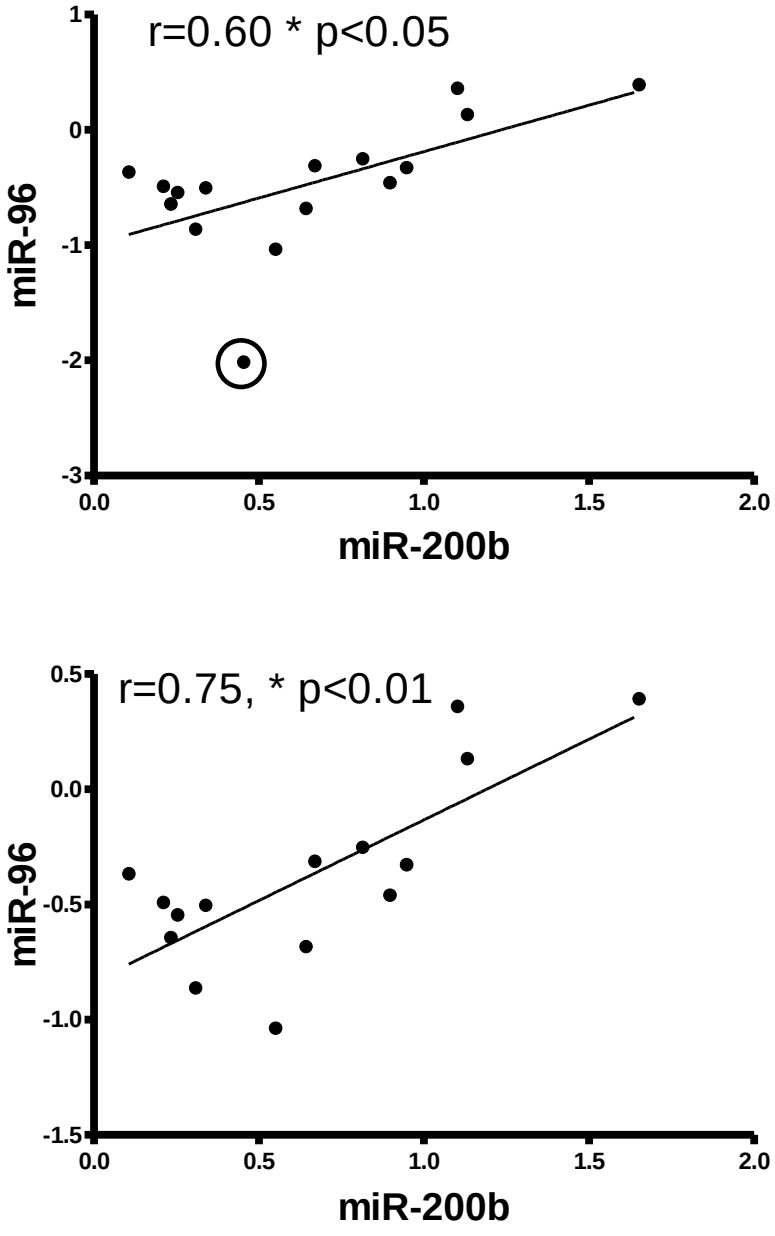


Fig. 4.6 Mean log2 hybridisation signal of each treatment is plotted on each axis. Pearson's correlation analysis was used. On the top panel all points were plotted. Circled is outlier point, day 1 control. The day 1 control sample was removed in the lower panel.

It was found that the expression of miR-96 in the day one control was an outlier and for that reasons was removed from further analysis.

4.5 Identifying differentially expressed miRNAs

4.5.1 MiRNAs deregulated by mitogenic PB doses

SAM was used to distinguish miRNAs that were differentially expressed between the mitogenic treatments (500 and 1000 ppm) and the non-mitogenic treatments (50 ppm and control) at the different time points (**Figure 4.7**).

Figure 4.7 *Common effects of mitogenic phenobarbital doses on microRNAs.*

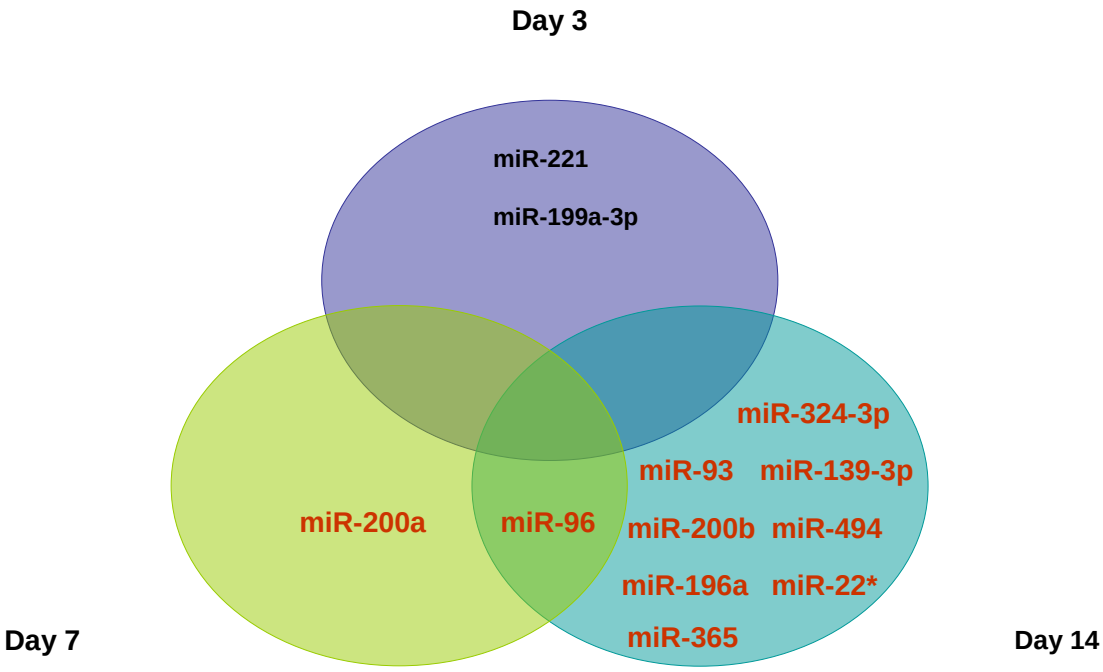


Fig. 4.7 The Venn diagram shows the miRNAs which were differentially expressed between hepatic non-proliferating (control and 50 ppm PB) and proliferating (500 and 1000 ppm PB) treatments**.** SAM was used with an FDR<0.001. Indicated as red are miRNAs with increased expression in the 1000 ppm treated animals, while coloured black are miRNAs with decreased expression in the 1000 ppm animals. No altered miRNAs were found on the first day of treatment.

The Ki67 data support a transient induction of hepatic turnover on the third day of exposure by the 500 and the 1000 ppm PB doses. A reasonable assumption to make was that miRNAs which were involved in PB-induced proliferation should also show a transient deregulation at the early timepoints (days 1-3) by the two mitogenic doses, but not by the 50 ppm PB dose. Only two miRNAs were found to be statistically significant between the mitogenic and mitogenic treatments at either 1 or 3 days. One was miR-199a-3p which was downregulated by the 500 ppm (0.74±0.03 fold) and 1000 ppm (0.81±0.08 fold) doses at day 3. The other was miR-221 which was downregulated by the 500 ppm (0.90±0.10) and 1000 ppm (0.63±0.13) at the same time. Their expression across all doses and timepoints can be seen in **Figure 4.8**.

Figure 4.8 *Fold Changes in expression of miR-199a-3p and miR-221 across doses and time.*

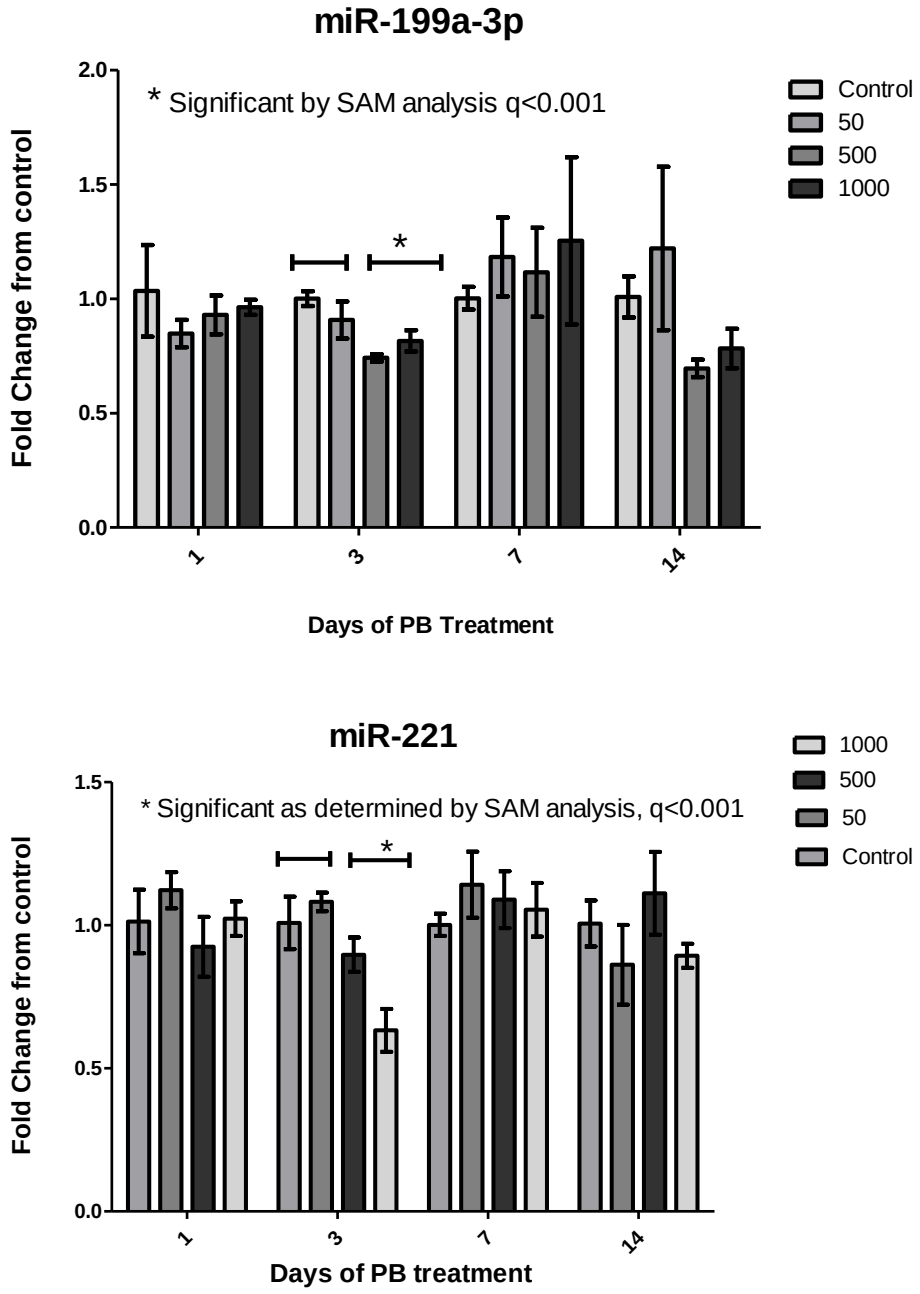


Fig. 4.8 Values shown are average fold change from control per treatment; n=3 and error bars are S.D. Significance tested by SAM analysis of control + 50 ppm Vs 500 and 1000 ppm).

The changes in the expressions of both miRNAs after 3 days were relatively modest. However, since the changes in expression were calculated for the whole liver, while only a small fraction of hepatic cells are induced to proliferate in response to PB

treatment (**Table 4.4**), it is possible that the magnitude of change for these miRNAs was underestimated. A number of miRNAs have been previously associated with hepatocyte proliferation. After 2/3 partial hepatectomy, induction of miR-21 and loss of miR-378 has been linked to the transition of hepatocytes from G1 to S phase (Song *et al.*, 2010). Let-7c is a miRNA that suppresses cell growth and is repressed in the liver of mice following Wy-14,643 treatment (Shah *et al.*, 2007). Another miRNA that has been linked to hepatocyte proliferation is miR-26a. This miRNA targets cyclins D2 and E2 in liver cells and can repress cancer cell proliferation *in vivo* (Kota *et al.*, 2009). The expressions of these miRNAs were examined at the early timepoints (**Table 4.10**), but were not affected by PB treatment.

Table 4.10 Changes in expression of selected microRNAs involved in proliferation

DAY	**1**			**3**		
Dose(ppm)	**50**	**500**	**1000**	**50**	**500**	**1000**
miR-21	0.99±0.01	0.85±0.10	1.1±0.23	0.95±0.33	0.81±0.08	0.93±0.24
miR-378	0.84±0.21	1.02±0.05	0.84±0.06	1.01±0.04	0.91±0.13	0.98±0.17
Let-7c	0.92±0.2	0.93±0.01	0.91±0.11	0.85±0.09	0.82±0.10	0.96±0.16
miR-26a	0.86±0.13	0.97±0.02	0.94±0.03	0.87±0.12	0.84±0.04	0.94±0.08

Fold change from control; ± S.D. n=3 animals per group

4.5.2 MiRNAs deregulated by carcinogenic 1000 ppm PB dose

After 14 days of PB treatment the carcinogenic 1000 ppm dose results in a characteristic miRNA hepatic profile. In order to identify the miRNAs which discriminate the carcinogenic dose, a SAM analysis was performed of 1000 ppm Vs control; 1000 ppm Vs 50 ppm; and 1000 ppm Vs 500 ppm (**Figure 4.9**).

Figure 4.9 *Specific effects of a carcinogenic phenobarbital dose on microRNAs.*

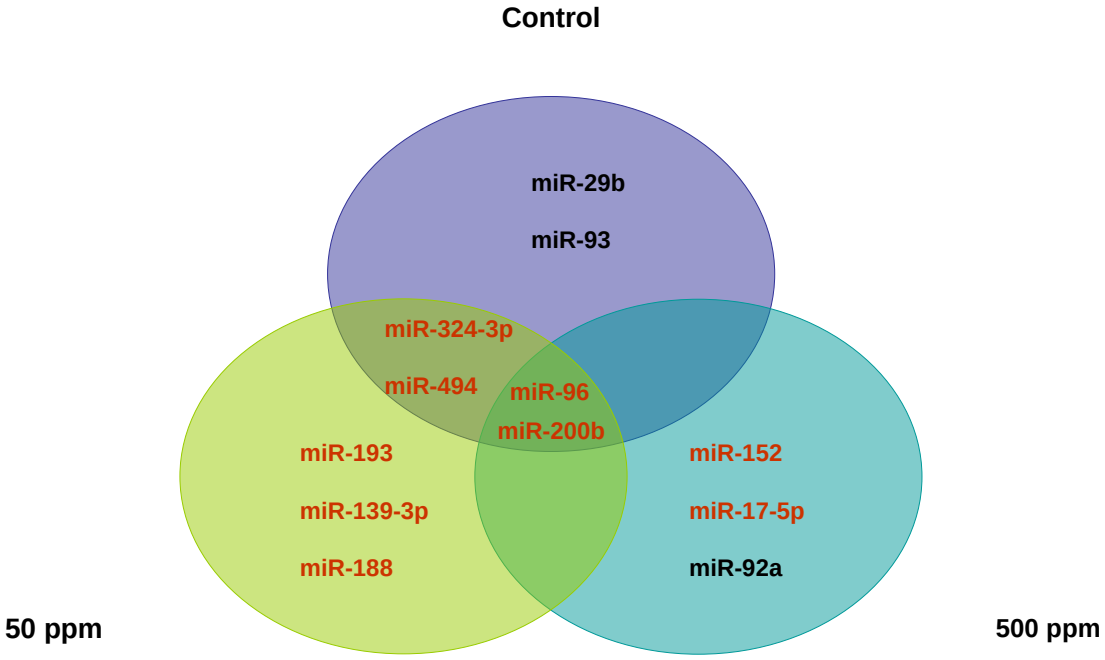


Fig. 4.9 The Venn diagram showing the miRNAs differentially expressed between 1000 ppm treated animals and other groups at 14 days. SAM was used with an FDR<0.001. Indicated as red are miRNAs with increased expression in the 1000 ppm treated animals, while coloured black are miRNAs with decreased expression in the 1000 ppm animals.

One interesting miRNA was miR-29b, which was downregulated to similar levels by all three PB doses at 14 days (**Figure 4.10**).

Figure 4.10 *Effect of phenobarbital doses on expression of miR-29b at 14 days*

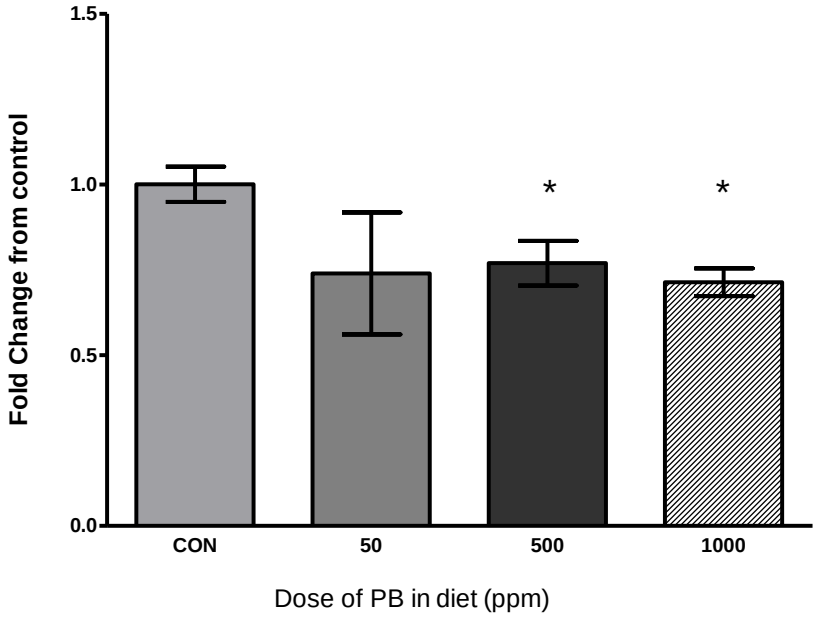


Fig. 4.10 Values shown are mean fold change; error bars are S.D.; n=3 animals per group. * Significant by SAM analysis of treatment Vs control, q<0.0001.

This miRNA has been shown previously to target DNA methyltransferases (DNMT) 3A and 3B (Garzon *et al.*, 2009), hence could be involved in regulation of methylation patterns of the liver. The two miRNAs that were significantly induced by the 1000 ppm dose compared to all other treatments at day 14 were miR-200b and miR-96 (**Figure 4.11**).

Figure 4.11 *Dose dependent effect of phenobarbital on miR-200b and miR-96*

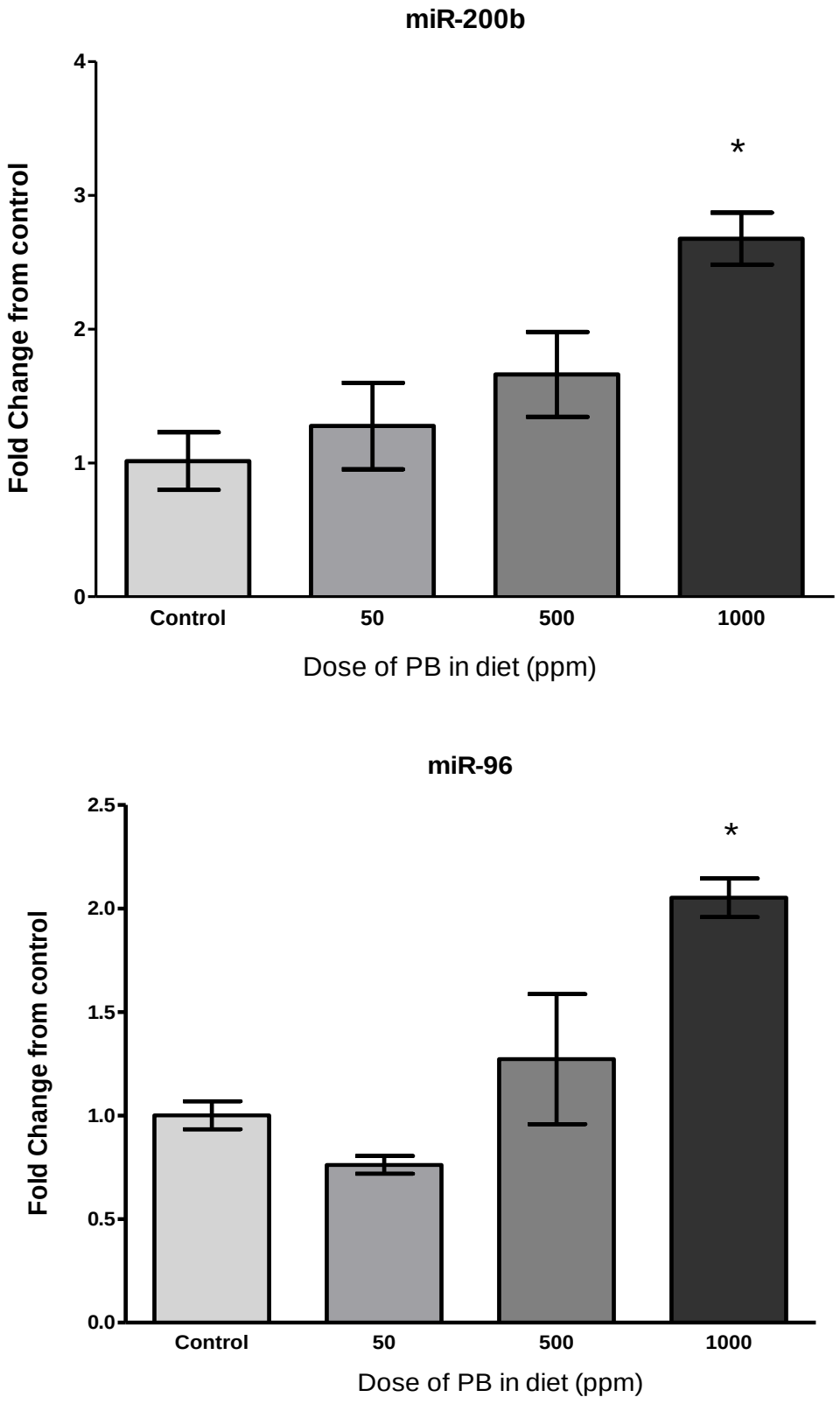


Fig. 4.11 Values are mean fold change; error bars are S.D.; n=3 animals per group. * Significant by SAM analysis of treatment Vs control, q<0.0001.

4.5.3 Progressive induction of miR-200b and miR-96 from 14 to 90 days

Six hepatic miRNAs were significantly differentially expressed between control and 1000 ppm treated animals at 14 days at FDR<0.01. The change in their expression from control animals was then compared at 14 and 90 days to determine whether the deregulation of these miRNAs was a consistent or transient event (**Table 4.11**)

Table 4.11 Comparison of effects of phenobarbital at 14 and 90 days

miRNA Id	Fold change from control day 14	Fold change from control day 90
miR-200b	2.7±0.2	4.5±0.4
miR-96	2.1±0.1	3.6±0.1
miR-494	1.7±0.05	0.6±0.2
miR-324-3p	1.5±0.2	0.8±0.4
miR-93	1.4±0.1	1.1±0.1
miR-29b	0.7±0.04	1.1±0.2

Fold change from control; values are mean fold change ± S.D. n=3

MiR-200b and miR-96 are the only miRNAs displaying progressive deregulation by miRNA treatment between 14 and 90 days. The other four miRNAs (miR-93, miR-324-3p, miR-93, and miR-29b) were expressed at similar levels as in the control animals at 90 days. The progressive inductions of miR-200a/miR-200b and miR-96 over the 90 days of PB treatment can be clearly seen in **Figure 4.12** and **Figure 4.13** below.

Figure 4.12 *progressive inductions of miR-200a and miR-200b with 1000 ppm phenobarbital treatment*

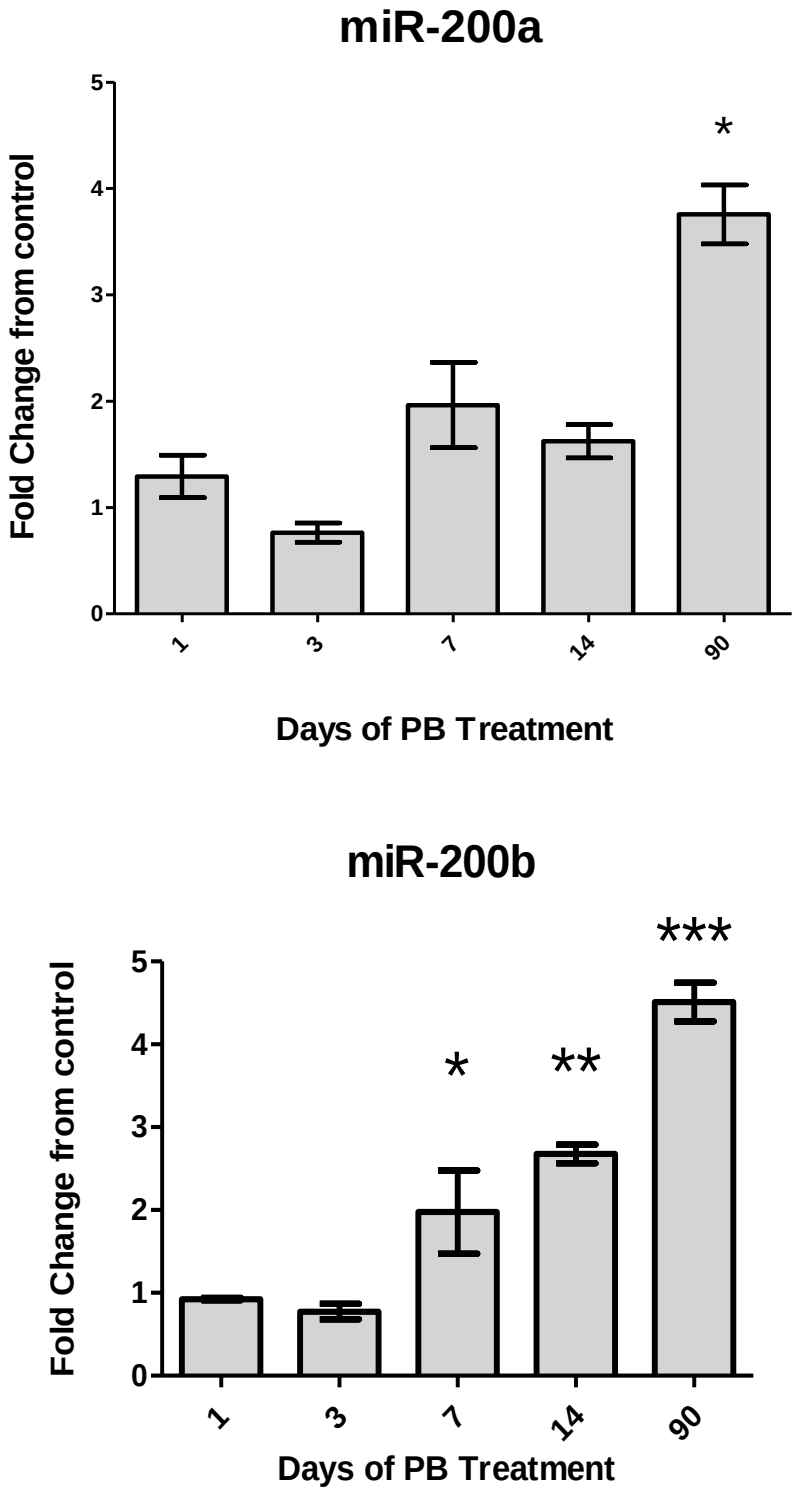


Fig. 4.12 Values shown are mean fold change from day control for that treatment; error bars are S.D. n=3 except for control samples at 90 days were n=5. Statistics performed were one-way ANOVA with Dunnett's post-test comparison against expression at day 1. * $p<0.05$ ** $p<0.01$ *** $p<0.0001$.

Figure 4.13 *Progressive induction of miR-96 with 1000 ppm phenobarbital treatment.*

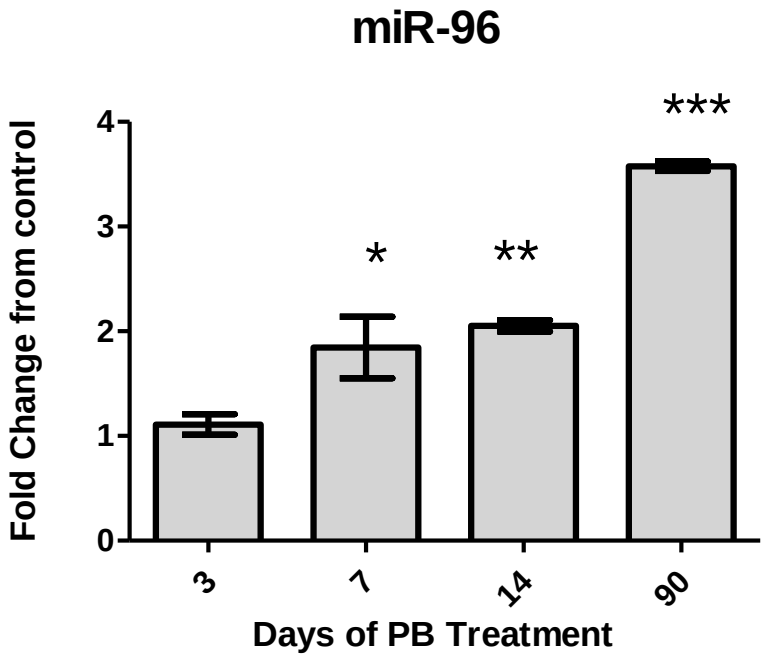


Fig. 4.13 Values are mean fold change from day control for that treatment; error bars are S.D; n=3. Statistics performed were one-way ANOVA with Dunnett's post-test comparison against day 3. Day 1 data were removed because they were clear outliers.

4.6 Correlations of miR-200b and miR-96 with hepatic parameters

The availability of data on the effect of PB doses on the liver allowed an examination of the association between these hepatic parameters and the expression of PB-induced miRNAs miR-200a, miR-200b and miR-96. In Table 4.12 the non-parametric correlation between the microsomal PROD activity (Table 4.1), relative liver weight (Table 4.2), and Ki67 hepatic labelling index (Table 4.3) and the expressions of these three miRNAs are tabulated.

Table 4.12 Correlation between CAR-induced microRNAs and liver parameters

	miR-200a	**miR-200b**	**miR-96**
Relative liver weight	r=0.58, *p<0.05	r=0.67, **p<0.01	r= 0.63, **p<0.01
PROD activity	r=0.73, **p<0.01	r=0.67, **p<0.01	r= 0.67, **p<0.01
Ki67 LI	r=-0.51, *p<0.05	r=-0.51, *p<0.05.	r=-0.18, n.s.

r is Spearman correlation; n=3 for microarrays and n=5 for hepatic parameters

A significant correlation was observed for relative liver weight and PROD activity with the levels of the three PB-induced miRNAs. On the other hand, the correlation between the expression of these miRNAs and the Ki 67 hepatic LI was not significant.

4.7 qPCR validation of observed changes

MiR-200b was the miRNA showing the biggest significant fold-change in expression at 14 days. The dose and temporal nature of miR-200b induction by PB in the day 14 PB study was examined by qPCR (**Figure 4.13**).

Figure 4.14 *QPCR analysis of miR-200b in 14 day PB study.*

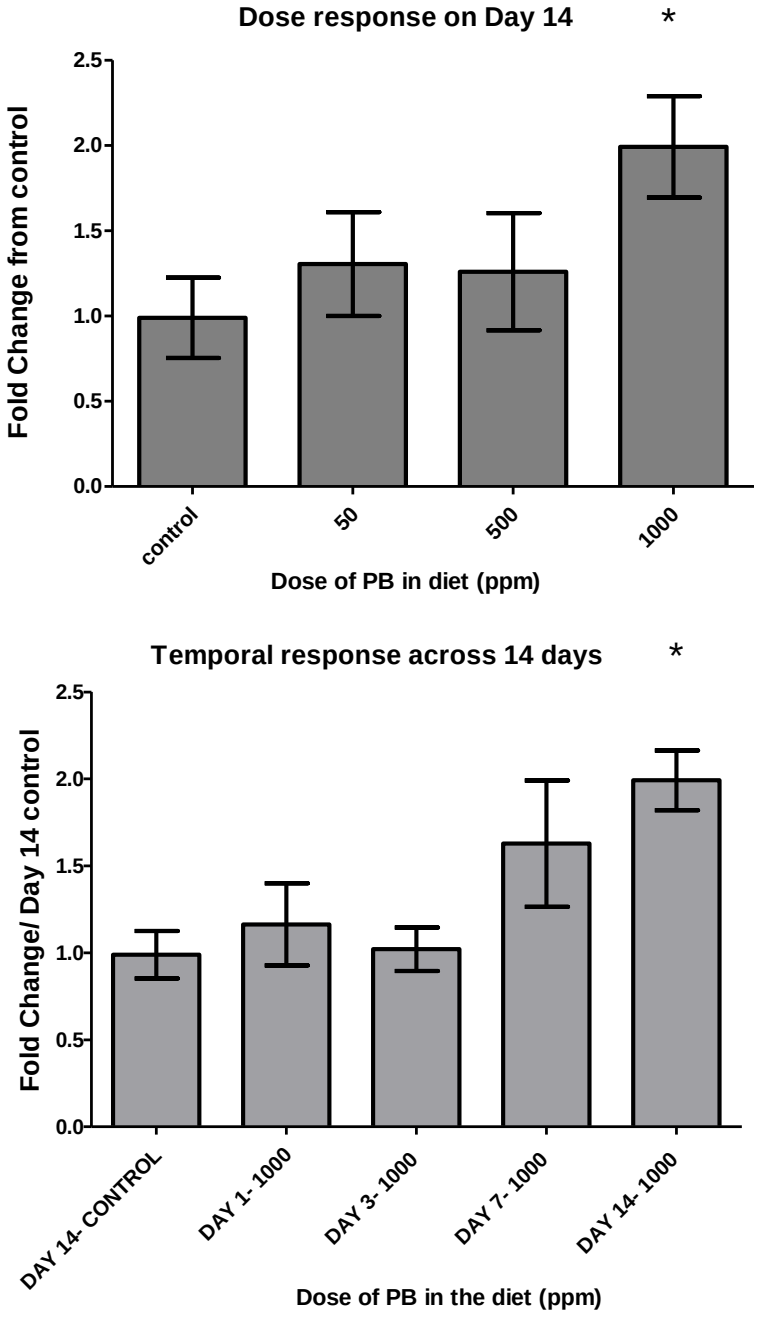


Fig. 4.14 Plotted on the Y-axis are mean fold-changes from day 14 control samples as calculated by qPCR. N=3 animals per group; errors bars signify S.D. One way ANOVA with Dunnett's post-test, * $p<0.05$.

The qPCR data agreed with the microarray data in showing a significant induction of miR-200b specifically by the carcinogenic PB dose at 14 days. The correlation between the microarrays and qPCR for the expression of miR-200b in the examined samples was highly significant (**Figure 4.15**)

Figure 4.15 ***Correlation of microRNA fold changes calculated by qPCR and microarrays for miR-200b***

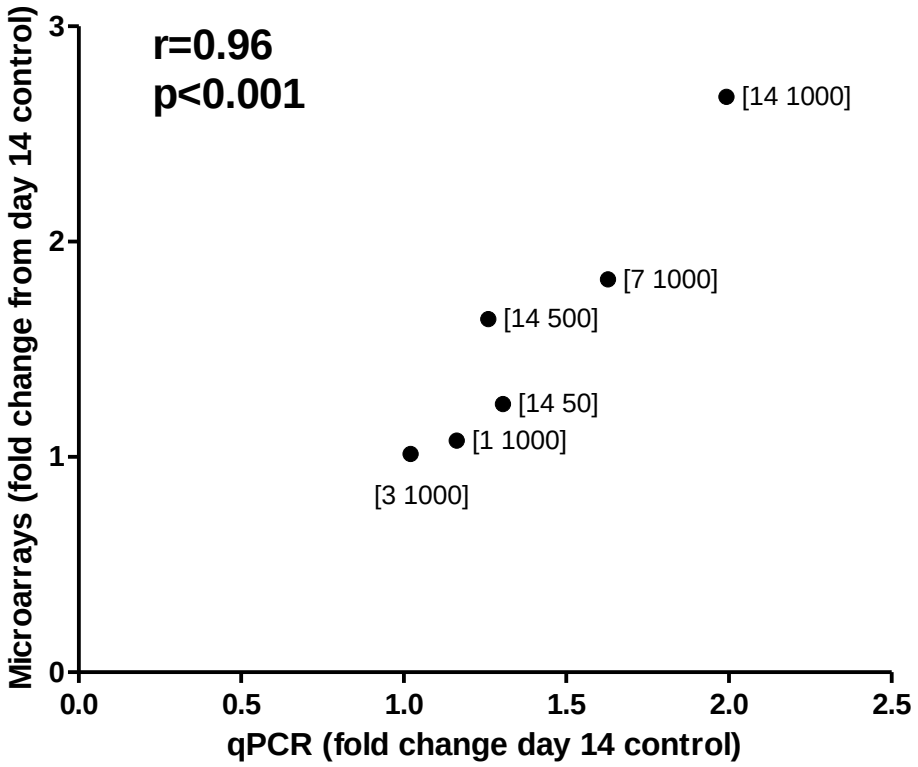


Fig. 4.15 On the y-axis the fold change in expression from day 14 control (calculated by comparative C_T method) and on the x-axis the fold change in expression from day 14 control calculated by microarrays. Expression plotted is the average of three animals; Pearson's correlation was used to measure correlation of the two methods. Next to each plotted sample the label identifies the dose and time of exposure to PB.

4.8 Discussion

The importance of both the dose and temporal parameters of chemical treatments is a fundamental tenet of toxicology. Indeed, the data generated here indicate that the effects of PB on the hepatic miRNAome are dependent on both the dose and temporal aspects of the chemical treatment (for example the distinct miRNA profiles of PB doses and the progressive induction of the miR-200a/200b/429 and miR-96/182 clusters). Unfortunately, for the other compounds examined in this study miRNA profiles were only generated for a single dose and timepoint (**Chapter 3**). Profiling of livers from animals exposed to multiple dose levels would also allow an evaluation of whether the observed effects of chemicals are restricted to the very high doses used here or are also relevant at lower doses. Temporal information would be important for the determination of the optimal timepoint at which miRNA profiles are the most informative. Together, the availability of dose and temporal data would also greatly facilitate the investigation of the functional effects of miRNA deregulations.

Waterman *et al.* (2010) previously performed transcriptomic profiling of the same PB-treated liver samples that were examined here. Their analysis revealed that protein-coding genes affected by PB displayed three major patterns: consistent induction or repression; transient early induction (days 1-3); and genes deregulated at later stages (days 7-14). Many miRNAs are embedded within protein-coding genes and are co-transcribed with them. In addition, transcription factors and cellular signalling pathways regulate both mRNAs and miRNAs. A reasonable assumption then would be that the effects of PB on miRNAs would follow similar patterns to that of protein-coding genes. However, in fact miRNAs displayed a different profile of response. Minimal changes in the miRNA profile were determined after 1, 3, and 7 days. The biggest changes could be seen at 14 days of treatment by which point

carcinogenic and non-carcinogenic doses of PB could be discriminated based on their hepatic miRNA profiles.

The results of the miRNA profiling indicate that hepatic miRNAs are initially (1-3 days of treatment) more resistant to deregulation by chemical treatment than protein-coding genes. The reason behind this discrepancy between miRNAs and mRNAs responses is currently not clear. However, after continual chemical treatment (>one week) miRNA deregulations are also prominent. In confirmation of this finding, it has been recently reported that the genotoxic hepatocarcinogen N-ethyl-N-nitrosourea (ENU) also displays progressive deregulation of miRNAs, with minimal changes at 1 and 3 days, with maximum changes after 7 and 14 days (Li *et al.*, 2010b). It is important to note that the two reported studies of carcinogens which did not affect hepatic miRNAs looked at acute effects i.e. Benzo[a]pyrene and TCDD caused minimal effects in the liver after three and four days of treatment respectively (Moffat *et al.*, 2007, Yauk *et al.*, 2010). *Therefore, the currently available data indicates that it is important that the effect of chemicals on hepatic liver miRNAome must take into consideration both dose and temporal aspects.*

The mitogenic properties of PB are considered a key event in its carcinogenic mechanism (Holsapple *et al.*, 2006). It is well established that miRNAs can regulate cellular proliferation. Nevertheless, studies of conditional Dicer1-knockout in hepatocytes have shown that liver cells can proliferate even in the absence of functional miRNAs (Sekine *et al.*, 2009, Song *et al.*, 2010). Hence, it is possible that PB can induce proliferation without affecting miRNAs. Two miRNAs were deregulated by the two mitogenic PB doses at day 3, miR-199a-3p and miR-221.

Interestingly, downregulation of miR-199a-3p in HCC has been recently linked to activation of the PAK4/Raf/MEK/ERK pathway that facilitates tumour growth (Hou *et al.*, 2011). It will be interesting to examine in future studies whether the downregulation of this miRNA is involved in the mitogenic stimulation of PB. Other miRNAs which have been previously linked to hepatocyte proliferation (miR-21, miR-378, and let-7c) were not changed by mitogenic PB exposure.

Treatment with CAR activators for 90 days (PB, BP, and possibly DEHP) had the common effect of the coordinated induction of the two miRNA clusters, miR-200a/200b/429 and miR-182/96. Examination of the 14 day PB treated samples revealed that the induction of these miRNA clusters (a) occurs after one-two weeks of treatment (b) is progressive in nature (c) is dose-dependant. These PB-induced effects were also confirmed by qPCR. A progressive induction of these two miRNA clusters between 12 and 24 weeks was also reported for 2-AAF (Pogribny *et al.*, 2009a). It was found here that the induction of these miRNA clusters occurs after the transient induction of proliferation has ceased. In the present study, a significant correlation was found between the expression of these miRNA clusters with both the relative liver weight and microsomal PROD activity. Both of these parameters are mediated by activation of CAR, suggesting a potential link.

Besides its other effects, CAR also affects hepatic metabolism. Activation of CAR in ob/ob mice attenuated diabetes progression, increases insulin-induced repression of gluconeogenesis, and decreased hepatic lipid content (Dong *et al.*, 2009). Similar beneficial results of CAR activation on hepatic glucose and lipid metabolism were also reported for mice fed high fat diets (Gao *et al.*, 2009). CAR can block FOXO1 by

directly binding to it (Kodama *et al.*, 2004). CAR also antagonises HNF-4 by competing for its coactivators and binding sites (Miao *et al.*, 2006). It is possible that the induction of the miR-200a/200b/429 and of the miR-96/182 clusters by CAR activators may be another way by which such chemicals can affect hepatic metabolism. MiR-182 and miR-96 have been shown to repress expression of FOXO proteins in T-cells (Stittrich *et al.*, 2010), ovarian (Myatt *et al.*, 2010), and breast cancer (Guttilla & White, 2009). A recent paper has shown that members of the miR-200 family cooperate in targeting FOG-2, resulting in greater activity of the PI3K pathway (Hyun *et al.*, 2009). Therefore inductions of these two clusters are predicted to target gluconeogenesis by repressing FOXO directly (miR-182/miR-96) and indirectly (miR-200) (**Figure 4.16**).

Figure 4.16 *Proposed model for metabolic regulation of gluconeogenesis by CAR activators*

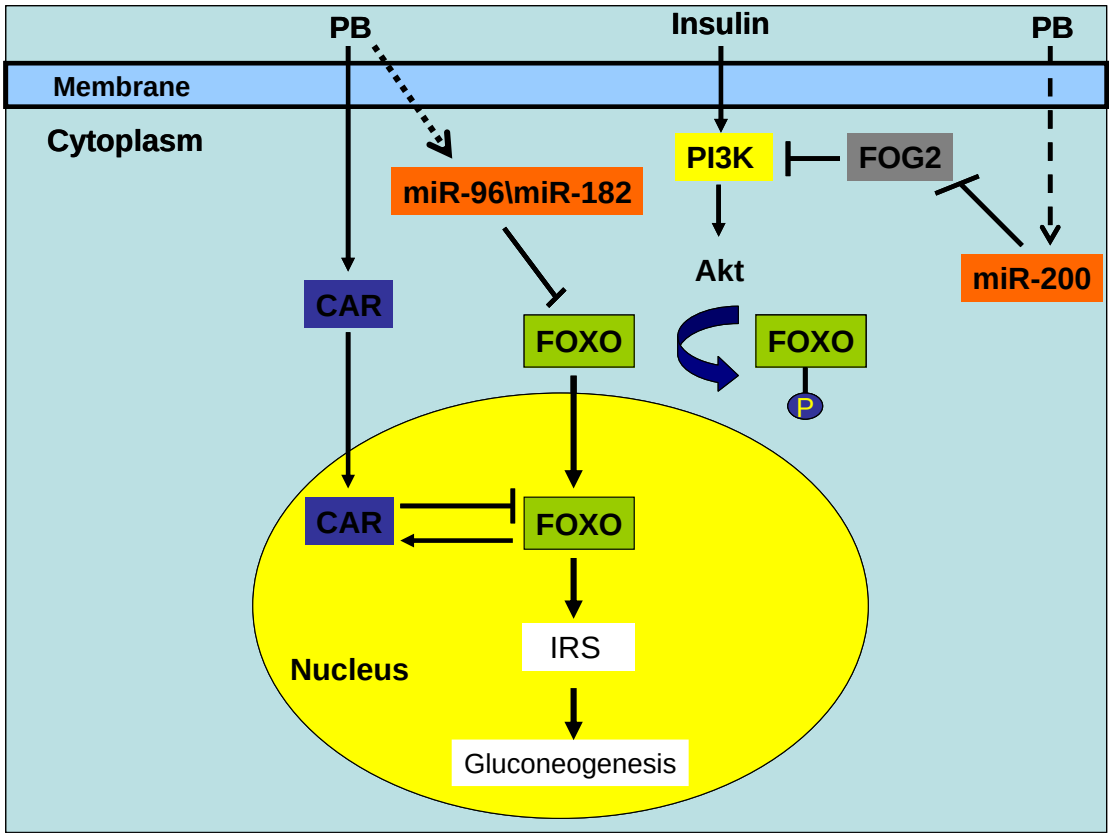


Fig. 4.16 FOXO promotes gluconeogenesis by binding to the insulin response sequence (IRS) of genes. Insulin causes the phosphorylation of FOXO by activating the PI3K-Akt pathway. The phosphorylation of FOXO represses gluconeogenesis by causing the localisation of FOXO outside the nucleus reducing its ability to bind IRS. FOG2 is a recently discovered inhibitor of the PI3K pathway. PB treatment results in translocation of CAR to the nucleus and the induction of the miR-200a/200b/429 and miR-182/96 miRNA clusters. Within the nucleus CAR inhibits FOXO by direct binding onto it. MiR-96 and miR-182 target and repress FOXO protein. The miR-200a/miR-200b, and miR-429 target FOG2 releasing the PI3K-Akt pathway. The dashed arrows indicate an associated but undefined linkage.

Clearly more work is needed to examine whether these miRNAs are involved in metabolic effects of CAR activation on the liver.

Chapter 5:
Causes and consequences of chemical-treatment induced miRNA deregulation

5.1 Introduction

In chapter 3 a set of liver miRNAs that were commonly deregulated after short-term treatment with chemical hepatocarcinogens are described. In chapter 4 the time and dose dependent effects of PB on the liver miRNAome were revealed. The work presented so far therefore had focused on profiling the liver miRNAome and on the identification of altered miRNAs. The studies reported in this chapter used this information and focused on investigating (**1**) the mechanisms underlying miRNA deregulation (**2**) the consequences of the observed miRNA deregulation. Particular emphasis was placed on the investigation of cause/effect associated with miRNAs affected by PB treatment.

Altered DNA methylation patterns are frequently associated with deregulated gene expression in disease, including cancers (Sharma *et al.*, 2010). Interestingly, among the identified "biomarker" miRNAs affected by hepatocarcinogens (see **Table 3.12**) almost all have been reported to be dysregulated in cancers due to altered methylation- miR-203 (Kozaki *et al.*, 2008, Furuta *et al.*, 2010, Lodygin *et al.*, 2008), miR-200a/200b/429 cluster (Li *et al.*, 2010), and miR-193 (Kozaki *et al.*, 2008)]. Moreover, hepatocarcinogens can affect both gene-specific and global DNA methylation in the liver (Pogribny *et al.*, 2008). Disruption of the normal methylation pattern is hence a mechanism by which chemical treatments can potentially affect the expression of liver miRNAs. CpG islands are guanine-cytosine rich genomic regions containing clusters of CpG dinucleotides whose methylation represses gene expression. The identification of "CpG islands" in the promoters of miRNAs indicates that methylation could potentially be involved in their regulation. The methylation of

CpG sites within the CpG islands can then be compared to the expression of the miRNA to determine if there is a relationship between the two.

Just as methylation patterns can affect expression of miRNAs, so too some miRNAs themselves regulate the epigenetic machinery. Three known enzymes are involved in methylation of CpG sites in mammals: DNMT1, DNMT3A, and DNMT3B. DNMT1 is the enzyme that is responsible for maintaining existing methylation patterns, while DNMT3A and DNMT3B perform *de novo* methylation (Jones, 2002). The members of the miR-29 family of miRNAs (miR-29a, miR-29b, and miR-29c) repress the expression of DNMT3A and DNMT3B (Fabbri *et al.*, 2007, Filkowski *et al.*, 2010, Takada *et al.*, 2009). It has also been shown that miR-29b can indirectly repress DNMT1 through the DNMT1-regulator Sp1 (Garzon *et al.*, 2009). In acute amyloid leukaemia (AML) cells, overexpression of miR-29b results in global DNA hypomethylation (Garzon *et al.*, 2009). Interestingly the expression of miR-29b was found to be transiently repressed at 14 days of PB treatment (**Figure 4.10**). Based on this observation the global hepatic DNA methylation was examined to determine whether an association exists between the deregulation of this miRNA and global hepatic DNA methylation.

Besides methylation patterns, the expression of miRNAs can also be regulated by the binding of transcription factors (e.g. (Shah *et al.*, 2007, Chang *et al.*, 2007, Fazi *et al.*, 2005, Bracken *et al.*, 2008). The research here has shown diverse chemicals affect a common set of miRNAs. This finding suggests that these miRNAs may be regulated by important cellular pathways that are disrupted by chemical treatments. Several computational tools have been developed for the purpose of identifying transcription

factor binding motifs within DNA sequences. The identification of transcription factor binding motifs in the promoter sequences of a miRNA is an indication that the protein may regulate the expression of that miRNA.

Ultimately, the functional significance of the deregulation of a miRNA depends on the proteins, which are regulated by it. Each miRNA can potentially affect hundreds or even thousands of proteins (Selbach *et al.*, 2008, Baek *et al.*, 2008). Unfortunately, a very small fraction of the predicted targets of miRNAs have been experimentally verified so far. Consequently, the use of computational methods is necessary to predict the genes regulated by miRNAs. Although imperfect, several *in silico* methods have been developed that can predict miRNA targets with moderate success (Bartel, 2009). The phenotypic consequences of the observed deregulation of miRNAs can then be explored through the proteins that they affect. Particularly important for most computational methods of predicting miRNA-targets, is the strength of the Watson-Crick complementarity between the miRNA seed region (nucleotides 2-8 from 5' end) and sites within mRNAs, especially within their 3' UTR. Hence, the seed regions of miRNAs are the most significant aspect in the prediction of their regulated genes. The phenotypic consequences of miRNA deregulation can then be investigated by consideration of the proteins/pathways that they are predicted to regulate.

An important confounding factor in determining the functional consequences of the observed miRNA deregulations was the lack of information regarding the identity of the cells in which these deregulations were taking place. In this project miRNA profiles were determined for liver segments removed from treated animals. This was a reasonable approach to start this study, allowing a determination of whether effects of

chemicals on the liver miRNAome could be detected. An obvious weakness of this methodology was its treatment of the liver as a uniform organ, ignoring the complex architecture and multiple cell types that constitute the liver (see **Section 1.3.1**). It is well established that the effects of chemicals are often restricted to a small fraction of hepatic cells. Consequently, the magnitude of miRNA deregulations determined here are expected to be underestimated, perhaps considerably so. Additionally, recently studies have shown that deregulations of miRNAs within specific hepatic cells can have important phenotypic consequences. One study reported that chronic alcohol treatment results in the induction of miR-155 in KC *in vivo*, which subsequently regulates TNFα production (Bala *et al.*, 2011), whilst activation of HSCs alters their miRNA profiles (Guo *et al.*, 2009). Hence potentially useful information on the hepatic cell types with affected miRNAome after chemical treatment could not be deduced from the microarray data generated here.

5.2. Analysis of miRNA promoters

5.2.1 Identification of CpG islands within the promoters of miRNAs

At present the definition of what parameters constitute a "true" CpG islands is somewhat arbitrary. Here a formal definition of CpG island was used (regions bigger than 500 base pairs; G/C content greater than 55%; expected/observed ratio of CpG dinucleotides greater than 0.65) which has the benefit of excluding GC-rich units, such as Alu repeats (Takai & Jones, 2002). The presence of CpG islands in the promoter regions of the identified biomarker miRNAs (**Table 3.12**) was examined using the CpG island searcher (http://cpgislands.usc.edu/, Takai & Jones, 2002) with the parameters set as described above. Besides rats, the bioinformatic search was also performed for the promoter region of their mouse and human homologs, to determine whether the detected CpG islands were conserved across species.

Given the fact that pri-miRNAs can be tens of kilobases in length (Chang *et al.*, 2007, Ozsolak *et al.*, 2008) it was decided to examine regions from 30 kilobases upstream to 1 kilobase downstream of each miRNA for the presence of CpG islands. DNA sequences were extracted from the UCSC genome browser (http://genome.ucsc.edu/) for the regions of interest. Using the criteria mentioned above CpG islands were identified in the promoters of most of the biomarker miRNAs (**Table 5.1**).

Table 5.1 CpG islands in the promoters of "biomarker" miRNAs.

MiRNA	Rat CpG islands?	Mouse CpG islands?	Human CpG islands?	Location of CpG islands
miR-203	YES	YES	YES	Overlapping miRNA
miR-34a	YES	YES	YES	~22Kb upstream (rodents) ; ~30Kb (Human)
miR-96/182/183	YES	YES	YES	~3.5-7 Kb upstream ; mice and humans also at 9-11 Kb
miR-193	YES	YES	YES	One overlapping miRNA and one at 7-10 Kb upstream
miR-200b/200a/429	NONE	NONE	YES	~3-5 Kb upstream in humans
miR-99a	NONE	NONE	NONE	NONE

Genomic regions up to 30 kilobases upstream of pre-miRNA and up to 1 kilobase downstream were examined; in humans the promoter of miR-193a was examined as it is more similar to the rat homolog miR-193

Only for miR-99a was a CpG island not detected in any of the three examined species. Conserved CpG islands were detected in all three species for miR-203, miR-34a, miR-193, and the miR-96/182 cluster. Interestingly, for the miR-200a/200b/429 cluster CpG islands were identified in the human promoter, but not for mice or rodents within the examined region. The CpG islands identified here show that methylation could potentially be involved in the regulation of several of the miRNAs identified in the present study.

5.2.2 P53 binding sites are located in the promoter of miR-34a

MiR-34a is a miRNA whose expression is directly activated by binding of p53 to a consensus p53 binding site (GGGCTTGCCTGGGCTTGTT) close to the transcriptional start site (TSS) of the miRNA (Chang *et al.*, 2007, He *et al.*, 2007). Examination of the genomic sequence up to 30Kb upstream of miR-34a in the rat

identified a single instance of this p53 binding site, which was located within the identified CpG island. Examination of the DNA sequences of the other biomarker miRNAs did not identify this p53 binding site in regions up to 30 kilobases upstream. Hence miR-34a is expected to be particularly responsive to p53 activity. 2-AAF, which was the only GNTX chemical used in this study, would be predicted to cause activation of the p53 signalling pathway. Examination of transcriptomic data from the liver samples at 90 days (Richard Currie personal communication) revealed an induction of p53 associated genes (e.g. *Bax*, *cdkn1a*, *ccng1*, and *mdm2*) specifically for 2-AAF treated rat liver samples. In accordance with these observations, the induction of hepatic miR-34a by 2-AAF treatment is much more pronounced than for other treatments (**Figure 3.13**).

5.2.3 Analysis of miR-200a/200b/429 cluster proximal promoter

The human promoter of the miR-200a/200b/429 cluster has been studied extensively (Bracken *et al.*, 2008). The authors identified a putative TSS at 4277 bases upstream of miR-200b, with the proximal miR-200a/200b/429 promoter being located within the region of -320/+120 of the TSS. The expression of the miR-200a/200b/429 cluster was shown to be induced by five out of six tested carcinogens at 90 days, with the biggest effect observed following carcinogenic PB treatment (**Figure 3.10**).

ZEB1 and ZEB2 transcription factor proteins, characterised by widely separated regions containing zinc-finger clusters, are the best established regulators of the miR-200 family (Burk *et al.*, 2008, Bracken *et al.*, 2008). They repress the expression of the miR-200a/200b/429 cluster by direct binding to paired E-box sequences which are conserved within the human, rat, mouse, and canine genomes proximal miR-

200a/200b/429 promoters. Besides transcriptionally regulating the expression of the miR-200a/200b/429 these transcription factors are also the best established targets of the miRNA members of the miR-200a/200b/429 cluster (Gregory *et al.*, 2008a, Christoffersen *et al.*, 2007, Korpal & Kang, 2008, Park *et al.*, 2008). Therefore a negative feedback loop relationship between the miR-200 family and the ZEB1/ZEB2 proteins exist (Burk *et al.*, 2008, Bracken *et al.*, 2008) (**Figure 5.1**).

Figure 5.1 *Negative feedback loop regulating the expression of the miR-200a/200b/429 cluster and the ZEB1/ZEB2 transcription factors.*

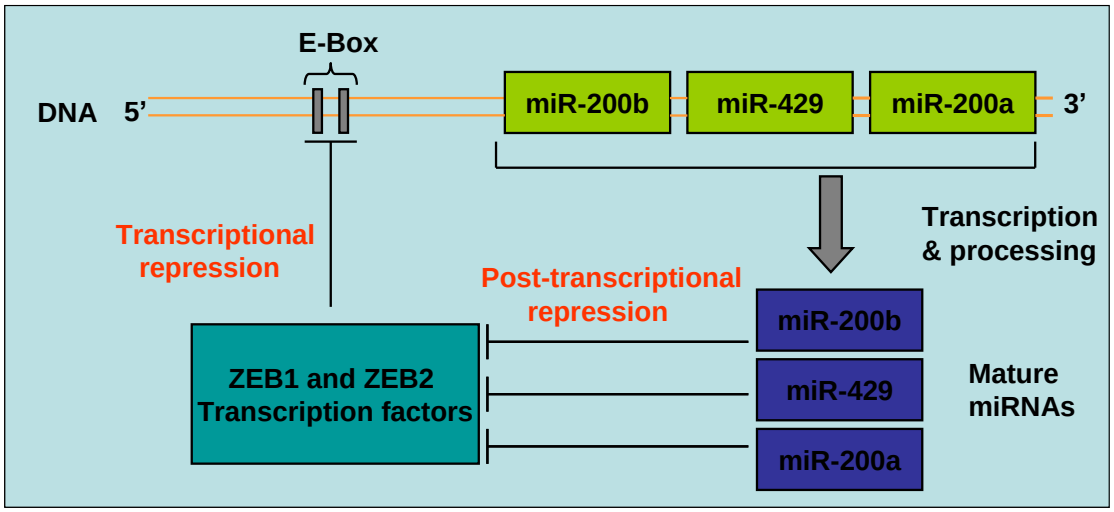


Fig. 5.1 ZEB1 and ZEB2 bind onto E-box sites found in the promoter region of the miR-200a/200b/429 cluster and repress the transcription of the unit. At the same time, all three miRNA target and post-transcriptionally regulate ZEB1 and ZEB2.

However, neither *zeb1* nor *zeb2* displayed altered expression in the rat liver transcriptomic data (R.A. Currie, personal communication). This finding indicated that these proteins are not involved in the observed induction of miR-200a, miR-200b, and miR-429 in the rat liver after chemical treatment. Since the biggest induction of the hepatic miR-200a/200b/429 cluster was caused by PB treatment, transcription

factors that are affected by the chemical were examined as potential mediators of that effect. Activation of CAR is a main mechanism by which PB propagates its cellular effects (Ueda *et al.*, 2002). Two other transcription factors that are known to be affected by PB treatment or CAR activation are the hepatic nuclear factor 4 alpha (hnf4α) (Bell & Michalopoulos, 2006) and c-myc (Blanco-Bose *et al.*, 2008). The genomic region up to 5 kilobases upstream of the miR-200b was examined for HNF4α, CAR, and c-myc binding sites.

DNA sequences recognised by nuclear receptors are repeats of distinct hexamers at particular orientations to each other. These orientations include inverted repeats (IR), direct repeats (DR), and everted repeats (ER). NUBISCAN, a computational tool that was developed specifically to identify binding sites of nuclear receptors (Podvinec *et al.*, 2002), was used to identify potential HNF4α binding sites within the promoter of miR-200b (**Table 5.2**).

Table 5.2 Putative HNF4α a binding sites up to 5000 bases upstream of rat miR-200b

Position upstream of miR-200b	p. value	Strand	Score	Repeat	SEQUENCE
4292	0.01	+	0.98	IR3	AGGCCAcacTGTCCT
4225	0.01	+	0.94	ER5	TGACCTgtgccAGTTCA
3870	0.01	+	0.93	DR10	GGGGCAggcaggagggAGGCCA
142	0.02	+	0.93	ER9	TGCCCTccagaatgaAGGGCT
4220	0.02	-	0.92	DR8	AGGTCAacaactcgGGGACA
3854	0.03	+	0.92	IR3	AGGCCAgacAGGCCT
3854	0.03	+	0.92	DR3	AGGCCAgacAGGCCT
345	0.03	+	0.92	DR8	AGGCCAggtttggcTGGACA
1865	0.04	+	0.88	IR6	TGGGCAcctaccTGCCCC
4552	0.04	-	0.92	DR0	AGGCCCAGGCCA
3880	0.04	+	0.88	DR4	GGGGCAggtaGGGGCA
1335	0.04	+	0.87	ER5	TGCCCTtttctGGTGCA
1702	0.05	+	0.91	DR2	AGGACAcaGGGACA

Putative HNf4α binding sites within 5000 base pairs upstream of the rat miR-200b, with a calculated $p<0.05$ were identified using NUBISCAN. Scores were calculated by the NUBISCAN algorithm, with the highest scores indicating a better match. Also shown is the identified repeat element. Under the sequence heading are shown the genomic sequences containing putative HNF4α binding sequence, with the hexamer sequences shown in capitals.

Interestingly, the top two predicted HNF4α binding sites were located at 4225 (IR3 elements) and 4292 bases (ER5 element) upstream of miR-200b, within the identified proximal promoter of the miRNA cluster. Next, the PROMO tool for prediction of putative transcription factor binding sites (Messeguer *et al.*, 2002) was used to identify c-myc and CAR/RXR binding sites in the same genomic region by comparing the genomic DNA sequences to known transcription factor binding sites (**Table 5.3**).

Table 5.3 Putative c-myc and CAR/RXR binding sites upstream of miR-200b

Transcription factor	Putative binding sites
c-myc	40, 620, 670, 830, 1430, 2780, 3630, 3990, 4110, 4240, 4840
CAR/RXR	50, 790, 2170, 3610

Putative binding sites were predicted in region up to 5000 bases upstream of the pre-miR-200b using the PROMO software. Threshold was set as 15% dissimilarity between genomic sequence and transcription factor matrix.

Therefore potential HNF4α and c-myc binding sites are present close to the proximal promoter of the miR-200a/200b/429 cluster and could regulate its expression.

5.2.4 Examination of genomic region upstream of miR-96/182

A very strong correlation was observed for the miR-200a/200b/429 cluster and the miR-96/182 cluster in liver samples treated with PB, BP, and DEHP at 90 days (**Figure 3.15**). A similar association between these two miRNA clusters has also been reported in the literature to occur in breast and pancreatic cells (Shimono *et al.*, 2009, Wellner *et al.*, 2009). These findings suggest the activity of a common factor in the induction of these two miRNA clusters. It was decided to examine the genomic region upstream of the miR-96/182 cluster for the presence of binding sites that could also regulate the miR-200a/200b/429 cluster (i.e. CAR/RXR, ZEB1/ZEB2, HNF4α, and c-myc).

In order to narrow down the genomic area to be examined, the FirstEF software was used, a computational tool developed with the aim of predicting gene promoters by taking into account structural and functional elements of DNA sequences (Davuluri *et*

al., 2001). Examination of the region 30 kilobases upstream of the rat miR-96 identified two possible promoter regions in the anti-sense strand: at 6156-6725 upstream of miR-96 with a high probability (with p of 0.9979) and at 27699 to 28268 with a much lower probability (p 0.4966). The first putative promoter, and the one with the highest probability, was localised within the area (3.5-7.5 kilobases) upstream of miR-96 that contains large CpG islands. CpG islands upstream of eukaryotic genes often indicate the presence of regulatory elements (Fickett & Hatzigeorgiou, 1997). RNA from rat liver samples could be amplified by RT-PCR using PCR primers designed to anneal to sequences 4478-5336 upstream of miR-96, demonstrating that this region is indeed transcribed in the rat liver (**Figure 5.2**).

Figure 5.2 *RT-PCR amplification upstream of miR-96*

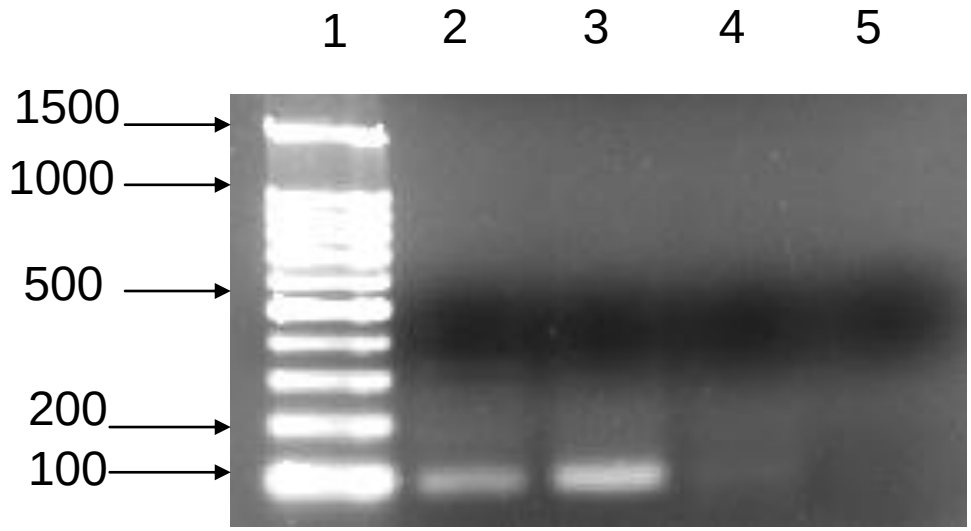


Fig. 5.2 Genomic region amplified was 3426-3519 bases upstream of miR-96. Expected product is 93 bases in size. In lane 1 the DNA ladder can be seen with molecular weights of 1500, 1000, and incremental decreases of 100 bases for each band from then on. In lane 2 and lane 3 cDNA is amplified at 55^{o}C and 60^{o}C. In lane 4 PCR amplification was performed on no reverse transcriptase control and in lane 5 PCR amplification was performed on a no template control sample. PCR amplification was performed for 35 cycles for each sample.

Based on these observations the genomic region that contained the evolutionary conserved CpG islands (3.5-7.5 kilobases upstream of miR-96) was considered to be potentially important for the regulation of this miRNA cluster, and was chosen to be examined for potential ZEB1/ZEB2, CAR/RXR, HNF4α, and c-myc binding sites. This analysis identified a number of putative binding sites for these transcription factors within the CpG-containing genomic area upstream of miR-96 (**Table 5.4**)

Table 5.4 selected binding motifs in CpG islands upstream of miR-96

Binding Motiff	Position upstream of miR-96
CAR/RXR	5760 bases
E-boxes (zeb1/zeb2 binding)	4343, 4645, and 4876 bases
c-myc	3910, 4140, 4440, 4530, 4670, 5190, 6280
HNF4α	5420

Transcription factor binding motifs were detected using PROMO and NUBISCAN computational tools.

In an attempt to better define the promoter region of the miR-96/182 cluster, rapid amplification of cDNA ends (RACE) was performed using the Generacer kit as described in materials & methods (**Section 2.6**). This method allows the identification of TSS of genes, and has been used previously for analysis of pri-miRNA transcripts (Chang *et al.*, 2007). As a positive control Hela RNA template was processed simultaneously with sample RNA. PCR primers were then used to specifically amplify *β-ACTIN* modified by the attachment at the 5' end of the Generacer RNA oligo from the generated Hela cDNA template (**Figure 5.3**).

Figure 5.3 *PCR amplification of positive control for Generacer*

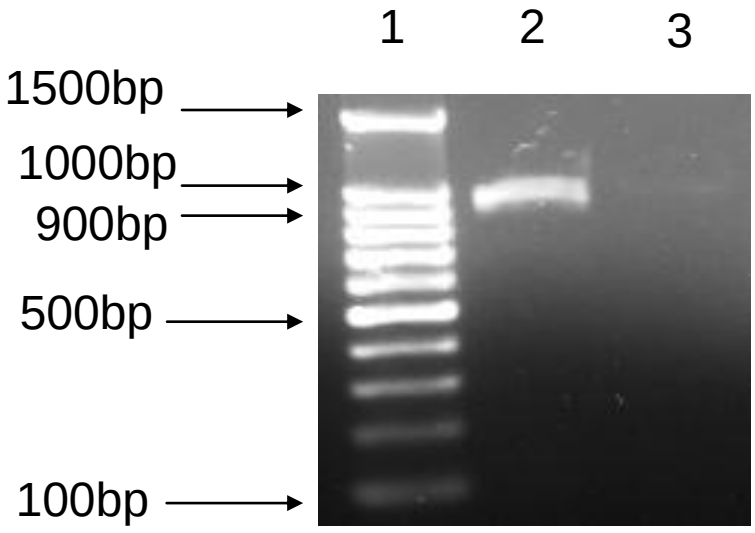


Fig. 5.3 Control Hela RNA was processed simultaneously with sample RNA using the Generacer kit. On lane 1 from left the same ladder was used as in figure 5.2. In lane 2 amplification of *β-actin*, modified by the addition to its 5' end of Generacer oligo, was performed using manufacturer's primers and 30 PCR cycles. The expected product was 872 base pairs (828 of β-actin + 44 bases of attached RNA oligo). In lane 3 the same PCR reaction was performed on a no template control sample. For PCR amplification 5 PCR cycles were performed at 72^{o}C, 5 PCR cycles at 70^{o}C, and 20 PCR cycles at 68^{o}C.

The positive control reaction demonstrated that the processing of the RNA (dephosphorylation, decapping, attachment of RNA oligo, and reverse transcription) worked as expected. For RLM-RACE PCR amplification is performed on modified RNA using one fixed primer that anneals to the attached oligo, and one custom primer that anneals at some known region further downstream. PCR amplification was performed using the reverse primer used in **Figure 5.2**. Unfortunately the identification of the TSS of this miRNA cluster using the Generacer kit was not successful. Despite repeated attempts at optimising the PCR process (e.g. different set of primers, range of temperatures and PCR cycles, amount of starting cDNA, normal and touchdown PCR) a RACE PCR product could not be generated for the pri-miRNA containing miR-182 and miR-96. The reason for difficulty in the amplification of the pri-miRNA was not clear. It is possible that a rapid turnover of

the pri-miRNA to a pre-miRNA renders its detection by RACE more difficult than for other genes.

Interestingly, a publicly available cloned cDNA (AK044240) (Carninci & Hayashizaki, 1999) ((Jan 2010) has been isolated from the CpG island containing genomic region from the mouse retina, an organ known to have high expression of the miR-182/96 cluster (Xu *et al.*, 2007). This clone corresponds to the region that is 3342-5562 bases upstream of the pre-miR-96, within the CpG island region, but corresponds to the opposite strand to that on which the pre-miR-96 is located. It has been reported that for some miRNA loci both strands can be transcribed (Tyler *et al.*, 2008). Amplification by RT-PCR using two distinct primer sets could amplify cDNA corresponding to this region (**Figure 5.4**).

Figure 5.4 *RT-PCR amplification of complementary strand to miR-96*

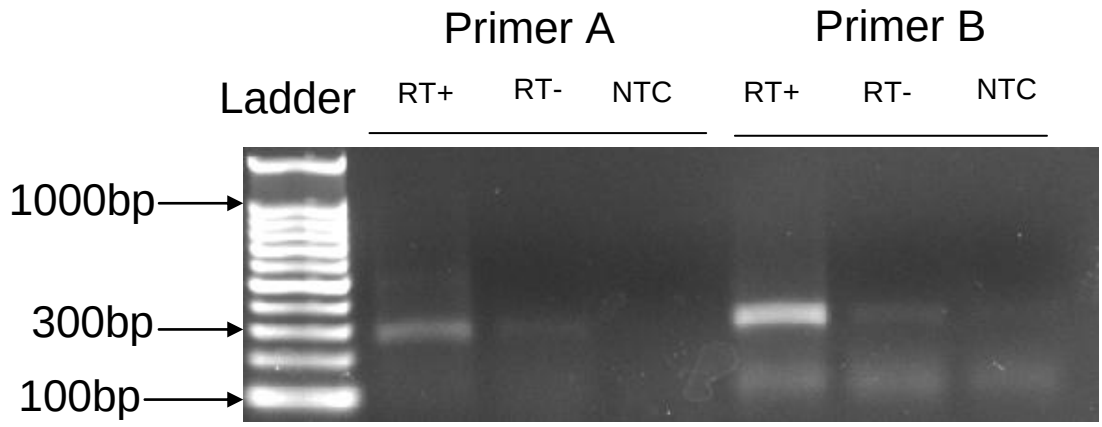


Fig. 5.4 Amplification of sequences within the cloned cDNA (AK044240) corresponding to the opposite strand to miR-96**.** On the first lane from the left the DNA ladder can be seen with molecular weights of 1500, 1000, and incremental decreases of 100 bases for each band from then on. Primer A (4478-4727 upstream of miR-96) and primer B (5117-5336 upstream of miR-96) were custom designed. The expected size of the PCR product was 249 bases for primer A and 260 bases for primer B. For each primer three PCRs were performed: Normal PCR (RT+), no reverse transcriptase control (RT-), and a no template control (NTC). For each PCR reaction 35 cycles at 55^{o}C were performed.

5.3 Consequences of miRNA deregulation

5.3.1 Correlation between miR-29b and global DNA Methylation

The microarray data for the PB treatment over 90 days indicated a transient repression in the expression of miR-29b, but not of miR-29a or miR-29c, specifically at 14 days (**Figure 5.5**).

Figure 5.5 *Expression of hepatic miR-29b in control and 1000 ppm PB-treated animals after 7, 14, and 90 days*

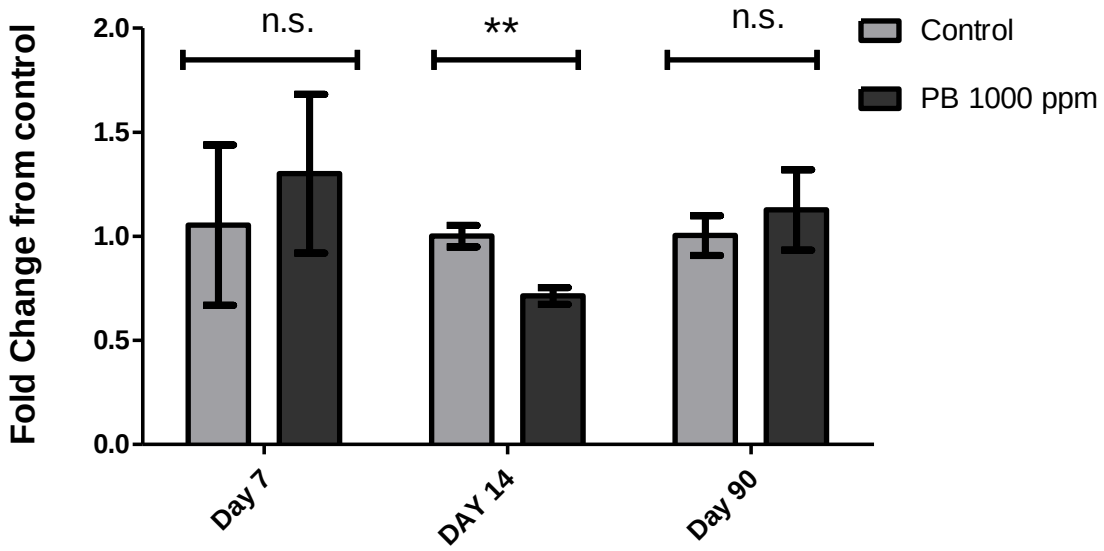


Fig. 5.5 The expression of miR-29b was quantified with microarrays and for each treated group the mean fold change from the control samples was calculated. SAM analysis was performed at each time between control and 1000 ppm PB treated animals ** q<0.01 and error bars indicate S.D. n=3 except for 90 day control samples were n=5.

This miRNA was not affected at 90 days by any other chemical treatment studied here. It has been reported that this miRNA can regulate global DNA methylation (Garzon *et al.*, 2009, Takada *et al.*, 2009, Blum *et al.*, 2010). The Methylamp Global DNA Methylation Quantification Ultra Kit (Epigentek) was used to evaluate whether the observed repression of miR-29b by PB affected the methylation status of the liver. This kit quantifies the amount of methylated DNA based on the binding of an antibody to methylated cytosine. A standard curve showed that this kit could be used to quantify the amount of methylated DNA reliably (**Figure 5.6**).

Figure 5.6 ***Standard curve for Methylamp kit***.

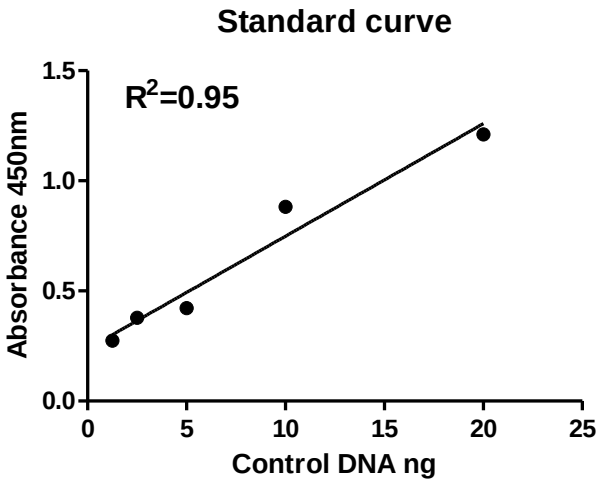


Fig. 5.6 Control DNA supplied by manufacturer (synthesized polynucleotide methylated at every 5-cytosine) was diluted to known concentrations and was measured using the methylamp kit. Line was automatically drawn using least squares.

The methylation of genomic DNA from samples treated with the 1000 ppm PB dose after 7, 14, and 90 days was then examined. This analysis found that global DNA methylation was the inverse to the expression of miR-29b, being transiently induced only at 14 days of PB treatment (**Figure 5.7**).

Figure 5.7 *Global DNA methylation in 1000 ppm PB-treated samples at days 7, 14, and 90.*

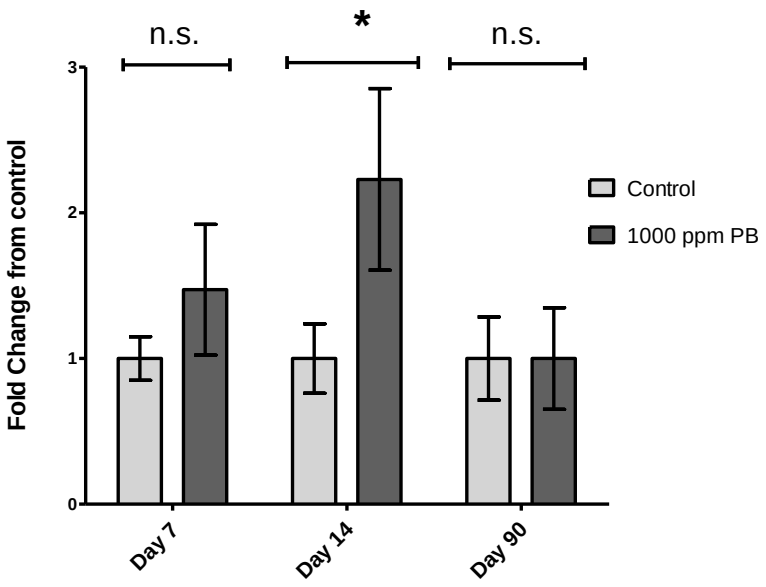
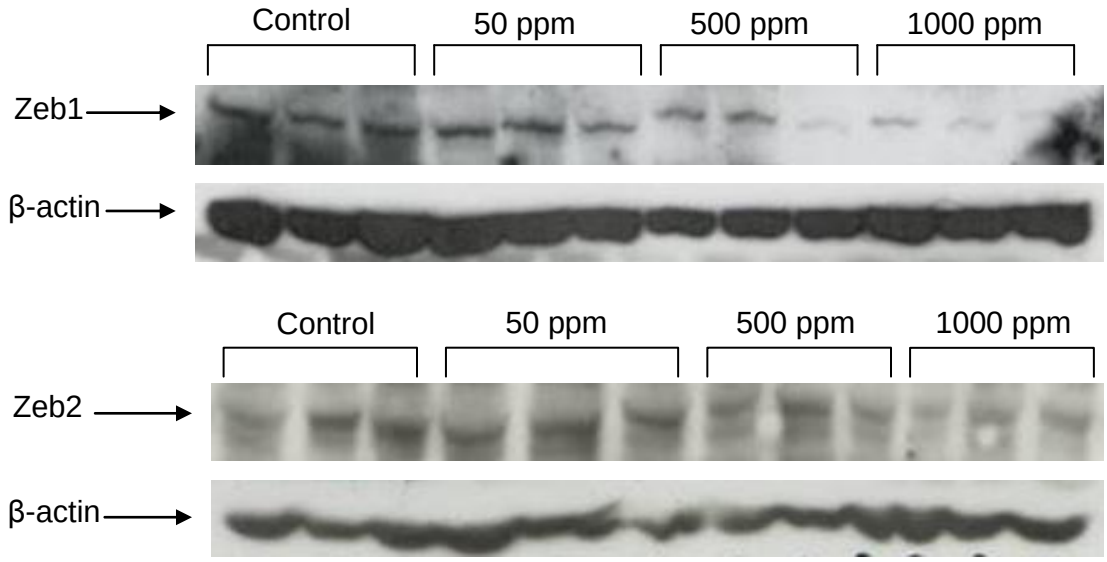


Fig. 5.7 Methylation was measured using the Methylamp kit. The values for all treatments was normalised to their respective day control. Three animal liver DNA samples were examined per group, error bars represent S.D. T-test was performed between samples treated with 1000 ppm PB and their respective day control samples, * signifies $p<0.05$.

5.3.2 Effect of miR-200a/200b/429 dose-dependent induction on the expression of zeb1/zeb2 and epithelial markers

Immunoblotting was used to quantify the expression of the zeb1 and zeb2 proteins in the control and PB-treated samples at 14 days of treatment (**Figures 5.8**).

Figure 5.8 *Immunoblotting of zeb1, zeb2, and b-actin in control and phenobarbital-treated samples at 14 days.*



N=3 animals per group.

Quantification of the immunoblots then confirmed the specific downregulation of zeb1 and zeb2 by the carcinogenic dose of PB at 14 days (**Figure 5.9**).

Figure 5.9 *Quantification of zeb1 and zeb2 proteins in livers treated with phenobarbital doses for 14 days.*

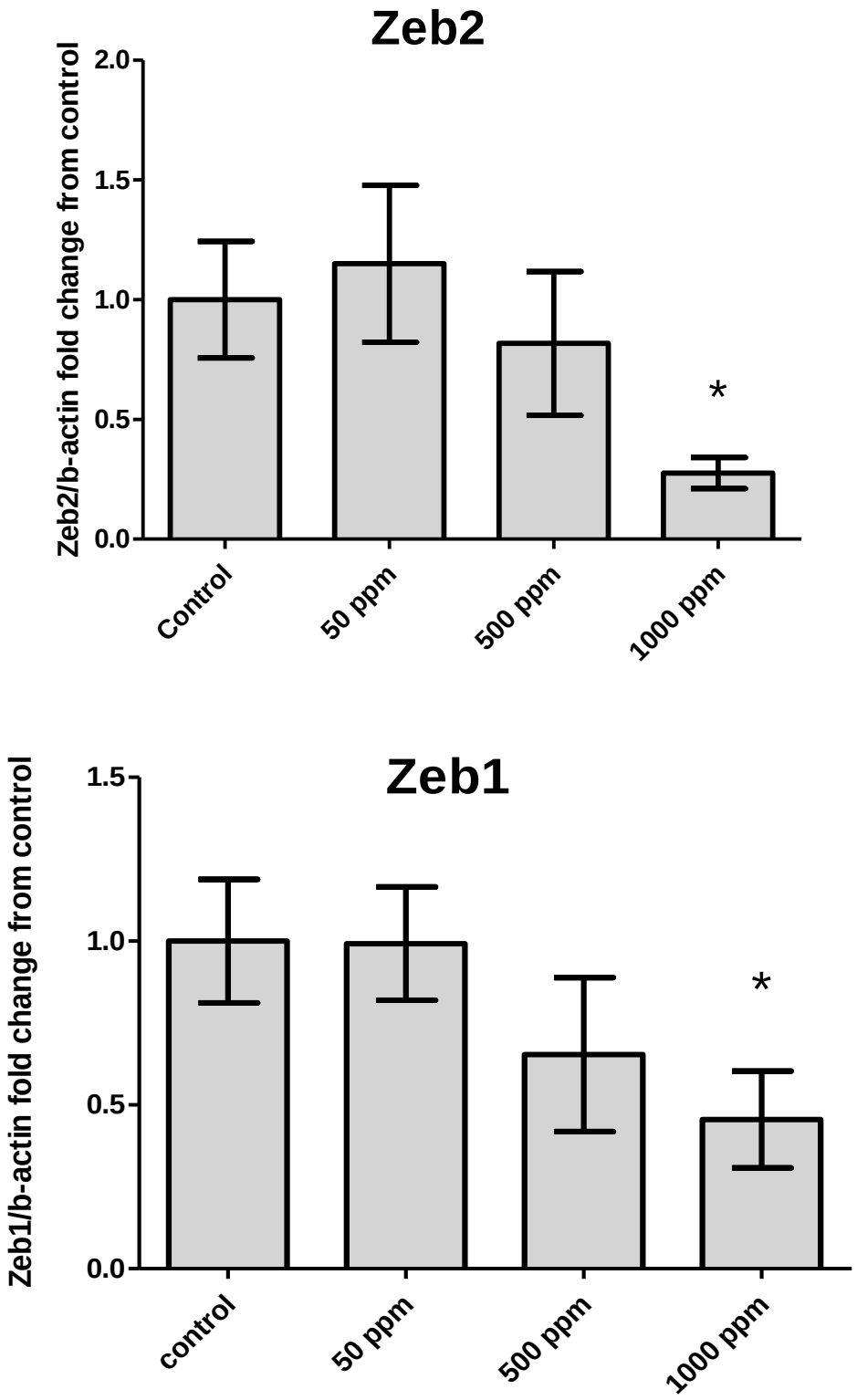
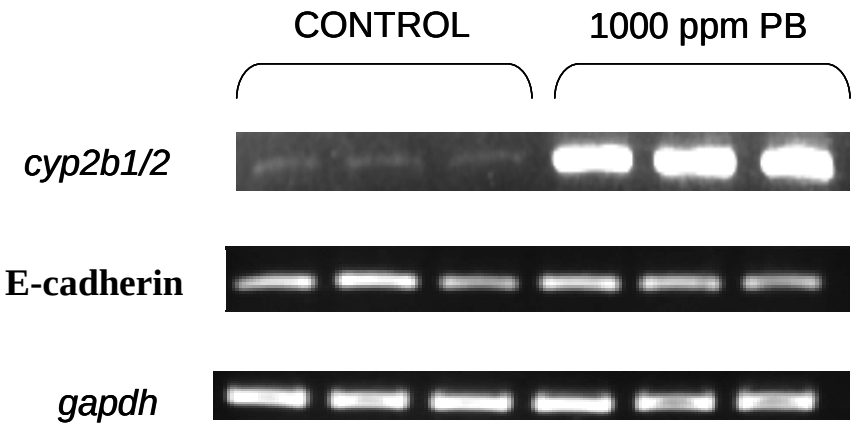


Fig. 5.9 Shown is the mean fold change from control. Protein expression was normalised to b-actin. N=3 per group, * $p<0.05$ one-way ANOVA with Dunnett's post-test. Error bars are S.D.

Zeb1 and zeb2, targets of the miR-200 family of miRNAs, are major regulators of epithelial to mesenchymal transition (EMT), a complex process whereby epithelial cells are reorganised into more motile mesenchymal ones (Berx *et al.*, 2007) . The proteins repress the expression E-cadherin gene (*CDH1*), a cell adhesion protein that is found on the plasma membrane of normal epithelial cells, and also induce the upregulation of the mesenchymal markers vimentin and N-cadherin (Vandewalle *et al.*, 2009). However, examination of the transcriptomic data (personal communication R.A. Currie) did not reveal an association between induction of the miR-200a/200b/429 cluster and deregulation of these three EMT marker genes (*cdh1, vim, cdh2*). At 90 days the only one of these genes showing a significant deregulation more than 1.5-fold was N-cadherin, and that was only observed in one treatment (-1.6 fold in DEHP treated animals).

To confirm the microarray data the expression of *E-cadherin* was examined in control and 1000 ppm PB treated samples at 90 days by semi-quantitative PCR. This timepoint was chosen as the maximum induction of the miR-200a/200b/429 cluster was observed at that stage. As a positive control the expression of c*yp2b1/2* was examined in the same samples by end-point PCR (**Figure 5.10**).

Figure 5.10 *PCR amplifications of cyp2b1/2, cdh1, and gapdh in control and phenobarbital-treated samples at 90 days*



N=3, 30 PCR cycles were performed

Quantification of the PCR gels showed a pronounced induction of *cyp2b1/2* (as expected), but no change in the levels of *cdh1* (**Figure 5.11**).

Figure 5.11 *Quantification of E-cadherin and cyp2b1/2 genes in livers treated with 1000 ppm phenobarbital at 90 days*

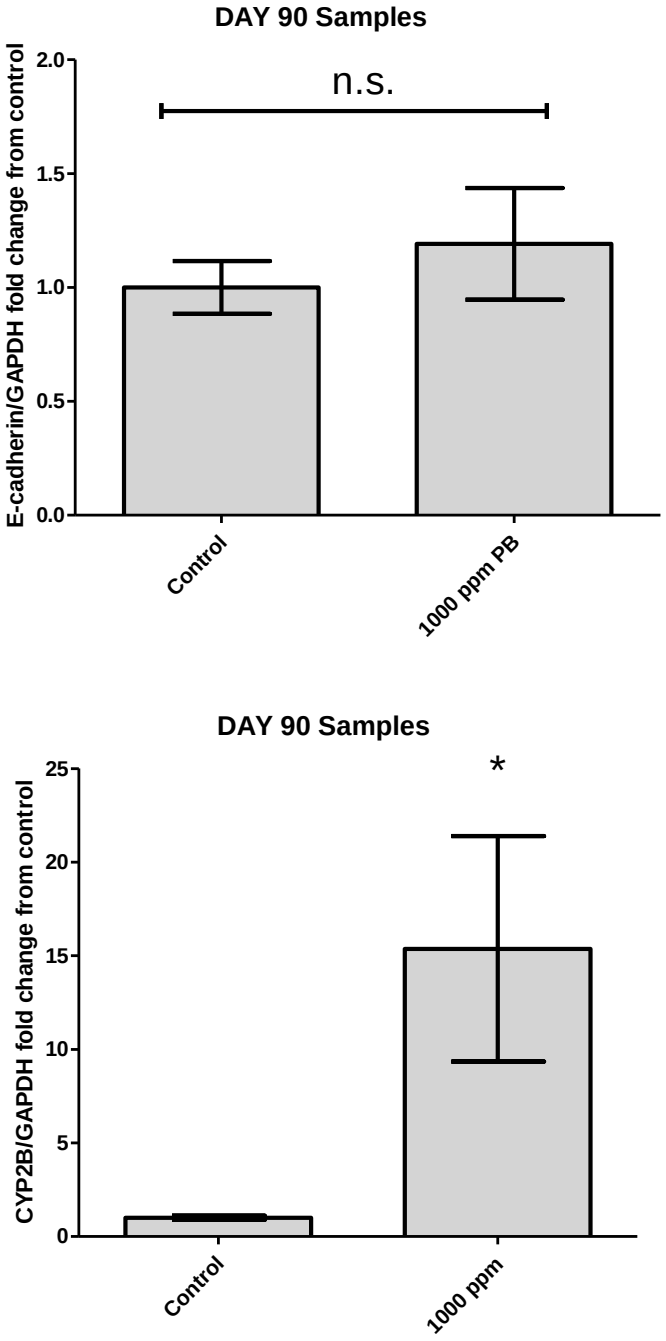


Fig. 5.11 Expression of genes was normalised to that of *gapdh*. N=3 per group, error bars indicate S.D. * $p<0.05$ T-test.

Hence examination by end-point PCR agreed with the microarray data in that the expression of the *E-cadherin* gene was not changed at 90 days, despite the downregulation of zeb1 and zeb2.

5.3.3 Pathways enriched for predicted gene targets of biomarker miRNAs

As an alternative approach to focusing on candidate protein targets of miRNAs, their regulatory roles can be investigated through a consideration of the multiple proteins that they can regulate. The first step in such an analysis is the use of computational methods for prediction of potential gene targets. Most of these methods rely heavily on the sequence of the mature miRNAs, which can be seen in **Table 5.5**.

Table 5.5 Mature sequence of biomarker microRNAs

microRNA	sequence (5' to 3')
miR-200a	uaacacugucugguaacgaugu
miR-200b	uaauacugccugguaaugaugac
miR-429	uaauacugucugguaaugccgu
miR-96	uuuggcacuagcacauuuuugcu
miR-182	uuuggcaaugguagaacucacaccg
miR-34a	uggcagugucuuagcugguugu
miR-203	gugaaauguuuaggaccacuag
miR-99a	aacccguagauccgaucuugug
miR-193	aacuggccuacaaagucccagu

Mature sequences were obtained from miRBase (http://www.mirbase.org/). Highlighted in red is the seed region (nucleotides 2-8) for each miRNA. MiRNAs belonging to the same family are coloured the same colour and grouped in brackets.

As can be seen miRNAs belonging to the same families have very similar mature sequences, supporting their overlapping regulatory roles. MiR-200b and miR-429 in particular have exactly the same seed sequence (aauacug), while they differ from

miR-200a by a single base. Similarly, the seed regions of miR-96 and miR-182 differ by only one base.

TargetScan is a widely used *in silico* tool for the prediction of miRNA targets which takes into account a number of parameters (the strength of the seed pairing, the number of complementary sites between the miRNA and the mRNA, the location of complementary sites, etc.) (Bartel, 2009). Although imperfect, this computational method can identify most regulatory targets of miRNAs. It has been experimentally shown to be more successful than other miRNA target-prediction tools (Baek *et al.*, 2008).

Here the latest release of TargetScan (5.1) was used to predict the genes that are regulated by the biomarker miRNAs. For miR-200b and miR-429 the same genes are predicted gene targets due to the fact that the miRNAs have the same seed sequence. The generated lists of predicted gene targets were then analysed within the PANTHER database (Thomas *et al.*, 2006). This classification system groups genes according to their known functions, families, pathway etc. Binomial statistics were used to compare the classification of the predicted miRNA-target genes with that of genes within the reference rat genome. Pathways for which there was an over-representation of predicted miRNA gene targets could then be highlighted. The full list of the allocation of predicted miRNA targets to pathways can be viewed in the **Appendix III** section. In **Table 5.6** the numbers of genes and the top five over-represented pathways within the TargetScan generated lists for the biomarker miRNAs are shown.

Table 5.6 Top pathways enriched with predicted targets of biomarker microRNAs

miRNA	# of predicted targets	Top five over-represented pathways ranked by p value
miR-96	672	PI3K (18, p<0.0001); EGF receptor (16, p<0.0001), Integrin (18, p<0.0001); insulin/IGF (12, p<0.0001), FGF (14, p<0.0001)
miR-182	659	PI3K (15, p<0.0001); EGF (16, p<0.0001), FGF (13, p<0.0001); TGF-beta (13, p=0.0001), metabotropic glutamate receptor III (9,p=0.0001)
miR-200b/429	637	PDGF (20, p<0.0001); Ras (14, p<0.0001); PI3K (16, p<0.0001); EGF (16, p<0.0001); FGF (15, p<0.0001)
miR-200a	438	Insulin/IGF (11, p<0.0001); PI3K (12, p<0.0001); TGF-beta (12, p<0.0001); EGF (11, p<0.0001); interleukin (11, p=0.0001)
miR-203	415	Integrin (12, p<0.0001); angiogenesis (11, p<0.0001); p53 pathway feedback loops 2 (6, p<0.0001); insulin/IGF (7, p<0.0001); PI3K (6, p<0.0001)
miR-34a	376	TGF-beta (12, p<0.0001); PI3K (9, p<0.0001); metabotropic glutamate receptor III (7, p<0.0001); metabotropic glutamate receptor II (6, p=0.0002); insulin/IGF (7, p=0.0003)
miR-193	99	EGF (6, p<0.0001); FGF (5, p=0.0001); Ras (4, p=0.0003); PDGF (5, p=0.0003); Inflammation mediated by cytokine and chemokine signalling (4, p=0.004)
miR-99a	36	WNT(4, p=0.0004), Angiogenesis (3, p=0.002), presenilin (2, 0.01), Cadherin signalling (2, p=0.02); p53 pathway by glucose deprivation (1, p=0.03)

MiRNA targets were predicted using TargetScan v5.1 and allocated to pathways using the PANTHER database. Top five pathways per miRNA are shown as ranked by p. value. Within brackets are shown the number of predicted miRNA-target genes that belong to that pathway and the calculated p. value.

As can be seen there is a considerable variability in the number of proteins that are predicted to be regulated by miRNAs (from several hundreds to only 36 for miR-99a, see **Appendix III**). Interestingly, the analysis above clearly shows that the predicted miRNA target genes are enriched for genes belonging to the same signalling pathways. These pathways are thought to be important in the development of HCC e.g. PI3K, TGF-beta, WNT, Ras (Llovet & Bruix, 2008).

5.3.4 Synergistic activities of PB-induced miRNAs

Next the focus was placed on examining the predicted effects of PB deregulated miRNAs on cellular pathways. Often individual miRNAs have a relatively modest effect on the levels of protein expression (Selbach *et al.*, 2008, Baek *et al.*, 2008). However, it is reasonable to assume that even small effects of miRNAs on protein expression can be additive when miRNAs target several proteins of the same signalling pathway. Moreover, a protein will be much more effectively silenced when it is targeted by several miRNAs at the same time. Since the main effect of PB on the liver miRNAome is the coordinated progressive induction of the two miRNA clusters (miR-96/182 and miR-200a/200b/429), the possibility of overlap in their regulatory roles was examined. Indeed, analysis showed that miRNA members of the two clusters are predicted to target several targets within the PI3K, TGF-beta, Ras, and EGF pathways (**Tables 5.7-5.10**).

Table 5.7 Predicted gene targets of phenobarbital-induced microRNAs in the PI3K pathway

	miR-200a	miR-200b/429	miR-96	miR-182
AKT2	-	X	-	-
CCND2	-	-	X	X
FOXA1	X	-	-	-
FOXA2	X	-	-	-
FOXF2	-	-	X	X
FOXG1	-	X	-	-
FOXJ3	X	-	-	-
FOXK1	-	X	-	-
FOXK2	-	X	X	X
FOXN2	X	X	-	-
FOXN3	X	X	X	X
FOXO1	-	-	X **(Verified)**	X **(Verified)**
FOXO3		X	X **(Verified)**	X **(Verified)**
FOXO4	-	-	X	-
FOXP1	X	X	-	-
FOXP2	X	X	X	X
FOXQ1	-	-	X	X
GNAI3	-	X	X	X
GNAQ	-	-	X	X
GRB2	X	-	X **(Verified)**	X **(Verified)**
INPP5A	-	-	X	X
IRS1	-	-	X **(Verified)**	-
IRS2	X	-	-	-
KRAS	-	X	X **(Verified)**	-
PDPK1	-	X	-	-
PIK3R1	-	-	X	X
PTEN	X	X	-	-
RPS6KB1	-	X	-	-
SOS1	-	-	X	X
YWHAB	-	X	-	-
YWHAG	X	X	X	X
YWHAZ	X	-	-	-

Genes are marked with "X" if they were predicted to be regulated by that miRNA by TargetScan. "Verified" signifies that the miRNA has been reported to regulate that gene by luciferase assays.

Table 5.8 Predicted gene target of phenobarbital-induced microRNAs in the TGF-beta pathway

	miR-200a	miR-200b/429	miR-96	miR-182
ACVR1	-	-	X	X
ACVR2A	X	X	-	-
ACVR2B	-	X	-	-
BMP1	X	-	-	-
CITED2	X	-	-	X
CREBBP	X	-	-	-
FOXA1	X	-	-	-
FOXA2	X	-	-	-
FOXF2	-	-	X	X
FOXG1	-	X	-	-
FOXJ3	X	-	-	-
FOXK1	-	X	-	-
FOXK2	-	X	X	X
FOXN2	X	X	-	-
FOXN3	X	X	X	X
FOXO1	-	-	X **(Verified)**	X **(Verified)**
FOXO3		X	X **(Verified)**	X **(Verified)**
FOXO4	-	-	X	-
FOXP1	X	X	-	-
FOXP2	X	X	X	X
FOXQ1	-	-	X	X
GDF6	X	-	-	X
JUN	-	X	-	-
KRAS		X	X **(Verified)**	
MAPK1	-	-	-	X
SMAD1	-	-	-	X
SMAD7	-	-	X	X
SMURF2		X		
TGFB2	X **(Verified)**	-	-	-
TGFBR1	X	-	-	-
TLL1	-	-	X	-

Genes are marked with "X" if they were predicted to be regulated by that miRNA by TargetScan. "Verified" signifies that the miRNA has been reported to regulate that gene by luciferase assays.

Table 5.9 Predicted gene target of phenobarbital-induced microRNAs in the Ras pathway

	miR-200a	miR-200b/429	miR-96	miR-182
AKT2	-	X	-	-
CDC42	X	-	-	-
ETS1	-	X **(Verified)**	-	-
GRB2	X	-	X **(Verified)**	X **(Verified)**
JUN	-	X	-	-
KRAS	-	X	X **(Verified)**	-
MAPK1	-	-	-	X
MAP2K1	-	-	X	X
MAP2K3	-	-	X	-
MAP2K4	X	-	-	-
PAK1	-	-	X	-
PAK6	-	X	-	-
PAK7	-	X	-	-
PDPK1	-	X	-	-
PLD1	-	-	-	X
RAC1	-	X	X	X
RALGDS	-	-	-	X
RGL1	-	X	-	-
RHOA	-	X	-	-
RPS6KA2	-	X	-	-
RPS6KA3	-	X	-	-
SHC1	-	X	X	-
SOS1	-	-	X	X
SRF	-	X	-	-
TIAM1	X	-	-	-

Genes are marked with “X” if they were predicted to be regulated by that miRNA by TargetScan. “Verified” signifies that the miRNA has been reported to regulate that gene by luciferase assays.

Table 5.10 Predicted gene target of phenobarbital-induced microRNAs in the EGF pathway

	miR-200a	miR-200b/429	miR-96	miR-182
AKT2	-	X	-	-
CBL	X	X	-	-
CDC42	X	-	-	-
GAB2	-	-	X	X
GAB4	-	-	-	X
GRB2	X		X **(Verified)**	X **(Verified)**
HBEGF	-	-	X	-
KRAS	-	X	X **(Verified)**	-
MAPK1	-	-	-	X
MAP2K1	-	-	X	X
MAP2K3	-	-	X	-
MAP2K4	X	-	-	-
MAP3K3	X	-	X	X
MAP3K5	-	X	-	-
MRAS	-	-	X	X
NF1	-	-	X	X
PI3K2CA	-	-	X	-
PLCG1	-	X **(Verified)**	X	X
PPP2CA	X	X	-	-
PPP2R5C	-	X	-	-
PPP2R5E	-	X	-	-
PRKCA	-	X	-	-
PRKCE	X	-	X	X
PRKD1	-	X	-	X
RAC1	-	X	X	X
RAP1B	-	X	-	-
RAP2C	-	X	-	-
RASA1	-	-	-	X **(Verified)**
SHC1	-	X	X	-
SOS1	-	-	X	X
SPRY4	X	-	-	X
STAT5B	X	-	-	-
YWHAB	-	X	-	-
YWHAG	X	X	X	X
YWHAZ	X	-	-	-

Genes are marked with "X" if they were predicted to be regulated by that miRNA by TargetScan. "Verified" signifies that the miRNA has reported to regulate that gene by luciferase assays.

This observation suggests that the induction of these miRNAs can have significant effects on the functioning of these signalling pathways which are important for the regulation of cell proliferation and apoptosis.

5.4 Discussion

Given the important roles of miRNAs in physiology and disease it is expected that they will be under strict transcriptional regulation. Altered methylation patterns are one possible mechanism that could drive the observed hepatic miRNA deregulations observed after chemical treatments. It has been estimated that CpG islands are found in the promoter regions of 40% of mammalian genes (Larsen *et al.*, 1992). CpG islands were also identified upstream of all the potential biomarker miRNAs, with the exceptions of miR-99a and miR-200a/200b/429. The presence of CpG islands in their promoters is an indication that methylation could be an important factor regulating their expression. For the miR-200a/200b/429 cluster a CpG island was only identified in the human promoter, but not for its rodent counterparts. It should be noted that methylation of this CpG island has been shown to be important for the regulation of this cluster in humans (Li *et al.*, 2010). Future research should examine whether the absence of a CpG island in rodents compared to humans is an important aspect of the regulation of these miRNAs.

Chemical carcinogens could also deregulate miRNAs by affecting the activities of transcription factors that regulate them. Amongst the biomarker miRNAs, a conserved consensus p53 binding motif was found only in the promoter of miR-34a. It seems probable that its pronounced activation observed in 2-AAF treated samples is due to the DNA damage caused by the chemical stimulating miR-34a *via* p53 activation. Toxicogenomic studies have previously shown that GNTX and NGTX carcinogens have different gene expression profiles (Ellinger-Ziegelbauer *et al.*, 2005). The distinctive characteristic of GNTX carcinogens was that they affected cellular pathways involved in DNA damage response, proliferation, and survival. The

profound 2-AAF-induced upregulation of miR-34a can then be interpreted as part of the DNA damage response.

Since the miR-200a/200b/429 cluster was induced at 90 days by several chemical treatments (PB, BP, DEHP, 2-AAF, and Chl. Ac), it was reasonable to assume that the factor mediating the induction of this miRNA cluster would be a common target of these chemicals. The biggest induction of this cluster was clearly observed after PB treatment. Moreover, a more modest induction of the cluster was also observed in the livers of BP-treated animals. Given that BP appears to be a weaker inducer of PB-type responses in the liver (e.g. smaller induction of *cyp2b1* gene) it is probable that the miRNA cluster is induced as a consequence of the deregulation of a gene pathway that is strongly affected by PB. Activation of CAR is a main mechanism by which PB propagates its effects (Ueda *et al.*, 2002). However a number of observations indicated that CAR is not the mediator of the activation of these miRNAs: (1) the other chemical treatments which affected the miR-200a/200b/429 cluster in this study (i.e. 2-AAF, MP HCL, Chl.Ac) are not thought to activate CAR (2) PB treatment activates CAR activity immediately, while miR-200a/200b/429 only become induced after 1-2 weeks.

Hence, the two other transcription factors that are affected by PB (c-myc and HNF4α) were considered as better candidates for factors regulating the expression of the miR-200a/200b/429 cluster. C-myc is commonly activated in the liver by treatments with NGTX carcinogens (Nie *et al.*, 2006, Lee & Edwards, 2003). C-myc has also been reported to regulate the miR-200a/miR-200b/429 cluster in murine embryonic stem cells (Lin *et al.*, 2009). A potential link between HNF4α and the expression of the

miR-200 family is suggested by the fact that HNF4α is a regulator of EMT in the liver (Ning *et al.*, 2010, Yue *et al.*, 2010, Parviz *et al.*, 2003). Interestingly, binding motifs for both c-myc and HNF4α were identified within the proximal promoter region of the miR-200a/200b/429 cluster. It is important to note that identification of putative transcription factor binding sites is not proof that the miRNAs are regulated by the transcription factors. Experimental validation is required to verify the proposed sites of regulation (e.g. chromosomal immunoprecipitation and electrophoretic mobility shift assay). However, the identification of the putative binding sites is a starting point indicating that these transcription factors could regulate these miRNAs.

For many miRNAs it has been shown that they can have pronounced effects through their regulation of a single or a few protein targets. One of them is miR-29b which by repressing DNA methyltransferases can affect global DNA methylation. Here an inverse relationship was found between the expression of miR-29b in PB-treated liver and the global hepatic DNA methylation. What is the functional significance of the altered methylation observed in PB-treated livers? One possibility is that the increased activity of DNMT enzymes results in the aberrant methylation of tumour suppressor genes, which persists after global DNA levels return back to normal. A second possibility however, is that the transient induction of global DNA methylation may be an adaptive mechanism protecting the organ from excessive demethylation. A recent study found an association between the downregulation of members of miR-29c, a miRNA that also regulates DNMT enzymes, and resistance to disruption of global hepatic methylation (Pogribny *et al.*, 2010). Older studies had shown that global hypomethylation is more pronounced in PB-treated livers of mice strains susceptible to PB-treated carcinogenesis (Counts *et al.*, 1996). The repression of miR-29b in PB-

treated rats, and the resultant increase in the activity of DNMT enzymes, may protect the animal from excessive demethylation and be part of the reason that rats are relatively resistant (compared to some mice strains) to PB-induced tumours.

In a similar vein it has also been shown that members of the miR-200 family of miRNAs are master-regulators of EMT in various cell types *via* regulation of the Zeb1/Zeb2 proteins (Korpal & Kang, 2008, Gregory *et al.*, 2008b). EMT is a very important event during embryonic development, as well as for wound healing. Evidence accumulated in recent years supports that EMT could be an important step in the acquisition of more invasive and malignant properties by neoplastic hepatocytes (van Zijl *et al.*, 2009, Choi & Diehl, 2009). A reciprocal association between the expression of miRNA members of the miR-200 family and the expression of the zeb1/zeb2 proteins in the rat liver has been reported previously (Pogribny *et al.*, 2009a, Pogribny *et al.*, 2010, Tryndyak *et al.*, 2009). Moreover, overexpression of miR-200b in primary mouse hepatocytes induces a higher expression of the E-cadherin protein (Pogribny *et al.*, 2010). Therefore, the induction of the hepatic miR-200a/200b/429 cluster by PB treatment would be expected to elevate the expression of epithelial genes in the liver. It is interesting to note the Phillips *et al.*, 2009 study, which treated CAR WT and CAR KO mice with a carcinogenic PB dose for 23 weeks (precancerous tissue) and 32 weeks (100% liver tumours). They found that at 23 weeks of PB treatment hepatic *cdh1* was induced only in the CAR WT mice. This was reversed at 32 weeks in liver tumours. This finding is in agreement with an early induction of the miR-200 family of miRNAs.

Here it was found that, as expected, the induction of miR-200a/200b/429 was inversely correlated to the expression of the zeb1/zeb2 proteins. However, despite the

observed repression of the Zeb1/Zeb2 proteins, no evidence was found that the observed induction of miR-200a/200b/429 cluster affected the expression of hepatic epithelial or mesenchymal markers. It is possible that downregulation of zeb1/zeb2 is not sufficient by itself to cause a stable induction of hepatic *cdh1* because other regulatory factors are important e.g. methylation of E-cadherin promoter and activity of other transcription factors such as snail, twist, etc. Alternatively, the induction of miR-200a/200b/429 may act to protect the epithelial status of hepatic cells from other signalling pathways that promote EMT.

Computational tools were then used to identify pathways that were enriched amongst proteins that are predicted to be regulated by the biomarker miRNAs. Of course any prediction tool that is used is imperfect, resulting in the misprediction of some miRNA-targets and overlooking some real targets of miRNAs. Nevertheless, since target-prediction tools (such as TargetScan) can pick up multiple true targets of miRNAs (Baek *et al.*, 2008), they can be used to elicit the regulatory roles of miRNAs. Examination of the targets predicted for the biomarker miRNAs that are altered by the carcinogens in the present study revealed that they target pathways that are important in HCC e.g. PI3K, EGF, etc.

Intriguingly, examination of the predicted targets of the PB-induced miRNAs indicated that they could be involved in a long known observation about PB: its biphasic effect on the liver. Many liver tumour promoters (such as PB or TCDD) stimulate a transient hepatic cell proliferation, followed by a sustained induction of a mitoinhibitory state (Andersen *et al.*, 1995). It was first shown that inclusion of PB in the diet resulted in the suppression of hepatic proliferation in mice fed a choline-

deficient diet (Abanobi *et al.*, 1982). Shortly after a second study found that long term PB treatment (four weeks and 12 months) inhibited the mitogenic response in rat liver after 2/3 partial hepatectomy. This effect was progressive, with a stronger inhibitory effect at 12 months than at four weeks, and no effect of short term PB treatment (Barbason *et al.*, 1983). It has also been shown that hepatocytes isolated from livers after chronic PB treatment are less responsive to mitogenic factors (Fletcher *et al.*, 1999, Fletcher *et al.*, 1997, Tsai *et al.*, 1991). In addition, chronic PB administration (1000 ppm PB) for eight weeks resulted in a decreased activation of PKC by treatment with Phorbol ester (Brockenbrough *et al.*, 1991).

EGF is a prosurvival and mitogenic pathway that frequently displays aberrant activation in HCC (Llovet & Bruix, 2008, Liu *et al.*, 2006). In accordance with the mitoinhibitory effects of long-term PB treatment, members of the two miRNA clusters induced by the chemical target several key proteins which are involved in the transmission of EGF signalling (**Figure 5.12**).

Figure 5.12*: Predicted targets of the miR-200a/200b/429 and miR-96/182 cluster found in EGF signalling pathway*

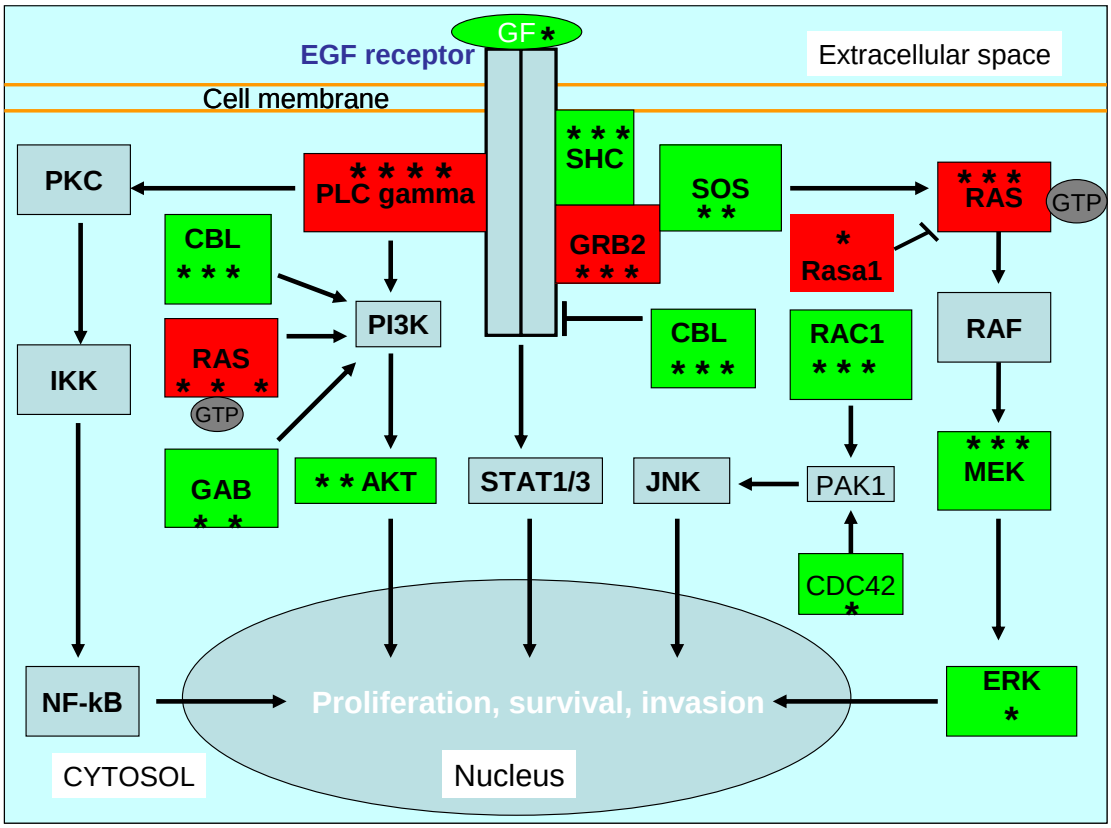


Fig. 5.12 Red boxes are verified miRNA targets from literature, green boxes are predicted targets by TargetScan. * Within red or green boxes signify how many of the screened miRNAs (miR-200a/200b/429/182/96) are predicted to regulate that gene. EGF signalling pathway is initiated by the binding of a growth factor (EGF, TGF-α, HB-EGF) onto the EGF receptor, resulting in activation of the tyrosine kinase activity of the receptor. Intracellular signalling eventually affects cellular proliferation, survival, invasion etc.

Some of these predicted targets have been experimentally confirmed in previously reported studies e.g. that GRB2 is regulated by miR-96 and miR-182 (Xu & Wong, 2008); KRAS by miR-96 (Yu *et al.*, 2010); PLCG1 by the miR-200b and miR-429 miRNAs (Uhlmann *et al.*, 2010). Importantly, several of the predicted targets are important upstream and/or linkage parts of the pathway. GRB2, SOS1, and SHC link growth factors to the activation of the RAS/RAF/MEK/ERK pathway (Dharmawardana *et al.*, 2006). It has also been known for some time that PB

promotion acts to negatively select Ha-ras mutations (Aydinlik *et al.*, 2001). PLC gamma is important for the activation of PI3K and PKC by the EGF pathway (Wells, 1999).

The induction of the two miRNA clusters by PB also agrees temporally with the initiation of the shift in PB-treated liver from a proliferating to a mitoinhibitory state. Ki67 data suggest that the PB-stimulated entrance of quiescent hepatic cells into the cell cycle occur in day 3 and is completed by day 7 (Table 4.4). The two clusters are induced after one week of PB treatment, when the transient hyperplastic effect of PB ceases. **Therefore**, **examination of the predicted targets of the PB-induced miRNAs suggests that they are involved in the shift of the liver to a mitoinhibitory state**.

In conclusion, the work performed here suggests that altered methylation or deregulation of transcription factors could underlie the observed changes in the expression of hepatic miRNAs. An inverse association was shown here between the expression of miR-200a/200b/429 and zeb1/zeb2, important protein regulators of EMT, as well as between the expression of miR-29b and global DNA methylation. Moreover, computational analysis suggests that the miRNAs that were found here to be targeted by chemical hepatocarcinogens regulate the activity of key cellular pathways.

Chapter 6:
Discussion

6.1 MiRNA signatures are a promising approach to facilitate chemical risk assessment

As was described in the Introduction (**Section 1.4**), there is a growing need for the development of alternative strategies for the assessment of chemical compounds. The principal hypothesis of this project was that hepatic miRNA profiles can contribute to the development of such alternative strategies. This project generated several important findings concerning the hypothesis of this project. These results were extensively discussed previously (see **Discussions Chapters 3-5**). Briefly, among the key findings were:

(a) That all the tested carcinogenic treatments -which included chemicals with diverse MOA and carcinogenic potencies- resulted in the deregulation of hepatic miRNAs after 90 days in the pre-neoplastic liver tissue.

(b) That there is possibly a link between hepatic miRNA profiles and the MOA of the chemical treatments -as indicated by the very similar hepatic miRNA profiles of PB and BP.

(c) That although some miRNA perturbations were chemical-specific, the most significant effects of chemical treatments could be seen on a small set of "biomarker" miRNAs. These miRNAs are predicted to affect pathways that are important in the development of HCC.

(d) That dose and temporal parameters greatly affect the hepatic miRNAome responses to the chemical treatment. Carcinogenic dose of PB had distinct hepatic miRNA profiles from non-carcinogenic doses. It was also found that effects of continuous PB treatment on the hepatic miRNAome were becoming progressively more pronounced through 90 days of treatment.

To the best of my knowledge this was the first study that has investigated in depth the effects of treatments with chemical hepatocarcinogens on the liver miRNAome. The data generated support the hypothesis that hepatic miRNA profiles can facilitate the earlier prediction and an improved mechanistic understanding of chemical hepatocarcinogens. Therefore, the utilisation of hepatic miRNAs profiles for the risk assessment of chemicals is a promising strategy that qualifies for further investigation.

6.2 MiRNA deregulations in the pre-neoplastic liver treated with hepatocarcinogens: Extraneous, adaptive or adverse?

Given that chemical treatments affect the liver miRNAome, what is the phenotypic importance of these changes? There are three possible answers to this question:

(1) **The miRNA changes are an extraneous response to chemical treatments**. One possibility is that the observed changes in miRNA expressions are functionally irrelevant, not affecting the activity of proteins that are important for HCC development. In such a case the deregulation of miRNAs may be occurring passively due to the perturbation of upstream cellular pathways or as side-effects of the histopathological effects induced by the chemical treatments. It should be noted that even if that were the case, miRNA signatures would still be highly useful, so long as they possess sufficient specificities and sensitivities. However, in that case the mechanistic insight that could be gained from the study of these miRNAs would be more limited.

(2) **The miRNA changes contribute to the adaption of the liver to the chemical treatments.** Adaptive liver responses to xenobiotic exposure are those which do not affect organ or animal viability, are reversible, maintain homeostasis, and enhance the ability of the organ to respond to the xenobiotic stress (Williams & Iatropoulos, 2002). The livers which were examined here had been exposed to the chemical treatments for considerable time (up to 90 days), hence it is reasonable to expect that liver should have adapted to the chemical treatments by that point. Given the known regulatory role of miRNAs over key toxicological events (e.g. proliferation and

apoptosis), the changes in miRNA expressions could contribute to the eventual adaptation of the liver to the chemical responses.

(3) **The miRNA changes are adverse responses to the chemical treatments that contribute to the eventual carcinogenesis**. Ample evidence supports the important role of miRNAs in HCC (see section **1.3.5**). Hence the deregulated miRNAs in the rat liver could contribute to the eventual development of HCC by the disruption of key cellular pathways. These changes could facilitate the eventual development of mutations or offer a selective advantage to initiated pre-malignant cells. In addition, there is some evidence that disruptions of cellular pathways can occur firstly by epigenetic defects, followed later on by genetic mutations (Baylin & Ohm, 2006), hence early deregulations of miRNAs can possibly lead to an "addiction" of the incipient cancer cells to dysregulated pathways.

It is certainly possible that all three cases may be true at the same time, with some of the observed miRNA deregulations being adaptive responses, some having adverse consequences, while others could be extraneous events. Clarifying which of the three possibilities is the case for individual miRNAs is a challenging task, since several important aspects of miRNA functioning have still not been elucidated. For example, it is still uncertain whether the main effects of miRNAs are mediated by the regulation of a few key proteins or through the totality of their effects on the proteome. A second uncertainty is whether effects have a threshold. There is evidence to support the latter suggestion (Brown *et al.,* 2007, Sarasin-Filipowicz *et al.,* 2009). Therefore, it is still vague whether the observed hepatic miRNA deregulations after chemical treatment in the rat have to exceed certain threshold (1.5-fold? 5-fold? 10-fold?) before they have significant effects. Closely related to this issue is the question of whether modest

deregulation of highly expressed miRNAs or considerable deregulations of miRNAs with lower expression are more important functionally. It is known that there is wide diversity in the expression levels of hepatic miRNAs (Hou *et al.*, 2011). For instance, the hybridisation signal for miR-99a determined in this study was 45 times higher than that for miR-34a. Yet, for miR-99a the maximum deregulation by chemical treatment in the rat liver was measured at 1.6-fold, while for miR-34a that value was 27.9 fold. At present it is not clear which of the two deregulated miRNAs would have the most important phenotypic significance.

A further confusing factor is that the effects of miRNAs vary in different cellular contexts. This is a reasonable consequence of the fact that miRNAs propagate their cellular effects through the proteins that they regulate, while the protein milieu varies between cell types, developmental stage, and according to environmental conditions. For example in pancreatic cancers a tumour suppressive role has been suggested for miR-96 through regulation of KRAS (Yu *et al.*, 2010), whilst in ovarian cancers miR-96 is thought to act as an oncogene by suppressing the expression of FOXO proteins (Myatt *et al.*, 2010). Hence, extrapolation of miRNA effects determined in one cell type to effects in a different cell type can be misleading. This is an uncertainty in the current study.

Despite all these confounding factors, cautionary examination of the phenotypic consequences of miRNA deregulation has been attempted (**Chapter 5**). For the set of "biomarker" miRNAs (**Table 3.13**), the most probable interpretation is that their deregulation has some functional importance. This conclusion is based on their frequent deregulation by chemical carcinogens, their deregulation in various cancers

including HCC (**Table 3.15**), and the bioinformatic prediction that they regulate pathways which are important in cancer development (**Table 5.6**).

Two of the proposed biomarker miRNAs were miR-99a and miR-203, which had been previously reported to be downregulated in HCC compared to normal liver (Furuta *et al.*, 2010, Hou *et al.*, 2011). MiR-99a is a miRNA with relatively high expression in normal liver (sixth highest in normal human liver according to Hou *et al.*, 2011, who used a deep sequencing approach). This miRNA showed the biggest downregulation trend following treatment with the two most potent carcinogens, 2-AAF and MP HCl. It has been reported that downregulation of this miRNA can induce mTOR signalling (Doghman *et al.*, 2010). Interestingly, few targets are predicted for miR-99a using TargetScan (**Table 5.6**). Among its targets were FZD5 and FZD8, two receptors of WNT ligands, suggesting that this miRNA may also be involved in the dysregulation of β-catenin signalling in HCC. Importantly, both the mTOR and beta-catenin pathways are important in HCC development (Whittaker *et al.*, 2010). MiR-203 has been reported to be downregulated by aberrant methylation in HCC (Furuta *et al.*, 2010) as well as in several other cancers (Kozaki *et al.*, 2008, Bueno *et al.*, 2008, Bo *et al.*, 2011, Chiang *et al.*, 2011). In different cellular contexts, miR-203 has been reported to repress cellular proliferation and to promote differentiation (Furuta *et al.*, 2010, Bueno *et al.*, 2008, Yi *et al.*, 2008). Hence a logical explanation is that the deregulation of these two miRNAs contributes to the development of chemical-induced HCC.

Another miRNA that was frequently found to be affected by chemical carcinogens was miR-34a. In various cell types miR-34a has been shown to induce apoptosis and

senescence (Chang *et al.*, 2007, He *et al.*, 2007). This fact suggests a tumour-suppressive role for miR-34a. In accordance with this, expression of this miRNA is often lost in various tumours due to either chromosomal deletions or epigenetic silencing (Hermeking, 2010, Hermeking, 2007). As has been discussed previously (section 5.4), the potent induction of miR-34a by 2-AAF is most probably the result of DNA damage. However, miR-34a was also induced- though much more modestly- by three presumed NGTX carcinogens (MON, Chl.Ac, MP HCL). For these carcinogens transcriptomic data did not indicate activation of the p53 or apoptotic pathway (R.A Currie, personal communication). It will be interesting to establish whether the induction of this miRNA by the three NGTX carcinogens is associated with DNA damage.

Despite the fact that miR-34a has been reported to be a tumour-suppressor miRNA in several types of cancers, there is also a possibility that it can have oncogenic effects during HCC development. In the liver miR-34a has been reported to be progressively upregulated between normal to cirrhotic and cirrhotic to HCC (Pineau *et al.*, 2010). Interestingly, it has been reported that in the livers of rat treated with 2-AAF the miR-34a-p53 feedback loop is disrupted (Pogribny *et al.*, 2009a). The effects of miR-34a on cellular proliferation have been reported to be cell-type and context dependent (Dutta *et al.*, 2007). A recent study suggests that in conditions of myc overexpression miR-34a can be anti-apoptotic (Sotillo *et al.*, 2011). Recently it has also been reported that miR-34a targets HNF4α (Takagi *et al.*, 2010) which is a tumour suppressor in HCC (Ning *et al.*, 2010). Hence the role of miR-34a in HCC development needs to be examined further.

The most common effect of chemical treatments on the liver miRNAome though was the induction of the miR-200a/200b/429 cluster. It is interesting to note that members of the miR-200 family are expressed predominantly in epithelial cells. Of the most abundant hepatic cells, hepatocytes and the SECs are epithelial, while KC and HSCs are mesenchymal (Bhunchet & Wake, 1992). This suggests that the induction of miR-200a/200b/429 occurs within hepatic epithelial cell populations. The miR-200 family has been one of the most extensively studied in recent years due to its central role in the regulation of several key signalling pathways **(Figure 6.1)**.

Figure 6.1 *Central regulatory role of the miR-200 family*

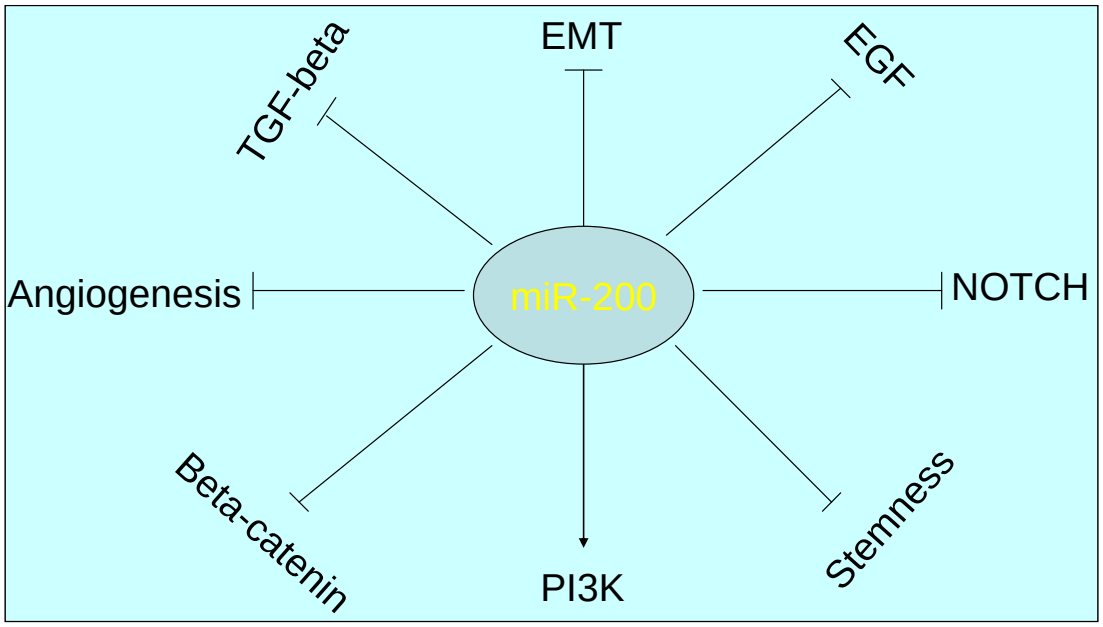


Fig. 6.1 In recent years this miRNA family has been shown to be involved in the regulation of key signalling pathways: EMT (Burk *et al.*, 2008, Gregory *et al.*, 2008b); PI3K (Hyun *et al.*, 2009); EGF (Uhlmann *et al.*, 2010, Adam *et al.*, 2009); Notch (Brabletz *et al.*, 2011, Vallejo *et al.*, 2011); Stemness (Shimono *et al.*, 2009, Wellner *et al.*, 2009); Beta-catenin (Xia *et al.*, 2010); Angiogenesis (Chan *et al.*, 2011); and TGF-beta (Wang *et al.*, 2011)

These regulatory roles suggest that the early induction of the miR-200a/200b/429 may be an adaptive response of the liver, regulating hepatocyte proliferation, invasion, survival, and angiogenesis (through the regulation of EGF pathway, beta-catenin,

notch etc.) after continued treatment with chemical carcinogens. This regulatory role is in agreement with the fact that five out of six hepatocarcinogenic treatments induced this miRNA cluster in the liver. In addition, expression of these miRNAs is downregulated in HCC compared to normal liver (Gramantieri *et al.*, 2007, Jiang *et al.*, 2008, Ladeiro *et al.*, 2008, Ura *et al.*, 2009). It was also hypothesised that members of the miR-200a/200b/429 and miR-96/182 clusters can cooperate to more effectively regulate cellular pathways (**Tables 5.7-5.10; Figure 5.12**)

6.3 Future work

Reflection on the main findings of this project, points to several further research avenues that can be pursued. A first obvious line of research to be followed would be the examination of hepatic miRNA profiles of animals treated with more chemicals, at different timepoints, and with various doses. Examination of larger sets of liver samples would address several of the limitations discussed previously (for example a more accurate estimation of sensitivities and specificities of miRNA signatures or the optimal timepoint to use these signatures to predict carcinogenicity).

The most complete dataset on chemical-induced hepatic miRNA expressions was generated here for PB, a prototype of NGTX carcinogens (**Chapter 3 and 4**). The main effect of the chemical on the hepatic miRNAome was found to be the progressive induction of the miR-200a/200b/429 and miR-96/182 clusters. It will be important to examine further the effects of PB on the liver miRNAome. This could include examining the miRNAome of hepatic foci and tumours generated by chronic PB treatment. This would allow determination of whether the effects of PB treatment on miRNAs, and the miR-200a/220b/429 and miR-96/182 clusters specifically,

continue until tumour development or whether they reverse at later stages. Examination of the miRNAome of livers from animals in which PB treatment was terminated some time before animal sacrifice will also demonstrate whether the miRNA deregulations are stable or reversible. In view of the reversible nature of chemical-induced NGTX carcinogenesis, such studies will be important

As discussed previously, it is important to determine the identity of the hepatic cellular populations in which miRNA deregulation take place to assess cell specificity. Several different methodologies are currently available which attempt to localise changes in gene expressions within selective tissues. One such methodology is *in situ* hybridisation, which is based on the binding of labelled probes onto target sequences. For example, the localisation of miRNA deregulation of miR-21 in cardiac disease occurs within cardiac fibroblasts (Thum *et al.*, 2008). A second possible method is to examine directly the expression of miRNAs in purified hepatic cell fractions. Protocols are available that allow the isolation of specific cell types from whole liver tissues (e.g. hepatocytes, HSCs, KC). Hence, the examination of hepatic miRNAs in isolated cell fractions from chemically-treated livers could inform the localisation of miRNA deregulation. It is also important to note that in this project most probably only a small fraction of the complete rat miRNAome examined. In January 2010, 350 rat miRNAs were known compared to 700 mouse and 1000 human miRNAs. No fundamental reason is known for why the number of miRNAs should vary between species by such a degree; hence it is expected that a large number of rat miRNAs remain to be identified. Indeed, a single recent study identified 61 new rat miRNAs (Linsen *et al.*, 2010). Therefore, it is probable that this project overlooked a large fraction of hepatic miRNAs. However, it is important to note that the first

miRNAs to be identified in rats, and which were examined here, represent ones which are highly conserved across eukaryotes and which thus possibly have the most important regulatory roles. A more complete examination of the effects of chemicals on the liver miRNAome can be performed in the future when a larger proportion of the rat miRNAome would have been identified.

At present considerable challenges exist in linking the expressions of miRNA with that of mRNA. For example, it is not possible to predict *a priori* whether a gene target of a miRNAs is regulated by mRNA degradation or repression of translation (Fillipowitz *et al.*, 2008). In addition, the bioinformatic tools for predicting the genes which are regulated by miRNAs are currently imperfect (Bartel *et al*., 2009). It is expected that in the future as this and other issues are addressed, it will be possible to perform a much more robust integration of mRNA with miRNA data.

An exciting possibility, if still somewhat controversial, is the use of circulating miRNAs found in bodyfluids as biomarkers of diseases (Gilad *et al.*, 2008, Kosaka *et al.*, 2010, Fabbri, 2010). Normally RNA species are rapidly degraded in biofluids due to the presence of ribonucleases, however circulating miRNAs are highly stable because they are located within lipid or lipoprotein complexes (Kosaka *et al.*, 2010). Several studies have shown that levels of circulating miRNAs are altered in cancer (Mitchell *et al.*, 2008, Taylor & Gercel-Taylor, 2008, Huang *et al.*, 2010). A recent study has found that selected miRNAs which are often deregulated in HCC (miR-122, miR-21, and miR-223) are detectable in the plasma of patients with chronic hepatitis or HCC (Xu *et al.*, 2011). Another study has found serum miRNA signatures that can identify Hepatitis B -infected and Hepatitis B -positive HCC (Li *et al.*, 2010a). One preliminary study has also indicated that serum miRNA levels are altered from the

early stages of exposure of rats to hepatocarcinogens (Sukata *et al.*, 2011). Circulating miRNA biomarkers have obvious benefits as biomarkers of disease in being more readily accessible and less invasive.

Elucidating the importance of chemical-induced deregulation of the liver miRNAome is likely to be an area of intense future research. One powerful tool that can be utilised is that of genetically modified animals. For example, animals in which hepatic *dicer1* has been specifically knocked down, and hence lack miRNA expression in hepatocytes, have been already described (Sekine *et al.*, 2009, Song *et al.*, 2010). It will be very interesting to examine how animals with miRNA-depleted livers will respond to treatment with chemical carcinogens. The effects of selected miRNAs can also be investigated *in vitro* by targeted deletions or overexpression in hepatic cells. It is also possible, as a few ambitious studies have demonstrated, to manipulate hepatic miRNA levels *in vivo* (Hou *et al.*, 2011, Kota *et al.*, 2009).

Another long term goal would be to examine whether chemical-mediated effects on the liver miRNAome are involved in species-specificity. Species-specific effects of miRNAs could result either from differential regulation of the miRNAs or in divergences in the proteins that are regulated by a given miRNA in a particular species. For instance, the large CpG island that is involved in regulation of the miR-200a/200b/429 cluster is humans (Li *et al.*, 2010), is not conserved in rodents (**Table 5.1**), suggesting likely differences in their regulation. Various approaches could be taken to examine species-specific chemical effects emerging from different miRNA activities such as computational methods, comparisons of rodent and human hepatocytes, and use of humanised animal models.

6.4 Conclusions: Opening the black box

The rodent bioassay has been developed in more or less its present form about sixty to seventy years ago (Weisburger & Williams, 1981, Weisburger, 1983). At its most basic, this assay can be considered as a typical "black box system": there is a defined input (the chemical treatment) which is examined; a black box (the animal itself); and a measurable output (cancer incidence). Hence the rodent bioassay can measure the carcinogenicity of chemicals in rodents. Whether this relates to similar processes in humans continues to be a very controversial debate. Today in the post-genomic era, with the availability of powerful "omic" and molecular biology technologies, after decades of extensive research, the complexity of cancer is still baffling (Hanahan & Weinberg, 2011). Moreover, the assessment of chemical hepatocarcinogens is rendered even more difficult due to the fact that the liver is possibly the second most complex mammalian organ, after the brain (**Section 1.3**). Simplicity was, and to a large extent still is, the main advantage of the rodent lifetime assay.

In the pursuit for replacements or refinements of the rodent lifetime bioassays, some approaches focus on measuring different "outputs" (e.g. histopathological effects of chemicals or indeed miRNAs signatures generated after short-term exposure), or even using a different "black box" (e.g. performing the assay on transgenic mice which respond faster to carcinogenic treatment) (**Section 1.4.2**). If these strategies prove successful they will allow considerable savings in time and costs, whilst largely side-stepping the issue of *how* the chemicals induce liver tumours. Yet, there are considerable benefits to elucidating the mechanisms of chemical carcinogenesis (Blomme *et al.*, 2009): the improved extrapolation of human health hazard from animal carcinogenicity data; the safer design of new drugs and compounds;

illumination of early stages of HCC development; and perhaps more information on the aetiologies of human HCC.

At present the utilisation of mechanistic information to predict carcinogenicity is limited. Instead mechanistic examination is often used only *after* the detection of a positive in the lifetime bioassay, in order to discriminate false positives (Boobis *et al.*, 2009a). This state of affairs is not surprising given the gaps of knowledge regarding the process of chemical carcinogenesis. As a case in point, the effects of chemical carcinogens on miRNAomes have been barely examined up to this point. Evidence gathered by this project suggests that examination of the hepatic miRNAome will reveal important aspects of chemical hepatocarcinogenesis. Of course, while deregulation of hepatic miRNAs could be an important step in chemical carcinogenesis, it will not be the whole story. Deep mechanistic understanding of chemical carcinogenesis will ultimately require the examination and integration of multiple layers of information: conventional toxicological, transcriptomic, proteomic, and metabonomic data (Waters & Fostel, 2004, Bugrim *et al.*, 2004, Plant, 2008). Recent decades have seen tremendous progress in the field of biomedical sciences and cancer research (Hanahan & Weinberg, 2011). It is noteworthy that miRNAs had not even been properly identified until ten years ago (Lagos-Quintana *et al.*, 2001, Lau *et al.*, 2001, Lee & Ambros, 2001). The rapid pace of progress of biomedical sciences underlies the optimism that the black box of chemical carcinogenesis will be eventually opened.

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APPENDICES

Appendix I

Table S.1:Expression of all detected miRNAs in control male Fischer rats

miRNA	Log_2	SD	miRNA	Log_2	SD	miRNA	Log_2	SD
rno-miR-122	10.9	0.4	rno-miR-378	3.8	0.1	rno-miR-25	1.8	0.3
rno-let-7f	7.3	0.4	rno-miR-30b-5p	3.6	0.4	rno-miR-199a-3p	1.8	0.2
rno-miR-21	7.0	0.3	rno-miR-30c	3.5	0.2	rno-miR-22*	1.8	0.4
rno-let-7a	6.9	0.4	rno-let-7i	3.4	0.2	rno-miR-223	1.7	0.3
rno-miR-192	6.5	0.4	rno-miR-195	3.4	0.4	rno-let-7e	1.7	0.4
rno-miR-29a	6.5	0.1	rno-miR-107	3.4	0.1	rno-miR-193	1.6	0.5
rno-miR-22	6.5	0.2	rno-miR-143	3.4	0.1	rno-miR-196a	1.4	1.0
rno-miR-26a	6.3	0.1	rno-miR-19b	3.3	0.4	rno-miR-30e	1.2	0.2
rno-let-7c	5.9	0.4	rno-miR-146a	3.2	0.2	rno-miR-140*	1.1	0.3
rno-miR-26b	5.7	0.4	rno-miR-30a	3.1	0.2	rno-miR-148b-3p	1.0	0.2
rno-miR-23b	5.3	0.2	rno-miR-30d	3.0	0.3	rno-miR-126*	0.9	0.2
rno-miR-125b-5p	5.3	0.4	rno-miR-15b	2.8	0.2	rno-miR-322	0.8	0.3
rno-miR-29c	5.1	0.2	rno-miR-31	2.8	0.4	rno-miR-152	0.7	0.3
rno-let-7b	5.1	0.3	rno-miR-10a-5p	2.7	0.4	rno-miR-150	0.7	0.3
rno-miR-126	5.0	0.2	rno-miR-497	2.7	0.2	rno-miR-139-3p	0.6	0.9
rno-miR-99a	5.0	0.1	rno-miR-324-3p	2.7	0.4	rno-miR-30e*	0.6	0.3
rno-miR-194	4.9	0.1	rno-miR-29b	2.6	0.1	rno-miR-17	0.5	0.2
rno-let-7d	4.9	0.3	rno-miR-20a	2.6	0.3	rno-miR-30a*	0.5	0.2
rno-miR-16	4.8	0.3	rno-miR-98	2.5	0.2	rno-miR-203	0.5	0.2
rno-miR-23a	4.6	0.2	rno-miR-103	2.4	0.2	rno-miR-196b	0.5	0.7
rno-miR-451	4.4	0.3	rno-miR-130a	2.4	0.5	rno-miR-345-5p	0.4	0.1
rno-miR-365	4.2	0.3	rno-miR-151	2.3	0.1	rno-miR-652	0.3	0.3
rno-miR-494	4.2	0.6	rno-miR-352	2.3	0.2	rno-miR-93	0.3	0.2
rno-miR-27b	4.1	0.3	rno-miR-196c	2.2	0.8	rno-miR-125a-5p	0.3	0.3
rno-miR-24	4.1	0.2	rno-miR-27a	2.1	0.2	rno-miR-199a-5p	0.3	0.1
rno-miR-101b	3.9	0.1	rno-miR-101a	2.1	0.1	rno-miR-28	0.3	0.2
rno-miR-92a	3.9	0.3	rno-miR-100	1.9	0.3	rno-miR-743a	0.2	0.9

Mean log_2 normalised hybridisation signal in control animals from multicompound study for the 167 miRNAs detected in 50% of animals in any treatment group (Chapter 3)

Table S.1 (continued) :Expression of all detected miRNAs in control male Fischer rats

miRNA	Log_2	SD	miRNA	Log_2	SD	miRNA	Log_2	SD
rno-miR-142-3p	0.2	0.4	rno-miR-664	-0.9	0.4	rno-miR-181d	-1.9	1.5
rno-miR-361	0.2	0.2	rno-miR-200a	-0.9	0.3	rno-miR-483	-2.1	0.3
rno-miR-145	0.1	0.1	rno-miR-409-3p	-1.0	0.2	rno-miR-7a*	-2.1	0.4
rno-miR-878	0.1	0.8	rno-miR-128	-1.0	0.3	rno-miR-142-5p	-2.2	0.6
rno-miR-333_v11.0	0.1	0.7	rno-miR-1*	-1.0	0.3	rno-miR-429	-2.2	0.5
rno-miR-221	0.0	0.5	rno-miR-505	-1.0	0.6	rno-miR-324-5p	-2.3	0.2
rno-miR-106b	0.0	0.1	rno-miR-125a-3p	-1.1	0.6	rno-miR-340-5p	-2.3	0.6
rno-miR-186	0.0	0.3	rno-let-7b*	-1.1	0.3	rno-miR-874	-2.3	0.8
rno-miR-140	0.0	0.2	rno-miR-200c	-1.1	0.6	rno-miR-92b	-2.4	0.4
rno-miR-188	-0.1	1.0	rno-miR-872	-1.1	0.4	rno-miR-484	-2.4	0.3
rno-miR-378*	-0.1	0.3	rno-miR-338	-1.2	0.2	rno-miR-350	-2.5	0.2
rno-miR-500	-0.2	0.2	rno-miR-99b	-1.2	0.3	rno-miR-532-3p	-2.5	0.8
rno-miR-10b	-0.2	0.3	rno-miR-466b	-1.3	0.3	rno-miR-374	-2.5	0.4
rno-miR-7a	-0.4	0.3	rno-miR-29c*	-1.3	0.4	rno-miR-674-3p	-2.5	0.6
rno-miR-96	-0.5	0.3	rno-miR-450a	-1.5	0.3	rno-miR-340-3p	-2.6	0.5
rno-miR-335	-0.5	0.5	rno-miR-331	-1.5	0.4	rno-let-7d*	-2.7	0.5
rno-miR-200b	-0.5	0.4	rno-miR-532-5p	-1.5	0.2	rno-miR-598-3p	-2.8	0.5
rno-miR-34a	-0.5	0.3	rno-miR-296*	-1.7	0.3	rno-miR-347	-2.9	0.6
rno-miR-342-3p	-0.5	0.3	rno-miR-328	-1.7	0.3	rno-miR-99a*	-2.9	0.9
rno-miR-214	-0.6	0.4	rno-miR-144	-1.7	0.7	rno-miR-290	-3.0	0.4
rno-miR-345-3p	-0.6	1.0	rno-miR-193*	-1.7	0.1	rno-miR-30c-1*	-3.1	0.7
rno-miR-425	-0.6	0.2	rno-miR-185	-1.7	0.4	rno-miR-146b	-3.1	0.8
rno-miR-210	-0.6	0.3	rno-miR-139-5p	-1.8	0.6	rno-miR-322*	-3.2	1.0
rno-miR-181a	-0.6	0.3	rno-miR-298	-1.9	0.1	rno-miR-99b*	-3.2	0.9
rno-miR-212	-0.7	0.6	rno-miR-129*	-1.9	0.4	rno-miR-877	-3.3	0.8
rno-miR-19a	-0.7	0.3	rno-miR-375	-1.9	0.6	rno-miR-326	-3.3	0.6
rno-miR-320	-0.9	0.6	rno-miR-30c-2*	-1.9	0.2			

Mean log_2 normalised hybridisation signal in control animals from multicompound study for the 167 miRNAs detected in 50% of animals in any treatment group (Chapter 3)

Appendix II

Table S.2: Differentially expressed microRNAs at 90 days treatment

microRNA	Corrected p.value	DETU	BP	PB	DEHP	MON	Chl. Ac.	MP HCL	2-AAF
miR-34a	>0.01	-1.1	-1.3	1	-1.2	3.9	2.3	1.7	27.9
miR-96	>0.01	1.3	2	3.6	1.6	1.6	-1.5	1	-1.3
miR-221	>0.01	-1.5	-1.8	-1.1	3	-1.2	1.5	1	-1.1
miR-200a	>0.01	-1.2	2	3.7	1.7	1.1	2	1.7	2
miR-107	>0.01	-1.1	1.1	-1.1	2.0	1	1.1	-1.2	-1.3
miR-200b	>0.01	-1	2.5	4.5	2.0	1.2	2.3	1.6	2
miR-429	>0.01	-1.1	2.4	3.8	1.9	1.2	2.3	1.8	2.7
miR-182	>0.01	2.4	4.3	6.3	3.1	3.3	1.1	1.3	1.4
miR-375	>0.01	-2	-2.3	-2	1.8	2.2	1.7	-2.1	-1.5
miR-193	>0.01	-1.3	-1.1	1.2	-1.7	-2.4	-4.9	-1.9	-1.3
miR-17	>0.01	-1.1	1.1	-1.1	1.1	1	-1.6	-1.2	-1.2
miR-497	>0.01	1	-1.1	-1.1	-1.2	-1.3	-1.7	-1.3	-1
miR-203	>0.01	-1.2	-1.1	-1.4	-1.4	-1.2	-2	-1.2	-1.6
miR-31	>0.01	1.1	1.1	-1	1.3	-1	1.3	-1.4	-1.5
miR-29a	>0.01	-1.1	-1	1.1	1.3	1	-1.2	-1.2	-1.2
miR-29c	>0.01	-1.1	1	1.2	1.3	-1	-1.3	-1.2	-1.2
miR-99a	>0.01	-1	-1.3	-1.3	-1.3	-1.5	-1.3	-1.5	-1.6
miR-20a	>0.01	-1	1	-1.1	1.1	-1	-1.6	-1.2	-1.3
miR-139-5p	>0.01	1.3	-1.1	1.1	1.3	1.3	3.2	1.3	-1.3
miR-28	>0.01	1.1	1	1.1	-1.1	1	1.4	1	-1.3
miR-505	>0.01	-1.1	1.1	1.5	1.5	1.1	2.8	1.2	-1.6
miR-210	>0.01	-1	-1.1	1.1	-1.2	-1.3	-2.7	1.1	1.1
miR-494	>0.01	-1.1	1.6	-1.6	-1.2	-1.7	-1.7	-1.5	3.7
miR-199a-5p	>0.01	1	-1.1	-1	-1.1	-1.1	-1	1.3	1.3
miR-327	>0.01	2.1	1.9	-1.6	-1.2	-1.3	-2.6	-2.7	6.1
miR-29b	0.01	1	1.2	1.1	1.3	-1.1	-1.8	-1.1	-1.1
miR-126*	0.01	-1.4	-1.2	-1	-1.1	-1.1	-1.1	-1.2	-1.8
miR-342-3p	0.01	1	-1.1	-1.5	-1.2	1	1.3	-1	1.1
miR-101b	0.01	-1.1	-1	-1	-1.5	-1.2	-1.5	-1.3	-1.3
miR-152	0.01	1	-1	1	1.2	1	1.2	-1.1	-1.2
miR-146a	0.01	1.2	1	-1.2	-1	1.3	1.5	1.2	-1.1
miR-30b-5p	0.01	-1.3	-1.2	-1.2	-1.2	-1.4	-1.9	-1.5	-1.5
miR-345-5p	0.01	-1	1	1.1	-1.5	-1	-1.3	-1.1	-1.1
miR-10a-5p	0.01	-1.2	-1.3	-1.2	-1.1	-1.1	-1	-1.2	-1.6
miR-144	0.01	1.5	1.5	-1.4	-1.7	-1.2	-3.8	-1.1	1.8
miR-186	0.01	1.1	1	1.1	1.1	1.1	1.5	1.1	-1.2
miR-212	0.01	1.1	-1.1	-1.7	-1.5	-2	-7.6	-2.3	1.2

MiRNAs were identified by one-way ANOVA with Benjamini-Hochbeg $p<0.05$.
Shown is the mean fold change from control;; n=3-5 animals per treatment

Table S.2 (continued): Differentially expressed microRNAs at 90 days treatment

microRNA	Corrected p.value	DETU	BP	PB	DEHP	MON	Chl. Ac.	MP HCL	2-AAF
miR-598-3p	0.01	1	1.2	1.2	1.5	1.1	1.6	-1.1	-1.7
miR-130a	0.02	1.1	1.1	1.1	-1.3	1.1	-1.4	1.1	1.2
miR-27a	0.02	1.1	-1.1	-1	-1.1	1.1	1.1	1	1.1
miR-365	0.02	-1.1	-1	1	1.1	1.1	1.1	-1.2	-1.5
miR-103	0.02	-1	1.2	-1	1.2	1.2	-1	-1.1	-1.2
miR-142-3p	0.02	1.3	-1.1	-1.3	-1.3	-1.2	-1.6	1.2	1.4
miR-451	0.02	-1	1.2	-1.3	-1.7	1.1	-1.3	-1.2	-1
miR-352	0.02	-1	1.1	-1.1	1.2	1.2	1.2	-1.1	-1.4
miR-338	0.02	1.1	1.1	1	-1.2	-1.5	-1.6	-1.1	-1
miR-19a	0.02	1.1	-1	-1.1	-1.1	-1.2	-2.5	-1	-1.1
miR-125b-5p	0.02	-1	-1.3	-1.3	-1.3	-1.3	-1.2	-1.5	-1.7
miR-30b-3p	0.02	2	1.5	1.3	1.9	1.3	1.9	1.1	1.3
miR-345-3p	0.02	-1.2	-1.6	-1.3	-1.7	-2.5	-10.3	-2.1	-1.3
miR-324-3p	0.02	-1.1	-1.1	-1.2	-1.9	-1.5	-3.9	-1.6	-1.2
miR-878	0.02	-1.1	-1.5	-1.5	-1.7	-2.9	-9.3	-2.1	-1.4
miR-7a*	0.02	-1.1	-1.2	1.1	-1.3	-1.1	1.2	-1.3	-1.6
miR-30c-1*	0.02	2.1	1.4	-1.3	-1.4	1.1	-1.6	-3.3	2.4
miR-378*	0.02	-1	1.1	1	1.4	-1	1.3	-1.1	-1.3
miR-100	0.03	1	-1.2	-1.2	-1.2	-1.3	-1.1	-1.3	-1.4
miR-150	0.03	1.1	-1.1	-1.5	-1.1	1.1	1.3	-1.1	-1.1
miR-101a	0.03	-1.1	-1.1	-1	-1.3	-1.3	-1.6	-1.3	-1.3
miR-7a	0.03	-1.1	-1.1	-1	-1.2	-1.1	1.2	1.1	-1.4
miR-331	0.03	1.2	1.2	1.1	1.6	1.3	1.9	1.2	1.2
miR-425	0.03	-1	1.1	1.1	1.2	-1.1	1	1	-1.3
miR-743a	0.03	-1.4	-1.5	-1.6	-2	-3	-9.7	-2.2	-1.6
miR-128	0.04	1	1	-1	1.3	1	1.4	-1	-1.3
miR-10b	0.04	-1.2	-1.3	-1.3	-1.1	-1.1	1	-1.1	-1.4
miR-125a-5p	0.04	1	-1.1	-1.2	-1	1.1	1.5	1	-1
miR-30d	0.04	1	1	-1	1.2	1.2	1.4	-1.1	-1.2
miR-93	0.04	1.1	1.2	1.1	1.4	1.1	1	1.1	1
let-7f	0.04	-1.1	-1.1	-1.1	1.1	1.1	1.2	-1.3	-1.5
let-7i	0.04	-1	-1	-1.1	1.2	1	1.3	-1	-1.2
miR-148b-3p	0.04	-1	-1.1	-1	1.1	-1	1.1	-1.1	-1.4

MiRNAs were identified by one-way ANOVA with Benjamini-Hochbeg $p<0.05$.
Shown is the mean fold change from control;; n=3-5 animals per treatment

Appendix III: Significantly over-represented pathways for "biomarker" miRNAs

Table S.3: Significantly enriched pathways for rno-miR-200a

Pathways	Rat genome	expected	predicted	p.value
Insulin/IGF pathway-protein kinase B signaling cascade	87	1.39	11	0.00
PI3 kinase pathway	118	1.88	12	0.00
TGF-beta signaling pathway	149	2.37	12	0.00
EGF receptor signaling pathway	143	2.28	11	0.00
Interleukin signaling pathway	161	2.56	11	0.00
p53 pathway	158	2.52	10	0.00
Parkinson disease	122	1.94	8	0.00
FGF signaling pathway	129	2.05	8	0.00
Wnt signaling pathway	338	5.38	14	0.00
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	32	0.51	4	0.00
Metabotropic glutamate receptor group I pathway	34	0.54	4	0.00
Synaptic_vesicle_trafficking	40	0.64	4	0.00
Hedgehog signaling pathway	23	0.37	3	0.01
Axon guidance mediated by Slit/Robo	23	0.37	3	0.01
Metabotropic glutamate receptor group III pathway	77	1.23	5	0.01
Integrin signalling pathway	183	2.91	8	0.01
Ionotropic glutamate receptor pathway	57	0.91	4	0.01
p53 pathway feedback loops 2	58	0.92	4	0.01
PDGF signaling pathway	162	2.58	7	0.02
Muscarinic acetylcholine receptor 1 and 3 signaling pathway	68	1.08	4	0.02
Axon guidance mediated by semaphorins	40	0.64	3	0.03
Cadherin signaling pathway	155	2.47	6	0.04
Inflammation mediated by chemokine and cytokine signaling pathway	288	4.59	9	0.04
Ras Pathway	84	1.34	4	0.05

MiRNA targets for miR-200a were predicted using TargetScan V5.1 and allocated to pathways using the PANTHER database. The numbers shown in the table indicate the total number of known genes belonging to a pathway in the rat; the expected number of genes within the miRNA predicted targets that should belong to that pathway; the actual number of predicted miRNA-targets that belong to that pathway; and the calculated p.value. of enriched pathways using binomial statistics. Pathways shown are those with p. values less than < 0.05

Table S.4: significantly enriched pathways for rno-miR-200b/429

Pathways	Rat genome	expected	predicted	p.value
PDGF signaling pathway	159	5.2	20	0.00
Ras Pathway	79	2.58	14	0.00
PI3 kinase pathway	115	3.76	16	0.00
EGF receptor signaling pathway	135	4.41	16	0.00
FGF signaling pathway	124	4.05	15	0.00
Hedgehog signaling pathway	25	0.82	7	0.00
Interleukin signaling pathway	161	5.26	17	0.00
TGF-beta signaling pathway	145	4.74	16	0.00
Angiogenesis	191	6.24	18	0.00
Insulin/IGF pathway-protein kinase B signaling cascade	89	2.91	11	0.00
Integrin signalling pathway	181	5.92	16	0.00
GABA-B_receptor_II_signaling	40	1.31	7	0.00
Axon guidance mediated by semaphorins	43	1.41	7	0.00
Inflammation mediated by chemokine and cytokine signaling	283	9.25	20	0.00
p53 pathway	113	3.69	11	0.00
Metabotropic glutamate receptor group III pathway	73	2.39	8	0.00
Beta2 adrenergic receptor signalling pathway	44	1.44	6	0.00
Huntington disease	167	5.46	13	0.00
Hypoxia response via HIF activation	32	1.05	5	0.00
Wnt signaling pathway	317	10.4	20	0.00
Cytoskeletal regulation by Rho GTPase	98	3.2	9	0.01
Parkinson disease	100	3.27	9	0.01
Metabotropic glutamate receptor group II pathway	51	1.67	6	0.01
p53 pathway feedback loops 2	52	1.7	6	0.01
Apoptosis signaling pathway	123	4.02	10	0.01
Transcription regulation by bZIP transcription factor	53	1.73	6	0.01
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	166	5.43	12	0.01
p53 pathway by glucose deprivation	25	0.82	4	0.01
Endothelin signaling pathway	91	2.98	8	0.01
VEGF signaling pathway	75	2.45	7	0.01
Oxytocin receptor mediated signaling pathway	60	1.96	6	0.02
Beta1 adrenergic receptor signaling pathway	44	1.44	5	0.02
5HT1 type receptor mediated signaling pathway	44	1.44	5	0.02
Interferon-gamma signaling pathway	29	0.95	4	0.02
Muscarinic acetylcholine receptor 2 and 4 signaling pathway	62	2.03	6	0.02
T cell activation	102	3.33	8	0.02
5HT2 type receptor mediated signaling pathway	69	2.26	6	0.03
Ubiquitin proteasome pathway	70	2.29	6	0.03
Synaptic_vesicle_trafficking	38	1.24	4	0.04
Histamine H2 receptor mediated signaling pathway	25	0.82	3	0.05

Same methodology as for **Table S.3**

Table S.5 Significantly enriched pathways for rno-miR-96

Pathways	Rat genome	expected	predicted	p.value
PI3 kinase pathway	118	2.9	18	0.00
EGF receptor signaling pathway	143	3.5	16	0.00
Integrin signalling pathway	183	4.5	18	0.00
Insulin/IGF pathway-protein kinase B signaling cascade	87	2.1	12	0.00
FGF signaling pathway	129	3.2	14	0.00
Interleukin signaling pathway	161	4	15	0.00
5HT2 type receptor mediated signaling pathway	78	1.9	10	0.00
Nicotinic acetylcholine receptor signaling pathway	98	2.4	11	0.00
Oxytocin receptor mediated signaling pathway	68	1.7	9	0.00
Metabotropic glutamate receptor group III pathway	77	1.9	9	0.00
Alpha adrenergic receptor signaling pathway	32	0.8	6	0.00
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase	32	0.8	6	0.00
Endothelin signaling pathway	96	2.4	10	0.00
Angiogenesis	200	4.9	15	0.00
Metabotropic glutamate receptor group I pathway	34	0.8	6	0.00
TGF-beta signaling pathway	149	3.7	12	0.00
Thyrotropin-releasing hormone receptor signaling pathway	71	1.7	8	0.00
Metabotropic glutamate receptor group II pathway	56	1.4	7	0.00
PDGF signaling pathway	162	4	12	0.00
Ras Pathway	84	2.1	8	0.00
Muscarinic acetylcholine receptor 1 and 3 signaling pathway	68	1.7	7	0.00
B cell activation	88	2.2	8	0.00
T cell activation	135	3.3	10	0.00
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway	137	3.4	10	0.00
Axon guidance mediated by semaphorins	40	1	5	0.00
Cadherin signaling pathway	155	3.8	10	0.01
Muscarinic acetylcholine receptor 2 and 4 signaling pathway	70	1.7	6	0.01
5HT3 type receptor mediated signaling pathway	17	0.4	3	0.01
Gamma-aminobutyric acid synthesis	6	0.2	2	0.01
Axon guidance mediated by netrin	34	0.8	4	0.01
Beta2 adrenergic receptor signaling pathway	53	1.3	5	0.01
Beta1 adrenergic receptor signaling pathway	53	1.3	5	0.01
Histamine H1 receptor mediated signaling pathway	54	1.3	5	0.01
Parkinson disease	122	3	8	0.01
Cytoskeletal regulation by Rho GTPase	100	2.5	7	0.01
Wnt signaling pathway	338	8.3	15	0.02
GABA-B_receptor_II_signaling	44	1.1	4	0.02
Oxidative stress response	67	1.7	5	0.03
Inflammation mediated by chemokine and cytokine signaling	288	7.1	13	0.03
5HT1 type receptor mediated signaling pathway	49	1.2	4	0.03

Same methodology as for **Table S.3**

Table S.6: enriched pathways for rno-miR-182

Pathways	Rat genome	expected	predicted	p.value
PI3 kinase pathway	118	2.87	15	0.00
EGF receptor signaling pathway	143	3.48	16	0.00
FGF signaling pathway	129	3.14	13	0.00
TGF-beta signaling pathway	149	3.62	13	0.00
Metabotropic glutamate receptor group III pathway	77	1.87	9	0.00
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	32	0.78	6	0.00
PDGF signaling pathway	162	3.94	13	0.00
Insulin/IGF pathway-protein kinase B signaling	87	2.12	9	0.00
Angiogenesis	200	4.86	14	0.00
Endothelin signaling pathway	96	2.33	9	0.00
Interleukin signaling pathway	161	3.92	12	0.00
5HT2 type receptor mediated signaling pathway	78	1.9	8	0.00
Oxytocin receptor mediated signaling pathway	68	1.65	7	0.00
B cell activation	88	2.14	8	0.00
Thyrotropin-releasing hormone receptor signaling	71	1.73	7	0.00
Integrin signalling pathway	183	4.45	12	0.00
Inflammation mediated by chemokine and cytokine	288	7	16	0.00
Ionotropic glutamate receptor pathway	57	1.39	6	0.00
Ras Pathway	84	2.04	7	0.00
T cell activation	135	3.28	9	0.01
5HT1 type receptor mediated signaling pathway	49	1.19	5	0.01
Alpha adrenergic receptor signaling pathway	32	0.78	4	0.01
5HT3 type receptor mediated signaling pathway	17	0.41	3	0.01
Metabotropic glutamate receptor group I pathway	34	0.83	4	0.01
Axon guidance mediated by netrin	34	0.83	4	0.01
Histamine H1 receptor mediated signaling pathway	54	1.31	5	0.01
Nicotinic acetylcholine receptor signaling pathway	98	2.38	7	0.01
Cytoskeletal regulation by Rho GTPase	100	2.43	7	0.01
Metabotropic glutamate receptor group II pathway	56	1.36	5	0.01
5HT4 type receptor mediated signaling pathway	40	0.97	4	0.02
GABA-B_receptor_II_signaling	44	1.07	4	0.02
Muscarinic acetylcholine receptor 1 and 3 signaling	68	1.65	5	0.03
Notch signaling pathway	46	1.12	4	0.03
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	173	4.21	9	0.03
Cadherin signaling pathway	155	3.77	8	0.04
Wnt signaling pathway	338	8.22	14	0.04
Leucine biosynthesis	2	0.05	1	0.05
Alanine biosynthesis	2	0.05	1	0.05

Same methodology as for **Table S.3**

Table S.7: enriched pathways for rno-miR-34a

Pathways	Rat genome	expected	predicted	p.value
TGF-beta signaling pathway	149	2.08	12	0.00
PI3 kinase pathway	118	1.65	9	0.00
Metabotropic glutamate receptor group III pathway	77	1.07	7	0.00
Metabotropic glutamate receptor group II pathway	56	0.78	6	0.00
Insulin/IGF pathway-protein kinase B signaling cascade	87	1.21	7	0.00
Synaptic_vesicle_trafficking	40	0.56	5	0.00
Interleukin signaling pathway	161	2.24	9	0.00
Nicotinic acetylcholine receptor signaling pathway	98	1.37	7	0.00
Notch signaling pathway	46	0.64	5	0.00
Thyrotropin-releasing hormone receptor signaling pathway	71	0.99	6	0.00
5HT2 type receptor mediated signaling pathway	78	1.09	6	0.00
EGF receptor signaling pathway	143	1.99	8	0.00
Oxytocin receptor mediated signaling pathway	68	0.95	5	0.00
Angiogenesis	200	2.79	8	0.01
5HT3 type receptor mediated signaling pathway	17	0.24	2	0.02
Plasminogen activating cascade	18	0.25	2	0.03
PDGF signaling pathway	162	2.26	6	0.03
Parkinson disease	122	1.7	5	0.03
5HT1 type receptor mediated signaling pathway	49	0.68	3	0.03
Alzheimer disease-presenilin pathway	129	1.8	5	0.04
Beta2 adrenergic receptor signaling pathway	53	0.74	3	0.04
Beta1 adrenergic receptor signaling pathway	53	0.74	3	0.04
Blood coagulation	55	0.77	3	0.04
Cell cycle	24	0.33	2	0.04
Integrin signalling pathway	183	2.55	6	0.05
Ionotropic glutamate receptor pathway	57	0.79	3	0.05

Same methodology as for **Table S.3**

Table S.8: enriched pathways for rno-miR-193

Pathways	Rat genome	expected	predicted	p.value
EGF receptor signalling pathway	143	0.5	6	0.00
FGF receptor signalling pathway	129	0.5	5	0.00
Ras pathway	84	0.3	4	0.00
PDGF signalling pathway	162	0.6	5	0.00
Inflammation mediated by chemokine and cytokine signalling pathway	288	1	5	0.00
B cell activation	88	0.3	3	0.00
Interferon-gamma signalling pathway	31	0.1	2	0.01
Angiogenesis	200	0.7	4	0.01
Wnt signalling pathway	338	1.2	5	0.01
PI3K kinase pathway	118	0.4	3	0.01
Alzheimer disease-presenilin pathway	129	0.5	3	0.01
p53 pathway feedback loops 2	58	0.2	2	0.02
Interleukin signalling pathway	161	0.6	3	0.02
Integrin signalling pathway	183	0.7	3	0.03
VEGF signalling pathway	82	0.3	2	0.04
Cytoskeletal regulation by Rho GTPase	100	0.4	2	0.05

Same methodology as for **Table S.3**

Table S.9: significantly enriched pathways for rno-miR-203

Pathways	Rat genome	expected	predicted	p.value
Integrin signalling pathway	183	2.74	12	0.00
Angiogenesis	200	3	11	0.00
p53 pathway feedback loops 2	58	0.87	6	0.00
Insulin/IGF pathway-protein kinase B signaling cascade	87	1.3	7	0.00
PI3 kinase pathway	118	1.77	8	0.00
Endothelin signaling pathway	96	1.44	7	0.00
EGF receptor signaling pathway	143	2.14	8	0.00
VEGF signaling pathway	82	1.23	6	0.00
TGF-beta signaling pathway	149	2.23	8	0.00
Wnt signaling pathway	338	5.07	12	0.01
p53 pathway by glucose deprivation	25	0.37	3	0.01
Circadian clock system	9	0.13	2	0.01
p53 pathway	158	2.37	7	0.01
Interleukin signaling pathway	161	2.41	7	0.01
Interferon-gamma signaling pathway	31	0.46	3	0.01
PDGF signaling pathway	162	2.43	7	0.01
Inflammation mediated by chemokine and cytokine signaling pathway	288	4.32	10	0.01
Alpha adrenergic receptor signaling pathway	32	0.48	3	0.01
Methionine biosynthesis	1	0.01	1	0.01
Ras Pathway	84	1.26	4	0.04
B cell activation	88	1.32	4	0.04
FGF signaling pathway	129	1.93	5	0.05
Hedgehog signaling pathway	23	0.34	2	0.05
Axon guidance mediated by Slit/Robo	23	0.34	2	0.05

Same methodology as for **Table S.3**

Table S.10: Significantly enriched pathways for rno-miR-99a

Pathways	Rat genome	expected	predicted	p.value
Wnt signalling pathway	338	0.44	4	0.00
Angiogenesis	200	0.26	3	0.00
Alzheimer disease-presenilin pathway	129	0.17	2	0.01
Cadherin signaling pathway	155	0.2	2	0.02
p53 pathway by glucose deprivation	25	0.03	1	0.03
Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	32	0.04	1	0.04
Hypoxia response via HIF activation	33	0.04	1	0.04

Same methodology as for **Table S.3**

Appendix IV: Conference Abstracts

Koufaris, C., Wright, J., Currie, R.A., and Gooderham, N.J, (2011) Dysregulation of hepatic microRNA profiles are associated with early stages of chemical carcinogenesis. Society of Toxicology international meeting march 2011, Washington DC, US. Oral presentation

Koufaris, C., Wright, J., Currie, R.A., and Gooderham, N.J, (2011) A characteristic set of microRNAs are deregulated in pre-neoplastic liver exposed to chemical carcinogens. American Association of cancer research, april 2011, orlando, US. Poster presentation

Koufaris, C., Wright, J., Currie, R.A., and Gooderham, N.J, (2010) The non-genotoxic hepatocarcinogen Phenobarbital causes persistent changes in the expression of liver microRNAs in the male Fischer rat. British Toxicology Society, march 2009, university of Warwick. Poster presentation

Koufaris, C., Wright, J., Currie, R.A., and Gooderham, N.J, (2010) Hepatocarcinogenic Phenobarbital treatments are associated with early, persistent alterations in the expression of the miR-200 family in the liver of male rat. Society of Toxicology international meeting march 2010, Baltimore, US. Poster presentation.

Koufaris, C., Wright, J., Currie, R.A., and Gooderham, N.J, (2009) Carcinogenic And Non-Carcinogenic Doses Of Phenobarbital Cause Distinct MicroRNA Profiles In Male Fischer Rats After 14 Days Of Dietary Exposure, march 2008, university of Surrey. Poster presentation.