THE UNIVERSITY of ADELAIDE

MicroRNA MEDIATED GENE REGULATION IN CANCER

By Qingqing Wang

M.Sc., B.Sc.

Breast Cancer Genetics Group, Centre for Personalised Cancer Medicine

Dame Roma Mitchell Cancer Research Laboratories

School of Medicine

The University Of Adelaide

This thesis is submitted to the University of Adelaide in the fulfilment of the requirements for the degree of

Doctor of Philosophy

August 2017

Table of contents

Overview

Cancer, as the second cause of death worldwide, requires further understanding of its mechanism to improve patient survival and quality of life. MicroRNAs are important regulators of translation and play major roles in normal cellular functions as well as cancer pathobiology. The broad aim of my study was to provide new insight into microRNA-mediated gene regulation in cancer. Although miRNAs account for the post-transcriptional regulation of more than 60% of human protein-coding genes, this work is mainly focused on two molecular pathways: the p53 signalling pathway and the androgen receptor signalling pathway, each of which have been shown to be strongly connected to tumorigenesis.

p53, a transcription factor that participates in multiple cellular functions, is considered the most important tumour suppressor and is mutated in ~50% of cancers. Previous evidence suggests that post-transcriptional deregulation of p53 by microRNAs contributes to tumorigenesis, tumour progression and therapeutic resistance. We found that the microRNA miR-766 was aberrantly expressed in breast cancer, and that over-expression of miR-766 caused accumulation of wild-type p53 protein in multiple cancer cell lines. Supporting its role in the p53 signalling pathway, miR-766 decreased cell proliferation and colony formation in several cancer cell lines, and cell cycle analyses revealed that miR-766 causes G2 arrest. At a mechanistic level, we demonstrate that miR-766 enhances p53 signalling by directly targeting MDM4, an oncogene and negative regulator of p53. Analysis of clinical genomic data from multiple cancer types supports the relevance of miR-766 in p53 signalling. Collectively, our study demonstrates that miR-766 can function as a novel tumour suppressor by enhancing p53 signalling.

Moreover, we have reported miR-9 as a novel miRNA that specifically down-regulates the expression of misssense p53 R248Q and R273H in multiple cell lines, while the wild-type p53 is upregulated and other p53 mutations are unaffected. We also identified a potential binding site within *TP53* ORF. A few potential mechanisms behind this unique observation are discussed. This part of my work provides novel evidence in the miRNA-mediated mutant p53 regulation and discusses the weakness of current miRNA target study.

Androgen receptor (AR) is a transcription factor that is the key driver of prostate cancer growth and progression. As such, AR and its downstream pathways are a critical target for prostate cancer treatment. MiRNAs participate in the regulation of these pathways by targeting AR itself or downstream genes. In our study, we identified miR-375 as a direct negative regulator of androgen receptor and its signalling pathways. Over-expression of miR-375 results in down-regulation of AR protein and mRNA levels and AR target genes *FKBP51* and *KLK3*, accompanied by growth inhibition of prostate cancer cells. Over-expression of AR rescued the effect of miR-375 over expression. Interestingly, AR binds the promoter region of the MIR375 gene and upregulates its expression. Thus, my work identifies a new feedback loop that balances the endogenous level of AR and miR-375 in prostate cells.

Overall, this work provides further understanding of how miRNAs regulate important gene pathways in different cancers.

Publications

Original research paper:

- Wang Q, Selth LA, and Callen DF. *MiR-766 induces p53 accumulation and G2/M arrest by directly targeting MDM4.* Oncotarget, 2017. 8(18): p. 29914-29924.
- Wang Q, Townley S, Paltoglou S, Callen DF, and Selth LA. *A novel miR-375-androgen receptor feedback loop regulates growth of prostate cancer cells.* Text in manuscript.

Review paper:

- Wang Q, Callen DF, and Selth LA. *The microRNA-p53 network: a game of balance.* Text in manuscript.

Presentation:

- Presentation: Wang Q, Callen DF. *Over-expression of miR-766 up-regulates p53 and represses cell proliferation by reducing MDM4.* Adelaide RNA Special Interest Group seminar, 31 October 2014, Adelaide, Australia
- Poster: Wang Q, Callen DF. *Over-expression of miR-766 up-regulates p53 and represses cell proliferation by reducing MDM4.* Florey International Postgraduate Research Conference, 25 September 2014, Adelaide, Australia.

- Poster: Wang Q, Callen DF. *Over-expression of miR-766 up-regulates p53 and represses cell proliferation by reducing MDM4.* 27th Lorne Cancer Conference 2015, 12-14 February 2015, Lorne, Australia.
- Poster: Wang Q, Selth LA, and Callen DF. *MiR-766 induces p53 accumulation and G2/M arrest by directly targeting MDM4.* Florey International Postgraduate Research Conference, 24 September 2015, Adelaide, Australia.

Acknowledgement

I would first like to express my sincere gratitude to my principal supervisor Prof. David Frederick Callen for his kind support and patient guidance throughout the past four years since the first day that I arrived in Australia in 2013. Prof. David Callen not only gave me this great opportunity to conduct my PhD at the University of Adelaide; he also generously provided me with a job in the lab as a research assistant even prior to starting my PhD candidature. During the years working under Prof. David Callen's supervision, his unsurpassed knowledge in cancer biology and research experience always broadened my horizon in the field and improved the quality and efficiency of my research. He also helped me improve my academic writing with extraordinary patience. I especially appreciated Prof. David Callen's open attitude towards unexpected small findings, which inspires me to remain curious while exploring the unknown field. Prof. David Callen encouraged me to embrace emerging new technologies in cancer genetics research, and supported me for the 2015 Winter School in Mathematical & Computational Biology at the University of Queensland, and the RStudio workshop at the University of Adelaide; both events opened a new direction in my research career.

I am also extremely grateful to Dr. Luke Selth for being my co-supervisor and supporting me through the second half of my PhD when my previous project was facing difficulty. Dr. Luke Selth is a very talented scientist with remarkable enthusiasm and immense knowledge in the non-coding RNA field. With the deep understanding of the molecular mechanisms, his insightful and enlightening comments helped me plan, scale and develop my project more efficiently. I enjoyed the discussions with Dr. Luke Selth that strengthened my precise logic in science, which will be essential for my future

research. Dr. Luke Selth introduced me to the field of bioinformatics, and helped me to develop data digging, meta-analysis and programming techniques. I also benefited from his skill in comprehensive data interpretation and precise scientific writing.

I am indebted to Dr. Paul Matthew Neilson, Dr. Kathleen Irene Pishas and Dr. Andrew Turner, who have worked in the Personalized Cancer Therapeutics Laboratory (PCTL) as postdoctoral fellows and have given me great advice and help during my study. Dr. Paul Neilson was co-supervising me when I first started working in the group, and I can never thank him enough for the inspiring and passionate discussions that we had in the beginning of my project. He greatly extended my understanding in non-coding RNA structure and function. Dr. Paul Neilson had the ability to influence people with his enthusiasm and faith when facing any challenges. Without his support, I would have had great difficulty in gaining confidence to begin independent research. Dr. Kathleen Pishas was my co-supervisor for a short period, and supported my work with thoughtful comments and suggestions. Dr. Andrew Turner joined PCTL in my third year of PhD, and managed the general lab issues as well as helped me with experiment design and material preparation. In particular, I also give my thanks to Dr. Andrew Turner for proofreading the paper and thesis via his editing business (according to the ASEP standards).

During my PhD candidature, I was lucky to work with a group of friendly and passionate young scientist. Many thanks goes to my good friends and colleagues Dr. Yu Feng, Dr. Alaknanda Adwal, Dr. Sheng Lei and Dr. Reshma Shakya, who are always will to help or discuss any issue that we are interested in. Their friendship fulfilled my

journey of PhD. Dr. Yu Feng and I both worded in the non-coding RNA area. Her solid knowledge in miRNA isoforms and RNA structure enhanced my confidence in the miR-9 research project. Dr. Alaknanda Adwal was warm-hearted and provided generous assistance in flow cytometry experiments.

In addition, I would like to thank Prof. Wayne Tilley for kindly giving me access to the Dame Roma Mitchell Cancer Research Laboratory (DRMCRL) and providing various resources for my work. While working in DRMCRL, I could never thank lab manager Adrienne Hanson enough for the lenti-virus training and her assistance in every aspect of work. My thanks also goes to the researchers and lab management staffs in DRMCRL. Dr. Rajdeep Das and Dr. Gerard Tarulli both helped me with reverse transwell invasion experiment and the confocal microscope training. Scott Townley and Steve Paltoglou both helped with the preparation of ChIP experiment. I would also like to extend my appreciation to research manager Suzanne Atkins for her important emotional support.

I believe it would not have been possible to complete my PhD without the Research Training Program Scholarship (formerly International Postgraduate Research Scholarship and Australian Post-graduate Award, which was generously granted in 2014 by the Australian government; this significantly eased financial pressures and allowed me to focus on my studies.

My heartfelt thanks goes to my parents. Although far away in a different country, they have always been unconditionally supporting all my decisions and goals with endless love, well wishes and encouragement. Finally, I would like to convey my deepest gratitude to my partner, Travis Roe, who has been my most trustworthy supporter, who has been the comfort in the hard times and the joy in the good days, and always endowed me with understanding and love.

Declaration

I, Qingqing Wang, certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. I acknowledge that copyright of published works contained within this thesis resides with the copyright holder(s) of those works. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Qingqing Wang

27 August 2017

Chapter 1.

Introduction

Chapter 1. Introduction

1.1. What is cancer?

1.1.1. Definition of cancer

Cancer, the emperor of all maladies, is a collection of diseases in which altered cells exhibit uncontrolled growth and the potential to spread to other parts of the body. To distinguish malignant cells from normal cells, ten key characteristics have been used to describe the aberrant growth and invasive behavior of cancer cells [1]. These "hallmarks of cancer" are: sustaining proliferative signalling, resistance to growth repression, avoidance of apoptosis, enabled replicative immortality, evading immune destruction, tumour-inducing inflammation, promoted angiogenesis, activation of invasion and metastasis, deregulation of cellular metabolism and genome instability/mutation [1] (Figure 1.1).

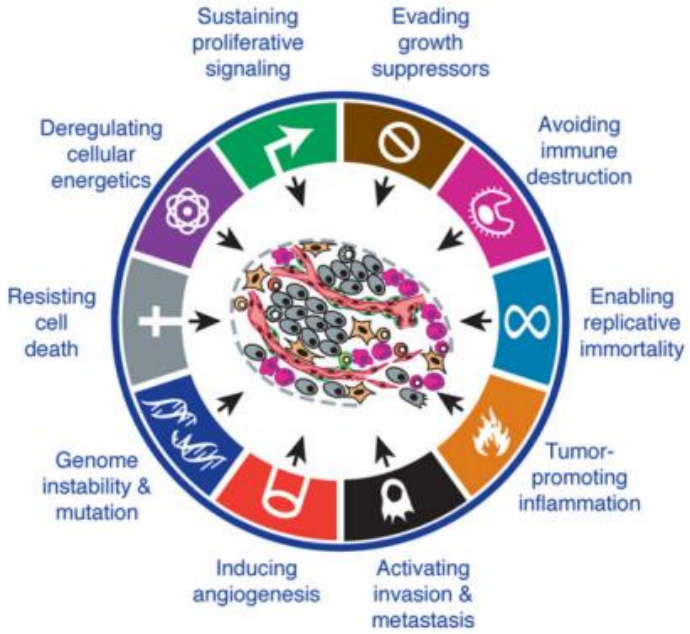


Figure 1.1. Hallmarks of cancer [1].

In general, cancers are named for the originating organ or tissue, and can be divided into categories based on the originating cell type. The major categories of cancer by cell types include carcinoma, sarcoma, leukemia, lymphoma, multiple myeloma, melanoma, brain and spinal cord tumours, germ cell tumours, neuroendocrine tumours, and carcinoid tumours [2]. Carcinoma is the most common type of cancer in humans, originating from epithelial cells, and can be further divided into adenocarcinoma, basal cell carcinoma, squamous cell carcinoma and transitional cell carcinoma. Sarcoma is a type of cancer that forms in soft tissues and bones. Leukemia, lymphoma and multiple myeloma are all cancers that originate from cells of the bone marrow and blood. Brain and spinal cord tumours are a group of cancers that originate from neurons or neuroglia cells. The other major cancer types (melanoma, germ cell tumours, neuroendocrine tumours, and carcinoid tumours) are defined by their origin from a specific cell type.

1.1.2. Cancer development

The origin of cancer is a complex process that is primarily driven by genetic alterations, which can be either inherited or somatic. Somatic mutations can arise from environmental factors such as exposure to radiation, tobacco smoking or alcohol. Other lifestyle factors can also play a role with obesity, diet and lack of physical exercise being implicated [3]. Certain viruses are also linked to oncogenesis [4]. For example, Epstein–Barr virus (EBV) is a member of the herpes family, and is associated with Hodgkin's lymphoma [5], Burkitt's lymphoma [6], gastric cancer [7] and nasopharyngeal carcinoma [8]. Hepatitis B virus (HBV) is the cause of hepatitis B, and also strongly related to the development of hepatocellular carcinoma [9]. Localized chronic irritation also

potentially contributes to oncogenesis by inducing hyperplasia, which may develop into dysplasia and if not treated, subsequently cancer [10].

Oncogenesis in humans is a multistep process which varies between different cancer types, but normally share similar core key pathological steps. This process normally begins with certain lesions of certain cancer-relevant genes, such as mutation, chromosome translocation, loss of heterozygosity and other dysregulations of tumour suppressors (i.e. *TP53* and *PTEN*) and proto-oncogenes (i.e. *RAS* and *MDM2*) (will be further discussed in section 1.2). Following the occurrence of the initial genetic lesions in normal cells, additional pre-malignant genetic events are required before the cells evolve into invasive cancer.

For example, in epithelial malignancies, initial genetic changes accelerate proliferation allowing otherwise normal appearing cells to form a fast growing tissue (hyperplasia). Second, as genetic lesions accumulate, the cells in the fast growing tissue start to present an aberrant appearance and disturb normal tissue organisation, i.e. hyperplasia develops into dysplasia. This step is a serious condition but not yet malignant, and does not necessarily need treatment. In the next step, additional genome alterations lead to further disruption of tissue structure. This is termed carcinoma *in situ*, where cells are malignant but infiltration is retained within the original abnormal tissue. Carcinoma *in situ* frequently progresses to invasive cancer and usually requires treatment[11] (Figure 1.2).

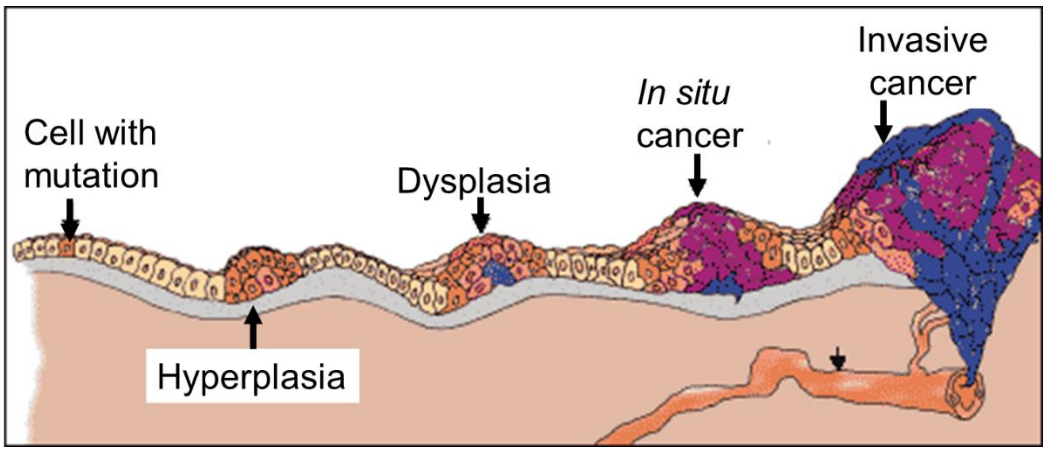


Figure 1. 2. Progression of cancer. Image obtained from internet, accessed on 25 August 2017

(Source: http://www.ndhealthfacts.org/wiki/Oncology_%28Cancer%29).

If carcinoma *in situ* is not treated a malignant tumour may form in the original organ as primary cancer. The primary tumour typically grows in an uncontrolled manner and angiogenesis, the formation of new blood vessels, is promoted. The induced angiogenesis promotes the transport of oxygen and energy and the export of metabolic wastes, facilitating tumour cell growth and survival [12]. Moreover, the enhanced tumour vasculature increases the ability of tumour cells to invade and metastasize. Metastasis is the final step of cancer development in which cancers spread to other parts of the body. Metastasis begins with malignant cells escaping from the primary tumour and invading adjacent normal tissue, followed by passage through the walls of blood vessels or lymph glands and subsequent distribution by a hematogenous or lymphatic route to other parts of the body [13, 14]. Following this distribution, the cancer cells can "seed" in a lymph node or foreign tissue to initially form micro-metastases; subsequent local angiogenesis can yield new macro-metastases [15]. Although a primary tumour can cause massive organ damage and other severe symptoms, the prognosis of primary cancer is almost

always better compared to metastatic disease. Indeed, ~90% of cancer-related mortality is caused by metastases [16].

1.1.3. Cancer grade, stage and prognosis

Clinically, cancer is assessed by stages and grades that guide treatment and prognosis. Classification of the stage of a cancer is defined by the growth of the primary tumour and the level of metastasis, and commonly divided into five stages (Table 1.1). Patients usually have poorer survival and prognosis when diagnosed with higher stage cancers compared with lower stages. For example, in the USA the 5 year relative survival rate for stage 1 breast cancer is nearly 100% while this drops to 72% for stage 3 breast cancer patients, and only 22% for patients with stage 4 cancer [17]. A grading system is also used for diagnostic purposes. Tumour grade is an estimate of the level of alteration in cell morphology exhibited by tumour cells compared to normal cells (Figure 1.3). A tumour that is more organized and closer to the normal state is considered well-differentiated and usually predicts better outcomes and prognosis. Poorly differentiated tumours, which lack distinctive cell morphology compared with normal tissue, are usually considered more dangerous and have a poorer prognosis.

Table 1.1. Cancer stages.

Stage	Description
Stage 0	Carcinoma in situ: malignant cells retained within the original abnormal tissue.
Stage 1	A relatively small malignant tumour that is contained within the organ it started in the organ where it started.
Stage 2	Cancer has not started to spread into surrounding tissue but with larger tumour than in stage 1.
Stage3	Cancer with larger tumour and may have started to spread into surrounding tissues and lymph nodes.
Stage4	Cancer that have spread from the originate organ to another body organ (metastasis cancer).

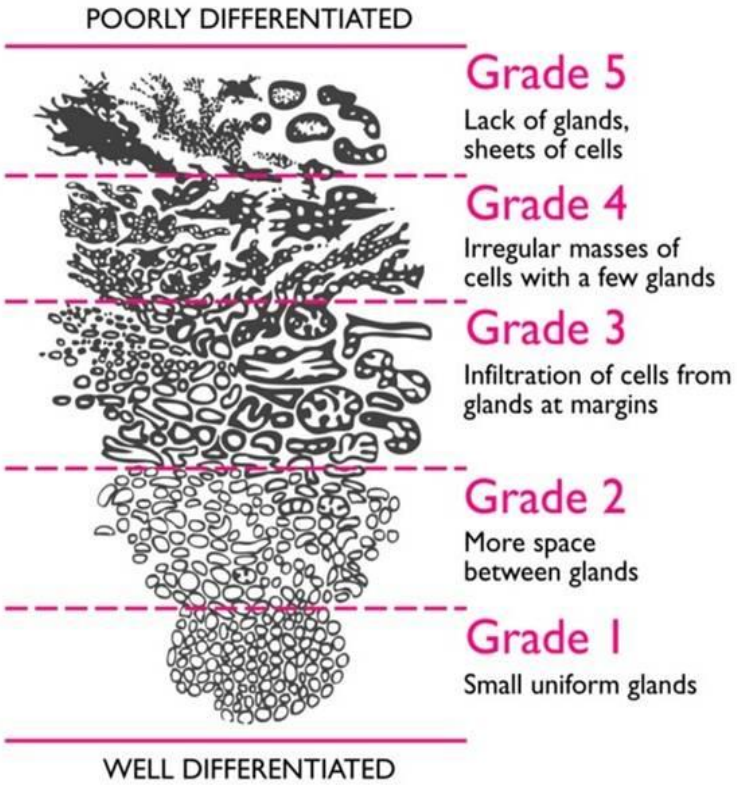


Figure 1.3 Cancer grades and cell differentiation. Image obtained from Google Image on 27 November 2017. (Source: http://www.ustoo.org/images/Gleason.jpg)

1.1.4. Cancer statistics

Cancer has been recognized as one of the leading causes of death in modern society. According to data provided by the World Health Organization (WHO), approximately 14 million new cases of cancer occurred worldwide in 2012, and a rise of new cases by 70% is expected in the next twenty years. In Australia, cancer incidence has increased dramatically from 47440 new cases in 1982 to more than 120000 new cases in 2012. This number is estimated to further increase to 134174 in 2017, with over 45% of cases being either breast cancer, prostate cancer, colorectal carcinoma, melanoma and lung cancer [18].

Nearly 1 in every 6 deaths in Australia is caused by cancer, making it the second-leading cause of death in this country; this disease killed 44171 Australians in 2014. In 2017, the chance of death by cancer by the age of 85 is estimated to be 1 in 5. Although mortality rates are decreasing (from 209/100,000 in 1982 to 161/100,000 in 2017, according to *Cancer in Australia 2017*), the overall 5-year-survival rate in Australia is still ~68%; thus, vast improvements in diagnosis and treatment are still required to improve outcomes [18].

1.1.5. Recent progress in cancer treatment

Modern medicine has developed multiple options for the treatment of cancer since the 19th century. Surgery is the most direct treatment for non-haematological cancers, with the aim to physically remove the entire tumour and potential metastasis affected lymph nodes. Other types of treatment are mainly to kill or limit cancer cell growth, in order to control cancer development or shrink the tumour. Radiotherapy is one of the

main cancer treatment options, where ionizing radiation is used to kill cancer cells. Chemotherapy also aims to kill cancer cells using chemical anti-cancer drugs. For haematological cancers, bone marrow or stem cell transplant is widely used after high doses of destructive chemotherapy or radiation.

More recently, targeted therapy [19, 20], immunotherapy [21, 22], interventional radiation and other cancer treatments have been introduced into clinical use and are raising new hopes as more effective alternatives to surgery, radiotherapy and chemotherapy [23]. Targeted therapies mainly aim to repress cancer development or kill cancer cells via specific targets. For example, hormone therapy is a targeted therapy, and has been proven effective for controlling hormone-sensitive cancers (such as ovarian [24, 25], breast [26, 27] and prostate cancers [28]). Immunotherapy is focused on arousing the immune system of the patient to fight cancer cells, such as NK cell-based immunotherapy [21].

1.2. Molecular mechanism of cancer: oncogenes and tumour suppressors

Much research has identified genes that can regulate cancer development and growth. These genes can be broadly grouped into oncogenes and tumour suppressor genes.

1.2.1. Oncogenes

Oncogenes contribute to the initiation and progression of cancer, and can be extrinsic (i.e. viral oncogenes), or derived from proto-oncogenes within the human

genome [29]. Proto-oncogenes are usually human genes that function to regulate cell growth and survival, but can become an oncogene when hyper-activated, which can occur via mutation, transcriptional upregulation or post-translational mechanisms [30]. Mutation within the proto-oncogene coding region can lead to gain-of-function of the protein product and provide constitutive oncogenic activity, or disturb post-translational regulation and lead to increased protein stability and activity. For example, the *RAS* genes (*HRAS, NRAS* and *KRAS*) serve as GTPases in normal cells, but somatic mutations of *HRAS, NRAS* and *KRAS* have been frequently found in human cancers (~25%), and function as oncogenes [31]. Mutations within the regulatory region of a proto-oncogene or localized gene amplification can result in increased transcription and protein expression, such as MDM2, which has been found to be amplified in 70% of well-differentiated liposarcoma [32], and 31% of breast cancers [33]. Chromosome translocation may also lead to over-expression of a gene when the upstream regulatory element is altered. This mechanism may also produce fusion genes and relates to outcome of cancer, such as the TMPRSS2-ERG fusion, which is a frequent event in prostate cancer and linked to poorer prognosis [34, 35]. Some other factors may also participate in the conversion of proto-oncogenes, such as aberrant epigenetic modification of the gene and/or dysregulation of the proteins that regulate the proto-oncogenes.

Broadly, oncogenes disturb normal cell growth regulation or programmed cell death [36]. Researchers have identified thousands of genes that potentially relate to oncogenic roles and have divided them into six groups according to their functions: transcription factors, chromatin remodelers, growth factors, growth factor receptors, signal transductors and apoptosis regulators [37]. Examples include the *RAS* family that functions as signal transductors and contribute to cell proliferation, low differentiation

and survival when mutated in cancer [31]. VEGF-A can be upregulated by other oncogenes and encodes a ligand that contribute to tumour angiogenesis [38], upregulated VEGF-A expression has been associated with metastasis risk and poor prognosis in several cancers [39-41]. HER2/neu is a growth factor receptor encoded by *ERBB2* gene. HER2/neu plays a significant role in breast cancer progression [42], and has been found to be amplified or over expressed in many other cancers, including ovarian carcinoma, lung adenocarcinoma and gastric cancer [43]. Some oncogenes are more specifically related to certain cancer types, for example the androgen receptor plays a key role in prostate cancer [44] and breast cancer [45] (see Sections 3.5.2 for more detail). Some oncogenes are also found to facilitate cell migration and tumour metastasis by inducing epithelial-mesenchymal transition and angiogenesis, such as the *RAS* family and *Scr* family [46-49].

Oncogenes represent potential therapeutic targets for clinical cancer treatment, and some targeted drugs have already been developed, including the HER2 receptor inhibitor Trastuzumab [50] and the VEGF-A inhibitor Bevacizumab [51].

1.2.2. Tumour suppressors

Tumour suppressors are the opposite to oncogenes, being a group of genes that repress oncogenesis [52]. According to their function, tumour suppressors can be catalogued into three groups: “gatekeepers”, “caretakers” and “landscapers” [53, 54]. The gatekeepers are the genes that negatively control cell growth and survival, stopping the cell from potentially entering a malignant growth phase. The caretakers are genes that help maintain genetic stability and suppress mutations of other genes by arresting the cell

cycle to allow gene repair, or by triggering apoptosis if the damaged DNA cannot be repaired. The landscapers are genes that modify the cell microenvironment by regulating extracellular matrix proteins, cellular surface markers and growth factors [53, 54]. Loss of function of tumour suppressors is commonly observed in cancers due to mutation, deletion, loss-of-heterozygosity or dysregulation. Random mutations within the coding region of a tumour suppressor, or chromosome translocation, may lead to altered function of the protein product, thus impairing or abolishing its biological function. Aberrant epigenetic modification and mutations within the promoter region can reduce the transcription of the gene, whereas shortened protein stability due to mutation or other dysregulated factors can also lead to decreased protein expression and activity.

Since tumour suppressors are "preventers and stoppers" of cancer, strategies to regain their expression and function has become a heated area in cancer research. For example, encoded by *TP53*, p53 is a gatekeeper and a caretaker, and the most important tumour suppressor that participates in the regulation of apoptosis, cell cycle arrest and DNA repair (see more details in section 1.4). The tumour suppressor *PTEN* regulates cell division by repression cell cyle transition, and is frequently mutated or lost in human cancer [55-57]. *PTEN* is also an examples of landscaper genes which create an anti-growth extracellular stroma and repress the proliferation and invasion of cancer cells [58].

1.2.3. Non-coding RNAs in cancer

1.2.3.1. Non-coding RNAs

In addition to classic protein-coding oncogenes and tumour suppressors, non-coding RNAs (ncRNAs) can also have equivalent functions. An ncRNA is an RNA transcript that does not translate into a protein molecule. The ncRNA family comprises multiple types characterized by the biogenesis length, function and structure of the ncRNA (Figure 1.4). Transfer RNA (tRNA) is a type of ncRNA that carries the amino acid to the the ribosomal for translation. rRNA is another type of ncRNA that composes the subunits of ribosome and is essential for protein synthesis. Small nuclear RNA (snRNA) are found in the Cajal bodies of the nucleus and participate in pre-mRNA maturation. A subtype of snRNA is small nucleolar RNA (snoRNA), which is highly similar to snRNAs but participates in modification of ncRNAs (such as tRNAs and rRNAs) instead of pre-mRNAs. Small Cajal body-specific RNAs (scaRNAs) belong to the snoRNA family but are only involved in the modification of RNA polymerase II transcribed spliceosomal RNAs [59].

While the average length of tRNAs, snRNAs, snoRNAs and scaRNAs is approximately 70~200nt [60], there are small ncRNAs that play roles in RNA silencing consisting of approximately 30 nucleotides. The largest classes of small ncRNAs in animals are piwi-interacting RNAs (piRNAs), which are ~28-33nt in length [61]; microRNAs (miRNAs), small single strand RNAs of approximately 18-25 nucleotides [62]; and small interfering RNAs (siRNAs), double-stranded RNAs that also serve as RNA silencers.

Longer ncRNAs also exist and participate in in a broad range of cellular functions. Long-non coding RNA (lncRNA) are ncRNAs with a length of more than 200nt, and

have been reported to regulate the process of DNA transcription by interacting with different elements of the process. Another class of longer ncRNAs is circular RNA (circRNA), which is usually composed of 1-5 exons and can be 1500nt in length [63]. Different from linear RNAs, a circRNA is a single RNA strand that forms a circle by joining the 5' and 3' ends and is believed to function as a regulator of other linear RNAs.

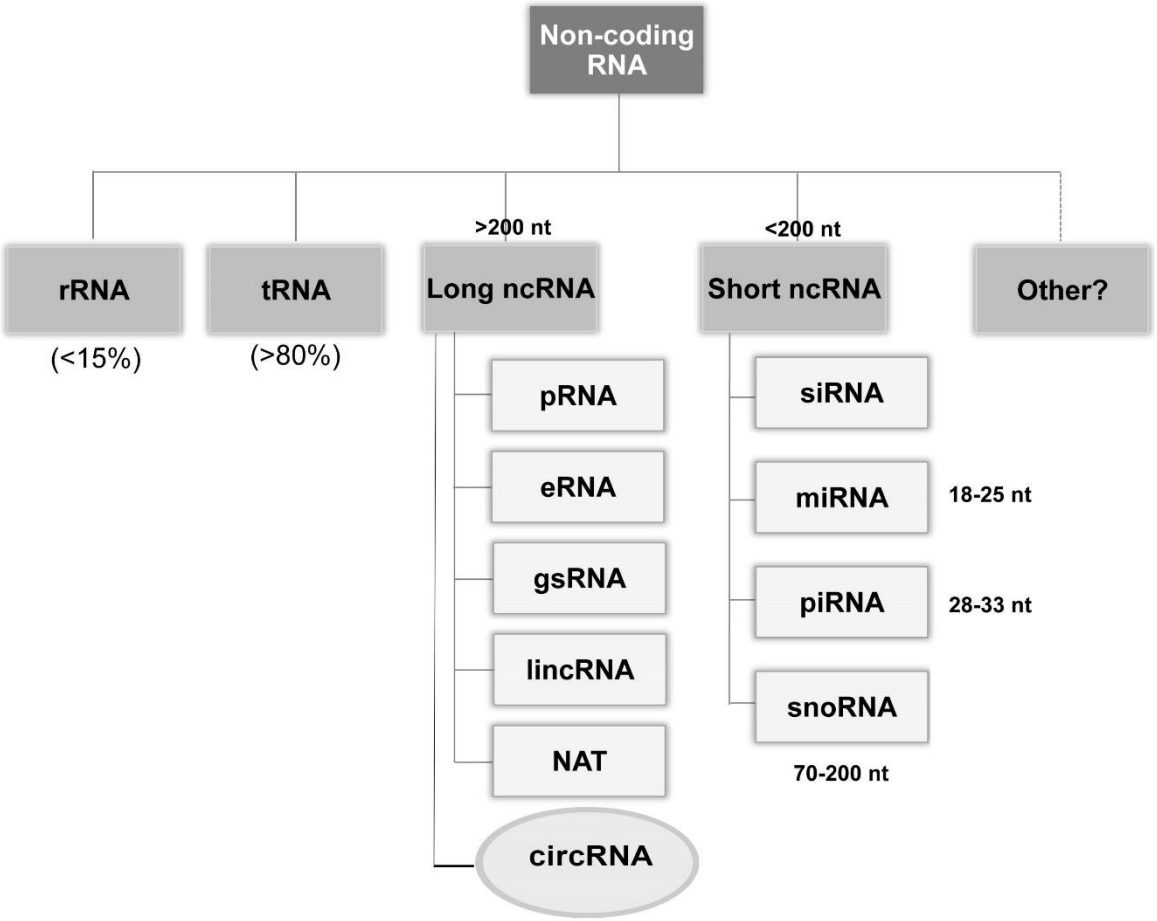


Figure 1.4. Non-coding RNAs grouped by length and function. rRNA and tRNA were each grouped by function. ncRNAs with more than 200 nucleotides were grouped as long ncRNAs and ncRNAs with less than 200 nucleotides were grouped as short ncRNAs. Image adapted from *"Role of Non-coding RNAs in Cystic Fibrosis"* [64].

1.2.3.2. non-coding RNAs in cancer

The roles of ncRNAs in cancer are being gradually revealed. LncRNAs are considered important components in cancer biology, participating in cancer cell related signalling with dysregulated expression observed in cancer [65, 66]. SiRNAs are currently proposed as tools in targeted cancer therapies that aim to prohibit the expression of crucial oncogenes. Evidence also points to an association between circRNA expression and multiple cancers, mainly by functioning as miRNA sponge inhibitors [67]. A miRNA sponge inhibitor is a vector that contains repetitive short sequences that binds to a certain miRNA, and therefore functions as a competitive inhibitor [67]. MicroRNAs (miRNAs) are well characterised short ncRNAs and have been revealed as key regulators of oncogenesis in the past two decades. This work is focused on the participation of miRNAs in oncogenic regulation.

1.3. MicroRNA and cancer

1.3.1. What is a microRNA?

As previously described, miRNAs are a type of small RNA of approximately 22 nucleotides that are abundantly expressed across organs and species. 2588 mature miRNAs have been officially identified in the human genome [68]. An additional 3707 novel mature miRNAs had been identified in 2015 with potentially more still to be found [69]. Typically in animals, a miRNA gene is first transcribed by RNA polymerase II into primary miRNA (pri-miRNA), which is a long single strand RNA of approximately 300 to 1000nt. Drosha (a member of the RNA polymerase III family), together with DGCR8, binds and processes the pri-miRNA into hairpin structures enabling cleavage of these hairpins to produce a precursor miRNA (pre-miRNA) of ~70nt. The pre-miRNA is then

exported from the nucleus to the cytoplasm by Exportin5 (XPO5) and then cleaved into a ~22bp miRNA/miRNA* duplex by Dicer. One strand of the miRNA/miRNA* duplex binds the miRNA induced silencing complex (miRISC) and functions as mature miRNA, while the other stand is normally degraded (Figure 1.4) [70].

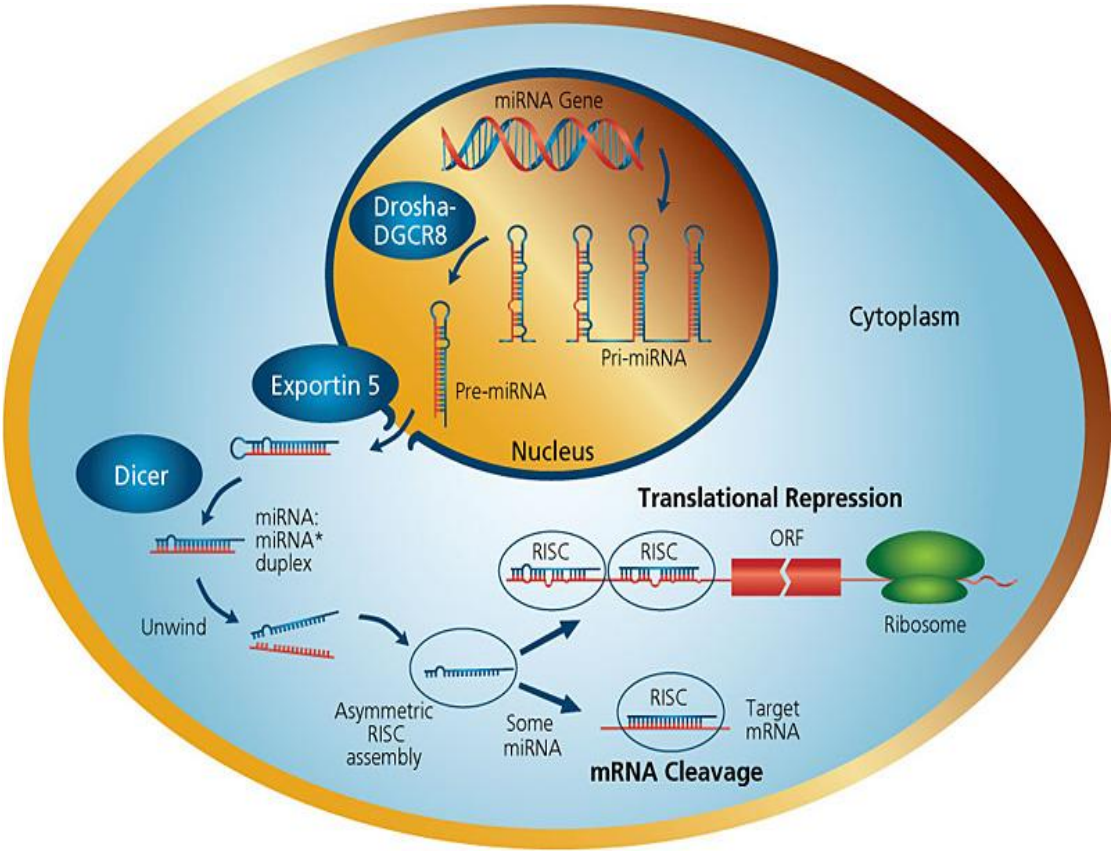


Figure 1.5. Biogenesis and function of miRNAs [71].

Mature miRNAs function primarily as negative regulators of gene expression by binding to specific target sequences located in the 3' untranslated region (3'UTR) of target mRNAs, which promotes transcript degradation or inhibits mRNA translation [71]. Transcript degradation occurs in the case of perfect pairing between the miRNA seed sequence (which is essential for miRNA-mRNA binding, usually located 2-7 nt from the

miRNA 5' end) and the binding site, whereas translational repression of the mRNA by the miRISC complex occurs if the seed sequence only partially matches the binding site. The miRISC protein complex, which is composed of argonaute 2 (AGO2) and GW182 in mammals, plays a key role in directing and binding the miRNA to its target mRNAs [71]. Later studies revealed that in rare cases, miRNAs could also bind to the open reading frame (ORF) [72, 73] and the 5' untranslated region (5'UTR) [74] of genes. Interestingly, unlike the other types of binding between miRNA and target mRNA that result in reduced protein expression, 5'UTR binding can lead to enhanced target gene expression [74].

MiRNA expression and activity can be regulated at different levels. About 60% of miRNAs are located in a host protein-coding gene and co-transcribed with their host genes. The transcription of some miRNAs can be also activated independently from host genes. In addition, some miRNAs are not located outside of protein coding genes and are transcriptionally regulated via their own promoters. The regulation of miRNA biogenesis also accounts for the modulation of miRNA expression, where the repression or activation of Drosha, Dicer and XPO5 can affect the overall level of mature miRNAs. After being processed into mature miRNAs, the stability and degradation of miRNAs can be affected by RNA editing [75, 76] or small RNA degradation nucleases (SDNs) [77, 78]. Moreover, the targeting efficiency of miRNAs can also be regulated by Argonaute activity and by RNA binding proteins, which may block the binding site from the miRISC complex and thus inhibit the function of miRNAs.

1.3.2. OncomiRs and tumour repressor miRNAs

Similar to oncogenes, oncomiRs are miRNAs that facilitate the initiation and development of cancer. Over-expression of oncomiRs down-regulates the expression of target tumour suppressor genes and represses anti-oncogenic pathways. For example, levels of miR-17-92 were found to be increased in aggressive small cell lung cancer in 2005, caused by frequent amplification of the miR-17-92 cluster. Later studies identified that the miR-17-92 cluster promotes cancer by targeting tumour suppressors, including *CDKN1A* and *PTEN,* as direct targets [79]. miR-155, miR-21 and miR-221 also have been reported as oncomiRs which target *PTEN*, *TP53* and other tumour suppressors [80].

Tumour suppressor miRNAs are miRNAs that target oncogenes and thereby repress oncogenesis and cancer progression. The endogenous expression of these miRNAs is usually down regulated in cancers, accompanied by elevated expression of target oncogenes and signalling pathways. One example is the let-7 family, which targets multiple oncogenes including *EGFR, RAS, MYC* and *CCND2* [81-83]. MiR-15 and miR-16 have been shown to induce apoptosis by targeting *BCL2* [84]. By targeting VEGF-A and PIK3R2, miR-126 also represses cancer progression [80, 85].

Notably, since miRNAs have multiple targets, their biological roles are highly context-dependent and can play dual roles in cancer according to the cellular microenvironment and tissue type. For example, miR-125b has been recognized as an oncomiR in haematological cancers [86, 87] but as a tumour suppressor in some solid tumours [88-91]. The experimentally verified targets of miR-125b include both

oncogenes and tumour suppressors, which explains the contradicting outcome [92]. Similarly, mir-663 has been reported to be an oncomiR that targets HSPG2 and induces chemo resistance in breast cancer [93], while functioning as a tumour suppressor by targeting p21 in nasopharyngeal carcinoma [94]. To determine the overall effect of a miRNA in cancer development, valid target genes of the miRNA should be grouped by functions, and the abundance of these targets will be important for the holistic analysis. Extrinsic factors should also be taken into consideration of this network [92]. The general impact of miRNAs can also vary due to the dual functions of major target genes, which commonly occurs in cancer related immune signalling [95, 96] and tumour stroma [97, 98]. Moreover, organ environment specificity and the regulatory network of miRNA expression also contribute to the complexity of the functional effect(s) of a miRNA.

1.3.3. Clinical utilization of miRNAs in cancer

Since many miRNAs show distinctive expression patterns in cancer, researchers have proposed their use as potential biomarkers for cancer diagnosis and prognosis [99]. In breast cancer for example, miR-21, miR-106 and miR-155 were found to be highly expressed in malignant tissue specimens compared with normal tissue specimens, while miR-126, miR-199 and miR-335 were significantly decreased [100]. MiR-21 was also suggested as prognostic marker for breast cancer metastasis [101]. Importantly for their use as biomarkers, miRNAs can be readily detected in serum, plasma and other readily accessible body fluids. Enders et al [102] suggested that plasma miR-451 and miR-145 could be used as combined markers for early stage breast cancer screening. Another study analysed the serum miRNA profile in breast cancer patients with receiver operating characteristic (ROC) curve analysis and found that serum miR-145, miR-155 and miR-

382 could distinguish breast cancer patients from normal healthy people [103]. The detection of circulating miRNA markers may provide an easy, early screening and monitoring strategy for cancers, and provide more personalized insight into individual cancer incidence.

MicroRNAs could also be targeted as a therapeutic approach. Hypomethylating agents can be used to reverse the epigenetic silencing of tumour suppressor miRNAs [104, 105], and oligonucleotide based approaches may also be used to restore the level of such miRNAs [106, 107]. To block the activity of oncomiRs, multiple oligonucleotide based methods have been developed, including LNA [108], tiny LNA [109], antagomiRs [110], miRNA sponges [111] and small molecule targeting miRNAs (SMIRs) [112]. A LNA-based antisense molecule targeting miR-122 has been developed by Santaris Pharma and evaluated in clinical trials for hepatitis C treatment, which could also reduce rates of hepatocellular carcinoma [108]. However, as the functional target of miRNA can be highly context dependent, the potential for off-target effects of miRNAs in clinical use has raised concerns [113].

1.4. The miRNA-mediated moderation of the p53 signalling network.

This part of introduction is written as a manuscript of review for publication.

Statement of Authorship

Title of Paper	The microRNA-p53 network: a game of balance
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Manustript prepared for publication

Principal Author

Name of Principal Author (Candidate)	Qingqing Wang
Contribution to the Paper	Reviewed the literature background and the progress of the field and wrote the manuscript.
Overall percentage (%)	80%
Certification:	This paper reviewed the literature background and the progress of the field during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 28/8/17

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Luke Selth
Contribution to the Paper	Supervised the progress, helped with manuscript preparation.
Signature	Date 28/8/17

Name of Co-Author	David Callen
Contribution to the Paper	Supervised the progress, helped with manuscript preparation.
Signature	Date 28/8/17.

The microRNA-p53 network: a game of balance

Qingqing Wang[1,2], Luke A Selth[2, 3], David F Callen[1]

[1] *Breast Cancer Genetics Group, Centre for Personalised Cancer Medicine, Adelaide Medical School, University of Adelaide, South Australia.*

[2] *Dame Roma Mitchell Cancer Research Laboratories, Adelaide Medical School, University of Adelaide, South Australia.*

[3] *Freemasons Foundation Centre for Men's Health, Adelaide Medical School, University of Adelaide, South Australia.*

CORRESPONDING AUTHORS

David Callen: Breast Cancer Genetics Group, Centre for Personalised Cancer Medicine, School of Medicine, Level 8 AHMS Building, The University of Adelaide, SA 5000, Australia. Phone: +618 8222 3145. E-mail: david.callen@adelaide.edu.au

Luke Selth: Dame Roma Mitchell Cancer Research Laboratories, School of Medicine, Level 8 Level 8 AHMS Building, The University of Adelaide, SA 5000, Australia. Phone: +618 8222 3618. Fax: +618 8222 3217. E-mail: luke.selth@adelaide.edu.au

Keywords: microRNA, p53, mutant p53, cancer

Abstract

Tumour protein p53, a transcription factor that participates in multiple cellular functions, is considered the most important tumour suppressor in the cell. The dysregulation of p53 abundance and activity is closely related to tumorigenesis and tumour progression; indeed, the *TP53* gene is mutated in over 50% of cancers. Emerging evidence suggests that MicroRNAs (miRNAs) represent another important mechanism controlling the expression and activity of p53 in cancer. This relationship is two-way, since both wild-type and mutant p53 participate in the regulation of miRNAs at the level of biogenesis and transcription. In this review, we will summarize the state of play of miRNA: p53 regulatory networks, and discuss the implication of such regulation in cancer. Furthermore, we explore the progress in miRNA mediated regulation of mutant p53, and highlighted the influence of polymorphism and 3'UTR mutations of p53.

1. MiRNA biosynthesis and function in cancer

MicroRNAs (miRNAs) are a type of non-coding RNA of approximately 22 nucleotides that mainly function as negative regulators of gene expression (Bartel 2004). Initially, miRNA genes are transcribed into primary miRNAs (pri-miRNAs), which assemble into hairpin structures with the assistance of DGCR8, Drosha, p68 and p72, ultimately resulting into cleavage of pri-miRNAs into precursor miRNAs (pre-miRNAs). By binding Exportin 5 (XPO5), pre-miRNAs are then exported into the cytoplasm where they are processed into ~22 bp mature miRNA by Dicer, and bind the miRNA induced silencing complex (miRISC). miRISC, which is comprised of argonaute 2 (AGO2) and GW182 in mammals, plays a key role in directing binding of the mature miRNA to the binding site on the target mRNA, generally located at the 3' untranslated region (3'UTR) (Bartel 2004). If perfect pairing exists between the miRNA seed sequence and its binding site, the interaction can result in degradation of the mRNA by AGO2. Alternatively, if there is partial matching between the seed sequence and the binding site the result is translational repression of the mRNA.

Accumulating evidence indicates the important role of miRNAs in cancer related regulation. Some miRNAs are aberrantly expressed in cancer and have been nominated as potential tumour markers. For instance, miR-21 (Krichevsky and Gabriely 2009; Liu, Lu et al. 2017) and miR-155 (Mashima 2015; Due, Svendsen et al. 2016; Kapodistrias, Mavridis et al. 2017) are both found highly expressed in multiple cancers, and their levels associate with disease reoccurrence and poor prognosis. Functionally, miRNAs have now been shown to participate in all the classic hallmarks of cancer (Hanahan and Weinberg 2011), with assigned roles as tumour suppressor miRNAs or oncomiRs by targeting

known oncogenes or tumour suppressors respectively. Several cancer related signalling pathways, including the p53 signalling pathway, have been shown to be regulated by miRNAs through multiple factors in the pathway. However, since a single miRNA can influence the expression of multiple genes with opposing functions in a pathway the resulting impact of these miRNAs in a particular cancer cell can be difficult to ascertain and be context dependent.

2. Tumour suppressor p53

p53 is a nuclear phosphoprotein of 393 amino-acids, which is encoded by the *TP53* gene located at 17p13.1 (Isobe, Emanuel et al. 1986). Wild-type p53 contains 5 domains: the N-terminal transactivation domain (TAD), the proline-rich region (PRR) (Venot, Maratrat et al. 1998), the central DNA-binding core domain (DBD), the C-terminal domain and the homo-oligomerisation domain (OD) (Harms and Chen 2005; Joerger and Fersht 2010). The DBD is essential for p53 to perform its function as a transcription factor.

Although p53 was initially considered as an oncogene (Crawford 1985; Jenkins, Rudge et al. 1985), Baker's group discovered the common existence of *TP53* mutations in 75% of colorectal cancer and first reported the association between cancer and inactivation of wild-type p53 (Baker, Fearon et al. 1989). In 1990, patients with constitutional *TP53* mutations were found to have greatly increased risks of cancer (Malkin, Li et al. 1990; Srivastava, Zou et al. 1990). Since then, somatic *TP53* mutations has been found to occur at a frequency of around 50% in all cancer types (Levine 1997), and the role of wild-type p53 confirmed as a key tumour suppressor. The most common

mutation of p53 in cancer is the missense mutation, which is a single nucleotide mutation located in the DBD of p53. Missense mutations of p53 often result in gain-of-function or loss-of-function, and enable mutant p53 to act like an oncogene. The major tumour suppressor role of p53 is driven by response to a variety of cellular stress signals that results in transcriptional activation or repression of downstream effectors (Ho and Benchimol 2003). As a tumour suppressor, one of the most important functions of p53 is as a cell cycle regulator to prevent inappropriate cell proliferation. p53 negatively regulates cell G1/S and G2/M transition through multiple targets, including p21 (el-Deiry, Tokino et al. 1993), GTES1 (Brown, Boswell et al. 2007), GADD45A (Taylor and Stark 2001) and 14-3-3sigma (Hermeking and Benzinger 2006; Kuroda, Aishima et al. 2007). The second core function of wild-type p53 is to induce programmed cell death (apoptosis). The pro-apoptotic Bcl2 family, consisting of Bax (Miyashita, Krajewski et al. 1994), PUMA (Nakano and Vousden 2001), Noxa (Oda, Ohki et al. 2000) and Bid (Sax, Fei et al. 2002), are transcriptionally activated by p53, leading to activation of cytochrome-C and apoptosis (Juin, Hunt et al. 2002). Other pro-apoptotic effectors, such as Apaf-1 (Kannan, Kaminski et al. 2001; Robles, Bemmels et al. 2001), p53DINP1(Okamura, Arakawa et al. 2001), p53AIP1 (Oda, Arakawa et al. 2000) and PIDD (Attardi, Reczek et al. 2000), also contribute to the apoptosis mediated by p53 (Fridman and Lowe 2003). p53 can also facilitate apoptosis in a transcript-independent manner by translocating to the mitochondria and directly inducing cell apoptosis (Caelles, Helmberg et al. 1994; Marchenko, Zaika et al. 2000). Recent studies reveal diverse additional functions of p53, including functions in cell stemness, tumour metastasis, epithelial–mesenchymal transition (EMT) (Chang, Chao et al. 2011; Schubert and Brabletz 2011), tumour angiogenesis and cellular senescence (Burns, D'Ambrogio et al. 2011).

3. MiRNAs regulated by wild-type p53

As a transcription factor, p53 plays a key role in the regulation of miRNAs at both the level of transcription and maturation.

Several miRNA have been shown to be induced by p53 and, in common with p53, also have tumour suppressor function. The miR-34 family, consisting of miR-34a/b/c, was reported as the first p53-induced miRNA via direct promoter activation. These induced miR-34 family members also enhance p53-induced growth inhibition and apoptosis by targeting Bcl2, cyclin E2, CDK4 and CDK6 (He, He et al. 2007; Raver-Shapira, Marciano et al. 2007).

Similarly, miR-107 expression is also directly regulated by p53 and can repress tumour growth and angiogenesis by targeting hypoxia inducible factor-1beta (HIF-1beta) *in vitro* and *in vivo* (Yamakuchi, Lotterman et al. 2010). Wild-type p53 also directly activates the expression of miR-145, which functions as a tumour suppressor by targeting c-Myc (Sachdeva, Zhu et al. 2009). MiR-26a and miR-16 has been shown to be another targets of p53, and enhance the p53 mediated response to genotoxic stress by targeting Chk1 and Wee1 (Lezina, Purmessur et al. 2013). Wild-type p53 is able to reduce cell growth and EMT by reducing expression of ZEB1, ZEB2 and multiple cell cycle regulators, which can be mediated by p53 up-regulating the miR-200 family (miR-200a/b/c, mir-141 and miR-429) and the miR-192 family (miR-192,194,215) that target ZEB1 and ZEB2 (Kim, Veronese et al. 2011; Hermeking 2012). MiR-1246 is also a p53-induced miRNA and represses the cell proliferation of human hepatocellular carcinoma

cells by targeting NFIB (Zhang, Liao et al. 2011; Zhang, Cao et al. 2015). Although additional miRNAs have been shown to be positively regulated by wild-type p53, such as miR-375 (Hao, Lou et al. 2017), miR-17-5p (Hao, Wei et al. 2017), miR-1915-3p (Wan, Cui et al. 2017), miR-199a-3p (Yang, Xu et al. 2017), miR-7 and miR-182 (Blume, Hotz-Wagenblatt et al. 2015), and would be predicted to be tumour suppressors by contributing to the regulation of p53 downstream pathways, functionally they have been reported as oncomiRs by targeting tumour suppressor genes in the cellular context (Figure 1).

A few miRNAs have been found negatively regulated by p53. The well-known oncomiRs miR-17-92 cluster is a direct target of p53, and is transcriptionally repressed in hypoxic environment to facilitate the DNA damage response (Yan, Xue et al. 2009). MiR-224 and miR-502 are repressed targets of p53, and their down-regulation results in blockage of cell proliferation (Hermeking 2012; Liang, Yao et al. 2013). miR-100, miR-146a, miR-221 and miR-150 are also found negatively regulated by p53 via disturbed NFκB p65/RelA activity in HeLa cells, but their function in cancer remains to be verified (Ghose and Bhattacharyya 2015) (Figure 1).

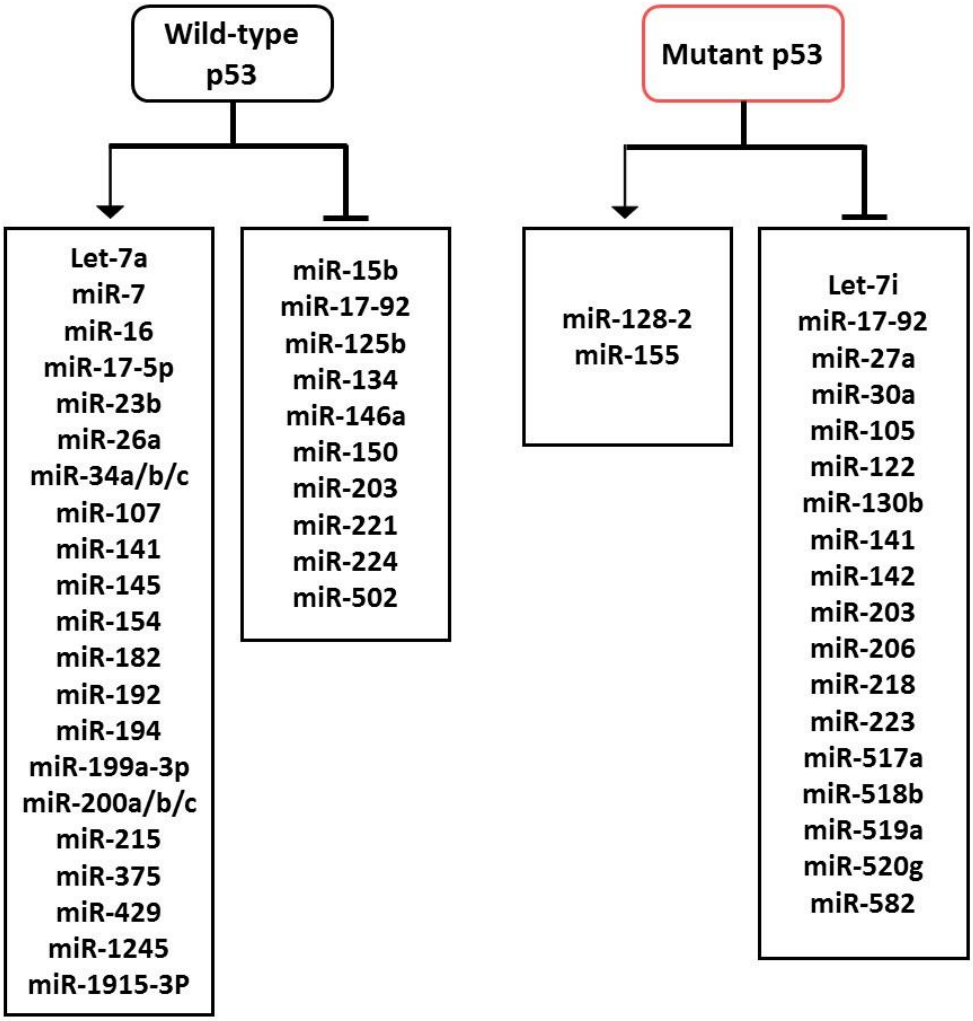


Figure 1. MicroRNA are regulated by wild-type and mutant p53.

4. MiRNAs participate in the regulation of wild-type p53

As an essential gatekeeper that maintains normal cell function, the level and activity of p53 is strictly modulated by multiple factors including miRNAs.

4.1. MiRNAs that directly regulate p53

Post-transcriptionally, several miRNAs and miRNA families have been reported to directly regulate p53 (Figure 2). MiR-125b directly binds to the 3'UTR of *TP53* mRNA to reduce its expression (Le, Teh et al. 2009). A further study explored the role of miR-125b in p53 signaling across species by using a gain- and loss-of-function screen. This study identified 20 novel targets of miR-125b that were also down-stream factors of p53 signaling, suggesting that miR-125b also participates in the down-stream pathways of p53 to repress p53 network activity (Le, Shyh-Chang et al. 2011). Subsequently, there have been multiple additional miRNAs reported that directly target the p53 3'UTR and decrease p53 expression (Figure 2). These miRNAs, such as miR-125b, miR-380-5p (Swarbrick, Woods et al. 2010) and miR-24 (Chen, Luo et al. 2016), usually have functions as oncomiRs, which is consistent with the inactivation of p53-anticancer pathways. However, several other miRNAs that target p53 have reported dual roles in cancer. For example, miR-504 functions as an oncomiR by targeting p53 (Hu, Chan et al. 2010), but was also found to be a tumour suppressor and repress cell proliferation in human glioma (Cui, Guan et al. 2016). MiR-33 decreases sensitivity to radiation and stem cell self-renew by repressing p53 (Herrera-Merchan, Cerrato et al. 2010; Wolfe, Bambhroliya et al. 2016), but reduces oncogenesis in melanoma by targeting HIF-1a (Zhou, Xu et al. 2015). Similarly, miR-150 (Zhang, Wei et al. 2013; Wang, Ren et al. 2015; Xu, Zhou et al. 2016), miR-375 (Liu, Xing et al. 2013), miR-181a (Galluzzi,

Morselli et al. 2010; Cao, Zhao et al. 2017; Chang, Chen et al. 2017) and miR-214 (Wang, Lv et al. 2015; Jiang, Yao et al. 2016) have oncomiR roles by targeting p53 in lung, breast and gastric cancer models, but also function as tumour-suppressors in other cancer models. These opposing roles of micro-RNAs may result from the particular molecular context of organ-specific cellular environments, and the overall biological outcomes of the miRNA would be ideally verified by *in vivo* experiments.

Interestingly, self-regulatory feedback loops have been reported in miRNA-p53 regulation. MiR-150 (Ghose and Bhattacharyya 2015) and miR-141 (Gordon, Yan et al. 2015) are both direct targets of p53 and activated by p53 but also both target and reduce p53 expression. Thus, miR-150 and miR-141 are upregulated after p53 activation, leading to subsequent p53 repression, and therefore contribute to the transient nature of p53 expression and activity in the normal cellular environment.

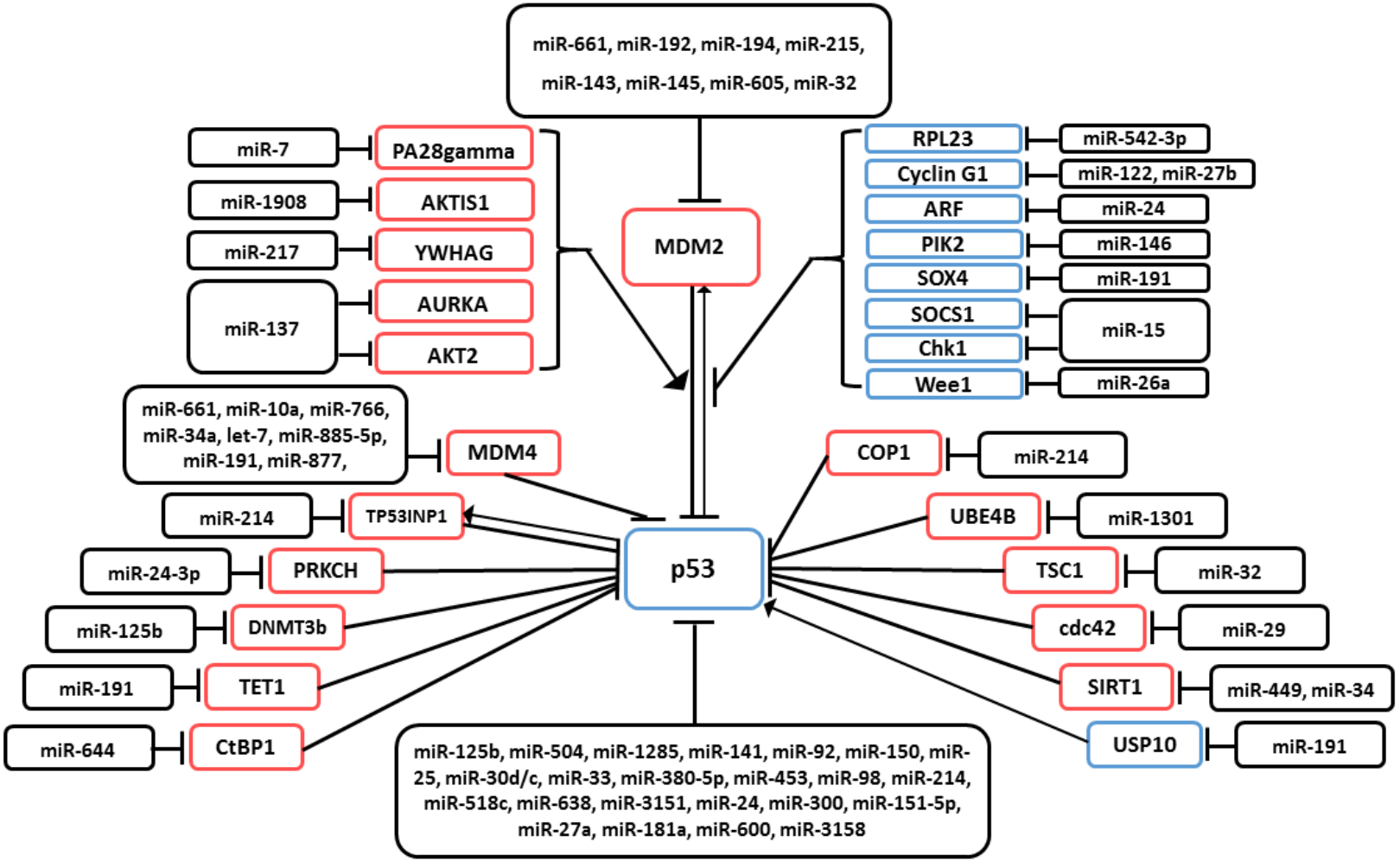


Figure 2. MiRNAs regulate p53 directly and indirectly.

4.2. MiRNAs that indirectly regulate p53

There has been described miRNAs indirectly involved in the regulation of p53 network via the modulation of other post-transcriptional regulators of the p53 signalling pathway (Figure 2).

4.2.1. Regulation of p53 by direct miRNA targeting of MDM2 and MDM4

Wild-type p53 is a very unstable protein with a half-life of only a few minutes (Rogel, Popliker et al. 1985). This ensures low levels of p53 in the absence of cellular stresses. The degradation of p53 is proteasome-dependent and requires the ubiquitination of p53 via the specific E3 ubiquitin ligase MDM2. MDM2 is activated by p53, and this feed-back loop plays a key role in regulating the stability of p53 (Nag, Qin et al. 2013). The C-terminal RING finger domain of MDM2 provides the E3 ubiquitin ligase activity and ubiquitinates p53 (Fang, Jensen et al. 2000; Nakamura, Roth et al. 2000; Lai, Ferry et al. 2001) by direct binding to the N terminal BOX1 domain of p53 (Kubbutat, Ludwig et al. 1998; Bottger, Bottger et al. 1999). MDM4, a protein sharing structural homology with MDM2, is also intimately involved in determining p53 stability by binding directly to p53, inhibiting p53 activity as a transcription factor, and thus influencing the MDM2-p53 feedback loop (Shvarts, Steegenga et al. 1996; Stad, Ramos et al. 2000). MDM4 also stabilizes MDM2 by forming a heterodimer via the MDM2 RING domain (Sharp, Kratowicz et al. 1999; Tanimura, Ohtsuka et al. 1999) leading to enhanced p53 degradation (Linares, Hengstermann et al. 2003; Wang, Wang et al. 2011). The MDM2-MDM4-p53 axis allows an efficient activation of p53 during cellular stress, and also allows p53 levels to rapidly return to normal when the stress is removed (Toledo and Wahl 2007; Nag, Qin et al. 2013).

Recently a group of miRNAs were described that contribute to the post-transcriptional regulation of p53 via regulation of MDM2 and/or MDM4. The inhibition of both MDM2 and MDM4 by these miRNAs results in upregulation of p53 and strengthening of p53 signalling. For example, MDM2 is directly regulated by the miR-192 family (Moore, Ooi et al. 2015), miR-605 (Xiao, Lin et al. 2011) and several other miRNAs (Figure 2), and the repressed MDM2 expression leads to increased p53 expression and activation of p53 downstream genes. Similarly, MDM4 is directly targeted by miR-10a (Ovcharenko, Stolzel et al. 2011), miR-766 (Wang, Selth et al. 2017) and let-7 (Xie, Chen et al. 2015), and the decreased MDM4 level consequently causes the activation of p53 transactivation. MiR-885-3p is also involved in the MDM4 regulation, but elevates MDM4 expression by targeting the 5'UTR of MDM4, and results in suppression of p53 and p53-dependent apoptosis (Huang, Chuang et al. 2011). MiR-661 also functions as a p53 activator by targeting both MDM2 and MDM4, which explains its association with good prognosis in breast cancer (Hoffman, Bublik et al. 2014) (Figure 2). Interestingly, a recent study shows single nucleotide polymorphism (SNP) variation of MDM4 can create an illegitimate 3'UTR binding site for miR-191, and allows miR-191 mediated MDM4-p53 regulation in ovarian cancer (Wynendaele, Bohnke et al. 2010). Furthermore, another MDM4 SNP rs4245739, allows direct repression of MDM4 by miR-191-5p and miR-877 in prostate cancer (Stegeman, Moya et al. 2015). These studies highlight the impact of genetic variation on miRNA mediated regulation.

In addition to the direct miRNA-p53 regulatory feedback loops, the miR-192 family, miR-143/145 and miR-605 have been reported to increase p53 levels by targeting MDM2 (Xiao, Lin et al. 2011; Zhang, Sun et al. 2013; Moore, Ooi et al. 2015; Xu, Zhang et al. 2017), while being also regulated by p53 (Sachdeva, Zhu et al. 2009; Kim, Veronese et al. 2011; Zhou, Zhang et al. 2014; Cabrita, Bose et al. 2017). The p53 induced miR-34a also directly targets MDM4 via a binding site located in the MDM4 ORF, and functions as a activator of p53 in a miR-34a-MDM4-p53 feedback loop (Mandke, Wyatt et al. 2012).

4.2.2. Regulation of p53 by indirect miRNA targeting of MDM2 and MDM4

By targeting MDM2, the 60S ribosomal protein L23 (RPL23) blocks MDM2 mediated p53 ubiquitination and degradation, and therefore influences p53 stability and consequently its activity (Dai, Zeng et al. 2004). Thus, over-expressed RPL23 *in vitro* leads to p53 dependent cell cycle arrest (Jin, Itahana et al. 2004). Wang et al reported RPL23 as a direct target of miR-542-3p and over expression of this miRNA upregulated p53 signalling in cancer cells (Wang, Huang et al. 2014).

Another protein indirectly influencing p53 levels is PA28gamma, a proteasome activator that enhances MDM2-induced p53 degradation (Zhang and Zhang 2008). MiR-7 increases p53 expression by targeting PA28gamma in breast and lung cancer (Xiong, Zheng et al. 2014; Shi, Luo et al. 2015). Another protein indirectly involved in the p53 axis is cyclin G1 which interacts with MDM2 to protect p53 from MDM2 mediated degradation (Kimura and Nojima 2002). By targeting cyclin G1, miR-122 inhibits the binding of phosphatase 2A (PP2A) which is capable of dephosphorylating MDM2 and

enhancing the p53 pathway in hepatocellular carcinoma cells (Fornari, Gramantieri et al. 2009). It is also reported that miR-27b can increase the sensitivity to p53 related chemotherapy in SGC7901/VCR cells via the same Cyclin G1-MDM2-p53 mechanism (Shang, Feng et al. 2016).

The ARF tumour suppressor is another important p53 regulator that binds to MDM2 and forms a $p14^{ARF}$-MDM2-p53 complex (Stott, Bates et al. 1998), that both disturbs the MDM2 mediated nuclear export of p53 and stabilizes p53 (Holmberg Olausson, Nister et al. 2012). MiR-24, as well as directly targeting p53 (Chen, Luo et al. 2016), also targets $p14^{ARF}$ resulting in decreased levels of this protein, thereby suppressing p53 signalling (To, Pajovic et al. 2012), and further reinforcing the p53 inhibition effect of miR-24 *in vitro* (Chen, Luo et al. 2016).

There are several additional reports of miRs that target p53 indirectly. These include Plk2, a protein that can stabilize p53 via the p16Ink4a/p19Arf pathway in mice (Deng, Wang et al. 2017) that is targeted by miR-146. A further example is miR-137, a FoxD3 regulated miRNA that is down-regulated in hepatocellular carcinoma. The decrease of miR-137 leads to enhanced MDM2 activity via its target gene *AKT2* with subsequent repression of p53 *in vitro* and *in vivo* (Abraham and O'Neill 2014; Liu, Lu et al. 2014). In the same pathway, AKT1 substrate 1 (*AKT1S1*), a negative regulator of p53 via MDM2-p53 regulation, is also a target of miR-1908, which identifies miR-1908 as a potential tumour suppressor miRNA in non-small cell lung cancer (Ma, Feng et al. 2016).

A recently described DNA damage sensor, sex-determining region Y-related high-mobility group (HMG) box 4 (SOX4), was found to respond to DNA damage stress in an ATM/ATR dependent manner (Pan, Zhao et al. 2009). SOX4 activates and stabilizes p53 by binding the regulatory domain (359–393 amino acids) of p53 and disrupting the MDM2-p53 interaction (Pan, Zhao et al. 2009). Targeting *SOX4* is miR-191 and is reported to repress p53 signalling and cell apoptosis in breast cancer (Sharma, Nagpal et al. 2017). However in ovarian cancer (Wynendaele, Bohnke et al. 2010) and intrahepatic cholangiocarcinoma (Li, Zhou et al. 2017) miR-191 has been shown to have the opposite role in p53 regulation by targeting *MDM4* and *TET1*, suggesting that the decision making of p53 regulation by miR-191 may vary depend on organ specificity and cell context. As all the three studies of miR-191 were established in cell lines only, how this miRNA will modulate p53 signaling *in vivo* is unclear.

The YWHAG protein is an enhancer that can accelerate MDM4 phosphorylation and thereby increase the degradation of p53. By targeting *YWHAG*, miR-217 protects p53 from MDM4 mediated inhibition and reduces glioblastoma cell proliferation *in vitro* and *in vivo* (Wang, Zhi et al. 2017).

4.2.3. MiRNA regulation of p53 post-translational modifications

The phosphorylations of p53, and therefore the relevant kinases, play major roles in the regulation of p53. These kinases, such as ATM/Chk2 and ATR/Chk1, induce the phosphorylation of p53 under DNA damage stress causing structural transition of p53 resulting in its reduced binding to MDM2 (Shieh, Ikeda et al. 1997; Chehab, Malikzay et al. 2000), blockade of p53 ubiquitination and nuclear export (Zhang and Xiong 2001;

Shiloh and Ziv 2013). AURKA is a protein that represses the ATM/Chk2 mediated p53 phosphorylation, and is reported to be a direct target of miR-137 (Qin, Zhang et al. 2017). In hepatocellular carcinoma, miR-137 is often down-regulated, is related to poor prognosis of HCC, and is confirmed to target AURKA directly. Overexpression of miR-137 results in reactivation of the ATM/Chk2 pathway and consequently increased p53 signaling *in vitro* and *in vivo* (Qin, Zhang et al. 2017).

Additional miRNAs reported to influence p53 modifications include miR-19, miR-155, miR-15 miR-17-5p and miR-106b-5p. MiR-19 and miR-155 inhibit p53 signalling by directly targeting SOCS1, which contributes to p53 activation and phosphorylation on serine 15 by forming a ternary complex with ATM or ATR in myeloma cells (Mignacca, Saint-Germain et al. 2016). In addition, the p53-induced miRNAs, miR-15 family (Pouliot, Chen et al. 2012; Mei, Su et al. 2015) and miR-26a (Lezina, Purmessur et al. 2013), were shown to negatively regulate Chk1 and Wee1. The reduction of Chk1 and Wee1 by these miRNAs was accompanied by upregulated p53 signaling and sensitized response to genotoxic therapies in a positive miR-p53 feedback loop. TRIM8 also stabilizes, and hence increases p53 activity, via p53 phosphorylation which results in cell cycle arrest (Caratozzolo, Micale et al. 2012). The N-Myc regulated miR-17-5p and miR-106b-5p were found to directly target TRIM8 and reduced p53-dependent chemotherapy sensitivity in clear cell Renal Cell Carcinoma and colorectal cancer (CRC) (Mastropasqua, Marzano et al. 2017), revealing additional aspect in Myc mediated inhibition of p53 phosphorylation.

Acetylation of the p53 protein functions to stabilize p53 from oncogene-mediated repression. The silent information regulator 1 (SIRT1) induces deacetylation of p53 and reduces p53-mediated apoptosis. MiR-34a has been identified to target SIRT1, thus up-regulating p53 protein levels and rescuing the p53 dependent expression of Bax and Bcl2 resulting in increased apoptosis (Yan, Wang et al. 2016). Within the miR-34a-SIRT-p53 axis, miR-34a has a tumour suppressor role, and provides additional mechanism to the miR-34a-p53 self-regulatory feedback loop (Mandke, Wyatt et al. 2012).

4.2.4. Other miscellaneous mechanisms whereby miRs influence p53 function

The ubiquitination factor E4B (UBE4B) is an E3 and E4 ubiquitin ligase, which can ubiquitinate and degrade p53 independent of MDM2. MiR-1301 was found to directly target UBE4B in breast and prostate cancer, and increases p53 levels and activity (Wang, Wu et al. 2017). In breast cancer, miR-214 targets COP1, an E3 ligase that enables the proteasomal degradation of p53 independent of MDM2 (Zhang, Su et al. 2016). In addition to targeting MDM2, miR-32 also has been reported to target TSC1 and therefore stabilize p53 via mTORC1 activation in the cancer glioblastoma multiforme (Suh, Yoo et al. 2012). Additional reports suggest other miRs are involved in the varied controls of p53 activity. MiR-885-5p has been reported to activate p53 signalling pathways by targeting CDK2 (Afanasyeva, Mestdagh et al. 2011). The high-risk HPV-16 oncogenic proteins are known for repressing the expression of p53, p21 and p16. MiR-139-3p has been shown to be a defender against virus induced oncogenesis by targeting the HPV-16 oncogenic proteins and restoring the p53 anti-cancer pathways (Sannigrahi, Sharma et al. 2017).

Furthermore, several miRNAs mediate indirect p53 regulation by targeting multiple genes that share similar functions, or via a positive feedback loop. The Rho GTPases Cdc42 mediates p53 ubiquitination independent of MDM2 (Ma, Xue et al. 2013), and Cdc42 is targeted by miR-29 (Park, Lee et al. 2009). Interestingly, miR-29 also targets two negative regulators of p53, p85 (Park, Lee et al. 2009) and PPM1D (Ugalde, Ramsay et al. 2011). In addition, by targeting CNOT6, miR-29c-3p also enhances the p53-p21 and p16-pRB pathways (Shang, Yao et al. 2016). The p53 dependent induction of miR-29, together with the multiple targets that are negative regulators of p53, suggests a strong functional feedback loop between miR-29 and p53 expressions. Another example of a feedback loop involves the miR-19a/b and its target the tumour protein 53-inducible nuclear protein 1 (TP53INP1) which is transcriptionally induced by p53. This generates a positive feedback loop for restoring p53 function (Wang, Wang et al. 2016).

MiRNAs also participate in the transcriptional regulation of p53. In adenoid cystic carcinomas, miR-24-3p increase the p53-p21 pathway activity by targeting the protein kinase C eta (*PRKCH*), which restore the PRKCH mediated transcriptional repression of p53 (Zhang, Zhang et al. 2016), and thereby consistent with a tumour suppressor role for miR-24-3p. This is consistent with the reported oncomiR function of miR-24 in hepatocellular carcinoma (Wang, Lv et al. 2015).

The expression of p53 can also be epigenetically regulated, since DNA methylation at the CpG rich island region of *TP53* promoter plays an essential role in its expression. Aberrant methylation of the p53 promoter has been frequently reported in cancer. For

example the ten-eleven translocation 1 (TET1) can demethylate the *TP53* promoter and activate p53 transcription and thus anti-cancer pathways. The over-expressed miR-191 targets TET1 and consequently represses the p53 response by retaining the methylation status of *TP53* (Li, Zhou et al. 2017). DNA (cytosine-5)-methyltransferase 3b (DNMT3b) induces the hypermethylation of *TP53* and epi-genetically repress its expression, and is negatively regulated by miR-125b as a direct target. The repressed DNMT3b by miR-125b results in increased p53 expression and activated pathways in a vascular smooth muscle cell model (Cao, Zhang et al. 2016). However, miR-125b was previously reported as a negative regulator of p53 in cancer. These data suggest that miR-125b may have opposite functions in cancer and normal tissue.

Moreover, there are reports that several oncomiRNAs, including miR-3646 (Tao, Liu et al. 2016), miR-17-92 (Tavakoli, Vakilian et al. 2017) and miR-21 (Liu, Lu et al. 2017), show a negative correlation with p53 expression and p53 signalling strength in cancer, however their overall impact on the regulatory mechanism of p53 is unclear and requires further clarification.

5. MiRNAs and mutant p53

TP53 mutations have been found in more than 50% of human cancers. The most common alterations to p53 in cancer are missense mutations in the DNA binding domain (DBD). These mutations lead to structural deformation of the p53 DBD, resulting in either abolishment of DNA binding or a shift in p53 DNA binding patterns. Several missense mutation hotspots have been found across cancers, including DNA contact mutations R248 and R273, and conformational mutations R175, G245, R249, and R282

(Freed-Pastor and Prives 2012). It has been confirmed that mutant p53 proteins often lose the ability to transactivate the downstream genes of wild-type p53, which is described as a loss-of-function (LOF) (Blagosklonny 1997). On the other hand, previous studies have also indicated that certain missense p53 mutations exhibit oncogenic gain-of-function (GOF) activities by activating a new set of target genes. Such p53 mutations include R273H (Coffill, Muller et al. 2012; Kalo, Kogan-Sakin et al. 2012; Guo, Chen et al. 2016), R248Q (Igarashi, Hirano et al. 2014), R175H (Lang, Iwakuma et al. 2004), G245S and R273C (Nesslinger, Shi et al. 2003).

In this section, we will discuss the current level of understanding of the involvement of miRNAs in the mutant p53 landscape.

5.1. Mutant p53 can influence miRNA regulation

Mutations of p53 participate in the regulation of miRNAs distinctly from wild-type p53 for both LOF and GOF. Wild-type p53 participates in the maturation of miRNAs via interaction with p68, consequently increases Drosha activity, and therefore facilitate the maturation process of miRNAs. Loss of wild-type p53 function has been shown to decrease expression of miR-15a, miR-16-1, miR-143, miR-145, miR-122 and miR-199a (Feng, Zhang et al. 2011; Hermeking 2012). Whether this effect exists in the regulation of other miRNAs as a general response to Drosha, however, is unclear. Lack of wild-type p53 activity may also affect the targeting efficiency of miRNAs by inducing the RNA-binding-motif protein 38 (RBM38). This protein enables both the RNA accessibility for miRNAs (Leveille, Elkon et al. 2011) and facilitates the interaction between AGO2 and mature miRNAs, which allows miRNAs to locate and bind to potential binding sites on

the mRNA (Krell, Stebbing et al. 2016). Moreover in a mutant p53 scenario, those miRNAs that are direct targets of wild-type p53, are likely to experience reduction of expression levels, while the miRNAs down-regulated by wild-type p53 may become upregulated (Figure 1).

Because mutant p53 often loses the normal binding ability and function of wild-type p53, mutant p53 has been shown to impair the maturation of miRNAs by interrupting the interaction between Drosha, p68, p72/82 and pri-miRNAs. The affected miRNAs include miR-517a, miR-519a, miR-105 and miR-218 have been reported (Muller, Trinidad et al. 2014; Garibaldi, Falcone et al. 2016; Gurtner, Falcone et al. 2016). On the other hand, mutant p53 GOF itself can also contribute to miRNA regulation by modulating/influencing a distinct set of miRNA targets. For example, the p53 R175H mutant binds to the host gene of miR-128-2 and induces its expression (Donzelli, Fontemaggi et al. 2012). In addition, miR-223 (Masciarelli, Fontemaggi et al. 2014) and miR-27a (Li, Jones et al. 2014) have both shown to be directly silenced by mutant p53 binding at their respective promoter.

Mutant p53 has also been shown to suppress the maturation of miR-130b, miR-203 and miR-206 by blocking the p63-Dicer activation (Su, Chakravarti et al. 2010; Li, Jones et al. 2014; Li, Han et al. 2015) . Interestingly, the interaction between mutant p53 and AGO2 dissociates AGO2 from miRNAs, which leads to the destabilization and reduction of let-7s levels, while wild-type p53 induce the opposite result by increasing the AGO2-miRNA association (Krell, Stebbing et al. 2016). Mutant p53, through its activation of p63, can also upregulate miR-155 which has an established role as an oncomiR (Neilsen,

Noll et al. 2013). Other miRs the have been shown to be influenced by mutant p53 include miR-520g (Zhang, Geng et al. 2015) and let-7i which are repressed by mutant p53. There is also evidence that miR-30a is down-regulated by p53 R273H via the IGF-1R/AKT pathway, but whether this is specifically related to this particular mutant is unknown (Guo, Chen et al. 2016).

5.2. MiRNAs that regulate mutant p53

MiR-600 has been shown to target mutant p53 in the colorectal cancer cell lines SW480 (R273H and P309S), SW620 (R273H and P309S) and DLD-1 (S241F) (Zhang, Zuo et al. 2017). The p73 activated miR-3158 also directly targets mutant p53 and decreased its expression in the breast cancer cell lines MDA-MB-231 and MDA-MB-468 (Galtsidis, Logotheti et al. 2017). Although it is not yet confirmed if miR-600 and miR-3158 can influence expression of wild-type p53, it is possible that miRNAs could specifically target mutant p53s. It is unlikely that miRNAs can discriminate mutant p53 from wild-type p53 if the specific miRNA binding site on the p53 mRNA is unchanged. However, the mutation of p53 may create a new miRNA binding site or alter mRNA 3D structure and influence miRNA binding accessibility or efficiency. Alternatively, the observed effect may be indirect, with the miRNA influencing expression of a protein that specifically modulates expression of mutant p53 or wild-type p53. However, it is yet to be resolved if miRNA can specifically target a mutant p53.

MiRNAs can also regulate mutant p53 indirectly via other specific regulators of mutant p53. While E3 ligases reduce mutant p53 levels by inducing mutant p53 ubiquitination (Lukashchuk and Vousden 2007; Sheng, Laister et al. 2008), USP10 a

deubiquitinating enzyme, has the opposite effect as it deubiquitinates and stabilizes both wild-type and mutant p53 (Yuan, Luo et al. 2010). MiR-191 directly targets USP10 and impairs the USP10 mediated p53 protection in the pancreatic cancer cell lines PANC-1 (R273H) and SW-1990 (p53 wild-type) (Liu, Xu et al. 2014). Another example of an indirect target of mutant p53 is the transcriptional co-repressor CtBP1 (Bergman, Birts et al. 2009), and its confirmed target miR-644 (Raza, Saatci et al. 2016). By targeting CtBP1, miR-644 has been shown to induce up-regulation of mutant p53 and promote NOXA mediated cell apoptosis in the breast cancer cell lines MDA-MB-231 and MCF7 (Raza, Saatci et al. 2016).

Moreover, MDM2, the key regulator of wild-type p53, partially retains the ability to bind missense p53 and when enforcedly expressed, induces the ubiquitination and degradation of missense p53 (Haupt, Maya et al. 1997; Lukashchuk and Vousden 2007). CHIP, Pirh2 and COP1 are all E3 ligases that have been shown to contribute to mutant p53 ubiquitination, thus consequently reducing mutant p53 levels (Lukashchuk and Vousden 2007; Sheng, Laister et al. 2008). Considering the shared regulatory mechanisms, miRNAs that have been reported to regulate wild-type p53 via MDM2, CHIP, Pirh2 and COP1 pathways are also potentially capable of performing similar roles in the regulation of mutant p53, although there is no current experimental evidence.

5.3. p53 genetic variations and 3'UTR mutations can influence miRNA mediated repression.

Recently, several reports have identified genetic polymorphisms of p53 as a novel mechanism that can influence miRNA mediated p53 regulation. Until 2012, the focus of

p53 sequencing was its mRNA coding sequence (Deng, Becker et al. 2014). However, since this time germ-line polymorphisms within the DNA encoding the p53 3'UTR were reported (Stacey, Sulem et al. 2011; Enciso-Mora, Hosking et al. 2013; Diskin, Capasso et al. 2014; Macedo, Araujo Vieira et al. 2016; Zhao, Li et al. 2016). The single nucleotide polymorphism (SNP) *rs78378222* is located in the 3′UTR of *p53* mRNA and changes the polyadenylation signal from AATAAA to AATACA (Stacey, Sulem et al. 2011). This polymorphism introduces a novel miRNA binding site, which is a perfect match for miR-125b and provides additional strength to the miR-125b mediated p53 repression (Zhao, Li et al. 2016). This *rs78378222* SNP has been shown to be associated with increased risk of skin basal cell carcinoma (Stacey, Sulem et al. 2011), prostate cancer, colorectal adenoma, glioma (Egan, Nabors et al. 2012; Enciso-Mora, Hosking et al. 2013), neuroblastoma (Diskin, Capasso et al. 2014) and esophageal squamous cell carcinoma (Zhou, Yuan et al. 2012; Li, Gordon et al. 2013) and it is possible that the basis of this association is an increased repression of p53.

Cancer specific genetic variations of p53 3'UTR have been reported from genetic sequencing of diffuse large B-cell lymphoma (DLBCL) patients (Li, Gordon et al. 2013). Regardless of the p53 coding sequence, a number of novel mutations were found in the 3'UTR of p53 with a higher frequency than mutations in the coding sequence. Some of these mutations are located at potential miRNA binding sites and therefore it is predicted that there may be alteration of miRNA-p53 targeting. Furthermore, clinical data shows that DLBCL patients with the 3'UTR mutations have a better overall prognosis, suggesting the 3'UTR mutations may function as a positive regulatory factor in p53 signalling network by evading miRNA mediated p53 repression (Li, Gordon et al. 2013).

6. Concluding remarks

Described as a “fine tuner of gene expression” (Seki and Hata 2017), many miRNAs have been identified in cancer to have oncomiRs or tumour suppressor properties. This review focusses on miRNAs that can influence p53 expression and functions in cancer. This miRNA-mediated regulation can be either direct or indirect and can influence either wild-type or mutant p53 or the expression of both.

It is apparent that p53, both wild-type and mutant, has a highly complex interconnecting web of controls that include both classical genes and multitudes of miRNAs. The presence of p53-miRNA and p53-miRNA-p53 regulator feedback loops enables the strict maintenance of the protein level and activity of p53 during normal cellular processes. When these feedback loops are compromised, this imbalances p53 regulation which can lead to oncogenesis.

There are reports of miRNAs (i. e. miR-375 (Costa-Pinheiro, Ramalho-Carvalho et al. 2015) and miR-766 (Li, Li et al. 2015; Wang, Selth et al. 2017)) with conflicting properties, with some reports suggesting an oncogenic role while others a tumour suppressor role. Due to the complexity of the genetic elements that control p53 expression and the impact of the other targets of the miRNA in the cell, the role of a miRNA in cancer is likely to be context specific, which is dependent on the particular molecular environment of the cancer cell.

The majority of the miRNAs reported to interact with the p53 network are from single studies where the expression of miRNAs was modulated in a limited number of cancer cell lines. In some cases the miRNAs are reported to modulate the expression of a protein involved in p53 regulation but the assessment of any effect on p53 expression was not determined. The extent to which such mechanisms can be translated into the complex *in vivo* situation is unclear. The endogenous abundance of the miRNA is likely to effect the biological significance *in vivo*. The functions and activity of the multiple targets of the miRNA may vary due to the interactive network amongst themselves, which is also expected to lead to different *in vivo* outcomes from in vitro results. For example, the miRNA that targets a series of genes that belong to the same pathway *in vitro* is more likely to present the same function *in vivo*.

With the increased understanding of mutant p53 gain-of-function, it is apparent that there are genetic regulatory pathways both limited to mutant p53 and in common with wild-type p53. Such pathways can contribute to the development of cancer at different levels. Disturbing miRNA expression and activity by mutant p53 can bring more down-stream factors as targets of the affected miRNAs into the mutant p53 network, which may further explain the biological impact of mutant p53 gain- and loss- function.

A recent finding, and an aspect that may be of particular impact, is that genetic variation can impact miRNA function. This has been described at the genetic level where disease associated SNPs have been shown to create additional potential binding sites for miRNAs (Zhou, Zhang et al. 2013; Gao, Xiong et al. 2015; Stegeman, Moya et al. 2015). This may provide a functional explanation where disease associated SNPs are located in

chromosomal regions devoid of classical protein encoding genes. Detailed analysis of cancer somatic mutations that are not located in gene ORFs shows that these mutations may have the potential to modulate miRNA activity (Li, Gordon et al. 2013). These mutations may create or delete specific miRNA binding sites thus enhancing or impairing miRNA-mediated gene regulation. Additional detailed analysis of non-coding mutations in cancer integrated with a focus on miRNA binding sites will be required to ascertain if this is a commonly occurring mechanism in cancer.

This review summarizes the current progress in the understanding of the complex network of miRNAs involved in wild-type and mutant p53 signalling and major outstanding issues are highlighted below.

7. Outstanding questions

1) Since AGO2 is an essential for miRNA stability and miRNA targeting activity, how does the interaction between p53 and AGO2 affect global miRNA expression and function?

2) Several miRNAs play dual roles in p53 regulation according to the function of their target genes. What is the cancer molecular context that determines the impact of these miRNAs on p53 function?

3) Several miRNAs are reported to target proteins involved in p53 regulation, but there is no direct evidence showing their impact on p53 protein level and activity. The identity of these miRNAs and their targets and in what context they influence p53 signalling is yet to be resolved.

4) There are overlaps in the mechanisms and pathways between the regulation of wild-type and mutant p53s with numerous miRNAs reported to be directly or indirectly involved. The current studies of these miRNAs do not comprehensively investigate the impacts across both wild-type and the different mutant p53s. How do the regulatory effects of these miRNAs crosstalk between wild-type and mutant p53 and how do they influence p53 related cellular functions when the p53 status is different?

5) Do the cancer mutations in the p53 3'UTR as reported by Li et al. (Li, Gordon et al. 2013) create novel binding sites for miRNAs that is not previously predicted to target p53?

6) The p53 3'UTR mutation was proposed as a self-defence mechanism during oncogenesis of DLBCL (Li, Gordon et al. 2013). How frequent and broadly does occur across different cancers and what is the impact on cancer prognosis?

References

Abraham, A. G. and E. O'Neill (2014). "PI3K/Akt-mediated regulation of p53 in cancer." Biochem Soc Trans **42**(4): 798-803.

Afanasyeva, E. A., P. Mestdagh, et al. (2011). "MicroRNA miR-885-5p targets CDK2 and MCM5, activates p53 and inhibits proliferation and survival." Cell Death Differ **18**(6): 974-984.

Attardi, L. D., E. E. Reczek, et al. (2000). "PERP, an apoptosis-associated target of p53, is a novel member of the PMP-22/gas3 family." Genes Dev **14**(6): 704-718.

Baker, S. J., E. R. Fearon, et al. (1989). "Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas." Science **244**(4901): 217-221.

Bartel, D. P. (2004). "MicroRNAs: genomics, biogenesis, mechanism, and function." Cell **116**(2): 281-297.

Bergman, L. M., C. N. Birts, et al. (2009). "CtBPs promote cell survival through the maintenance of mitotic fidelity." Mol Cell Biol **29**(16): 4539-4551.

Blagosklonny, M. V. (1997). "Loss of function and p53 protein stabilization." Oncogene **15**(16): 1889-1893.

Blume, C. J., A. Hotz-Wagenblatt, et al. (2015). "p53-dependent non-coding RNA networks in chronic lymphocytic leukemia." Leukemia **29**(10): 2015-2023.

Bottger, V., A. Bottger, et al. (1999). "Comparative study of the p53-mdm2 and p53-MDMX interfaces." Oncogene **18**(1): 189-199.

Brown, L., S. Boswell, et al. (2007). "Transcriptional targets of p53 that regulate cellular proliferation." Crit Rev Eukaryot Gene Expr **17**(1): 73-85.

Burns, D. M., A. D'Ambrogio, et al. (2011). "CPEB and two poly(A) polymerases control miR-122 stability and p53 mRNA translation." Nature **473**(7345): 105-108.

Cabrita, M. A., R. Bose, et al. (2017). "The p53 protein induces stable miRNAs that have the potential to modify subsequent p53 responses." Gene **608**: 86-94.

Caelles, C., A. Helmberg, et al. (1994). "p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes." Nature **370**(6486): 220-223.

Cao, C., H. Zhang, et al. (2016). "miR-125b targets DNMT3b and mediates p53 DNA methylation involving in the vascular smooth muscle cells proliferation induced by homocysteine." Exp Cell Res **347**(1): 95-104.

Cao, Y., D. Zhao, et al. (2017). "MicroRNA-181a-5p Impedes IL-17-Induced Nonsmall Cell Lung Cancer Proliferation and Migration through Targeting VCAM-1." Cell Physiol Biochem **42**(1): 346-356.

Caratozzolo, M. F., L. Micale, et al. (2012). "TRIM8 modulates p53 activity to dictate cell cycle arrest." Cell Cycle **11**(3): 511-523.

Chang, C. J., C. H. Chao, et al. (2011). "p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs." Nat Cell Biol **13**(3): 317-323.

Chang, S., B. Chen, et al. (2017). "Long non-coding RNA XIST regulates PTEN expression by sponging miR-181a and promotes hepatocellular carcinoma progression." BMC Cancer **17**(1): 248.

Chehab, N. H., A. Malikzay, et al. (2000). "Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53." Genes Dev **14**(3): 278-288.

Chen, L., L. Luo, et al. (2016). "MicroRNA-24 increases hepatocellular carcinoma cell metastasis and invasion by targeting p53: miR-24 targeted p53." Biomed Pharmacother **84**: 1113-1118.

Coffill, C. R., P. A. Muller, et al. (2012). "Mutant p53 interactome identifies nardilysin as a p53R273H-specific binding partner that promotes invasion." EMBO Rep **13**(7): 638-644.

Costa-Pinheiro, P., J. Ramalho-Carvalho, et al. (2015). "MicroRNA-375 plays a dual role in prostate carcinogenesis." Clin Epigenetics **7**(1): 42.

Crawford, L. (1985). "Human p53 and human tumours." Bioessays **3**(3): 117-120.

Cui, R., Y. Guan, et al. (2016). "A tumor-suppressive microRNA, miR-504, inhibits cell proliferation and promotes apoptosis by targeting FOXP1 in human glioma." Cancer Lett **374**(1): 1-11.

Dai, M. S., S. X. Zeng, et al. (2004). "Ribosomal protein L23 activates p53 by inhibiting MDM2 function in response to ribosomal perturbation but not to translation inhibition." Mol Cell Biol **24**(17): 7654-7668.

Deng, Q., L. Becker, et al. (2014). "The dichotomy of p53 regulation by noncoding RNAs." J Mol Cell Biol **6**(3): 198-205.

Deng, S., H. Wang, et al. (2017). "MicroRNA-146a Induces Lineage-Negative Bone Marrow Cell Apoptosis and Senescence by Targeting Polo-Like Kinase 2 Expression." Arterioscler Thromb Vasc Biol **37**(2): 280-290.

Diskin, S. J., M. Capasso, et al. (2014). "Rare variants in TP53 and susceptibility to neuroblastoma." J Natl Cancer Inst **106**(4): dju047.

Donzelli, S., G. Fontemaggi, et al. (2012). "MicroRNA-128-2 targets the transcriptional repressor E2F5 enhancing mutant p53 gain of function." Cell Death Differ **19**(6): 1038-1048.

Due, H., P. Svendsen, et al. (2016). "miR-155 as a Biomarker in B-Cell Malignancies." Biomed Res Int **2016**: 9513037.

Egan, K. M., L. B. Nabors, et al. (2012). "Rare TP53 genetic variant associated with glioma risk and outcome." J Med Genet **49**(7): 420-421.

el-Deiry, W. S., T. Tokino, et al. (1993). "WAF1, a potential mediator of p53 tumor suppression." Cell **75**(4): 817-825.

Enciso-Mora, V., F. J. Hosking, et al. (2013). "Low penetrance susceptibility to glioma is caused by the TP53 variant rs78378222." Br J Cancer **108**(10): 2178-2185.

Fang, S., J. P. Jensen, et al. (2000). "Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53." J Biol Chem **275**(12): 8945-8951.

Feng, Z., C. Zhang, et al. (2011). "Tumor suppressor p53 meets microRNAs." J Mol Cell Biol **3**(1): 44-50.

Fornari, F., L. Gramantieri, et al. (2009). "MiR-122/cyclin G1 interaction modulates p53 activity and affects doxorubicin sensitivity of human hepatocarcinoma cells." Cancer Res **69**(14): 5761-5767.

Freed-Pastor, W. A. and C. Prives (2012). "Mutant p53: one name, many proteins." Genes Dev **26**(12): 1268-1286.

Fridman, J. S. and S. W. Lowe (2003). "Control of apoptosis by p53." Oncogene **22**(56): 9030-9040.

Galluzzi, L., E. Morselli, et al. (2010). "miR-181a and miR-630 regulate cisplatin-induced cancer cell death." Cancer Res **70**(5): 1793-1803.

Galtsidis, S., S. Logotheti, et al. (2017). "Unravelling a p73-regulated network: The role of a novel p73-dependent target, MIR3158, in cancer cell migration and invasiveness." Cancer Lett **388**: 96-106.

Gao, F., X. Xiong, et al. (2015). "A Regulatory MDM4 Genetic Variant Locating in the Binding Sequence of Multiple MicroRNAs Contributes to Susceptibility of Small Cell Lung Cancer." PLoS One **10**(8): e0135647.

Garibaldi, F., E. Falcone, et al. (2016). "Mutant p53 inhibits miRNA biogenesis by interfering with the microprocessor complex." Oncogene **35**(29): 3760-3770.

Ghose, J. and N. P. Bhattacharyya (2015). "Transcriptional regulation of microRNA-100, -146a, and -150 genes by p53 and NFkappaB p65/RelA in mouse striatal STHdh(Q7)/ Hdh(Q7) cells and human cervical carcinoma HeLa cells." RNA Biol **12**(4): 457-477.

Gordon, M. W., F. Yan, et al. (2015). "Regulation of p53-targeting microRNAs by polycyclic aromatic hydrocarbons: Implications in the etiology of multiple myeloma." Mol Carcinog **54**(10): 1060-1069.

Guo, F., H. Chen, et al. (2016). "Mutation R273H confers p53 a stimulating effect on the IGF-1R-AKT pathway via miR-30a suppression in breast cancer." Biomed Pharmacother **78**: 335-341.

Gurtner, A., E. Falcone, et al. (2016). "Dysregulation of microRNA biogenesis in cancer: the impact of mutant p53 on Drosha complex activity." J Exp Clin Cancer Res **35**: 45.

Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.

Hao, J., Q. Lou, et al. (2017). "MicroRNA-375 Is Induced in Cisplatin Nephrotoxicity to Repress Hepatocyte Nuclear Factor 1-beta." J Biol Chem **292**(11): 4571-4582.

Hao, J., Q. Wei, et al. (2017). "Induction of microRNA-17-5p by p53 protects against renal ischemia-reperfusion injury by targeting death receptor 6." Kidney Int **91**(1): 106-118.

Harms, K. L. and X. Chen (2005). "The C terminus of p53 family proteins is a cell fate determinant." Mol Cell Biol **25**(5): 2014-2030.

Haupt, Y., R. Maya, et al. (1997). "Mdm2 promotes the rapid degradation of p53." Nature **387**(6630): 296-299.

He, L., X. He, et al. (2007). "A microRNA component of the p53 tumour suppressor network." Nature **447**(7148): 1130-1134.

Hermeking, H. (2012). "MicroRNAs in the p53 network: micromanagement of tumour suppression." Nat Rev Cancer **12**(9): 613-626.

Hermeking, H. and A. Benzinger (2006). "14-3-3 proteins in cell cycle regulation." Semin Cancer Biol **16**(3): 183-192.

Herrera-Merchan, A., C. Cerrato, et al. (2010). "miR-33-mediated downregulation of p53 controls hematopoietic stem cell self-renewal." Cell Cycle **9**(16): 3277-3285.

Ho, J. and S. Benchimol (2003). "Transcriptional repression mediated by the p53 tumour suppressor." Cell Death Differ **10**(4): 404-408.

Hoffman, Y., D. R. Bublik, et al. (2014). "miR-661 downregulates both Mdm2 and Mdm4 to activate p53." Cell Death Differ **21**(2): 302-309.

Holmberg Olausson, K., M. Nister, et al. (2012). "p53 -Dependent and -Independent Nucleolar Stress Responses." Cells **1**(4): 774-798.

Hu, W., C. S. Chan, et al. (2010). "Negative regulation of tumor suppressor p53 by microRNA miR-504." Mol Cell **38**(5): 689-699.

Huang, Y., A. Y. Chuang, et al. (2011). "Phospho-DeltaNp63alpha/miR-885-3p axis in tumor cell life and cell death upon cisplatin exposure." Cell Cycle **10**(22): 3938-3947.

Igarashi, H., H. Hirano, et al. (2014). "Anti-apoptotic roles for the mutant p53R248Q through suppression of p53-regulated apoptosis-inducing protein 1 in the RA-derived fibroblast-like synoviocyte cell line MH7A." Clin Immunol **150**(1): 12-21.

Isobe, M., B. S. Emanuel, et al. (1986). "Localization of gene for human p53 tumour antigen to band 17p13." Nature **320**(6057): 84-85.

Jenkins, J. R., K. Rudge, et al. (1985). "The cellular oncogene p53 can be activated by mutagenesis." Nature **317**(6040): 816-818.

Jiang, Z., L. Yao, et al. (2016). "miRNA-214 inhibits cellular proliferation and migration in glioma cells targeting caspase-1 involved in pyroptosis." Oncol Res.

Jin, A., K. Itahana, et al. (2004). "Inhibition of HDM2 and activation of p53 by ribosomal protein L23." Mol Cell Biol **24**(17): 7669-7680.

Joerger, A. C. and A. R. Fersht (2010). "The tumor suppressor p53: from structures to drug discovery." Cold Spring Harb Perspect Biol **2**(6): a000919.

Juin, P., A. Hunt, et al. (2002). "c-Myc functionally cooperates with Bax to induce apoptosis." Mol Cell Biol **22**(17): 6158-6169.

Kalo, E., I. Kogan-Sakin, et al. (2012). "Mutant p53R273H attenuates the expression of phase 2 detoxifying enzymes and promotes the survival of cells with high levels of reactive oxygen species." J Cell Sci **125**(Pt 22): 5578-5586.

Kannan, K., N. Kaminski, et al. (2001). "DNA microarray analysis of genes involved in p53 mediated apoptosis: activation of Apaf-1." Oncogene **20**(26): 3449-3455.

Kapodistrias, N., K. Mavridis, et al. (2017). "Assessing the clinical value of microRNAs in formalin-fixed paraffin-embedded liposarcoma tissues: Overexpressed miR-155 is an indicator of poor prognosis." Oncotarget **8**(4): 6896-6913.

Kim, T., A. Veronese, et al. (2011). "p53 regulates epithelial-mesenchymal transition through microRNAs targeting ZEB1 and ZEB2." J Exp Med **208**(5): 875-883.

Kimura, S. H. and H. Nojima (2002). "Cyclin G1 associates with MDM2 and regulates accumulation and degradation of p53 protein." Genes Cells **7**(8): 869-880.

Krell, J., J. Stebbing, et al. (2016). "TP53 regulates miRNA association with AGO2 to remodel the miRNA-mRNA interaction network." Genome Res **26**(3): 331-341.

Krichevsky, A. M. and G. Gabriely (2009). "miR-21: a small multi-faceted RNA." J Cell Mol Med **13**(1): 39-53.

Kubbutat, M. H., R. L. Ludwig, et al. (1998). "Regulation of Mdm2-directed degradation by the C terminus of p53." Mol Cell Biol **18**(10): 5690-5698.

Kuroda, Y., S. Aishima, et al. (2007). "14-3-3sigma negatively regulates the cell cycle, and its down-regulation is associated with poor outcome in intrahepatic cholangiocarcinoma." Hum Pathol **38**(7): 1014-1022.

Lai, Z., K. V. Ferry, et al. (2001). "Human mdm2 mediates multiple mono-ubiquitination of p53 by a mechanism requiring enzyme isomerization." J Biol Chem **276**(33): 31357-31367.

Lang, G. A., T. Iwakuma, et al. (2004). "Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome." Cell **119**(6): 861-872.

Le, M. T., N. Shyh-Chang, et al. (2011). "Conserved regulation of p53 network dosage by microRNA-125b occurs through evolving miRNA-target gene pairs." PLoS Genet **7**(9): e1002242.

Le, M. T., C. Teh, et al. (2009). "MicroRNA-125b is a novel negative regulator of p53." Genes Dev **23**(7): 862-876.

Leveille, N., R. Elkon, et al. (2011). "Selective inhibition of microRNA accessibility by RBM38 is required for p53 activity." Nat Commun **2**: 513.

Levine, A. J. (1997). "p53, the cellular gatekeeper for growth and division." Cell **88**(3): 323-331.

Lezina, L., N. Purmessur, et al. (2013). "miR-16 and miR-26a target checkpoint kinases Wee1 and Chk1 in response to p53 activation by genotoxic stress." Cell Death Dis **4**: e953.

Li, H., Z. Q. Zhou, et al. (2017). "MicroRNA-191 acts as a tumor promoter by modulating the TET1-p53 pathway in intrahepatic cholangiocarcinoma." Hepatology.

Li, H., Z. Q. Zhou, et al. (2017). "MicroRNA-191 acts as a tumor promoter by modulating the TET1-p53 pathway in intrahepatic cholangiocarcinoma." Hepatology **66**(1): 136-151.

Li, W., W. Han, et al. (2015). "P53-dependent miRNAs mediate nitric oxide-induced apoptosis in colonic carcinogenesis." Free Radic Biol Med **85**: 105-113.

Li, X. L., M. F. Jones, et al. (2014). "Mutant p53 exerts oncogenic effects through microRNAs and their target gene networks." FEBS Lett **588**(16): 2610-2615.

Li, Y., M. W. Gordon, et al. (2013). "Single nucleotide variation in the TP53 3' untranslated region in diffuse large B-cell lymphoma treated with rituximab-CHOP: a report from the International DLBCL Rituximab-CHOP Consortium Program." Blood **121**(22): 4529-4540.

Li, Y. C., C. F. Li, et al. (2015). "MicroRNA-766 targeting regulation of SOX6 expression promoted cell proliferation of human colorectal cancer." Onco Targets Ther **8**: 2981-2988.

Liang, M., G. Yao, et al. (2013). "Transcriptional cooperation between p53 and NF-kappaB p65 regulates microRNA-224 transcription in mouse ovarian granulosa cells." Mol Cell Endocrinol **370**(1-2): 119-129.

Linares, L. K., A. Hengstermann, et al. (2003). "HdmX stimulates Hdm2-mediated ubiquitination and degradation of p53." Proc Natl Acad Sci U S A **100**(21): 12009-12014.

Liu, H., X. F. Xu, et al. (2014). "MicroRNA-191 promotes pancreatic cancer progression by targeting USP10." Tumour Biol **35**(12): 12157-12163.

Liu, L. L., S. X. Lu, et al. (2014). "FoxD3-regulated microRNA-137 suppresses tumour growth and metastasis in human hepatocellular carcinoma by targeting AKT2." Oncotarget **5**(13): 5113-5124.

Liu, Y., R. Xing, et al. (2013). "miR-375 targets the p53 gene to regulate cellular response to ionizing radiation and etoposide in gastric cancer cells." DNA Repair (Amst) **12**(9): 741-750.

Liu, Z., Y. Lu, et al. (2017). "Upregulation of miR-21 expression is a valuable predicator of advanced clinicopathological features and poor prognosis in patients with renal cell carcinoma through the p53/p21cyclin E2Bax/caspase-3 signaling pathway." Oncol Rep **37**(3): 1437-1444.

Lukashchuk, N. and K. H. Vousden (2007). "Ubiquitination and degradation of mutant p53." Mol Cell Biol **27**(23): 8284-8295.

Ma, J., Y. Xue, et al. (2013). "Role of activated Rac1/Cdc42 in mediating endothelial cell proliferation and tumor angiogenesis in breast cancer." PLoS One **8**(6): e66275.

Ma, Y., J. Feng, et al. (2016). "miR-1908 Overexpression Inhibits Proliferation, Changing Akt Activity and p53 Expression in Hypoxic NSCLC Cells." Oncol Res **24**(1): 9-15.

Macedo, G. S., I. Araujo Vieira, et al. (2016). "Rare germline variant (rs78378222) in the TP53 3' UTR: Evidence for a new mechanism of cancer predisposition in Li-Fraumeni syndrome." Cancer Genet **209**(3): 97-106.

Malkin, D., F. P. Li, et al. (1990). "Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms." Science **250**(4985): 1233-1238.

Mandke, P., N. Wyatt, et al. (2012). "MicroRNA-34a modulates MDM4 expression via a target site in the open reading frame." PLoS One **7**(8): e42034.

Marchenko, N. D., A. Zaika, et al. (2000). "Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling." J Biol Chem **275**(21): 16202-16212.

Masciarelli, S., G. Fontemaggi, et al. (2014). "Gain-of-function mutant p53 downregulates miR-223 contributing to chemoresistance of cultured tumor cells." Oncogene **33**(12): 1601-1608.

Mashima, R. (2015). "Physiological roles of miR-155." Immunology **145**(3): 323-333.

Mastropasqua, F., F. Marzano, et al. (2017). "TRIM8 restores p53 tumour suppressor function by blunting N-MYC activity in chemo-resistant tumours." Mol Cancer **16**(1): 67.

Mei, Z., T. Su, et al. (2015). "The miR-15 family enhances the radiosensitivity of breast cancer cells by targeting G2 checkpoints." Radiat Res **183**(2): 196-207.

Mignacca, L., E. Saint-Germain, et al. (2016). "Sponges against miR-19 and miR-155 reactivate the p53-Socs1 axis in hematopoietic cancers." Cytokine **82**: 80-86.

Miyashita, T., S. Krajewski, et al. (1994). "Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo." Oncogene **9**(6): 1799-1805.

Moore, R., H. K. Ooi, et al. (2015). "MiR-192-Mediated Positive Feedback Loop Controls the Robustness of Stress-Induced p53 Oscillations in Breast Cancer Cells." PLoS Comput Biol **11**(12): e1004653.

Muller, P. A., A. G. Trinidad, et al. (2014). "Mutant p53 regulates Dicer through p63-dependent and -independent mechanisms to promote an invasive phenotype." J Biol Chem **289**(1): 122-132.

Nag, S., J. Qin, et al. (2013). "The MDM2-p53 pathway revisited." J Biomed Res **27**(4): 254-271.

Nakamura, S., J. A. Roth, et al. (2000). "Multiple lysine mutations in the C-terminal domain of p53 interfere with MDM2-dependent protein degradation and ubiquitination." Mol Cell Biol **20**(24): 9391-9398.

Nakano, K. and K. H. Vousden (2001). "PUMA, a novel proapoptotic gene, is induced by p53." Mol Cell **7**(3): 683-694.

Neilsen, P. M., J. E. Noll, et al. (2013). "Mutant p53 drives invasion in breast tumors through up-regulation of miR-155." Oncogene **32**(24): 2992-3000.

Nesslinger, N. J., X. B. Shi, et al. (2003). "Androgen-independent growth of LNCaP prostate cancer cells is mediated by gain-of-function mutant p53." Cancer Res **63**(9): 2228-2233.

Oda, E., R. Ohki, et al. (2000). "Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis." Science **288**(5468): 1053-1058.

Oda, K., H. Arakawa, et al. (2000). "p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53." Cell **102**(6): 849-862.

Okamura, S., H. Arakawa, et al. (2001). "p53DINP1, a p53-inducible gene, regulates p53-dependent apoptosis." Mol Cell **8**(1): 85-94.

Ovcharenko, D., F. Stolzel, et al. (2011). "miR-10a overexpression is associated with NPM1 mutations and MDM4 downregulation in intermediate-risk acute myeloid leukemia." Exp Hematol **39**(10): 1030-1042 e1037.

Pan, X., J. Zhao, et al. (2009). "Induction of SOX4 by DNA damage is critical for p53 stabilization and function." Proc Natl Acad Sci U S A **106**(10): 3788-3793.

Park, S. Y., J. H. Lee, et al. (2009). "miR-29 miRNAs activate p53 by targeting p85 alpha and CDC42." Nat Struct Mol Biol **16**(1): 23-29.

Pouliot, L. M., Y. C. Chen, et al. (2012). "Cisplatin sensitivity mediated by WEE1 and CHK1 is mediated by miR-155 and the miR-15 family." Cancer Res **72**(22): 5945-5955.

Qin, Y., S. Zhang, et al. (2017). "Epigenetic silencing of miR-137 induces drug resistance and chromosomal instability by targeting AURKA in multiple myeloma." Leukemia **31**(5): 1123-1135.

Raver-Shapira, N., E. Marciano, et al. (2007). "Transcriptional activation of miR-34a contributes to p53-mediated apoptosis." Mol Cell **26**(5): 731-743.

Raza, U., O. Saatci, et al. (2016). "The miR-644a/CTBP1/p53 axis suppresses drug resistance by simultaneous inhibition of cell survival and epithelial-mesenchymal transition in breast cancer." Oncotarget **7**(31): 49859-49877.

Robles, A. I., N. A. Bemmels, et al. (2001). "APAF-1 is a transcriptional target of p53 in DNA damage-induced apoptosis." Cancer Res **61**(18): 6660-6664.

Rogel, A., M. Popliker, et al. (1985). "p53 cellular tumor antigen: analysis of mRNA levels in normal adult tissues, embryos, and tumors." Mol Cell Biol **5**(10): 2851-2855.

Sachdeva, M., S. Zhu, et al. (2009). "p53 represses c-Myc through induction of the tumor suppressor miR-145." Proc Natl Acad Sci U S A **106**(9): 3207-3212.

Sannigrahi, M. K., R. Sharma, et al. (2017). "Role of Host miRNA Hsa-miR-139-3p in HPV-16-Induced Carcinomas." Clin Cancer Res.

Sax, J. K., P. Fei, et al. (2002). "BID regulation by p53 contributes to chemosensitivity." Nat Cell Biol **4**(11): 842-849.

Schubert, J. and T. Brabletz (2011). "p53 Spreads out further: suppression of EMT and stemness by activating miR-200c expression." Cell Res **21**(5): 705-707.

Seki, N. and A. Hata (2017). "Noncoding RNAs: a new fine-tuner is a key player of human pathogenesis." J Hum Genet **62**(1): 1.

Shang, J., Y. Yao, et al. (2016). "miR-29c-3p promotes senescence of human mesenchymal stem cells by targeting CNOT6 through p53-p21 and p16-pRB pathways." Biochim Biophys Acta **1863**(4): 520-532.

Shang, Y., B. Feng, et al. (2016). "The miR27b-CCNG1-P53-miR-508-5p axis regulates multidrug resistance of gastric cancer." Oncotarget **7**(1): 538-549.

Sharma, S., N. Nagpal, et al. (2017). "P53-miR-191-SOX4 regulatory loop affects apoptosis in breast cancer." RNA.

Sharp, D. A., S. A. Kratowicz, et al. (1999). "Stabilization of the MDM2 oncoprotein by interaction with the structurally related MDMX protein." J Biol Chem **274**(53): 38189-38196.

Sheng, Y., R. C. Laister, et al. (2008). "Molecular basis of Pirh2-mediated p53 ubiquitylation." Nat Struct Mol Biol **15**(12): 1334-1342.

Shi, Y., X. Luo, et al. (2015). "miR-7-5p suppresses cell proliferation and induces apoptosis of breast cancer cells mainly by targeting REGgamma." Cancer Lett **358**(1): 27-36.

Shieh, S. Y., M. Ikeda, et al. (1997). "DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2." Cell **91**(3): 325-334.

Shiloh, Y. and Y. Ziv (2013). "The ATM protein kinase: regulating the cellular response to genotoxic stress, and more." Nat Rev Mol Cell Biol **14**(4): 197-210.

Shvarts, A., W. T. Steegenga, et al. (1996). "MDMX: a novel p53-binding protein with some functional properties of MDM2." EMBO J **15**(19): 5349-5357.

Srivastava, S., Z. Q. Zou, et al. (1990). "Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome." Nature **348**(6303): 747-749.

Stacey, S. N., P. Sulem, et al. (2011). "A germline variant in the TP53 polyadenylation signal confers cancer susceptibility." Nat Genet **43**(11): 1098-1103.

Stad, R., Y. F. Ramos, et al. (2000). "Hdmx stabilizes Mdm2 and p53." J Biol Chem **275**(36): 28039-28044.

Stegeman, S., L. Moya, et al. (2015). "A genetic variant of MDM4 influences regulation by multiple microRNAs in prostate cancer." Endocr Relat Cancer **22**(2): 265-276.

Stott, F. J., S. Bates, et al. (1998). "The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2." Embo j **17**(17): 5001-5014.

Su, X., D. Chakravarti, et al. (2010). "TAp63 suppresses metastasis through coordinate regulation of Dicer and miRNAs." Nature **467**(7318): 986-990.

Suh, S. S., J. Y. Yoo, et al. (2012). "MicroRNAs/TP53 feedback circuitry in glioblastoma multiforme." Proc Natl Acad Sci U S A **109**(14): 5316-5321.

Swarbrick, A., S. L. Woods, et al. (2010). "miR-380-5p represses p53 to control cellular survival and is associated with poor outcome in MYCN-amplified neuroblastoma." Nat Med **16**(10): 1134-1140.

Tanimura, S., S. Ohtsuka, et al. (1999). "MDM2 interacts with MDMX through their RING finger domains." FEBS Lett **447**(1): 5-9.

Tao, S., Y. B. Liu, et al. (2016). "miR-3646 promotes cell proliferation, migration, and invasion via regulating G2/M transition in human breast cancer cells." Am J Transl Res **8**(4): 1659-1677.

Tavakoli, R., S. Vakilian, et al. (2017). "The role of miR-17-92 cluster in the expression of tumor suppressor genes in unrestricted somatic stem cells." Biologicals **46**: 143-147.

Taylor, W. R. and G. R. Stark (2001). "Regulation of the G2/M transition by p53." Oncogene **20**(15): 1803-1815.

To, K. H., S. Pajovic, et al. (2012). "Regulation of p14ARF expression by miR-24: a potential mechanism compromising the p53 response during retinoblastoma development." BMC Cancer **12**: 69.

Toledo, F. and G. M. Wahl (2007). "MDM2 and MDM4: p53 regulators as targets in anticancer therapy." Int J Biochem Cell Biol **39**(7-8): 1476-1482.

Ugalde, A. P., A. J. Ramsay, et al. (2011). "Aging and chronic DNA damage response activate a regulatory pathway involving miR-29 and p53." EMBO J **30**(11): 2219-2232.

Venot, C., M. Maratrat, et al. (1998). "The requirement for the p53 proline-rich functional domain for mediation of apoptosis is correlated with specific PIG3 gene transactivation and with transcriptional repression." Embo j **17**(16): 4668-4679.

Wan, Y., R. Cui, et al. (2017). "Identification of Four Oxidative Stress-Responsive MicroRNAs, miR-34a-5p, miR-1915-3p, miR-638, and miR-150-3p, in Hepatocellular Carcinoma." Oxid Med Cell Longev **2017**: 5189138.

Wang, B., H. Wu, et al. (2017). "MicroRNA-1301 suppresses tumor cell migration and invasion by targeting the p53/UBE4B pathway in multiple human cancer cells." Cancer Lett **401**: 20-32.

Wang, F., P. Lv, et al. (2015). "microRNA-214 enhances the invasion ability of breast cancer cells by targeting p53." Int J Mol Med **35**(5): 1395-1402.

Wang, H., H. Zhi, et al. (2017). "MiR-217 promoted the proliferation and invasion of glioblastoma by repressing YWHAG." Cytokine **92**: 93-102.

Wang, Q., L. A. Selth, et al. (2017). "MiR-766 induces p53 accumulation and G2/M arrest by directly targeting MDM4." Oncotarget **8**(18): 29914-29924.

Wang, X., Y. Ren, et al. (2015). "Down-regulation of 5S rRNA by miR-150 and miR-383 enhances c-Myc-rpL11 interaction and inhibits proliferation of esophageal squamous carcinoma cells." FEBS Lett **589**(24 Pt B): 3989-3997.

Wang, X., J. Wang, et al. (2011). "MdmX protein is essential for Mdm2 protein-mediated p53 polyubiquitination." J Biol Chem **286**(27): 23725-23734.

Wang, X., L. Wang, et al. (2016). "A positive feedback loop of p53/miR-19/TP53INP1 modulates pancreatic cancer cell proliferation and apoptosis." Oncol Rep **35**(1): 518-523.

Wang, Y., J. W. Huang, et al. (2014). "p53 is positively regulated by miR-542-3p." Cancer Res **74**(12): 3218-3227.

Wolfe, A. R., A. Bambhroliya, et al. (2016). "MiR-33a Decreases High-Density Lipoprotein-Induced Radiation Sensitivity in Breast Cancer." Int J Radiat Oncol Biol Phys **95**(2): 791-799.

Wynendaele, J., A. Bohnke, et al. (2010). "An illegitimate microRNA target site within the 3' UTR of MDM4 affects ovarian cancer progression and chemosensitivity." Cancer Res **70**(23): 9641-9649.

Xiao, J., H. Lin, et al. (2011). "miR-605 joins p53 network to form a p53:miR-605:Mdm2 positive feedback loop in response to stress." EMBO J **30**(24): 5021.

Xie, C., W. Chen, et al. (2015). "MDM4 regulation by the let-7 miRNA family in the DNA damage response of glioma cells." FEBS Lett **589**(15): 1958-1965.

Xiong, S., Y. Zheng, et al. (2014). "PA28gamma emerges as a novel functional target of tumour suppressor microRNA-7 in non-small-cell lung cancer." Br J Cancer **110**(2): 353-362.

Xu, D. D., P. J. Zhou, et al. (2016). "miR-150 Suppresses the Proliferation and Tumorigenicity of Leukemia Stem Cells by Targeting the Nanog Signaling Pathway." Front Pharmacol **7**: 439.

Xu, S., B. Zhang, et al. (2017). "miR-194 functions as a novel modulator of cellular senescence in mouse embryonic fibroblasts." Cell Biol Int **41**(3): 249-257.

Yamakuchi, M., C. D. Lotterman, et al. (2010). "P53-induced microRNA-107 inhibits HIF-1 and tumor angiogenesis." Proc Natl Acad Sci U S A **107**(14): 6334-6339.

Yan, H. L., G. Xue, et al. (2009). "Repression of the miR-17-92 cluster by p53 has an important function in hypoxia-induced apoptosis." EMBO J **28**(18): 2719-2732.

Yan, S., M. Wang, et al. (2016). "MicroRNA-34a affects chondrocyte apoptosis and proliferation by targeting the SIRT1/p53 signaling pathway during the pathogenesis of osteoarthritis." Int J Mol Med **38**(1): 201-209.

Yang, R., X. Xu, et al. (2017). "p53 induces miR199a-3p to suppress SOCS7 for STAT3 activation and renal fibrosis in UUO." Sci Rep **7**: 43409.

Yuan, J., K. Luo, et al. (2010). "USP10 regulates p53 localization and stability by deubiquitinating p53." Cell **140**(3): 384-396.

Zhang, J., B. Su, et al. (2016). "miR-214 promotes apoptosis and sensitizes breast cancer cells to doxorubicin by targeting the RFWD2-p53 cascade." Biochem Biophys Res Commun **478**(1): 337-342.

Zhang, J., Q. Sun, et al. (2013). "Loss of microRNA-143/145 disturbs cellular growth and apoptosis of human epithelial cancers by impairing the MDM2-p53 feedback loop." Oncogene **32**(1): 61-69.

Zhang, M. X., J. Zhang, et al. (2016). "miR-24-3p Suppresses Malignant Behavior of Lacrimal Adenoid Cystic Carcinoma by Targeting PRKCH to Regulate p53/p21 Pathway." PLoS One **11**(6): e0158433.

Zhang, N., X. Wei, et al. (2013). "miR-150 promotes the proliferation of lung cancer cells by targeting P53." FEBS Lett **587**(15): 2346-2351.

Zhang, P., Z. Zuo, et al. (2017). "miR-600 inhibits cell proliferation, migration and invasion by targeting p53 in mutant p53-expressing human colorectal cancer cell lines." Oncol Lett **13**(3): 1789-1796.

Zhang, Q., L. Y. Cao, et al. (2015). "p53-induced microRNA-1246 inhibits the cell growth of human hepatocellular carcinoma cells by targeting NFIB." Oncol Rep **33**(3): 1335-1341.

Zhang, Y., L. Geng, et al. (2015). "MicroRNA-520g confers drug resistance by regulating p21 expression in colorectal cancer." J Biol Chem **290**(10): 6215-6225.

Zhang, Y., J. M. Liao, et al. (2011). "p53 downregulates Down syndrome-associated DYRK1A through miR-1246." EMBO Rep **12**(8): 811-817.

Zhang, Y. and Y. Xiong (2001). "A p53 amino-terminal nuclear export signal inhibited by DNA damage-induced phosphorylation." Science **292**(5523): 1910-1915.

Zhang, Z. and R. Zhang (2008). "Proteasome activator PA28 gamma regulates p53 by enhancing its MDM2-mediated degradation." EMBO J **27**(6): 852-864.

Zhao, Y., X. Li, et al. (2016). "rs78378222 polymorphism in the 3'-untranslated region of TP53 contributes to development of age-associated cataracts by modifying microRNA-125b-induced apoptosis of lens epithelial cells." Mol Med Rep **14**(3): 2305-2310.

Zhou, C. H., X. P. Zhang, et al. (2014). "Involvement of miR-605 and miR-34a in the DNA damage response promotes apoptosis induction." Biophys J **106**(8): 1792-1800.

Zhou, J., D. Xu, et al. (2015). "miR-33a functions as a tumor suppressor in melanoma by targeting HIF-1alpha." Cancer Biol Ther **16**(6): 846-855.

Zhou, L., Q. Yuan, et al. (2012). "A functional germline variant in the P53 polyadenylation signal and risk of esophageal squamous cell carcinoma." Gene **506**(2): 295-297.

Zhou, L., X. Zhang, et al. (2013). "Association of a genetic variation in a miR-191 binding site in MDM4 with risk of esophageal squamous cell carcinoma." PLoS One **8**(5): e64331.

1.5. The regulation of Androgen Receptor (AR) signalling pathways by miRNAs in prostate cancer

1.5.1. Prostate cancer

Prostate cancer (PCa) is a type of carcinoma originating in the prostate. Prostate cancer is the most common male cancer diagnosed and the second most common cause of male cancer death in Australia (Table 1.2). By 2017, it is estimated that more than 185,000 Australian men will be living with PCa [114]. 90% of patients are diagnosed at an early stage that only presents as local disease; the 5-year-survival of these patients is nearly 100%. However, a small minority of patients are diagnosed with metastatic prostate cancer, and an additional subset of patients (~30%) fail therapy for localised disease and progress to metastatic disease. For these men, the 5-year-survival rate is less than 30% [115].

The diagnosis of prostate cancer is mainly based on the microscopic assessment of a biopsy, which is requested after an abnormal serum prostate specific antigen (PSA) measurement or digital rectal exam. PSA is a kallikrein-like serine protease encoded by Kallikrein Related Peptidase 3 (*KLK3*) gene and produced exclusively by prostatic epithelial cells. The serum level of PSA has been recognized as a biomarker of PCa and is broadly used in PCa detection [116]. Assessment of the biopsy provides a primary and secondary Gleason grade based on the histological pattern of the prostate tissue. The Gleason grading system is used to determine prognosis by dividing prostate cancer into 5 strata [117]. MRI imaging can also be used to identify potential lesions in the prostate, which can allow better targeting of subsequent biopsies [118].

Table 1.2. The ten most commonly diagnosed cancers(a) among males and all persons, Australia, 2009 [119].

Males				Persons			
Cancer type (ICD-10-AM code)	**New cases**	**Crude rate[b]**	**Per cent[c]**	**Cancer type (ICD-10-AM code)**	**New cases**	**Crude rate[b]**	**Per cent[c]**
Prostate (C61)	21,808	201.2	32.9	Prostate (C61)	21,808	100.1	18.8
Bowel (C18–C20)	7,848	72.4	11.8	Bowel (C18–C20)	14,214	65.3	12.3
Melanoma (C43)	6,571	60.6	9.9	Breast (C50)	13,855	63.6	12.0
Lung (C33–C34)	6,062	55.9	9.1	Melanoma (C43)	11,264	51.7	9.7
Non-Hodgkin Lymphoma (C82–C85)	2,447	22.6	3.7	Lung (C33–C34)	10,241	47.0	8.8
Kidney (C64)	1,773	16.4	2.7	Non-Hodgkin Lymphoma (C82–C85)	4,367	20.1	3.8
Bladder (C67)	1,727	15.9	2.6	Kidney (C64)	2,762	12.7	2.4
Unknown primary site (C80)	1,386	12.8	2.1	Unknown primary site (C80)	2,696	12.4	2.3
Pancreas (C25)	1,327	12.2	2.0	Pancreas (C25)	2,567	11.8	2.2
Stomach (C16)	1,289	11.9	1.9	Bladder (C67)	2,344	10.8	2.0
Other[d]	*14,122*	*121.1*	*19.8*	*Other[d]*	*29,779*	*136.7*	*25.7*
Total	**66,360**	**612.4**	**100.0**	**Total**	**115,897**	**532.2**	**100.0**

(a) Most commonly diagnosed for males and for all persons, excluding basal and squamous cell carcinomas of the skin.

(b) Crude rates are based on the 2011 Census preliminary rebased estimated resident population for 30 June 2009 and expressed per 100,000 males.

(c) Proportion of the total for each group (males and persons).

(d) Includes all other cancers not listed, but excludes basal and squamous cell carcinomas of the skin.

Source: AIHW Australian Cancer Database 2009

The treatment for localized and metastatic PCa varies, taking into account the risk of cancer and the side effects of treatment. For localized PCa, patients will have the option to monitor cancer progress without treatment if detected with a lower Gleason grade/score and PSA level [114, 120] (Table 1.3). For patients with localized PCa but higher Gleason grade/score and PSA levels, surgical removal of tumour or radiation will be recommended [117]. For patients diagnosed with metastatic disease and for the ~30% of men who experience disease recurrence after surgery/radiation therapy, androgen deprivation therapy (ADT) is the mainstay treatment. Most men respond to ADT initially but, in all cases, the therapy fails and progresses to castration-resistant prostate cancer (CRPC), which leads to poor prognosis with a median survival of only ~15-36 months [115, 121]. CRPC is treated with second generation AR-targeting therapies (i.e. Enzalutamide (MDV3100) and Abiraterone Acetate) [122] and chemotherapies.

Table 1.3. Recommended selection criteria for prostate cancer active surveillance (adapted from "*Active Surveillance for Prostate Cancer: How to Do It Right*" [123])

Table 2. Selection Criteria for Active Surveillance

	Clinical Stage	PSA Level	Gleason Score on Biopsy	PSA Density	Number of Positive Cores on Biopsy
University of Toronto[19]	T1c/T2a	≤ 10–15 ng/mL	≤ 3+3=6	NI	NI
Multicenter European study (PRIAS)[21]	T1c/T2a	≤ 10 ng/mL	≤ 3+3=6	≤ 0.2 ng/mL/cm^3	2
Johns Hopkins[20]	T1c	NI	≤ 3+3=6	< 0.15 ng/mL/cm^3	2
Canary Collaboration[49]	T1/T2	Any	≤ 7	Any	Any

NI = not included; PRIAS = Prostate Cancer Research International Active Surveillance; PSA = prostate-specific antigen.

1.5.2. AR signalling in prostate cancer

1.5.2.1. AR

The androgen receptor (AR), is a member of the hormone receptor superfamily and a ligand-dependent transcription factor of 110kDa encoded by the *AR* gene on chromosome X [124, 125]. The AR protein is composed of four functional domains: an amino-terminal domain (NTD), containing transcriptional activation function-1 and 5 (TAU1 and TAU5) [126, 127]; a carboxy-terminal ligand-binding domain (LBD) [126]; a DNA binding domain (DBD), which is highly conserved and responsible for AR-DNA binding and homodimer formation via two zinc fingers [128]; and a flexible hinge region which joins the LBD and DBD and contains a nuclear localization signal [129].

As shown in Figure 6, AR is primarily located in the cytoplasm in association with protective chaperone proteins [130]. When binding to its native agonists DHT and testosterone, AR dissociates from the chaperone complex and translocates to the nucleus where it regulates target genes. The translocation of AR is mediated by the hinge region interacting with cytoskeletal proteins and importin-a. The nuclear AR, acting as a homodimer, then recognizes androgen response elements within gene promoters and enhancers via its DBD. The AR target genes contribute to cell proliferation, differentiation and survival [131].

1.5.2.2. AR in prostate cancer

AR signalling has been found to be significantly reprogrammed in PCa compared to normal cells [132]. During malignant transformation and cancer progression, AR-

regulated cell proliferation, survival and apoptosis becomes imbalanced due to the over-expression or mutation of AR [133]. More than 85 AR mutations have been found in CRPC, mostly being somatic mutations with single base substitutions in the LBD [134]. Evidence suggests that certain mutations in AR enable it to be activated by other steroids, or be hypersensitive to androgen stimulation, which may explain the active AR downstream signaling in androgen-independent PCa [135].

1.5.2.3. The regulation of AR

The expression and activation of AR is strictly regulated during development. The primary regulator of AR is its ligands: in most organs, the transcription of AR is repressed by high doses of androgens, whereas low doses increase the stability and phosphorylation of AR, which allows AR signaling to be controlled in a self-regulating mechanism. Interestingly, despite the native limitation of ligand binding, AR undergoing androgen deprivation may also be activated in a ligand-independent manner. Several cell surface receptors including EGFR, IL-6 and Her2/neu, as well as intracellular kinases such as MAPK, SRC, PI3K/Akt and ERK, have shown the ability to activate or sensitise AR to further downstream activation [136].

MiRNAs also participate in post-transcriptional regulation of AR by directly binding to the 3'UTR of AR mRNA. MiR-185 binds to the AR 3'UTR and reduces its expression, consequently resulting in repression of AR-mediated tumorigenesis both *in vitro* and *in vivo* [137]. Several other miRNAs have also been identified as direct

regulators of AR and function as tumour suppressors, including miR-31, miR-654-5p, miR-203/205, miR-488* and let-7c [138-143].

1.5.3. MiRNAs in prostate cancer

1.5.3.1. Tumour suppressor miRNAs in prostate cancer

MiRNAs have been found to be dysregulated in PCa, with some being described as tumour suppressors. For example, the miR-200 family represses EMT and metastasis in PCa via SNAL2, SLUG and PDGF-D pathways [144-146]. MiR-15/16 repress PCa cell proliferation by targeting BCL2, CCND1 and CDK1/2 [84, 147, 148]. Both miR-34a/c [149] and miR-205 [150] target AR signalling and inhibit AR-driven PCa progression. Let-7 family members are often down regulated in PCa and can repress PCa cell growth by targeting CCND2 and E2F2 [151]. Moreover, by targeting MYC and LIN28, let-7c is also able to down-regulate AR expression and repress cancer development [143].

1.5.3.2. OncomiRs in prostate cancer

MiR-106b-25 and miR-153 are upregulated in PCa and facilitate oncogenesis by targeting *PTEN* and *FOXO1*. Several miRNAs that are androgen-regulated have shown the ability to promote PCa progress as oncomiRs. MiR-21 is an androgen-regulated miRNA that functions as an oncogene. Inhibition of miR-21 leads to higher sensitivity to apoptosis and less invasion by PC3 and DU145 cells, which may be mediated by its target genes *MARCKS, BTG2, PTEN, RECK* and *PDCD4* [152]. MiR-221 and miR-222 are also upregulated by AR and induce androgen-independent growth *in vitro* and *in vivo*

by targeting p27kip1, *HECTD2* and *RAB1A* [153-155]. MiR-125b targets and down-regulates the tumour suppressor p53, and inhibition of miR-125b induces profound growth blockade in PCa [88]. MiR-32 is another androgen regulated miRNA that is elevated in PCa and inhibits cell apoptosis by targeting *BIM* [156]. Other miRNAs that are upregulated by AR and function in cell growth, invasion and survival, include miR-141, miR-23a/27/24-2 miR-19a and miR-133b [157-159]. These androgen-activated oncomiRs in PCa highlight that the involvement of AR in gene regulation also occurs at the post-transcriptional level.

1.5.4. miR-375 in prostate cancer

MiR-375 is a miRNA that is positively correlated with androgen receptor levels during DNMT induced methylation [160]. As a potential biomarker of PCa, miR-375 has been found to be very highly expressed in tumours and serum from patients with prostate cancer [161-165]. My supervisor's group has shown that circulating miR-375 levels are elevated in PCa metastasis patients compared to primary PCa patients, and can be used as a biomarker and improve the prediction of PCa status when combined with serum PSA levels [166]. Moreover, by targeting Sec23A and CIC, miR-375 functions as an oncogene in PCa [164, 167-169]. However, miR-375 increased apoptosis and reduced the invasion and survival of PC3 and 22Rv1 cells [170]. By targeting YAP1, miR-375 also represses PCa cell invasion while serum levels of miR-375 positively correlate with circulating tumour cells, suggesting a dual role of miR-375 in PCa [171]. Therefore, the precise role of miR-375 in PCa remains to be clarified.

1.6. Overall aims and research questions

With the background in mind, my PhD aims to investigate how oncogenes and tumour suppressors are regulated by miRNAs, and how this regulation influences the development and progression of cancer.

Each of the chapters investigates a distinct cancer-associated miRNA: mRNA regulation event. In Chapter 2, I identify miR-766-mediated regulation of p53; in Chapter 3, I investigate how miR-9 differentially regulates mutant and wild-type p53; and in Chapter 4 I uncover an AR-miR-375 regulatory feedback interaction that influences prostate cancer growth.

1.7. References

1. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
2. Berman, J.J., *Tumor taxonomy for the developmental lineage classification of neoplasms.* BMC Cancer, 2004. **4**: p. 88.
3. Whiteman, D.C. and L.F. Wilson, *The fractions of cancer attributable to modifiable factors: A global review.* Cancer Epidemiol, 2016. **44**: p. 203-221.
4. Tashiro, H. and M.K. Brenner, *Immunotherapy against cancer-related viruses.* Cell Res, 2017. **27**(1): p. 59-73.
5. Vockerodt, M., et al., *Epstein-Barr virus and the origin of Hodgkin lymphoma.* Chin J Cancer, 2014. **33**(12): p. 591-7.
6. Rowe, M., L. Fitzsimmons, and A.I. Bell, *Epstein-Barr virus and Burkitt lymphoma.* Chin J Cancer, 2014. **33**(12): p. 609-19.
7. Ribeiro, J., et al., *Epstein-Barr virus gene expression and latency pattern in gastric carcinomas: a systematic review.* Future Oncol, 2017. **13**(6): p. 567-579.
8. Raab-Traub, N., *Nasopharyngeal Carcinoma: An Evolving Role for the Epstein-Barr Virus.* Curr Top Microbiol Immunol, 2015. **390**(Pt 1): p. 339-63.
9. Wang, M., D. Xi, and Q. Ning, *Virus-induced hepatocellular carcinoma with special emphasis on HBV.* Hepatol Int, 2017. **11**(2): p. 171-180.
10. Aggarwal, B.B., et al., *Inflammation and cancer: how hot is the link?* Biochem Pharmacol, 2006. **72**(11): p. 1605-21.
11. Crissman, J.D. and R.J. Zarbo, *Dysplasia, in situ carcinoma, and progression to invasive squamous cell carcinoma of the upper aerodigestive tract.* Am J Surg Pathol, 1989. **13 Suppl 1**: p. 5-16.
12. Raica, M., A.M. Cimpean, and D. Ribatti, *Angiogenesis in pre-malignant conditions.* Eur J Cancer, 2009. **45**(11): p. 1924-34.
13. Zeidman, I., *Metastasis: a review of recent advances.* Cancer Res, 1957. **17**(3): p. 157-62.
14. Paduch, R., *The role of lymphangiogenesis and angiogenesis in tumor metastasis.* Cell Oncol (Dordr), 2016. **39**(5): p. 397-410.
15. Hart, I.R., *'Seed and soil' revisited: mechanisms of site-specific metastasis.* Cancer Metastasis Rev, 1982. **1**(1): p. 5-16.

16. Spano, D., et al., *Molecular networks that regulate cancer metastasis.* Semin Cancer Biol, 2012. **22**(3): p. 234-49.
17. Society, A.C., *Cancer Facts and Statistics.* 2017.
18. Australia, C., 2017.
19. Kim, J.Y., C.H. Cho, and H.S. Song, *Targeted therapy of ovarian cancer including immune check point inhibitor.* Korean J Intern Med, 2017.
20. Chan, Y.C. and M. Hsiao, *Protease-activated nanomaterials for targeted cancer theranostics.* Nanomedicine (Lond), 2017.
21. Fang, F., W. Xiao, and Z. Tian, *NK cell-based immunotherapy for cancer.* Semin Immunol, 2017.
22. Sahin, B., *Enlighting the Shadow for Advanced Hepatocellular Carcinoma: Immunotherapy with Immune Checkpoint Inhibitors.* J Gastrointest Cancer, 2017.
23. Yoo, B.C., et al., *Clinical multi-omics strategies for the effective cancer management.* J Proteomics, 2017.
24. Guidozzi, F., *Estrogen therapy in gynecological cancer survivors.* Climacteric, 2013. **16**(6): p. 611-7.
25. Wen, Y., et al., *The safety of postoperative hormone replacement therapy in epithelial ovarian cancer patients in China.* Climacteric, 2013. **16**(6): p. 673-81.
26. Khan, M.H., R. Allerton, and L. Pettit, *Hormone Therapy for Breast Cancer in Men.* Clin Breast Cancer, 2015. **15**(4): p. 245-50.
27. Glaser, R. and C. Dimitrakakis, *Testosterone and breast cancer prevention.* Maturitas, 2015. **82**(3): p. 291-5.
28. Cui, Y., et al., *The effect of testosterone replacement therapy on prostate cancer: a systematic review and meta-analysis.* Prostate Cancer Prostatic Dis, 2014. **17**(2): p. 132-43.
29. Land, H., L.F. Parada, and R.A. Weinberg, *Cellular oncogenes and multistep carcinogenesis.* Science, 1983. **222**(4625): p. 771-8.
30. Torry, D.S. and G.M. Cooper, *Proto-oncogenes in development and cancer.* Am J Reprod Immunol, 1991. **25**(3): p. 129-32.
31. Malumbres, M. and M. Barbacid, *RAS oncogenes: the first 30 years.* Nat Rev Cancer, 2003. **3**(6): p. 459-65.

32. Ito, M., et al., *Comprehensive mapping of p53 pathway alterations reveals an apparent role for both SNP309 and MDM2 amplification in sarcomagenesis.* Clin Cancer Res, 2011. **17**(3): p. 416-26.
33. Lam, S., et al., *Role of Mdm4 in drug sensitivity of breast cancer cells.* Oncogene, 2010. **29**(16): p. 2415-26.
34. Kron, K.J., A. Murison, and S. Zhou, *TMPRSS2-ERG fusion co-opts master transcription factors and activates NOTCH signaling in primary prostate cancer.* 2017.
35. Demichelis, F. and M.A. Rubin, *TMPRSS2-ETS fusion prostate cancer: biological and clinical implications.* J Clin Pathol, 2007. **60**(11): p. 1185-6.
36. Fischinger, P.J. and V.T. DeVita, Jr., *Governance of science at the National Cancer Institute: perceptions and opportunities in oncogene research.* Cancer Res, 1984. **44**(10): p. 4693-6.
37. Croce , C.M., *Oncogenes and Cancer*. New England Journal of Medicine, 2008. **358**(5): p. 502-511.
38. Carmeliet, P., *VEGF as a key mediator of angiogenesis in cancer.* Oncology, 2005. **69 Suppl 3**: p. 4-10.
39. Kuerti, S., et al., *VEGF-C expression attributes the risk for lymphatic metastases to ovarian cancer patients.* Oncotarget, 2017. **8**(26): p. 43218-43227.
40. Ma, J., et al., *Role of activated Rac1/Cdc42 in mediating endothelial cell proliferation and tumor angiogenesis in breast cancer.* PLoS One, 2013. **8**(6): p. e66275.
41. Veselaj, F., et al., *Prognostic Value of Vascular Endothelial Growth Factor A in the Prediction of the Tumor Aggressiveness in Clear Cell Renal Cell Carcinoma.* Open Access Maced J Med Sci, 2017. **5**(2): p. 167-172.
42. Burstein, H.J., *The distinctive nature of HER2-positive breast cancers.* N Engl J Med, 2005. **353**(16): p. 1652-4.
43. Buza, N., D.M. Roque, and A.D. Santin, *HER2/neu in Endometrial Cancer: A Promising Therapeutic Target With Diagnostic Challenges.* Arch Pathol Lab Med, 2014. **138**(3): p. 343-50.
44. Miyahira, A.K. and H.R. Soule, *The 23rd Annual Prostate Cancer Foundation Scientific Retreat report.* Prostate, 2017.

45. Wu, Y. and J.V. Vadgama, *Androgen Receptor as a Potential Target for Treatment of Breast Cancer.* Int J Cancer Res Mol Mech, 2017. **3**(1).
46. Lu, H., et al., *Oncogenic BRAF-Mediated Melanoma Cell Invasion.* Cell Rep, 2016. **15**(9): p. 2012-24.
47. Greenberg, A.H., S.E. Egan, and J.A. Wright, *Oncogenes and metastatic progression.* Invasion Metastasis, 1989. **9**(6): p. 360-78.
48. Uekita, T., et al., *Oncogenic Ras/ERK signaling activates CDCP1 to promote tumor invasion and metastasis.* Mol Cancer Res, 2014. **12**(10): p. 1449-59.
49. Summy, J.M. and G.E. Gallick, *Src family kinases in tumor progression and metastasis.* Cancer Metastasis Rev, 2003. **22**(4): p. 337-58.
50. Pharmacists., T.A.S.o.H.-S., *Trastuzumab.* 2016.
51. Pharmacists., T.A.S.o.H.-S., *Bevacizumab.* 2016.
52. GM, C., *Tumor Suppressor Genes.* The Cell: A Molecular Approach. 2nd edition., 2000.
53. Deininger, P., *Genetic instability in cancer: caretaker and gatekeeper genes.* Ochsner J, 1999. **1**(4): p. 206-9.
54. Macaluso, M., M.G. Paggi, and A. Giordano, *Genetic and epigenetic alterations as hallmarks of the intricate road to cancer.* Oncogene, 2003. **22**(42): p. 6472-8.
55. Smith, J.R., et al., *Major susceptibility locus for prostate cancer on chromosome 1 suggested by a genome-wide search.* Science, 1996. **274**(5291): p. 1371-4.
56. Podsypanina, K., et al., *Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems.* Proc Natl Acad Sci U S A, 1999. **96**(4): p. 1563-8.
57. Chen, Z., et al., *Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis.* Nature, 2005. **436**(7051): p. 725-30.
58. Hopkins, B.D. and R.E. Parsons, *Molecular pathways: intercellular PTEN and the potential of PTEN restoration therapy.* Clin Cancer Res, 2014. **20**(21): p. 5379-83.
59. Eddy, S.R., *Non-coding RNA genes and the modern RNA world.* Nat Rev Genet, 2001. **2**(12): p. 919-29.
60. Marz, M., et al., *Animal snoRNAs and scaRNAs with exceptional structures.* RNA Biol, 2011. **8**(6): p. 938-46.
61. Huang, Y., J.Y. Bai, and H.T. Ren, *PiRNAs biogenesis and its functions.* Bioorg Khim, 2014. **40**(3): p. 320-6.

62. Starega-Roslan, J., et al., *Structural basis of microRNA length variety.* Nucleic Acids Res, 2011. **39**(1): p. 257-68.
63. Jeck, W.R. and N.E. Sharpless, *Detecting and characterizing circular RNAs.* Nat Biotechnol, 2014. **32**(5): p. 453-61.
64. Jessica Varilh, J.B.a.M.T.-C., *Chapter 12: Role of Non-coding RNAs in Cystic Fibrosis*, in *Cystic Fibrosis in the Light of New Research*, D. Wat, Editor. 2015, Intech.
65. Peng, W.X., P. Koirala, and Y.Y. Mo, *LncRNA-mediated regulation of cell signaling in cancer.* Oncogene, 2017.
66. Prensner, J.R. and A.M. Chinnaiyan, *The emergence of lncRNAs in cancer biology.* Cancer Discov, 2011. **1**(5): p. 391-407.
67. Greene, J., et al., *Circular RNAs: Biogenesis, Function and Role in Human Diseases.* Front Mol Biosci, 2017. **4**: p. 38.
68. MiRBase.org, *miRBase 21*. 2014.
69. Londin, E., et al., *Analysis of 13 cell types reveals evidence for the expression of numerous novel primate- and tissue-specific microRNAs.* Proc Natl Acad Sci U S A, 2015. **112**(10): p. E1106-15.
70. Krol, J., I. Loedige, and W. Filipowicz, *The widespread regulation of microRNA biogenesis, function and decay.* Nat Rev Genet, 2010. **11**(9): p. 597-610.
71. Bartel, D.P., *MicroRNAs: genomics, biogenesis, mechanism, and function.* Cell, 2004. **116**(2): p. 281-97.
72. Mandke, P., et al., *MicroRNA-34a modulates MDM4 expression via a target site in the open reading frame.* PLoS One, 2012. **7**(8): p. e42034.
73. Xie, C., et al., *MDM4 regulation by the let-7 miRNA family in the DNA damage response of glioma cells.* FEBS Lett, 2015. **589**(15): p. 1958-65.
74. Huang, Y., A.Y. Chuang, and E.A. Ratovitski, *Phospho-DeltaNp63alpha/miR-885-3p axis in tumor cell life and cell death upon cisplatin exposure.* Cell Cycle, 2011. **10**(22): p. 3938-47.
75. Jones, M.R., et al., *Zcchc11-dependent uridylation of microRNA directs cytokine expression.* Nat Cell Biol, 2009. **11**(9): p. 1157-63.
76. Katoh, T., et al., *Selective stabilization of mammalian microRNAs by 3' adenylation mediated by the cytoplasmic poly(A) polymerase GLD-2.* Genes Dev, 2009. **23**(4): p. 433-8.

77. Chatterjee, S. and H. Grosshans, *Active turnover modulates mature microRNA activity in Caenorhabditis elegans.* Nature, 2009. **461**(7263): p. 546-9.
78. Ramachandran, V. and X. Chen, *Degradation of microRNAs by a family of exoribonucleases in Arabidopsis.* Science, 2008. **321**(5895): p. 1490-2.
79. Olive, V., Q. Li, and L. He, *mir-17-92: a polycistronic oncomir with pleiotropic functions.* Immunol Rev, 2013. **253**(1): p. 158-66.
80. Inamura, K., *Major Tumor Suppressor and Oncogenic Non-Coding RNAs: Clinical Relevance in Lung Cancer.* Cells, 2017. **6**(2).
81. Su, J.L., et al., *Function and regulation of let-7 family microRNAs.* Microrna, 2012. **1**(1): p. 34-9.
82. Chiu, S.C., et al., *Therapeutic potential of microRNA let-7: tumor suppression or impeding normal stemness.* Cell Transplant, 2014. **23**(4-5): p. 459-69.
83. Wagner, S., et al., *Role of miRNA let-7 and its major targets in prostate cancer.* Biomed Res Int, 2014. **2014**: p. 376326.
84. Cimmino, A., et al., *miR-15 and miR-16 induce apoptosis by targeting BCL2.* Proc Natl Acad Sci U S A, 2005. **102**(39): p. 13944-9.
85. Kong, R., et al., *The crucial role of miR-126 on suppressing progression of esophageal cancer by targeting VEGF-A.* Cell Mol Biol Lett, 2016. **21**: p. 3.
86. Knackmuss, U., et al., *MAP3K11 is a tumor suppressor targeted by the oncomiR miR-125b in early B cells.* Cell Death Differ, 2016. **23**(2): p. 242-52.
87. Li, Q., et al., *miR-125b regulates cell progression in chronic myeloid leukemia via targeting BAK1.* Am J Transl Res, 2016. **8**(2): p. 447-59.
88. Amir, S., et al., *Oncomir miR-125b suppresses p14(ARF) to modulate p53-dependent and p53-independent apoptosis in prostate cancer.* PLoS One, 2013. **8**(4): p. e61064.
89. Budd, W.T., et al., *Dual Action of miR-125b As a Tumor Suppressor and OncomiR-22 Promotes Prostate Cancer Tumorigenesis.* PLoS One, 2015. **10**(11): p. e0142373.
90. Tang, X.Y., et al., *miR-125b acts as a tumor suppressor in chondrosarcoma cells by the sensitization to doxorubicin through direct targeting the ErbB2-regulated glucose metabolism.* Drug Des Devel Ther, 2016. **10**: p. 571-83.

91. Zhang, J., et al., *MicroRNA-125b suppresses the epithelial-mesenchymal transition and cell invasion by targeting ITGA9 in melanoma.* Tumour Biol, 2016. **37**(5): p. 5941-9.
92. Svoronos, A.A., D.M. Engelman, and F.J. Slack, *OncomiR or Tumor Suppressor? The Duplicity of MicroRNAs in Cancer.* Cancer Res, 2016. **76**(13): p. 3666-70.
93. Hu, H., et al., *The overexpression of hypomethylated miR-663 induces chemotherapy resistance in human breast cancer cells by targeting heparin sulfate proteoglycan 2 (HSPG2).* J Biol Chem, 2013. **288**(16): p. 10973-85.
94. Yi, C., et al., *MiR-663, a microRNA targeting p21(WAF1/CIP1), promotes the proliferation and tumorigenesis of nasopharyngeal carcinoma.* Oncogene, 2012. **31**(41): p. 4421-33.
95. Wu, C., et al., *MiR-30d induces apoptosis and is regulated by the Akt/FOXO pathway in renal cell carcinoma.* Cell Signal, 2013. **25**(5): p. 1212-21.
96. Gaziel-Sovran, A., et al., *miR-30b/30d regulation of GalNAc transferases enhances invasion and immunosuppression during metastasis.* Cancer Cell, 2011. **20**(1): p. 104-18.
97. Mitra, A.K., et al., *MicroRNAs reprogram normal fibroblasts into cancer-associated fibroblasts in ovarian cancer.* Cancer Discov, 2012. **2**(12): p. 1100-8.
98. Yin, Y., et al., *Tumor-secreted miR-214 induces regulatory T cells: a major link between immune evasion and tumor growth.* Cell Res, 2014. **24**(10): p. 1164-80.
99. Bahrami, A., et al., *The prognostic and therapeutic application of microRNAs in breast cancer: Tissue and circulating microRNAs.* J Cell Physiol, 2017.
100. Wang, F., et al., *Correlation and quantitation of microRNA aberrant expression in tissues and sera from patients with breast tumor.* Gynecol Oncol, 2010. **119**(3): p. 586-93.
101. Asaga, S., et al., *Direct serum assay for microRNA-21 concentrations in early and advanced breast cancer.* Clin Chem, 2011. **57**(1): p. 84-91.
102. Ng, E.K., et al., *Circulating microRNAs as specific biomarkers for breast cancer detection.* PLoS One, 2013. **8**(1): p. e53141.
103. Mar-Aguilar, F., et al., *Serum circulating microRNA profiling for identification of potential breast cancer biomarkers.* Dis Markers, 2013. **34**(3): p. 163-9.
104. Galm, O., J.G. Herman, and S.B. Baylin, *The fundamental role of epigenetics in hematopoietic malignancies.* Blood Rev, 2006. **20**(1): p. 1-13.

105. Melo, S., et al., *Small molecule enoxacin is a cancer-specific growth inhibitor that acts by enhancing TAR RNA-binding protein 2-mediated microRNA processing.* Proc Natl Acad Sci U S A, 2011. **108**(11): p. 4394-9.
106. Bader, A.G., et al., *Developing therapeutic microRNAs for cancer.* Gene Ther, 2011. **18**(12): p. 1121-6.
107. Kota, J., et al., *Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model.* Cell, 2009. **137**(6): p. 1005-17.
108. Motavaf, M., S. Safari, and S.M. Alavian, *Targeting microRNA-122: walking on cutting edge of hepatitis C virus infection therapy.* Acta Virol, 2014. **58**(4): p. 301-8.
109. Obad, S., et al., *Silencing of microRNA families by seed-targeting tiny LNAs.* Nat Genet, 2011. **43**(4): p. 371-8.
110. Krutzfeldt, J., et al., *Silencing of microRNAs in vivo with 'antagomirs'.* Nature, 2005. **438**(7068): p. 685-9.
111. Kluiver, J., et al., *Rapid generation of microRNA sponges for microRNA inhibition.* PLoS One, 2012. **7**(1): p. e29275.
112. Young, D.D., et al., *Small molecule modifiers of microRNA miR-122 function for the treatment of hepatitis C virus infection and hepatocellular carcinoma.* J Am Chem Soc, 2010. **132**(23): p. 7976-81.
113. Ling, H., M. Fabbri, and G.A. Calin, *MicroRNAs and other non-coding RNAs as targets for anticancer drug development.* Nat Rev Drug Discov, 2013. **12**(11): p. 847-65.
114. *Prostate Cancer Foundation of Australia and Cancer Council Australia PSA Testing Guidelines Expert Advisory Panel. Clinical practice guidelines PSA Testing and Early Management of Test-Detected Prostate Cancer*. 2016; Available from: http://wiki.cancer.org.au/australiawiki/index.php?oldid=134877.
115. Nandana, S. and L.W. Chung, *Prostate cancer progression and metastasis: potential regulatory pathways for therapeutic targeting.* Am J Clin Exp Urol, 2014. **2**(2): p. 92-101.
116. Duskova, K. and S. Vesely, *Prostate specific antigen. Current clinical application and future prospects.* Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub, 2015. **159**(1): p. 18-26.

117. Epstein, J.I., et al., *The 2014 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma: Definition of Grading Patterns and Proposal for a New Grading System.* Am J Surg Pathol, 2016. **40**(2): p. 244-52.
118. Woo, S., et al., *Diagnostic Performance of Prostate Imaging Reporting and Data System Version 2 for Detection of Prostate Cancer: A Systematic Review and Diagnostic Meta-analysis.* Eur Urol, 2017.
119. Welfare, A.I.o.H.a., *Prostate Cancer In Australia.* Cancer series no. 79. , 2013. **Cat. no. CAN 76. Canberra: AIHW**.
120. Filson, C.P., L.S. Marks, and M.S. Litwin, *Expectant management for men with early stage prostate cancer.* CA Cancer J Clin, 2015. **65**(4): p. 265-82.
121. Crawford, E.D., D. Petrylak, and O. Sartor, *Navigating the evolving therapeutic landscape in advanced prostate cancer.* Urol Oncol, 2017. **35S**: p. S1-S13.
122. Ahmed, A., S. Ali, and F.H. Sarkar, *Advances in androgen receptor targeted therapy for prostate cancer.* J Cell Physiol, 2014. **229**(3): p. 271-6.
123. Garisto, J.D. and L. Klotz, *Active Surveillance for Prostate Cancer: How to Do It Right.* Oncology (Williston Park), 2017. **31**(5): p. 333-40, 345.
124. Chang, C.S., J. Kokontis, and S.T. Liao, *Molecular cloning of human and rat complementary DNA encoding androgen receptors.* Science, 1988. **240**(4850): p. 324-6.
125. Tilley, W.D., et al., *Characterization and expression of a cDNA encoding the human androgen receptor.* Proc Natl Acad Sci U S A, 1989. **86**(1): p. 327-31.
126. Jenster, G., et al., *Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization.* Mol Endocrinol, 1991. **5**(10): p. 1396-404.
127. He, B., J.A. Kemppainen, and E.M. Wilson, *FXXLF and WXXLF sequences mediate the NH2-terminal interaction with the ligand binding domain of the androgen receptor.* J Biol Chem, 2000. **275**(30): p. 22986-94.
128. Shaffer, P.L., et al., *Structural basis of androgen receptor binding to selective androgen response elements.* Proc Natl Acad Sci U S A, 2004. **101**(14): p. 4758-63.
129. Ozanne, D.M., et al., *Androgen receptor nuclear translocation is facilitated by the f-actin cross-linking protein filamin.* Mol Endocrinol, 2000. **14**(10): p. 1618-26.

130. Smith, D.F. and D.O. Toft, *Minireview: the intersection of steroid receptors with molecular chaperones: observations and questions.* Mol Endocrinol, 2008. **22**(10): p. 2229-40.
131. Zhu, M.L. and N. Kyprianou, *Androgen receptor and growth factor signaling cross-talk in prostate cancer cells.* Endocr Relat Cancer, 2008. **15**(4): p. 841-9.
132. Wang, Q., et al., *Androgen receptor regulates a distinct transcription program in androgen-independent prostate cancer.* Cell, 2009. **138**(2): p. 245-56.
133. Crumbaker, M., L. Khoja, and A.M. Joshua, *AR Signaling and the PI3K Pathway in Prostate Cancer.* Cancers (Basel), 2017. **9**(4).
134. Gottlieb, B., et al., *Nuclear receptors and disease: androgen receptor.* Essays Biochem, 2004. **40**: p. 121-36.
135. Taplin, M.E., et al., *Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer.* N Engl J Med, 1995. **332**(21): p. 1393-8.
136. Dai, C., H. Heemers, and N. Sharifi, *Androgen Signaling in Prostate Cancer.* Cold Spring Harb Perspect Med, 2017.
137. Qu, F., et al., *MicroRNA-185 suppresses proliferation, invasion, migration, and tumorigenicity of human prostate cancer cells through targeting androgen receptor.* Mol Cell Biochem, 2013. **377**(1-2): p. 121-30.
138. Ostling, P., et al., *Systematic analysis of microRNAs targeting the androgen receptor in prostate cancer cells.* Cancer Res, 2011. **71**(5): p. 1956-67.
139. Sikand, K., et al., *miR 488* inhibits androgen receptor expression in prostate carcinoma cells.* Int J Cancer, 2011. **129**(4): p. 810-9.
140. Boll, K., et al., *MiR-130a, miR-203 and miR-205 jointly repress key oncogenic pathways and are downregulated in prostate carcinoma.* Oncogene, 2013. **32**(3): p. 277-85.
141. Hagman, Z., et al., *miR-205 negatively regulates the androgen receptor and is associated with adverse outcome of prostate cancer patients.* Br J Cancer, 2013. **108**(8): p. 1668-76.
142. Lin, P.C., et al., *Epigenetic repression of miR-31 disrupts androgen receptor homeostasis and contributes to prostate cancer progression.* Cancer Res, 2013. **73**(3): p. 1232-44.

143. Nadiminty, N., et al., *MicroRNA let-7c suppresses androgen receptor expression and activity via regulation of Myc expression in prostate cancer cells.* J Biol Chem, 2012. **287**(2): p. 1527-37.
144. Kong, D., et al., *miR-200 regulates PDGF-D-mediated epithelial-mesenchymal transition, adhesion, and invasion of prostate cancer cells.* Stem Cells, 2009. **27**(8): p. 1712-21.
145. Liu, Y.N., et al., *MiR-1 and miR-200 inhibit EMT via Slug-dependent and tumorigenesis via Slug-independent mechanisms.* Oncogene, 2013. **32**(3): p. 296-306.
146. Slabakova, E., et al., *TGF-beta1-induced EMT of non-transformed prostate hyperplasia cells is characterized by early induction of SNAI2/Slug.* Prostate, 2011. **71**(12): p. 1332-43.
147. Bonci, D., et al., *The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities.* Nat Med, 2008. **14**(11): p. 1271-7.
148. Takeshita, F., et al., *Systemic delivery of synthetic microRNA-16 inhibits the growth of metastatic prostate tumors via downregulation of multiple cell-cycle genes.* Mol Ther, 2010. **18**(1): p. 181-7.
149. Kashat, M., et al., *Inactivation of AR and Notch-1 signaling by miR-34a attenuates prostate cancer aggressiveness.* Am J Transl Res, 2012. **4**(4): p. 432-42.
150. Wang, N., et al., *miR-205 is frequently downregulated in prostate cancer and acts as a tumor suppressor by inhibiting tumor growth.* Asian J Androl, 2013. **15**(6): p. 735-41.
151. Dong, Q., et al., *MicroRNA let-7a inhibits proliferation of human prostate cancer cells in vitro and in vivo by targeting E2F2 and CCND2.* PLoS One, 2010. **5**(4): p. e10147.
152. Ayub, S.G., D. Kaul, and T. Ayub, *Microdissecting the role of microRNAs in the pathogenesis of prostate cancer.* Cancer Genet, 2015. **208**(6): p. 289-302.
153. Galardi, S., et al., *miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1.* J Biol Chem, 2007. **282**(32): p. 23716-24.
154. Sun, T., et al., *The role of microRNA-221 and microRNA-222 in androgen-independent prostate cancer cell lines.* Cancer Res, 2009. **69**(8): p. 3356-63.

155. Mercatelli, N., et al., *The inhibition of the highly expressed miR-221 and miR-222 impairs the growth of prostate carcinoma xenografts in mice.* PLoS One, 2008. **3**(12): p. e4029.
156. Ambs, S., et al., *Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer.* Cancer Res, 2008. **68**(15): p. 6162-70.
157. Waltering, K.K., et al., *Androgen regulation of micro-RNAs in prostate cancer.* Prostate, 2011. **71**(6): p. 604-14.
158. Mo, W., et al., *Identification of novel AR-targeted microRNAs mediating androgen signalling through critical pathways to regulate cell viability in prostate cancer.* PLoS One, 2013. **8**(2): p. e56592.
159. Fletcher, C.E., et al., *Androgen-regulated processing of the oncomir miR-27a, which targets Prohibitin in prostate cancer.* Hum Mol Genet, 2012. **21**(14): p. 3112-27.
160. Chu, M., et al., *Androgen receptor is negatively correlated with the methylation-mediated transcriptional repression of miR-375 in human prostate cancer cells.* Oncol Rep, 2014. **31**(1): p. 34-40.
161. Haldrup, C., et al., *Profiling of circulating microRNAs for prostate cancer biomarker discovery.* Drug Deliv Transl Res, 2014. **4**(1): p. 19-30.
162. Huang, X., et al., *Exosomal miR-1290 and miR-375 as prognostic markers in castration-resistant prostate cancer.* Eur Urol, 2015. **67**(1): p. 33-41.
163. Li, X., et al., *An eight-miRNA signature as a potential biomarker for predicting survival in lung adenocarcinoma.* J Transl Med, 2014. **12**: p. 159.
164. Tiryakioglu, D., et al., *miR-141 and miR-375 induction and release are different from PSA mRNA and PCA3 upon androgen stimulation of LNCaP cells.* Biomed Rep, 2013. **1**(5): p. 802-806.
165. Wach, S., et al., *The combined serum levels of miR-375 and urokinase plasminogen activator receptor are suggested as diagnostic and prognostic biomarkers in prostate cancer.* Int J Cancer, 2015. **137**(6): p. 1406-16.
166. Roberts, M.J., et al., *Diagnostic performance of expression of PCA3, Hepsin and miR biomarkers inejaculate in combination with serum PSA for the detection of prostate cancer.* Prostate, 2015. **75**(5): p. 539-49.

167. Choi, N., et al., *miR-93/miR-106b/miR-375-CIC-CRABP1: a novel regulatory axis in prostate cancer progression.* Oncotarget, 2015. **6**(27): p. 23533-47.
168. Hart, M., et al., *Comparative microRNA profiling of prostate carcinomas with increasing tumor stage by deep sequencing.* Mol Cancer Res, 2014. **12**(2): p. 250-63.
169. Wach, S., et al., *MicroRNA profiles of prostate carcinoma detected by multiplatform microRNA screening.* Int J Cancer, 2012. **130**(3): p. 611-21.
170. Costa-Pinheiro, P., et al., *MicroRNA-375 plays a dual role in prostate carcinogenesis.* Clin Epigenetics, 2015. **7**(1): p. 42.
171. Selth, L.A., et al., *A ZEB1-miR-375-YAP1 pathway regulates epithelial plasticity in prostate cancer.* Oncogene, 2017. **36**(1): p. 24-34.

Chapter 2.

MiR-766 induces p53 accumulation and G2/M arrest by directly targeting MDM4.

Preface

This chapter presents the work aiming to investigate miRNA-mediated regulation of p53. In this chapter, miR-766 is identified as a novel positive regulator of wild-type p53 by directly targeting MDM4. MiR-766 is also found to inhibit cell proliferation and G2/M transition in multiple cancer cells, and positively correlate with p53 downstream targets in lung cancer patients (data from TCGA). This work first identifies miR-766 as a tumour suppressor by enhancing the downstream signalling of p53.

This chapter is presented as a manuscript prepared for submission to a journal for publication.

Contribution of the candidate: All figures, all tables and the manuscript writing.

Statement of Authorship

Title of Paper	MiR-766 induces p53 accumulation and G2/M arrest by directly targeting MDM4
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Published paper. Wang, Q., L.A. Selth, and D.F. Callen, MiR-766 induces p53 accumulation and G2/M arrest by directly targeting MDM4. Oncotarget, 2017. 8(18): p. 29914-29924.

Principal Author

Name of Principal Author (Candidate)	Qingqing Wang
Contribution to the Paper	Designed and conducted all the experiments and analysis in this work, interoperated the data and wrote the manuscript.
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 23/08/2017

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Luke Selth
Contribution to the Paper	Supervised the progress of the work, helped with data interpretation and manuscript preparation.
Signature	Date 23/8/17

Name of Co-Author	David Callen
Contribution to the Paper	Supervised the progress of the work, helped with data interpretation and manuscript preparation.
Signature	Date 23/08/17

Abstract

p53, a transcription factor that participates in multiple cellular functions, is considered the most important tumor suppressor. Previous evidence suggests that post-transcriptional deregulation of p53 by microRNAs contributes to tumorigenesis, tumor progression and therapeutic resistance. In the present study, we found that the microRNA miR-766 was aberrantly expressed in breast cancer, and that over-expression of miR-766 caused accumulation of wild-type p53 protein in multiple cancer cell lines. Supporting its role in the p53 signalling pathway, miR-766 decreased cell proliferation and colony formation in several cancer cell lines, and cell cycle analyses revealed that miR-766 causes G2 arrest. At a mechanistic level, we demonstrate that miR-766 enhances p53 signalling by directly targeting MDM4, an oncogene and negative regulator of p53. Analysis of clinical genomic data from multiple cancer types supports the relevance of miR-766 in p53 signalling. Collectively, our study demonstrates that miR-766 can function as a novel tumor suppressor by enhancing p53 signalling.

Introduction

p53 is a nuclear phosphoprotein encoded by the *TP53* gene (Isobe, Emanuel et al. 1986) that is widely recognized to be the most important tumor suppressor in the cell. As a transcription factor, p53 responds to various stresses and activates multiple pathways including apoptosis, cell cycle arrest and DNA repair – hence its designation as the "guardian of the genome". Recent studies reveal diverse additional functions of p53, including functions in cell stemness (Park, Lee et al. 2015), epithelial–mesenchymal transition (EMT) and tumor metastasis (Chang, Chao et al. 2011; Schubert and Brabletz 2011), tumor angiogenesis (Chakraborty, Adhikary et al. 2014) and cellular senescence (Burns, D'Ambrogio et al. 2011). Given its critical role as a tumor suppressor, it is not surprising that *TP53* is frequently mutated in cancer. Indeed, mutation of this gene occurs in over 50% of cancers (Levine 1997), enabling malignant cells to escape wild-type p53-dependent growth inhibition and cell death.

The expression and activity of p53 is tightly regulated by, amongst other mechanisms, ubiquitination, phosphorylation and nuclear/cytoplasmic translocation. Two key negative regulators of p53 are MDM2 and MDM4. MDM2 is a specific E3 ligase for p53, promoting its polyubiquitination and subsequent degradation by the proteasome (Honda, Tanaka et al. 1997; Nag, Qin et al. 2013). MDM4, through its BOX1 domain, binds directly to p53 and inhibits its activity (Shvarts, Steegenga et al. 1996; Stad, Ramos et al. 2000). MDM4 also interacts with MDM2 directly (Sharp, Kratowicz et al. 1999; Tanimura, Ohtsuka et al. 1999) to enhance MDM2-mediated ubiquitination and p53 degradation (Linares, Hengstermann et al. 2003).

MicroRNAs (miRNAs), small non-coding RNAs that negatively regulate gene expression by binding to complementary sequences in their targets (Bartel 2004), have been shown to play an important role in the post-transcriptional regulation of p53. For example, several miRNAs and miRNA families directly regulate p53, including miR-125b (Le, Teh et al. 2009), miR-504 (Hu, Chan et al. 2010), miR-380-5p (Swarbrick, Woods et al. 2010), miR-25 and miR-30d (Kumar, Lu et al. 2011). Other miRNAs can upregulate p53 expression and activity by targeting MDM2 and MDM4. For example, miR-34a directly targets MDM4 by binding to a site in its open reading frame (ORF) (Mandke, Wyatt et al. 2012; Okada, Lin et al. 2014). Interestingly, miR-34a is also a downstream target of p53 (Chang, Wentzel et al. 2007; Raver-Shapira, Marciano et al. 2007; Hermeking 2009), and therefore represents a positive feedback loop for p53 through MDM4 inhibition. Similarly, miR-605, miR-192 and miR-215, which directly target MDM2, are also downstream targets of p53 (Xiao, Lin et al. 2011). Moreover, miR-339-5p (Jansson, Damas et al. 2015) and miR-661 (Hoffman, Bublik et al. 2014) also promote p53 activity and stabilities by targeting MDM2 and/or MDM4, while miR-122 (Fornari, Gramantieri et al. 2009), miR-885-5p (Afanasyeva, Mestdagh et al. 2011) and miR-542-3p stabilise p53 in cancer cells by disrupting MDM2-mediated p53 degradation (Wang, Huang et al. 2014).

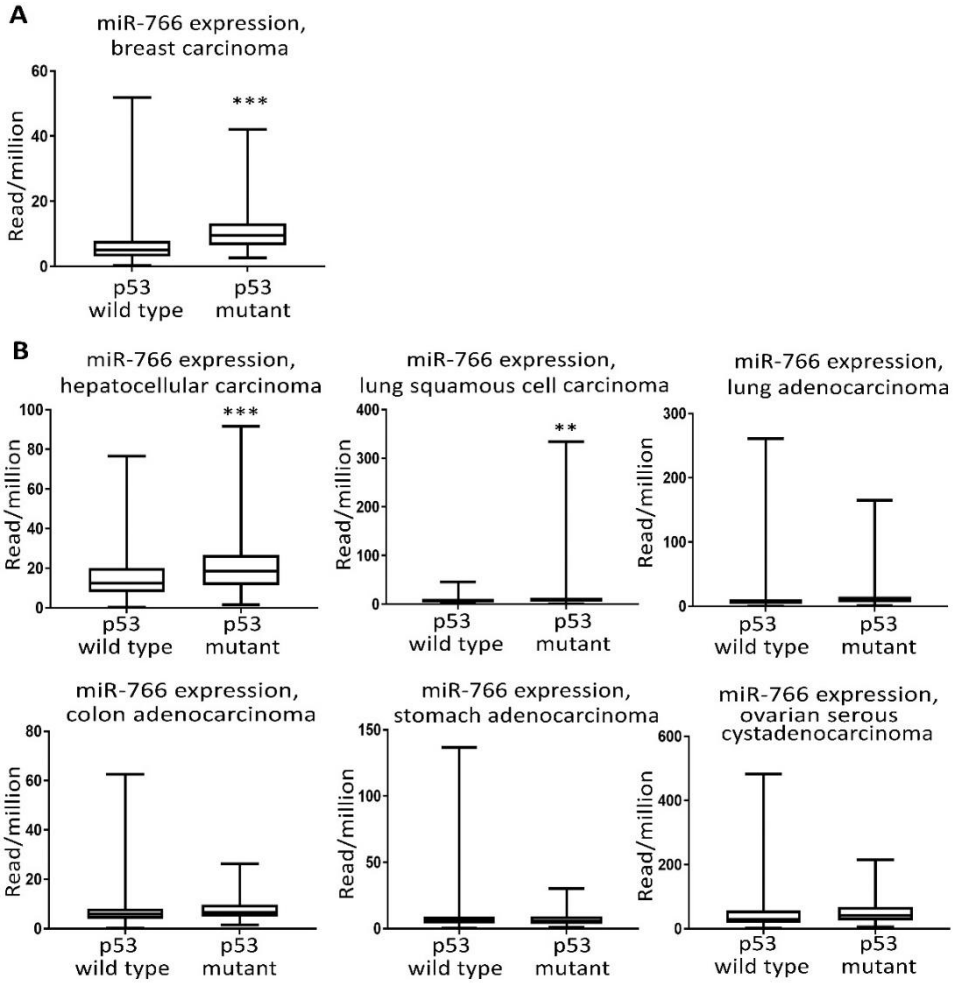
MiR-766-3p (miR-766) is a microRNA residing within an intron of the *SEP6* gene. Several studies indicated that miR-766 expression was highly expressed in cutaneous squamous cell carcinoma biopsies (Sand, Skrygan et al. 2012), lung adenocarcinoma (LUAD) (Li, Shi et al. 2014) and acute promyelocytic leukemia cells (Liang, Li et al. 2013). In this present study, we analyzed small RNA sequencing data from The Cancer Genome Atlas (TCGA) and identified miR-766 as a putative post-transcriptional

regulator of p53. We demonstrated that miR-766 stabilised p53 by targeting the 3'UTR of MDM4, leading to repression of cell growth and cell cycle arrest in cancer cells and enhancing the p53 signalling pathway. Overall, our study indicates that miR-766 is a new and important regulator of p53-dependent tumor suppression.

Results

MiR-766 induces wild-type p53 protein accumulation and cell growth repression.

We analyzed small RNA deep sequencing data of breast cancer tumors expressing wild-type p53 (228 tumors) or missense p53 (57 tumors) downloaded from TCGA. Compared to tumors with wild-type p53, miR-766 was elevated in mutant p53 tumor samples (Supplementary Figure 1A). More data was collected from TCGA across different cancer types (including hepatocellular carcinoma, lung squamous cell carcinoma, lung adenocarcinoma, colon adenocarcinoma, stomach adenocarcinoma and ovarian serous cystadenocarcinoma), and a trend of increased miR-766 expression in p53 mutant groups was found (Supplementary Figure 1B). Given that highly expressed wild-type p53 induces cell proliferation arrest or cell programmed death, cancer cells tend to suppress factors that activate it (Hoffman, Bublik et al. 2014). We hypothesized that the down-regulation of miR-766 in wild-type p53 tumors compared to mutant p53 may indicate a potential connection between miR-766 and p53 function.



Supplementary Figure 1. A, Small RNA deep sequencing data of 228 breast cancer tumours expressing wild type p53 and 57 breast cancer tumours expressing mutant p53 were downloaded from TCGA; **B,** Small RNA deep sequencing data were downloaded from TCGA, including hepatocellular carcinoma (310 expressing wild type p53, 114 expressing mutant p53), lung squamous cell carcinoma (141 expressing wild type p53, 382 expressing mutant p53), lung adenocarcinoma (308 expressing wild type p53, 253 expressing mutant p53), colon adenocarcinoma (337 expressing wild type p53, 70 expressing mutant p53), stomach adenocarcinoma (253 expressing wild type p53, 177 expressing mutant p53) and ovarian serous cystadenocarcinoma (184 expressing wild type p53, 277 expressing mutant p53). (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

To test this hypothesis, we ectopically over-expressed miR-766 in a panel of wild-type p53 cancer cell lines derived from breast cancer, lung cancer and sarcoma (Supplementary Figure 2) to observe its effect on p53 levels. In all cell lines examined, we observed increased p53 protein levels following transfection of miR-766 mimic (Figure 1A). Conversely, inhibition of miR-766 using an LNA inhibitor reduced p53 protein in SBC3 and U2OS cells, further supporting the concept that this miRNA maintains p53 expression (Figure 1B). To determine if these effects were transcriptional or post-transcriptional, p53 mRNA was measured following miR-766 over-expression in MCF10A, SBC3 and U2OS cells. No significant change in p53 mRNA was observed (Supplementary Figure 3), suggesting that p53 protein levels were increased by miR-766 at a post-transcriptional level.

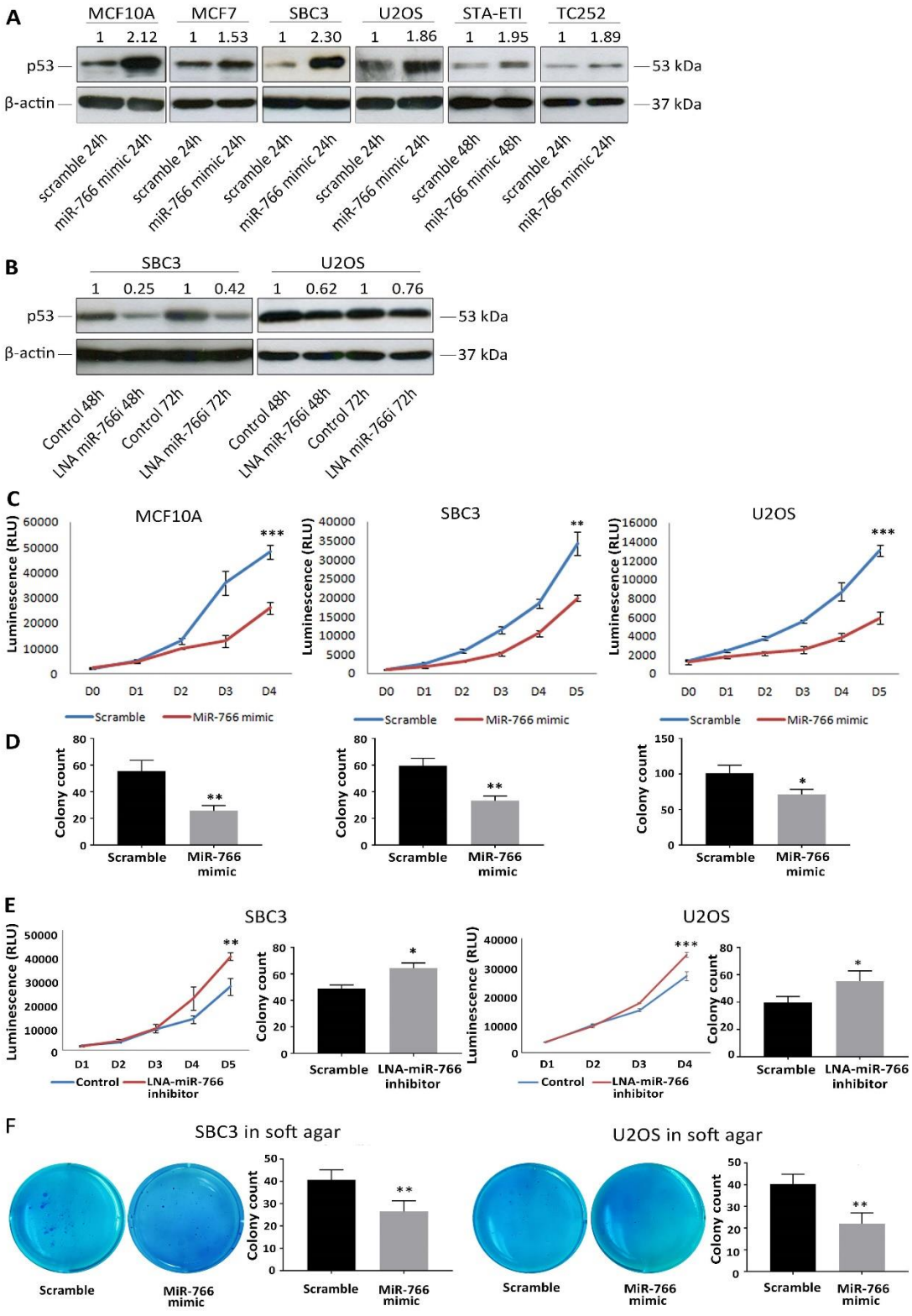
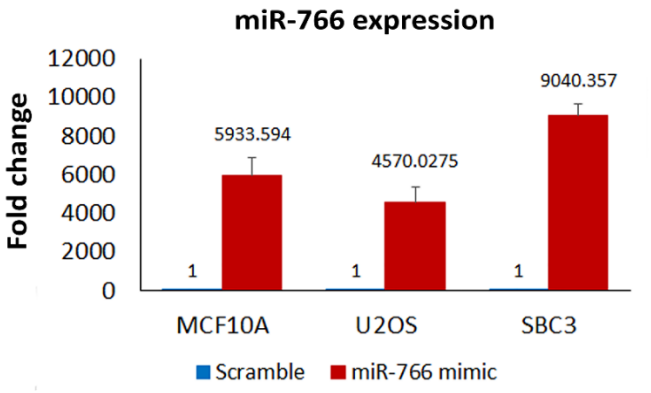
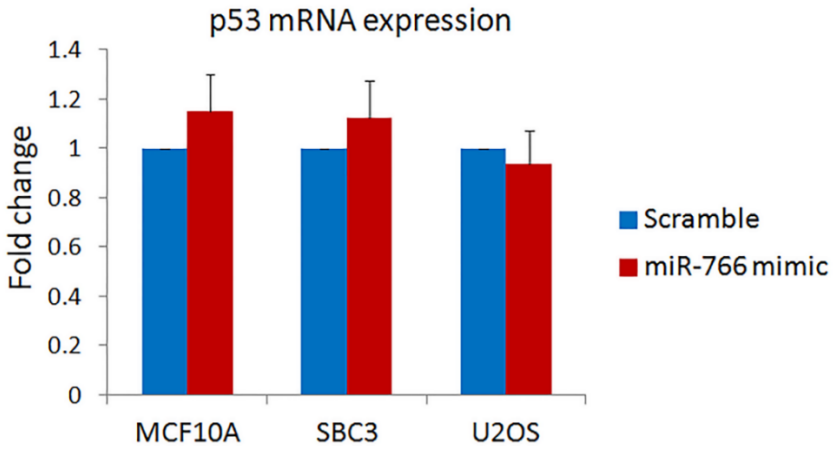

A
MCF10A
MCF7
SBC3
U2OS
STA-ETI
TC252
1 2.12
1 1.53
1 2.30
1 1.86
1 1.95
1 1.89
p53
β-actin
53 kDa
37 kDa
scramble 24h
miR-766 mimic 24h
scramble 48h
miR-766 mimic 48h
B
SBC3
U2OS
1 0.25 1 0.42
1 0.62 1 0.76
Control 48h
LNA miR-766i 48h
Control 72h
LNA miR-766i 72h
C
MCF10A
SBC3
U2OS
Luminescence (RLU)
D0 D1 D2 D3 D4 D5
Scramble
MiR-766 mimic
D
Colony count
Scramble
MiR-766 mimic
E
SBC3
U2OS
D1 D2 D3 D4 D5
Control
LNA-miR-766 inhibitor
F
SBC3 in soft agar
U2OS in soft agar

Figure 1. MiR-766 upregulates p53 expression and blocks cancer cell growth. **(A)** Whole protein lysates were collected 24 and 48 hours following transfection with miR-766 mimic or a "scramble" control miRNA mimic. p53 protein levels were detected by Western blotting (β-Actin was used as a loading control). **(B)** Whole protein lysates were collected 24 and 48 hours following LNA miR-766 inhibitor or LNA control transfection. p53 protein levels were detected by Western blotting (β-Actin was used as a loading control). **(C)** Cell proliferation was determined by Cell-TiterGlo Assay from in U2OS, SBC3 and MCF10A cells following transfection with miR-766 mimic or a "scramble" control miRNA mimic. **(D)** Colony formation assay was performed on U2OS, SBC3, MCF7 and MCF10A cells following transfection with miR-766 mimic or a "scramble" control miRNA mimic. **(E)** Cell proliferation was determined by Cell-TiterGlo Assay in SBC3 cells following transfection with LNA miR-766-inhibitor or LNA control. Colony formation assay was performed on SBC3 cells following transfection with LNA miR-766-inhibitor or LNA control. **(F)** Soft agar colony formation assay was performed using miR-766 mimic transfected SBC3 and U2OS cells in 0.7%-0.3% agar gel and incubated for 3 weeks. For all assays, p values (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) were determined by Student's t-test.



Supplementary Figure 2. MiR-766 over expression in MCF10A, U2OS and SBC3 cell lines with miRNA mimic. Total RNA was collected 24 hours after transfection and miR-766 levels were detected by Taqman real-time PCR. Results were normalized by GAPDH.



Supplementary Figure 3. MCF10A, SBC3 and U2OS cells were transfected with miR-766 mimic and scramble. Total RNA was collected 24 and 48 hours after transfection and p53 mRNA levels were detected by real-time PCR, results normalized by GAPDH.

We next asked whether the miR-766-mediated increase in p53 protein was associated with enhanced p53 cellular activity. Cell proliferation assays demonstrated that the growth of MCF10A, SBC3 and U2OS cells was significantly suppressed after ectopic over-expression of miR-766 (Figure 1C). Moreover, using a colony formation assay, we found that miR-766 decreased colonogenesis in MCF10A, SBC3 and U2OS cells (Figure 1D). Conversely, inhibition of miR-766 using an LNA inhibitor increased cell proliferation and colony formation of SBC3 and U2OS cells (Figure 1E). A soft agar colony formation assay was used to further investigate the anti-growth capacity of miR-766. As shown in Figure 1F, the ability of SBC3 and U2OS cell lines to generate spheres was significantly impaired after miR-766 over expression in both S. Collectively, these data reveal that miR-766 increases wild-type p53 levels, resulting in reduced cell proliferation and colony formation.

MiR-766 promotes G2/M arrest in U2OS and SBC3 cells.

To further explore the biological function of miR-766, flow cytometry was used to analyze its impact on cell cycle regulation. We observed a significantly increased proportion of cells in G2 phase 48 hours after miR-766 mimic transfection in U2OS and SBC3 cells (Figure 2A). Specifically, the proportion of cells blocked at the G2/M checkpoint increased from 17.9% to 33.8% in U2OS cells and 8.6% to 18.1% in SBC3 cells. Conversely, a decrease of G2 phase cells was observed in U2OS (24.1% to 14.3%) and SBC3 (16.4% to 9.9%) cells after miR-766 inhibition (Figure 2B).

To detect molecular alterations in the p53 pathway associated with changes in miR-766 expression, we characterized the mRNA levels of *GADD45A*, *SFN* and *GEST1* genes (as these genes are p53 downstream targets involved in cell cycle regulation) following transfection of miR-766 mimic. As expected, all of these genes were significantly induced by miR-766 (Figure 2C), which is consistent with observed increased expression of p53 and G2/M phase.

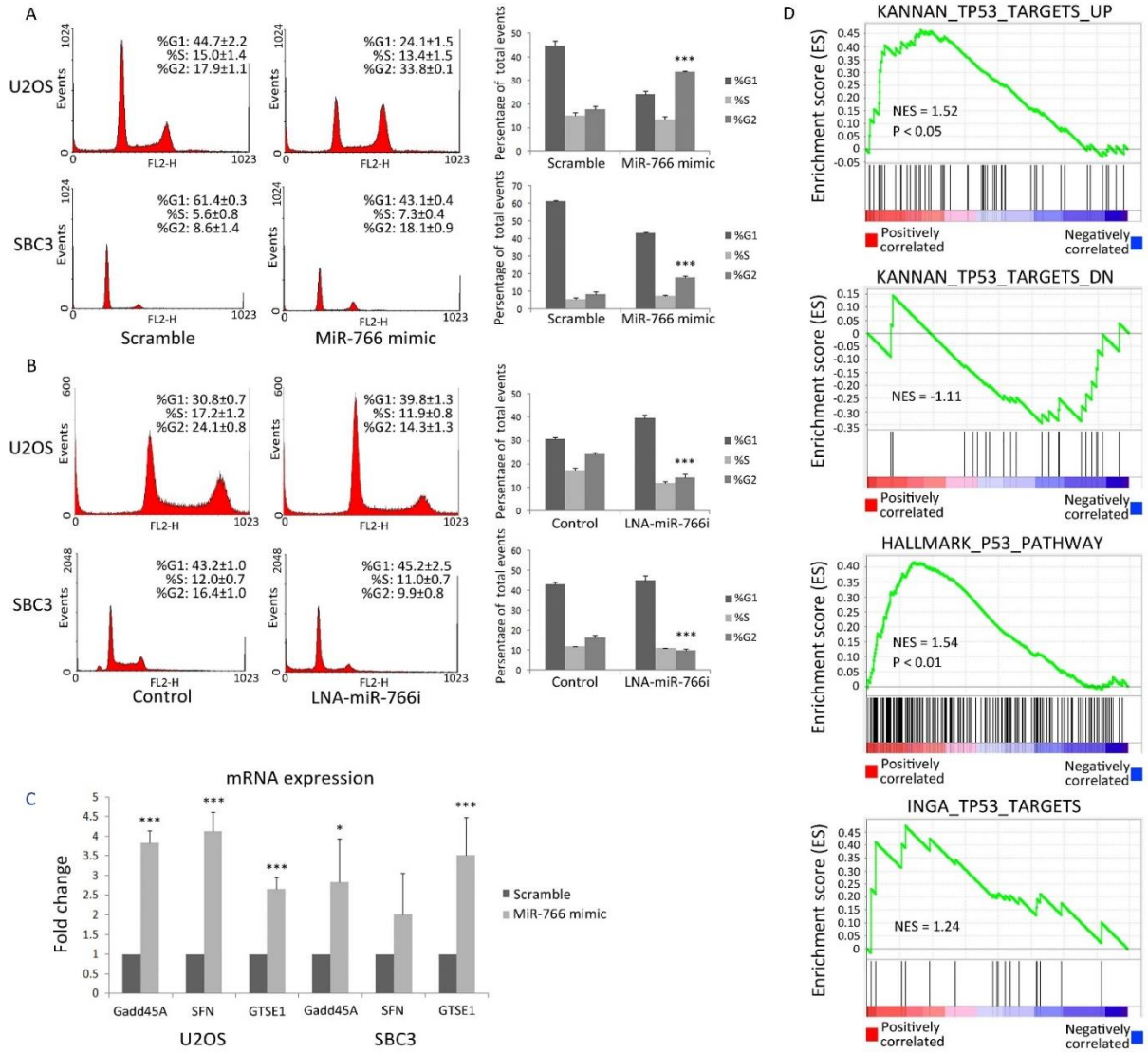


Figure 2. miR-766 is positively correlated with active p53 signalling. **(A-B)** Cell cycle profiles were analyzed through PI staining in U2OS and SBC3 48 hours after (A) miR-766 over-expression and (B) miR-766 inhibition. **(C)** Total RNA was collected from SBC3 and U2OS cells following transfection with miR-766 mimic or a "scramble" control miRNA mimic. *GADD45A*, *SFN* and *GSTE1* mRNA levels were detected by qRT-PCR (normalized using *GAPDH*). Results are the average of three independent experiments. p values (* $p<0.05$, *** $p<0.001$) were determined by Student's t-test.

(D) MiR-766 is positively correlated with p53 signalling in lung cancer, as determined by gene set enrichment analysis. The correlation between expression levels of miR-375 and 20,531 genes was calculated using matched miRNA and mRNA data from 102 lung tumors. Genes were subsequently ranked according to Pearson correlation coefficient (r) value (shown by a heat map), and GSEA (Preranked analysis) was implemented using the Broad Institute's public GenePattern server, using default parameters.

To explore the association between miR-766 and p53 signalling in clinical samples, we utilized gene set enrichment analysis (GSEA). By analysing lung cancer samples from TCGA, we identified a positive association between miR-766 expression and genes upregulated by p53 in 3 distinct gene sets, and a negative association between miR-766 and genes down-regulated by p53 in one gene set (Figure 2D). These associations further support the concept that miR-766 enhances p53 activity in cancer.

MDM4 is a direct target of miR-766

Since target genes of miRNAs are expected to be downregulated, we speculated that miR-766 may directly target one or more negative regulators of p53. The online miRNA target prediction tool miRDB.org lists 5 putative binding sites for miR-766 in the 3'UTR of *MDM4* mRNA (Figure 3A). To validate this potential interaction, sequences carrying the predicted miR-766 recognition sites were cloned downstream of luciferase and used for reporter assays. Sites 2, 3 and 4 were strongly repressed by miR-766, site 5 weakly, and site 1 was not regulated (Figure 3B). Having confirmed a direct interaction between miR-766 and sites in the *MDM4* 3'UTR, we subsequently transfected cells with miR-766 mimic and measured MDM4 expression. As shown in Figure 3C and 3D, miR-766 over-expression reduced both MDM4 protein and mRNA levels 48 hours in MCF10A, SBC3 and U2OS cells. Conversely, miR-766 inhibition with an LNA increased MDM4 protein (Figure 3C).

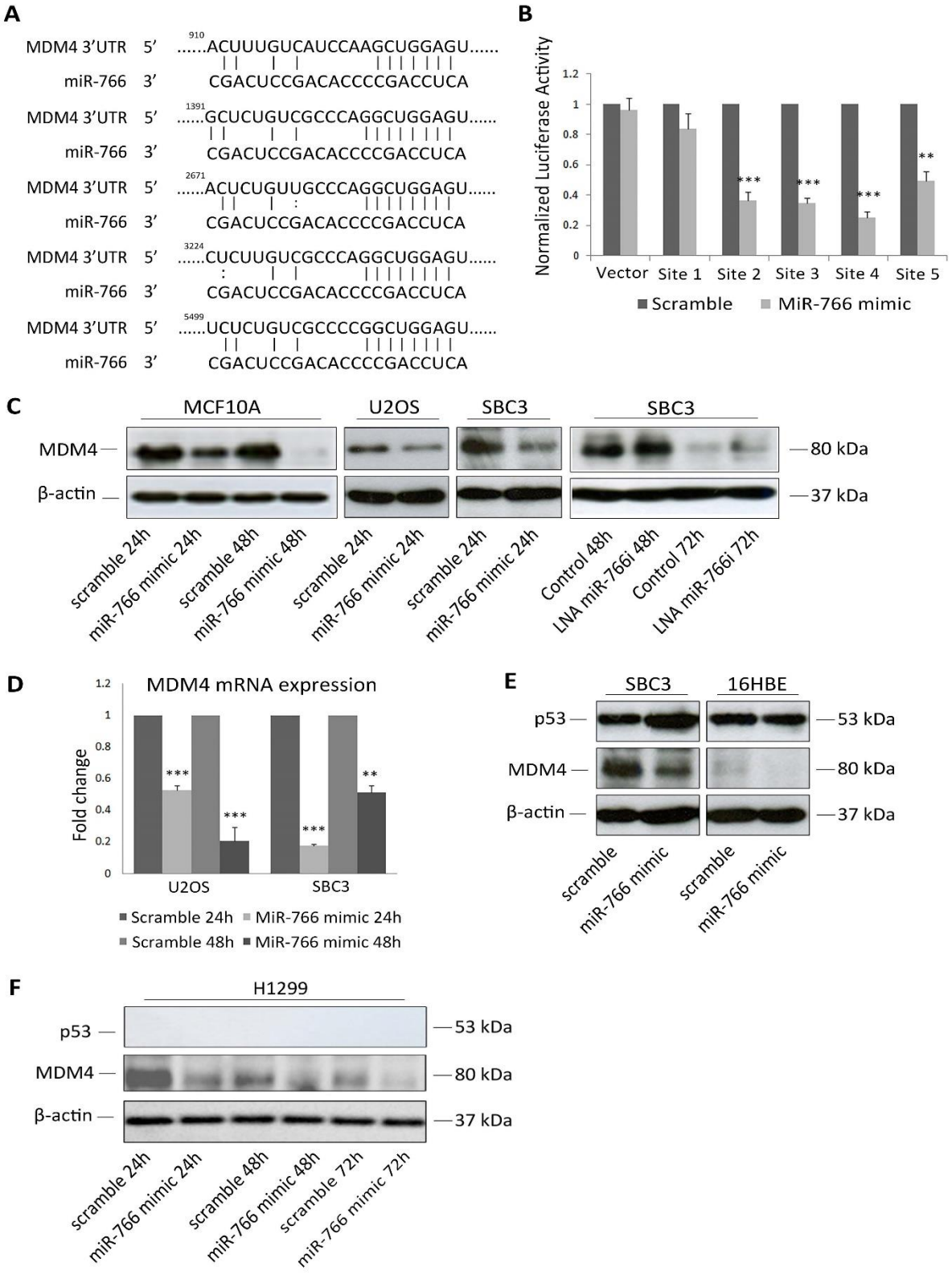
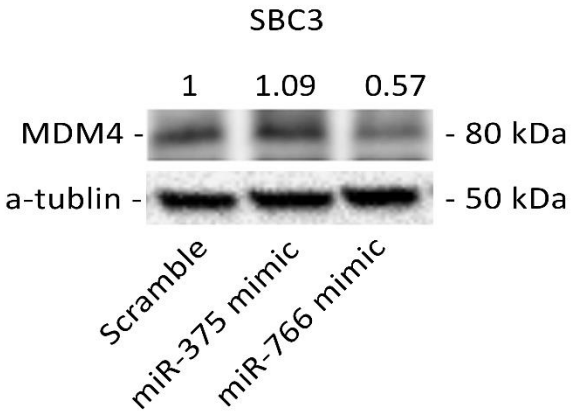

A
MDM4 3'UTR 5' 910 .....ACUUUGUCAUCCAAGCUGGAGU.....
miR-766 3' CGACUCCGACACCCCGACCUCA
MDM4 3'UTR 5' 1391 .....GCUCUGUCGCCCAGGCUGGAGU.....
miR-766 3' CGACUCCGACACCCCGACCUCA
MDM4 3'UTR 5' 2671 .....ACUCUGUUGCCCAGGCUGGAGU.....
miR-766 3' CGACUCCGACACCCCGACCUCA
MDM4 3'UTR 5' 3224 .....CUCUUGUCGCCCAGGCUGGAGU.....
miR-766 3' CGACUCCGACACCCCGACCUCA
MDM4 3'UTR 5' 5499 .....UCUCUGUCGCCCCGGCUGGAGU.....
miR-766 3' CGACUCCGACACCCCGACCUCA
B
Normalized Luciferase Activity
Vector
Site 1
Site 2
Site 3
Site 4
Site 5
Scramble
MiR-766 mimic
C
MCF10A
U2OS
SBC3
SBC3
MDM4
β-actin
80 kDa
37 kDa
scramble 24h
miR-766 mimic 24h
scramble 48h
miR-766 mimic 48h
scramble 24h
miR-766 mimic 24h
scramble 24h
miR-766 mimic 24h
Control 48h
LNA miR-766i 48h
Control 72h
LNA miR-766i 72h
D
MDM4 mRNA expression
Fold change
U2OS
SBC3
Scramble 24h
MiR-766 mimic 24h
Scramble 48h
MiR-766 mimic 48h
E
SBC3
16HBE
p53
MDM4
β-actin
53 kDa
80 kDa
37 kDa
scramble
miR-766 mimic
scramble
miR-766 mimic
F
H1299
p53
MDM4
β-actin
53 kDa
80 kDa
37 kDa
scramble 24h
miR-766 mimic 24h
scramble 48h
miR-766 mimic 48h
scramble 72h
miR-766 mimic 72h

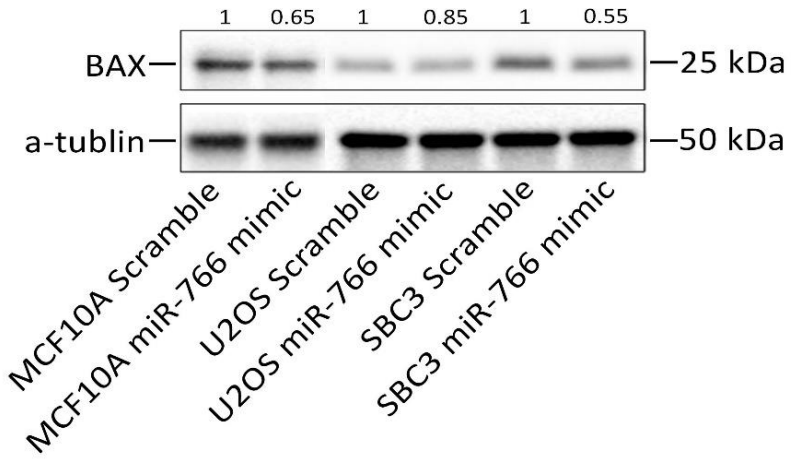
Figure 3. MDM4 is a direct target of miR-766. **(A)** Putative miR-766-binding sequence in the 3'UTR of *MDM4* mRNA. **(B)** Luciferase reporter assays 48 h after transfection with miR-766 mimics and reporter plasmids. Results are the average of 3 independent experiments. p values (* $p<0.05$, *** $p<0.001$) were determined by determined by unpaired *t* tests. **(C)** MCF10A, U2OS, SBC cells were transfected with miR-766 mimic or LNA miR-766-inhibitor and control. Whole protein lysates were collected 24 and 48 hours after transfection and p53 and MDM4 protein levels were detected by Western blotting (β-Actin was used as a loading control). **(D)** Total RNA was collected after miR-766-mimic transfection in U2OS and SBC3 cells. *MDM4* mRNA levels were detected by qRT-PCR (normalized using *GAPDH*). Results are the average of 3 independent experiments. p values (* $p<0.05$, *** $p<0.001$) were determined by Student's t-test. **(E-F)** SBC3 and 16HBE (E) and H1299 (F) cells were transfected with miR-766 mimic and a scramble control. Whole protein lysates were collected 24, 48 and 72 hours after transfection and p53 and MDM4 protein levels were detected by Western blotting (β-Actin and a-tublin were used as loading controls).

To confirm the specificity of MDM4 targeting by miR-766, an unrelated miRNA, miR-375, was used as an additional negative control (Supplementary Figure 4). Finally, to further validate the activity of miR-766, we assessed its effect on the expression of a previously reported target gene, BAX (Liang, Li et al. 2013): as expected, miR-766 caused a significant reduction of Bax protein (Supplementary Figure 5).

To further assess the relevance of the miR-766-MDM4 interaction in p53 signalling, we employed the 16HBE lung epithelial cell line, which expresses wild-type p53 but very low levels of MDM4 compared with SBC3 (Figure 3E). Although MDM4 was down-regulated by miR-766 in this line, there was no effect on p53, which can be explained by the very low basal levels of MDM4, while p53 was strongly increased in SBC3 following MDM4 inhibition. Considering the negative feedback regulation of the p53-MDM2-MDM4 axis (Gu, Kawai et al. 2002), we also examined miR-766-mediated targeting of MDM4 in a p53-negative line, H1299, to exclude the possibility that the change in MDM4 after miR-766 over expression was a result of p53 accumulation (Figure 3F). Although MDM4 protein was low in general in this model system, it was further reduced by miR-766 transfection, verifying the direct link between these two factors.



Supplementary Figure 4. SBC3 cells were transfected with miR-375 mimic, miR-766 mimic and scramble as control. Cell lysis was collected 48 hours after transfection and protein levels were detected by western blot. A-tublin was used as loading control.



Supplementary Figure 5. MCF10A, U2OS and SBC3 cells were transfected miR-766 mimic and scramble as control. Cell lysis was collected 48 hours after transfection and protein levels were detected by western blot. A-tublin was used as loading control.

To validate the impact of MDM4 knockdown in our model systems, U2OS and SBC3 cells were transfected with MDM4 siRNA. Consistent with our hypothesis, knockdown of MDM4 (Figure 4A) pheno-copied miR-766 over expression: more specifically, it induced significant cell growth repression (Figure 4B), decreased soft agar colonogenesis (Figure 4C) and caused an increase in G2/M phase cells (Figure 4D).

To further validate the biological relevance of the miR-766-MDM4-p53 regulatory pathway, a vector expressing the MDM4 cDNA without its 3'UTR was constructed. Since expression of MDM4 protein from this vector would be independent of miR-766, it was used in "rescue" experiments. Over-expression of MDM4 reversed both G2/M arrest (Figure 4E) and p53 accumulation (Figure 4F) induced by miR-766 over expression in SBC3 cells. This experiment confirms that MDM4 is likely to be the key target by which miR-766 enhances the p53 signalling pathway.

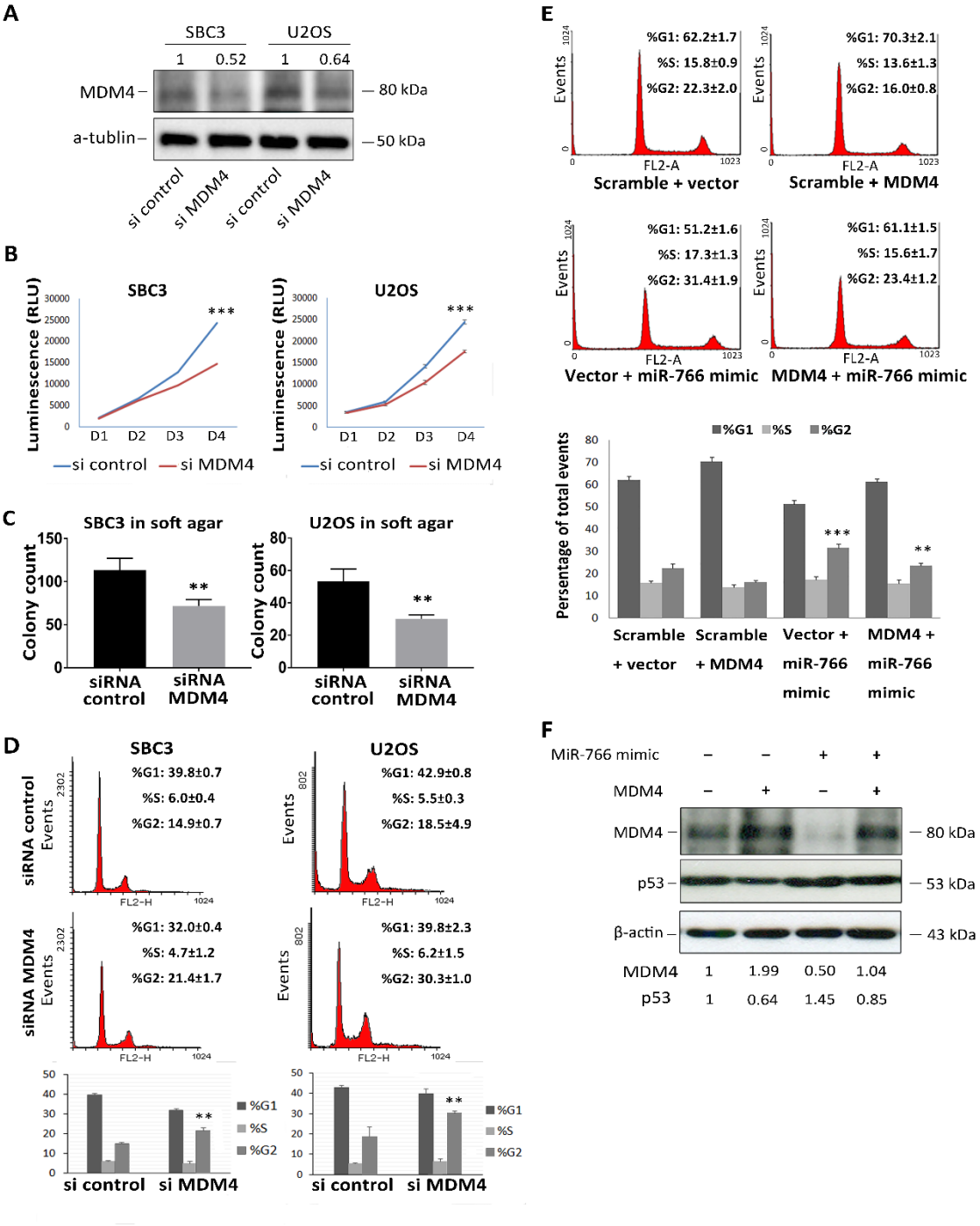

A
SBC3
U2OS
1
0.52
1
0.64
MDM4
a-tublin
80 kDa
50 kDa
si control
si MDM4
si control
si MDM4
B
SBC3
U2OS
Luminescence (RLU)
***
D1
D2
D3
D4
si control
si MDM4
C
SBC3 in soft agar
U2OS in soft agar
Colony count
**
siRNA control
siRNA MDM4
D
SBC3
U2OS
siRNA control
siRNA MDM4
Events
%G1: 39.8±0.7
%S: 6.0±0.4
%G2: 14.9±0.7
%G1: 42.9±0.8
%S: 5.5±0.3
%G2: 18.5±4.9
%G1: 32.0±0.4
%S: 4.7±1.2
%G2: 21.4±1.7
%G1: 39.8±2.3
%S: 6.2±1.5
%G2: 30.3±1.0
FL2-H
%G1
%S
%G2
si control
si MDM4
E
%G1: 62.2±1.7
%S: 15.8±0.9
%G2: 22.3±2.0
%G1: 70.3±2.1
%S: 13.6±1.3
%G2: 16.0±0.8
FL2-A
Scramble + vector
Scramble + MDM4
%G1: 51.2±1.6
%S: 17.3±1.3
%G2: 31.4±1.9
%G1: 61.1±1.5
%S: 15.6±1.7
%G2: 23.4±1.2
Vector + miR-766 mimic
MDM4 + miR-766 mimic
Persentage of total events
Scramble + vector
Scramble + MDM4
Vector + miR-766 mimic
MDM4 + miR-766 mimic
F
MiR-766 mimic
MDM4
p53
β-actin
80 kDa
53 kDa
43 kDa
MDM4 1 1.99 0.50 1.04
p53 1 0.64 1.45 0.85

Figure 4. MDM4 is a biologically relevant target of miR-766. **(A-D)** SBC3 and U2OS cells were transfected with MDM4 siRNA, **(A)** whole protein lysates were collected 48 hours after transfection, MDM4 protein levels were detected by Western blotting (a-tublin used as loading control); **(B)** Cell proliferation was determined by Cell-TiterGlo Assay following transfection; **(C)** transfected SBC3 and U2OS cells were plated in 0.7%-0.3% soft agar and incubated for 3 weeks; **(D)** Cell cycle profiles were analyzed through PI staining 48 hours after transfection. Results are the average of three independent experiments. p values (**P<0.01; *** *p<0.001)* were determined by Student's t-test. **(E)** SBC3 cells were co-transfected with miR-766 mimic and a MDM4 over-expression vector or a control vector. Cell cycle profiles were analyzed through PI staining 48 hours after transfection. Results are the average of three independent experiments. p values (**P<0.01; *** *p<0.001)* were determined by Student's t-test. **(F)** Whole protein lysates were collected from cells 48 hours after transfection as described in E. p53 and MDM4 protein levels were detected by Western blotting (β-Actin was used as a loading control).

Discussion

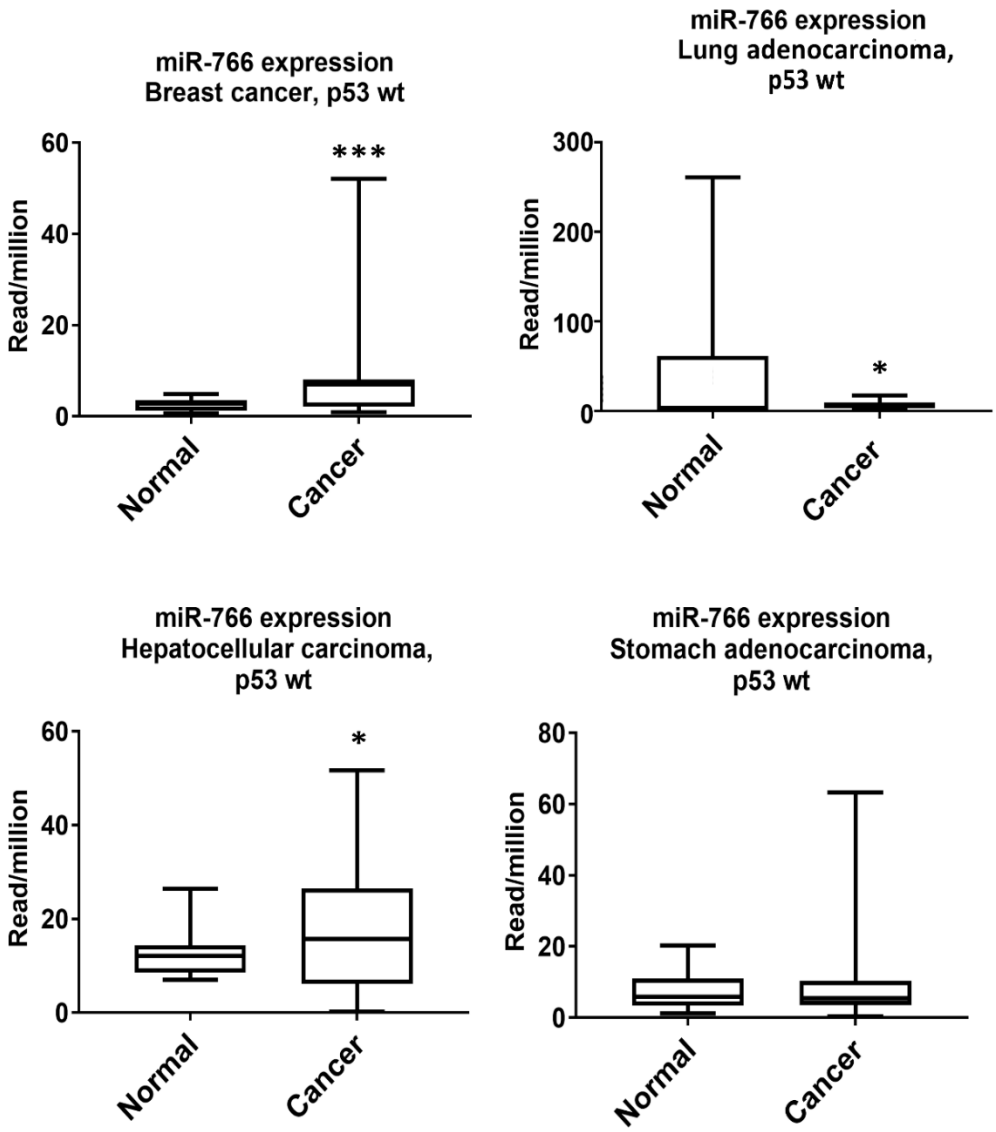
An increasing number of miRNAs have been recognized to participate in the post-transcriptional regulation of p53 and its downstream pathways. In this study, we have shown for the first time that miR-766 enhances p53 levels and signalling activity by directly targeting the p53 repressor, MDM4. By targeting MDM4, miR-766 enhances p53-mediated cell cycle arrest and repression of proliferation.

Cell cycle regulation is one of the core functions of p53. By transactivating downstream targets of p53, including *CDKN1A*, *GADD45A*, *SFN* and *GTSE1*, p53 induces cell cycle arrest, primarily at the G1/S and G2/M check points (Brown, Boswell et al. 2007). MiRNAs have been previously reported to participate in p53-related cell cycle regulation. In our study, *GADD45A, SFN* and *GTSE1* were significantly elevated by miR-766. Given that GADD45A (Taylor and Stark 2001) and SFN (Hermeking, Lengauer et al. 1997) function as inhibitors of G2/M progression, and GTSE1 is a p53-inducible gene that specifically delays the G2/M transition, we hypothesize that these genes are involved in the observed cell cycle arrest mediated by miR-766 over-expression.

While a number of studies describing roles for miR-766 in different cancer types have been published, overall its function in malignancy is poorly characterized. In acute promyelocytic leukemia cells, miR-766 was reported to be down-regulated following arsenic trioxide (As2O3) treatment, resulting in elevation of the miR-766 target *BAX* and enhanced cell apoptosis (Liang, Li et al. 2013). In contrast, our study shows miR-766 is a positive regulator of p53 via directly targeting MDM4, and participates in cell cycle regulation. Interestingly, the *BAX* gene is also a downstream target of p53 (Miyashita and

Reed 1995). This raises the interesting possibility that a miR-766-p53-Bax axis may exist such that miR-766 maintains p53-mediated cell cycle regulation while concomitantly suppressing Bax-induced cell death. MiR-766 has been shown to be upregulated in colorectal cancer and to be associated with promotion of cell proliferation and anchorage-independent growth by targeting SOX6 in the colorectal adenocarcinoma line SW480 (Li, Li et al. 2015). The results from this study define oncogenic roles to miR-766, which contrasts with our findings. The frequency of p53 mutations in colorectal cancer is over 50%, and the cell line SW480 is p53 mutant. We speculate that miR-766 can function as a tumor suppressor in a wild-type p53 environment but can switch to an oncomiR when p53 is inactivated by mutation.

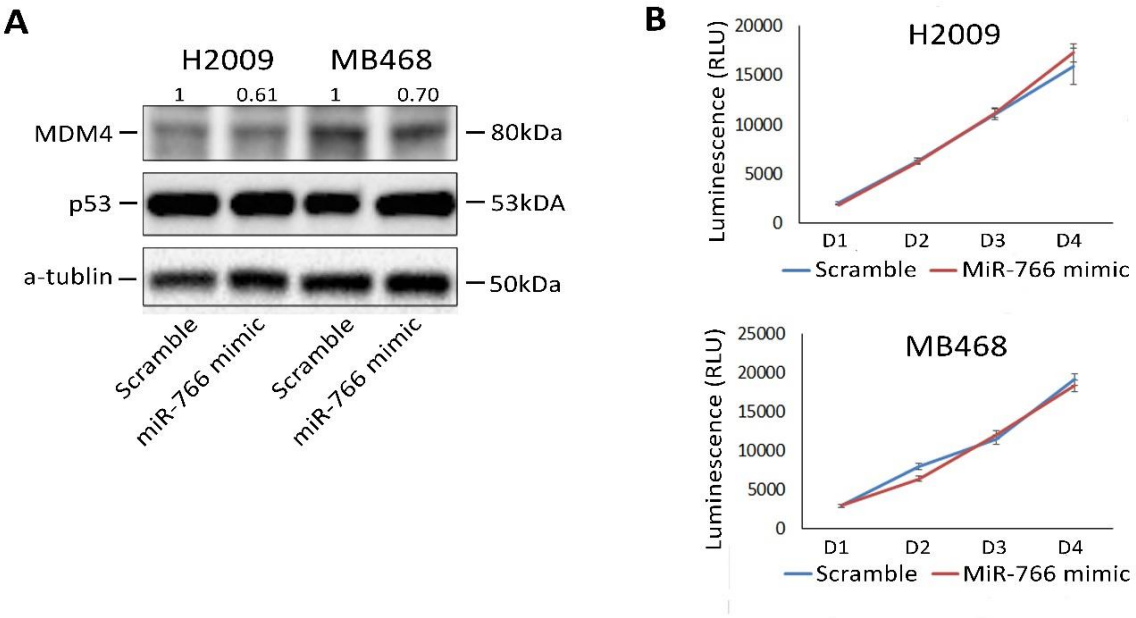
There are previous reports showing increased miR-766 expression in cancers. MiR-766 was found to be significantly up-regulated in 7 pairs of cutaneous squamous cell carcinoma biopsies (Sand, Skrygan et al. 2012) and 372 lung adenocarcinoma cases correlating with late stage and poorer prognosis (Li, Shi et al. 2014). The status of p53 and MDM4 was not specified in those studies but it is known that the frequency of p53 mutations is high in both cutaneous squamous cell carcinoma (40%-50%) (Owens 2007) and lung adenocarcinoma (63%) (Bian, Li et al. 2015). This knowledge, in combination with our finding that miR-766 is elevated in p53-mutant cancers (Supplementary Figure 1) complicates the association between miR-766 and malignancy identified in the earlier studies of lung adenocarcinoma (Li, Shi et al. 2014) and cutaneous squamous cell carcinoma (Sand, Skrygan et al. 2012). We examined the association between miR-766 and cancer specifically in wild-type p53 tumours of multiple cancer types. While miR-766 was upregulated in breast and liver, it was not significantly changed in stomach cancer and was down-regulated in lung adenocarcinoma (Supplementary Figure 6). With



Supplementary Figure 6. Small RNA deep sequencing data of different cancers expressing wild type p53 were downloaded from TCGA and matched with normal tissue from the same patient, including breast cancer (65 patients), lung adenocarcinoma (21 patients), hepatocellular carcinoma (36 patients), and stomach adenocarcinoma (33 patients) (* $p<0.05$, **$p<0.01$, *** $p<0.001$).

our new findings in mind, the links between miR-766 and malignancy, and hence its previous designation as an oncomiR (Liang, Li et al. 2013; Li, Li et al. 2015), are ambiguous.

Since miRNAs have multiple targets, their roles in normal biology and disease are highly context-dependent. Indeed, many examples exist where specific miRNAs have dichotomous functions in different tissues or disease states (Yi, Wang et al. 2012). With this concept in mind, what our study does conclusively indicate is that p53 status will influence the biological functions of miR-766 in tumours. Specifically, miR-766 can target MDM4 and thereby act as a tumor suppressor when MDM4 levels are high, but fails to regulate p53 levels or activity in cells with low expression of MDM4. Thus, in contexts where MDM4 levels are low, or in mutant p53 tumors where the normal transactivation activities of p53 are compromised, miR-766 may potentially exhibit oncogenic behaviour. Having said that, we failed to observe any significant impact on either p53 level or cell proliferation after miR-766 over expression in p53 mutant cell lines (H2009 expressing p53R273L and MDA-MB-468 expressing p53R273H) (Supplementary Figure 7).



Supplementary Figure 7. H2009 and MDA-MB-468 cells were transfected miR-766 mimic and scramble as control. **A,** Cell lysis was collected 48 hours after transfection and protein levels were detected by western blot. A-tublin was used as loading control; **B,** cell proliferation was determined by Cell-TiterGlo Assay from in H2009 and MDA-MB-468 cells.

MiR-766 was reported to increase in esophageal carcinoma cell lines following 5-fluorouracil treatment (Hummel, Wang et al. 2011). 5-fluorouracil is known to induce the accumulation of p53 and thereby activate p53 dependent pathways (Ju, Schmitz et al. 2007). Thus, we speculate that in addition to the known mechanism of p53 stabalization via MDM2 due to its activation of DNA damage pathways, miR-766 is up-regulated and this also contributes to stabilization of p53 via modulation of MDM4. In normal cells, elevated miR-766 levels can provide an additional mechanism that contributes to the stabilization of p53 after external insults. However, in cancer, this function of miR-766 is absent where p53 is mutated or through disruption of other pathways, resulting in miR-766 engaging in alternative functions, some of which may be oncogenic. Based on these results, it is likely that high levels of miR-766 in the context of wild-type p53 may provide an approach for identifying cancers with greater sensitivity to DNA damaging agents.

In summary, our study is the first to identify miR-766 as a novel p53 activator that functions by targeting MDM4 and thereby enhancing the p53 signalling axis. We propose that miR-766 could serve as a potential marker for MDM4 and wild-type p53 based cancer therapies such as MDM4 siRNA and 5-fluorouracil treatment.

Materials and methods

Cell lines and reagents

MCF10A, MCF7, HEK293T, MDA-MB468 and U2OS cell lines were purchased from the American Type Culture Collection (ATCC). Other sarcoma cell lines used were supplied by G. Hamilton (University of Vienna, Austria) (TC252) and P. Ambros (St. Anna Children's Hospital, Austria) (STA-ET-1). SBC-3 and H2009 were kindly given as a gift from Dr. Sandra Hodge (University of Adelaide, Australia). SBC 3 was maintained in DMEM with 10% FBS, HEPES and PSG. H2009 was maintained in RPMI-1640 with 10% FBS. All other cell lines were cultured as previously described (Pishas, Al-Ejeh et al. 2011; Noll, Jeffery et al. 2012; Yu, Bracken et al. 2015).

Reverse-transcription PCR (RT-PCR) and real-time RT-PCR

RNA extraction from cells was performed using the RNAeasy mini kit (Qiagen, Valencia, CA USA) and MIReasy mini kit (Qiagen). ND-1000 NanoDrop spectrometer (Thermo Scientific, Wilmington, DE USA) were used to measure RNA concentration. Total RNA and small RNA were reverse-transcribed into cDNA as previously described using Moloney Murine Leukaemia Virus (M-MLV) Reverse Transcriptase (Promega, Madison, WI USA) and random 6'mer primers (Promega) (Yu, Bracken et al. 2015).

To determine Real-time PCR reactions were performed using IQ SYBR Green Supermix (BioRad) on a BioRad iCycler (BioRad) as previously described (Pishas, Al-Ejeh et al. 2011). Primers used for Real-time PCR were listed in table 2. Relative expression levels of specific mRNAs were subsequently determined by the ΔΔCT method and normalized with GAPDH (Primers see Supplementary Table 2.6.1).

Supplementary Table 2.6.1. Primers used for real-time PCR

Gene	Primer	Sequence (5'-3')
MDM4	Forward	TCTCGCTCTCGCACAGGATCACA
	Reverse	AACCACCAAGGCAGGCCAGCTA
GADD45A	Forward	AAAGGATGGATAAGGTGGGGG
	Reverse	TGATGTCGTTCTCGCAGCA
SFN	Forward	CTGGACAGCCACCTCATCAA
	Reverse	GACGGAAAAGTTCAGGGCCA
GTSE1	Forward	GGGAGAAGTTCGTGGAGGTG
	Reverse	ATTCCCTGGGCAAAGCATGA
TP53	Forward	GGGCTCCGGGGACACTTTGCG
	Reverse	TGGCAGTGACCCGGAAGGCAGT
GAPDH	Forward	AGCCTCCCGCTTCGCTCTCTGC
	Reverse	ACCAGGCGCCCAATACGACCAAA

Vector construction

For luciferase assay, MDM4 3'UTR parts with miR-766 binding sites were obtained by synthetic single chain oligo annealing (oligo sequences see Supplementary Table 2.6.2), and were connected into psiCHECK2 dual-luciferase vector (Promega).

For MDM4 over expression, pENTR223.1 with MDM4 cDNA clone was purchased from Mybiosource. pcDNA3.2-DEST-MDM4 and control were constructed using pcDNA™3.2/GW/D-TOPO® Expression Kit from Raman Sharma (University of Adelaide, Australia) and pENTR223.1-MDM4.

Transient transfection

Asynchronously growing cells were seeded at 3 x 10^5 cells/well in six-well plates or 1 x 10^5 cells/well in 24-well plates. Transfection of cells with 50 nM mimic miR-766 (Genepharma, Shanghai, CN) or 50 nM LNA anti-miR (EXIQON, Denmak) or 100nM siRNA (Sigma Aldrich) was performed using Lipofectamine RNAiMAX (Invitrogen, CA, USA). Transfection of cells with 1ug/mL with psiCHECK2 dual-luciferase reporter vectors or pcDNA3.2-DEST-MDM4 and control was performed using Lipofectamine 2000 (Invitrogen).

Cell proliferation assay

Cells were plated at 1000 cells/well in 96-well plates 24 hours after transfection and were cultured for 1, 2, 3, 4 and/or 5 days. On the indicated days, cells were incubated with CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega) and fluorescence was measured by LUMIstar Galaxy luminometer (BMG Labtech).

Supplementary Table 2.6.2 Oligo sequences for annealing

MiR-766 binding site	Oligo	Sequence
Binding site 1	Forward (5'-3')	TCGAGTGAGAGACGGTCTCACTTTGTCATCCAAGCTGGAGTGCAGTGGTGCAAACACGGCCCACC TCCTGGGCTCGC
	Reverse (5'-3')	GGCCGCGAGCCCAGGAGGTGGGCCGTGTTTGCACCACTGCACTCCAGCTTGGATGACAAAGTGAG ACCGTCTCTCAC
Binding site 2	Forward (5'-3')	TCGAGGACGGTCTCACTTTGTCATCCAAGCTGGAGTGCAGTGGTGCAAACACGGCCCACCTCCTG GGCTCAAGTGGC
	Reverse (5'-3')	GGCCGCCACTTGAGCCCAGGAGGTGGGCCGTGTTTGCACCACTGCACTCCAGCTTGGATGACAAA GTGAGACCGTCC
Binding site 3	Forward (5'-3')	TCGAGGACAGTCTCACTCTGTTGCCCAGGCTGGAGTGCAATGGCATGATCTCTGCTCACCGCAAC CTCTGCCTCCGC
	Reverse (5'-3')	GGCCGCGGAGGCAGAGGTTGCGGTGAGCAGAGATCATGCCATTGCACTCCAGCCTGGGCAACAG AGTGAGACTGTCC
Binding site 4	Forward (5'-3')	TCGAGTACTTGAGATGGAGTTTTGCTCTTGTCGCCCAGGCTGGAGTGCAGTGGAGTGATCTCGGCT CACTGCAACGC
	Reverse (5'-3')	GGCCGCGTTGCAGTGAGCCGAGATCACTCCACTGCACTCCAGCCTGGGCGACAAGAGCAAAACTC CATCTCAAGTAC
Binding site 5	Forward (5'-3')	TCGAGTAGACGGAGTCTCTCTCTGTCGCCCCGGCTGGAGTGCAGTGGCGCGATCTCGGCTCACTG CAACCTCCGC
	Reverse (5'-3')	GGCCGCGGAGGTTGCAGTGAGCCGAGATCGCGCCACTGCACTCCAGCCGGGGCGACAGAGAGAG ACTCCGTCTAC

Cell clonogenic assay

500 cells were placed in 6-well plates 24 hours after transfection, and cultured in complete medium for 10 days (MCF10A and SBC3) and 15 days (U2OS). Colonies were fixed with methanol, stained with 0.1% crystal violet, and counted.

Soft agar colony formation assay

15000 cells were mixed with 0.35% soft agar and placed in 6-well plates coated with 0.7% soft agar 24 hours after transfection, and cultured for 3 weeks (SBC3) and 5 weeks (U2OS). Colonies were fixed with ethanol, stained with 0.1% crystal violet, and counted.

Cell cycle analysis

48 and 72 hours after transfections, cells were harvested and fixed as previously described for cell cycle analysis (Neilsen, Noll et al. 2011). Fixed cells were stained with 100 μg/mL RNase A (Sigma Aldrich) and 50 μg/mL propidium iodide solution (Sigma Aldrich) in PBS for 45 minutes at 37°C. DNA content was determined by a FACSCalibur™ flow cytometer (BD, CA, USA). Cell cycle profiles were analyzed using WinMDI v2.8 software (Scripps Research Institute).

Western blot and antibodies

Western blot assay was performed as previously described (Yu, Bracken et al. 2015). Antibodies used include: mouse anti-p53 DO-1 (Santa Cruz Biotechnology, Santa Cruz, CA), Rabbit anti-MDM4 (Bethyl Laboratories), mouse anti-BAX (Invitrogen), mouse anti-a-tublin (Santa Cruz) and mouse anti-β-actin (Sigma Aldrich).

Luciferase assay

For the luciferase reporter assay, 293T cells were plated in a 24-well plate and then cotransfected with 50 μM of either miR-766 mimic or negative control, and 1 ug of psiCHECK2-MDM4-3'UTR-BS1, psiCHECK2-MDM4-3'UTR-BS2, psiCHECK2-MDM4-3'UTR-BS3, psiCHECK2-MDM4-3'UTR-BS4 or psiCHECK2-MDM4-3'UTR-BS5, using Lipofectamine 2000 (Invitrogen). Cells were collected 48 h after transfection and fluorescence was meassured using the Dual-Luciferase Reporter Assay System (Promega). Both Renilla and Firefly luminescence were measured with a GloMax 20/20 Luminometer (Promega). Renilla luciferase was used as an internal control for any differences in transfection and harvesting efficiencies. Transfections were performed in duplicate and repeated at least three times in independent experiments.

Gene set enrichment analysis

The Pearson correlation coefficient (r) between miR-766 and 20,501 genes was calculated using sample matched miRNA and mRNA expression profiles from 102 wild-type p53 lung tumors (data was obtained from the TCGA data portal). Genes were subsequently ranked by r value. GSEA Preranked analysis was implemented using the Broad Institute's public GenePattern server, using default parameters and 3 distinct p53-associated gene sets: the Kannan_TP53_Targets gene sets contain 58 up-regulated and

24 down-regulated p53 gene targets; the Inga_TP53_Targets gene set contains 17 direct p53 targets that were verified to be strongly activated following p53 expression; the Hallmark p53 gene set covers a broader range of both direct and indirect targets of p53.

Statistical analysis

Data are presented as means ± SD from at least three independent experiments. Student's t-test was performed using replicate values to compare between groups and indicate significance. Values of $p<0.05$ were considered statistically significant (as labeled as * in figures), while values of $p<0.01$ were labeled as ** and $p<0,001$ as ***.

References

Afanasyeva, E. A., P. Mestdagh, et al. (2011). "MicroRNA miR-885-5p targets CDK2 and MCM5, activates p53 and inhibits proliferation and survival." Cell Death Differ **18**(6): 974-984.

Bartel, D. P. (2004). "MicroRNAs: genomics, biogenesis, mechanism, and function." Cell **116**(2): 281-297.

Bian, C., Z. Li, et al. (2015). "Clinical outcome and expression of mutant P53, P16, and Smad4 in lung adenocarcinoma: a prospective study." World J Surg Oncol **13**: 128.

Brown, L., S. Boswell, et al. (2007). "Transcriptional targets of p53 that regulate cellular proliferation." Crit Rev Eukaryot Gene Expr **17**(1): 73-85.

Burns, D. M., A. D'Ambrogio, et al. (2011). "CPEB and two poly(A) polymerases control miR-122 stability and p53 mRNA translation." Nature **473**(7345): 105-108.

Chakraborty, S., A. Adhikary, et al. (2014). "Capsaicin-induced activation of p53-SMAR1 auto-regulatory loop down-regulates VEGF in non-small cell lung cancer to restrain angiogenesis." PLoS One **9**(6): e99743.

Chang, C. J., C. H. Chao, et al. (2011). "p53 regulates epithelial-mesenchymal transition and stem cell properties through modulating miRNAs." Nat Cell Biol **13**(3): 317-323.

Chang, T. C., E. A. Wentzel, et al. (2007). "Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis." Mol Cell **26**(5): 745-752.

Fornari, F., L. Gramantieri, et al. (2009). "MiR-122/cyclin G1 interaction modulates p53 activity and affects doxorubicin sensitivity of human hepatocarcinoma cells." Cancer Res **69**(14): 5761-5767.

Gu, J., H. Kawai, et al. (2002). "Mutual dependence of MDM2 and MDMX in their functional inactivation of p53." J Biol Chem **277**(22): 19251-19254.

Hermeking, H. (2009). "MiR-34a and p53." Cell Cycle **8**(9): 1308.

Hermeking, H., C. Lengauer, et al. (1997). "14-3-3sigma is a p53-regulated inhibitor of G2/M progression." Mol Cell **1**(1): 3-11.

Hoffman, Y., D. R. Bublik, et al. (2014). "miR-661 downregulates both Mdm2 and Mdm4 to activate p53." Cell Death Differ **21**(2): 302-309.

Hoffman, Y., D. R. Bublik, et al. (2014). "MiR-661 downregulates both Mdm2 and Mdm4 to activate p53." Cell Death and Differentiation **21**(2): 302-309.

Honda, R., H. Tanaka, et al. (1997). "Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53." FEBS Lett **420**(1): 25-27.

Hu, W., C. S. Chan, et al. (2010). "Negative regulation of tumor suppressor p53 by microRNA miR-504." Mol Cell **38**(5): 689-699.

Hummel, R., T. Wang, et al. (2011). "Chemotherapy-induced modification of microRNA expression in esophageal cancer." Oncol Rep **26**(4): 1011-1017.

Isobe, M., B. S. Emanuel, et al. (1986). "Localization of gene for human p53 tumour antigen to band 17p13." Nature **320**(6057): 84-85.

Jansson, M. D., N. D. Damas, et al. (2015). "miR-339-5p regulates the p53 tumor-suppressor pathway by targeting MDM2." Oncogene **34**(15): 1908-1918.

Ju, J., J. C. Schmitz, et al. (2007). "Regulation of p53 expression in response to 5-fluorouracil in human cancer RKO cells." Clin Cancer Res **13**(14): 4245-4251.

Kumar, M., Z. Lu, et al. (2011). "Negative regulation of the tumor suppressor p53 gene by microRNAs." Oncogene **30**(7): 843-853.

Le, M. T., C. Teh, et al. (2009). "MicroRNA-125b is a novel negative regulator of p53." Genes Dev **23**(7): 862-876.

Levine, A. J. (1997). "p53, the cellular gatekeeper for growth and division." Cell **88**(3): 323-331.

Li, X., Y. Shi, et al. (2014). "An eight-miRNA signature as a potential biomarker for predicting survival in lung adenocarcinoma." J Transl Med **12**: 159.

Li, Y. C., C. F. Li, et al. (2015). "MicroRNA-766 targeting regulation of SOX6 expression promoted cell proliferation of human colorectal cancer." Onco Targets Ther **8**: 2981-2988.

Liang, H., X. Li, et al. (2013). "MicroRNAs contribute to promyelocyte apoptosis in As2O3-treated APL cells." Cell Physiol Biochem **32**(6): 1818-1829.

Linares, L. K., A. Hengstermann, et al. (2003). "HdmX stimulates Hdm2-mediated ubiquitination and degradation of p53." Proc Natl Acad Sci U S A **100**(21): 12009-12014.

Mandke, P., N. Wyatt, et al. (2012). "MicroRNA-34a modulates MDM4 expression via a target site in the open reading frame." PLoS One **7**(8): e42034.

Miyashita, T. and J. C. Reed (1995). "Tumor suppressor p53 is a direct transcriptional activator of the human bax gene." Cell **80**(2): 293-299.

Nag, S., J. Qin, et al. (2013). "The MDM2-p53 pathway revisited." J Biomed Res **27**(4): 254-271.

Neilsen, P. M., J. E. Noll, et al. (2011). "Mutant p53 uses p63 as a molecular chaperone to alter gene expression and induce a pro-invasive secretome." Oncotarget **2**(12): 1203-1217.

Noll, J. E., J. Jeffery, et al. (2012). "Mutant p53 drives multinucleation and invasion through a process that is suppressed by ANKRD11." Oncogene **31**(23): 2836-2848.

Okada, N., C. P. Lin, et al. (2014). "A positive feedback between p53 and miR-34 miRNAs mediates tumor suppression." Genes Dev **28**(5): 438-450.

Owens, D. M. (2007). "p53, chemokines, and squamous cell carcinoma." J Clin Invest **117**(7): 1752-1755.

Park, E. K., J. C. Lee, et al. (2015). "Transcriptional repression of cancer stem cell marker CD133 by tumor suppressor p53." Cell Death Dis **6**: e1964.

Pishas, K. I., F. Al-Ejeh, et al. (2011). "Nutlin-3a is a potential therapeutic for ewing sarcoma." Clin Cancer Res **17**(3): 494-504.

Raver-Shapira, N., E. Marciano, et al. (2007). "Transcriptional activation of miR-34a contributes to p53-mediated apoptosis." Mol Cell **26**(5): 731-743.

Sand, M., M. Skrygan, et al. (2012). "Microarray analysis of microRNA expression in cutaneous squamous cell carcinoma." J Dermatol Sci **68**(3): 119-126.

Schubert, J. and T. Brabletz (2011). "p53 Spreads out further: suppression of EMT and stemness by activating miR-200c expression." Cell Res **21**(5): 705-707.

Sharp, D. A., S. A. Kratowicz, et al. (1999). "Stabilization of the MDM2 oncoprotein by interaction with the structurally related MDMX protein." J Biol Chem **274**(53): 38189-38196.

Shvarts, A., W. T. Steegenga, et al. (1996). "MDMX: a novel p53-binding protein with some functional properties of MDM2." EMBO J **15**(19): 5349-5357.

Stad, R., Y. F. Ramos, et al. (2000). "Hdmx stabilizes Mdm2 and p53." J Biol Chem **275**(36): 28039-28044.

Swarbrick, A., S. L. Woods, et al. (2010). "miR-380-5p represses p53 to control cellular survival and is associated with poor outcome in MYCN-amplified neuroblastoma." Nat Med **16**(10): 1134-1140.

Tanimura, S., S. Ohtsuka, et al. (1999). "MDM2 interacts with MDMX through their RING finger domains." FEBS Lett **447**(1): 5-9.

Taylor, W. R. and G. R. Stark (2001). "Regulation of the G2/M transition by p53." Oncogene **20**(15): 1803-1815.

Wang, Y., J. W. Huang, et al. (2014). "p53 Is Positively Regulated by miR-542-3p." Cancer Res **74**(12): 3218-3227.

Xiao, J., H. Lin, et al. (2011). "miR-605 joins p53 network to form a p53:miR-605:Mdm2 positive feedback loop in response to stress." Embo j **30**(3): 524-532.

Yi, C., Q. Wang, et al. (2012). "MiR-663, a microRNA targeting p21(WAF1/CIP1), promotes the proliferation and tumorigenesis of nasopharyngeal carcinoma." Oncogene **31**(41): 4421-4433.

Yu, F., C. P. Bracken, et al. (2015). "p53 Represses the Oncogenic Sno-MiR-28 Derived from a SnoRNA." PLoS One **10**(6): e0129190.

Chapter 3.

Overexpression of miR-9 leads to mutation specific knockdown of missense p53

3.1. Preface

As introduced in chapter 1.4, mutant p53 is found in over 50% of human cancers and presents as a highly stable protein. Certain missense p53 mutations have been reported to have properties consistent with an oncogene due to an associated GOF property. However, the regulation of missense p53 remains largely unclear. Our group has been interested in the participation of miRNAs in the post-transcriptional regulation of missense p53 and its influence in cancer. This part of the thesis investigates the miRNA-mediated regulation of missense p53 and discusses the potential mechanism behind our novel observation.

3.2. Introduction

Somatic *TP53* mutations have been broadly found in 50% of human cancers with missense mutations in the DNA binding domain (DBD) as the most common alternations to p53. Missense p53 loses its function as a tumour suppressor due to the structural changes of the p53 protein [1], which may prevent the interaction between the missense p53 and the normal targets of wild-type p53. Interestingly, missense p53 is also found capable of inducing selected wild-type p53 downstream targets via p63, but unlike the transitory effect of wild-type p53, this missense p53-mediated induction is constant in the cell [2, 3]. Moreover, some missense p53 mutations are found to gain oncogenic functions (GOF) by trans-activating a set of downstream genes that are different from wild-type p53 [4-6].

The mechanisms of post-transcriptional regulation of missense p53 are of importance due to its oncogenic functions. Similar to wild-type p53, E3 ligase mediated ubiquitination and degradation is the core mechanism of missense p53 regulation. MDM2, CHIP, Pirh2 and COP1, the essential E3 ligases of wild-type p53, are also able to ubiquitinate missense p53 [7, 8]. In contrast to wild-type p53, the MDM2-mediated ubiquitination of missense p53 does not necessarily lead to rapid degradation of the protein, which partially explains the stability of missense p53 in cancers [7, 9]. USP10, on the other hand, is a deubiquitinating enzyme of both missense and wild-type p53 that can help p53 evade the E3-ligase-mediated regulation [10]. Several genes have also been revealed to regulate mutant p53, including Hsp90 [11, 12] and Hsp70 [13], which protect missense p53 from CHIP mediated ubiquitination. However, overall the post-transcriptional regulation of missense p53 is still unclear compared to wild-type p53.

As discussed, miRNAs are extensively involved in the regulation wild-type p53 but there is also emerging evidence of participation in the regulation of missense p53. There have been reports of miR-600s and miR-3158 directly targeting missense p53 in colorectal and breast cancer cell lines [14, 15]. MiR-644 also regulates missense p53 indirectly by targeting CtBP1 in breast cancer [16]. However, the crosstalk of miRNA-mediated regulation in mutant and wild-type p53 remains largely unexplored.

The MiR-9 family consists of three members: miR-9-1, miR-9-2, miR-9-3, which are derived from three different locations in the human genome (chromosomes 1, 5 and 15) [17]. Initially, miR-9 was found to participate in neurogenesis by regulating the survival and development of neuronal progenitor cells [18]. Latter studies reported aberrant miR-9 levels in multiple cancers and suggested its involvement in human cancers. Overexpression of miR-9 was found in primary brain cancer [19], lung cancer [20, 21], lymphoma [22, 23] and breast cancer [24-26]. The high expression of miR-9 is associated with higher invasion ability and metastasis potential in these cancers. Moreover, in 2016 a systematic meta-analysis reviewed the prognostic value of miR-9 in cancers and indicated overexpression of miR-9 could predict poor overall survival of osteosarcoma, lung cancer and breast cancer [27].

In contrast, the opposite function of miR-9 was found in ovarian cancer [28, 29], colorectal cancer [30] and renal clear cell carcinoma [31] with reduced expression of miR-9 and a tumour suppressor behaviour in cancer cell proliferation and invasion. Meta-analysis also suggested that overexpressed mir-9 was associated with good overall

survival, and the low levels of tissue miR-9 could predict poor progression-free survival in ovarian carcinoma patients [27].

In this chapter, our work identifies the distinctive expression patterns of miR-9 in tumours expressing wild-type or missense p53 across different cancers, and observed an opposite impact of miR-9 on missense p53 levels compared with wild-type p53 in cell lines. This is the first report of a miRNA that can specifically regulate a particular p53 mutation. This may challenge the current understanding of miRNA-mRNA interactions. Hypothesises are proposed to provide a basis for this observation although their confirmation will require further exploration in the future.

3.3. Results

3.3.1. MiR-9 is aberrantly expressed in tumours expressing missense p53.

The previously described analysis of small RNA deep sequencing data from TCGA revealed several miRNAs that showed ectopic expression in missense p53 tumours compared to wild-type p53 tumours (Chapter 2). MiR-9, including miR-9-1, miR-9-2 and miR-9-3, was one of those miRNAs that presented significant elevation in the missense p53 group of breast cancers (Figure 3.1A). Additional data from different cancers was analysed, including lung squamous cell carcinoma, ovarian carcinoma, renal clear cell carcinoma and colon cancer (Figure 3.1). MiR-9 showed the same trend between missense and wild-type p53 in lung squamous cell carcinoma as in breast cancer, but the difference was not significant (Figure 3.1A). By contrast, miR-9 was downregulated in missense p53 tumours in ovarian carcinoma, renal clear cell carcinoma and colon cancer (Figure 3.1B).

Considering the reported pro-metastasis role of miR-9 in breast cancer and lung cancer and the anti-cancer function of miR-9 in the other three cancers, the trend of miR-9 expression in the missense p53 group of both cancers suggests that there is a potential link between miR-9 expression and p53 status relating to mutant p53 in cancer progression in these cancers.

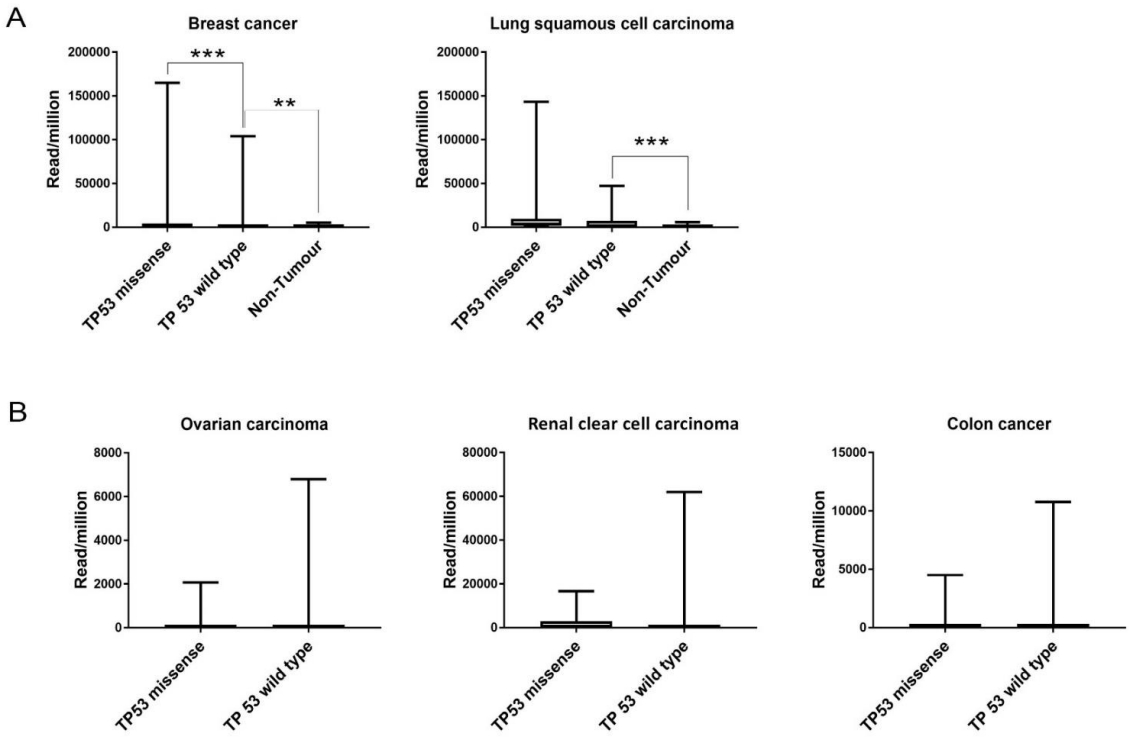


Figure 3.1. Dysregulation of miR-9 expression levels in different cancers between tumours expressing missense p53 and tumours expression wild-type p53. **A,** miRNA expression profile of 1055 breast cancer samples (204 missense p53 tumours ,748 wild-type p53 tumours and 103 non-tumour samples with wild-type p53) and 372 lung squamous cell carcinoma samples (258 missense p53 tumours , 69 wild type p53 tumours and 45 non-tumour samples with wild type p53) was collected and analyzed. **B,** miRNA expression profile of 359 ovarian carcinoma samples (175 missense p53 tumours and 184 wild type p53 tumours), 509 renal clear cell carcinoma samples (9 missense p53 tumours and 500 wild type p53 tumours) and 899 colon cancer samples (122 missense p53 tumours and 777 wild type p53 tumours) was collected and analyzed. p values (* $p<0.05$, ** $p<0.01$, *** $p<0.001$) were determined by Student's t-test.

3.3.2. Overexpression of miR-9 induces accumulation of wild-type p53

To explore the link between miR-9 expression and p53 mutation status, we first investigated the impact of miR-9 on wild-type p53. Three cancer cell lines (MCF10A, SBC3 and U2OS) expressing wild-type p53 (Table 3.1) were transfected with miR-9 mimic or a negative control. A significant increase of wild-type p53 protein level was observed in all three cell lines upon ectopic expression of miR-9. The increased wild-type p53 activity in these cells was measured by induction of MDM2 and p21, the classic down-stream targets of wild-type p53 (Figure 3.2A).

p53 mRNA levels were also examined by qRT-PCR from the transfected cells to determine whether the increase of p53 protein level was a result of interrupted p53 degradation or a result of activated p53 expression. As shown in figure 3.2B, overexpression of miR-9 induced *TP53* transcription significantly in all three cell lines, indicating that the miR-9-mediated wild-type p53 induction was at least partially at the transcriptional level.

Since one of the key function of wild-type p53 is cell growth repression, the increased p53 cellular activity was further confirmed by the growth curve of the transfected cells. Cell-TiterGlo assay was used to quantify the cell proliferation. Results showed significant repression of proliferation in MCF10A and SBC3 (Figure 3.2C). The repressed proliferation was consistent with the elevated wild-type p53 signalling, but conflicting with the previously reported oncogenic roles in breast and lung cancer. However, in the H1299 WT cells (a p53 inducible cell line derived from the p53 null cell line H1299), miR-9 failed to cause any significant growth inhibition (Figure 3.2C).

Scratch wound assay was also used to examine the impact of miR-9 on cell migration, but no obvious changes were observed. However, cell morphology changes of H1299 wild-type cells after miR-9 overexpression suggested increased EMT activity (Supplementary figure 3.1).

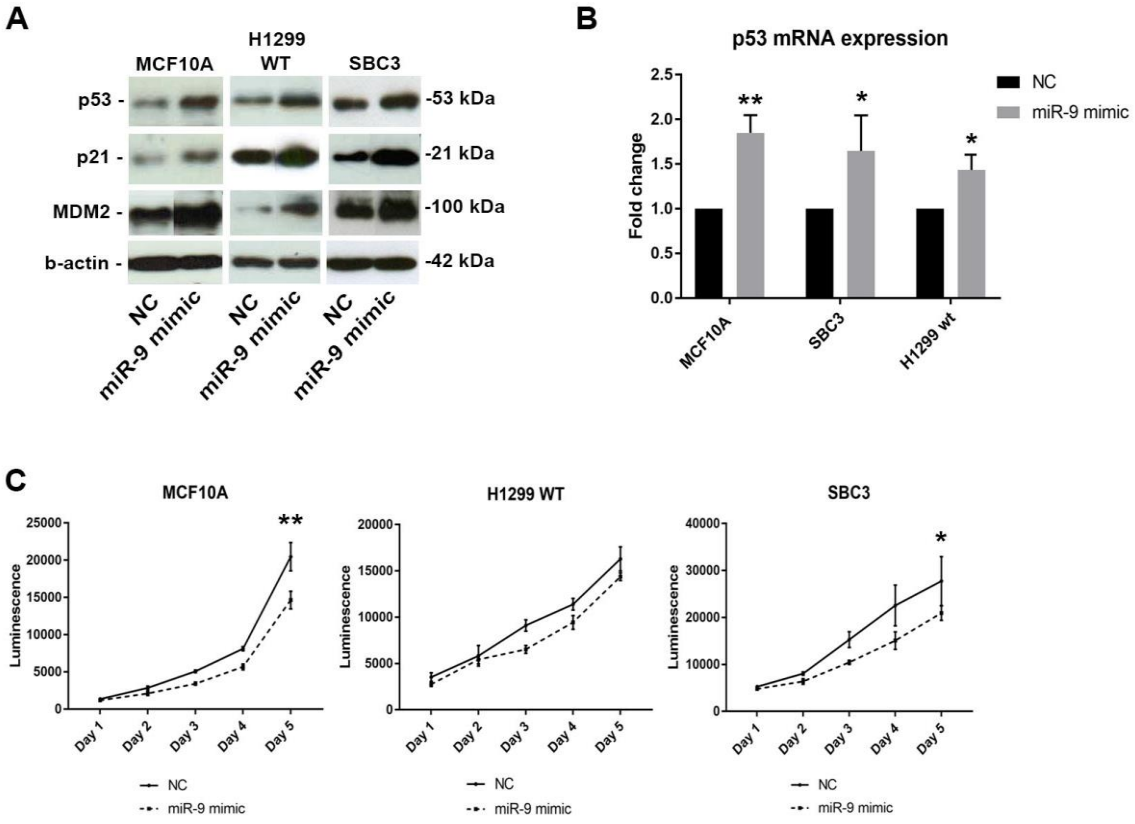
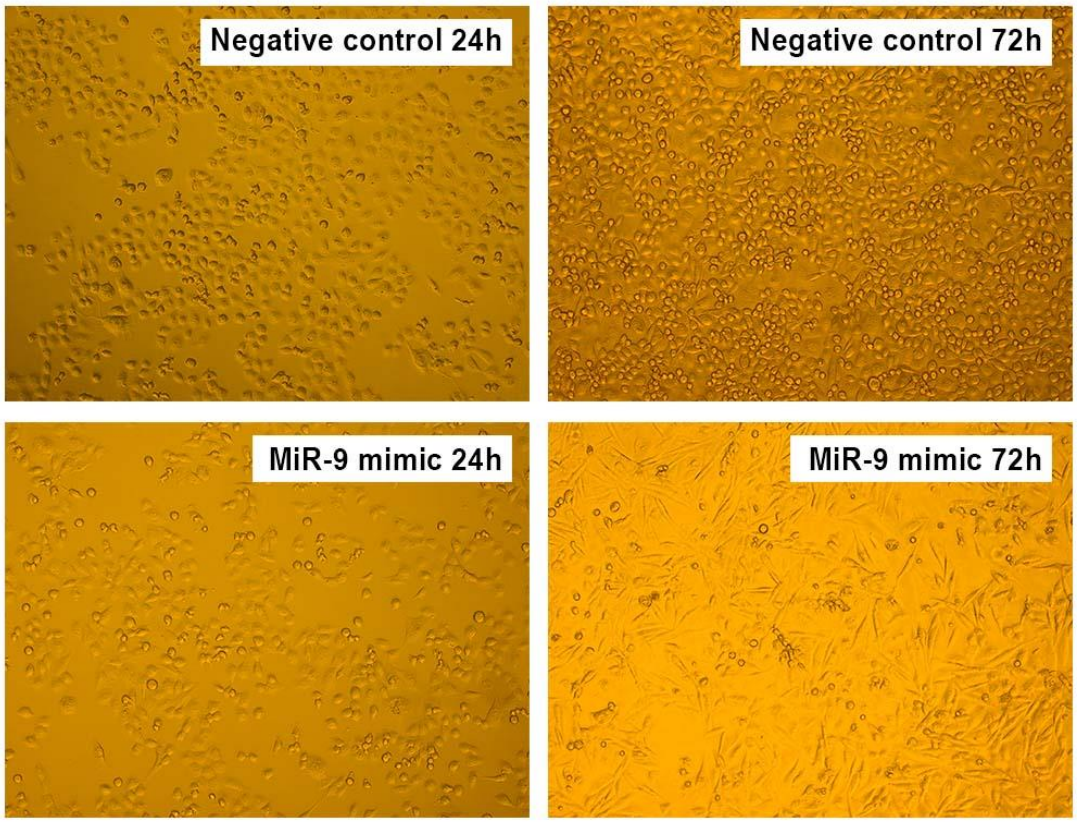


Figure 3.2. Overexpression of miR-9 enhances p53 signaling in wild-type p53 cell lines. **(A-C)** Wild-type p53 cell lines MCF10A, H1299-wild-type and SBC3 were transfected with miR-9 mimic or a negative control; H1299-wild-type was treated with PonA. **(A)** Whole protein lysate was collected 48 hours following transfection, p53, MDM2 and p21 protein levels were measured by western blotting (beta-actin as a loading control). **(B)** Total RNA was collected from transfected cells 48 hours following transfection. *TP53* mRNA levels were detected by qRT-PCR (normalized by *GAPDH*). **(C)** Cell proliferation rate was determined by Cell-TiterGlo Assay in the transfected cells. For all data, results were repeated in more than three independent experiments, p values (* $p<0.05$, ** $p<0.01$) were determined by Student's t-test.

Table 3.1. Cell lines expressing wild-type p53

Cell line	Tissue	*TP53* status
MCF10A	Breast	Wild-type
H1299 wild-type	Lung	Wild-type
SBC3	Lung	Wild-type



Supplementary Figure 3.1. MiR-9 overexpression induces cell morphology transformation. H1299-wild-type was transfected with miR-9 mimic or a negative control while treated with PonA. Cell morphology was recorded under microscope after 24 and 72 hours.

3.3.3. Overexpression of miR-9 reduces missense p53 R248Q and R273H protein levels

The impact of miR-9 overexpression on p53 regulation was also explored in a panel of cell lines expressing missense p53 (Table 3.2). Surprisingly, the protein levels of missense p53 decreased dramatically in MDA-MB-468 (R273H), H1299 R248Q and OVCAR3 (R248Q) cell lines following enforced miR-9 overexpression (Figure 3.3A). Thus, both p53 R248Q and p53 R273H were negatively regulated by miR-9, while wild-type p53 was positively regulated. This miRNA-mediated opposite effect on wild-type and missense p53 has not been previously reported.

To further understand the cause of the missense p53 reduction mediated by miR-9, the p53 mRNA levels were detected using qRT-PCR from the transfected MDA-MB-468, H1299 R248Q and OVCAR3. Results showed reduction of mRNA levels of p53 R273H in MDA-MB-468 cells following miR-9 overexpression, which would explain the observed miR-9 driven protein level changes of this missense mutation (Figure 3.3B). However, the mRNA levels of p53 R248Q in both H1299 R248Q and OVCAR3 showed a significant increase after miR-9 overexpression (Figure 3.3B). The diverse mRNA expression patterns in response to miR-9 indicates that miR-9 regulates the two missense p53 mutations and wild-type p53 via different pathways.

Cell proliferation rates of these three cell lines were also measured to determine the possible cellular function of miR-9 overexpression in the missense p53 environment. Following miR-9 overexpression, repressed cell growth was observed in the H1299 R248Q and OVCAR3 cell lines, but not in MDA-MB-468 (Figure 3.3C).

Table 3.2. Cell lines expressing missense p53.

Cell line	Tissue	*TP53* status
MDA-MB-468	Breast	Missense R273H
MDA-MB-231	Breast	Missense R280K
OVCAR3	Ovary	Missense R248Q
H1299 R248Q	Lung	Missense R248Q
H1299 R175H	Lung	Missense R175H

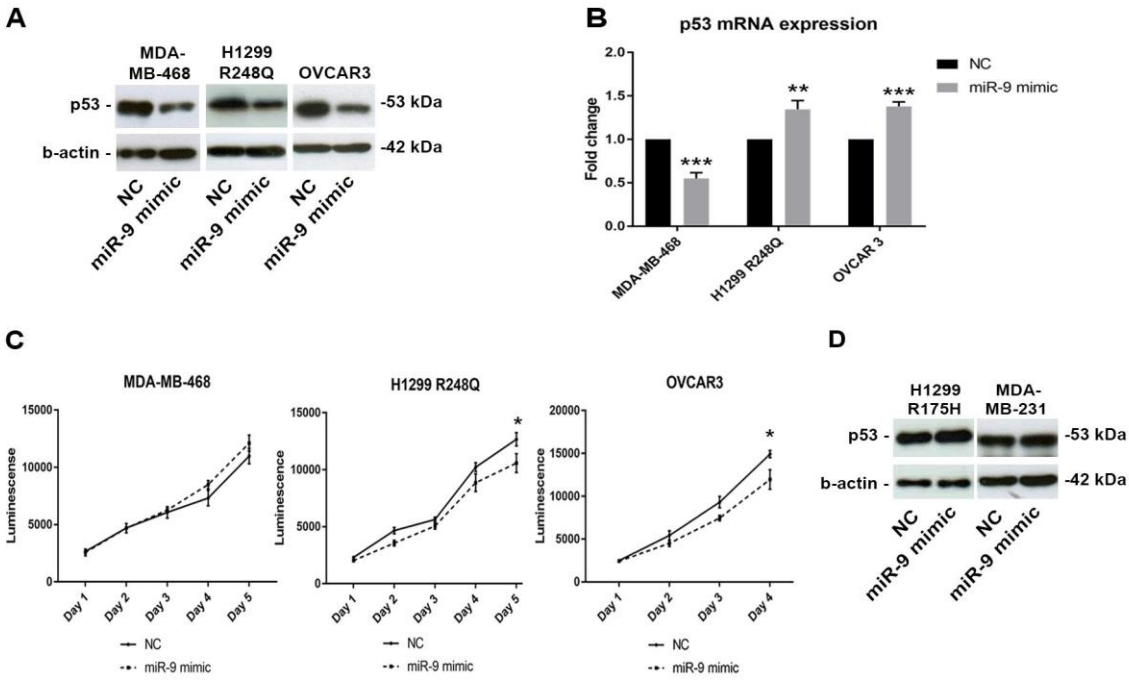


Figure 3.3. MiR-9 overexpression represses the protein levels of missense p53 R248Q and R273H. **(A-C)** Missense p53 cell lines MDA-MB-468, H1299 R248Q and OVCAR3 were transfected with miR-9 mimic or a negative control. **(A)** Whole protein lysate was collected 48 hours following transfection, p53 protein levels were measured by western blotting (beta-actin was used as a loading control). **(B)** Total RNA was collected from transfected cells 48 hours following transfection. *TP53* mRNA levels were detected by qRT-PCR (results were normalized using *GAPDH*). Results were repeated in three independent experiments. p values (** $p<0.01$, *** $p<0.001$) were determined by Student's t-test. **(C)** Cell proliferation rate was determined by Cell-TiterGlo Assay in the transfected cells. p values (* $p<0.05$) were determined by Student's t-test. **(D)** Missense p53 cell lines H1299 R175H and MDA-MB-231 were transfected with miR-9 mimic or a negative control. Whole protein lysate was collected 48 hours following transfection. p53 protein levels were detected by western blotting (beta-actin was used as a loading control).

No significant changes were observed in cell migration or morphology when miR-9 was overexpressed.

Interestingly, miR-9 overexpression did not influence the expression of two additional missense p53 mutations, H1299 R175H and R280K in MDA-MB-231, (Figure 3.3D), indicating that the miR-9-mediated p53 regulation is specific to particular p53 mutations.

3.3.4. Potential binding site of miR-9 within ORF of p53 mRNA

A possible explanation of the down-regulation of p53 R248Q and p53 R273H is miRNA-mediated post-transcriptional inhibition. A sequence-based analysis of p53 revealed a potential binding site of miR-9 within the ORF of p53. The potential binding site encodes amino acids 318 and 319 of the p53 protein, which is located between the C-terminal and the DNA binding domain of p53 and in close proximity to the missense mutation sites that have been examined in this study (Figure 3.4). However, the validation of this potential miR-9 binding site could not be completed due to time restraints.

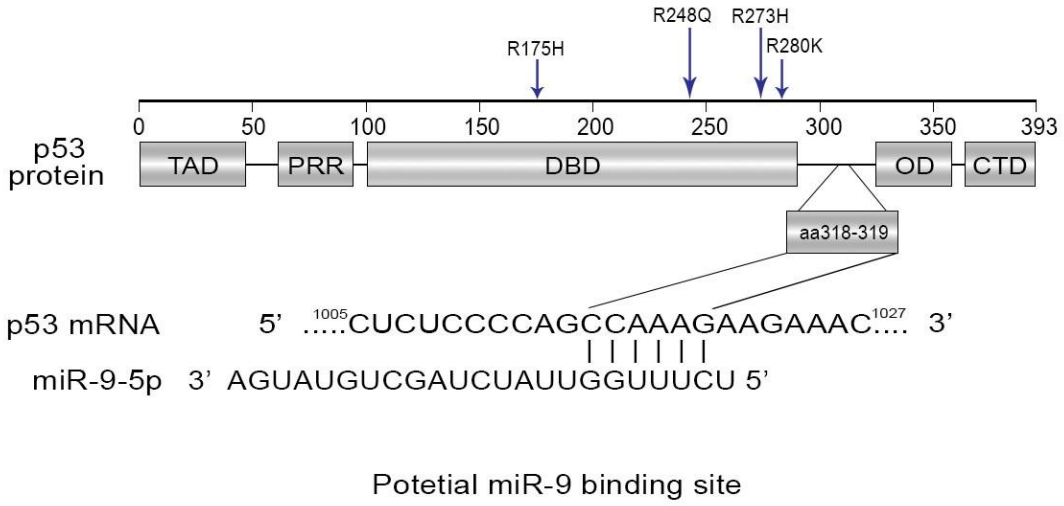


Figure 3.4. Putative miR-9-binding sequence in the ORF of *TP53* mRNA and the location of the corresponding amino acids.

3.4. Discussion

The accumulation of missense p53 protein has been observed in many cancers due to its higher stability compared with wild-type p53 protein, but the regulation of missense p53 is not as yet as well understood as wild-type p53. In this presented work, we identified distinctive expression patterns of miR-9 in tumours with missense or wild-type p53. Furthermore, we explored the miR-9-mediated regulation of both wild-type and missense p53 in cancer cells which revealed a novel p53 mutation specific regulation. This evidence suggests that miRNAs can regulate genes in an allele specific mechanism.

Wild-type p53 participates in the process of miRNA maturation by interacting with p68, which consequently increases the activity of Drosha and mature miRNA expression [32, 33]. Moreover, wild-type p53 and its downstream genes may function as transcriptional factors that regulate miRNA transcription. Thus, lack of wild-type p53 can result in reduction of multiple miRNAs [34]. On the other hand, missense p53 has also been reported to directly bind to the promoter of target miRNAs and regulate its expression [35-37].

In ovarian cancer, renal clear cell carcinoma and colorectal cancer there were low levels of miR-9 in missense p53 tumours. Possible explanations are disturbed miRNA maturation due to the absence of wild-type p53, or missense p53 mediated miRNA silencing via promotor binding. To investigate these possibilities, miR-9 expression could be determined following p53 knockdown and *in silico* prediction of possible miR-9 promoter binding sites that could be directly or indirectly bound by mutant p53.

However, the genes encoding the three members of the miR-9 family are located on different chromosomes but all show similar expression patterns in ovarian cancer, renal clear cell carcinoma and colorectal cancer. Therefore the decreased expression of all three miR-9s in these missense p53 tumours is unlikely to be based on promoter-mediated transcriptional regulation.

In addition, this theory does not provide an explanation for the observed elevated miR-9 expressions in missense p53 breast cancers and lung cancers. Given that the overexpression of miR-9 induced accumulation of wild-type p53, the observed lower levels of miR-9 in wild-type p53 tumours may be a consequence of selection against such wild-type p53 expressing cancer cells. Although miR-9 is described as an oncomiR in these two cancers for its ability to promote invasion and metastasis at the late stage of disease development [24, 26], it may perform a repressive role during the early malignant transformation of the cell. An alternative possibility is that miR-9 may be repressed by wild-type p53 directly or indirectly in the particular molecular environment of breast cancer and lung cancer, but not in the other three cancers.

This study also demonstrates the impact of miR-9 overexpression on wild-type p53 protein levels. The up-regulation of wild-type p53 upon miR-9 overexpression can be caused by miR-9 disturbing post-translational regulation of p53 (i.e. ubiquitination), or by a miR-9 induced signalling cascade that activates p53 transcription, for example MAPK signalling. The elevation of p53 mRNA levels following enforced miR-9 expression favours the second hypothesis. The defensive activation of wild-type p53 in response to the expression of an oncogene has been reported [38]. This hypothesis also

implies that the induction of wild-type p53 is a cellular defensive response to the internal stress induced by miR-9, which complies with the previously described oncogenic role of miR-9 [20, 22, 24, 39, 40].

Our novel finding is the impact of miR-9 on two particular missense p53 mutations, R248Q and R273H, differs from that on wild-type p53 or other missense mutations of p53. This unique observation may result from miR-9 induced change of a protein that can specifically regulate the missense p53 R248Q and R273H, but not wild-type or other mutations. However, to our current understanding, none of the reported regulators of p53 have p53 mutation specific functions. High-throughput screening to detect interacting proteins with a p53 mutant specific function would be of interest to explore this possibility.

An alternative possible explanation of the miR-9 driven decrease in levels of p53 R248Q and R273H protein is that miR-9 decreases the protein expression by specifically targeting the 3'UTR of these missense p53 mRNAs. Prediction algorithms did not find any potential binding site for miR-9 in the 3'UTR NCBI sequence of wild-type p53. However, mutations have been found in the 3'UTR of p53 with a higher frequency than in the coding sequence [41], and some of these variations may alter miRNA-mediated p53 regulation by altering a miRNA binding site, for example SNP *rs78378222* [42, 43]. It is possible that there are 3'UTR SNPs or mutations in the p53 R248Q and R273H mRNA, and these variants create a potential binding site for miR-9. The full-length mRNA transcript of p53 in the tested cell lines MDA-MB-468 and OVCAR3 should be sequenced to confirm whether any potential miR-9 binding

sites exist. However, H1299 R248Q is a cell line with inducible expression of mutant p53 and does not contain the 3'UTR of p53. Thus H1299 R248Q cannot have any regulation based on p53 3'UTR targeting. Therefore, at least the observation in one of the cell lines cannot be explained by this hypothesis.

Interestingly, a potential binding site of miR-9 was identified at the ORF of p53 mRNA (Figure 3.4), which is ~120 nt away from mutant site of R273H and ~195 nt away from R248Q. As reported in previous studies, miRNA can repress target gene expression by binding to the ORF of the target gene [44]. Thus, it is possible that miR-9 may participate in the regulation of p53 via this binding site at the ORF. However, this ORF-targeting theory does not provide an explanation to the observed absence of miR-9 regulation of p53 R248Q and R273H. We speculate that a possibility is that the higher-order-structure of RNA is a crucial factor in miRNA-mRNA interaction, which is the basis for the observed conflicting impacts of miR-9 on the regulation of the different missense and wild-type p53s in this study. The 3-D configuration of RNA is known to determine the biological function of the RNA molecule, i.e. tRNA and circRNA [45, 46]. Formed by dynamic packing of base-paired nucleotides, the 3-D structure of RNA enables the RNA molecule to interact with other molecules [47]. It is possible that a single mutation in missense p53 mRNA may specifically alter the higher-order structure of the mRNA molecule and allow it to expose different sites for interaction with other protein or nucleic acids. In our hypothesis, the conformational change of mRNA caused by R248Q and R273H mutations exposes the potential miR-9 binding site for miR-9 targeting, which results in the reduction of missense p53 protein level regardless of the change of mRNA levels. This hypothesis may explain why the reduction of p53 protein only occurred in p53 R248Q and R273H specifically following

miR-9 overexpression, while other missense mutations are unaffected and wild-type p53 is up-regulated.

To further explore this hypothesis, computational modelling of the 3-D configuration of p53 mRNA with different missense mutations will be essential. Accounting for non-canonical base pairing [48], ion-dependent folding [49, 50] and long-range tertiary interaction [51], *in silico* modelling tools have been developed, including miREN [52] and Sfold [53, 54]. Physical and biochemical methods have also been developed as probes to obtain specific data of RNAs. For example, the Selective 2'-Hydroxyl Acylation analysis by Primer Extension (SHAPE) assay analyses the probability of nucleotide pairing [55, 56], while cross-linking assay [57, 58] and tethered hydroxyl radical probing (t-HRP) assay [59] provide evidence of inter-nucleotide distances. In contrast, solution hydroxyl radical probing (solution HRP) evaluates the backbone solvent accessibility for most nucleotides of the RNA [60]. The bias discrete molecular dynamics (DMD) study can be used to combine the experimental results and computational analyses to generate structural ensembles consistent with experiment data. However, the inaccuracy of the 3-D structure prediction declines as the length of the RNA molecules increase, and the effective analysis of the 3-D structure of p53 mRNA and its impact on miRNA-mRNA interaction may need further development in bioinformatics.

Altogether, this chapter describes a novel phenomenon in miRNA-p53 regulation, and highlights the potent impact of target gene mutation on miRNA-regulated regulations. Future avenues of research are highlighted which will further explore the molecular basis for these observations.

3.5. Materials and methods

Cell lines and reagents

MCF10A, OVCAR3, MDA-MB-468 and MDA-MB-231 cell lines were purchased from the American Type Culture Collection (ATCC). SBC3 was kindly given as a gift from Dr. Sandra Hodge (University of Adelaide, Australia). MCF10A was cultured in DMEM/F12 with 5% horse serum, 20 ng/mL human EGF, 0.5mg/ml hydrocortisone, 100ng/ml cholera toxin and 10 ug/mL insulin. OVCAR3 was cultured in RPMI-1640 with 10% FBS and PSG. SBC3, MDA-MB-468 and MDA-MB-231 were maintained in DMEM with 10% FBS, HEPES and PSG. H1299 wild-type, H1299 R248Q, H1299 R175H cell lines are maintained in DMEM with 10% FBS and 2.5ng/mL of puromycin [2]. Wild-type and mutant p53 expression in H1299 inducible cells were induced by 2.5 ug/mL ponasterone A (PonA) as previously described [2].

Reverse-transcription PCR (RT-PCR) and real-time PCR (qRT-PCR)

RNA extraction from cells was performed using the RNAeasy mini kit (Qiagen, Valencia, CA USA). ND-1000 NanoDrop spectrometer (Thermo Scientific, Wilmington, DE USA) was used to measure RNA concentration. Total RNA was reverse-transcribed into cDNA as previously described using Moloney Murine Leukaemia Virus (M-MLV) Reverse Transcriptase (Promega, Madison, WI USA) and random 6'mer primers (Promega) [61].

Real-time PCR reactions were performed using IQ SYBR Green Supermix (BioRad, USA) on a BioRad iCycler (BioRad) as previously described [62]. Relative

expression levels of p53 mRNAs were determined by the ΔΔCT method and normalized using GAPDH. All primers used in qRT-PCR are listed in Table 3.4

Table 3.4.

Gene		Primer sequence
TP53	Forward	GGGCTCCGGGGACACTTTGCG
	Reverse	TGGCAGTGACCCGGAAGGCAGT
GAPDH	Forward	AGCCTCCCGCTTCGCTCTCTGC
	Reverse	ACCAGGCGCCCAATACGACCAAA

Transient transfection

Asynchronously growing cells were seeded at 3 x 10^5 cells/well in six-well plates or 1.2 x 10^5 cells/well in 24-well plates. Transfection of cells with 50 nM/well (six-well plate) or 12.5 nM/well (24-well plate) miR-9 mimic (Genepharma, Shanghai, CN) was performed using Lipofectamine RNAiMAX as previously described (Invitrogen, CA, USA).

Cell proliferation assay

Cells were plated at 1500 cells/well in 96-well plates 24 hours after transfection and were cultured for 1, 2, 3, 4 and 5 days. On the indicated days, cells were incubated

with 50% CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega) and 50% media. Fluorescence was measured by LUMIstar Galaxy luminometer (BMG Labtech).

Western blot and antibodies

Western blot assay was performed as previously described [61]. Antibodies used include: mouse anti-p53 DO-1 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-MDM2 (Santa Cruz), rabbit anti-p21 (Santa Cruz) and mouse anti-β-actin (Sigma Aldrich).

Statistical analysis

Data are presented as means ± SD from at least three independent experiments. Student's t-test was performed using replicate values to compare between groups and indicate significance. Values of $p<0.05$ were considered statistically significant ($p<0.05$ as *, $p<0.01$ as ** and $p<0,001$ as ***).

3.6. References

1. Blagosklonny, M.V., *Loss of function and p53 protein stabilization.* Oncogene, 1997. **15**(16): p. 1889-93.
2. Neilsen, P.M., et al., *Mutant p53 uses p63 as a molecular chaperone to alter gene expression and induce a pro-invasive secretome.* Oncotarget, 2011. **2**(12): p. 1203-17.
3. Neilsen, P.M., et al., *Mutant p53 drives invasion in breast tumors through up-regulation of miR-155.* Oncogene, 2013. **32**(24): p. 2992-3000.
4. Coffill, C.R., et al., *Mutant p53 interactome identifies nardilysin as a p53R273H-specific binding partner that promotes invasion.* EMBO Rep, 2012. **13**(7): p. 638-44.
5. Guo, F., et al., *Mutation R273H confers p53 a stimulating effect on the IGF-1R-AKT pathway via miR-30a suppression in breast cancer.* Biomed Pharmacother, 2016. **78**: p. 335-41.
6. Igarashi, H., et al., *Anti-apoptotic roles for the mutant p53R248Q through suppression of p53-regulated apoptosis-inducing protein 1 in the RA-derived fibroblast-like synoviocyte cell line MH7A.* Clin Immunol, 2014. **150**(1): p. 12-21.
7. Lukashchuk, N. and K.H. Vousden, *Ubiquitination and degradation of mutant p53.* Mol Cell Biol, 2007. **27**(23): p. 8284-95.
8. Sheng, Y., et al., *Molecular basis of Pirh2-mediated p53 ubiquitylation.* Nat Struct Mol Biol, 2008. **15**(12): p. 1334-42.
9. Haupt, Y., et al., *Mdm2 promotes the rapid degradation of p53.* Nature, 1997. **387**(6630): p. 296-9.
10. Yuan, J., et al., *USP10 regulates p53 localization and stability by deubiquitinating p53.* Cell, 2010. **140**(3): p. 384-96.
11. Li, D., et al., *Functional inactivation of endogenous MDM2 and CHIP by HSP90 causes aberrant stabilization of mutant p53 in human cancer cells.* Mol Cancer Res, 2011. **9**(5): p. 577-88.
12. Peng, Y., et al., *Inhibition of MDM2 by hsp90 contributes to mutant p53 stabilization.* J Biol Chem, 2001. **276**(44): p. 40583-90.

13. King, F.W., et al., *Co-chaperones Bag-1, Hop and Hsp40 regulate Hsc70 and Hsp90 interactions with wild-type or mutant p53.* EMBO J, 2001. **20**(22): p. 6297-305.
14. Zhang, P., et al., *miR-600 inhibits cell proliferation, migration and invasion by targeting p53 in mutant p53-expressing human colorectal cancer cell lines.* Oncol Lett, 2017. **13**(3): p. 1789-1796.
15. Lankenau, M.A., et al., *MicroRNA-3151 inactivates TP53 in BRAF-mutated human malignancies.* Proc Natl Acad Sci U S A, 2015. **112**(49): p. E6744-51.
16. Bergman, L.M., et al., *CtBPs promote cell survival through the maintenance of mitotic fidelity.* Mol Cell Biol, 2009. **29**(16): p. 4539-51.
17. Sheng, Y. and C. Previti, *Genomic features and computational identification of human microRNAs under long-range developmental regulation.* BMC Genomics, 2011. **12**: p. 270.
18. Yuva-Aydemir, Y., et al., *MicroRNA-9: functional evolution of a conserved small regulatory RNA.* RNA Biol, 2011. **8**(4): p. 557-64.
19. Nass, D., et al., *MiR-92b and miR-9/9* are specifically expressed in brain primary tumors and can be used to differentiate primary from metastatic brain tumors.* Brain Pathol, 2009. **19**(3): p. 375-83.
20. Chen, X., et al., *Oncogenic miR-9 is a target of erlotinib in NSCLCs.* Sci Rep, 2015. **5**: p. 17031.
21. Xu, T., et al., *Up-regulation of miR-9 expression as a poor prognostic biomarker in patients with non-small cell lung cancer.* Clin Transl Oncol, 2014. **16**(5): p. 469-75.
22. Leucci, E., et al., *Inhibition of miR-9 de-represses HuR and DICER1 and impairs Hodgkin lymphoma tumour outgrowth in vivo.* Oncogene, 2012. **31**(49): p. 5081-9.
23. Zhu, M., et al., *MicroRNA and gene networks in human Hodgkin's lymphoma.* Mol Med Rep, 2013. **8**(6): p. 1747-54.
24. Ma, L., et al., *miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis.* Nat Cell Biol, 2010. **12**(3): p. 247-56.
25. Iorio, M.V., et al., *MicroRNA gene expression deregulation in human breast cancer.* Cancer Res, 2005. **65**(16): p. 7065-70.
26. Lehmann, U., et al., *Epigenetic inactivation of microRNA gene hsa-mir-9-1 in human breast cancer.* J Pathol, 2008. **214**(1): p. 17-24.

27. Sun, H., et al., *Prognostic value of microRNA-9 in cancers: a systematic review and meta-analysis.* Oncotarget, 2016. **7**(41): p. 67020-67032.
28. He, L., et al., *miR-9 functions as a tumor inhibitor of cell proliferation in epithelial ovarian cancer through targeting the SDF-1/CXCR4 pathway.* Exp Ther Med, 2017. **13**(4): p. 1203-1208.
29. Tang, H., et al., *miR-9 functions as a tumor suppressor in ovarian serous carcinoma by targeting TLN1.* Int J Mol Med, 2013. **32**(2): p. 381-8.
30. Park, Y.R., et al., *MicroRNA-9 suppresses cell migration and invasion through downregulation of TM4SF1 in colorectal cancer.* Int J Oncol, 2016. **48**(5): p. 2135-43.
31. Hildebrandt, M.A., et al., *Hsa-miR-9 methylation status is associated with cancer development and metastatic recurrence in patients with clear cell renal cell carcinoma.* Oncogene, 2010. **29**(42): p. 5724-8.
32. Feng, Z., et al., *Tumor suppressor p53 meets microRNAs.* J Mol Cell Biol, 2011. **3**(1): p. 44-50.
33. Hermeking, H., *MicroRNAs in the p53 network: micromanagement of tumour suppression.* Nat Rev Cancer, 2012. **12**(9): p. 613-26.
34. Cabrita, M.A., et al., *The p53 protein induces stable miRNAs that have the potential to modify subsequent p53 responses.* Gene, 2017. **608**: p. 86-94.
35. Donzelli, S., et al., *MicroRNA-128-2 targets the transcriptional repressor E2F5 enhancing mutant p53 gain of function.* Cell Death Differ, 2012. **19**(6): p. 1038-48.
36. Masciarelli, S., et al., *Gain-of-function mutant p53 downregulates miR-223 contributing to chemoresistance of cultured tumor cells.* Oncogene, 2014. **33**(12): p. 1601-8.
37. Wang, W., et al., *Mutant p53-R273H gains new function in sustained activation of EGFR signaling via suppressing miR-27a expression.* Cell Death Dis, 2013. **4**: p. e574.
38. Horn, H.F. and K.H. Vousden, *Coping with stress: multiple ways to activate p53.* Oncogene, 2007. **26**(9): p. 1306-16.
39. Barbano, R., et al., *Stepwise analysis of MIR9 loci identifies miR-9-5p to be involved in Oestrogen regulated pathways in breast cancer patients.* Sci Rep, 2017. **7**: p. 45283.

40. Gomez, G.G., et al., *Suppression of microRNA-9 by mutant EGFR signaling upregulates FOXP1 to enhance glioblastoma tumorigenicity.* Cancer Res, 2014. **74**(5): p. 1429-39.
41. Li, Y., et al., *Single nucleotide variation in the TP53 3' untranslated region in diffuse large B-cell lymphoma treated with rituximab-CHOP: a report from the International DLBCL Rituximab-CHOP Consortium Program.* Blood, 2013. **121**(22): p. 4529-40.
42. Stacey, S.N., et al., *A germline variant in the TP53 polyadenylation signal confers cancer susceptibility.* Nat Genet, 2011. **43**(11): p. 1098-103.
43. Zhao, Y., X. Li, and S. Zhu, *rs78378222 polymorphism in the 3'-untranslated region of TP53 contributes to development of age-associated cataracts by modifying microRNA-125b-induced apoptosis of lens epithelial cells.* Mol Med Rep, 2016. **14**(3): p. 2305-10.
44. Mandke, P., et al., *MicroRNA-34a modulates MDM4 expression via a target site in the open reading frame.* PLoS One, 2012. **7**(8): p. e42034.
45. Greene, J., et al., *Circular RNAs: Biogenesis, Function and Role in Human Diseases.* Front Mol Biosci, 2017. **4**: p. 38.
46. Hasler, D. and G. Meister, *From tRNA to miRNA: RNA-folding contributes to correct entry into noncoding RNA pathways.* FEBS Lett, 2016. **590**(15): p. 2354-63.
47. Dokholyan, F.D.a.N.V., *RNA Three-Dimensional Structure Determination Using Experimental Constraints* in *RNA Nanotechnology and Therapeutics*, P.G.a.F. Haque, Editor. 2013, CRC. p. 159-171.
48. Das, R., J. Karanicolas, and D. Baker, *Atomic accuracy in predicting and designing noncanonical RNA structure.* Nat Methods, 2010. **7**(4): p. 291-4.
49. Draper, D.E., *Folding of RNA tertiary structure: Linkages between backbone phosphates, ions, and water.* Biopolymers, 2013. **99**(12): p. 1105-13.
50. Grilley, D., A.M. Soto, and D.E. Draper, *Mg2+-RNA interaction free energies and their relationship to the folding of RNA tertiary structures.* Proc Natl Acad Sci U S A, 2006. **103**(38): p. 14003-8.
51. Gherghe, C.M., et al., *Native-like RNA tertiary structures using a sequence-encoded cleavage agent and refinement by discrete molecular dynamics.* J Am Chem Soc, 2009. **131**(7): p. 2541-6.

52. Leoni, G. and A. Tramontano, *A structural view of microRNA-target recognition.* Nucleic Acids Res, 2016. **44**(9): p. e82.
53. Long, D., C.Y. Chan, and Y. Ding, *Analysis of microRNA-target interactions by a target structure based hybridization model.* Pac Symp Biocomput, 2008: p. 64-74.
54. Long, D., et al., *Potent effect of target structure on microRNA function.* Nat Struct Mol Biol, 2007. **14**(4): p. 287-94.
55. Wilkinson, K.A., et al., *High-throughput SHAPE analysis reveals structures in HIV-1 genomic RNA strongly conserved across distinct biological states.* PLoS Biol, 2008. **6**(4): p. e96.
56. Wilkinson, K.A., E.J. Merino, and K.M. Weeks, *Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE): quantitative RNA structure analysis at single nucleotide resolution.* Nat Protoc, 2006. **1**(3): p. 1610-6.
57. Harris, M.E., et al., *Use of photoaffinity crosslinking and molecular modeling to analyze the global architecture of ribonuclease P RNA.* EMBO J, 1994. **13**(17): p. 3953-63.
58. Pinard, R., et al., *The hairpin ribozyme substrate binding-domain: a highly constrained D-shaped conformation.* J Mol Biol, 2001. **307**(1): p. 51-65.
59. Das, R., et al., *Structural inference of native and partially folded RNA by high-throughput contact mapping.* Proc Natl Acad Sci U S A, 2008. **105**(11): p. 4144-9.
60. Cate, J.H., et al., *Crystal structure of a group I ribozyme domain: principles of RNA packing.* Science, 1996. **273**(5282): p. 1678-85.
61. Yu, F., et al., *p53 Represses the Oncogenic Sno-MiR-28 Derived from a SnoRNA.* PLoS One, 2015. **10**(6): p. e0129190.
62. Pishas, K.I., et al., *Nutlin-3a is a potential therapeutic for ewing sarcoma.* Clin Cancer Res, 2011. **17**(3): p. 494-504.

Chapter 4.

A novel miR-375-androgen receptor feedback loop regulates growth of prostate cancer cells

Preface

This chapter demonstrates a AR-miR-375 self regulatory feedback loop as AR induces miR-375 expression and secretion by binding *MIR375* promoter, while miR-375 represses AR signalling by targeting AR 3'UTR. This finding identifies a new mechanism of AR regulation in prostate cancer.

This chapter is presented as a manuscript prepared for submission to a journal for publication.

Contribution of the candidate: All figures, all tables and the manuscript writing.

Statement of Authorship

Title of Paper	A novel miR-375-androgen receptor feedback loop regulates growth of prostate cancer cells
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Manuscript prepared for publication

Principal Author

Name of Principal Author (Candidate)	Qingqing Wang
Contribution to the Paper	Designed and conducted all the experiments except the preparation of ChIP DNA, analysis and interpreted the data in this work, interoperated the data and wrote the manuscript.
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 28.8.17

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Scott Townley
Contribution to the Paper	Constructed the pGL3 luciferase reporter vectors containing miR-375 promoter and performed the DHT treatment for LNCaP cells and prepared ChIP DNA.
Signature	Date 28.8.17

Name of Co-Author	Steve Paltoglou
Contribution to the Paper	Performed the DHT treatment for LNCaP cells and prepared ChIP DNA
Signature	Date 23/8/17

Name of Co-Author	Luke Selth
Contribution to the Paper	Supervised the progress of the work. helped with data interpretation and manuscript preparation.
Signature	Date 23/8/17

Name of Co-Author	David Callen
Contribution to the Paper	Supervised the progress of the work. helped with data interpretation and manuscript preparation.
Signature	Date 23/08/17.

A novel miR-375-androgen receptor feedback loop regulates growth of prostate cancer cells

Qingqing Wang[1,2], Scott Townley[2, 3], Steve Paltoglou[2, 3], David F Callen[1] and Luke A Selth[2, 3]

[1] *Breast Cancer Genetics Group, Centre for Personalised Cancer Medicine, School of Medicine, University of Adelaide, South Australia.*

[2] *Dame Roma Mitchell Cancer Research Laboratories, School of Medicine, University of Adelaide, South Australia.*

[3] *Freemasons Foundation Centre for Men's Health, Discipline of Medicine, University of Adelaide, South Australia.*

CORRESPONDING AUTHORS

Luke Selth: Dame Roma Mitchell Cancer Research Laboratories, School of Medicine, Level 8 Level 8 AHMS Building, The University of Adelaide, SA 5000, Australia. Phone: +618 8222 3618. Fax: +618 8222 3217. E-mail: luke.selth@adelaide.edu.au

David Callen: Breast Cancer Genetics Group, Centre for Personalised Cancer Medicine, School of Medicine, Level 8 AHMS Building, The University of Adelaide, SA 5000, Australia. Phone: +618 8222 3145. E-mail: david.callen@adelaide.edu.au

KEYWORDS: MicroRNA, androgen receptor, prostate cancer

RUNNING TITLE: The miR-375-AR feedback loop in prostate cancer

Abstract

Prostate cancer is the most commonly diagnosed male non-skin cancer in the Western world and a major cause of cancer death. Androgen receptor (AR), a ligand-dependent transcription factor, is the key driver of prostate cancer development and progression. As such, inhibition of AR signalling by androgen deprivation therapy is the mainstay therapeutic strategy for metastatic disease. Despite intensive research, the mechanisms that regulate AR activity in prostate cancer remain to be fully elucidated. In particular, post-transcriptional regulation of AR expression by microRNAs appears to be an important mechanism controlling prostate cancer growth. In the present study, we identified microRNA-375 (miR-375) as a direct target of AR: AR binds to the *MIR375* promoter and upregulates its expression. Converse regulation also occurs, since miR-375 can directly target AR by binding to a single recognition sequence in the AR 3'UTR. Thus, AR-miR-375 represents a novel self-regulatory feedback loop that controls AR signalling in prostate cancer cells. Supporting the relevance of this feedback loop, ectopic delivery of miR-375 suppressed proliferation of prostate cancer cells, but this effect could be rescued by over-expression of AR. Altogether, this study identifies a novel mechanism by which AR self-regulates its activity in prostate cancer.

Introduction

Prostate cancer is the most commonly diagnosed male non-skin cancer in the Western world and a major cause of cancer death. (Siegel, Miller et al. 2017). Although the 5-year survival rate is nearly 100% in patients with local disease, for men with metastatic disease the 5-year survival rate is less than 30%, and this poor prognosis has not improved in the past 25 years (Wu, Fish et al. 2014).

The key driver of prostate cancer is the androgen receptor (AR) (Massie, Lynch et al. 2011, Dai, Heemers et al. 2017), a member of the hormone receptor superfamily and a ligand-dependent transcription factor encoded by the *AR* gene on chromosome X (Chang, Kokontis et al. 1988, Tilley, Marcelli et al. 1989). The normal functions of AR include development and maintenance of the male sexual phenotype (Sinisi, Pasquali et al. 2003), induction of skeletal muscle growth (MacKrell, Yaden et al. 2015) and maintaining male bone integrity (Sinnesael, Claessens et al. 2012) in response to male hormones. Dysregulation of AR is related to multiple diseases, including prostate (Massie, Lynch et al. 2011, Dai, Heemers et al. 2017), breast (Rahim and O'Regan 2017) and ovarian cancer cancers (van Kruchten, van der Marel et al. 2015). In prostate cancer cells, AR activates a transcriptional program that contributes to proliferation, differentiation and survival (Zhu and Kyprianou 2008, Waltering, Porkka et al. 2011). Given its critical role in the growth of prostate cancer, androgen deprivation therapy (ADT) is the mainstay therapeutic strategy for metastatic disease. However, ADT is never curative and patients will progress to a lethal form of the disease termed castration-resistant prostate cancer (CRPC) (Nandana and Chung 2014). Interestingly, AR still remains the key driver of tumour growth in CRPC, and multiple mechanisms by which AR continues to signal in the

face of ADT have been elucidated (Koochekpour 2010, Dai, Heemers et al. 2017). With this in mind, strategies to target AR signalling independent of ADT are of particular interest to improve patient outcomes.

MicroRNAs (miRNAs, miRs) are a group of small non-coding RNAs of ~22 nt that negatively regulate gene expression by binding to complementary sequences in their targets (Krol, Loedige et al. 2010). Multiple miRNAs have been reported to participate in AR signalling. MiR-185, miR-31, miR-654-5p, miR-203/205, miR-488* and let-7c are direct inhibitors of AR via 3'UTR targeting, and are considered as tumour suppressors in prostate cancer (Ostling, Leivonen et al. 2011, Sikand, Slaibi et al. 2011, Nadiminty, Tummala et al. 2012, Boll, Reiche et al. 2013, Hagman, Haflidadottir et al. 2013, Lin, Chiu et al. 2013, ChunJiao, Huan et al. 2014). On the other hand, miRNAs such as miR-21, miR-32, miR-221/222, miR-141, miR-23a/27/24-2 miR-19a and miR-133b are trans-activated by AR and act as downstream oncogenic effectors (Volinia, Calin et al. 2006, Ambs, Prueitt et al. 2008, Sun, Wang et al. 2009, Waltering, Porkka et al. 2011, Fletcher, Dart et al. 2012, Mo, Zhang et al. 2013). In short, the interplay between AR and miRNAs appears to play a major role in prostate cancer, but the details of this important regulatory network are yet to be completely elucidated.

In the present work, we identify miR-375 as a direct AR target. This miRNA is a known marker of prostate cancer in both tissues and in the circulation, and has been proposed as a potential prognostic and/or predictive biomarker (Selth, Townley et al. 2012, Huang, Yuan et al. 2015, Kachakova, Mitkova et al. 2015, Wach, Al-Janabi et

al. 2015). Importantly, we identify a novel feedback loop whereby AR-regulated miR-375 directly represses AR. This work yields new insight into the regulation of AR signalling in prostate cancer and provides a rationale for the elevated levels of miR-375 observed in this disease.

Results

AR regulates miR-375 expression in prostate cancer cells

Based on our earlier finding that miR-375 is associated with active androgen signalling in prostate cancer (Selth, Das et al. 2017), we tested whether it was an AR-regulated miRNA. Knockdown of AR using a siRNA reduced the endogenous levels of miR-375 in the prostate cancer C42B and LNCaP cell line models (Figure 1A i). By contrast, over-expression of AR significantly increased the expression of endogenous miR-375 in both cell lines (Figure 1A ii).

To evaluate whether *MIR375* is a direct AR target gene, we interrogated AR ChIP-seq data from prostate tumours (Pomerantz, Li et al. 2015). We identified 2 potential AR binding sites within the promoter region of the *MIR375* gene (Figure 1C). To validate AR binding at these sites, we conducted ChIP-qPCR in LNCaP cells. As shown in Figure 1D, we could confirm a reproducible AR binding event at the *MIR375* promoter.

To validate that the *MIR375* promoter was AR-regulated, we cloned two regions encompassing this regulatory element upstream of luciferase and conducted transactivation assays in 293T cells. Both promoter regions were activated by AR, whereas a control site was not (Figure 1E). Collectively, these data identify miR-375 as a direct target of AR in prostate cancer cells.

As aberrant levels of circulating miR-375 have been reported in prostate cancer, we examined the impact of AR on miR-375 secretion. Secreted miR-375 levels increased significantly after AR over-expression in both C42B and LNCaP cells, whereas AR knockdown by siRNA decreased miR-375 secretion (Figure 1B). These

results suggest that elevated levels of miR-375 in serum/plasma of patients with prostate cancer are at least partly due to active AR signalling in prostate tumours.

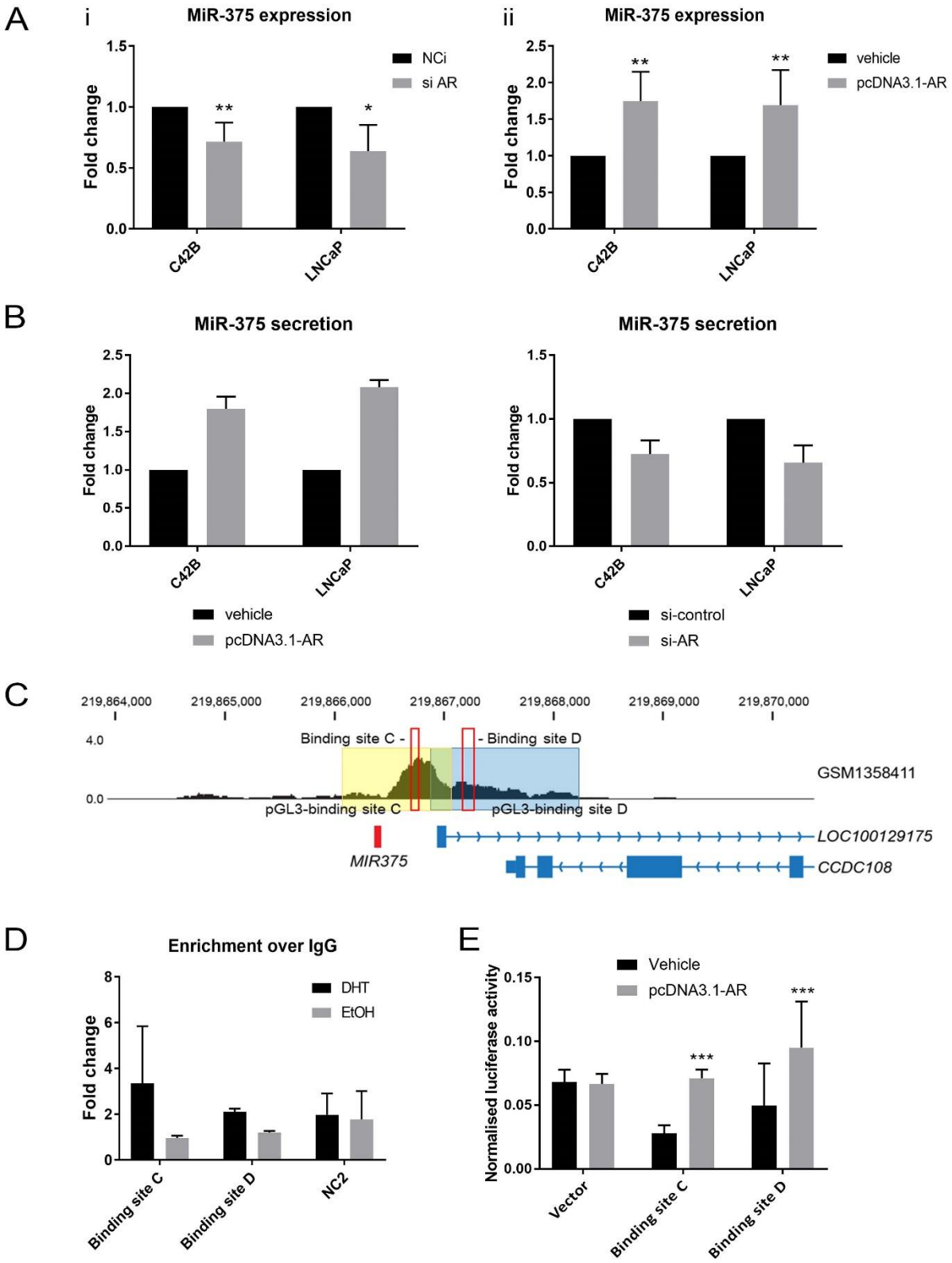

A
i
MiR-375 expression
Fold change
NCi
si AR
C42B
LNCaP
ii
MiR-375 expression
vehicle
pcDNA3.1-AR
B
MiR-375 secretion
MiR-375 secretion
si-control
si-AR
C
219,864,000
219,865,000
219,866,000
219,867,000
219,868,000
219,869,000
219,870,000
Binding site C
Binding site D
GSM1358411
pGL3-binding site C
pGL3-binding site D
MIR375
LOC100129175
CCDC108
D
Enrichment over IgG
DHT
EtOH
Binding site C
Binding site D
NC2
E
Vehicle
pcDNA3.1-AR
Normalised luciferase activity
Vector
Binding site C
Binding site D

Figure 1. AR transactivates miR-375 expression and increases miR-375 secretion. **A**, i, C42B and LNCaP cells were transfected with AR siRNA or control and total RNA was prepared after 72 hours. MiR-375 expression was detected 48 and 72 hours after transfection by qRT PCR; RNU48 was used as internal control. ii, C42B and LNCaP cells were transfected with pcDNA3.1-AR or vehicle and total RNA was prepared after 72 hours. MiR-375 expression was detected by qRT-PCR; RNU48 was used as internal control. Results are the average of three independent experiments. p values (** *P<0.01;* *** *p<0.001*) were determined by Student's t-test. **B.** Transfections were performed as described in **A**. Transfected cells were washed and incubated in fresh serum free media for 72 hours before secretom collection. The secreted RNA was purified from secretom. MiR-375 levels were detected using qRT PCR and cel-miR-39 was used as internal control, and normalized by the ratio of living cells and total cells, represent the mean (±SEM) of 3 replicates; results are representative of 2 independent experiments. **C**, AR binding sites (from ChIP-seq) proximal to the AR binding sites (from ChIP-seq) proximal to the *MIR375* gene in nonmalignant and prostate tumor samples gene in nonmalignant and prostate tumor samples (Pomerantz, Li et al. 2015). **D**, Validation of three putative AR binding sites (shown in the ChIP-seq) by ChIP qPCR. Error bars, ±SEM. NC2 was used as a negative control. **E**, 293T cells were transfected with pRL and plasmids expressing AR and a *MIR375* promoter reporter. Transcriptional activity values as assessed by luciferase assays represent the mean (±SEM) of at least 3 independent experiments; An unpaired t test was used to assess the effect of AR on *MIR375* activity. p values (* *p<0.05,* ** *p<0.01,* *** *p<0.001)*

AR is a direct target of miR-375

To determine whether miR-375 can influence the androgen signalling axis, we assessed the consequences of modulating this miRNA in prostate cancer cells. Interestingly, miR-375 over-expression significantly reduced the expression of AR and its targets, PSA and FKBP51, at both the protein (Figure 2A) and RNA (Figure 2D) level. In contrast, suppression of miR-375 using a locked nucleic acid (LNA) inhibitor resulted in upregulation of AR and its targets (Figure 2B) (Figure 2D). Since circulating PSA is used as a marker of prostate cancer, the secretion of this protein was also examined after miR-375 overexpression in C42B cells. A significant reduction in PSA secretion was observed at both 72 and 96 hours after delivery of miR-375 (Figure 2C). Collectively, these results suggest that AR can self-regulate its own activity via miR-375, and that this self-regulation results in miR-375 influencing the levels of clinically-relevant markers of AR activity such as PSA.

To determine whether miR-375 can directly target AR mRNA, we examined the AR 3'UTR for miR-375 recognition sequences. Two potential binding sites for miR-375 were identified in the extended 3'UTR of AR (Figure 2E). Using luciferase reporter assays, we demonstrated that both binding sites were significantly repressed by miR-375 overexpression (Figure 2F).

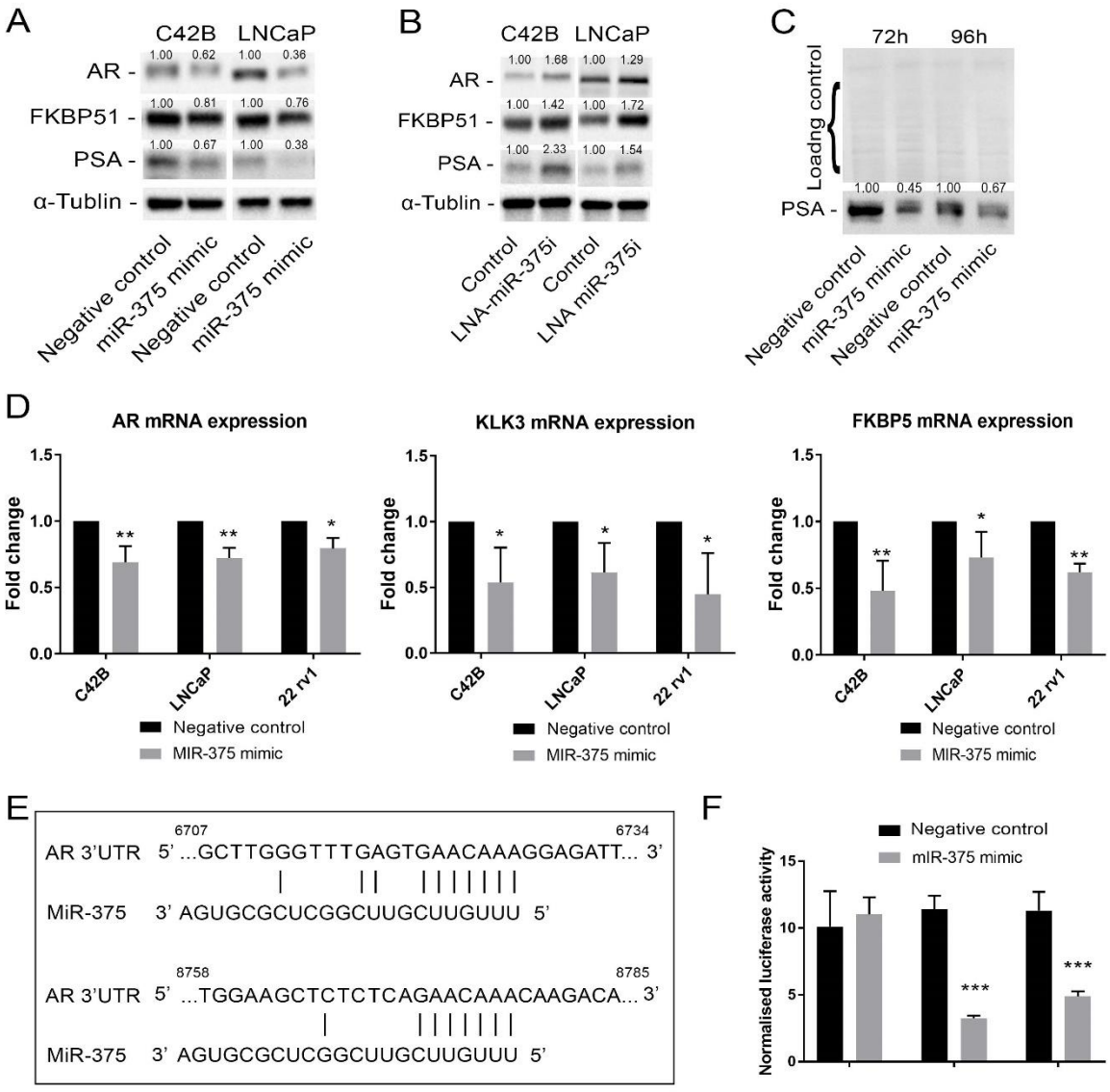

A
C42B
LNCaP
AR -
FKBP51 -
PSA -
α-Tublin -
1.00 0.62 1.00 0.36
1.00 0.81 1.00 0.76
1.00 0.67 1.00 0.38
Negative control
miR-375 mimic
Negative control
miR-375 mimic
B
C42B
LNCaP
AR -
FKBP51 -
PSA -
α-Tublin -
1.00 1.68 1.00 1.29
1.00 1.42 1.00 1.72
1.00 2.33 1.00 1.54
Control
LNA-miR-375i
Control
LNA miR-375i
C
72h
96h
Loadng control
PSA -
1.00 0.45 1.00 0.67
Negative control
miR-375 mimic
Negative control
miR-375 mimic
D
AR mRNA expression
KLK3 mRNA expression
FKBP5 mRNA expression
Fold change
1.5
1.0
0.5
0.0
C42B
LNCaP
22 rv1
Negative control
MIR-375 mimic
E
6707
6734
AR 3'UTR 5' ...GCTTGGGTTTGAGTGAACAAAGGAGATT... 3'
MiR-375 3' AGUGCGCUCGGCUUGCUUGUUU 5'
8758
8785
AR 3'UTR 5' ...TGGAAGCTCTCTCAGAACAAACAAGACA... 3'
MiR-375 3' AGUGCGCUCGGCUUGCUUGUUU 5'
F
Negative control
mIR-375 mimic
Normalised luciferase activity
15
10
5
0
***
***

Figure 2. MiR-375 suppresses AR signalling by directly targeting AR. **A**, C42B and LNCaP cells were transfected with miR-375 mimic or a negative control. After 48 hours, the levels of proteins were examined by Western blotting. α-tublin was used as an internal control. Blot values were determined by ImageJ. **B**, C42B and LNCaP cells were transfected with LNA-miR-375 inhibitor and control, whole lysis were collected 48 hours after transfection and protein levels were examined by western blot with α-tublin as internal control. **C**, C42B cells were transfected with miR-375 mimic and or a negative control. After 96 hours, total protein was extracted from the media and assessed by Western blotting **D**, C42B, LNCaP and 22rv1 cells were transfected with miR-375 mimic and negative control, total mRNA were collected 48 hours after transfection. *AR* and downstream gene mRNA levels were detected by real-time PCR with *GAPDH* as internal control. Results are the average of three independent experiments. p values (**P<0.01; *** *p<0.001)* were determined by Student's t-test. **E**, Potential miR-375 binding site located within the 3'UTR of AR. **F**, HEK239T cells were transfected with reporter plasmids and miR-375 mimic or a negative miRNA control. Dual luciferase reporter assay was performed 48 hours after miR-375 mimic/negative control transfection. Results are the average of 3 independent experiments. p values (*** *p<0.001)* were determined by determined by unpaired *t* tests.

Over-expression of AR rescues miR-375 induced repression of AR signalling and cell growth

MiR-375 has previously been shown to inhibit prostate cancer growth and invasion (Costa-Pinheiro, Ramalho-Carvalho et al. 2015, Selth, Das et al. 2017). To assess whether this tumour suppressive activity is related to targeting of AR, we assessed AR signalling outputs and growth after cells were transfected with miR-375 and a non-targetable form of the AR mRNA. As expected, over-expression of AR reversed the miR-375-mediated down-regulation of PSA and FKBP51 (Figure 3A). Similarly, expression of the non-targetable AR rescued miR-375-mediated suppression of C4-2B cell growth (Figure 3B). These experiments indicate that the ability of miR-375 to suppress prostate cancer cell growth is at least partly effected by targeting of AR.

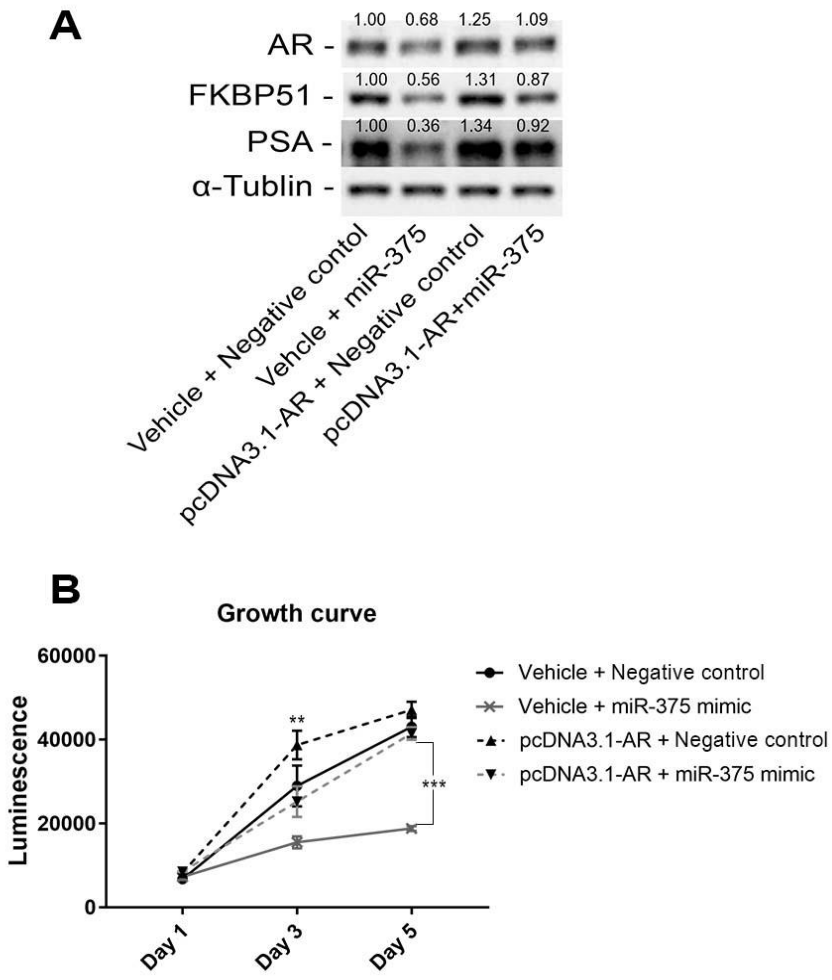


Figure 3. Over expression of AR rescues miR-375-induced phenotypesn. **A,** C42B cells were transfected with negative control, miR-375 mimic, AR expression vector pcDNA-AR and empty vehicle, whole lysis and total mRNA were collected 48 hours after transfection. AR and downstream gene expression were analysed by western blot with α-tublin as internal control. Blot values were determined by ImageJ. **B,** C42B cells were transfected with negative control, miR-375 mimic, AR expression vector pcDNA-AR and empty vehicle. Transfected cells were seeded in 96 well plates; cell proliferation was examined by CellTiter-Glo assay at day 1, 3, 5. Results are the average of three independent experiments. p values (*** *p<0.001)* were determined by Student's t-test.

Discussion

AR is a critical oncogene in prostate cancer. In this present work, we provide new insight into the regulation of AR by identifying a feedback loop with miR-375, whereby AR-induced miR-375 directly inhibits AR expression.

As a transcription factor, AR has been reported to trans-activate the expression of several miRNAs which behave as oncomiRs in prostate cancer. For example, miR-32 is a direct target of AR, and the AR-induced expression of miR-32 leads to increased cell proliferation by targeting BTG2 in castration-resistant prostate cancer (Ambs, Prueitt et al. 2008). Additional miRNA targets of AR have been found, including miR-221, miR-222, miR-141, miR-23a/27/24-2 miR-19a, and miR-133b, which all function to promote cell growth, invasion and survival (Sun, Wang et al. 2009, Waltering, Porkka et al. 2011, Fletcher, Dart et al. 2012, Mo, Zhang et al. 2013). By contrast, this study and previous work indicate that miR-375 performs an anti-cancer role after being induced by AR in our study. With this in mind, we propose that induction of miR-375 by AR could be more prominent in the normal prostate, where miR-375 would serve to enhance the organ's differentiation status Given the ability of miR-375 to induce mesenchymal-epithelial transition in prostate cancer cell model (Selth, Das et al. 2017), this AR induced miR-375 expression may contribute to the androgen dependent prostate cell differentiation and the organ development.

Regulation of miR-375 by the AR signalling axis has previously been reported: more specifically, Chu et al. found that miR-375 was upregulated due to decreased promoter hypermethylation as a consequence of AR repressing the activity of DNA

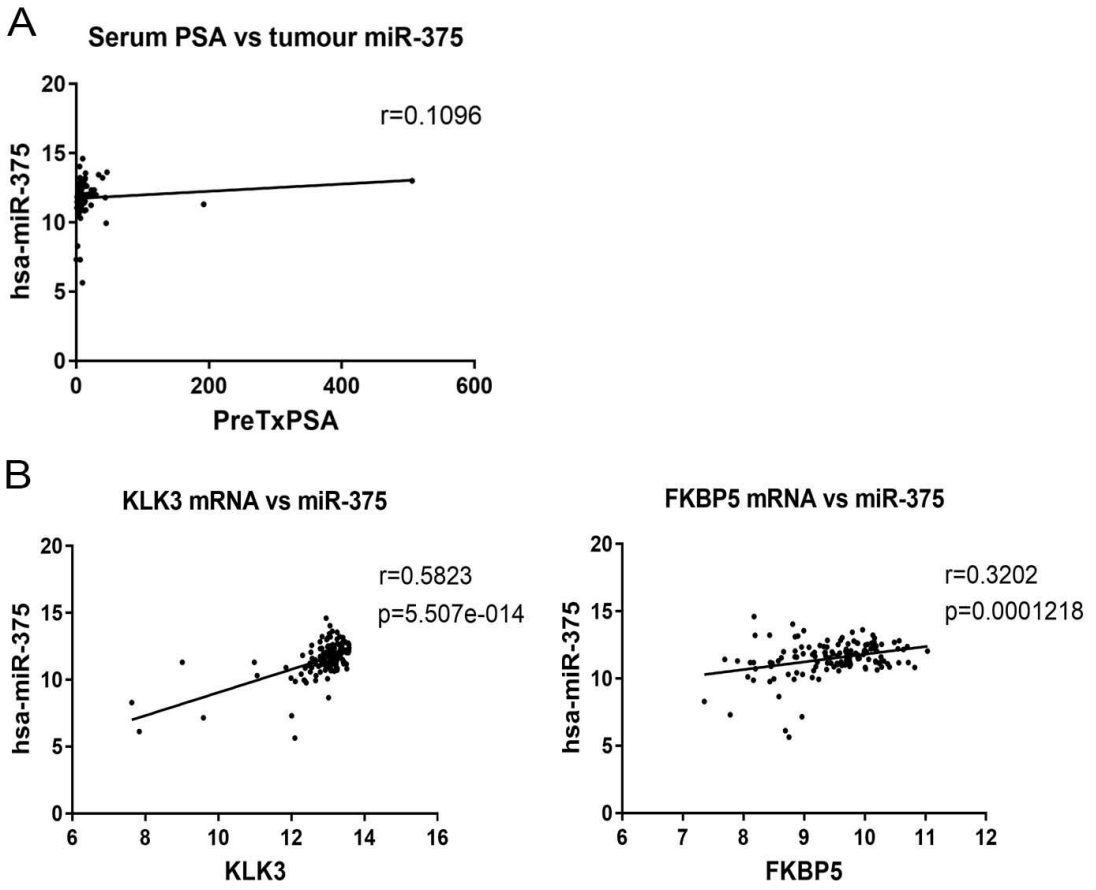
methyltransferases (DNMTs) (Chu, Chang et al. 2014). Our study has identified a novel, direct mechanism by which AR regulates miR-375 expression. Importantly, the AR binding site in the *MIR375* promoter that regulates expression of the gene was identified in tissue samples, suggesting that this regulation is biologically relevant.

The expression and activity of AR is strictly regulated by androgen stimulation; indeed, activated AR binds its own promoter and represses *AR* gene expression as a self-regulatory negative feedback loop (Cai, He et al. 2011). In prostate cancer, this self-regulation is diminished or lost, resulting in over-expression of AR and aberrant activation of its downstream pathways (Cai, He et al. 2011). Our work reveals a novel miRNA-mediated feedback loop as an additional self-restrictive regulation of AR, which would normally function to constrain AR signalling in the prostate. We propose that this regulatory axis would also be disrupted in prostate cancer development and progression, such that miR-375 targeting of AR becomes a minor component of the axis and AR upregulation of miR-375 becomes the major component. Supporting this idea, miR-375 is positively associated with AR signalling in malignant prostate cancer (Selth, Das et al. 2017). Indeed, by analysing tumour and blood samples from 111 prostate cancer patients (Taylor, Schultz et al. 2010), we found a weak positive correlation between tumour miR-375 expression and circulating PSA levels, while tumour KLK3 and FKBP5 mRNA levels showed significant positive correlation with miR-375 (Supplementary Figure 1).

MiR-375 has been described to have a dual role in prostate cancer: on the one hand, multiple studies, including one from my supervisor's lab, have found that it can

suppress cell growth and invasion and increase apoptosis (Costa-Pinheiro, Ramalho-Carvalho et al. 2015, Selth, Das et al. 2017); by contrast, other studies have shown that it can promote proliferation by targeting CIC and CBX7t (Choi, Park et al. 2015, Pickl, Tichy et al. 2016). Notably, in these latter studies, a pro-proliferative role for miR-375 was only observed in the PC3 cell model, which is AR-negative. Our study sheds new light on the actions of miR-375 in prostate cancer, providing further evidence for an anti-proliferative function that is at least partly mediated by suppression of AR signalling.

Despite the apparently contradictory roles of miR-375 in prostate cancer growth and progression, this miRNA has consistently been identified as both a tumour and circulating marker of prostate cancer (Szczyrba, Nolte et al. 2011, Chu, Chang et al. 2014, Choi, Park et al. 2015, Costa-Pinheiro, Ramalho-Carvalho et al. 2015, Huang, Yuan et al. 2015, Kachakova, Mitkova et al. 2015, Pickl, Tichy et al. 2016). Circulating levels of miR-375 are associated with higher grade primary cancers and can predict overall survival in the CRPC context (Huang, Yuan et al. 2015, Wach, Al-Janabi et al. 2015). Our study provides a possible explanation for these earlier findings: enhanced AR signalling in prostate cancer cells leads to enhanced expression and secretion of miR-375, which is consistent with earlier work (Tiryakioglu, Bilgin et al. 2013). This finding further strengthens the potential rationale for utilisation of this miRNA as a biomarker in the clinic.



Supplementary figure 1. Correlation between miR-375, serum PSA, KLK3 and FKBP5 mRNA levels in prostate cancer. **A,** Serum PSA level and tumour mRNA/small RNA deep sequencing data was obtained from 111 prostate cancer patients (Taylor, Schultz et al. 2010). Serum PSA levels and tumour miR-375 expression showed weak trend of positive correlation. **B,** With the same dataset, tumour miR-375 levels showed positive correlation with tumour mRNA levels of *KLK3* and *FKBP5*.

Methods and material

Cell lines and reagents

LNCaP, C42B and 22Rv1 human prostate carcinoma cells and HEK293T cells were purchased from the American Type Culture Collection (ATCC). LNCaP, C42B and 22Rv1 cells were cultured in RPMI-1640 media with 10% fetal bovine serum. HEK293T cells were maintained in DMEM media with 10% fetal bovine serum and 1% PSG.

Reverse-transcription PCR (RT-PCR) and real-time RT-PCR

Endogenous RNA extraction from cells was performed using the RNAeasy mini kit (Qiagen, Valencia, CA USA) and MIReasy mini kit (Qiagen). ND-1000 NanoDrop spectrometer (Thermo Scientific, Wilmington, DE USA) was used to measure total RNA concentration. Media miRNA extraction was performed using the GenElute™ Plasma/Serum RNA Purification Mini Kit (Sigma, USA) following the manufacturer's instructions. MiRNAs were reverse-transcribed into cDNA using TaqMan assay. Total RNA and small RNA were reverse-transcribed into cDNA as previously described (ref) using Moloney Murine Leukaemia Virus (M-MLV) Reverse Transcriptase (Promega, Madison, WI USA) and random 6'mer primers (Promega) (Yu, Bracken et al. 2015).

Real-time PCR reactions were performed using TaqMan assays following the manufacturer instruction, or IQ SYBR Green Supermix (BioRad) on a BioRad iCycler (BioRad) as previously described (Pishas, Al-Ejeh et al. 2011). Primers used for Real-time PCR are listed in Supplementary Table 1. Relative expression levels of miR-375 was determined by the ΔΔCT method after normalization with RNU48 (for endogenous miR-375) or cel-miR-39 (for media miR-375). Relative expression levels

of specific mRNAs were determined by the ΔΔCT method after normalization with GAPDH (All primers see Supplementary Table 1).

Vector construction

The reporter vectors pMIR-binding-site-1, pMIR-binding-site-2, pGL3-miR-375-promoter-1, pGL3-miR-375-promoter-2 and AR expression plasmid pcDNA3.1-AR were a kind gift from (senior author on that paper) (Gillis, Selth et al. 2013).

Transient transfection

Asynchronously growing cells were seeded at 3 x 10^5 cells/well in six-well plates or 1 x 10^5 cells/well in 24-well plates. Transfection of cells with 20 nM mimic miR-375 (Genepharma, Shanghai, CN) or 50 nM LNA anti-miR (EXIQON, Denmak) or 100nM siRNA (Sigma Aldrich) was performed using Lipofectamine RNAiMAX (Invitrogen, CA, USA). Transfection of cells with 1ug/mL with reporter vectors or pcDNA3.1-AR and control was performed using Lipofectamine 2000 (Invitrogen).

Cell proliferation assay

C42B cells were plated at 1500 cells/well in 96-well plates 24 hours after transfection and were cultured for 1, 3 and 5 days. On the indicated days, cells were incubated with CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega) and fluorescence was measured by LUMIstar Galaxy luminometer (BMG Labtech).

Supplementary Table 1. Primers

Gene	Primer	Sequence (5'-3')
AR	Forward	CCTGGCTTCCGCAACTTACAC
	Reverse	GGACTTGTGCATGCGGTACTC
KLK3	Forward	GCCTGGATCTGAGAGAGATATCATC
	Reverse	ACACCTTTTTTTTTCTGGATTGTTG
FKBP5	Forward	AAAAGGCCAAGGAGCACAAC
	Reverse	TTGAGGAGGGGCCGAGTTC
GAPDH	Forward	AGCCTCCCGCTTCGCTCTCTGC
	Reverse	ACCAGGCGCCCAATACGACCAAA
AR binding-site-C	Forward	GAGTCAATATTTGCCCCGAGC
	Reverse	GGGCTCCGTGTGCTCTTAT
AR binding-site-D	Forward	GTTCTTCCTCCCCAGCCTCT
	Reverse	CACAGTAACTGAGTGCCAGG
NC2	Forward	GTGAGTGCCCAGTTAGAGCATCTA
	Reverse	GGAACCAGTGGGTCTTGAAGTG

Western blot and antibodies

Western blot assay was performed as previously described (Yu, Bracken et al. 2015). Antibodies used include: goat anti-KLK3 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-FKBP51 (Santa Cruz), rabbit anti-AR (Santa Cruz) and mouse anti-α-tublin (Santa Cruz).

Luciferase assay

For validation of AR binding miR-375 promoter, HEK293T cells were plated in a 24-well plate and then cotransfected with 0.5 ug of reporter vectors pGL3-miR-375-promoter-1, pGL3-miR-375-promoter-2 and 0.5 ug of AR expression plasmid pcDNA3.1-AR or their controls along with 0.25 ug of pRL as indicator of the transfection efficiency using Lipofectamine 2000 (Invitrogen).

For validation of miR-375 targeting AR 3'UTR, HEK293T cells were plated in a 24-well plate and then cotransfected with 50 μM of either miR-375 mimic or negative control, and 0.75 ug of pMIR-binding-site-1, pMIR-binding-site-2 or pMIR-control along with 0.25 ug of pRL as indicator of the transfection efficiency using Lipofectamine 2000 (Invitrogen).

Transfected cells were collected 48 h after transfection and fluorescence was meassured using the Dual-Luciferase Reporter Assay System (Promega). Both Renilla and Firefly luminescence were measured with a GloMax 20/20 Luminometer (Promega). Renilla luciferase was used as an internal control for any differences in transfection and harvesting efficiencies. All transfections were performed in duplicate and repeated at least three times in independent experiments.

Chromatin immunoprecipitation and antibodies

LNCaP cells were plated 4,000,000 per 15cm plate in PRFRPMI + 10% CSS and serum-starved for 72 hrs prior to treatment with 10nM DHT/EtOH (1 plate per treatment) for 4 hrs. Cells were then X-linked as per usual, snap-frozen and pellets stored at -80. ChIP assays were performed as previously described (Schmidt, Wilson et al. 2009) using an AR antibody and an IgG antibody from Abcam. The known AR binding site on KLK3 promoter was used as positive control.

Statistical analysis

Data are presented as means ± SD from at least three independent experiments. Student's t-test and unpaired t-test were performed using replicate values to compare between groups and indicate significance. Values of $p<0.05$ were considered statistically significant (labeled as * in figures, while values of $p<0.01$ were labeled as ** and $p<0,001$ as ***).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

The results published here are in part based on the data obtained from The Cancer Genome Atlas, established by the National Cancer Institute and the National Human Genome Research Institute, and we are grateful to the specimen donors and relevant research groups associated with this project. This work was supported by: Young Investigator Awards from the Prostate Cancer Foundation (the Foundation 14 award; LAS) and grants from PCFA/Movember/Cancer Australia (Grant IDs 1012337 and 1043482; WDT, LAS and LMB) and the National Health and Medical Research Council (Grant ID 1083961) and the Prostate Cancer Foundation of Australia (PCFA)/Movember (YI 0810; LAS, YI 0412; MMC). QW is supported by a Research Training Program Scholarship from the Australia Government. The research programs of LAS are supported by the Movember Foundation and the Prostate Cancer Foundation of Australia through Movember Revolutionary Team Awards.

References

Ambs, S., R. L. Prueitt, M. Yi, R. S. Hudson, T. M. Howe, F. Petrocca, T. A. Wallace, C. G. Liu, S. Volinia, G. A. Calin, H. G. Yfantis, R. M. Stephens and C. M. Croce (2008). "Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer." Cancer Res **68**(15): 6162-6170.

Boll, K., K. Reiche, K. Kasack, N. Morbt, A. K. Kretzschmar, J. M. Tomm, G. Verhaegh, J. Schalken, M. von Bergen, F. Horn and J. Hackermuller (2013). "MiR-130a, miR-203 and miR-205 jointly repress key oncogenic pathways and are downregulated in prostate carcinoma." Oncogene **32**(3): 277-285.

Cai, C., H. H. He, S. Chen, I. Coleman, H. Wang, Z. Fang, S. Chen, P. S. Nelson, X. S. Liu, M. Brown and S. P. Balk (2011). "Androgen receptor gene expression in prostate cancer is directly suppressed by the androgen receptor through recruitment of lysine-specific demethylase 1." Cancer Cell **20**(4): 457-471.

Chang, C. S., J. Kokontis and S. T. Liao (1988). "Molecular cloning of human and rat complementary DNA encoding androgen receptors." Science **240**(4850): 324-326.

Choi, N., J. Park, J. S. Lee, J. Yoe, G. Y. Park, E. Kim, H. Jeon, Y. M. Cho, T. Y. Roh and Y. Lee (2015). "miR-93/miR-106b/miR-375-CIC-CRABP1: a novel regulatory axis in prostate cancer progression." Oncotarget **6**(27): 23533-23547.

Chu, M., Y. Chang, P. Li, Y. Guo, K. Zhang and W. Gao (2014). "Androgen receptor is negatively correlated with the methylation-mediated transcriptional repression of miR-375 in human prostate cancer cells." Oncol Rep **31**(1): 34-40.

ChunJiao, S., C. Huan, X. ChaoYang and R. GuoMei (2014). "Uncovering the roles of miRNAs and their relationship with androgen receptor in prostate cancer." IUBMB Life **66**(6): 379-386.

Costa-Pinheiro, P., J. Ramalho-Carvalho, F. Q. Vieira, J. Torres-Ferreira, J. Oliveira, C. S. Goncalves, B. M. Costa, R. Henrique and C. Jeronimo (2015). "MicroRNA-375 plays a dual role in prostate carcinogenesis." Clin Epigenetics **7**: 42.

Dai, C., H. Heemers and N. Sharifi (2017). "Androgen Signaling in Prostate Cancer." Cold Spring Harb Perspect Med.

Fletcher, C. E., D. A. Dart, A. Sita-Lumsden, H. Cheng, P. S. Rennie and C. L. Bevan (2012). "Androgen-regulated processing of the oncomir miR-27a, which targets Prohibitin in prostate cancer." Hum Mol Genet **21**(14): 3112-3127.

Gillis, J. L., L. A. Selth, M. M. Centenera, S. L. Townley, S. Sun, S. R. Plymate, W. D. Tilley and L. M. Butler (2013). "Constitutively-active androgen receptor variants function independently of the HSP90 chaperone but do not confer resistance to HSP90 inhibitors." Oncotarget **4**(5): 691-704.

Hagman, Z., B. S. Haflidadottir, J. A. Ceder, O. Larne, A. Bjartell, H. Lilja, A. Edsjo and Y. Ceder (2013). "miR-205 negatively regulates the androgen receptor and is associated with adverse outcome of prostate cancer patients." Br J Cancer **108**(8): 1668-1676.

Huang, X., T. Yuan, M. Liang, M. Du, S. Xia, R. Dittmar, D. Wang, W. See, B. A. Costello, F. Quevedo, W. Tan, D. Nandy, G. H. Bevan, S. Longenbach, Z. Sun, Y. Lu, T. Wang, S. N. Thibodeau, L. Boardman, M. Kohli and L. Wang (2015). "Exosomal miR-1290 and miR-375 as prognostic markers in castration-resistant prostate cancer." Eur Urol **67**(1): 33-41.

Kachakova, D., A. Mitkova, E. Popov, I. Popov, A. Vlahova, T. Dikov, S. Christova, V. Mitev, C. Slavov and R. Kaneva (2015). "Combinations of serum prostate-specific antigen and plasma expression levels of let-7c, miR-30c, miR-141, and miR-375 as potential better diagnostic biomarkers for prostate cancer." DNA Cell Biol **34**(3): 189-200.

Koochekpour, S. (2010). "Androgen receptor signaling and mutations in prostate cancer." Asian J Androl **12**(5): 639-657.

Krol, J., I. Loedige and W. Filipowicz (2010). "The widespread regulation of microRNA biogenesis, function and decay." Nat Rev Genet **11**(9): 597-610.

Lin, P. C., Y. L. Chiu, S. Banerjee, K. Park, J. M. Mosquera, E. Giannopoulou, P. Alves, A. K. Tewari, M. B. Gerstein, H. Beltran, A. M. Melnick, O. Elemento, F. Demichelis and M. A. Rubin (2013). "Epigenetic repression of miR-31 disrupts androgen receptor homeostasis and contributes to prostate cancer progression." Cancer Res **73**(3): 1232-1244.

MacKrell, J. G., B. C. Yaden, H. Bullock, K. Chen, P. Shetler, H. U. Bryant and V. Krishnan (2015). "Molecular targets of androgen signaling that characterize skeletal muscle recovery and regeneration." Nucl Recept Signal **13**: e005.

Massie, C. E., A. Lynch, A. Ramos-Montoya, J. Boren, R. Stark, L. Fazli, A. Warren, H. Scott, B. Madhu, N. Sharma, H. Bon, V. Zecchini, D. M. Smith, G. M. Denicola, N. Mathews, M. Osborne, J. Hadfield, S. Macarthur, B. Adryan, S. K. Lyons, K. M. Brindle, J. Griffiths, M. E. Gleave, P. S. Rennie, D. E. Neal and I. G. Mills (2011). "The androgen

receptor fuels prostate cancer by regulating central metabolism and biosynthesis." EMBO J **30**(13): 2719-2733.

Mo, W., J. Zhang, X. Li, D. Meng, Y. Gao, S. Yang, X. Wan, C. Zhou, F. Guo, Y. Huang, S. Amente, E. V. Avvedimento, Y. Xie and Y. Li (2013). "Identification of novel AR-targeted microRNAs mediating androgen signalling through critical pathways to regulate cell viability in prostate cancer." PLoS One **8**(2): e56592.

Nadiminty, N., R. Tummala, W. Lou, Y. Zhu, J. Zhang, X. Chen, R. W. eVere White, H. J. Kung, C. P. Evans and A. C. Gao (2012). "MicroRNA let-7c suppresses androgen receptor expression and activity via regulation of Myc expression in prostate cancer cells." J Biol Chem **287**(2): 1527-1537.

Nandana, S. and L. W. Chung (2014). "Prostate cancer progression and metastasis: potential regulatory pathways for therapeutic targeting." Am J Clin Exp Urol **2**(2): 92-101.

Ostling, P., S. K. Leivonen, A. Aakula, P. Kohonen, R. Makela, Z. Hagman, A. Edsjo, S. Kangaspeska, H. Edgren, D. Nicorici, A. Bjartell, Y. Ceder, M. Perala and O. Kallioniemi (2011). "Systematic analysis of microRNAs targeting the androgen receptor in prostate cancer cells." Cancer Res **71**(5): 1956-1967.

Pickl, J. M., D. Tichy, V. Y. Kuryshev, Y. Tolstov, M. Falkenstein, J. Schuler, D. Reidenbach, A. Hotz-Wagenblatt, G. Kristiansen, W. Roth, B. Hadaschik, M. Hohenfellner, S. Duensing, D. Heckmann and H. Sultmann (2016). "Ago-RIP-Seq identifies Polycomb repressive complex I member CBX7 as a major target of miR-375 in prostate cancer progression." Oncotarget **7**(37): 59589-59603.

Pishas, K. I., F. Al-Ejeh, I. Zinonos, R. Kumar, A. Evdokiou, M. P. Brown, D. F. Callen and P. M. Neilsen (2011). "Nutlin-3a is a potential therapeutic for ewing sarcoma." Clin Cancer Res **17**(3): 494-504.

Pomerantz, M. M., F. Li, D. Y. Takeda, R. Lenci, A. Chonkar, M. Chabot, P. Cejas, F. Vazquez, J. Cook, R. A. Shivdasani, M. Bowden, R. Lis, W. C. Hahn, P. W. Kantoff, M. Brown, M. Loda, H. W. Long and M. L. Freedman (2015). "The androgen receptor cistrome is extensively reprogrammed in human prostate tumorigenesis." Nat Genet **47**(11): 1346-1351.

Rahim, B. and R. O'Regan (2017). "AR Signaling in Breast Cancer." Cancers (Basel) **9**(3).

Schmidt, D., M. D. Wilson, C. Spyrou, G. D. Brown, J. Hadfield and D. T. Odom (2009). "ChIP-seq: using high-throughput sequencing to discover protein-DNA interactions." Methods **48**(3): 240-248.

Selth, L. A., R. Das, S. L. Townley, I. Coutinho, A. R. Hanson, M. M. Centenera, N. Stylianou, K. Sweeney, C. Soekmadji, L. Jovanovic, C. C. Nelson, A. Zoubeidi, L. M. Butler, G. J. Goodall, B. G. Hollier, P. A. Gregory and W. D. Tilley (2017). "A ZEB1-miR-375-YAP1 pathway regulates epithelial plasticity in prostate cancer." Oncogene **36**(1): 24-34.

Selth, L. A., S. Townley, J. L. Gillis, A. M. Ochnik, K. Murti, R. J. Macfarlane, K. N. Chi, V. R. Marshall, W. D. Tilley and L. M. Butler (2012). "Discovery of circulating microRNAs associated with human prostate cancer using a mouse model of disease." Int J Cancer **131**(3): 652-661.

Siegel, R. L., K. D. Miller and A. Jemal (2017). "Cancer Statistics, 2017." CA Cancer J Clin **67**(1): 7-30.

Sikand, K., J. E. Slaibi, R. Singh, S. D. Slane and G. C. Shukla (2011). "miR 488* inhibits androgen receptor expression in prostate carcinoma cells." Int J Cancer **129**(4): 810-819.

Sinisi, A. A., D. Pasquali, A. Notaro and A. Bellastella (2003). "Sexual differentiation." J Endocrinol Invest **26**(3 Suppl): 23-28.

Sinnesael, M., F. Claessens, M. Laurent, V. Dubois, S. Boonen, L. Deboel and D. Vanderschueren (2012). "Androgen receptor (AR) in osteocytes is important for the maintenance of male skeletal integrity: evidence from targeted AR disruption in mouse osteocytes." J Bone Miner Res **27**(12): 2535-2543.

Sun, T., Q. Wang, S. Balk, M. Brown, G. S. Lee and P. Kantoff (2009). "The role of microRNA-221 and microRNA-222 in androgen-independent prostate cancer cell lines." Cancer Res **69**(8): 3356-3363.

Szczyrba, J., E. Nolte, S. Wach, E. Kremmer, R. Stohr, A. Hartmann, W. Wieland, B. Wullich and F. A. Grasser (2011). "Downregulation of Sec23A protein by miRNA-375 in prostate carcinoma." Mol Cancer Res **9**(6): 791-800.

Taylor, B. S., N. Schultz, H. Hieronymus, A. Gopalan, Y. Xiao, B. S. Carver, V. K. Arora, P. Kaushik, E. Cerami, B. Reva, Y. Antipin, N. Mitsiades, T. Landers, I. Dolgalev, J. E. Major, M. Wilson, N. D. Socci, A. E. Lash, A. Heguy, J. A. Eastham, H. I. Scher, V. E. Reuter, P. T. Scardino, C. Sander, C. L. Sawyers and W. L. Gerald (2010). "Integrative genomic profiling of human prostate cancer." Cancer Cell **18**(1): 11-22.

Tilley, W. D., M. Marcelli, J. D. Wilson and M. J. McPhaul (1989). "Characterization and expression of a cDNA encoding the human androgen receptor." Proc Natl Acad Sci U S A **86**(1): 327-331.

Tiryakioglu, D., E. Bilgin, S. Holdenrieder, N. Dalay and U. Gezer (2013). "miR-141 and miR-375 induction and release are different from PSA mRNA and PCA3 upon androgen stimulation of LNCaP cells." Biomed Rep **1**(5): 802-806.

van Kruchten, M., P. van der Marel, L. de Munck, H. Hollema, H. Arts, H. Timmer-Bosscha, E. de Vries, G. Hospers and A. Reyners (2015). "Hormone receptors as a marker of poor survival in epithelial ovarian cancer." Gynecol Oncol **138**(3): 634-639.

Volinia, S., G. A. Calin, C. G. Liu, S. Ambs, A. Cimmino, F. Petrocca, R. Visone, M. Iorio, C. Roldo, M. Ferracin, R. L. Prueitt, N. Yanaihara, G. Lanza, A. Scarpa, A. Vecchione, M. Negrini, C. C. Harris and C. M. Croce (2006). "A microRNA expression signature of human solid tumors defines cancer gene targets." Proc Natl Acad Sci U S A **103**(7): 2257-2261.

Wach, S., O. Al-Janabi, K. Weigelt, K. Fischer, T. Greither, M. Marcou, G. Theil, E. Nolte, H. J. Holzhausen, R. Stohr, V. Huppert, A. Hartmann, P. Fornara, B. Wullich and H. Taubert (2015). "The combined serum levels of miR-375 and urokinase plasminogen activator receptor are suggested as diagnostic and prognostic biomarkers in prostate cancer." Int J Cancer **137**(6): 1406-1416.

Waltering, K. K., K. P. Porkka, S. E. Jalava, A. Urbanucci, P. J. Kohonen, L. M. Latonen, O. P. Kallioniemi, G. Jenster and T. Visakorpi (2011). "Androgen regulation of micro-RNAs in prostate cancer." Prostate **71**(6): 604-614.

Wu, J. N., K. M. Fish, C. P. Evans, R. W. Devere White and M. A. Dall'Era (2014). "No improvement noted in overall or cause-specific survival for men presenting with metastatic prostate cancer over a 20-year period." Cancer **120**(6): 818-823.

Yu, F., C. P. Bracken, K. A. Pillman, D. M. Lawrence, G. J. Goodall, D. F. Callen and P. M. Neilsen (2015). "p53 Represses the Oncogenic Sno-MiR-28 Derived from a SnoRNA." PLoS One **10**(6): e0129190.

Zhu, M. L. and N. Kyprianou (2008). "Androgen receptor and growth factor signaling cross-talk in prostate cancer cells." Endocr Relat Cancer **15**(4): 841-849.

Chapter 5.

Discussion

As an important member of the ncRNA family, miRNAs have been revealed to play multifunctional roles in all cellular processes. Many human diseases are accompanied by aberrant expression of miRNAs, including cancer. The growing evidence of the participation of miRNAs in cancer highlights its significance for current cancer research. Focusing on the miRNA-mediated regulation of two important pathways in cancer, this present work extends the current knowledge of the miRNA-protein networks in cancer, and describes a novel observation of miRNA-mediated specific regulation of gene mutations.

5.1. MiR-766 induces p53 accumulation and G2/M arrest by directly targeting MDM4.

A detailed understanding of regulation of the tumour suppressor p53 is critical for determining the basis of cancer initiation and development, and subsequently for the development of new treatment approaches to target this pathway. The major core mechanisms of post-transcriptional regulation of p53 include phosphorylation, ubiquitination and degradation, with the MDM4-p53 axis being the essential player in this regulation. A complex network of the regulation of p53 signalling has now been established and this continues to be refined as new members of this network are discovered. In particular, a number of miRNAs have now been found that directly or indirectly regulate p53, many of which also contribute to p53-associated cancer progression.

The first part of my work identifies a previously unknown function of miR-766 in p53 regulation, distinguishing this miRNA as a novel element in the regulatory network

of p53 signalling. Previous studies of MDM4 predominately focused on its impact on p53 activity. In the work presented in this thesis, we also explore the impact of MDM4 on p53 protein expression, as well as p53's transcriptional activity and the downstream signaling. This work strengthens the evidence of MDM4-regulation of p53 protein levels and enhances our understanding of MDM4's role in the MDM2-MDM4-p53 regulatory axis.

An ongoing concern about the biological significance of some miRNAs is related to their low abundance *in vivo*. Low abundant miRNAs (i.e. < 15 reads per million, as determined by deep sequencing data) are considered less likely to have a significant impact on cellular events [1]. MiR-766 exhibits low abundance in the deep sequencing data across different solid tumours, but we believe it has the potential to significantly affect cellular processes by regulating the key tumour suppressor p53. Supporting this idea, we observed robust cellular effects not only when miR-766 was over-expressed but also when its activity was blocked by a LNA-miR-inhibitor in multiple cancer and normal cell lines. In summary, this work, together with other reports of low abundance miRNAs playing important roles in cancer [2-6], provide evidence that low abundance miRNAs can be biologically relevant and their possible roles should not be underestimated. Interestingly, the accuracy of miRNA relative abundance may be improved due to the methodological bias of sequencing technology. And it is possible that the reported relative abundance of miRNAs in current database may be modified in future.

Another issue related to miRNAs is determining their overall role in the cell when each miRNA has multiple valid target genes with different functions. Similar to many

other miRNAs, miR-766 has been reported to target genes that can lead to opposite cellular outcomes in different cellular contexts. Interestingly, while my work nominates miR-766 as a positive regulator of p53 by targeting MDM4, it is also confirmed to target BAX [7] and SOX6 [8]. Both can potentially compromise the MDM4-mediated effect of miR-766 on p53 signalling. Although my study provides solid evidence of the overall tumour suppressor behaviour of miR-766 in the cell line models, animal experiments (i.e. xenograft tumours in nude mice), analysis of more clinical data and further *in silico* analysis of miR-766 targeting network would significantly improve the biological relevance.

The upstream regulation of miR-766 is an aspect that has not been addressed in this work. Although this miRNA was initially selected for its distinct expression between tumours with wild-type and mutant p53, attempts to validate the effects of p53 on miR-766 expression failed due to a lack of consistent results (knockdown of p53 induced changes of miR-766 expression in cell lines could not be consistently repeated). Following p53 knockdown by p53 siRNA, the expression of miR-766 was detected using TaqMan assay in cell lines expressing wild-type or mutant p53, but no consistent or repeatable trend of change in miR-766 regulation. *In silico* investigation was also performed using promoter prediction and data from ChIP-seq, but the results revealed little possibility for the host gene of miR-766 to be a transcription target of wild-type p53. The observed upregulation of miR-766 in missense p53 tumours may be a result of an unknown missense p53 gain-of-function that activates the host gene SEPT6. Alternatively, the mechanism could be more indirect via regulation by a downstream target of mutant p53. These hypotheses could be verified in future studies by *in silico*

promoter analysis of miR-766, ChIP-seq and luciferase reporter assay to explain the trend of higher expression of miR-766 in missense p53 tumours across multiple tumour types.

5.2. Overexpression of miR-9 leads to mutation specific knockdown of missense p53

Chapter 3 of this thesis also focuses on the miRNA-involved p53 regulation, and highlights the post-transcriptional regulation of a specific p53 mutant. As described in chapter 3.1, ~80% of p53 mutations in cancer, including somatic and germline mutations, occur as missense mutations [9]. The missense p53 proteins are often more stable, and some variants transform its function from an inactivated tumour suppressor into an onco-protein. Although it has been shown that the protein-mediated post-transcriptional regulation of missense p53s differ from wild-type p53, little is known regarding the miRNA-mediated regulation of missense p53. The current major belief is that in the absence of 3'UTR variation of the mutant mRNA, miRNAs are incapable of discriminating between missense and wild-type p53, which is supported by previous studies [10, 11]. Surprisingly, my work found that overexpression of miR-9 could discriminate between specific missense p53 mutations and wild-type p53, leading to opposite consequences in p53 protein expression. To my knowledge, similar observations have not previously been described and therefore this finding is novel and significant.

To validate this observation, inhibition of miR-9 in the same cell lines to repeat the consistent result with miR-9 overexpression would be important, and a broader panel of cell lines with various p53 mutations should be used in further investigation. In addition,

clinical data relating outcomes with particular p53 mutations and miR-9 expression would significantly enhance the soundness of this work. Unfortunately, since R248Q and R273H are only a small proportion of the overall missense mutations, the TCGA deep sequencing data did not provide sufficient clinical data to accomplish this analysis. It is expected that data from a larger cohort of tumour samples will be available in the near future to undertake such analysis.

However, the molecular mechanism of this unique and unexpected observation, particularly how this miRNA was able to discriminate between a specific missense p53 over other missense mutations, is still unclear, opening a wide range of possibilities to explore in the future. A possible explanation of this miRNA-mediated mutation-specific regulation is that the secondary structure of an mRNA may influence the access to miRNA binding sites. In this scenario, changes of the secondary structure of a particular missense mutant p53 mRNA may expose a miRNA binding site within the ORF. This hypothesis suggests that mRNA higher order structure could be a new factor that determines miRNA targeting. This hypothesis could be validated by undertaking a bioinformatics analysis. For example, the hiCLIP approach could be used to reveal the structural impact of missense mutations in p53 mRNA. The use of computational models such as miREN [12] and Sfold [13, 14], which aim to analyse the folding structure of the target mRNAs and to improve the current *in silico* prediction methods, would be relevant to such a study. The Millar group in the Australian National University is currently undertaking a project aiming to identify the effects of target mRNA secondary structures on the repression efficiency of miRNA in plants. Surprisingly, although the analysis tools have been available and researches have reported the impact of mRNA secondary and tertiary structure in the miRNA-targeting interactions [15, 16], the secondary structure

of target mRNAs is rarely considered in previous functional miRNA studies in human diseases. Considering the high frequency of gene mutations in cancer, and the potential impact of the mutants on the tertiary folding structure of the mRNAs, the lack of understanding and awareness of this issue may result in neglecting many existing miRNA- regulations that may exist when gene mutation or variation occurs. Thus, the modelling and analysis of target mRNA structures and its impact on miRNA-AGO2 binding should be adopted as soon as possible into miRNA-related cancer research. To validate this hypothesis, specific gene editing using CRISPR/Cas9 would help determine the impact of certain mutations on miRNA-mRNA binding.

Another of our hypotheses to explain the mutation-specific impact of miR-9 relates to possible 3'UTR variation of missense p53 mRNA that may exist, particularly in cancer cells. One major issue with this hypothesis is that it fails to explain the miR-9 impact on H1299 R248Q cells as the p53 mRNA does not contain 3'UTR in this inducible cell line. Also it seems to be an unlikely coincidence of the same 3'UTR variation occurring in two unrelated cell lines (Ovcar3 and MDA-MB-468), which should be tested by sequencing for the 3'UTR of p53 in these two cell lines. To date, we know little about the connection between p53 missense mutation and 3'UTR variation, and there is minimal data regarding 3'UTR variation. A few researches have reported loss of miRNA binding sites due to 3'UTR variation or mRNA splicing [17, 18], yet it is unknown whether such variation could create new miRNA binding sites [19-21]. As a new area that remains largely unknown, I believe the importance of 3'UTR variation analysis should be highlighted in future miRNA research.

An alternative hypothesis to explain the mutation-specific effect of miR-9 is that it can somehow upregulate a molecular structure sensitive protein that recognises and degrades the specific mutant p53 proteins R248Q and R273H. To investigate this hypothesis, high-throughput screening of proteins and computer modelling of the protein folding structures would be required.

Similar to my miR-766 study, further study of the the upstream regulation of miR-9 will be of interest. The regulation of miR-9 has been reported in previous studies via activating the promoter of the miRNA but independent of the host gene [22-24]. However, whether and how the maturation of miR-9 is regulated still remains unclear. Since missense p53 has been revealed to affect miRNA maturation by regulating Dicer [25], it is of significant interest to investigate if the missense p53 protein has a role in the maturation of miR-9; which may also explain the distinctive expression patterns of miR-9 in missense and wild-type p53 tumours.

Both the miR-766 and miR-9 studies in this thesis add novel miRNA regulators to the complex network of p53 signalling, and reveal that both miR-766 and miR-9 contribute to the tumour suppressor role of wild-type p53. Our findings suggest these two miRNAs could be exploited to restore wild-type p53 function in future cancer treatment. For example, the impact of miR-9 on specific mutant p53 also suggests that miR-9 may be used as a wild-type p53 reactivator in the treatment of tumours with certain p53 heterozygous mutations.

5.3. A novel miR-375-androgen receptor feedback loop regulates growth of prostate cancer cells.

Apart from the regulation of the tumour suppressor p53, a miRNA involved in regulation of AR signalling in prostate cancer is also discussed in the second part of my work. AR is a critical player in the development and progression of prostate cancer, and as such is the major therapeutic target in this disease. MiRNAs participate in the regulation of AR signalling by directly targeting AR or AR downstream genes in the pathway [26].

This part of my work presents miR-375 as a miRNA that directly targets AR, and effectively regulates the downstream signalling of AR. This work extends the current knowledge of miRNA-AR regulation, and shows miR-375 is a negative factor in prostate cancer progression, which is consistent with the finding in our previous report [27]. While being a target of miR-375, AR also directly transactivates the miR-375 promoter, and induces the expression of miR-375. This work describes a novel miRNA-AR negative feedback loop that potentially contributes to the balance of AR signalling in healthy cells.

We identified AR as a direct target of miR-375 via the extended 6 kb 3'UTR of AR, which is not assessed in many available algorithms (i.e. TargetScan). Six other miRNAs, including miR-31, miR-654-5p, miR-203/205, miR-488* and let-7c, have also been reported to target AR by binding the extended 3'UTR [28-33], while only miR-185 is verified to target AR via the predicted 436 nt 3'UTR (identified by TargetScan) [34]. This suggests that the AR 3'UTR should be updated in publically-available algorithms.

Interestingly, this work illustrates a strong reduction of PSA secretion when miR-375 expression is enforced, which is in conflict with the previous analysis of miR-375 expression and serum PSA levels in prostate cancer [35]. The inconsistency of our *in vitro* result and clinical data may be explained by the targeting efficiency of the miRNA-mRNA interaction. My hypothesis is that AR-mediated induction of the *MIR375* gene is a far more robust process than miR-375 targeting of AR. As a result, AR and its target genes are positively correlated with miR-375 in prostate cancer, rather than negatively correlated as may be expected given that AR is a direct target of this miRNA. Our in vitro data also suggest that high tumour levels of miR-375 could profoundly influence serum PSA levels; as such, quantitation of both markers may improve the prediction of prostate cancer status [35].

5.4. Final comments

All three miRNAs studied in the work of this thesis exhibit dual-role roles in different cancer contexts. The data presented in this thesis is consistent with miR-766 as a potent tumour suppressor, while previous studies described it as an oncomiR. Both miR-9 and miR-375 have shown both oncogenic and anti-cancer abilities in different cancer models, and my work provides additional evidence supporting their tumour repressive roles. The details that possibly defines the cancer context are still to be further uncovered. To determine the general role of the miRNAs, meta-analysis of clinical data and *in vivo* experiments should be used for different cancer models. Additionally, once we more fully understand the complete repertoires of miRNA targets, we will be able to much more readily understand the context-dependent roles of miRNAs. Also, gene

mutation status should be acquired by deep sequencing to evaluate potential disruption in miRNA-mediated pathways; however, this will require larger cancer datasets.

Collectively, miRNAs have emerged as critical functional regulators of cancer cells. The work described in this thesis provides new evidence of miRNA: mRNA interactions in multiple cancer types, and enhances our understanding of the regulatory mechanisms underlying two essential signalling pathways in cancer. The three projects also each raise questions relating to the molecular mechanism of miRNA-RNA interactions and the translation from *in vitro* finding to *in vivo* and clinical studies. Multiple challenges to draw a clear picture of the complexity of miRNA-mRNA networks in cancer exist, but the types of studies presented here provide hope that we will eventually achieve this ambitious goal – a goal that will help us to better understand, control and defeat cancer as well as other diseases.

References

1. Farazi, T.A., et al., *MicroRNAs in human cancer.* Adv Exp Med Biol, 2013. **774**: p. 1-20.
2. Shen, H., et al., *MicroRNA-137 inhibits tumor growth and sensitizes chemosensitivity to paclitaxel and cisplatin in lung cancer.* Oncotarget, 2016. **7**(15): p. 20728-42.
3. Tung, M.C., et al., *Reduction of microRNA-184 by E6 oncoprotein confers cisplatin resistance in lung cancer via increasing Bcl-2.* Oncotarget, 2016. **7**(22): p. 32362-74.
4. Nadal, E., et al., *A MicroRNA cluster at 14q32 drives aggressive lung adenocarcinoma.* Clin Cancer Res, 2014. **20**(12): p. 3107-17.
5. Incoronato, M., et al., *miR-212 increases tumor necrosis factor-related apoptosis-inducing ligand sensitivity in non-small cell lung cancer by targeting the antiapoptotic protein PED.* Cancer Res, 2010. **70**(9): p. 3638-46.
6. Yin, M., et al., *Selective killing of lung cancer cells by miRNA-506 molecule through inhibiting NF-kappaB p65 to evoke reactive oxygen species generation and p53 activation.* Oncogene, 2015. **34**(6): p. 691-703.
7. Liang, H., et al., *MicroRNAs contribute to promyelocyte apoptosis in As2O3-treated APL cells.* Cell Physiol Biochem, 2013. **32**(6): p. 1818-29.
8. Li, Y.C., et al., *MicroRNA-766 targeting regulation of SOX6 expression promoted cell proliferation of human colorectal cancer.* Onco Targets Ther, 2015. **8**: p. 2981-8.
9. Soussi, T. and K.G. Wiman, *TP53: an oncogene in disguise.* Cell Death Differ, 2015. **22**(8): p. 1239-49.
10. Liu, H., et al., *MicroRNA-191 promotes pancreatic cancer progression by targeting USP10.* Tumour Biol, 2014. **35**(12): p. 12157-63.
11. Raza, U., et al., *The miR-644a/CTBP1/p53 axis suppresses drug resistance by simultaneous inhibition of cell survival and epithelial-mesenchymal transition in breast cancer.* Oncotarget, 2016. **7**(31): p. 49859-49877.
12. Leoni, G. and A. Tramontano, *A structural view of microRNA-target recognition.* Nucleic Acids Res, 2016. **44**(9): p. e82.
13. Long, D., C.Y. Chan, and Y. Ding, *Analysis of microRNA-target interactions by a target structure based hybridization model.* Pac Symp Biocomput, 2008: p. 64-74.

14. Long, D., et al., *Potent effect of target structure on microRNA function.* Nat Struct Mol Biol, 2007. **14**(4): p. 287-94.
15. Gan, H.H. and K.C. Gunsalus, *Tertiary structure-based analysis of microRNA-target interactions.* RNA, 2013. **19**(4): p. 539-51.
16. Bohmer, M., et al., *Structural analysis of microRNA-target interaction by sequential seed mutagenesis and stem-loop 3' RACE.* PLoS One, 2013. **8**(11): p. e81427.
17. Enciso-Mora, V., et al., *Low penetrance susceptibility to glioma is caused by the TP53 variant rs78378222.* Br J Cancer, 2013. **108**(10): p. 2178-85.
18. Li, Y., et al., *Single nucleotide variation in the TP53 3' untranslated region in diffuse large B-cell lymphoma treated with rituximab-CHOP: a report from the International DLBCL Rituximab-CHOP Consortium Program.* Blood, 2013. **121**(22): p. 4529-40.
19. Stegeman, S., et al., *A genetic variant of MDM4 influences regulation by multiple microRNAs in prostate cancer.* Endocr Relat Cancer, 2015. **22**(2): p. 265-76.
20. Wynendaele, J., et al., *An illegitimate microRNA target site within the 3' UTR of MDM4 affects ovarian cancer progression and chemosensitivity.* Cancer Res, 2010. **70**(23): p. 9641-9.
21. Gao, F., et al., *A Regulatory MDM4 Genetic Variant Locating in the Binding Sequence of Multiple MicroRNAs Contributes to Susceptibility of Small Cell Lung Cancer.* PLoS One, 2015. **10**(8): p. e0135647.
22. Laneve, P., et al., *A minicircuitry involving REST and CREB controls miR-9-2 expression during human neuronal differentiation.* Nucleic Acids Res, 2010. **38**(20): p. 6895-905.
23. Zhao, J., et al., *Ngn1 inhibits astrogliogenesis through induction of miR-9 during neuronal fate specification.* Elife, 2015. **4**: p. e06885.
24. Barbano, R., et al., *Stepwise analysis of MIR9 loci identifies miR-9-5p to be involved in Oestrogen regulated pathways in breast cancer patients.* Sci Rep, 2017. **7**: p. 45283.
25. Muller, P.A., et al., *Mutant p53 regulates Dicer through p63-dependent and -independent mechanisms to promote an invasive phenotype.* J Biol Chem, 2014. **289**(1): p. 122-32.
26. ChunJiao, S., et al., *Uncovering the roles of miRNAs and their relationship with androgen receptor in prostate cancer.* IUBMB Life, 2014. **66**(6): p. 379-86.

27. Selth, L.A., et al., *A ZEB1-miR-375-YAP1 pathway regulates epithelial plasticity in prostate cancer.* Oncogene, 2017. **36**(1): p. 24-34.
28. Ostling, P., et al., *Systematic analysis of microRNAs targeting the androgen receptor in prostate cancer cells.* Cancer Res, 2011. **71**(5): p. 1956-67.
29. Sikand, K., et al., *miR 488* inhibits androgen receptor expression in prostate carcinoma cells.* Int J Cancer, 2011. **129**(4): p. 810-9.
30. Boll, K., et al., *MiR-130a, miR-203 and miR-205 jointly repress key oncogenic pathways and are downregulated in prostate carcinoma.* Oncogene, 2013. **32**(3): p. 277-85.
31. Hagman, Z., et al., *miR-205 negatively regulates the androgen receptor and is associated with adverse outcome of prostate cancer patients.* Br J Cancer, 2013. **108**(8): p. 1668-76.
32. Lin, P.C., et al., *Epigenetic repression of miR-31 disrupts androgen receptor homeostasis and contributes to prostate cancer progression.* Cancer Res, 2013. **73**(3): p. 1232-44.
33. Nadiminty, N., et al., *MicroRNA let-7c suppresses androgen receptor expression and activity via regulation of Myc expression in prostate cancer cells.* J Biol Chem, 2012. **287**(2): p. 1527-37.
34. Qu, F., et al., *MicroRNA-185 suppresses proliferation, invasion, migration, and tumorigenicity of human prostate cancer cells through targeting androgen receptor.* Mol Cell Biochem, 2013. **377**(1-2): p. 121-30.
35. Roberts, M.J., et al., *Diagnostic performance of expression of PCA3, Hepsin and miR biomarkers inejaculate in combination with serum PSA for the detection of prostate cancer.* Prostate, 2015. **75**(5): p. 539-49.