The dysregulation of microRNAs due to mutant p53$^{R172H}$ expression in a mouse model of pancreatic ductal adenocarcinoma

Thesis submitted for the degree of
Doctor of Philosophy
At the University of Leicester

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July 2015
Abstract

The dysregulation of microRNAs due to mutant p53<sup>R172H</sup> expression in a mouse model of pancreatic ductal adenocarcinoma – Jack David Godfrey

Pancreatic cancer is one of the most aggressive of human malignancies, with incidence almost equalling mortality. The main reason for the poor prognosis is that the majority of pancreatic cancer cases are not diagnosed until the disease has metastasised. Mutations to p53 have been shown to have gain of function characteristics, which promote metastasis in pancreatic cancer. MicroRNAs are small endogenous molecules which inhibit gene expression, and have been shown to be dysregulated in a number of diseases, including cancer. Current therapeutic approaches to treatment of metastatic pancreatic cancer have poor clinical outcomes, with little hope of remission. Given this, it is necessary to understand the molecular events governing metastasis in pancreatic cancer, in order to develop novel therapeutic approaches for the treatment of this disease.

This study has implemented a mouse model of pancreatic cancer to profile microRNA expression in primary tumours which express mutant p53<sup>R172H</sup> (p53<sup>R175H</sup> in humans). The experiments showed that a subset of microRNAs were dysregulated in mutant p53<sup>R172H</sup> expressing tissues, with the majority being downregulated, including miR-148a-3p and miR-142-3p. Reverse trans-well invasion assays were employed to investigate how modulation of some of these microRNAs may influence the invasive ability of cells. The experiments show that miR-148a-3p inhibits invasion of pancreatic cancer cells. Additionally, this study presents evidence to suggest that the methyltransferase DNMT1, is upregulated in mutant p53<sup>R172H</sup> expressing tumours. Depletion of DNMT1 is sufficient to rescue expression of miR-142-3p in mutant p53<sup>R172H</sup> expressing primary cells.

This study presents data to suggest that a novel mechanism by which mutant p53<sup>R172H</sup> attains gain of function abilities, may be through promoting hypermethylation of microRNAs.
Acknowledgements

I would like to thank all members of the MRC Toxicology Unit for their support throughout this study, especially the members of Professor Martin Bushell’s group. Special thanks must be offered to Dr Ania Wilczynska, Dr Ewan Smith and Dr Wei-ting Lu for agreeing to proof read this thesis. I would particularly like to thank Professor Martin Bushell for his continued support, funding and for providing me with the opportunity to complete this study. Important thanks go to Professor Owen Sansom and Dr Jennifer Morton of the Beatson Institute in Glasgow, for providing me with the material required to carry out this study and for on-going support. I would also like to thank my family for their unwavering support and for helping to ensure that this thesis is intelligible, even while not understanding the content. Special thanks to Veronica for putting up with me during the more stressful times (very much including writing this thesis!) and for helping me to grow as a person.
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<td>2′-O-methyl</td>
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<td>ACSL4</td>
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<td>ADAR</td>
<td>Adenosine Deaminase Acting on RNA</td>
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<td>Component 3 of Promoter Of RISC</td>
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<td>CO₂</td>
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<td>CPSF</td>
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<tr>
<td>eIF</td>
<td>eukaryotic Initiation Factor</td>
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<td>FBS</td>
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<td>H-RAS</td>
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<tr>
<td>PDAC</td>
<td>Pancreatic Ductal Adenocarcinoma</td>
</tr>
</tbody>
</table>
PDCD4  Programmed Cell Death 4
Pen/Strep  Penicillin Streptomycin
PIC  Pre-Initiation Complex
PNACL  Protein and Nucleic Acid Chemistry Laboratory
PNPT1  Polyribonucleotide Nucleotidyltransferase 1
Poly(A)  Poly-adenosine
PR  Proline rich domain
pre-miRs  Precursor microRNAs
pri-miRs  Primary microRNAs
PTEN  Phosphatase and Tensin Homolog
PUMA  BCL2 Binding Component 3
RAC1  RAS-related C3 botulinum toxin substrate 1
RB  Retinoblastoma Protein
RISC  Ribonucleic acid Induced Silencing Complex
RNA  Ribonucleic acid
RNApol  RNA Polymerase
RNAse  Ribo-nuclease
RPMI  Roswell Park Memorial Institute
rRNA  Ribosomal Ribonucleic Acid
RRP41  Ribosomal RNA processing Protein 41
RT  Reverse transcription
RT-qPCR  Reverse Transcription quantitative Polymerase chain reaction
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>S. cerevisiae</td>
<td><em>Saccharomyces cerevisiae</em></td>
</tr>
<tr>
<td>S. pombe</td>
<td><em>Schizosaccharomyces pombe</em></td>
</tr>
<tr>
<td>S1PR1</td>
<td>sphingosine 1 phosphate receptor 1</td>
</tr>
<tr>
<td>SAM</td>
<td>Sterile Alpha Motif</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficient</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>siRNA</td>
<td>small-interfering Ribonucleic Acid</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Sirtuin 1</td>
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<tr>
<td>SLUG</td>
<td>Snail Family Zinc Finger 2</td>
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<td>SMAD4</td>
<td>Mothers Against Decapentaplegic Homolog 4</td>
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<td>SNAIL</td>
<td>Snail Family Zinc Finger 1</td>
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<td>snoRNA</td>
<td>small-nucleolar Ribonucleic Acid</td>
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<td>SP1</td>
<td>Specificity Protein 1</td>
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<tr>
<td>SV40</td>
<td>Simian Virus 40</td>
</tr>
<tr>
<td>TA</td>
<td>Trans-activating domain</td>
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<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA buffer</td>
</tr>
<tr>
<td>TAMs</td>
<td>Tumour Associated Macrophages</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA-binding protein</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-Buffered Saline with Tween</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N′,N′′-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TFI1</td>
<td>General Transcription factors</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor β</td>
</tr>
<tr>
<td>TNRC6</td>
<td>Tri-nucleotide Repeat Containing 6</td>
</tr>
<tr>
<td>TOP</td>
<td>Terminal Oligopyrimidine</td>
</tr>
<tr>
<td>TRBP</td>
<td>Transactivating Response RNA-Binding Protein</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer Ribonucleic Acid</td>
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<tr>
<td>TWIST</td>
<td>Twist Family BHLH Transcription Factor</td>
</tr>
<tr>
<td>U6</td>
<td>U6 small nuclear RNA</td>
</tr>
<tr>
<td>USP25</td>
<td>Ubiquitin Specific Peptidase 25</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated Region</td>
</tr>
<tr>
<td>UTRN</td>
<td>Utrophin</td>
</tr>
<tr>
<td>VAMP</td>
<td>Vesicle-Associated Membrane Protein</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth factor</td>
</tr>
<tr>
<td>XPO5</td>
<td>Exportin 5</td>
</tr>
<tr>
<td>XRN</td>
<td>5′-3′ exo-ribonuclease</td>
</tr>
<tr>
<td>ZEB</td>
<td>Zinc Finger E-Box Binding Homeobox</td>
</tr>
<tr>
<td>ΔN</td>
<td>N-terminal truncation</td>
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Chapter 1  Introduction

1.1 The pancreas
To understand the complexities of pancreatic disease, we first need an understanding of the structure and function of the healthy tissue. The pancreas is an abdominal secretory organ located close to the duodenum, which has both hormone producing (endocrine) and digestive enzyme producing (exocrine) function. The endocrine pancreas consists of three distinct cell types located in structures called the islets of Langerhans: alpha cells secrete glucagon, which has the primary function of signalling for stored glycogen to be metabolised in order to increase glucose concentration in the blood; beta cells secrete insulin, which has the opposing effect of promoting the absorption of glucose from the blood into storage; and delta cells, which secrete somatostatin, an enzyme with inhibitory effects on a variety of hormones, including glucagon and insulin (Quesada, Todorova et al. 2006).

Figure 1-1: The location of the pancreas and surrounding structures. This image has been reproduced with permission courtesy of dream designs at FreeDigitalPhotos.net with annotation carried out by Jack Godfrey.
The exocrine pancreas consists of clusters of acini cells, which secrete digestive enzymes, known as pancreatic “juice” into pancreatic ducts, which then flow into larger ducts and eventually to the main pancreatic duct, and into the duodenum. The major components of pancreatic “juice” are: amylase, pancreatic proteases and pancreatic lipases. The major function of amylase is to metabolise starch into more simple disaccharides and trisaccharides, which are then further metabolised into glucose for use in energy production. Pancreatic proteases consist of a number of enzymes, though trypsin and chymotrypsin are the major constituents. Both are produced as the inactive precursors, trypsinogen and chymotrypsinogen, which are activated through proteolytic cleavage upon entry to the duodenum. Trypsinogen is cleaved by enterokinase, and once activated, is able to cleave and activate other trypsinogen molecules as well as chymotrypsinogen. Pancreatic lipase is able to metabolise triglyceride into monoglyceride and free fatty acid, aiding in absorption of these nutrients (Pandol 2010).

These functions outline the diverse and vital role the pancreas plays in maintaining the health of an organism. Insufficient endocrine pancreatic function is linked to diabetes, a condition responsible for 5.2% of global mortality in 2000 (Roglic, Unwin et al. 2005). Loss of exocrine pancreatic function can lead to a condition known as Exocrine Pancreatic Insufficiency (EPI) which can lead to loss of nutrient uptake causing severe discomfort and weight loss. The most serious pancreas related maladies are: cystic fibrosis, pancreatitis and pancreatic cancer (Hart, Conwell 2015). Pancreatitis is caused by inflammation of the pancreas and may be acute or chronic. Both disorders are conditions of pancreatic damage, but while acute pancreatitis is reversible, chronic pancreatitis is defined by non-reversible damage to the pancreas (Sarles 1986). Importantly, chronic pancreatitis is a predictor of pancreatic cancer, possibly due to the inflammatory response caused by chronic pancreatitis (Lowenfels, Maisonneuve et al. 1993, Whitcomb, Pogue-Geile 2002). Pancreatic cancer will be discussed in detail in section 1.3 following a general introduction into cancer.
1.2 Cancer

1.2.1 What is cancer?
Cancer is a group of diseases, defined by uncontrolled cell growth and proliferation, which may be as ancient as multicellular organisms. Given that cancer is defined by uncontrolled cellular division, as soon as multicellular organisms evolved, where cell division is tightly controlled, there is the opportunity for loss of control and unchecked cell growth. Tumours have been found in the bones of dinosaurs dating back as far as 70 million years (Rothschild, Tanke et al. 2003). The oldest documented human malignancy is recorded in the Edwin Smith papyrus which dates back to ~3000BC, and describes a number of cases of a disease presenting as a lump in the breast of patients which was described as incurable (Breasted 1984). While this early documentation is relatively vague in its description of the disease, the “father of medicine”, Hippocrates, was the first to study it in detail, and give it a name, Karakininos, the ancient Greek word for crab, due to the fact that blood vessels surrounding tumours look like the claws of a crab. He was also able to designate different types of tumour and name them: carcinos, a benign tumour; carcinoma, a malignant tumour and cancer, a malignant ulcer. He also described tumours in a variety of different tissues (Littre 1846).

The underlying cause of cancer and the way the disease develops, represent major avenues of scientific endeavour. Several key experimental findings underlie our modern understanding of cancer development. That environmental factors may cause cancer has been known since the 18th century, where John Hill observed that excessive use of tobacco snuff correlated with the incidence of nasal cancer (Hill 1764). Additionally, Percival Pott showed a high incidence of scrotal skin cancers in chimney sweeps (Pott 1775). This was further supported by evidence that when chimney sweeps were told to wash after work, a dramatic reduction in the incidence of this tumour was observed. More direct evidence came about in the early 20th century when Katsusaburo Yamagiwa was able to directly induce skin cancer in rabbits, by painting their ears with coal tar (Yamagiwa, Ichikawa 1918). Another landmark finding in the understanding of cancer came in 1927 when Herman Muller showed that X-rays were able to induce mutations in the genome of drosophila melanogaster (Muller
Later, large scale experiments by Bruce Ames were able to show that many chemical compounds which were known to be carcinogenic, were also mutagenic (Ames, McCann et al. 1975), suggesting a causal link between genetic mutation and cancer. It is now almost universally accepted that cancer is a genetic disease, with a huge bibliography of evidence to support the claim (Dunning, Healey et al. 1999, Calin, Croce 2006, Lohmueller, Pearce et al. 2003, Vogelstein, Kinzler 2004, Stratton, Campbell et al. 2009, Hirschhorn, Lohmueller et al. 2002).

A landmark study was carried out by Peyton Rous in the early 20th century. Peyton began his study using breast sarcomas from chickens, which he implanted into healthy chickens and observed the formation of a sarcoma. He took this further by homogenising the sarcoma tissue, before filtering it through a very fine filter to ensure no chicken or bacterial cells would be in the filtrate. He then injected the filtrate it into healthy chickens, which then developed sarcomas at the site of injection. He also showed that this process could be repeated using the secondary tumour to infect other healthy chickens (Rous 1911). Rous observed that smaller amounts of filtrate were required to induce sarcoma formation in later generations of infection, suggesting that the carcinogen was increasing in concentration. As a chemical carcinogen has no way of amplifying itself, it would be expected that it would be titrated down through generations of infection, and a greater amount of filtrate would be required to induce sarcoma formation. With the opposite being observed, it lead to the hypothesis that cancer could be caused by viral infection. Additionally the fact that the filtrate which was injected into chickens had been filtered through pores too fine for either chicken or bacterial cells to pass through, suggesting that neither prokaryotic nor eukaryotic cells could be responsible. This virus was the first to be shown to be directly carcinogenic and was named the Rous Sarcoma Virus (RSV). It was not until 1977 that the gene responsible for RSV induced oncogenesis was identified as Schmidt-Ruppin A-2 (SRC) (Brugge, Erikson 1977). Now described as an oncogene, SRC is a tyrosine kinase, known to drive proliferative signalling cascades.

While genetic mutations may cause cancer, it is seldom true that a single mutation is sufficient to promote oncogenesis. A milestone study by Alfred Knudson in 1971 showed that familial retinoblastoma presents at a very young age and often affects
both eyes, while sporadic retinoblastoma presents more frequently in adults and affects only one eye. Knudson applied a statistical approach to show that sporadic retinoblastoma was consistent with two mutational events, while hereditary retinoblastoma was consistent with a single mutational event. These findings lead to the development of the Knudson’s two hit hypothesis, and the identification of tumour suppressor genes (Knudson 1971).

Following the above observations, many tumour suppressor genes and oncogenes have been observed to be mutated in a variety of types of cancer. Generally, mutations to oncogenes cause hyperactivity or amplified expression, while mutations to tumour suppressor genes typically cause loss of function of the gene product.

A more recent key review defined cancers as diseases which: have self-sufficiency in growth signals, are insensitive to anti-growth signals, invade and metastasise, have limitless replicative potential, sustain angiogenesis and are able to evade apoptosis (Hanahan, Weinberg 2000). This was later expanded to include the ability to avoid the immune system, and the dysregulation of normal cellular energetics (Hanahan, Weinberg 2011). These publications provide both a definition of what cancer is, as well as identifying avenues for therapeutic intervention. For example, the ability to reverse the self-sufficiency of growth signals or insensitivity to anti-growth signals would result in regaining control of proliferation, essentially halting tumour growth. Also, stopping cells evading the immune system or apoptosis would result in tumour cell death and a reduction of tumour burden.

Cancer may occur anywhere within an organism, from organs such as the lungs, to structural components such as bone, as well as connective tissues, nerves and blood. The most critical aspect of a tumour is the sustained and uncontrolled growth and proliferation of cells. While these diseases share phenotypic characteristics, the genetic mutations which cause them vary significantly between tumour types. More recently it has been shown that cancers of a specific organ may also have widely varied genetic mutations associated with them. For example, breast cancer cells may express receptors for oestrogen, progesterone or human epidermal growth factor receptor 2 (HER2). These cells respond differently to endogenous stimuli, but more
importantly, require distinct therapeutic strategies. For example, the drug trastuzumab (Herceptin) is effective in treating HER2 positive breast cancers, while having limited, to no effect on other types (Moja, Tagliabue et al. 2012, Hudis 2007).

As previously mentioned, cancer is a genetic disease, which results in the transformation of normal cells into tumour cells, through the accumulation of genetic mutations which allow sustained growth and proliferation (Hanahan, Weinberg 2011, Hanahan, Weinberg 2000). Tumours may be benign, meaning they do not spread from the primary site, or malignant, which involves the metastatic spread of tumour cells to distant sites. The processes involved in invasion and metastasis will be discussed in the next sections.

1.2.2 Epithelial Mesenchymal Transition
Epithelial Mesenchymal Transition (EMT) is a critical step in tumour progression and is believed to be an early step in the transition from benign to malignant transformation. As the name suggests, EMT is a phenotypic change in cellular architecture, between the ordered and structured epithelial organisation, to the more fibroblastic and motile mesenchymal organisation (Kalluri, Weinberg 2009). An array of molecular events occurs within a cell undergoing EMT, including: losing basal-apical polarity, reorganisation of the structural components of a cell, a loss of cell-cell junctions including adherens junctions (Oft, Peli et al. 1996), tight junctions (Ikenouchi, Matsuda et al. 2003), desmosomes (Vandewalle, Comijn et al. 2005) and gap junctions (Bax, Pijnappels et al. 2011). Changes in gene expression are also frequently observed with: Zinc Finger E-Box Binding Homeobox 1 & 2 (ZEB1 & 2) (Kim, Veronese et al. 2011), Snail Family Zinc Finger 1 & 2 (SNAIL & SLUG) (Medici, Hay et al. 2008) and Twist Family BHLH Transcription Factor (TWIST) (Yang, Hsu et al. 2010) frequently being found to be dysregulated. The process of EMT is not inherently harmful, and is required for normal cell functions, such as wound healing (Yan, Grimm et al. 2010) and embryonic development (Caramel, Papadogeorgakis et al. 2013), but the process is frequently hijacked by cancer cells in order to promote a more invasive phenotype. A converse event known as Mesenchymal to Epithelial Transition (MET) is another
normal cellular function required for the transition of fibroblast to induced pluripotent stem cells (Li, Liang et al. 2010), which is also hijacked by cancer cells to establish secondary sites of tumour growth during metastatic spread (Chaffer, Brennan et al. 2006, Chao, Shepard et al. 2010).

MicroRNA expression has been shown to be critically involved in maintaining the epithelial architecture of cells and suppression of EMT (see section 1.4 for details of microRNAs). The miR-200 family and miR-205 have been shown to be critical inhibitors of EMT through their targets: ZEB1&2, SIP1 and SLUG (Liu, Yin et al. 2013, Park, Gaur et al. 2008, Gregory, Bert et al. 2008). MiR-34a has also been shown to target SNAIL and regulate EMT (Siemens, Jackstadt et al. 2011, Hahn, Jackstadt et al. 2013). These microRNAs are frequently lost in cancer and correlate with poor prognosis and an increased invasive phenotype (Korpal, Lee et al. 2008, Burk, Schubert et al. 2008, Wu, Zhu et al. 2009, Liu, Kelnar et al. 2011).

Both EMT and MET are used by cancer cells during metastasis: EMT to produce invasive tumour cells which can reach secondary sites, and MET for cells to establish secondary colonies and progress to a metastatic disease.

1.2.3 Metastasis
Metastasis is a complex, multi-step procedure where tumour cells migrate from the primary tumour site and develop secondary sites of tumour growth. It is the major cause of cancer related morbidity and mortality (Chaffer, Weinberg 2011, Valastyan, Weinberg 2011). Due to its complexity, metastasis is an inherently inefficient process with very few invading tumour cells (<0.01%) surviving to establish secondary sites (Fidler 1970). This is perhaps not surprising as there is no selective pressure for cells which are becoming invasive at the site of a primary tumour, to evolve mechanisms to aid their survival in the vascular system. Indeed, they require phenotypes directly opposed to those required to establish secondary sites of growth, such as reduced motility and sustained growth.

The basic steps of metastasis are: increased invasion and migration, often through EMT (See section 1.2.2 for details on EMT); intravasation, the entry of invading cells
into the vascular system; dissemination, the spread of cancer cells through the vascular system; extravasation, the exit of tumour cells from the vascular system, and the establishment of secondary tumour sites, as may be seen in figure 1.2.

**Figure 1-2** A basic schematic of the steps of metastasis. Mutations cause EMT which promotes invasion in cells. This leads to cells invading the vascular system, disseminating to distant sites and establishing secondary tumours.

After undergoing EMT, the tumour cells are more motile and invasive, and are able to migrate towards blood vessels. It has been suggested that Tumour Associated Macrophages (TAMs) influence this process (Wyckoff, Wang et al. 2007). New blood vessels caused by angiogenesis around the tumour site have been shown to have weak cell-cell junctions, which allow invading tumour cells easy access into the vascular system. Transforming Growth Factor β (TGFβ) and Vascular Endothelial Growth factor (VEGF) have both been implicated as being involved in this process (Anderberg, Cunha et al. 2013).
Once the tumour cells have entered the blood vessels, they must survive the inhospitable environment of the vascular system and avoid detection by the immune system. This is thought to occur due to interactions with other cells within the vascular system, most notably platelets. Tumour cells within the vascular system are thought to form aggregates with platelets, which protect them from the stress of the vascular system as well as blocking immune cell interactions (Nieswandt, Hafner et al. 1999). Interestingly, high platelet counts correlate with poor prognosis in patients with colorectal cancer (Lin, Huang et al. 2012), oesophageal carcinoma (Aminian, Karimian et al. 2011), non-small cell lung cancer (Ji, Sheng et al. 2014) and pancreatic cancer (Suzuki, Aiura et al. 2004). Additionally, antiplatelet therapies have been successful in the treatment of metastatic diseases in animal models (Zhang, Dang et al. 2012, Bando, Yamashita et al. 1984). An interesting study found that dual treatment with the antiplatelet drug dipyridamole and RA-233 (an analogue of dipyridamole) resulted in a 70% reduction in the number of hepatic metastases in a mouse model of PDAC (Tzanakakis, Agarwal et al. 1993). As well as protecting the disseminating tumour cells, the platelet/metastasising cancer cell aggregates are also more likely to become stuck in smaller blood vessels, allowing the tumour cells to extravasate and establish secondary tumour sites.

Tumour cells of a particular origin appear to have a propensity to metastasise to specific organs. For example, the majority of pancreatic metastases are observed in the liver (Kayahara, Nagakawa et al. 1993, Kamisawa, Isawa et al. 1995) or lungs (Deeb, Haque et al. 2015), while breast cancer typically metastasises to the brain (Tham, Sexton et al. 2006), lung (Minn, Gupta et al. 2005) or bone (Solomayer, Diel et al. 2000). Two competing theories exist as to why certain types of cancers tend to disseminate to specific sites. The first, hypothesised in 1889, is called the “seed and soil” hypothesis, which suggests that cancer cells of a specific origin are better suited to establish secondary tumours in specific tissues (Paget 1889). Another hypothesis suggested that the organisation of the vascular and lymphatic systems were sufficient to explain the effect (Ewing 1928). A benchmark study investigating melanoma metastasis was able to shed some light onto this controversy. Melanoma cells have a propensity to metastasise to the lung (Tas 2012). By grafting lung tissue or a control
of renal tissue into identical locations of mice, and injecting them with B-16 melanoma cells, the study showed that there were preferential metastases to the lung grafts rather than the renal grafts, supporting the “seed and soil” hypothesis (Hart, Fidler 1980).

Extravasation may occur when tumour cells which have become trapped in a blood vessel, begin to grow, and upon reaching a required size, physically burst the blood vessel, gaining access to the surrounding tissue (Al-Mehdi, Tozawa et al. 2000). An alternate hypothesis is that tumour cells which have become stationary within blood vessels are able to invade between the endothelial cells of blood vessels in order to establish a secondary tumour. This is similar to the process of intravasation described above, but moving from the inside of the blood vessels to the outside. While this may be correct in a broad sense, the microenvironment within blood vessels is dramatically dissimilar to that outside the blood vessels, with no TAMs or other tumour-associated cells being present. Also, specific genes have been implicated as being involved with extravasation but having no impact on intravasation, such as Angiopoietin-Like Protein 4 (ANGPTL4) which promotes extravasation of breast cancer cells to the lungs (Padua, Zhang et al. 2008). This data suggests distinct mechanism control the intravasation and extravasation processes.

Once tumour cells have emerged from the vascular system, they must adjust to survive in the alien environment in which they then find themselves. As the environment from which a metastasising cell began may be vastly different to the location they find themselves following extravasation, they must modify the environment to allow them to grow successfully. One hypothesis postulates that primary tumours are able to prime distant sites by forming a “pre-metastatic niche” which allows metastasising tumour cells to settle into their new environment more easily. The inflammatory chemoattractants S100A8 and S100A9 have been shown to be induced by primary tumours, which attract myeloid cells to pre-metastatic location in the lungs, priming them for metastatic invasion (Hiratsuka, Watanabe et al. 2006, Hiratsuka, Watanabe et al. 2008). Additionally, VEGFR1 expressing haematopoietic progenitor cells have been shown to translocate to pre-metastatic sites and form clusters which aid metastasising tumour cells in establishing new colonies (Kaplan,
Lysyl oxidase (LOX) is secreted by hypoxic primary tumours, which crosslinks collagen IV at pre-metastatic sites, allowing the establishment of CD11b+ myeloid cells, which secrete Matrix Metalloproteinases (MMPs) and degrade the basement membrane, allowing metastasising tumour cells to establish themselves (Erler, Bennewith et al. 2009). These data suggest that complex mechanisms and co-operation of multiple cell types are involved in the establishment of the “pre-metastatic niche”, which facilitates the establishment of secondary sites of tumour growth. An important recent study showed that PDAC derived exosomes cause Kupffer cells (a liver-specific, specialised macrophage) to secrete TGFβ, resulting in hepatic stellate cells secreting fibronectin. The resulting fibrotic environment allows the recruitment of bone marrow-derived macrophages (BMDM) and the establishment of a “pre-metastatic niche” for PDAC metastasis (Costa-Silva, Aiello et al. 2015).

As previously mentioned, metastasis is the major cause of cancer-related mortality, as it is very difficult to treat metastatic disease. The development of novel therapeutic approaches, such as modulation of microRNA expression, may reduce metastasis related mortality and increase the survival of cancer patients.

1.3 Pancreatic cancer

1.3.1 Types of pancreatic cancer

Both the endocrine and exocrine pancreas are susceptible to cancer formation, however, the majority of pancreatic cancers evolve in the exocrine pancreas (Warshaw, Castillo 1992). The most common exocrine pancreatic cancer is adenocarcinoma, with most of these being Pancreatic Ductal Adenocarcinoma (PDAC) which makes up >85% of pancreatic cancer cases (Warshaw, Castillo 1992), and is the focus of this study. Some more rare exocrine pancreatic cancers exist, such as Pancreatic Acina Cell Carcinoma (PACC). These arise in the acinar cells, which produce the digestive “juice” of the pancreas, and make up ~1% of pancreatic cancers (Callata-Carhuapoma, Pato Cour et al. 2015). Endocrine tumours of the pancreas are quite rare and may be split into two categories: functional or non-functional, with functional
tumours expressing hormones and non-functional tumours not expressing hormones (Halfdanarson, Rubin et al. 2008). These tumours may arise from alpha, beta or delta cells, and are named after the hormone primarily produced by the progenitor cell. For example, tumours derived from alpha cells are called glucagonomas, those from beta cells are insulinomas and those from delta cells are somatostatinomas (Halfdanarson, Rubin et al. 2008). When these tumours are functional they may cause increased expression of these hormones, which may have physiological effects. Other rare forms of pancreatic cancer include: Pancreatoblastoma, a childhood cancer which may derive from multiple cell types and have both endocrine and exocrine components (Kawamoto, Matsuo et al. 1985); Pancreatic sarcoma, a tumours derived from the connective tissue in the pancreas (Ambe, Kautz et al. 2011) and pancreatic lymphoma, which develop in lymphatic tissues located in the pancreas (Piesman, Forcione 2007).

The following sections will discuss in detail specific characteristics of PDAC.

1.3.2 Epidemiology of PDAC
The most up to date epidemiological data is based on United States statistics, which states that in 2012 incidence of pancreatic cancer was 12.94 cases per 100,000. The incidence was slightly higher for males, with an incidence of 14.47 per 100,000 and 11.66 per 100,000 for females. The mortality associated with pancreatic cancer for 2012 was 11.01 per 100,000. Again, a greater number of deaths were observed in men, with 12.65 per 100,000 and women, with 9.64 per 100,000, giving an almost equal number of new cases to deaths. Incidence of pancreatic cancer is very low in the under 50s but increases significantly with age. The latest survival figures suggest that <5% of people survive for 10 years with the disease (Howlader, Noone et al. 2015). A number of genetic conditions may predispose people to pancreatic cancer including type 1 diabetes (Bosetti, Rosato et al. 2012) and chronic pancreatitis (Raimondi, Lowenfels et al. 2010).

Tobacco use has been linked to an increased incidence in pancreatic cancer, with one meta-analysis finding that smoking cigarettes causes a 75% increased risk of development of pancreatic cancer, which persists for 10 years after stopping smoking.
Excessive alcohol consumption (Pezzilli 2015) and a poor diet (Ghorbani, Hekmatdoost et al. 2015) have also been shown to correlate with PDAC incidence.

The high mortality and morbidity rates of PDAC may be attributed to the difficulty in its diagnosis. Pancreatic cancer is often symptomless until it has become metastatic, and due to the location of the pancreas, it is very difficult to discern palpable lumps (Chari 2007). In the cases of functional endocrine tumours, excess hormone levels, or sudden onset diabetes, may act as early indicators (Pannala, Basu et al. 2009). Ductal adenocarcinomas in the head of the pancreas may lead to a blockage of the bile duct, which in turn may cause jaundice (Du Plessis 1970). However, in the majority of cases, pancreatic cancers are either diagnosed late in the disease progression, at which point it is often metastatic, or diagnosed during independent clinical procedures, such as baseline tests.

1.3.3 Characteristics of PDAC

PDAC is defined as a tumour with a histological resemblance to ductal cell morphology (Esposito, Konukiewitz et al. 2014). The disease typically occurs in the head of the pancreas (see figure 1.1) and invades into local tissue, with the most common primary site of metastasis being the liver (Kamisawa, Isawa et al. 1995). PDACs are highly fibrotic, dense and desmoplastic with significant contribution of inflammatory cells and fibroblasts (Pandol, Edderkaoui et al. 2009). Pancreatic stellate cells (pancreas specific fibroblast-like cells thought to become active following pancreatic injury), have been linked to the development of desmoplasia, as when activated, they secrete Extracellular Matrix (ECM) proteins (Jaster 2004). PDACs are often poorly vascularised, resulting in a hypoxic environment, which likely contributes to the high inflammatory response observed in the disease (Farrow, Evers 2002). Three precursor lesions have been identified which can lead to a fully invasive PDAC: Mucinous cystic neoplasm (MCN), Intraductal Papillary Mucinous neoplasm (IPMN) and Pancreatic Intraepithelial Neoplasias (PanINs). MCNs develop from mucin producing epithelial cells with an ovarian-type stroma, which have specific, focused sites of invasion, and
are 20 times more frequently observed in females than in males (Hruban, Maitra et al. 2007). IPMNs often occur on the main pancreatic ducts and are defined by the tall columnar epithelial cells which produce mucin (Sohn, Yeo et al. 2004). PanINs begin in small pancreatic ducts and may be categorised as either: PanIN-1, PanIN-2 or PanIN-3. PanIN-1 lesions consist of a columnar epithelial architecture with round, basally located nuclei. In PanIN-2 lesions, the nuclei are less organised and may vary in size as well as losing their basal polarity. In PanIN-3 lesions, ductal cells have more disorganised nuclei and exhibit cellular dysplasia, with some budding off of cells from the epithelium (Hruban, Maitra et al. 2008). All of these precursor lesions have the potential to develop into invasive PDACs but PanINs are the most frequently observed and the most well described.

1.3.4 Current therapeutic approaches

Current treatment of pancreatic cancer may involve surgery, chemotherapy, radiotherapy or a combination of these. However, pancreatic cancer is highly resistant to these conventional methods (Zalatnai, Molnár 2007, Wang, Li et al. 2011). Surgery may be an effective treatment if the tumour is diagnosed at an early stage, but due to the fact that pancreatic cancer is more prevalent in persons aged over 60, surgery may cause unacceptable trauma, and is often considered too invasive. The most common chemotherapeutic agent used to treat pancreatic cancer is the antimetabolite, gemcitabine (Burris, Moore et al. 1997). Gemcitabine is a modified deoxycytidine analogue, which is phosphorylated upon entering a cell to produce a defective nucleotide. When incorporated into newly synthesised DNA, it will only allow the addition of a single nucleotide to follow it. This leads to premature DNA strand termination and subsequent apoptosis (Plunkett, Huang et al. 1995). It also inhibits the action of ribonucleoside reductase, reducing the amount of deoxynucleotides within a cell, and increasing its own uptake into newly synthesised DNA (Mini, Nobili et al. 2006). The rapid proliferation of PDAC cells requires them to replicate their DNA frequently, making drugs such as Gemcitabine attractive therapeutic options. While Gemcitabine may be the best chemotherapeutic for treatments of PDAC, a positive clinical response is seen in only 23.8% of patients
This may be due to the hypovascular nature of PDAC, as well as the dense tumour stroma consisting of ECM (Extra Cellular Matrix) components, stellate cells and immune cells, which surround the lesion, inhibiting the uptake of chemotherapeutic agents into the primary tumour.

1.3.5 Genetics

In >90% of PDAC cases, the initiating mutation is to Kirsten Rat Sarcoma Viral Oncogene Homolog (K-RAS) (Morris, Wang et al. 2010, Kanda, Matthaei et al. 2012) (see section 1.3.5.1 for details). Following this, the mutations become more heterogeneous, though have been observed in cyclin-dependent kinase inhibitor 2A (INK4/p16) (Wilentz, Geradts et al. 1998), Mothers Against Decapentaplegic Homolog 4 (SMAD4) (Bardeesy, Cheng et al. 2006, Schneider, Schmid 2003) and the NOTCH signalling pathway (De La O, Emerson et al. 2008). However, the most frequent of these common mutations are to the p53 gene, with the most aggressive, not resulting in loss of p53, but instead resulting in a functional protein with both dominant negative and gain of function characteristics (See section 1.3.5.2 for details).

1.3.5.1 K-RAS

K-RAS is a ubiquitously expressed, small GTPase belonging to the RAS superfamily of small GTPases. Importantly K-RAS is upstream of various signal transduction pathways controlling growth and proliferation (discussed further below). Small GTPases are molecular switches which cycle between an active GTP bound form and an inactive GDP bound form (Bourne, Sanders et al. 1990). As their name suggests, they possess an intrinsic GTPase activity which hydrolyses bound GTP into GDP, inactivating signalling (Bourne, Sanders et al. 1990). Accessory proteins called Guanine nucleotide Exchange Factors (GEFs) facilitate GDP dissociation from the GTPase, which allows uptake of GTP, aiding in the activation of GTPases (Quilliam, Khosravi-Far et al. 1995). Another group of proteins called GTPase Activating Proteins (GAPs) catalyse the intrinsic GTPase activity, increasing turnover of GTP to GDP and turning off small GTPases signalling (Boguski, McCormick 1993).
K-RAS, along with Harvey Rat Sarcoma Viral Oncogene homologue (H-RAS) and Neuroblastoma Rat Sarcoma Viral Oncogene homologue (N-RAS), make up the “classical” RAS family. The RAS genes were first identified in two oncogenic viruses: Kirstin Sarcoma virus (K-RAS) and Harvey sarcoma virus (H-RAS). Shortly after, mammalian homologues of these genes were identified in the genomes of rats (DeFeo, Gonda et al. 1981), mice (Ellis, DeFeo et al. 1982) and humans (Chang, Gonda et al. 1982). Mutations to these genes were soon identified in diseases including bladder cancer and lung cancer (Der, Krontiris et al. 1982) and later in pancreatic cancer (Hirai, Okabe et al. 1985). Later studies have found very high incidence of K-RAS mutations in early stage pancreatic cancer (Kanda, Matthaei et al. 2012, Almoguera, Shibata et al. 1988).

Mutations to K-RAS have been shown to occur in >90% of PDAC cases and represent 90% of the initiating mutations in PDAC (Morris, Wang et al. 2010, Kanda, Matthaei et al. 2012). Mutations to K-RAS frequently occur at “hotspots” such as codons 12, 13 and 61 (Tabin, Bradley et al. 1982, Taparowsky, Suard et al. 1982, Capon, Chen et al. 1983). These mutations were found to impair the intrinsic GTPase activity of RAS proteins, with one study finding that mutation to codon 12 of H-RAS from a Glycine (G) to a Valine (V) reduced GTPase activity by ~4 fold (Gibbs, Sigal et al. 1984). While mutations to RAS are an important step in numerous tumour types, alone they are not sufficient to drive transformation. An interesting study found that mutations to RAS result in cellular senescence, and that this senescence could be prevented by inactivation of p53 or p16 (Serrano, Lin et al. 1997). These data support the theory that multiple mutations are required to causes oncogenic transformation (Nordling 1953). A later study found that as well as loss of p53, functional p53 mutants were also able to overcome K-RAS induced senescence and allow pre-malignant lesions to form invasive PDACs (Morton, Timpson et al. 2010).

RAS proteins are post transcriptionally modified through palmitoylation of their C-terminus, which facilitates their attachment with the internal cell membrane (Sefton, Trowbridge et al. 1982). Subsequent studies showed that this lipid modification and membrane association is vital for RAS function (Srivastava, Lacal et al. 1985). The membrane binding of RAS allows it to function as an upstream component of a
number of signalling pathways. RAF1, a component on the MAPK/ERK signalling cascade was shown to directly interact with both activating mutant and GTP bound RAS, and activate MAPK/ERK signalling (Moodie, Willumsen et al. 1993, Zhang, Settleman et al. 1993). Later, the PI3K signalling pathway was implicated as being a direct target of RAS (Rodriguez-Viciana, Warne et al. 1994). Downstream signalling of RAS to PI3K has been implicated in a number of mechanisms including: cytoskeletal reorganisation (Rodriguez-Viciana, Warne et al. 1997), cell growth and survival through PKB/AKT signalling (Marte 1997), and cell matrix adhesion, also through PKB/AKT signalling (Khwaja 1997). More recently, numerous other RAS effectors have been identified including: RAL (Chien, White 2003), Tiam1 (Malliri, van der Kammen et al. 2002) and PLC (Kelley 2001).

As previously mentioned, one of the primary events required for transformation is hyperactive proliferation and cell growth. In the majority of PDAC cases, this is facilitated through K-RAS hyperactivity (Morris, Wang et al. 2010, Kanda, Matthaei et al. 2012). All tissue and cell lines samples used in this study have an initiator mutation to K-RAS which results in benign PanIN formation. Further mutations to p53 are required for progression of these lesions into invasive PDACs (Morton, Timpson et al. 2010).

1.3.5.2 The p53 family and p53 mutation
The now ubiquitously known p53 protein was first observed in 1979 as being a protein within host cells infected with the oncogenic simian virus 40 (SV40), which co-immunoprecipitated with the SV40 large T antigen (Lane, Crawford 1979). Since its identification, it has been shown to be involved in a number of cellular processes, as well as in a large number of diseases, especially cancer, and has been given the moniker “the guardian of the genome” due to its potent role as a tumour suppressor gene (Lane 1992). The most frequently studied function of p53 is as a transcription factor, where it forms a homotetramer which binds DNA at two palindromic sequences separated by up to 15 base pairs with the sequence RRRCW/WGY (R is a purine, Y is a pyrimidine and W is an A or a T). Successful transcription requires
binding of all four p53 sub-units to the consensus sequence (Funk, Pak et al. 1992, El-Deiry, Kern et al. 1992).

In non-stressed cells, p53 expression is in a constant state of flux where it is being transcribed and then degraded by the proteasome (Honda, Tanaka et al. 1997), Haupt, Maya et al. 1997). The E3 ubiquitin ligase, Mouse Double Minute 2 (MDM2), has been shown to be a critical regulator of p53 expression. Mouse embryos which are deficient for MDM2 suffer lethality at ~E6.5, which can be rescued if p53 is also deleted (Jones, Roe et al. 1995, Montes de Oca Luna, R, Wagner et al. 1995) showing a vital role for MDM2 in regulation of p53 expression. Indeed, MDM2 has been shown to be a direct transcriptional target of p53 (Barak, Juven et al. 1993) suggesting a feedback loop, where p53 is able to regulate its own expression. Under non-stressed conditions, MDM2 mono-ubiquitinates lysine residues in the C-terminus of p53 (Lai, Ferry et al. 2001). Following this, a complex of MDM2 and CREB-binding protein (CBP) is able to extend the ubiquitin chains on p53, leading to its degradation by the proteasome (Grossman, Perez et al. 1998). Upon detection of cellular stress, such as DNA damage, MDM2 is phosphorylated by ATM (Khosravi, Maya et al. 1999, Maya, Balass et al. 2001), which interferes with its ability to bind and ubiquinate p53 (Hay, Meek 2000). Additionally, p53 itself it phosphorylated by ATM (Canman, Lim et al. 1998) as well as CHK1 and CHK2 (Ou, Chung et al. 2005) which further inhibits its ubiquitination and increases its stability. Stable p53 is able to function as a transcription factor for myriad transcriptional targets, with a diverse array of cellular effects, such as inducing senescence through p16\textsuperscript{INK4} (Lin, Barradas et al. 1998); initiating apoptosis, through inducing BCL2 Binding Component 3 (PUMA) (Yu, Wang et al. 2003), BCL2-Associated X Protein (BAX) and PMA-Induced Protein 1 (NOXA) (Shibue, Takeda et al. 2003); initiating DNA repair mechanisms, through induction of Growth Arrest And DNA-Damage-Inducible (GADD45) (Smith, Chen et al. 1994) and initiating cell cycle arrest through induction of Cyclin-Dependent Kinase Inhibitor 1A (p21\textsuperscript{cip1}) (el-Deiry, Tokino et al. 1993). Both p53 and MDM2 have been implicated in inhibition of invasion through regulation of SLUG (Wang, Wang et al. 2009) and SNAIL (Lim, Kim et al. 2010), further highlighting its impressive ability as a tumour suppressor.
More recently, p53 has been shown to have transcriptional control over a number of microRNAs, most notably, the miR-34 family. Details into p53 control over microRNAs will be discussed in detail in section 1.4.9.

P53 is part of a larger family of transcription factors called the p53 family, which consists of p53, p63 and p73. While there is a high degree of sequence homology between family members, a significant degree of functional autonomy has been shown (figure 1.3). It is important to note, that there is also a degree of functional redundancy within the family (Levrero, De Laurenzi et al. 2000). Both p63 and p73 have two isoforms: a full length TA isoform and an N terminally truncated ΔN isoform, which is transcribed from an independent promoter (Yang, Kaghad et al. 1998, Yang, Walker et al. 2000). As a general rule, the TA isoform is a functional transcription factor while the ΔN isoform functions as a dominant negative regulator of the TA isoform (Grob, Novak et al. 2001, Yang, Kaghad et al. 1998). More recent studies have shown that the ΔN isoforms have functions independent of dominant negative regulation of the TA isoforms (Dohn, Zhang et al. 2001, Wu, Nomoto et al. 2003). All full length members of the p53 family have an N-terminal trans-activating domain (ΔN isoforms lack this domain), followed by a proline rich domain, a DNA binding domain and an oligomerisation domain. While p53 ends at the oligomerisation domain, p63 and p73 have a second proline rich domain followed by a Sterile Alpha Motif (SAM).

**Figure 1-3: The domain structure of the major p53 family members.** This comparison is annotated as: Transactivating domain (TA), Proline rich domain (PR), DNA binding domain (DBD), (OD), and Sterile Alpha Motif (SAM) – adapted from Melino, De Laurenz et al. 2002.
Both the TA and ΔN isoforms of p63 and p73 also undergo C-terminal splicing events resulting in proteins with independent functionality, which have the notation α, β and γ (Ghioni, Bolognese et al. 2002).

The major role of p63 has been in epidermal development and differentiation, where the ΔNp63 isoform has been shown to be of critical importance (Lena, Cipollone et al. 2010). Mouse knockout models of p63 show significant developmental defects, including maldeveloped or missing limbs and skin, with post-utero animals tending to only survive for a few days (Mills, Zheng et al. 1999, Yang, Schweitzer et al. 1999). A number of germline p63 mutations have been observed in humans, which tend to result in developmental defects such as: Ectrodactyly–ectodermal dysplasia–cleft syndrome (EEC) (van Bokhoven, Hamel et al. 2001), a condition which results in malformed hands and feet and facial clefts; ankyloblepharon–ectodermal dysplasia–clefting syndrome (AEC) (van Bokhoven, Hamel et al. 2001), a group of conditions where the embryonic ectoderm does not properly develop, resulting in poor development of skin, hair nail and teeth, and Limb Mammary Syndrome (LMS) (van Bokhoven, Jung et al. 1999) which is defined by severe deformities of the hands, feet and mammary glands. In contrast to p53 mutations, p63 mutations do not predispose sufferers to spontaneous tumour development (Keyes, Vogel et al. 2006). The human patient data, along with the observations in mouse models, suggest a potent role for p63 in normal epidermal development.

The p73 protein is predominantly expressed in the developing brain, though interestingly, the ΔNp73 isoform is the principally expressed isoform, which has been shown to be required to inhibit the p53 apoptotic response in the developing mouse brain (Pozniak, Radinovic et al. 2000). This suggests that the ΔNp73 isoform may have a dominant negative function on wild type p53 function, a hypothesis supported by later studies (Zaika, Slade et al. 2002). Mice deficient for p73 also suffer developmental disorders though they are more related to neural development, with mice suffering social and sexual behavioural abnormalities (Zaika, Slade et al. 2002). Like p63 deficient mice, p73 deficient mice are not predisposed to spontaneous tumour development (Yang, Walker et al. 2000).
It is interesting that while p53 has been shown to be a potent tumour suppressor, its family proteins, p63 and p73, which share significant sequence and structural homology, have no tumour suppressor activity. While loss of p63 or p73 alone does not result in spontaneous tumour formation, mice which are hemizygous for both p63 and p73 do form spontaneous tumours (Flores, Sengupta et al. 2005). This may suggest that both p63 and p73 are tumour suppressor genes, but have sufficient functional redundancy to compensate for one another.

A number of previous studies have implicated loss of p63 in promotion of cancer invasion and metastasis, through loss of microRNA expression (Muller, Trinidad et al. 2013, Tucci, Agostini et al. 2012, Su, Chakravarti et al. 2010). The role of p63 in development may account for some of the observed functions in metastasis, as during development, cells are frequently undergoing EMT and MET, mechanisms which are also implicated in tumour development. Additionally, p63 has been implicated as being important in stemness (Senoo, Pinto et al. 2007), another characteristic of metastasis. It may be that p63 is able to revert cells to a stem like state to induce metastasis.

Mutations to p53 are observed in >50% of all sporadic human cancers (Soussi, Ishioka et al. 2006). While it may be assumed that mutations to this tumour suppressor gene may result in loss of function, the majority of mutations (>80%) result in a functional protein with both dominant negative and gain of function characteristics (Dittmer, Pati et al. 1993). Many functional p53 mutants have mutations to specific “hot spots” in the DNA binding domain, which may either be to residues which directly bind DNA, such as the p53R273H mutation, or may affect the structure of the DNA binding domain, such as the p53R175H mutation (Sigal, Rotter 2000). Both types of mutation negatively impact the DNA binding ability of the protein (Cho, Gorina et al. 1994). This causes the dominant negative characteristics associated with these mutants, as if a single mutant p53 protein is incorporated into the tetramer, the whole complex will lose its transcriptional activity (Willis, Jung et al. 2004). Importantly, due to its dominant negative function, p53 mutants do not transactivate MDM2 to reduce p53 levels, which leads to significant accumulation of mutant p53 in cells (Oren, Rotter 2010).
While some of the biological effects of mutant p53 can be explained by loss of wild type p53 function, numerous studies have observed phenotypic changes which cannot be explained by p53 loss alone. Many of these gain of function mutations result in the development of tumours which are not associated with loss of p53 function (Olive, Tuveson et al. 2004). Additionally, increased speed of development from premalignant lesions to invasive carcinoma (Heinlein, Krepulat et al. 2008), and a decrease in chromosomal stability through inhibition of ATM (Liu, Song et al. 2010) has also been observed. One very important publication showed that mutant p53 was able to overcome K-RAS induced senescence and induce the rapid onset of PDAC from PanIN lesions. The study also showed that while there was no significant change in survival (128 days for the p53 hemizygous and 134 days for the mutant p53 mice), there was a significant increase in the number of mice which exhibited metastasis (0/20 in the p53 hemizygous and 13/20 in the mutant p53 mice) (Morton, Timpson et al. 2010). Other studies have also shown that mutant p53 induces metastasis but does not alter survival (Lang, Iwakuma et al. 2004) suggesting an important aspect mutant p53 expressing tumours is not an increased ability to promote invasion or proliferation, but instead in the ability to specifically promote metastasis. These phenotypic changes suggest that mutant p53 overtly promotes tumorigenesis, rather than simply perturbing wild type p53 function.

A number of hypotheses have been suggested to explain the gain of function associated with mutant p53. Some studies have shown that mutant p53 is able to interfere with p63 and p73 activity (Strano, Fontemaggi et al. 2002, Gaiddon, Lokshin...
et al. 2001, Martynova, Pozzi et al. 2012, Stindt, Muller et al. 2014, Melino 2011). It has been shown that p63 is able to regulate Dicer and thus processing of microRNAs which suppress metastasis (Su, Chakravarti et al. 2010). A later study went on to show that mutant p53 was able to inhibit p63 transcriptional activity, which results in loss of Dicer expression. This study went on to show that while mutant p53 is indeed able to inhibit Dicer through suppression of p63, there was also a p63 independent mechanism which inhibited dicer (Muller, Trinidad et al. 2013). Interestingly, loss of Dicer has been shown to induce metastasis in a number of types of cancer, such as prostate (Zhang, Chen et al. 2013) and breast cancer (Martello, Rosato et al. 2010). Two previous studies showed that mutant p53 is able to promote integrin recycling (Muller, Caswell et al. 2009) and MET receptor trafficking (Muller, Trinidad et al. 2013) through inhibition of p63 to promote invasion and cell scattering.

The numerous and diverse data surrounding mutant p53 suggest that it may induce metastasis through a number of different mechanisms. With so many studies implicating p63 and Dicer, it would suggest that the microRNA pathway is of great importance for mutant p53-dependent tumour development, especially in the onset of metastasis. The interactions between microRNAs and mutant p53 will be discussed in detail in 1.4.10.

The mutation this study has focused on is the p53<sup>R175H</sup> mutation, though in mice this corresponds to the p53<sup>R172H</sup> mutation. As mutant p53 has previously been shown to impact processing of microRNAs, this study has focussed on profiling which microRNAs are dysregulated in PDAC due to expression of mutant p53<sup>R172H</sup>. In order to understand the consequences of microRNA dysregulation, it is first important to understand what they are and how they function.

### 1.4 MicroRNAs

MicroRNAs are small ~22nt RNA molecules which are able to regulate gene expression (please see section 1.7 for details on translation). They function within a protein complex known as the RNA Induced Silencing Complex (RISC), where they act as targeting molecules through Watson-Crick base pairing with the 3’ Untranslated
Regions (UTRs) of mRNAs, which results in the downregulation of the expression of the target gene by both translation inhibition and mRNA destabilisation (Bartel 2004). Some estimates have suggested that microRNAs may regulate ~30% of all protein coding genes in *Homo sapiens (H. sapiens)* (Berezikov, Guryev et al. 2005, Lewis, Burge et al. 2005). They have been implicated in a wide variety of biological processes such as DNA damage response, proliferation and apoptosis, as well as having more nefarious effects in diseases such as cancer. This section will provide an overview of what microRNAs are and how they function.

1.4.1 MicroRNA nomenclature
The naming conventions used to designate microRNAs (miRs) may not initially seem obvious, but in fact describe the microRNA in detail. The three letter prefix is used to denote the species the microRNA is found in. For example the prefix *hsa* shows that the microRNA is of human origin, while *mmu* identifies it to be of mouse origin. As microRNAs are discovered, they are given a numerical value which represents the hairpin or pre-microRNA which they are associated with. For example miR-21 was discovered before miR-22. A letter following the numerical microRNA identification number is used to differentiate mature microRNAs with highly similar sequences, but which are expressed from independent genomic locations. These are considered to form members of microRNA families, for example, miR-22a and miR-22b represent independent pre-microRNAs which form the miR-22 family. If more than one identical mature microRNA exists within a given genome, they are differentiated by numbers following the microRNA identification number and letter, in the order in which they were discovered. For example miR-22a-1 was identified before miR-22a-2. While both the mature microRNAs are identical, they are found at independent genomic loci (Ambros 2003). This value is often not present, as identical mature microRNAs are not frequently observed in the genome. Following this value, a 3p or 5p is used to represent which arm of the pre-microRNA hairpin the mature sequence is derived from. Initially, “s” (sense) was used to represent the “target strand”, the mature arm which is preferentially incorporated into the RISC, while “as” (antisense) was used to designate the “passenger strand”, which is the mature arm least frequently associated
with the RISC. Following this, “as” was replaced with an *, to denote the passenger strand. As tissue and cell specificity was found to affect the choice of which mature microRNA was incorporated into the RISC, the 3p and 5p annotation has been established as the preferred nomenclature (Griffiths-Jones, Hui et al. 2011). An example may be seen in figure 1.5.

![Figure 1-5: An example of current microRNA naming conventions.](image)

Some microRNAs do not follow this naming convention, such as the let-7 family and lin-4. The let-7 family was discovered early in microRNA research, where it was shown that loss of let-7 caused death in *Caenorhabditis elegans* (*C. elegans*) (Reinhart, Slack et al. 2000), thus the naming lethal 7 (let-7). Lin-4 was the first microRNA discovered in *C. elegans*, which has been shown to regulate development of the nematode (Lee, Feinbaum et al. 1993). Following discovery of this microRNA in higher organisms, it was re-named miR-125, with lin-4 now only representing the *C. elegans* microRNA. However, the vast majority of microRNAs follow the naming conventions discussed above.

1.4.2 History

In 1993, two laboratories identified a small RNA molecule in *C. elegans* called lin-4, which shared complementarity with the 3’ UTR of the mRNA of the protein coding gene lin-14 (Lee, Feinbaum et al. 1993, Wightman, Ha et al. 1993). Both laboratories
showed that lin-4 was able to attenuate the expression of the lin-14 protein, which was shown to be critical for *C. elegans* development. Soon after this discovery, another small RNA molecule, let-7, was found to function via the same mechanism as Lin-4 in *C. elegans* (Reinhart, Slack et al. 2000). Let-7 was soon found to be conserved between large numbers of species (Pasquinelli, Reinhart et al. 2000), which sparked intense interest in this mechanism of gene expression control. Currently there are 35,828 mature microRNAs annotated, in 223 species, with 2588 mature sequences (Kozomara, Griffiths-Jones 2013).

### 1.4.3 MicroRNA biogenesis
MicroRNAs are first transcribed into primary transcripts (pri-miRs), which can range in size from a few hundred, to tens of thousands of base pairs and contain hairpin structures at the locus of the mature microRNAs. Transcription of microRNAs is predominantly RNA polymerase 2 (RNApolII) dependent (Lee, Kim et al. 2004), with a minority being dependent on RNApolIII (Borchert, Lanier et al. 2006), and results in a transcript with a 5' 7-methylguanosine cap and a 3' poly(A) tail (Lee, Kim et al. 2004) (see section 1.6 for details on transcription).

Following transcription, pri-miRs undergo processing by the RNase III enzyme, Drosha, which processes the pri-miR into a ~65nt, double-stranded hairpin structure, with a 2nt 3' overhang, known as precursor microRNAs (pre-miR) (Lee, Ahn et al. 2003), as shown in figure 1.6. Drosha was first discovered as a component of a large complex which fractionated at ~650KDa, along with another protein called DiGeorge Syndrome Critical Region 8 (DGCR8). This complex was shown to be able to process pri-miRs (Han, Lee et al. 2004) into smaller, ~65nt hairpins. The complex is now known as the microprocessor and has since been shown to contain a number of other proteins (Shiohama, Sasaki et al. 2007, Wen, Tannukit et al. 2008).

Following Drosha processing, pre-miRs are translocated to the cytoplasm for further processing, as shown in figure 1.6. A 2003 study showed that depletion of Exportin 5 (XPOS) inhibited the function of ectopically expressed microRNA precursors which required Drosha processing, while having no effect on microRNA precursors which did
not. (Yi 2003). This suggested that XPO5 is not a microRNA effector molecule, but instead is important in their biogenesis downstream of Drosha. A later study found that the nuclear export of microRNAs could be inhibited through nuclear injection of RAN-GAPs, suggesting that XPO5 functions in a RAN-GTP dependent manner (Bohnsack 2004). These studies identified an important role of XPO5 in microRNA biogenesis.

Following export to the cytoplasm by XPO5, pre-miRs are further processed by another RNase III enzyme, Dicer (DCR), to form a short RNA duplex with a 2nt 3’ overhang (Ketting 2001, Hutvagner 2001, Knight 2001), as shown in figure 1.6. Dicer has a preference for double-stranded RNA molecules which have a 2nt 3’ overhang, such as those processed by Drosha (Zhang, Kolb et al. 2004). Dicer is thought to measure mature microRNAs from the 2nt 3’ overhang produced by Drosha, to ensure the resulting mature microRNA is ~22nt in length (MacRae 2006). Mammalian Dicer is also able to detect the 5’ phosphorylated end of the pre-miR and measure 22nt in a process known as the 5’ counting rule (Park, Heo et al. 2011). These processes together ensure accurate processing of the pre-miR into the short RNA duplex.

Following Dicer processing, the short RNA duplex is loaded into an AGO protein which incorporates it into the RISC, as shown in figure 1.6. Here, one of the strands must be removed in order to allow substrate mRNA binding. In some cases, AGO2 itself has been shown to cleave one of the strands, which is then removed by Component 3 of Promoter Of RISC (C3PO) (Rand, Petersen et al. 2005, Liu, Ye et al. 2009). Other studies have shown that Dicer, Transactivating Response RNA-binding Protein (TRBP) or PKR activating protein (PACT) are required for passenger strand removal (Noland, Doudna 2013). It may be that numerous mechanisms dictate how the AGO loaded duplex is processed and that different microRNAs, and different AGOs, may require distinct mechanisms.

The selection of which strand of the duplex is employed as the target strand, and which is to be removed as the passenger strand, is not fully understood, though some fly studies have suggested that a U at the 5’ of a mature microRNA promotes RISC incorporation (Seitz, Tushir et al. 2011) and that the thermodynamic stability of the 5’
of the RNA molecule may also be significant in strand selection (Schwarz, Hutvágner et al. 2003). Interestingly, cell type specificity of strand selection has been observed, suggesting these processes cannot be the only important mechanisms (Biasiolo, Sales et al. 2011, He, Liu et al. 2012).

AGO proteins were first identified in *Saccharomyces cerevisiae* (*S. cerevisiae*) though their role in RNA interference was not recognised (Ahmad, Nasrin et al. 1985). Later they were found in *Arabidopsis thaliana*, where they were shown to regulate development (Bohmert 1998). Since then, AGO proteins have been shown to be highly conserved and have been found in an array of organisms from *Schizosaccharomyces pombe* (*S. pombe*) (Sigova 2004) to *H. sapiens* (Schürmann, Trabuco et al. 2013). The mammalian genome contains 4 AGO proteins, while *S. pombe* has only one. Interestingly, the single AGO protein in *S. pombe* has been shown to be involved in both transcriptional and post-transcriptional gene silencing (Sigova, Rhind et al. 2004), while the majority of studies investigating mammalian AGO have focused on the post-transcriptional silencing abilities of the genes. However, some data support the hypothesis that AGO1 and AGO2 may also be involved in transcriptional regulation (Janowski, Huffman et al. 2006).

MicroRNAs have been found to be associated with all four AGO proteins (Burroughs, Ando et al. 2011), however AGO2 is the only mammalian AGO protein with an intrinsic RNase activity, known as “slicer” activity, and as such is the only AGO protein able to cleave mRNAs (Yekta, Shih et al. 2004). For this reason, it is believed that AGO2 is the only effector of the siRNA pathway in which small RNAs with perfect complementarity to a target mRNA, lead to cleavage and subsequent degradation of the transcripts. A previous study found that reintroduction of any of the AGO proteins into AGO deficient cells was able to rescue microRNA-mediated repression (Su, Trombly et al. 2009), suggesting some functional redundancy between AGO proteins. Another study found that mature microRNAs bound to AGO1 are frequently found to be the “passenger strand” of a hairpin, while those bound to AGO2 tend to be the “target strand” of the hairpin (Turchinovich, Burwinkel 2012), suggesting that AGO2 may be the vital component in microRNA-mediated repression. Additionally, “slicer” activity has been shown to be important in removal of the “passenger strand”. Finally, AGO2
is the only AGO protein known to interact with Glycine-Tryptophan Protein Of 182 KDa (GW182/TNRC6) proteins, critical components of the microRNA-mediated repression machinery, believed to be the major effector of microRNA-mediated repression (Takimoto, Wakiyama et al. 2009). It may be that numerous mechanisms dictate how the AGO-loaded duplex is processed and that different microRNAs, and different AGO proteins, may require distinct mechanisms.

**Figure 1-6: A schematic outlining microRNA processing.** This schematic shows the cell localisation as well as the major proteins involved in microRNA biogenesis.

MicroRNAs may be found at numerous genomic locations with some being intergenic (Kim 2005) and others intragenic (Ambros, Lee et al. 2003). Intragenic microRNAs may be transcribed from the same promoter as their host gene (BASKERVILLE 2005), or from independent promoters (Ramalingam, Palanichamy et al. 2013). The most
frequent location of intragenic microRNAs is introns, though some have been found in the 5’ and 3’ UTRs and even the coding region of other genes (Godnic, Zorc et al. 2013). Intron microRNAs with independent promoters are able to undergo processing in the usual manner (discussed above), but intronic microRNAs which are transcribed as part of a functional mRNA must be processed slightly differently. One early study found that Drosha processing of these microRNAs occurs prior to splicing and does not affect the ability of the spliceosome to process the mRNA (Kim, Kim 2007). Another study found that the microprocessor complex is recruited to RNAPol-II-dependent host genes while they are being transcribed, also suggesting that Drosha cleavage may occur during transcription (Morlando, Ballarino et al. 2008). Other studies have observed that DGCR8 co-immunoprecipitated with components of the spliceosome (Shiohama, Sasaki et al. 2007, Wen, Tannukit et al. 2008) suggesting some functional interplay between the 2 processing units. These data strongly suggest that resection of the microRNA occurs during the early stages of transcriptional elongation (see section 1.6 for details on transcription).

Another type of intragenic microRNA has been identified known as the “mirtron”. These microRNAs are found in very small introns of a host gene, and have a distinct mechanism for their early biogenesis. These microRNAs bypass Drosha cleavage, as splicing (see section 1.6 for details) itself is able to process them into hairpins, which are recognised by Dicer. This was first observed in invertebrates (Okamura, Hagen et al. 2007, Ruby, Jan et al. 2007), though computational analysis suggests such mirtrons may be conserved in the mammalian genome (Berezikov, Chung et al. 2007).

There have also been reports of microRNAs which do not require Dicer processing such as miR-451. Following normal transcription and processing by Drosha, miR-451 has only an 18bp duplex in its precursor hairpin, which is too short for Dicer binding. Instead, this microRNA is passed directly to AGO2 which cleaves the 3’ arm of the hairpin before an unknown nuclease trims the 3’ arm until a mature AGO2-associated microRNA of 23nt remains (Cifuentes, Xue et al. 2010, Cheloufi, Dos Santos et al. 2010, Yang, Maurin et al. 2010).
MicroRNA processing is a complex and multi-step procedure which results in a single stranded AGO2 bound microRNA, which is able to be used as a targeting molecule by the RISC complex.

1.4.4 Repression mechanism
As has already been mentioned, microRNAs incorporated into the RISC complex are able to regulate gene expression (Bartel 2004). MicroRNAs do not have any biochemical activity themselves, but function as simple targeting molecules for the RISC. Indeed, tethering of AGO2 alone to the 3’UTR of a reporter mRNA is able to cause translational repression (Pillai 2004). While the majority of documented microRNA response elements (MREs) are in the 3’ UTR of the target mRNA, a number of studies have found functional microRNA binding to the coding sequence (CDS) (Hausser, Syed et al. 2013, Fang, Rajewsky et al. 2011) and the 5’ UTR (Lee, Ajay et al. 2009). Even more unexpectedly, another study found that miR-10a is able to bind to the 5’ UTR of Terminal Oligopyrimidine (TOP) mRNAs and stimulate translation (Ørom, Nielsen et al. 2008).

In plants, microRNAs bind with almost complete complementarity to their target mRNA, which results in cleavage of the transcript (Hamilton, Baulcombe 1999). In mammalian microRNA-mediated repression, a degree of incomplete complementarity is tolerated and mRNA cleavage is not required (Bartel 2004); however, there are specific regions of the microRNA which do require sustained base pairing. The first 2-8 nucleotides are known as the seed region, and require a high degree of complementarity to its target, with the sequence in the 3’ of the microRNA being of less importance, though still having an influence on repression (Lewis, Burge et al. 2005). Given that only seven complementary nucleotides are required for microRNA:mRNA interactions, a large number of interacting mRNAs are predicted for each microRNA. The number of true targets of any microRNA is likely to be far fewer than the number predicted, making identification of bona-fide microRNA targets a challenge. Numerous prediction algorithms exist, though none have demonstrated high reliability.
A number of potential mechanisms have been postulated to explain microRNA-mediated repression, though it is still a topic of some dispute. MicroRNA function is thought to involve both destabilisation of the mRNA transcript as well as direct inhibition of translation. TNRC6 proteins are critical components of the microRNA-mediated repression machinery, which have been shown to interact with AGO2. (Takimoto, Wakiyama et al. 2009). They are also components of P-bodies, which are granular structures within cells where translationally silenced mRNAs are thought to be stored or degraded (Eystathioy 2003). As well as interacting with AGO2, GW182 proteins have been shown to recruit the deadenalase complex CCR4-NOT to microRNA bound targets to initiate deadenylation of mRNAs which leads to their destabilisation (Chekulaeva, Mathys et al. 2011). A study in D. melanogaster found that depletion of the de-capping proteins DCP1/2 leads to inhibition of microRNA-mediated repression and an enrichment of deadeylated mRNAs (Behm-Ansmant 2006). The prerequisite for decapping by DCP1/2 for microRNA-mediated repression to occur has not yet been shown in mammalian cell, but suggests the possibility that this is an important process in destabilisation downstream of deadenylation. While mRNA degradation is an important component of microRNA function, a number of studies have shown that it is not required for microRNA-mediated translational repression to occur (Fabian, Mathonnet et al. 2009, Djuranovic, Nahvi et al. 2012, Bazzini, Lee et al. 2012), suggesting that it may be a secondary step of microRNA function, following inhibition of translation.

As destabilisation of mRNA targets is not necessary for repression, there must be distinct processes which govern destabilisation and translational repression. A number of conflicting studies have been published which try to identify the point at which microRNA-mediated translational repression occurs. Some studies have suggested that microRNAs function post initiation by inducing ribosomal drop-off (Petersen, Bordeleau et al. 2006), or by impairing elongation (Nottrott, Simard et al. 2006). However, the majority of recent studies seem to point towards microRNAs functioning at the initiation step of protein synthesis. A previous study employed polysome fractionation, and showed that microRNA repressed targets shift to the lightest fractions of the gradient, suggesting that they have no translating ribosomes
attached (Pillai 2005). Another study found that Cationic Amino acid Transporter-1 CAT1, an mRNA usually repressed by miR-122, is shifted into the polysomes when stress causes inhibition of miR-122 function (Bhattacharyya, Habermacher et al. 2006). This suggests that repressed mRNAs do not have attached ribosomes, but upon rescue from repression, ribosomes are loaded onto the mRNA, again suggesting that the mRNAs are not being degraded, but rather repressed and stored for further use.

The use of Internal Ribosome Entry sites (IRES) has been widely implemented to investigate microRNA-mediated repression at the stage of initiation. IRES elements are RNA sequences which allow cap-independent ribosomal recruitment to an mRNA. They have been identified in a number of viruses such as the polio virus (Brown, Ehrenfield 1979) and the picornaviruses (Jang, Pestova et al. 1990). IRES elements are useful for molecular studies as they require different sets of initiation factors to initiate translation. An previous study implementing two IRES elements, The Cricket Paralysis Virus (CrPV) IRES, which requires no initiation factors for ribosome entry, and the encephalomyocarditis virus (EMCV) IRES, which requires all except the cap binding protein, eIF4E, to initiate translation (Pestova, Shatsky et al. 1996). The observation that both CrPV and ECMV IRES elements were both refractory to microRNA-mediated repression, suggest that initiation is the critical step in microRNA-mediated repression, and that eIF4E is not involved (Humphreys, Westman et al. 2005). A later study following these observations, which I was involved in, took this further by utilising the Hepatitis C Virus (HCV) IRES, which requires an initiator methionine, eIF2, eIF3, eIF5 and eIF5B in order to initiate translation (Lukavsky 2009), as well as using the ECMV and CrPV IRES elements. We found that both the HCV and ECMV IRES elements overcome microRNA-mediated translational repression (Meijer, Kong et al. 2013). These data suggest that microRNA-mediated repression occurs at a very early stage of translational initiation and involve members of the eIF4F complex (see 1.7 for details on translation). To follow this up, we used siRNA screens to deplete members of the eIF4F complex and assessed any changes in microRNA-mediated repression. A major finding of this study was that depletion of eIF4A2 was able to attenuate microRNA-mediated repression, while depletion of eIF4A1, eIF4G1, eIF4G2, eIF4B and eIF4H had no effect on microRNA-mediated repression (Meijer, Kong et al. 2013). As mentioned
in section 1.7, eIF4A proteins are components of the eIF4F complex, which function as helicases during translational initiation. This fascinating result highlighted two critical findings: that eIF4A1 and eIF4A2 have independent functions, and that microRNA-mediated repression occurs during translation initiation.

1.4.5 MicroRNA turnover
The turnover of microRNAs is not currently very well understood, though it has been observed that specific mechanisms are able to promote degradation or stabilisation of microRNAs. For example, miR-503, a member of the miR-16 family, is rapidly upregulated by cells entering into G0 phase of the cell cycle, but as cells transit into G1 phase, its expression is rapidly lost. This is believed to occur due to an inherent instability of this microRNA, possibly due to the nucleotides in its seed sequence. Due to this, cells must rapidly transcribe miR-503 to maintain high expression, but as cells transit into G1, transcription ceases, and the instability of the microRNA leads to rapid loss in its expression (Rissland, Hong et al. 2011). As well as the innate instability of some microRNAs affecting their turnover, some 5′-3′ and 3′-5′ ribonucleases have been shown to target microRNAs. 5′-3′ Exoribonuclease 1 (XRN1) and Ribosomal RNA processing Protein 41 (RRP41) have both been implicated as directly degrading miR-382 (Bail, Swerdel et al. 2010) and polyribonucleotide nucleotidyltransferase 1 (PNPT1) has been implicated in degrading miR-221 and miR-222 (Andrade, Arraiano 2008). Some viruses have also been shown to code for short RNA sequences which destabilise microRNAs (Cazalla, Yario et al. 2010, Libri, Helwak et al. 2011). Another interesting finding was that microRNAs are able to destabilise other microRNAs. It has been shown that miR-107 can form a duplex with let-7, leading to let-7 destabilisation and increased expression of let-7 targets (Lai, Wiel et al. 2004). Another fascinating study found that, in the retina, the stability of some microRNAs is regulated by the levels of light, with rapid degradation of microRNA occurring in darkness, and rapid transcription occurring upon light stimulation (Krol, Busskamp et al. 2010).

These findings suggest that a complex interplay between microRNAs and other RNA species and proteins, may add a further level of complexity to microRNA function.
While microRNAs are predominantly highly stable molecules, presumably due to their association with AGO2, some are less stable than others, and some have specialised mechanisms controlling their turnover.

1.4.6 MicroRNAs in cancer
With the large number of genes being predicted as targets of microRNAs, it is of little surprise that their dysregulation has deleterious effects. Dysregulation of microRNAs has been implicated in the vast majority of cancers with both over- and under-expression having been observed. Some microRNAs have been found to be overexpressed in a large number of tumour types and are considered to be oncogenic, while others are frequently downregulated and are considered to be tumour suppressors. While this may be broadly true, the repertoire of mRNA targets of a microRNA within a specific environment is what causes the microRNA to have oncogenic, or tumour suppressive abilities. For example, miR-31 is upregulated in cervical cancer, which promotes invasion and metastasis through modulation of ARID1A (Wang, Zhou et al. 2014). In this case, miR-31 is considered oncogenic, while in prostate cancer, hypermethylation leads to downregulation of miR-31, which leads to hyperactive androgen receptor signalling and promotion of tumorigenesis (Lin, Chiu et al. 2012). This pleiotropic nature of microRNAs makes it very difficult to place them into specific categories, and will be an important consideration when designing therapeutic approaches involving modulating their expression. That said, some microRNAs are consistently found to be up- or downregulated in multiple tumour types, so may still be considered broadly oncogenic or tumour suppressive.

MicroRNA genes are frequently found to be in cancer associated regions of the genome (Calin, Sevignani et al. 2004) adding weight to their importance in tumorigenesis. Additionally, global reduction in microRNA expression through siRNA targeting microRNA processing proteins: Drosha, DGCR8 and Dicer, leads to transformation and tumorigenesis (Kumar, Lu et al. 2007). The same study also showed that inhibition of Dicer with concomitant K-RAS mutation leads to increased
tumour progression in lung cancer. Another study showed that loss of Dicer leads to EMT (see section 1.2.2 for details on EMT) in pancreatic acinar cells, and that Dicer loss with concomitant K-RAS mutation, leads to accelerated PanIN development, as well as acinar-to-ductal metaplasia (ADM) (Wang, McAllister et al. 2014). Mutant p53 is well known for its ability to induce invasion and metastasis in tumours (see section 1.3.5.2 for details on mutant p53), including pancreatic cancer and has been shown to promote an invasive phenotype through inhibition of Dicer by inhibiting p63 transcriptional activity (Muller, Trinidad et al. 2013). Additionally, miR-103 and miR-107 have been found to directly target Dicer, leading to a loss of microRNA processing and increased invasion and metastasis in breast cancer when they are upregulated (Martello, Rosato et al. 2010). While most studies see an increase in tumour progression following Dicer loss, the opposite is observed in prostate cancer, where a reduction in tumour burden is observed with loss of Dicer. Interestingly, an increase in the invasive and migratory potential of prostate cancer cells is still observed with Dicer loss (Zhang, Chen et al. 2013).

Many of the studies where loss of Dicer resulted in increased tumour progression are tumour types where K-RAS mutations are frequently observed, such as pancreatic (Eser, Schnieke et al. 2014, di Magliano, Logsdon 2013) and lung cancer (Huncharek 1999, Ahrendt, Decker et al. 2001), or where K-RAS mutations are rare, but hyperactivity of K-RAS signalling pathways are frequent, such as breast cancer (McLaughlin, Olsen et al. 2013, Eckert, Repasky et al. 2004). Conversely, prostate cancer has a very low preponderance of K-RAS mutations (Moul, Friedrichs et al. 1992) and no increase in tumour growth is observed following Dicer loss. This may suggest a prerequisite for mutant K-RAS expression in the promotion of tumour growth as a result of loss of Dicer. While the effect of Dicer loss on tumour growth may not be entirely clear, as even in prostate cancer, Dicer loss is associated with increased invasion and metastasis, its activity in maintaining epithelial architecture and inhibiting invasion and metastasis appear to be consistent in different tissue and cell types, suggesting important roles for microRNAs in these mechanisms.
1.4.7 Oncogenic microRNAs
As previously mentioned, some microRNAs, such as miR-21, are consistently found to be overexpressed in an array of tumour types, which frequently correlates with poor prognosis. This microRNA is found to be upregulated in numerous types of cancer including: breast (Yan, Huang et al. 2008), ovarian (Iorio, Visone et al. 2007), cervical (Lui, Pourmand et al. 2007), lung (Gao, Shen et al. 2010), colorectal (Asangani, Rasheed et al. 2007) and pancreatic (Dillhoff, Liu et al. 2008, Hwang, Voortman et al. 2010). Of particular note with regards to the work presented here, hyperactivity of RAS signalling has been shown to induce miR-21 expression (Hatley, Patrick et al. 2010). In line with this, miR-21 upregulation has been found to be an early event in PDAC progression (du Rieu, Torrisani et al. 2010, Ryu, Matthaei et al. 2011).

The poor prognosis attributed to high miR-21 expression is likely due to its regulation of a number of tumour suppressor genes such as Phosphatase and tensin homolog (PTEN) (Meng, Henson et al. 2007, Xu, Zhang et al. 2015), retinoblastoma protein (RB) via Programmed Cell Death 4 (PDCD4) (Shen, Mo et al. 2014) and FBXO11 (Yang, Pfeffer et al. 2015). While miR-21 is not the only microRNA considered to be an oncogene, its role in cancer and tumorigenesis, serves as a good example of how increased expression of a microRNA can have detrimental effects on normal cellular function.

1.4.8 Tumour suppressor microRNAs
Members of the miR-200 family are an example of microRNAs frequently found to be downregulated in tumours. This family consists of five mature microRNAs derived from two pri-microRNAs. The first bears: miR-200c and miR-141, and the second: miR-200a, miR-200b and miR-429. The most well described mechanism by which downregulation of the miR-200 family promotes tumorigenesis is through upregulation of their target mRNAs’ encoding, the ZEB1 & ZEB2 proteins (Korpal, Lee et al. 2008, Asakura, Yamaguchi et al. 2015, Gregory, Bert et al. 2008). These proteins are both transcriptional repressors of the adherens junction component, Epithelial-Cadherin 1 (E-cadherin). Downregulation of E-cadherin is associated with tumorigenesis through induction of the EMT programme (see section 1.2.2 for details...
on EMT). Other targets of the miR-200 family which have been shown to promote EMT are ubiquitin specific peptidase 25 (USP25) (Li, Tan et al. 2014) and SLUG, another E-cadherin transcriptional repressor (Liu, Yin et al. 2012), suggesting that this family is inherently connected to maintaining epithelial integrity. The miR-200 family has been found to be downregulated in a number of tumour types including: breast (Neves, Scheel et al. 2010), colorectal (Chen, Liang et al. 2012), gastric (Kurashige, Kamohara et al. 2012) and pancreatic cancer (Lu, Lu et al. 2014, Li, VandenBoom et al. 2009). The miR-200 family is a good example of how the pleiotropic nature of microRNAs is important in cancer progression, as this family alone is able to affect a number of pathways, all involved in maintaining epithelial architecture.

1.4.9 MicroRNAs and p53
Given that p53 is a potent transcription factor, it is unsurprising that some of its transcriptional targets are microRNAs (see section 1.3.5.2 for details on p53). Indeed, p53 has been shown to transcriptionally regulate a number of microRNAs, such as: miR-145, another c-MYC targeting microRNA (Sachdeva, Zhu et al. 2009); miR-192 and miR-215, which are both able to induce cell cycle arrest (Georges, Biery et al. 2008) and miR-107, and inhibitor of angiogenesis and Hypoxia Inducible Factor-1 (HIF1) signalling (Yamakuchi, Lotterman et al. 2010). The most well described p53-dependent microRNAs are the miR-34 family. This family consists of two smaller sub-families: the miR-34 sub-family and the miR-449 subfamily. The miR-34 sub-family consists of miR-34a, miR-34b and miR-34c, with miR-34a being transcribed separately and miR-34b and miR-34c being transcribed on the same primary transcript. The miR-449 sub-family consists of miR-449a, miR-449b and miR-449c. While the miR-34 and miR-449 families share the same seed sequence, they have no sequence homology outside this region, as shown in figure 1.7.
The miR-34 family have been linked to p53 expression in a number of studies and have been shown to be a vital component of p53 mediated DNA damage response (Tarasov, Jung et al. 2007, Tazawa, Tsuchiya et al. 2007, Raver-Shapira, Marciano et al. 2007, He, He et al. 2007, Bommer, Gerin et al. 2007, Chang, Wentzel et al. 2007). The miR-34 family are believed to function largely through their regulation of a number of pro-proliferative genes including: c-MYC (Cannell, Kong et al. 2010), Cyclin Dependent Kinase 6 (CDK6) (Sun, Fu et al. 2008), Platelet Derived Growth Factor Receptor-β (PDGFR-β) (Chen, Li et al. 2014) and MET (Li, Fu et al. 2009). Given their ability to regulate cell cycle progression, it is unsurprising that the miR-34 family has been implicated in a number of types of cancer including colorectal (Rokavec, Öner et al. 2014), lung (Garofalo, Jeon et al. 2013), bladder (Sun, Tian et al. 2015), ovarian (Li, Shi et al. 2015) and pancreatic (Nalls, Tang et al. 2011, Ji, Hao et al. 2009). In addition to regulation of cell cycle following DNA damage, miR-34a has been shown to upregulate p53 expression, through targeting Siruitin 1 (SIRT1), a negative regulator of p53. Upon p53 stabilisation following stress, miR-34a is induced, which leads to inhibition of SIRT1 and subsequent increased expression of p53 (Yamakuchi, Ferlito et al. 2008).

As well as affecting microRNA expression through direct transcriptional activity, p53 has also been shown to promote the processing of a subset of primary microRNAs including: miR-16, miR-145 and miR-143, through direct interactions with Drosha and p68 following DNA damage (Suzuki, Yamagata et al. 2009). P53 is also a direct target
of miR-125b (Le, Teh et al. 2009), a microRNA known for its oncogenic effect when both over- (Klusmann, Li et al. 2010) and under-expressed (Feliciano, Castellvi et al. 2013). Another interesting observation is that loss of microRNA processing is able to induce a p53-dependent senescence programme (Mudhasani, Zhu et al. 2008), suggesting that consistent microRNA expression is required for normal cellular function.

1.4.10 Mutant p53 and microRNAs

Mutant p53 is observed in a variety of tumour types and is known for its potent effect in increasing metastasis (see section 1.2.3 for details on metastasis). The dominant negative activity, of mutant p53, it is likely able to reduce expression of all microRNAs which are direct transcriptional targets of p53. However, it is also able to influence the expression of microRNAs which have not been linked to wild type p53 function (see section 1.3.5.2 for details on p53). Mutant p53 has been shown to promote EMT through inhibiting expression of miR-130b which leads to increased expression of the miR-130b target, ZEB1, a transcriptional repressor of E-cadherin (Dong, Karaayvaz et al. 2012) (see sections 1.2.2 and 1.2.3 for details on EMT and metastasis). Interestingly, p63 response elements have been found in the promoter region of miR-130b (Rivetti di Val Cervo, Lena et al. 2012). Given that mutant p53 is thought to exert some of its gain of function though dominant negative regulation of p63 and p73 (see section 1.3.5.2 for details), this could suggest that mutant p53 may downregulate miR-130b by interfering with p63 transcriptional function. Another example of this is let-7i, which has been shown to be a direct p63 target gene, but is downregulated by mutant p53 expression (Subramanian, Francis et al. 2014). The ability of mutant p53 to affect the expression of microRNAs which are not transcriptional targets of wild type p53 adds weight to the argument that mutant p53 gains function through dominant negative inhibition of p63 function. An important previous study showed that mutant p53 inhibits p63 transcriptional activity resulting in loss of Dicer expression (Muller, Trinidad et al. 2013). This could cause a decrease in global microRNA expression levels, a phenomenon correlated with invasion and metastasis (see section 1.4.6 for details on microRNAs and cancer). Another important finding
was that wild type p53 is able to increase processing of a subset of primary microRNAs and that mutant p53 is able to inhibit this process, leading to reduced expression of miR-16, miR-145 and miR-143 (Suzuki, Yamagata et al. 2009). While a number of microRNAs are downregulated by mutant p53, some have been shown to be upregulated, such as miR-155 (Neilsen, Noll et al. 2012). TAp63 has been shown to be a negative regulator of miR-155 (Mattiske, Ho et al. 2013) suggesting that mutant p53 may inhibit TAp63 activity resulting in increased expression of miR-155. These data show that inhibition of p63 function by mutant p53 is able to cause both upregulation of downregulation of microRNAs.

The dominant negative activity of mutant p53 on other members of the p53 family means that the protein can influence a wide array of microRNAs with known effects on invasion and metastasis, which may in part explain mutant p53’s pro-metastatic character (see section 1.3.5.2 for details).

1.4.11 MicroRNAs in pancreatic cancer
As previously mentioned, dysregulation of miR-21, the miR-200 family and the miR-34 family have all been linked to pancreatic cancer (see sections 1.4.7, 1.4.8 and 1.4.9 for details). However, they are not the only microRNAs which have been implicated as being involved in PDAC development. An earlier study investigating microRNA profiles from patients who had undergone pancreatectoduodenectomy, implicated a large number of microRNAs as correlating with specific disease characteristics, such as lymph node metastasis, tumour regression and tumour stage. Most interestingly, this study demonstrated that high miR-21 and low miR-34a expression correlated very well with poor survival (Jamieson, Morran et al. 2011). A number of other microRNAs have been implicated in PDAC progression including: miR-10 (Ouyang, Gore et al. 2013), miR-146 (Ali, Ahmad et al. 2014), miR-429 (Song, Zheng et al. 2015), miR-545 (Song, Ji et al. 2014) and miR-148a (Zhang, Liu 2014) among others. MicroRNA expression profiles may also be used as diagnostic and prognostic tools. A previous study investigating microRNA profiles from Fine Needle Aspirate (FNA) and frozen tissue from PDAC patients show that miR-196 and miR-217 can be used to distinguish
malignant pancreatic tissue from benign tissue (Szafrańska, Doleshal et al. 2008). Another study demonstrated that microRNAs may be found in blood of PDAC patients, opening up new opportunities for less invasive diagnostic profiling (Wang, Chen et al. 2009). They identified miR-21, miR-210, miR-155, and miR-196a as being significantly elevated in PDAC patients compared to healthy controls. It appears that a number of different microRNAs have been implicated in being involved in PDAC development, but very few appear to be involved in all cases. This may be due to the diverse genetic backgrounds observed in PDAC (see section 1.3.5 for details). It also highlights the possibility that treatment regimens may require tailoring to the patient, depending on the genetic mutations observed in the patient’s disease. This current study aims to avoid the possibility of genetic heterogeneity impacting the microRNA profile of PDAC, by focusing on mutations to only two genes: K-RAS and p53 (See sections 1.3.5.1 and 1.3.5.2 for details). To my knowledge, this has not previously been carried out in pancreatic ductal adenocarcinoma.

1.4.12 MicroRNAs as therapeutic targets
Numerous proteins have been implicated in the disease progression of cancers. Drugs have then been developed which target those proteins, with the intention of providing relief from tumour burden. However, there are very few examples of highly efficacious drugs with occasional exceptions such as (Moja, Tagliabue et al. 2012, Hudis 2007). This may be due to the fact that targeting a single component of a pathway may not be sufficient to produce clinical efficacy. By targeting microRNAs, a drug could affect numerous proteins within a pathway, providing increased inhibition/relief of that pathway and making it more difficult for compensatory mechanisms to bypass the action of the drug, possibly potentiating the efficacy of the drug.

HCV is a single-stranded RNA virus, which primarily resides in the liver of infected hosts (Cuthbert 1990). The liver specific microRNA miR-122 has been shown to be expressed at very high levels in hepatic cells (Chang, Nicolas et al. 2004, Lagos-Quintana, Rauhut et al. 2002). The HCV viral genome contains two adjacent miR-122
binding sites within its 5’ UTR, which have been shown to be critical for HCV infection to progress, possibly through promotion of viral replication (Jopling 2008) or through stimulating viral translation (Henke, Goergen et al. 2008). This non-canonical use of microRNAs by a virus is currently being targeted for therapeutic intervention in a phase 2 human trial (NCT01200420), which has already shown that following five subcutaneous injections of an antisense inhibitor of miR-122, patients exhibited reduced viral load, which was maintained for 14 weeks in a small number of the patients. Most importantly, no unwanted side effects were observed, even in patients receiving higher doses of the drug, and no mechanisms to compensate for the drug were observed in any cases (Janssen, Reesink et al. 2013).

Currently, pre-clinical trials are on-going, involving targeting of microRNAs in the treatment of cancer. An interesting study which employed a xenograft of human H460 cells onto the back of Severe Combined Immunodeficient (SCID) mice followed by intratumoral injection, or viral infection of exogenous let-7, showed a reduction in tumour size (Trang, Medina et al. 2009). Another study found that systemic delivery of exogenous miR-34 was able to block tumour growth in a mouse model of Non-small cell lung cancer (NSCLC) (Wiggins, Ruffino et al. 2010). Systemic delivery of exogenous miR-16 has also been used to reduce tumour burden in prostate cancer (Takeshita, Patrawala et al. 2009). These studies highlight numerous opportunities where microRNA regulation has significantly affected tumour growth in-vivo, and posits the possibility that microRNAs may be useful therapeutic tools for the treatment of human cancer.

A major hurdle in implementing microRNA based therapeutic approaches is efficient targeting of the therapeutic to the tumour site. Mouse studies have shown successful targeting of siRNA based therapies into the lungs (Bitko, Musiyenko et al. 2004) and olfactory nerves through the nasal cavity (Renner, Frey et al. 2012). Given the similarities between siRNAs and microRNAs, it is certainly plausible that these mechanisms could be used to introduce exogenous microRNAs or microRNA inhibitors into the lungs and olfactory nerves. However, satisfactory delivery to other organs remains a impediment to siRNA and microRNA based therapies. An interesting study was able to show that conjugation of an siRNA to a bioactive molecule such as
cholesterol was able to significantly increase the uptake and bioavailability of injected siRNAs (Soutschek, Akinc et al. 2004). Similarly, delivery of recombinant antibody raised against an HIV envelope protein conjugated to an siRNA, showed expression of the siRNA, as well as downregulation of the siRNA targets only in HIV infected or HIV envelope expressing cells (Song, Zhu et al. 2005).

Nanoparticles have previously been used to target siRNAs and microRNAs to tumour sites through systemic delivery, which reduced tumour burden in a mouse model of lung cancer (Chen, Zhu et al. 2010). Another study found that a miR-34 mimic with a lipid based vehicle was able to successfully increase expression of miR-34 within the tumour site, as well as reduce expression of miR-34 targets, resulting in inhibition of tumour growth (Wiggins, Ruffino et al. 2010). These potential approaches offer exciting opportunities in providing microRNA based therapies in the clinic. It is likely that targeting specific organs and tumour sites may be more difficult than targeting others. For example cancers of the lungs are easily accessible through airways and cancers of the skin may be treated by local intervention.

A major aim of this study is to identify microRNAs which are dysregulated due to mutant p53R172H expression. However, the mechanisms behind their dysregulation are equally important to understand. A number of normal cellular function may be hijacked by mutant p53R172H, leading to microRNA dysregulation. A number of these potential mechanisms will be discussed below.

1.5 RNA editing

RNA editing is a process by which RNA nucleotides are modified post transcriptionally. The most common way this is achieved is through the enzymatic deamination of adenine residues, which converts them to Inosine, as shown in figure 1.8. This is carried out by Adenosine Deaminase Acting on RNA (ADAR) proteins, which consists of ADAR1, ADAR2 and ADAR3.
ADAR1 and ADAR2 are functional RNA deaminating enzymes, while ADAR3 has not currently been shown to have this activity (Chen, Cho et al. 2000). RNA editing can be within the coding sequence of genes, which may change the codon, and thus the protein sequence, though most RNA editing occurs in non-coding portions of genes (Park, Williams et al. 2012). Importantly for this current study, a subset of microRNAs have been found to be edited, which affects their processing. The primary transcript of miR-142 is a target of RNA editing at a number of residues, as shown in figure 1.9, which inhibits Drosha processing (Yang, Chendrimada et al. 2006). In a similar manner, the pre-miR-151 transcript is also edited, which leads to inhibition of Dicer processing (Kawahara, Zinshteyn et al. 2007).
Figure 1-9: The RNA editing sites on the miR-142 hairpin. This diagram shows the residues which have been validated as being targets of RNA editing – adapted from Yang, Chendrimada et al. 2006.

A previous study found that ADAR1 is amplified in oesophageal squamous cell carcinoma, which correlates with poor prognosis in patients (Qin, Qiao et al. 2014). In contrast, insufficient RNA editing has been observed in hepatocellular carcinoma which also correlated with poor prognosis (Chen, Li et al. 2013, Kang, Liu et al. 2015). These conflicting reports suggest the amount of RNA editing which occurs could be critical for normal cellular function, with hypo- or hyper-editing having detrimental effects. The relatively recent observation that RNA editing is involved in tumour progression could be a novel avenue for therapeutic intervention. As RNA editing can affect both the codon sequence and stability of RNA molecules, and has been observed in such a large proportion of genes, its potential influence on tumour progression should be taken seriously.

1.6 Eukaryotic transcription
Eukaryotic transcription is a complex process which varies depending upon whether the transcribed sequence is an mRNA, rRNA or tRNA. Transcription of ribosomal RNAs requires RNome, while mRNAs require RNAPolIII and tRNAs require RNAPolIII (Cramer, Armache et al. 2008). The majority of microRNAs are transcribed via RNAPolII (Lee, Kim et al. 2004), through some have been shown to require RNAPolIII (Borchert,
This section will concentrate on the transcription of mRNAs and microRNAs, so will focus on RNApolIII mediated transcription. In very general terms, transcription is the process by which an RNA template is made from DNA. This template is then used to create proteins through the process of translation (see section 1.7 for details on translation).

DNA which is not being actively transcribed is packaged into complexes consisting of DNA and proteins called nucleosomes. Nucleosomes consist of 146bp of DNA wrapped around an octameric complex made of histones. Histone octamers consist of two molecules of two distinct heterodimers; one heterodimer consists of H3 and H4 histones and the other of H2a and H2b histones (Luger, Mäder et al. 1997, Davey, Sargent et al. 2002).

Figure 1-10: The conformation of DNA wound around an octomeric histone complex – adapted from Tsankova, Renthal et al. 2007.

The packaging of DNA is not conducive to efficient transcription and a number of modifications must occur to allow transcription to occur. Histones may be modified by methylation of either arginine or lysine residues, which confer a variety of effects depending on the site of methylation and the number of methyl groups attached. For example H4K20 (methylation of histone H4 at Lysine 20) monomethylation results in a transcriptionally permissive conformation of DNA (Vakoc, Sachdeva et al. 2006) while dimethylation of H4K20 is required for DNA damage response (Botuyan, Lee et al. 2006) and trimethylation of H4K20 has been shown to be transcriptionally repressive (Wang, Zang et al. 2008). As well as methylation, histones may be acetylated, which
represses the active winding of DNA around histones, allowing a more transcriptionally permissive conformation.

Once the DNA is in a transcriptionally permissive state, it becomes possible for transcriptional proteins to bind the DNA at gene promoters and initiate transcription. Promoters consist of two regions: the core promoter element and a region which interacts with transcriptional regulators. The core promoter element consists of the transcriptional start site (TSS) with an AT rich region ~30bp upstream of the TSS, known as the TATA box which allows binding of TATA-binding protein (TBP), a protein required for the assembly of the transcriptional initiation complex (White, Rigby et al. 1992). Transcriptional regulators include: Upstream activating sequences (UAS), which allow binding of pro-transcriptional proteins; enhancers, which allow distant binding of pro-transcriptional proteins; Upstream Repressive Sequences, which allow binding of transcriptionally inhibitory proteins; and silencers, which allow distant binding of transcriptionally inhibitory proteins.

Formation of the transcriptional pre-initiation complex is carried out by the General Transcription Factors (GTFs): TFIIA, TFIB, TFIID, TFIE, TFIF, TFIIH and TBP. TBP and TFIID bind the DNA at the TATA box before TGIIA and TFIIB are recruited, which stabilises the binding with DNA. Following this, the catalytic subunit, RNApolII, is recruited along with TFIE and TFIF, before TFIIH is finally recruited. TFIIH then melts the DNA strands in an ATP dependent manner, which are held apart by TFIIB. Ribonucleotides are then sequentially added to the DNA strand being transcribed until a threshold of 10 nucleotides has been successfully added. At this point, TFIIH phosphorylates the RNApolIII subunit which allows the polymerase complex to leave the promoter region and continue on to the elongation phase of transcription (Wong, Jin et al. 2014).

As transcriptional elongation begins, the majority of GTFs are dissociated from the complex. RNApolIII then travels in the 3’-5’ direction, separating the complementary DNA strands and adding ribonucleotides to the nascent RNA strand from the 5’-3’ direction (Griffiths, Miller et al. 2000). As DNA is melted to allow elongation of the RNA molecule, the DNA which has been transcribed is re-annealed behind the
advancing RNAPolII. This creates a “transcriptional bubble” of ~25nt where the elongation is taking place (Fiedler, Timmers et al. 2001)

RNAPolII is able to progress past the end of the transcript, however, the nascent RNA strand is cleaved before RNAPolII finishes transcribing. This liberates the pre-mRNA and allows the 5'-exonuclease XRN2 to bind and begin degrading the excess RNA strand, until it reaches RNAPolII and facilitates its dissociation from the DNA (West, Gromak et al. 2004). The cleavage of the transcript is facilitated by a complex formed of Poly(A) polymerase (PAP), cleavage and polyadenylation specificity factor (CPSF) and cleavage stimulation factor (CSTF), which recognise the poly(A) sequence AAUAAA and cleave ~30nt downstream, before adding a poly(A) tail to the end of the nascent RNA molecule (Manley 1995)

During the beginning of transcriptional elongation, a modified G is added to the 5’ of the nascent RNA molecule. This capping process involves the dephosphorylation of a single phosphate group from the triphosphorylated most 5’ nucleotide in the sequence. mRNA guanylyltransferase then adds a guanosine monophosphate (GMP), to the 5’ of the sequence in a reverse, 5’-5’ conformation. Following this, the reversed guanosine is methylated to form the completed cap structure (Shatkin 1976). The formation of the cap structure is outlined in figure 1.11.

**Figure 1-11: schematic for the steps in the formation of the cap structure.** The 5’ terminal base is dephosphorylated before a guanosine base it ligated in a 5’-5’ conformation. This structure is then methylated resulting in the cap structure.
Splicing is another important part of mRNA synthesis which occurs during transcription, which results in the non-coding introns being excised from the pre-mRNA. This is carried out by the spliceosome, a very large multi-protein complex. Splicing requires cleavage at the 5’ of the intron being excised, followed by the binding of the 5’ of the intron to an adenosine, upstream of the 3’ splice site of the intron to forms a lariat structure. Following this, a new phosphodiester bond is formed between the exons and the intron is released (Moore, Sharp 1993). A similar mechanism called alternative splicing involves the removal or retention of specific exons, to yield different protein products (Rosenfeld, Lin et al. 1982).

1.7 Eukaryotic Translation
Following transcription, nascent mRNA molecules are used as templates for producing new protein molecules in a process called translation. Translation consists of 3 major steps: initiation, elongation and termination (Jackson, Hellen et al. 2010).

Initiation is the first step in translation and requires a number of specialised proteins and complexes known as eukaryotic initiation factors (eIFs). The first steps is the formation of the ternary complex (TC) consisting of a modified methionine, known as the initiator methionine (tRNAi^{Met}) and GTP bound eukaryotic initiation factor 2 (eIF2) complex (Cashion, Stanley 1974). The TC then binds to the 40S ribosomal subunit through interactions with eukaryotic initiation factor 1 (eIF1) and eukaryotic initiation factor 3 (eIF3) which load the tRNAi^{Met} into the P-site of the 40S ribosome forming the 43S pre-initiation complex (PIC) (Safer, Adams et al. 1975). The PIC then interacts with the cap binding complex known as eukaryotic initiation factor 4F complex (eIF4F) (Pause, Belsham et al. 1994). The eIF4F complex consists of; eukaryotic initiation factor 4E (eIF4E) which binds directly to the 5’ m7G cap of the mRNA, the DEAD box RNA helicase eukaryotic initiation factor 4A (eIF4A) and eukaryotic initiation factor 4G (eIF4G) which functions as a scaffold (Morley, Curtis et al. 1997). The RNA helicase eIF4A is then able to unwind secondary structure in the 5’UTR of the mRNA allowing discovery of the AUG start codon (Yoder-Hill, Pause et al. 1993). Upon detection of the start codon, the 48S initiation complex (IC) is formed consisting of eIF1, eIF1A eIF2
and eukaryotic initiation factor 5 (eIF5) (Pisareva, Pisarev 2014). At this point, eIF5 catalyses hydrolysis of the GTP bound eIF2 resulting in the release of the initiation factors. The 60S ribosomal subunit is then able to bind the 48S forming the 80S ribosome which is able to elongate and produce a polypeptide.

Once the initiator methionine is in place and initiation has been established, the ribosome continues to progress along the mRNA adding tRNAs and producing a polypeptide. This process is known as elongation and requires a number of specialised proteins known as elongation factors (eEFs). The 40S subunit of the ribosome has three sites where tRNAs are able to interact, known as the A (Aminoacyl), P (Peptidyl) and E (Exit) sites (Ramakrishnan 2002). tRNAs start in the A site then move to the P site, and finally to the E site, as new amino acids are incorporated into the polypeptide. New, amino-acyl tRNAs are brought to the 40S ribosome via eEF1 in its GTP bound form where they enter the ribosome via the A site. The 40S ribosome ensures correct codon:anticodon binding before hydrolysis of the GTP bound to eEF1 causes dissociation of eEF1 from the ribosome leaving the amino-acyl tRNA in the A site of the ribosome (Sasikumar, Perez et al. 2012). Any amino-acyl tRNA without the correct anticodon is rejected from the ribosome. The 60S ribosome then catalyses a peptide bond between the peptidyl amino acid in the P site with that of the amino-acyl amino acid in the A site. The peptidyl tRNA in the P sites then releases its amino acid and the ribosome, via eEF2, shifts along the mRNA until a new codon is revealed, shifting the now uncharged tRNA into the E site of the ribosome and the newly peptidyl amino acid to move into the P site, and allowing a new eEF1 associated amino-acyl tRNA to enter the A site of the ribosome (Ramakrishnan 2002). This process occurs until the A site contains a codon with the sequence: UAA, UAG or UGA to which there are no tRNA anticodons to bind. Instead, a protein called eukaryotic release factor 1 (eRF1) enters the A site of the ribosome and mediates peptide chain release and leading to dissociation of the 40S and 60S ribosomal units in a process called termination (Frolova, Le Goff et al. 1994).
1.8 DNA Methylation

The majority of the cells of an organism have the same library of DNA, the difference between cell types is which genes are actively transcribed within that cell. One of the mechanisms by which cells control the transcriptional activity of gene expression, including microRNAs, is through methylation of DNA, patterns of which vary between cell and tissue type (Ehrlich, Gama-Sosa et al. 1982). The earliest evidence to support the hypothesis that DNA methylation affects gene expression came in the late 1970s. Using methylation specific restriction enzymes, it was shown that B-globulin was expressed in cells types with no methylation, but was not expressed in cell types where the gene was methylated (McGhee, Ginder 1979). While prokaryotes have been shown to have both methylated adenosine (Fang, Munera et al. 2012) and cytosine (Casadesús, Low 2006) within their genomes, until relatively recently only cytosine methylation had been observed in eukaryotes. A number of publications have shown that adenosine methylation does occur in both plants (Pintor-Toro 1987) and lower eukaryotes (Hattman 2005), but no convincing data exist which has shown that methylated adenine is present in the mammalian genome. The major DNA substrate for methylation in higher eukaryotes is cytosine, which is methylated to produce 5-methylcytosine (Goll, Bestor 2005). The majority of cytosine methylation occurs in CpG islands, short sequences ~1000bp of CG rich DNA. The majority (>70%) of CpG islands occur at sites of transcriptional initiation (Saxonov, Berg et al. 2006), though many have been found in locations not yet known to be transcriptional initiation sites (Illingworth, Gruenewald-Schneider et al. 2010). However, it is possible that these sites will prove to be bona-fide transcriptional initiation sites in future studies, or that they and how redundant initiation sites for genes which no longer exist. Promoters which have CpG islands are very permissive and often lack the normal regulator motifs such as TATA boxes (Reynolds, Basu et al. 1984).
DNA methylation is carried out by the DNA methyltransferase (DNMT) family, which consists of DNMT1, DNMT3a and DNMT3b. DNMT2 shares sequence homology with other members of the family but has not been shown to have any DNA methyltransferase activity in humans (Dong, Yoder et al. 2001). Interestingly, in *drosophila melanogaster* (*D. melanogaster*) it has been shown to be able to methylate tRNAs and protect them from stress induced degradation (Jurkowski, Meusburger et al. 2008, Schaefer, Pollex et al. 2010), though this has not yet been seen in humans. DNMT1 has a preference for hemimethylated DNA, and has been designated as a maintenance methyltransferase (Yoder, Soman et al. 1997), while DNMT3a and DNMT3b have been shown to be involved in *de-novo* methylation (Okano, Bell et al. 1999).

Hypermethylation is often associated with repression of tumour suppressor genes and has been observed in, lung (Zöchbauer-Müller, Minna et al. 2002), colorectal (Kim, Lee et al. 2010) and pancreatic cancer (Kuroki, Tajima et al. 2004, Rozenblum, Schutte et al. 1997).

Hypomethylation is more frequently observed as leading to inappropriate expression of genes or activating oncogenes, and has been found in cancers of the, breast (Pakneshan, Szyf et al. 2004), liver (Stefanska, Huang et al. 2011) and prostate (Shukeir, Pakneshan et al. 2006). Methylation has also been shown to be important in maintaining the integrity of chromosomes. Breast cancer cell lines with low levels of
DNA methylation also have more highly rearranged genomes (Vilain, Vogt et al. 1999). Given that chromosomal instability is one of the hallmarks of cancer (Hanahan, Weinberg 2000), loss of methylation may be an important aspect to the development of some tumours.

Methylation of microRNAs has been observed in a number of tumour models, including pancreatic cancer where miR-124 has been shown to be hypermethylated which leads to metastasis through reduced repression of RAC1 (Wang, Chen et al. 2013). Additionally, DNMT1 has been shown to be a direct target of microRNAs, such as miR-148a-3p, in a number of models including PDAC (Zhan, Fang et al. 2015) and hepatocellular carcinoma (Gailhouste, Gomez-Santos et al. 2013). Additionally, miR-148a-3p has been shown to be hypermethylated by increased expression of DNMT1 (Xu, Jiang et al. 2012), suggesting a complex interplay between the two genes.

1.9 Mouse model

In order to study the roles of miRNAs in pancreatic adenocarcinoma, tissues and cell lines derived from mice that have the previously described mutations to K-RAS and p53 were used. These materials were a kind gift from Professor Owen Sansom of the Beatson Institute in Glasgow. In this section, the model systems which have been used will be described with regards their relevance to the study. This investigation has implemented mouse tissues with the following genotypes:

- **K-RAS\(^{G12D}\);p53\(^{+/−}\)**
- **K-RAS\(^{G12D}\);p53\(^{R172H/+}\)**

Primary tumour tissues are vital in this study as they allow investigation into the genuine molecular changes which are occurring in animals suffering from PDAC. This is important as cell lines grown in culture frequently do not recapitulate what is happening within animals, presumably due to the extreme differences in environment between an organism and a cell culture flask. As well as the tissues, primary cells lines derived from tissues were also provided by Professor Sansom. These are important as they consist only of tumour cells, rather than the complex, heterogeneous combination of structures and cell types which comprise tissues. This allows the
validation that changes which are observed in the tissues are genuine molecular changes which are occurring within the tumour cells. For example, if angiogenesis is hyperactive in the tumour tissue, it would be expected that microRNAs which are expressed at high levels in endothelial cells would be upregulated, simply due to the increase in the amount of blood vessels. By looking at microRNA expression in cell lines, it is possible to show that the observed changes are occurring due to biochemical changes within the tumour cells. The cell lines used in this study have the following genotypes:

- K-RAS\textsuperscript{G12D};p53\textsuperscript{+/+}
- K-RAS\textsuperscript{G12D};p53\textsuperscript{+/-}
- K-RAS\textsuperscript{G12D};p53\textsuperscript{+-}
- K-RAS\textsuperscript{G12D};p53\textsuperscript{R172H/+}

The addition of the K-RAS\textsuperscript{G12D};p53\textsuperscript{+/+} and K-RAS\textsuperscript{G12D};p53\textsuperscript{+/-} allow investigation into the effect of p53 dosage on the expression of microRNAs. This will help to differentiate between changes which are due to dominant negative, and gain of function effects of mutant p53\textsuperscript{R172H}. A final two cell lines which were derived from the K-RAS\textsuperscript{G12D};p53\textsuperscript{+/-} cells, which have been transfected with an expression vector for mutant p53\textsuperscript{R172H} and established as stable cell lines, were also provided by Professor Sansom, allowing further delineation between dominant negative and gain of function effects. As they are null for p53, any change in microRNA expression which occurs upon ectopic expression of mutant p53\textsuperscript{R172H}, cannot be due to a dominant negative effect and must be due to the gain of function of mutant p53\textsuperscript{R172H}.

As previously mentioned (section 1.3.5.1) the K-RAS mutation is able to induce benign lesions called PanINs. However, these lesions frequently senesce and do not develop into invasive carcinomas (Serrano, Lin et al. 1997). Previous studies have shown that the K-RAS\textsuperscript{G12D};p53\textsuperscript{+/-} mutant mice used in this study develop PanINs with extremely high frequency, which increase in number and progress to higher grade with age (Hingorani, Petricoin et al. 2003). This model was also used to show that animals with K-RAS mutations but wild type p53 expression, showed infrequent progression to PDAC, with only 1 in 29 animals progressing to develop invasive adenocarcinomas,
which spread to the liver and caused death at 6.25 months (Hingorani, Petricoin et al. 2003).

Further mutations are required for disease progression to advance, with the most frequently observed mutation being to p53. Both loss and mutation of p53 are able to bypass K-RAS induce senescence and lead to progression of invasive carcinoma (Morton, Timpson et al. 2010). The K-RAS\textsuperscript{G12D};p53\textsuperscript{+/−} and K-RAS\textsuperscript{G12D};p53\textsuperscript{−/−} mice have previously been shown to develop invasive disease, but do not frequently exhibit metastasis. The K-RAS\textsuperscript{G12D};p53\textsuperscript{R172H+/−} mice have been shown to have a high incidence of osteosarcomas and carcinomas with a high frequency of metastasis. In all cases, mice develop PanINs at roughly 6 weeks, which progress to invasive disease by 10 weeks, but only mice which express mutant p53\textsuperscript{R172H} exhibit metastasis (Morton, Timpson et al. 2010).

All of the above models accurately recapitulate human PDAC progression, and are therefore suitable models for investigation of both genotypic and phenotypic characteristics of the disease.
Chapter 2  Materials and methods

2.1 Mouse models
The four mouse models used in this investigation have the genotype K-RAS\textsuperscript{G12D}\textsuperscript{+/-};p53\textsuperscript{+/-}, K-RAS\textsuperscript{G12D}\textsuperscript{+/-};p53\textsuperscript{+/-}, K-RAS\textsuperscript{G12D}\textsuperscript{+/-};p53\textsuperscript{+/-}, K-RAS\textsuperscript{G12D}\textsuperscript{+/-};p53\textsuperscript{R172H}. The K-RAS mutation was achieved using site directed mutagenesis to cause a specific mutation in the K-RAS gene. This mutant allele is transcriptionally blocked by the insertion of a transcriptionally repressive STOP cassette flanked by loxP sites (Lox Stop Lox). A targeting vector containing the above mutations was transfected into mouse ESC (Embryonic Stem Cells), which were then screened for successful transfection using a specific transfection cassette. The process of homologous recombination then inserts the targeting vector into the genome. Sequencing was then required to ensure that the targeting vector has been inserted into the correct locus. These modified ECSSs were then transplanted into a mouse blastocyst and implanted into the uterus of a mouse, the offspring of which may contain the modified sequence. Further screening was carried out to ensure the sequence has been successfully incorporated into the offspring. These offspring were then crossed with mice containing the transgene PDX-1-Cre, which have high expression of Cre (Causes Recombination) in PDX-1 (Pancreatic and Duodenal homeobox 1) expressing pancreatic cells. This results in the STOP cassette being removed by PDX-1-Cre and the mutant allele of K-RAS being expressed. Similar processes were used to introduce the single nucleotide mutation and produce the p53\textsuperscript{R172H} mutant.

The p53\textsuperscript{+/-} and p53\textsuperscript{-/-} mice were produced using a similar procedure. However, LoxP sites were inserted into essential regions of the coding sequence of the gene. When mice containing these mutations were bred with PDX-1-Cre mice, essential areas of the gene were removed, rendering them inactive. The p53 mutant mice were then crossed with the K-RAS mutant mice, resulting in the model organisms involved in this investigation.

All animal husbandry was carried out under the supervision of Professor Owen Sansom following the institutional rules of The Beatson Institute. Mice were
2.2 RNA techniques

2.2.1 RNA extraction
All RNA extractions were carried out using Trizol reagent (Life Technologies - 15596-026). 10μg of mouse pancreatic tumour tissue was provided frozen in RNAlater (Life Technologies - AM7020). Each tissue sample was carefully thawed in order to remove them from RNAlater solution, before being quickly washed in ddH₂O and dried. Tissues were then flash frozen in liquid N₂ and homogenised using a pestle and mortar. 10ml of Trizol reagent was then added to the homogenised tissue and incubated at room temperature for 15 minutes.

Due to the extremely high levels of endogenous RNases in the pancreas, up to 200μg per gram of wet tissues have been reported (Beintema, Campagne et al. 1973), 10X the recommended volume of Trizol reagent was used for all RNA extractions from tissues. The Trizol/tissue solution was then separated into 10X Eppendorf tubes and 200μl of chloroform (Sigma Aldrich – C2432) was added to each tube, before being shaken vigorously for 15 seconds. The tubes were then allowed to settle for 5 minutes at room temperature, before being centrifuged at 12,000g for 15 minutes at 4°C. The aqueous layer (~500μl) from each tube was transferred into a fresh Eppendorf tube and 750μl of isopropanol (Fischer – P/7500/17) and 1μl of glycogen (Roche - 10901393001) were added, before the samples were precipitated overnight at -20°C. Samples were then centrifuged at 13,000g for 15 minutes at 4°C, and the supernatant was removed and discarded. The RNA pellet was then washed in 1ml of 75% ethanol (Fischer – E/0600DF/17 diluted to 75% in ddH₂O) and centrifuged at 13,000g for 15 minutes at 4°C. The 75% ethanol was then aspirated, and one of the RNA pellets was dissolved in 500μl ddH₂O. Once the RNA had dissolved, the RNA suspension was transferred to the next tube in order to pool and concentrate the samples. This was repeated until the RNA was pooled into a single tube in a total volume of 500μl. A 1:1 mixture of acid phenol (Sigma - P4682) and chloroform was produced and 500μl was
added to the RNA solution. This mixture was thoroughly agitated before being centrifuged at 12,000g for 15 minutes at 4°C. The aqueous layer was decanted into a fresh Eppendorf tube before 500µl of ddH2O was added to the phenol solution. This was again agitated thoroughly and centrifuged at 12,000g for 15 minutes at 4°C, and the aqueous layer was pooled with the previous aqueous layer, before 2.5ml of 100% ethanol, 100µl of 3M NaOAc and 1µl of glycogen were added. This product was precipitated at -20°C overnight, before being centrifuged at 13,000g for 15 minutes for 4°C. The resulting RNA pellet was then washed in 1ml of 75% ethanol, before being suspended in 50µl of ddH2O.

Cell lines were either lysed in 1ml of Trizol reagent directly on the plate the cells were cultured on, or scraped into PBS and pelleted through centrifugation in an Eppendorf tube, before being lysed in 1ml Trizol reagent and incubated at room temperature for 15 minutes. 200µl of chloroform was added per 1ml of Trizol reagent, before samples were vigorously shaken by hand for 15 seconds and incubated at room temperature for 5 minutes. Samples were then centrifuged at 12,000g for 15 minutes at 4°C, before the aqueous phase was decanted into a fresh Eppendorf tube and 1µl of glycogen and 750µl of isopropanol were added. This solution was precipitated overnight at -20°C, before being centrifuged at 13,000g for 15 minutes. The resulting RNA pellet was washed in 75% ethanol before being resuspended in 100µl ddH2O. 100µl of and 1:1 mixture of acid phenol and chloroform was added to the RNA solution, which was then thoroughly agitated before being centrifuged at 12,000g for 15 minutes at 4°C. The aqueous layer was then decanted into a fresh Eppendorf tube and 100µl of ddH2O was added to the acid phenol:chloroform mixture. This was then thoroughly agitated and centrifuged at 12,000g for 15 minutes at 4°C again. The aqueous layer was then pooled with the previous aqueous layer, resulting in ~180µl of RNA solution. To this, 18µl of NaOAc, 1µl of glycogen and 450µl of 100% ethanol were added and mixed well, before the sample was precipitated overnight at -20°C. The sample was then centrifuged at 13,000g for 15 minutes at 4°C, and the resulting RNA pellet was washed in 75% ethanol before being resuspended in 40µl ddH2O.

All RNA samples used in this study were analysed on a nanodrop spectrophotometer (Thermo Scientific - NanoDrop2000) to analyse the RNA concentration, 260:280 and
260:230 ratios. RNA was only used for downstream processes if it satisfied the following criteria:

- 260:280 - 2.00 ± 0.30
- 260:230 - 2.20 ± 0.30

2.2.2 Bioanalysis
Bioanalysis was carried out using the Agilent 2100 Bioanalyser (Agilent - G2939AA) with the Nano 6000 chips (Agilent - 5067-1512). For each sample to be analysed, 5µl of 100ng/µl of total RNA was heat denatured for 2 minutes at 70°C before being retained on ice. Unless otherwise specified, all reagents used for this procedure are part of the Nano chip kit (Agilent – 5067-1511). 550µl of gel matrix was filtered through the provided column at 1,500g for 10 minutes, before being separated into 65µl aliquots. The dye was thawed and agitated well, before 1µl was added to the 65µ gel aliquot, after which it was agitated well and centrifuged at 13,000g for 10 minutes. A Nano chip was placed into the chip priming station (Agilent - 065- 4401) and 9µl of gel was loaded into the assigned well. The priming station was then sealed and 1ml of air was plunged into the chip for 30 seconds in order to prime the chip with gel. The priming station was unsealed, before a further 9µl of gel was put into the 2 assigned wells. 5µl of Nano marker was then placed into each of the wells being used to analyse a sample, as well as into the ladder well. 1µl of ladder or denatured RNA sample was then placed into the appropriate well, before the chip was agitated for 1 minute. The electrodes of the Bioanalyser instrument were cleaned with RNA zap (Life technologies – AM9780) before being rinsed with ddH2O. The chip was then placed into the instrument and the Nano 6000 chip programme was selected to run.

2.2.3 Agilent microRNA microarrays
Unless otherwise specified, all reagents used for this technique come from the Agilent microRNA microarrays labelling kit (Agilent - 5190-0456). Spike-in controls (Agilent - 5190-1934) for both hybridisation and labelling, were separately prepared by diluting 2µl of the stock solution in 198µl of the provided dilution buffer, to produce the first
dilution mix. 2µl of this first dilution mix was then further diluted in 198µl ddH₂O to produce a second dilution mix. 2µl of the second dilution mix was then diluted a final time in 198µl ddH₂O to produce the working concentration of spike-ins. For each condition, 2µl of 50ng/µl total RNA was phosphatase treated for 30 minutes at 37°C in Calf Intestinal Phosphatase (CIP) Master Mix.

**CIP Master Mix (per sample):**

- CIP phosphatase buffer 0.4µl
- Calf Intestinal phosphatase 0.5µl
- Diluted labelling spike-in 1.1µl

Each sample was then denatured by incubating at 100°C for 7 minutes in the presence of 2.8µl of 100% DMSO, before being immediately transferred to an ice water bath. The sample was then labelled with labelling master mix for 2 hours at 16°C.

**Labelling master mix (per sample):**

- T4 RNA ligase buffer 1.0µl
- Cy3-pCp 3.0µl
- T4 RNA ligase 0.5µl

Each sample was then passed through Micro Bio-Spin 6 columns (BioRad-732-6203) in order to remove DMSO and free Cy3-pCp dye, before being placed into a vacuum concentrator at 45°C until completely dry (~1 hour). Each sample was then re-suspended in 17µl of ddH₂O, along with 1µl of diluted Hybridisation spike-in, 4.5µl of 10x GE Blocking Agent and 22.5µl of 2x Hi-RPM Hybridisation buffer. This product was well mixed, before being incubated at 100°C for 5 minutes, and transferred directly to an iced water bath for 5 minutes. The labelled RNA for each sample was then applied to one of the eight microRNA microarrays on each slide and sealed with a gasket cover, before being placed in a hybridisation oven at 55°C for 20 hours while rotating at 20rpm. After hybridisation, the slide and gasket were immersed in wash buffer 1 (Agilent - 5188-5325) at room temperature and separated, before the slide was placed into a fresh dish of wash buffer 1 on a magnetic stirring platform, and stirred at a low setting for 5 minutes at room temperature. The slide was then placed into 37°C wash
buffer 2 (Agilent - 5188-5326) on a magnetic heated platform at 37°C for 5 minutes with a small magnetic stirrer at low setting, before being carefully removed.

The labelled array slides were scanned using the Agilent G2565CA microarray scanner and the features were defined and extracted using Agilent feature extraction software (11.0). All fold changes and statistical analysis was carried out using the AgimicroRNA plug-in for the biostatistical programme R.

Data was first background corrected and normalised using the Robust Multichip Average (RMA) technique (Irizarry, Hobbs et al. 2003). This technique employs exponential (signal) and normal convolution model to background correct data, before using a median polish method to estimate the total gene signal from all replicate probes on an array (Tukey 1977). Undetected genes were filtered out, before Linear Models for Microarray Data (LIMMA) was used to fit a linear model to the data and carry out a moderated t-test for each gene (Smyth, Gentleman et al. 2005) (See supplemental figure 1 for R script).

2.2.4 Taqman microRNA RT-qPCR
MicroRNA RT-qPCR was carried out using the Taqman microRNA assays (Applied Biosystems) as manufacturer’s instruction. Unless otherwise specified, all reagents come from the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems – 4366597). 100ng of total RNA, or ddH2O as a no template control (NTC), was reverse transcribed using 3µl of 5X microRNA specific primer, as well as 0.25µl of 60X U6 reverse transcription primer (See table for assay details) in the following reaction solution:

Reverse transcription mastermix (per sample):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>100mM dNTPs</td>
<td>0.15µl</td>
</tr>
<tr>
<td>Reverse transcriptase (50U/µl)</td>
<td>1.00µl</td>
</tr>
<tr>
<td>10X Reverse transcription buffer</td>
<td>1.50µl</td>
</tr>
<tr>
<td>RNase Inhibitor (20U/µl)</td>
<td>0.19µl</td>
</tr>
<tr>
<td>ddH2O</td>
<td>3.91µl</td>
</tr>
</tbody>
</table>
The reaction was incubated at 16°C for 30 minutes, 42°C for 30 minutes and terminated at 85°C for 5 minutes. Each reverse transcription reaction was diluted 1:5 in ddH₂O to be used for the RT-qPCR reactions. A RT-qPCR mastermix for both the gene of interest and the normalisation gene, U6, was made for each experiment.

**RT-qPCR Master Mix:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan 2x Universal PCR Master Mix</td>
<td>10µl</td>
</tr>
<tr>
<td>Gene specific probe</td>
<td>1µl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>4µl</td>
</tr>
</tbody>
</table>

15µl of RT-qPCR Master mix was pipetted into wells of a 96 well optical plate (Applied Biosystems – 4346907) before 5µl of diluted cDNA was added for each condition in triplicate. Reaction mixes were run on an Applied Biosystems 7500 RT-qPCR thermocycler under default conditions:

- **Enzyme activation**: 95°C for 10 minutes
- **40 cycles**: 95°C for 15 seconds, 60°C for 60 seconds

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Assay name</th>
<th>Assay code</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-142-3p</td>
<td>hsa-miR-142-3p</td>
<td>000464</td>
</tr>
<tr>
<td>miR-142-5p</td>
<td>hsa-miR-142-5p</td>
<td>002248</td>
</tr>
<tr>
<td>miR-148a-3p</td>
<td>hsa-miR-148a</td>
<td>000470</td>
</tr>
<tr>
<td>miR-34a-5p</td>
<td>hsa-miR-34a</td>
<td>000426</td>
</tr>
<tr>
<td>miR-34b-5p</td>
<td>hsa-miR-34b</td>
<td>002102</td>
</tr>
<tr>
<td>miR-34c-5p</td>
<td>hsa-miR-34c</td>
<td>000428</td>
</tr>
<tr>
<td>miR-340-5p</td>
<td>hsa-miR-340</td>
<td>002258</td>
</tr>
<tr>
<td>miR-96-5p</td>
<td>mmu-miR-96</td>
<td>000186</td>
</tr>
<tr>
<td>U6</td>
<td>U6 snRNA</td>
<td>001973</td>
</tr>
</tbody>
</table>

**Table 2.1: Taqman assay detains.** The order code and name of all Taqman assays used in this study.
2.2.5 SYBR Green RT-qPCR
Reverse transcription was achieved using Superscript 3 (Invitrogen - 18080044) reverse transcriptase as manufacturer’s instruction. 100ng of total RNA from each sample being interrogated, or ddH$_2$O as a Non-template control (NTC), was combined with 250ng of random primers and 500µM. The reaction mix was then made up to a total volume of 13µl with ddH$_2$O and incubated at 65°C for 5 minutes. 7µl of cDNA synthesis mastermix was added to each sample before they were incubated at 50°C for 50 minutes and 85°C for 5 minutes.

cDNA synthesis mastermix (per sample):

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x First strand buffer</td>
<td>4µl</td>
</tr>
<tr>
<td>0.1M DTT</td>
<td>1µl</td>
</tr>
<tr>
<td>RNase Inhibitor (40U/µl)</td>
<td>1µl</td>
</tr>
<tr>
<td>Superscript 3 reverse transcriptase</td>
<td>1µl</td>
</tr>
</tbody>
</table>

Following reverse transcription, the product was diluted 1:5 in ddH$_2$O for RT-qPCR. Primers were designed using Primer3 on the NCBI website (http://www.ncbi.nlm.nih.gov/ tools/primer-blast), specific to the target of interest with the following specifications:

- the amplicon must be between 100 and 200 nucleotides
- one primer must span an exon:exon junction
- primer melting temperature must be between 57°C – 63°C
- the difference between the primer pairs melting temperature should be <3°C

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADCY9 Forward</td>
<td>CCTGTGTTCAGGACAGTCC</td>
</tr>
<tr>
<td>ADCY9 Reverse</td>
<td>GGAACCAGACCAAGAGGAGC</td>
</tr>
<tr>
<td>B-actin Forward</td>
<td>GTGGACAGTGAGGCAGGAT</td>
</tr>
<tr>
<td>B-actin Reverse</td>
<td>GATTACTGCTCTGGCTCCTAGCA</td>
</tr>
<tr>
<td>BAZ2A Forward</td>
<td>ACAGGACATAGAAATGGAGGCA</td>
</tr>
<tr>
<td>BAZ2A Reverse</td>
<td>CCTTGCTGGGGAAGTCTCAT</td>
</tr>
<tr>
<td>probe</td>
<td>sequence</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>BIM Forward</td>
<td>GCCAGGCTTCAACCACAT</td>
</tr>
<tr>
<td>BIM Reverse</td>
<td>TGCAACACCCCTCTTGTTG</td>
</tr>
<tr>
<td>CFL2 Forward</td>
<td>TATTCCTGGCTCTGAAAGTGC</td>
</tr>
<tr>
<td>CFL2 Reverse</td>
<td>GCGTCAGCGGTCCTTAATA</td>
</tr>
<tr>
<td>DDX6 Forward</td>
<td>TGGGAGCTTGAAGTCAACAA</td>
</tr>
<tr>
<td>DDX6 Reverse</td>
<td>ACTCATTCAATCCCACGCC</td>
</tr>
<tr>
<td>DNMT1 Forward</td>
<td>GTGCTCTCAACAGAGCCCC</td>
</tr>
<tr>
<td>DNMT1 Reverse</td>
<td>GGGTGCTTGACAGAAGGCTGCT</td>
</tr>
<tr>
<td>E2F3 Forward</td>
<td>CCACCGCAAAGCGAAGG</td>
</tr>
<tr>
<td>E2F3 Reverse</td>
<td>TTTGGAGTTTTTGGAATCGCGG</td>
</tr>
<tr>
<td>E2F7 Forward</td>
<td>GCAGCGACAATGGAGGTGAA</td>
</tr>
<tr>
<td>E2F7 Reverse</td>
<td>GGCTCGTTCATCGGTGT</td>
</tr>
<tr>
<td>GAPDH Forward</td>
<td>GACTGGCAAATCCGTTACA</td>
</tr>
<tr>
<td>GAPDH Reverse</td>
<td>TACGGCCAAATCCGTTACA</td>
</tr>
<tr>
<td>IL6 Forward</td>
<td>GCCCTTTTGGGACTGATGCT</td>
</tr>
<tr>
<td>IL6 Reverse</td>
<td>TGCCATGGCAACACTCTTTTCT</td>
</tr>
<tr>
<td>ITGA5 Forward</td>
<td>TTCTTGACGGGGAATACCA</td>
</tr>
<tr>
<td>ITGA5 Reverse</td>
<td>GATCCACAACGGGACACCAT</td>
</tr>
<tr>
<td>ITGAV Forward</td>
<td>ACTGTGAAGGCAGAATCA</td>
</tr>
<tr>
<td>ITGAV Reverse</td>
<td>GCTAAGGCTTGTGTTTCCG</td>
</tr>
<tr>
<td>MMP15 Forward</td>
<td>TGGCGCTCTAGCTTTACCTA</td>
</tr>
<tr>
<td>MMP15 Reverse</td>
<td>CCGTCTCATCCACGTTGCG</td>
</tr>
<tr>
<td>MYST2 Forward</td>
<td>ACATGAAGTGTCTACGCCG</td>
</tr>
<tr>
<td>MYST2 reverse</td>
<td>TCTGTCCTCTACCTTTGCG</td>
</tr>
<tr>
<td>p53 Forward</td>
<td>ATGCCCATGCTACAGAGGAG</td>
</tr>
<tr>
<td>p53 Reverse</td>
<td>AGACTGGCCTTTTCTTGTTCT</td>
</tr>
<tr>
<td>RAC1 Forward</td>
<td>AGAGTACATCCCCACCGTCT</td>
</tr>
<tr>
<td>RAC1 Reverse</td>
<td>TAAGAAACGCTCTGCTGCGG</td>
</tr>
<tr>
<td>RICTOR Forward</td>
<td>CAGCAAAGAATGCAACCGGTC</td>
</tr>
<tr>
<td>RICTOR Reverse</td>
<td>TCATAAACTGCTTGCGGTC</td>
</tr>
<tr>
<td>TGFBR1 Forward</td>
<td>CTGGATCAGGTTACCAGCTGCT</td>
</tr>
<tr>
<td>Primer Name</td>
<td>Primer Sequence</td>
</tr>
<tr>
<td>-------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>TGFBR1 Reverse</td>
<td>TG CCTCTCGGAACCATGAAC</td>
</tr>
<tr>
<td>TNRC6A Forward</td>
<td>CTCGCGAGCCTCGTTCC</td>
</tr>
<tr>
<td>TNRC6A Reverse</td>
<td>ACTAAATCCCTGCTAAGATTTCTCT</td>
</tr>
<tr>
<td>UTRN Forward</td>
<td>ACTATGACCCCTCAGTC</td>
</tr>
<tr>
<td>UTRN Reverse</td>
<td>ATCCTCACGTCTCGTTG</td>
</tr>
<tr>
<td>VAMP3 Forward</td>
<td>ACTGCAAGATGTGGCCGATA</td>
</tr>
<tr>
<td>VAMP3 Reverse</td>
<td>AAGCTCGATAACACGCGAGG</td>
</tr>
</tbody>
</table>

**Table 2.2: RT-qPCR primer details.** A list of all the primer sequences used for RT-qPCR in this study. Note that primer pairs which did not produce a suitable melt curve are not listed above.

A mastermix for each gene of interest and a normalisation gene (B-actin) was then made using the following recipe:

**RT-qPCR reaction mix:**

- 2X Sybr green master mix: 10µl
- Forward primer: 300nM final concentration
- Reverse primer: 300nM final concentration
- ddH₂O to a final volume of 15µl

15µl of RT-qPCR reaction mix was then plated into a 96 well optical plate in triplicate, per condition, before 5µl of diluted cDNA was added. The plate was run using default setting on the Applied Biosystems 7500 thermocycler.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95°C</td>
<td>20 S</td>
</tr>
<tr>
<td>40 cycles</td>
<td>95°C</td>
<td>3 S</td>
</tr>
<tr>
<td></td>
<td>60°C</td>
<td>30 S</td>
</tr>
</tbody>
</table>

### 2.2.6 Analysis of RT-qPCR data

The 7500 fast real time PCR system (Applied Biosystems – 4351106), along with the installed software package, were used for analysis of all data. Samples were prepared as previously described in 2.2.4 and 2.2.5, and analysed using the default settings on
the 7500 PCR system for either SYBR green or Taqman chemistries. Once the cycling completed, the cycle thresholds (CTs) were investigated to ensure that they lay within the exponential phase of the amplification plots, and adjusted if necessary. Following that, the baseline was checked to ensure that it was set to ~3 cycles before significant fluorescent signal was detected, and adjusted if necessary. Data was then exported as an excel file for further analysis.

For each sample, a mean CT and standard deviation was calculated from the triplicate measurements. The NTC was checked to ensure that it had a sufficiently low CT value (~6 CTs lower than the specific signal). Following that, any triplicate with a standard deviation >0.5 was discarded. The data was then normalised using the ΔCT technique (Livak, Schmittgen 2001), using either U6 for microRNA data, or β-actin for mRNA data.

For tissue data, sample 051152 was used as an “ultimate” control and a ΔΔCT was calculated for each sample compared to 051152 (Livak, Schmittgen 2001). A fold change was then calculated for each sample, by converting the ΔΔCT value into a ratio, using the equation $2^{\Delta\Delta CT}$. This value was then converted into log2, and a normalisation factor was calculated from the average of the log2 values of the K-RAS$^{G12D};p53^{+/−}$ samples. This value was then subtracted from all of the log2 values for each sample in order to baseline the average log2 values of the K-RAS$^{G12D};p53^{+/−}$ samples to 0. These normalised log values were used to produce box and whisker plots with the following characteristics:

- each spot represents a single sample
- the centre line represents the median value
- the upper and lower lines of the box represent the 75th and 25th percentiles respectively
- the upper and lower whiskers representing the highest and lowest values respectively
Two sample, two tailed unpaired t-tests were used to calculate significance for all RT-qPCR data investigating expression in tissues, with *<0.05, **<0.01 and ***<0.005.

For cell line data, an average ΔCT value and a standard deviation between the ΔCT values were calculated using at least three experimental repeats for each condition. The standard ΔΔCT method (Livak, Schmittgen 2001) was then used to compare the average ΔCT values for each group. An average fold change was then calculated for each condition by converting the ΔΔCT value into a ratio, using the equation $2^{-\Delta\Delta CT}$.

Error was determined by calculating the Maximum Relative Quantity (MaxRQ) and Minimum Relative Quantity (MinFQ) using the following equations:

- $\text{MaxRQ} = 2^{(\Delta\Delta CT + \text{standard deviation of } \Delta CTs \text{ for that condition})}$
- $\text{MinRQ} = 2^{(\Delta\Delta CT - \text{standard deviation of } \Delta CTs \text{ for that condition})}$

The positive error was then calculated by subtracting the MaxFQ for each condition from fold change for each condition, and the negative error was calculated by subtracting the fold change for each condition from the MinFQ for each condition. These values were used to represent the error in all bar charts representing RT-qPCR data. Two sample, two tailed, paired t-tests were used to calculate significance for all
RT-qPCR data investigating expression in cell lines, with *<0.05, **<0.01 and ***<0.005.

2.2.7 MicroRNA In-Situ Hybridisation
MicroRNA in-situ hybridisation was carried out using the miRCURY LNA microRNA ISH optimisation kit (Exiqon – 90002) as manufacturer’s instruction. Formalin Fixed Paraffin Embedded (FFPE) samples were provided by Professor Owen Sansom of the Beatson institute. Slides were deparaffinised by incubating 3 times in xylene for 5 minutes each. Slides were then rehydrated using a gradient of ethanol for 5 minutes each in: 100%, 96%, 70% and 50% before being immersed in Phosphate Buffered Saline (PBS) for 4 minutes. Sections were then treated with Proteinase K for 10 minutes at 37°C to reveal the epitopes. A range of concentrations were attempted between 5-200µg/ml, with 200µg/ml giving the best signal, while retaining tissue integrity (see results section 5.2.8 for optimisations). Sections were then washed well in PBS, before being dehydrated in an ethanol gradient for 1 minute each in: 70%, 96% and 100%, before being allowed to air dry at room temperature for 15 minutes. Once dry, the slides were pre-incubated with 50µl of diluted hybridisation buffer (Exiqon - 90000) for 30 minutes at 55°C. Following preincubation, the coverslip was carefully removed and 50µl of double DIG labelled specific probe (see table for probe details), diluted in hybridisation buffer, was placed on each section and a large cover slip was placed on top before the edges were sealed with fixogum (Marabu – 2901 10 000). U6 probes were used at a final concentration of 1nM while microRNA specific probes were used at 40nM. Sealed slides were placed into a hybridisation chamber and incubated for 1 hour at 55oC. Slides were removed from the hybridisation oven and placed into room temperature 5X Saline Sodium Citrate (SSC) before being washed for in 55°C 5xSSC once; 55°C, 1X SSC twice and 55°C, 0.2X SSC twice, before a final wash at room temperature in 0.2X SSC, with each wash lasting for 5 minutes. Slides were then maintained in room temperature PBS. A DAKO-pen (Vector – H-4000) was then used to make a hydrophobic barrier around the section on each slide before blocking buffer (PBS, 0.1% Tween, 2% sheep serum, 1% BSA) was applied to the tissue, ensuring that it was all covered, and was incubated at room temperature for 15 minutes. Blocking
buffer was then removed and anti-DIG antibody (Roche – 11 093 274 910) was diluted 1:800 in antibody diluant (PBS, 0.05% Tween, 1% sheep serum, 1% BSA), applied to the sections and incubated for 1 hour at room temperature. Slides were then washed 3 times at room temperature in PBS-T (PBS, 0.1% Tween). An Alkaline Phosphatase (AP) substrate solution was then prepared consisting of an NBT/BCIP tablet (Roche – 11 697 471 001), 0.2mM levamisole (Sigma – 31742) and 10ml ddH2O, and applied to each section, before the slides were placed into a hybridisation chamber for 2 hours at 30°C. The AP reaction was then quenched using KTBT (50mM Tris-HCL, 150mM NaCl, 10mM KCL in ddH2O) twice for 5 minutes at room temperature, before being washed twice in ddH2O for 1 minute each. Nuclear Fast Red (Sigma - N3020) was then applied to each section for 1 minute to counterstain before the slides were washed in running water for 10 minutes. Sections were then dehydrated in an ethanol gradient with: 70%, 96% and 100% ethanol, for 1 minute each, before a coverslip was attached using VectaMount (Vector – H5000). Mounting media was allowed to dry overnight before sections were analysed on a microscope.

Images were also taken at 5-20X magnification, with images taken from sequential slides, one for control and a second for the microRNA of interest. Each analysis of a section consists of 2 sequential slides, one with a scrambled control probe and the other with a microRNA specific probe.

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Probe number</th>
<th>RNA Tm</th>
<th>Hybridisation temperature</th>
<th>Probe sequence</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>U6</td>
<td>90002</td>
<td>84°C</td>
<td>54°C</td>
<td>5'-DIG/caggaatttgctgtcatcctt/DIG-3'</td>
<td>1nM</td>
</tr>
<tr>
<td>miR-142-3p</td>
<td>18043-15</td>
<td>80°C</td>
<td>50°C</td>
<td>5'-DIG/tccataagtaggaacactaca/DIG-3'</td>
<td>40nM</td>
</tr>
<tr>
<td>miR-145-5p</td>
<td>88068-15</td>
<td>84°C</td>
<td>54°C</td>
<td>5'-DIG/agggattcctgggaaaactggac/DIG-3'</td>
<td>40nM</td>
</tr>
<tr>
<td>miR-21-5p</td>
<td>90002</td>
<td>83°C</td>
<td>83°C</td>
<td>5'-DIG/taacatcagctgataagcta/DIG-3'</td>
<td>40nM</td>
</tr>
</tbody>
</table>

**Table 2.3 In-situ hybridisation probe details.** The order number and probe sequence of all the *in-situ* hybridisation probes used in this study.
2.3 DNA techniques

2.3.1 DNA extraction from cultured cells
DNA extraction was carried out using the DNeasy Blood and tissue kit (Qiagen – 69504). 3X10^6 K-RAS^{G12D};p53^{+/+} cells were centrifuged at 300g for 5 minutes, and resuspended in 200µl of PBS, before 20µl of proteinase K and 200µl of buffer AL were added. This mixture was incubated at 56°C for 10 minutes, before 200µl of 100% ethanol was added and mixed well. This reaction was then pipetted into a DNeasy spin column and centrifuged at 6000g for 1 minute at room temperature. The spin column was then placed into a fresh collection tube and 500µl of buffer AW1 was added, before the column was centrifuged at 6000g for 1 minute at room temperature. The flow through was discarded, before 500µl of buffer AW2 was added to the column and centrifuged at 20,000g for 3 minutes at room temperature. 200µl of buffer AE was then placed onto the membrane of the column and incubated for 1 minute at room temperature before being centrifuged at 6000g for 1 minute to elute. DNA was then stored at -20°C until required.

2.3.2 Agarose gel electrophoresis
Agarose gel electrophoresis was carried out to separate nucleic acids according to molecular weight. Molecular biology grade agarose (Sigma – MB1200) was added to 1X TAE buffer (40mM Tris acetate and 1mM EDTA), and dissolved by heating in a microwave. A typical amount of agarose was 1g per 100ml of TAE buffer. SybrSafe (Life technologies – S33102) was added in a ratio of 1:10,000 and the gel was allowed to cool and set. DNA samples were mixed with 5X DNA loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 0.1M EDTA, 30% glycerol) and loaded into the gel. Gels were electrophoresed, submerged in 1X TAE buffer, typically at 100V, and visualised on a UV transilluminator.

2.3.3 DNA extraction from agarose gel
DNA was extracted from agarose gel using the Zymoclean gel recovery kit (Zymo Research – D4008). A fragment of gel containing the DNA was excised from the gel
using a scalpel and placed into a clean Ependorf tube. The fragment was weighed, and 3X the volume of ADB buffer was added, before the sample was heated to 50°C for 10 minutes. The dissolved gel and ADB buffer were then passed through a Zymo-spin column by centrifuging at 13,000g for 60 seconds at room temperature. 200µl of DNA wash buffer was then passed through the column by centrifugation at 12,000g for 1 minute at room temperature. The column was then placed into a fresh Eppendorf tube and centrifuged again for 1 minute at 12,000g to ensure all flow through had passed through the column. The column was then placed into a clean Eppendorf tube and 10µl of warm ddH2O was placed onto the membrane of the column and incubated at room temperature for 1 minute. The DNA was then eluted by centrifugation at 12,000g for 1 minute at room temperature. DNA was then analysed using the Nanodrop spectrophotometer to investigate the concentration as well as 260:280 and 260:230 ratios. DNA was only used for downstream processes if it satisfied the following criteria:

- 260:280 - 1.80 ± 0.20
- 260:230 - 2.20 ± 0.20

2.3.4 DNA digestion
DNA was digested with restriction enzymes using buffers and quantities recommended by the manufacturers. The typical reaction volume was 20-50µl and the reaction mixture was incubated at the recommended temperature for 2-4 hours. For sequential digests requiring different buffers, the DNA was incubated with one enzyme, the enzyme inactivated by heat inactivation at 65°C for 10 minutes, and the DNA extracted by passing it through a QIAquick PCR cleanup column (Qiagen – 28104), before being incubated with the second enzyme. Following the final digestion, the restriction enzymes were deactivated by incubation at 65°C for 10 minutes.

2.3.5 Ligation
Ligations were achieved using T4 DNA ligase (Promega - M1801) and T4 DNA ligase buffer (C126A). A molar ratio of 1:3 plasmid to insert was used for each reaction, in a
total volume of 10µl, with the addition of ATP. Ligations were maintained in a thermocycler at 16°C for 16 hours to ensure effective ligation.

2.3.6 Transformations and DNA preparations
Following ligation, the vector was transformed into 50µl of Escherichia coli DH5α competent cells. The solution was maintained on ice for 20 minutes before the cells were heat shocked at 42°C for 1 minute, and immediately transferred to ice for 2 minutes. 1ml of antibiotic free Luria-Bertani (LB) broth (10g/L Bacto-tryptone, 5g/L yeast extract, 10g/L NaCl pH adjusted to 7.5 with NaOH) was added to the sample before it was incubated at 37°C for 1 hour with shaking. The sample was then centrifuged at 5000g for 1 minute and the LB was removed and the bacterial pellet was resuspended in 100µl of fresh LB broth, which was plated onto a pre-warmed LB agar plate containing the correct antibiotic for selection, and incubated overnight at 37°C. A single colony was picked and incubated in 10ml of LB medium overnight at 37°C with shaking.

Mini-preparation was performed using the SV Wizard Mini-prep kit (Promega - A1460) as described in the manufacturer’s instructions. Monoclonal cell culture was pelleted by centrifugation and re-suspended in 250µl Cell Resuspension Solution in a 1.5ml Eppendorf tube. 250µl of Cell Lysis Solution and 10µl of Alkaline Protease Solution were added and each sample before they were inverted 4 times to mix. This reaction was incubated for 5 minutes at room temperature, before 350µl of Neutralization Solution was added and the samples were mixed. Centrifugation was carried out at maximum speed for 10 minutes at room temperature. The cleared lysate was decanted into the provided column and centrifuged at 12,000g for 1 minute at room temperature, and the flow through discarded. The column was washed with 750µl of the provided wash solution and centrifuged at 12,000g for 1 minute before the wash step was repeated with 250µl of wash solution. A further centrifugation step was carried out for 2 minutes to fully remove remaining Wash solution, before the DNA was eluted with 100µl of ddH₂O.
2.3.7 Sequencing of p53 mutation
Total RNA from each sample was reverse transcribed using superscript 3 as described in section 2.2.5. A 498bp fragment of p53, which includes the site of mutation (nucleotide 672), was then amplified using primers designed using the annotated mouse p53 sequence (NM_011640.3). The amplified product was sent for sequencing analysis using a nested primer, at the Protein and Nucleic Acid Chemistry Laboratory (PNACL) of the University of Leicester. Sequences were aligned using the clone manager (Sci-Ed) alignment tool and analysis of residue 672 was undertaken.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 amplification primer Forward</td>
<td>ccctgtcatcttttgtcc</td>
</tr>
<tr>
<td>p53 amplification primer Reverse</td>
<td>ccagtgtgatgatggtaagg</td>
</tr>
<tr>
<td>p53 sequencing primer</td>
<td>aagctatctgccagcttgcc</td>
</tr>
</tbody>
</table>

Table 2.4: p53 sequencing primers. The sequences of the primer sequences used to amplify and sequence a 498bp fragment of p53 which contains the site of mutation.

2.3.8 MicroRNA expression vector construct
Overexpression of microRNAs was carried out using the pCMV-MIR vector system (Origene). The empty vector (Origene – PCMVMIR) and miR-142 expression vector (Origene - SC400778) were purchased from Origene, while the miR-34a vector was produced by Dr Adam Hall. The miR-148a vector was produced by designing primers to amplify a fragment of gDNA spanning 300nt upstream and downstream of the mature miR-148a locus, entailing a 694bp fragment. The forward primer contained a restriction site for sgf1 while the reverse primer contained a restriction site for mlu1.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-148a Forward primer</td>
<td>gaggccgatgccgtaacaggaccggaggagt</td>
</tr>
<tr>
<td>miR-148a Reverse primer</td>
<td>gcgacgcgtttgtgctttctctcttgctc</td>
</tr>
</tbody>
</table>

Table 2.5: miR-148a expression vector cloning primer sequences. Details of the primers used to produce the miR-148a expression vector. Restriction sites are annotated red.
Genomic DNA from K-RAS$^{G12D,p53^{+/+}}$ cells was denatured at 100°C for 5 minutes before being amplified using the above primers and Phusion polymerase (M0530) using the manufacturers instruction.

**Reaction mix per sample:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X Phusion HF buffer</td>
<td>10µl</td>
</tr>
<tr>
<td>10mM dNTPs</td>
<td>1µl (200µM final)</td>
</tr>
<tr>
<td>10µM forward primer</td>
<td>2.5µl (500nM final)</td>
</tr>
<tr>
<td>10µM reverse primer</td>
<td>2.5µl (500nM final)</td>
</tr>
<tr>
<td>Heat denatured gDNA</td>
<td>5µl (500ng final)</td>
</tr>
<tr>
<td>DMSO</td>
<td>1.5µl (3% final)</td>
</tr>
<tr>
<td>Phusion polymerase</td>
<td>0.5µl</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>27µl</td>
</tr>
</tbody>
</table>

**Cycling conditions:**

<table>
<thead>
<tr>
<th>Cycle Type</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>98°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>30 cycles</td>
<td>98°C</td>
<td>10 seconds</td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>20 seconds</td>
</tr>
<tr>
<td></td>
<td>72°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>10 minutes</td>
</tr>
</tbody>
</table>

The resulting amplified product was electrophoresed on a 1% agarose gel and the band corresponding to 694bp was excised and the DNA extracted using standard conditions (See sections 2.3.2 and 2.3.3 for details on electrophoresis and gel extraction). The gel extracted fragment and the pCMV-MIR vector were then digested sequentially first using sgf1 (Promega – R170B) in buffer C (Promega – R003) before digestion with mlu1 (New England Biolabs – R0198) in buffer 3.1 (New England Biolabs – B7203), with each digestion occurring for 3 hours and DNA being passed through a PCR clean-up column between digestions (see section 2.3.4 for details on digestions). The digested vector and insert were then electrophoresed and gel extracted using
standard conditions (see section 2.3.2 for details) before being ligated and used to transform DH5α bacterial cells to amplify the ligated construct using standard procedures (see section 2.3.5 for details). Four colonies were picked and miniprepped using standard procedures (see section 2.3.6) which were sequenced at PNACL using the VP1.5 sequencing primer which binds 50nt upstream of the Sgf1 restriction site.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP1.5 sequencing primer</td>
<td>ggaaccttccaaatgtcg</td>
</tr>
</tbody>
</table>

*Table 2.6: VP1.5 sequencing primer sequence.* Details of the VP1.5 sequencing primer used to sequence the miR-148a expression vector after cloning.

2.4 Protein techniques

2.4.1 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.4.1.a Cell lysis

Cells were lysed in either radioimmunoprecipitation assay buffer (RIPA) (50mM Tris, 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40) or, when more stringent lysis was required, AB lysis buffer (10mM Tris Ph 7.5, 50mM NaCl, 0.5% NP40, 0.5% sodium deoxycholate, 0.5% SDS, 10mM Iodoacetamide). Lysis buffers were always supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche - 04693116001) immediately prior to use. The Biorad protein assay (Biorad - 500-0001) was used to quantify protein to ensure equal loading.

2.4.1.b Electrophoresis

5X SDS PAGE sample buffer (62.5 mM Tris pH 6.8, 7% SDS, 20% sucrose, 5% β-mercaptoethanol, 0.01% bromphenol blue) was diluted to 1X in protein lysate, before they were heat denatured at 95°C for 10 minutes. Gels were made using various ratios of protogel (National Diagnostics - EC-890) and ddH2O with the addition of 4X resolving buffer (1.5M Tris, 1% SDS, pH 8.8) or 4X stacking buffer (0.25M Tris, 0.2% SDS, pH 6.8), diluted to 1X. 0.002% TEMED (N,N,N',N′-tetramethylethylenediamine) and 0.25% APS (Amonium Persulphate) were added to polymerise the gel. Fully polymerised gels were immersed in running buffer (25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3) and a suitable potential, typically ~100V was applied to migrate and separate the protein through the gel.
2.3.1.c Transfer of proteins onto nitrocellulose membranes
Following electrophoresis, the gels were placed onto a nitrocellulose membrane, sandwiched between 6 pieces of Whatman paper, and transferred to a Mini-Trans Blot cell (Biorad - 170-3930) wet transfer tank. An amperage of 250mA was applied for 1.5 hours to achieve sufficient transfer of proteins onto the membrane. Efficiency of transfer was analysed using Ponceau S staining, to visualise proteins, before the membrane was blocked for 1.5 hours in 5% milk in TBS-T (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6).

2.3.1.d Immunoblotting
Antibodies specific to the protein of interest were diluted in a suitable volume of 5% milk in Tris-Buffered Saline with Tween (TBS-T). This solution was applied to the transferred blotting membranes and incubated overnight at 4°C on a rolling platform. The blots were washed 3 times for five minutes in TBS-T before a secondary antibody, targeting the species in which the primary antibody was raised, was applied and incubated at room temperature for 1.5 hours. The secondary antibody was conjugated to a fluorescent reporter for use on the Odyssey imager (LI-COR).

<table>
<thead>
<tr>
<th>Final percentage of gel</th>
<th>4X Resolving Buffer (ml)</th>
<th>30% Acrylamide (ml)</th>
<th>dH₂O (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>2.5</td>
<td>2.5</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>2.5</td>
<td>3.33</td>
<td>4.17</td>
</tr>
<tr>
<td>12</td>
<td>2.5</td>
<td>4</td>
<td>3.5</td>
</tr>
<tr>
<td>15</td>
<td>2.5</td>
<td>5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Table 2.7: Acrylamide gel compositions. The recipes used to make acrylamide gels which were frequently used in this study.
### Table 2.8: Antibody details

A list of all of the antibodies used in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Developer</th>
<th>Product code</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGO2</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>ab32381</td>
</tr>
<tr>
<td>AGO2</td>
<td>Mouse</td>
<td>Abcam</td>
<td>ab57113</td>
</tr>
<tr>
<td>ATM</td>
<td>Mouse</td>
<td>Santa Cruz</td>
<td>sc-23921</td>
</tr>
<tr>
<td>Phospho ATM (ser1981)</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>ab81292</td>
</tr>
<tr>
<td>β-actin</td>
<td>Rabbit</td>
<td>Sigma Aldrich</td>
<td>A5060</td>
</tr>
<tr>
<td>Dicer</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>sc-30226</td>
</tr>
<tr>
<td>Digoxigenin (alkaline phosphatase conjugated)</td>
<td>Sheep</td>
<td>Roche</td>
<td>11 093 274 910</td>
</tr>
<tr>
<td>DNMT1</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>5032</td>
</tr>
<tr>
<td>Drosha</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>ab12286</td>
</tr>
<tr>
<td>FLAG</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>ab1162</td>
</tr>
<tr>
<td>p21</td>
<td>Goat</td>
<td>Santa Cruz</td>
<td>sc-397-G</td>
</tr>
<tr>
<td>p53</td>
<td>Mouse</td>
<td>Santa Cruz</td>
<td>sc-126</td>
</tr>
<tr>
<td>Phospho p53 (ser15)</td>
<td>Rabbit</td>
<td>Cell Signalling</td>
<td>9284</td>
</tr>
<tr>
<td>p63</td>
<td>Mouse</td>
<td>Santa Cruz</td>
<td>sc-8431</td>
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<tr>
<td>p73</td>
<td>Rabbit</td>
<td>Cell signalling</td>
<td>4662</td>
</tr>
<tr>
<td>PARP</td>
<td>Rabbit</td>
<td>Cell signalling</td>
<td>9542</td>
</tr>
</tbody>
</table>

#### 2.4.2 Endogenous AGO2 immunoprecipitation

AGO2 immunoprecipitations (IPs) were used to identify *bona-fide* targets of the microRNAs identified during this study. 2X10⁶ cells were plated onto 4X 15cm dishes and allowed to adhere overnight. 2 of the 15cm dishes were transfected with a 2’-O-Me for the microRNA being investigated, while the remaining 2X 15cm dishes were transfected with a control 2’-O-Me (See section 2.5.2 for details on transfections and 2’-O-Me details) and incubated at 37°C with 5% CO₂ for 24 hours.

120µl of packed Protein A/G plus beads (Santa Cruz - sc-2003) were washed twice in IP lysis buffer (20mM Tris pH 7.5, 150mM NaCl, 15mM MgCl₂, 0.5% NP40, 1mM EDTA) before being resuspended in 1ml of the same buffer. The bead suspension was then split into 2 clean Eppendorf tubes on ice. 30µg of AGO2 antibody (Abcam – ab57113) or 30µg of rabbit IgG (Santa Cruz – sc2025) was then added to the tubes before they were rotated end over end for at 4°C for 1 hour. Yeast tRNA fragments (Sigma - R4018) at a final concentration of 100µg/ml were then added to each tube and allowed to bind the beads for 30 minutes.
Cells were scraped into 10 ml of cold PBS and collected in 50ml falcon tubes. A further 10ml of ice cold PBS was used to wash the plate again and pooled together to ensure full recovery of cells. The tubes were then centrifuged at 1,500g for 10 minutes at 4°C before the PBS was discarded and the pellets briefly dried. 10ml of IP lysis buffer was supplemented with a mini protease inhibitor cocktail tablet (Roche – 11 836 170 001) and 400 units of Ribolock RNase inhibitor (Thermo Scientific – EO0381). Cells were resuspended in 1600µl of the supplemented lysis buffer and incubated on ice for 10 minutes. Each lysate was then transferred to a 2ml Eppendorf tube and centrifuged at 5000g for 10 minutes at 4°C. The supernatant was decanted into a fresh Eppendorf tube and the cell pellet was discarded. 80µl of lysate was collected as an input while the remaining lysate was split equally between the AGO2 antibody coated beads and the IgG coated beads. The tubes were then rotated end over end for 4 hours at 4°C.

The beads were pelleted at 1000g for 3 minutes at 4°C, before the supernatant was collected for analysis of AGO2 depletion. The beads were washed in 500µl of freshly prepared IP lysis buffer 3X before being split 1:5 into 2 Eppendorf tubes. The smaller component was then boiled in 1X SDS PAGE sample buffer with 5% β-mercaptoethanol and frozen at -80°C until required, while the larger component was placed into 1ml of Trizol reagent and a standard RNA extraction was carried out (see section 2.2.1 for details).

AGO2 IPs following microRNA overexpression were carried out as above, but with the following exceptions:

- 1X10⁶ cells were plated onto 4X 15cm dishes
- cells were incubated for 48 hours post transfection of the microRNA expression vector

2.4.3 FLAG tagged AGO2 immunoprecipitation

1X10⁶ cells were plated onto 2X 15cm dishes and allowed to adhere overnight. Both dishes were transfected (see section 2.5.3 for details on plasmid transfection) with 4µg of pIRESneo-FLAG/HA AGO2 (Addgene – 10822) and incubated 24 hours at 37°C. The plates were then transfected with either a 2′-O-Me against miR-142-3p or a
control 2'-O-Me (See section 2.5.2 for details on 2'-O-Me transfections) and incubated at 37°C for 24 hours. Cells were then scraped into cold PBS, before being centrifuged at 1500g for 10 minutes at 4°C. 10ml of IP lysis buffer was supplemented with a mini protease inhibitor cocktail tablet and 400 units of Ribolock RNase inhibitor. Cells were resuspended in 1100µl of the supplemented lysis buffer and incubated on ice for 10 minutes. Lysate was then transferred to a 2ml Eppendorf tube and centrifuged at 5000g for 10 minutes at 4°C. The supernatant was decanted into a fresh Eppendorf tube and the cell pellet was discarded. Equal quantities of M2 antibody conjugated magnetic beads (Sigma - M8823) were washed 3 times in IP lysis buffer and separated into 2 Eppendorf tubes, before being pelleted using a magnetic rack. 100µl of lysate was removed as an input, while the remaining was pipetted onto the M2 conjugated beads before the tubes were placed onto a daisy wheel for 4 hours at 4°C. The lysate was then removed and retained to assess FLAG-AGO depletion, and the beads were washed 3 times in IP lysis buffer, before they were split 1:5 for protein and RNA. Beads for protein were resuspended in 1X SDS PAGE sample buffer with 5% β-mercaptoethanol and boiled for 10 minutes before being stored at -80°C until required, while the remaining beads were resuspended in Trizol reagent, and processed using standard techniques (see section 2.2.1 for details of RNA extraction procedures).

2.5 Cell culture techniques

2.5.1 General cell culture

All cells, other than SAOS cells, were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco - 41966) supplemented with 10% FBS (Foetal Bovine Serum), 0.5% l-glut and 0.5% Penicillin/streptomycin. Cells stably expressing plasmids with a neomycin selection cassette were maintained in the above media with the addition of 0.1% G418 (Sigma – A1720). SAOS cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 (Gibco – 11875) supplemented with 10% FBS, 0.5% l-glut and 0.5% Pen/Strep. All cell lines were cultured at 37°C with a CO₂ content of 5%. 
2.5.2 Transfection of siRNAs and 2’-O-Me’s
Both siRNA and 2’-O-Me transfections were achieved using Dharmafect 1 transfection reagent (Dharmacon - T-2001). Unless stated otherwise, all siRNA and 2’-O-Me transfections were carried out as follows. Cells were sparsely plated into 6cm dishes so as to be ~10% confluent after 24 hours. Cells were washed with PBS, before being refreshed with antibiotic free media. For each condition, 200µl of optimum was placed into 2 Eppendorf tubes. A suitable amount of siRNA (20nM final concentration) or 2’-O-Me (50nM final concentration) was added to one of the tubes, while 5µl of Dharmafect 1 was added to the other. The tubes were incubated at room temperature for 5 minutes before being combined, mixed well through pipetting and incubated at room temperature for 20 minutes, before being added dropwise to the cells with antibiotic free media.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>siRNA number</th>
<th>5' sequence</th>
<th>3' sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMT1</td>
<td>4390771</td>
<td>caacggauuccuaacacutt</td>
<td>agugugauaggaucguugta</td>
</tr>
<tr>
<td>Control 1</td>
<td>4390844</td>
<td>Proprietary sequence</td>
<td>Proprietary sequence</td>
</tr>
</tbody>
</table>

**Table 2.9: siRNA details.** A list of the siRNAs used in this study. All siRNAs were purchased from Ambion.

<table>
<thead>
<tr>
<th>microRNA target</th>
<th>Sequence 5’ – 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-142-3p</td>
<td>mU/ZEN/mCmAmAmUmAmAmAmAmGmAmAmAmAmCmAmUmAmGm/3ZEN/</td>
</tr>
<tr>
<td>miR-148a-3p</td>
<td>mA/ZEN/mCmAmAmAmGmAmUmCmAmGmAmAmCmAmGmAmGmAm/3ZEN/</td>
</tr>
<tr>
<td>miR-34a-5p</td>
<td>mA/ZEN/mCmAmAmAmCmAmGmAmAmAmCmAmGmAmGmGmCm/3ZEN/</td>
</tr>
<tr>
<td>miR391-5p</td>
<td>mU/ZEN/mUmGmGmGmGmCmAmGmGmGmGmGmGmGmGmGmGmGmGmGm/3ZEN/</td>
</tr>
</tbody>
</table>

**Table 2.10: 2’-O-Me details.** A list of the 2’-O-Mes used in this study. All 2’-O-Mes were custom made by Integrated DNA Technologies. The miR-142-3p, miR-148a-3p and miR-34a-5p all target the murine microRNA sequence while the miR391-5p (control) targets an Arabidopsis thaliana microRNA.
2.5.3 Plasmid transfections

Unless stated otherwise, plasmid transfections were achieved using lipofectamine 2000 (Life technologies – 11668027). Cells were plated into 6cm dishes so as to be ~10% confluent after 24 hours. Cells were washed in cold PBS, before being refreshed in antibiotic free media. 200µl of optimum was placed into 2 Eppendorf tubes, a suitable amount of plasmid vector was added to one, while a suitable amount (1:3 ratio of DNA(µg) to lipofectamine 2000 (µl)) of lipofectamine 2000 was added to the other. The tubes were incubated at room temperature for 5 minutes, before being combined, mixed well and incubated for a further 20 minutes at room temperature. The transfection reaction was then added dropwise to the cells in antibiotic free media.

<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>Plasmid backbone</th>
<th>Made by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty vector (pCDNA3.1)</td>
<td>pCDNA3.1</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Wild type p53</td>
<td>pCDNA3.1</td>
<td>Dr Patricia Muller</td>
</tr>
<tr>
<td>Mutant p53R175H</td>
<td>pCDNA3.1</td>
<td>Dr Patricia Muller</td>
</tr>
<tr>
<td>TAp63</td>
<td>pCDNA3.1</td>
<td>Dr Patricia Muller</td>
</tr>
<tr>
<td>ΔNp63</td>
<td>pCDNA3.1</td>
<td>Dr Patricia Muller</td>
</tr>
<tr>
<td>TAp73</td>
<td>pCDNA3.1</td>
<td>Dr Patricia Muller</td>
</tr>
<tr>
<td>ΔNp73</td>
<td>pCDNA3.1</td>
<td>Dr Patricia Muller</td>
</tr>
<tr>
<td>Empty vector (pCMV-MIR)</td>
<td>pCMV-MIR</td>
<td>OriGene</td>
</tr>
<tr>
<td>miR-142</td>
<td>pCMV-MIR</td>
<td>Origene</td>
</tr>
<tr>
<td>miR-148a</td>
<td>pCMV-MIR</td>
<td>Jack Godfrey</td>
</tr>
<tr>
<td>miR-34a</td>
<td>pCMV-MIR</td>
<td>Dr Adam Hall</td>
</tr>
<tr>
<td>FLAG/HA Ago2</td>
<td>pIRESneo</td>
<td>AddGene</td>
</tr>
</tbody>
</table>

Table 2.11: Plasmid details. A list of the plasmids used in this study.

2.5.4 Invasion assays

2X10^5 cells were plated into 6cm dishes and allowed to adhere overnight. Cells were then transfected with a 2'-O-Me against the microRNA of interest using Genejuice (Merck – 70967) transfection reagent as follows. Cells were first washed with PBS before being refreshed with antibiotic free media. 200µl of optimum was placed into an Eppendorf tube with 3µl of 2'-O-Me and 5µl of Genejuice. The tube was agitated well and incubated at room temperature for 10 minutes, before the mixture was added, dropwise onto the cells in antibiotic free media. After 24 hours the cells were trypsinised, counted and a 350,000 cell/ml solution was made. 60µl of Matrigel
(Corning – 356234) was placed into the cell culture transwell inserts provided with the invasion chamber assay plates (Corning – 3422). The invasion chambers and plates were then incubated at 37°C with 5% CO₂ for 1 hour in order for the Matrigel to set. Once the Matrigel had set, the plate containing the Matrigel coated inserts was inverted, and 100µl of cell suspension was placed onto the base of the culture inserts, which were then incubated at 37°C with 5% CO₂ for 5 hours. The plates were then reverted and the chambers washed twice in serum free DMEM, before being placed into fresh wells containing 1ml of serum free media. 100µl of media with 10% serum and 10ng/ml Hepatocyte Growth Factor (HGF) as chemoattractants, was then placed into the transwell and the cells were incubated at 37°C and 5% CO₂ for the required length of time (48 hours for K-RAS<sup>G12D</sup>;p53<sup>+/R172H</sup> or 72 hours for K-RAS<sup>G12D</sup>;p53<sup>+/−</sup>). Cells were then stained with 4nM Calciene (Life Technologies - C1430) in serum free media for 1 hour before being imaged using a confocal microscope. For each experimental repeat, 2 transwells were used.

All non-invasive cells were removed from the base of the membrane using a sterile tissue, before the transwell was placed onto a large coverslip on the stage of a confocal microscope. Cells were first observed using and LED at 488nm wavelength in order to assess that they had invaded uniformly across the membrane. It is important to note that the centres of the transwells do not contain any invading cells due to the hypoxic environment of the assay, and therefore, no Z-stacks were taken from the centre of the membrane. For each transwell, 4 Z-stacks were taken at the positions outlined in figure 2.2. The same positions were taken for each transwell in order to reduce user bias. Each Z-stack had an interval of 15µm and a total of 10 images.
Each stack of images was opened in ImageJ and the fluorescence for each image of a stack was quantified. The fluorescent signal of cells passed 30μm was counted as invasive. The percentage of fluorescent signal passed 30μm was calculated as a percentage of the total fluorescent signal for each stack, and an average percentage of invasion was calculated for each transwell. Statistical analysis was carried out using a two sample, two tailed, unpaired t-test, comparing the average invasion of each transwell in a condition, across three experiments carried out on separate days.
Chapter 3  Profiling of microRNAs which are dysregulated by mutant p53<sup>R172H</sup> in pancreatic cancer

3.1 Introduction

Pancreatic cancer is an aggressive malignancy with extremely poor prognostic implications. With the majority of tumours only being diagnosed once the primary tumour has metastasised, it is of vital importance that therapeutic strategies be developed which are aimed at treating metastatic disease. Specific hotspot mutations to p53, such as p53<sup>R172H</sup> (p53<sup>R175H</sup> in humans), which is the focus of this study, have been shown to induce metastasis beyond that seen in p53 null tumours (Morton, Timpson et al. 2010). Mutant p53 has been shown to function in a dominant negative manner, but also to have gain of function characteristics (Dittmer, Pati et al. 1993). One interesting study was able to show that mutant p53 achieves some of its gain of function characteristics through dominant negative inhibition of p63 function, which leads to loss of Dicer, and presumably, loss of microRNA processing (Muller, Trinidad et al. 2013). As a higher rate of metastasis is observed in mutant p53 expressing tumours than those hemizygous, and null for p53, the gain of function characteristics have been implicated as the likely major cause of metastatic onset (see section 1.3.5.2 for details on mutant p53). This study has investigated the microRNA expression profiles of tumours tissues from two mouse models of Pancreatic Ductal Adenocarcinoma (PDAC), which had the following genotypes:

- K-RAS<sup>G12D</sup>;p53<sup>+/−</sup>
- K-RAS<sup>G12D</sup>;p53<sup>R172H+/−</sup>

As K-RAS mutations account for >90% of the initiating mutations in PDAC (Kanda, Matthaei et al. 2012, Morris, Wang et al. 2010), all mice and cell lines used in this study have activating mutations to K-RAS. Mice which express a mutant K-RAS, but wild type p53 develop very slow growing tumours and have a long survival (personal communication with Professor Owen Sansom). This results in them accruing random mutations as the disease develops. These mutations may affect microRNA expression,
which could lead to heterogeneity in the control group. For this reason the control group was engineered to be hemizygous for p53, which induces a much more aggressive phenotype with a considerably shorter survival, reducing the chance of random somatic mutations occurring. In addition, mutant p53 is known to have some dominant negative function on wild type p53, which may lead to changes in expression of p53-dependent microRNAs (see section 1.4.9 for details on p53-dependent microRNAs). It is hoped that making the control group hemizygous for p53 will reduce the number of microRNAs identified as differentially expressed, which would be due to the dominant negative function of mutant p53.

The initial profiling step of this study used tissues from 6X K-RAS$^{G12D}$;p53$^{+/−}$ and 6X K-RAS$^{G12D}$;P53$^{R172H/+}$ mice, which were a kind gift from Professor Owen Sansom of The Beatson Institute in Glasgow. Additionally, primary cell lines, which were derived from mouse primary pancreatic tumours, were also provided by Professor Sansom. As previously mentioned, the cell lines were useful in showing that the changes in microRNA expression were due to molecular changes within the tumour cells, rather than differences in populations of cell types which express the microRNAs (see section 1.9 for details). The cell lines used in this study had the following genotypes:

- K-RAS$^{G12D}$;p53$^{+/+}$
- K-RAS$^{G12D}$;p53$^{+/−}$
- K-RAS$^{G12D}$;p53$^{−/−}$
- K-RAS$^{G12D}$;p53$^{R172H/+}$

A final gift from Professor Sansom were two cell lines, which were the K-RAS$^{G12D}$;p53$^{−/−}$ cell line, which had been transfected with either an expression vector for mutant p53$^{R172H}$, or an empty vector, and established as stable cell lines. As these cells were null for p53, they were used to show that the changes in microRNA expression were not due to the dominant negative function of mutant p53$^{R172H}$, so must be due to its gain of function abilities.

All of the cell lines were interrogated using Taqman RT-qPCR assays to investigate the effect of mutant p53$^{R172H}$ on microRNA expression. While a number of studies have investigated the microRNA expression profile of PDAC, to the author’s knowledge, no.
study has looked specifically at how mutant p53R172H may alter microRNA expression on a mutant K-RAS background in PDAC. This study has identified microRNAs differentially expressed in PDACs which express mutant p53R172H, with the intention of identifying potential novel therapeutic, prognostic or diagnostic targets.

3.2 Results

3.2.1 Sample processing and sequencing
To ensure that all K-RASG12D;p53+/- tissues expressed only the wild type p53, and all K-RASG12D;p53R172H/+ tissues expressed the p53R172H mutant, a 498bp fragment, which contained the site of mutation, was reverse transcribed and amplified from total RNA extracted from each tissue (see methods and materials sections 2.3.7 for details). Here I show that the amplified fragment for both the K-RASG12D;p53+/- and K-RASG12D;p53R172H/+ tissue samples, show a clear, defined band at 498bp, which corresponds to the correct size of the fragment (figure 3.1-a). The p53R172H mutation was achieved by substituting a guanine to an adenine at base 672. This changes codon 172 from CGC, which codes for arginine (R), to CAC, which codes for histidine (H). Here I show that all of the samples designated K-RASG12D;p53+/- contain a G at base 672 and thus express wild type p53; and all samples designated K-RASG12D;p53R172H/+ apart from sample 120994, contain an A at base 672 and thus express the p53R172H mutant (figure 3.1-b). Sample 120994 has been designated a G at base 672, which would suggest that it expresses wild type p53, when it should be expressing the p53R172H mutant. Analysis of the chromatogram for sample 120994 shows two peaks at base 672, while all of the other K-RASG12D;p53R172H/+ samples have a single peak for A, and all of the other K-RASG12D;p53+/- samples have a single peak for G (figure 3.1-c). This double peak for sample 120994 suggests that both a wild type and mutant allele are being expressed. This may not be surprising, as it has previously been reported that heterozygous expression of mutant p53 often leads to Loss Of Heterozygosity (LOH) of the remaining wild type allele (Jacks, Remington et al. 1994). Our data would suggest that all samples, other than 120994, have undergone LOH of the wild type allele. As expression of a single allele of mutant p53 is sufficient to induce metastasis (Lang, Iwakuma et al. 2004), sample 120994 was retained in the analysis.
Figure 3-1 Sequencing of p53 in mouse primary tumour tissue. (a) A gel showing the 498bp fragment of p53 which contains the site of mutation was amplified for sequencing. (b) the sequences of the amplified fragments were aligned against the refseq sequence for mouse p53 (NM_011640.3). (c) The chromatograms showing examples of (i) wild type p53, (ii) mutant p53<sup>R172H</sup>, and (iii) sample 120994.
3.2.2 Bioanalysis and quality control
Once it had been shown that all tissue samples expressed the correct form of p53, it was important to ensure that the RNA was of sufficient quality for analysis. The nanodrop spectrophotometer was used to assess the yield and purity of the RNA samples (Table 3.1). For pure RNA, the 260/280 ratio should be close to 2.00, and a 260/230 ratio should be close to 2.20. A low 260/280 ratio is suggestive of contamination of the sample with proteins, possibly carried over from the tissue/cell line, while a low 260/230 suggests contamination with salts or phenol, perhaps carried over from the Trizol reagent. The Agilent bioanalyser was then implemented in order to attain qualitative information about the integrity of the RNA samples. The bioanalyser carries out capillary electrophoresis with laser induced fluorescence detection, to measure the amount of RNAs of various sizes within a sample, and uses a proprietary algorithm to calculate a RNA Integrity Number (RIN). The Bioanalyser assigns a RIN to each sample, ranging from 10 for intact to 1 for highly degraded. The electropherogram and RIN for each sample (figure 3.2-a), along with the pseudo-gel (figure 3.2-b) show qualitative information about the integrity of each sample.

To fulfil its role in digestion, the pancreas produces large amounts of RNases, making extraction of intact RNA from the pancreas very difficult (Gill, Aubin et al. 1996, Dastgheib, Irajie et al. 2014, Azevedo-Pouly, Elgamal et al. 2014). There have been a number of previous studies investigating how microRNAs are affected by total RNA degradation. One study, which used heat to denature total RNA samples, found little change in microRNA expression, even when mRNAs was highly degraded (Jung, Schaefer et al. 2010). Another, analysed total RNA extracted from formalin-fixed paraffin-embedded (FFPE) sections, and found that while the age of the sections correlated positively with increased degradation and loss of mRNAs, microRNAs were not affected (Hall, Taylor et al. 2012). This may not be surprising, as microRNAs will be bound to AGO2, which should stabilise them and block RNase activity as well as the fact that microRNAs are too short to be substrates for most RNases. Given the difficulty in extracting intact RNA from the pancreas, and the fact that microRNAs are likely to be intact, even when other RNA species are degraded, this current study
Figure 3-2: Bioanalysis of RNA derived from mouse primary tissue samples (a) The Agilent bioanalyser produces an electropherogram for each sample and assigns a RNA Integrity Number RIN (RIN). Any RIN below 6.00 is of insufficient quality to use in further analysis. (b) The pseudo-gel produced using to Agilent bioanalyser showing peaks for the 18S and 28S ribosomal RNAs. All suitable samples have a blue circle at the top of the gel while unsuitable samples have a red circle. Table 3.1 shows a summary of the quality control metrics for each tissue.

<table>
<thead>
<tr>
<th>Mouse number</th>
<th>Serotype</th>
<th>RIN</th>
<th>260/280</th>
<th>260/230</th>
</tr>
</thead>
<tbody>
<tr>
<td>05152</td>
<td>K-RASG12D;p53+/-</td>
<td>7.8</td>
<td>1.95</td>
<td>2.33</td>
</tr>
<tr>
<td>65552</td>
<td>K-RASG12D;p53+/-</td>
<td>7.6</td>
<td>1.99</td>
<td>2.19</td>
</tr>
<tr>
<td>9840</td>
<td>K-RASG12D;p53+/-</td>
<td>6.3</td>
<td>1.94</td>
<td>1.99</td>
</tr>
<tr>
<td>10494</td>
<td>K-RASG12D;p53+/-</td>
<td>6.2</td>
<td>2.05</td>
<td>2.12</td>
</tr>
<tr>
<td>10495</td>
<td>K-RASG12D;p53+/-</td>
<td>5.8</td>
<td>2.10</td>
<td>2.06</td>
</tr>
<tr>
<td>22407</td>
<td>K-RASG12D;p53+/-</td>
<td>8.5</td>
<td>2.04</td>
<td>1.95</td>
</tr>
<tr>
<td>120994</td>
<td>K-RASG12D;p53R172H/+</td>
<td>8.7</td>
<td>2.01</td>
<td>2.25</td>
</tr>
<tr>
<td>120982</td>
<td>K-RASG12D;p53R172H/+</td>
<td>8.3</td>
<td>2.03</td>
<td>2.22</td>
</tr>
<tr>
<td>29039</td>
<td>K-RASG12D;p53R172H/+</td>
<td>8.0</td>
<td>2.01</td>
<td>2.26</td>
</tr>
<tr>
<td>10579</td>
<td>K-RASG12D;p53R172H/+</td>
<td>8.1</td>
<td>2.11</td>
<td>1.99</td>
</tr>
<tr>
<td>38110</td>
<td>K-RASG12D;p53R172H/+</td>
<td>7.8</td>
<td>2.08</td>
<td>1.95</td>
</tr>
</tbody>
</table>
considers RNA with a RIN > 6.00 to be of sufficient quality to be taken forward for further analysis. The data I present here show that two samples, 10495 and 29039, fell below this threshold (1X K-RAS\textsuperscript{G12D};p53\textsuperscript{+/-} and 1X K-RAS\textsuperscript{G12D};P53\textsuperscript{R172H/+}), and were not taken forward for further analysis (figure 3.2 and table 3.1).

3.2.3 MicroRNA microarrays
Once the genotype of each tissue had been validated and the RNA found to be of good purity and of high integrity, Agilent microRNA microarrays were used to investigate the global changes in microRNA expression, brought about by mutant p53\textsuperscript{R172H}. Given that RNA from two mouse tissues were found to be of insufficient quality to investigate, RNA from 5X K-RAS\textsuperscript{G12D};p53\textsuperscript{+/-} and 5X K-RAS\textsuperscript{G12D};P53\textsuperscript{R172H/+} mice were used for global microRNA profiling using the Agilent microRNA microarray platform.

Total RNA from each tissue sample was labelled with a Cy3 dye and hybridised to Agilent microRNA microarrays as manufacturer’s instruction, before being scanned (See methods section 2.2.3 for details). Once the microarrays have been scanned, it is necessary to assign the relevant probe name to each spot on each array. Agilent’s Feature Extraction software automates this process and carries out quality control for each array. The six quality control metrics which are analysed are: “IsGoodGrid”, which checks that the grid file fits the array, ensuring that each spot is correctly annotated; “AddErrorEstimateGreen”, which calculates the additive error for green signal for each array; “AnyColorPrcentFeatPopnOL”, which calculates the percentage of spots which are population outliers; “gNonCtrlMedPrcntCVBGSubSig”, which calculates the median coefficient of variation for all non-outlier spots on each array; “LabelingSpike-InSignal”, which calculates the efficiency of the labelling reaction for each array using the labelling spike-in and “HybSpike-InSignal”, which calculates the efficiency of the hybridisation reaction using the hybridisation spike-in. The spike-in metrics are categorised as either good or evaluate, while the remaining metrics are categorised as either perfect, good or evaluate. A summary of the quality control metrics may be seen in Table 3.2, which shows that all arrays have the highest
Figure 3-3: Summary of the microarray quality control. (a) A pseudo-image of the microRNA microarrays, which shows that there are no edge effects, smudges or uneven hybridisation on any of the arrays used in this study. Table 3.2: An overview of the quality control metrics for each array in the analysis (full quality control metrics may be found in supplemental figure 2).
possible quality control metrics. There is also an image of each array, which highlights the fact that there are no edge effects or non-uniform areas of fluorescence on each array (figure 3.3-a). The full quality control results for all arrays may be found in supplemental figure 2.

3.2.4 MicroRNA profile of mutant p53 expressing mouse pancreatic tissues

Having shown that the microRNA microarrays were of high quality, analysis of microRNA expression was carried out. This was achieved using the AgiMicroRNA plugin for R, and compared the 5X K-RAS<sup>G12D</sup>,p53<sup>+/−</sup> samples to the K-RAS<sup>G12D</sup>,p53<sup>R172H/+</sup> samples, which had passed quality control, in order to establish which microRNAs are differentially expressed due to mutant p53<sup>R172H</sup> (see supplemental figure 1 for R script and methods section 2.2.3 for details). The microarrays detected 334 microRNAs, 29 of which were dysregulated by at least 2 fold (see supplemental figure 3 for full microarray results). From these 29 microRNAs, miR-3096 has recently been shown to be independent of Dicer and DGCR8 processing and does not immunoprecipitate with AGO2 (Castellano, Stebbing 2013). This gene is no longer categorised as a microRNA, so was not investigated any further in this study. Additionally, miR-1237 is not conserved in the <i>H. sapiens</i> genome, so was also removed from this study. Of the remaining 27 differentially expressed microRNAs, 6 (22%) were upregulated and 21 (78%) were downregulated (figure 3.4). Only 10 microRNAs were found to be statistically significantly, differentially expressed when using a p-value cut off of 0.05, all of which were downregulated. The microRNAs found to be differentially expressed by at least two fold, and which were statistically significant were: miR-101c, miR-141-3p, miR-142-3p, miR-142-5p, miR-146b-5p, miR-148a-3p, miR-340-5p, miR-34a-5p, miR-378b and miR-96-5p (figure 3.4).

In order to ensure that the microarray results were reliable, six microRNAs were chosen for validation by Taqman q-PCR assays (Applied Biosystems) (see Methods section 2.2.4 for details): miR-142-3p, miR-142-5p, miR-148a-3p, miR-34a-5p, miR-340-5p, and miR-96-5p. As was observed in the microarrays, all of the microRNAs
Figure 3-4: The results of the Agilent microRNA microarrays. For this comparison 5X K-RAS\(^{G12D}\);p53\(^{-/-}\) and 5x K-RAS\(^{G12D}\);p53\(^{R172H/+}\) mouse primary tissue samples were used. The microRNA microarrays were analysed using the AgiMicroRNA plugin for R (bioconductor). Please see methods section 2.2.3 for analysis parameters, and supplemental figure 1 for R script. The bar chart displays the 27 microRNAs which were found to be dysregulated by at least 2 fold in the K-RAS\(^{G12D}\);p53\(^{R172H/+}\) tissues. Statistical analysis was carried out using the AgiMicroRNA plugin for R. Statistical significance is represented as * (<0.05) ** (<0.01) and *** (<0.005).
Figure 3-5: RT-qPCR validation of the microRNA microarrays. Taqman RT-qPCR assays were used to validate the results of the microRNA microarrays using the same samples. The data is represented as box and whisker plots with the upper and lower band represent the highest and lowest value respectively. The top and base of the box represent the 75th and 25th percentile respectively, and the middle bar is the median value. Each dot represents a single sample. See methods section 2.2.6 for details on RT-qPCR analysis. A two sample, two tailed, unpaired t-test was used to compare the ΔCT values from each group. Statistical significance is represented as * (<0.05) ** (<0.01) and *** (<0.005).
were found to be downregulated in mutant p53 expressing tissues using this second technique (figure 3.5).

3.2.5 Cell line processing and sequencing
As previously mentioned, tissues may contain structures and cell types which are not parts of the tumour (see section 1.9 for details). This may cause changes in microRNA expression which are not brought about by molecular changes within the tumour cells, but instead may be due to differences in cell populations. In order to establish whether the changes in microRNA expression observed in the tumour tissues are due to changes within the tumour cells, primary cell lines derived from the tumours were investigated for microRNA expression using Taqman RT-qPCR. The cell lines used for this investigation have the following genotypes:

- K-RAS<sup>G12D</sup>;p53<sup>+/+</sup>
- K-RAS<sup>G12D</sup>;p53<sup>+/-</sup>
- K-RAS<sup>G12D</sup>;p53<sup>-/-</sup>
- K-RAS<sup>G12D</sup>;p53<sup>R172H/+</sup>

In order to ensure that only the K-RAS<sup>G12D</sup>;p53<sup>R172H/+</sup> cell line expressed mutant p53, and that each of the wild type p53 expressing cell lines expressed the correct level of p53, the same 498bp fragment of p53 used to genotype the tissue samples was reverse transcribed and amplified from total RNA from each cell line and sequenced. Agarose electrophoresis of a small amount of each sample shows that the K-RAS<sup>G12D</sup>;p53<sup>+/+</sup> and K-RAS<sup>G12D</sup>;p53<sup>R172H/+</sup> both have a defined band at 498bp, while K-RAS<sup>G12D</sup>;p53<sup>+/-</sup> has a band of approximately half the intensity, and K-RAS<sup>G12D</sup>;p53<sup>-/-</sup> has no band at all (figure 3.6-a). An alignment of K-RAS<sup>G12D</sup>;p53<sup>+/+</sup>, K-RAS<sup>G12D</sup>;p53<sup>+/-</sup> and K-RAS<sup>G12D</sup>;p53<sup>R172H/+</sup> sequence, against the annotated mouse p53 transcript (NM_011640.3) sequence, show that only the K-RAS<sup>G12D</sup>;p53<sup>R172H/+</sup> has a single nucleotide mutation at codon 672, where a G has been replaced with an A (figure 3.6-b). As previously mentioned, this corresponds to the expected sequence for mutant p53<sup>R172H</sup>. 

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Figure 3-6: Sequencing p53 in mouse primary cell lines. A 498bp fragment of p53, with includes the site of mutation was reverse transcribed and amplified from total RNA from the K-RAS<sup>G12D</sup>,p53<sup>+/+</sup>, K-RAS<sup>G12D</sup>,p53<sup>+/−</sup>, K-RAS<sup>G12D</sup>,p53<sup>−/−</sup> and K-RAS<sup>G12D</sup>,p53<sup>R172H+/−</sup> mouse primary cell lines. (a) Following amplification the samples were electrophoresed and the band, where present was excised and sequenced. (b) An alignment of the sequencing against the annotated mouse p53 sequence (NM_011640.3) shows that only the K-RAS<sup>G12D</sup>,p53<sup>R172H+/−</sup> cell line expresses mutant p53<sup>R172H</sup>. (c) The chromatograms of the site of mutation in each of the p53 expressing cell lines.
3.2.5a Cell line RT-qPCR investigating the effect of mutant p53<sub>R172H</sub> expression
The six microRNAs identified (figure 3.4) and validated (figure 3.5) as changing due to mutant p53<sup>R172H</sup> expression in the tumour tissues, were investigated in K-RAS<sup>G12D</sup>;p53<sup>+/+</sup> and K-RAS<sup>G12D</sup>;P53<sup>R172H/+</sup> cell lines, which correspond to the respective matching mouse tissue. Here I show a similar downregulation of: miR-142-3p, miR-148a-3p, miR-34a-5p, miR-340-5p and miR-96-5p (figure 3.7). The expression of miR-142-5p was below the detectable expression levels in the cell lines, so was not investigated any further in this study. These data suggest that the downregulation of miR-142-3p, miR-148a-3p, miR-34a-5p, miR-340-5p, and miR-96-5p are due to changes in the biochemistry of the tumour cells themselves, rather than being an artefact of the tissue heterogeneity.

3.2.5b Cell line RT-qPCR investigating the effect of p53 dosage on microRNA expression
Mutant p53 has been shown to function in both a dominant negative manner, as well as having gain of function abilities. As the gain of function has been shown to be the likely cause of mutant p53 induced metastasis (see introduction section 1.3.5.2 for details), it was important to show the effect of p53 dosage, on the expression of the microRNAs. This was investigated using Taqman RT-qPCR (see methods section 2.2.4 for details) in the following cell lines:

- K-RAS<sup>G12D</sup>;p53<sup>+/+</sup>
- K-RAS<sup>G12D</sup>;p53<sup>+/−</sup>
- K-RAS<sup>G12D</sup>;p53<sup>−/−</sup>

By examining how the expression of the microRNAs being investigated in this study changes with p53 dosage, it is possible to establish whether the changes are directly linked to p53, and therefore potentially constitute the dominant negative effect of mutant p53<sup>R172H</sup>. On the other hand, if the changes in expression do not correlate with p53 dosage, it may suggest that the changes in microRNA expression can be
Figure 3-7: RT-qPCR validation of the tissue microRNA microarrays in primary cell lines. The expression of the microRNAs identified in the microRNA microarrays were investigated in primary cell lines with the following genotypes, K-RAS\(^{G12D}\);p53\(^{+/−}\) and K-RAS\(^{G12D}\);p53\(^{R172H+/−}\), using RT-qPCR. The fold change represents the average fold change from three independent experimental replicates, with the error bars representing the minimum and maximum relative quantities (see methods section 2.2.6 for details). Two sample, two tailed, paired t-tests were carried out to investigate statistical significance, comparing the ΔCT values of the three repeat experiments. Statistical significance is represented as * <0.05, ** <0.01 and *** <0.005.
Figure 3-8: The effect of p53 dosage on microRNA expression. The expression of the microRNAs which were identified in the microRNA microarrays was investigated in the following primary cell lines, K-RAS\textsuperscript{G12D};p53\textsuperscript{+/+}, K-RAS\textsuperscript{G12D};p53\textsuperscript{+/-} and K-RAS\textsuperscript{G12D};p53\textsuperscript{--}. Fold change represents an average from three independent experimental repeats, with error bars representing the minimum and maximum relative quantities (see methods section 2.2.6 for details). Two sample, two tailed, paired t-tests were to investigate statistical significance comparing the \textDelta CT values of the three experimental repeats for each condition. The black bar represents significance tests are a comparison using the K-RAS\textsuperscript{G12D};p53\textsuperscript{+/+} cell line as a control, while the blue bar represent a comparison using K-RAS\textsuperscript{G12D};p53\textsuperscript{+/-} as a control. Statistical significance is represented as * <0.05, ** <0.01 and *** <0.005.
attributed to the gain of function properties associated with mutant p53R172H. Here I show that in the K-RASG12D;p53+/− cell line, miR-142-3p, miR-34a-5p and miR-96-5p are all downregulated, while miR-148a-3p and miR-340-5p are not (figure 3.8), when compared to a K-RASG12D;p53+/+ cell line. I have also shown that the K-RASG12D;p53−/− cells exhibit downregulation of miR-142-3p, miR-148a-3p, miR-34a-5p, miR-340-5p and miR-96 (figure 3.8), compared to K-RASG12D;p53+/+. A final comparison of the K-RASG12D;p53−/− cell lines to the K-RASG12D;p53+/+, reveal that all five of the microRNAs were downregulated: miR-142-3p, miR-148a-3p, miR-34a-5p, miR-340 and miR-96-5p, which might suggest a p53 dosage response correlation for all of the microRNAs being investigated in this study.

3.2.10 Investigation of microRNAs expression in p53 null cells with ectopic mutant p53R172H expression

Having shown that all of the microRNAs being investigated have at least some degree of response to p53 expression, it is possible that they are all downregulated simply due to the loss of p53 expression, and the dominant negative ability of mutant p53R172H. The aim of this study is to identify microRNAs which are dysregulated by mutant p53R172H, which may promote invasion and metastasis. As mutant p53 is thought to promote metastasis through its gain of function abilities, it is important to establish whether the changes in microRNA expression observed in the tissues and cell lines are due to the dominant negative or gain of function ability of mutant p53R172H.

In order to address question in detail, the p53 null cell lines which ectopically express mutant p53R172H or a control, (a kind gift from Professor Owen Sansom), were used. As no p53 is present in these cells, any change in microRNA expression cannot be attributed to dominant negative effect of mutant p53R172H, so must be due to its gain of function ability. Here I show that expression of p53 is only detectable in the cell lines which express ectopic mutant p53R172H (figure 3.9-a). Previously, I have shown that miR-340-5p was downregulated in tissues and cell lines which express mutant p53R172H (figures 3.4, 3.5 and 3.7). However, when this microRNA is investigated in p53 null cells with ectopic expression mutant p53R172H, it is found to be significantly upregulated (figure 3.9). Of the remaining microRNAs, miR-96-5p does not change in
Figure 3-9: MicroRNA expression following stable ectopic expression of mutant p53\textsuperscript{R172H}. Expression of the microRNA identified in the microRNA microarrays was investigated in p53 null cells lines with stable ectopic expression of mutant p53\textsuperscript{R172H}. (a) A representative western blot for p53 and β-actin for the control and mutant p53 expressing cell lines. (b) The fold change represents the average fold change from the independent experimental replicates, with the error bars representing the minimum and maximum relative quantities (see methods section 2.2.6 for details). Two sample, two tailed, paired t-tests were carried out to investigate statistical significance, comparing the ΔCT values of the three repeat experiments. Statistical significance is represented as * <0.05, ** <0.01 and *** <0.005.
expression, while miR-142-3p, miR-148a-3p and miR-34a-5p are all downregulated in the p53 null cell line which ectopically expresses mutant p53^{R172H} (figure 3.9).

So far the data in this chapter have identified that miR-142-3p, miR-148a-3p and miR-34a-5p are all downregulated due to mutant p53^{R172H}. This has been confirmed in primary tissues (figures 3.4 and 3.5), showing that this is a genuine change in expression in animals suffering from PDAC caused by mutant p53^{R172H} expression; primary cell lines (figure 3.7), showing that the change is due to genuine molecular changes within the tumour cells, rather than being artefact of the inherent heterogeneity of tissues; and p53 null cells which ectopically express mutant p53^{R172H} (figure 3.9), showing that the change in expression is due to a gain of function associated with mutant p53^{R172H}.

### 3.3 Discussion

This study has investigated how changes in microRNA expression may affect mutant p53^{R172H} driven metastasis. An important previous study was able to show that mutant p53^{R172H} is able to induce metastasis above that seen by loss of p53 in a mouse model of PDAC (Morton, Timpson et al. 2010), suggesting a gain of function to mutant p53^{R172H}. The role of microRNAs in mutant p53 function was elucidated in two important studies. The first, found that mutant p53 was able to reduce microRNA expression in a non-transcriptional manner, by interfering with the assembly of Drosha and the DEAD box helicase, p68, which leads to attenuation of primary microRNA processing (Suzuki, Yamagata et al. 2009). The second found that mutant p53 was able to inhibit another step of microRNA processing, through the repression of Dicer, via both p63 dependent and independent mechanisms (Muller, Trinidad et al. 2013). These data implicate microRNAs in mutant p53 function, and suggest that the gain of function of mutant p53 is required to induce metastasis. With these data in mind, the microRNA expression profile associated with mutant p53^{R172H} expression in PDAC was investigated in order to identify microRNAs which are dysregulated when mutant p53^{R172H} is expressed. Here I show that in mutant p53^{R172H} expressing tissues, the majority (78%) of the microRNAs which were differentially expressed by at least
two fold were downregulated, and that all of the statistically significant changes were downregulated (figure 3.4).

It is interesting that a large number of the microRNAs which were downregulated by at least two fold have been shown to be targets of hypermethylation in other models. The miR-200 family members, all five of which (miR-200a-3p, miR-200b-3p, miR-200c-3p, miR-141-3p and miR-429-3p) were found to be downregulated in the tumour tissues which express mutant p53R172H, have previously been shown to be hypermethylated (Vrba, Jensen et al. 2010, Lim, Wright et al. 2013, Neves, Scheel et al. 2010). This family is well described as being critical for inhibiting EMT (See introduction section 1.2.2 for details of EMT), through regulation of the E-cadherin transcriptional repressors: SNAIL, SLUG (Park, Gaur et al. 2008), ZEB1 (Chen, Liang et al. 2012) and ZEB2 (Kurashige, Kamohara et al. 2012). As well as the miR-200 family, miR-1224 (Dudziec, Miah et al. 2011), miR-142 (Abdul Razak, Baba et al. 2014), miR-143 (Dou, Zheng et al. 2012), miR-146b (Xiang, Birkbak et al. 2014), miR-148a (Zhu, Xia et al. 2011), miR-338 (Li, Chen et al. 2013), miR-375 (Chu, Chang et al. 2014), miR-34a (Cui, Zhao et al. 2014) and miR-378 (Deng, Guo et al. 2013) have all previously been shown to be hypermethylated in cancer. This corresponds to 15 of 21 (71%) of the mature microRNAs found to be downregulated in mutant p53R172H expressing tissues, having previously been found to be hypermethylated. This is a large percentage, and may suggest a role of methylation in mutant p53R172H gain of function. This hypothesis will be examined more closely in chapter 5.

In order to ensure that the microarray results were reliable, six microRNAs were chosen for validation by Taqman RT-qPCR assays (see Methods section 2.2.4 for details): miR-142-3p, miR-142-5p, miR-148a-3p, miR-34a-5p, miR-340-5p, and miR-96-5p. As was observed in the microarrays (figure 3.4), all of the microRNAs were found to be downregulated in tissue expressing mutant p53R172H (figure 3.5). The expression of these microRNAs was then investigated in primary cell lines to ensure that the changes observed in the tumour tissues were not due to the heterogeneity of the tissue samples. Here I show that all of the microRNAs which were downregulated in the tumour tissues which express mutant p53R172H, were also downregulated in the primary cell lines which express mutant p53R172H (figure 3.7). It is important to note
that miR-142-5p was not expressed at detectable levels in the cell lines, so was not investigated any further in this study. All of the microRNAs being investigated in this study exhibited some change in expression due to p53 dosage (figure 3.9). This may have suggested that the microRNAs were not changing due to the gain of function ability of mutant p53<sup>R172H</sup>, but instead may be due to the dominant negative effect of the mutation. In order to answer the question more thoroughly, it was necessary to investigate the change in a p53 null system, so as to remove all possibility of a dominant negative response. The expression of these microRNAs was investigated in p53 null primary cell lines which ectopically express mutant p53<sup>R172H</sup>, which found that only miR-142-3p, miR-148a-3p and miR-34a-5p were downregulated. As miR-96-5p expression did not change in this model, it may suggest that the change in its expression is due to the dominant negative effect of mutant p53<sup>R172H</sup>, and that as no wild type p53 was present in the cells, it was not possible for it to exert this effect. It is interesting that miR-340-5p was found to be downregulated by mutant p53<sup>R172H</sup> in the tissues (figure 3.4) and primary cells lines (figure 3.7), while it is upregulated in p53 null cells with ectopic expression of mutant p53<sup>R172H</sup> (figure 3.9). These conflicting data do not offer much insight into how this microRNA was regulated by mutant p53<sup>R172H</sup> so miR-340-5p was not investigated any further in this study.

This leaves this investigation with three microRNAs: miR-142-3p, miR-148a-3p and miR-34a-5p which are all downregulated in mutant p53<sup>R172H</sup> expressing primary tissues (figure 3.4 and 3.5), suggesting that the change in microRNA expression is a genuine change which occurs in animals suffering from the PDAC; primary cell lines (figure 3.7), suggesting that the change in microRNA expression is occurring in the tumour cells, rather than in other structures or cell types within the tissue; and p53 null cells with ectopic expression of mutant p53<sup>R172H</sup> (figure 3.9), suggesting that the change is not due to the dominant negative ability of mutant p53<sup>R172H</sup>. In order to further investigate these three microRNAs, it is first necessary to understand their role in previous studies.

As I have shown that miR-142-3p is downregulated in all three experiment systems being utilised in this study (figures 3.4, 3.5, 3.7 and 3.9), I will investigate the current literature involving miR-142-3p regulation in cancer. MiR-142-3p has been shown to
be able to target RAS-related C3 botulinum toxin substrate 1 (RAC1) in the Hepatocellular Carcinoma (HCC) cell lines, which promotes invasion (Wu, Cai et al. 2011). Another microRNA which targets RAC1, miR-124, has been shown to be hypermethylated in PDAC, which leads to increased RAC1 activity, invasion and metastasis (Wang, Chen et al. 2013). These data show that miR-142-3p regulates RAC1 expression, and that dysregulation of RAC1 promotes invasion and metastasis in PDAC, and thus identifies a possible mechanism by which mutant p53R172H is able to induce invasion and metastasis. Other targets of miR-142-3p which have been implicated in invasion are HMGAl in osteosarcoma (Xu, Wang et al. 2014) and FZD7 in cervical cancer (Deng, Zhang et al. 2015). Heat-Shock Proteins 70 (HSP70) has been shown to be upregulated in PDAC, where it is believed to drive tumorigenesis through inhibition of apoptosis (Aghdassi, Phillips et al. 2007). The drug Triptolide has been shown to suppress HSP70 expression in PDAC, where it causes increased cell death and a reduction in tumour burden (Phillips, Dudeja et al. 2007). Interestingly, Triptolide has been shown to function in PDAC through driving expression of miR-142-3p, which inhibits HSP70 protein synthesis through direct binding to HSP70’s 3’UTR (MacKenzie, Mujumdar et al. 2013). These data, in combination with this present study, suggest a possible mechanism by which miR-142-3p downregulation by mutant p53R172H, may inhibit apoptosis in PDAC cells. Unfortunately, Triptolide is poorly soluble in water, which limits its clinical use. Minnelide, a water-soluble pro-drug of Triptolide is currently in phase 1 clinical trials (NCT01927965) for the treatment of pancreatic cancer. These studies highlight the fact that miR-142-3p is able to regulate a number of genes involved in invasion, making it an interesting target to investigate in more detail. Additionally, it offers the possibility of using drugs to induce microRNA expression in the treatment of PDAC.

As miR-148a-3p has been shown to be downregulated in the three model systems being utilised in this study, it is important to understand the characteristics of this microRNA, in order to link it to mutant p53R172H driven invasion and metastasis. A 2010 study found that miR-148a hypermethylation is an early event in PDAC progression with both PanINs and PDACs having low expression of miR-148a (Hanoun, Delpu et al. 2010). A later study reinforced this data and found that miR-148a was
downregulated in early stage PanINs, and became further downregulated in later stage PanINs and PDACs, even suggesting that miR-148 expression could be suitable as a diagnostic marker to differentiate healthy, early and late stage pancreatic tumours (Xue, Abou Tayoun et al. 2013). Another study analysed microRNA expression and related it back to survival in 225 human PDAC patients and found that low miR-148a correlated strongly with short overall survival (Schultz, Andersen et al. 2012). Another recent study found that miR-148a was downregulated in PDACs resulting in increased expression of its targets, cholecystokinin-B receptor (CCKBR) and B cell lymphoma-2 (BCL-2) (Zhang, Li et al. 2013), promoting tumour progression.

MiR-148a-3p has been shown to target a number of genes involved in inhibition of invasion including: ROCK1 (Xu, Jiang et al. 2012), sphingosine 1 phosphate receptor 1 (S1PR1) (Zhang, Liu 2014, Wen, Zhao et al. 2015) and Metastasis suppressor-1 (MTSS1) (Parr, Jiang 2009). Given the large number of microRNAs which were found to be downregulated in the mutant p53R172H expressing tissues (figures 3.4 and 3.5), it is very interesting that DNMT1 has also been shown to be a direct target of miR-148a-3p (Xu, Jiang et al. 2012, Zhu, Xia et al. 2011), including in pancreatic cancer (Zhan, Fang et al. 2015). Additionally, miR-148a-3p has been shown to be hypermethylated in a number of cancer types (Li, Chowdhury et al. 2014, Hanoun, Delpu et al. 2010). It is intriguing to speculate that interplay between mutant p53R172H, miR-148a-3p and DNMT1 may be involved in the invasion and metastasis associated with mutant p53R172H in PDAC. This hypothesis will be expanded in Chapter 5.

MiR-34a-5p was found to be downregulated in all three experimental systems which were used to interrogate microRNAs in this study. It is important to understand the current evidence as to miR-34a-5p’s function, in order to establish a relationship between this microRNA and mutant p53R172H-driven invasion and metastasis. MiR-34a is a member of the miR-34 family comprising: miR-34a, miR-34b and miR-34c (see section 1.4.9 for details). The entire family shares an identical seed sequence and a high degree of conservation throughout the remaining sequence, which would suggest that they have similar mRNA targets and some degree of redundancy.
Given that the miR-34 family has been well documented as being regulated by p53 (He, He et al. 2007, Bommer, Gerin et al. 2007, Raver-Shapira, Marciano et al. 2007) it is unsurprising that I have found that miR-34a-5p is downregulated by mutant p53$^{R172H}$ expressing tissues (figure 3.4 and 3.5) and cell lines (figure 3.7). However, exogenous expression of mutant p53$^{R172H}$, in p53 null cells also leads to a significant reduction in miR-34a expression (figure 3.9), suggesting loss of miR-34a-5p is not entirely due to loss of p53. As the p53 family members (p53, p63, p73) have some functional redundancy (see section 1.3.5.2 for details), it is possible that upon loss of p53, they are able to function as transcription factors for miR-34a-5p. As mutant p53$^{R172H}$ is able to function in a dominant negative manner upon the other p53 family members, a further reduction in miR-34a-5p could be explained via dominant negative inhibition of other p53 family members. This hypothesis will be explored in detail in Chapter 5.

It is interesting to note, that miR-34b and miR-34c do not have any significant change in expression in the tissues (see supplemental figure 3 for full microRNA microarray results).

One very interesting study investigated global microRNA profiles from patients who had undergone surgical resection of PDAC. The study associated microRNA expression with clinical aspects of the disease and found that reduced expression of miR-34a correlates very significantly with poor survival, with patients expressing high miR-34a having an average survival of 43.1 months, while those expressing low miR-34a had an average survival of just 13.4 months (Morton, Timpson et al. 2010). Interestingly, miR-34b and miR-34c expression did not correlate with any aspect of the disease investigated in the study (Jamieson, Morran et al. 2011). Here we have shown that expression of mutant p53$^{R172H}$ leads to decreased expression of miR-34a (figures 3.4, 3.5, 3.7 and 3.9) but not miR-34b or miR-34c (see supplemental figure 3 for full microarray results), which correlates well with the Jamieson study.

Downregulation of miR-34a has been shown to lead to increased expression of SNAIL1 and a subsequent repression of E-cadherin expression and increased invasion and metastasis (Cano, Diaz-Lopez et al. 2014). miR-34a has also been shown to target Interleukin 6 receptor (IL6R) where downregulation of miR-34a leads to increased IL6 activity and a subsequent invasive and metastatic phenotype in colorectal cancer.
(CRC) patients (Rokavec, Öner et al. 2014). As loss of miR-34a has been linked to invasion in other cancer types, and poor survival in PDAC, further study into the biological effect of miR-34a-5p loss in this model of PDAC is certainly worthwhile.

Having shown that all three of the microRNAs which are downregulated in all three experimental systems used in this study, have all previous links to cancer invasion, it is vitally important that the biological consequences of their loss in this model of PDAC is investigated. This will be carried out in Chapter 4.

3.4 Summary
- Mutant p53R172H expression results in dysregulation of microRNA expression, with 78% of the dysregulated microRNAs being downregulated.
- A large percentage (71%) of downregulated microRNAs have previously been shown to be hypermethylated in cancer.
- miR-142-3p, miR-148a-3p and miR-34a-5p are consistently downregulated by mutant p53R172H in three experimental models.
Chapter 4 Biological consequences of microRNA dysregulation in Pancreatic Ductal Adenocarcinoma

4.1 Introduction
Mutant p53 has been shown to induce an invasive and metastatic phenotype in a number of cancer models, including pancreatic cancer (Morton, Timpson et al. 2010) (see introduction section 1.3.5.2 for details). Importantly, a seminal paper was able to show that mutant p53 is able to promote invasion and metastasis through regulation of Dicer via inhibition of p63 transcriptional activity (Muller, Trinidad et al. 2013). As Dicer is a key component of microRNA processing (See section 1.4.3 for details) this strongly implicates the microRNA pathway in mutant p53’s pro-metastatic abilities. A number of other studies have associated microRNAs in invasion and metastasis, most notably the miR-200 family, which has been shown to have a profound effect on invasion through their targeting of a number of transcriptional regulators of E-cadherin, which induces an EMT phenotype (see section 1.2.2 for details on EMT). With this in mind, it is important to establish whether the microRNAs found to be downregulated by mutant p53 R172H in chapter 3, miR-142-3p, miR-148a-3p and miR-34a-5p, play a role in invasion. Interestingly, SNAIL, a gene which promotes EMT, and is regulated by the miR-200 family, has also been shown to be a direct target of miR-34a (Siemens, Jackstadt et al. 2011), suggesting a possible mechanism by which loss of miR-34a-5p could promote invasion in PDAC. Additionally, miR-142-3p has predicted target sites in the 3’ UTR’s of ZEB1 and ZEB2 (figure 4.1), suggesting that it has the potential to modulate EMT and invasion.
Loss of miR-148a-3p has been implicated as being involved in invasion through dysregulation of its targets, ROCK1 (Zheng, Liang et al. 2011), Sphingosine 1 Phosphate Receptor 1 (S1PR1) (Zhang, Liu 2014, Wen, Zhao et al. 2015) and Metastasis suppressor-1 (MTSS1) (Parr, Jiang 2009), though to this author’s knowledge no studies have previously found a link between miR-148a-3p expression and invasion in PDAC. It is important to note that miR-148a-3p has been linked to other deleterious processes in PDAC (see section 3.3 for details). To test the hypothesis that mutant p53R172H drives invasion and metastasis through downregulation of either miR-142-3p, miR-148a-3p or miR-34a-5p, the expression of these microRNAs was modulated in the primary cell lines provided by Professor Owen Sansom:

- K-RAS\textsuperscript{G12D};p53\textsuperscript{+/−}
- K-RAS\textsuperscript{G12D};p53\textsuperscript{R172H/+}

As mutant p53 expression has previously been shown to induce metastasis and invasion (see section 1.3.5.2 for details), siRNA depletion of p53 in the mutant p53 expressing cells was also carried out, with the hypothesis that it would reduce the invasive potential of the cells.

In Chapter 3, miRNAs were identified that were dysregulated due to expression of mutant p53, while this is very important, microRNAs do not have any physiological effect alone. The effect of a microRNA is governed by which mRNAs it is targeting for translational repression within a specific system. Therefore, the next logical step in
Figure 4-2: Reverse trans-well invasion assay investigating inhibition of miR-142-3p, miR-148a-3p and miR-34a-5p in p53 hemizygous cells. The K-RAS^{G12D};p53^{+/-} cells were transfected with a 2'-O-Me microRNA inhibitor against miR-142-3p, miR-148a-3p, miR-34a-5p or a control 2'-O-Me and their invasive ability was investigated using reverse trans-well migration assays. (a) A representative example of the z-stack images taken from each condition. Cells were considered as being invasive if they had detectable fluorescent signal beyond 30µm. (b) Three repeat experiments each consisting of two trans-wells, each having four z-stack images taken, were used for each condition. The average invasion per trans-well was calculated as the average fluorescence beyond 30µm of the four stacks for each trans-well. The average of the six trans-wells for each condition was reported as the percentage of invading cells, with the error bars representing the standard deviation of each condition. Statistical analysis: two sample, two tailed, unpaired t-tests comparing the six repeat experiments of a test condition to the six repeat experiments to the Ctrl 2'-O-Me condition, with n.s representing no significance, p<0.05 (*), p<0.01 (**) and p<0.005 (***)
delineating the importance of the dysregulated microRNAs is to identify their targets within this system. In order to address this, AGO2 immunoprecipitations and overexpression of the microRNAs followed by investigation of the mRNA target abundance was carried out.

4.2 Results

4.2.1 Inhibition of miR-148a-3p, but not miR-142-3p and miR-34a-5p, was sufficient to increase the invasive potential of K-RAS\textsuperscript{G12D};p53\textsuperscript{+/-} mouse PDAC cells

Chapter 3 of this investigation identified three microRNAs: miR-142-3p, miR-148a-3p and miR-34a-5p, as being statistically significantly downregulated by mutant p53 in tissues and cell lines (figures 3.4, 3.5, 3.7 and 3.9). As mutant p53 has previously been shown to induce invasion through interfering with Dicer via p63 (Muller, Trinidad et al. 2013), which presumably leads to downregulation of microRNAs, it was hypothesised that loss of one of the microRNAs identified in this study may be responsible for the invasive phenotype associated with mutant p53. If this hypothesis were correct, then it may be assumed that inhibition of the microRNA could lead to increased invasion without the need for mutant p53 expression. To test this hypothesis, the microRNAs being investigated in this study were inhibited using 2’-O-Me’s, in the p53 hemizygous cells, and reverse trans-well migration assays were performed. Compared to control, inhibition of miR-142-3p and miR-34a-5p resulted in very slight increases in invasion, while inhibition of miR-148a-3p resulted in a modest, though statistically significant increase in invasion (figure 4.2). The fact that loss of a single microRNA is able to induce an invasive phenotype in cells is potentially very important, and may suggest a mechanism by which mutant p53\textsuperscript{R172H} is able to promote invasion.

4.2.2 Overexpression of miR-148a, but not miR-142 or miR-34a, was able to decrease the invasive potential of mutant p53\textsuperscript{R172H} expressing mouse PDAC cells

Having established that inhibition of miR-148a-3p was able to increase the invasive potential of cells which are minimally invasive, it was important to investigate whether
Figure 4-3: Reverse trans-well invasion assay investigating overexpression of miR-142-3p, miR-148a-3p and miR-34a-5p in mutant p53R172H expressing cells. The K-RASG12D;p53R172H/+ cells were transfected with 1μg of an expression vector for either miR-142, miR-148a or miR-34a or a control expression vector. (a) Taqman RT-qPCR were used to quantify the expression of miR-142-3p, miR-148a-3p and miR-34a-5p in each condition 24 hours after transfection of the microRNA overexpression vector. The fold change represents the average relative quantity of each microRNA, in each condition, relative to the Ctrl vector transfected cells. The error bars represent the maximum and minimum relative quantity calculated using the standard deviation between ΔCT values for each condition (see section 2.2.6 for details on error calculations). Statistical analysis: a two sample, two tailed, paired t-test was used comparing the ΔCT values of the Ctrl vector to those of the test condition. All significance is denoted as n.s representing no significance, p<0.05 (*), p<0.01 (**) and p<0.005 (***) (b) A representative example of the z-stack images taken from each condition. Cells were considered as being invasive if they had detectable fluorescent signal beyond 30μm. (c) Three repeat experiments each consisting of two trans-wells, each having four z-stack images taken, were used for each condition. The average invasion beyond 30μm was calculated as the average of the four stacks for each trans-well. The average of the six trans-wells for each condition was reported as the percentage of invading cells with the error bars representing the standard deviation of the six trans-wells. Statistical analysis: two sample, two tailed, unpaired t-tests comparing the six repeat experiments of a test condition to the six repeat experiments to the Ctrl 2’-O-Me condition.
overexpression of this microRNA was able to reduce invasion in the mutant p53<sup>R172H</sup> expressing cell line. While inhibition of miR-142-3p and miR-34a-5p did not promote invasion in the minimally invasive K-RAS<sup>G12D</sup>;p53<sup>+/−</sup> cells, it is still possible that their overexpression in the invasive K-RAS<sup>G12D</sup>;p53<sup>R172H/+</sup> cell line may affect invasion. Therefore, the impact on invasion of overexpression of these microRNAs in the invasive mutant p53<sup>R172H</sup> expressing cells was investigated. The cells were transfected with an expression vector which contains a fragment of the primary microRNA of either; miR-142, miR-148a or miR-34a which are processed into mature, functional microRNAs, or a control vector with no insert (see section 2.3.8 for details on how the plasmids were produced, and table 2.11 for details on plasmids). Figure 4.3-a shows that each mature microRNA was upregulated compared to endogenous levels, as judged by RT-qPCR. While the degree of overexpression varied greatly between the microRNAs, this is presumably due to the basal endogenous levels of each microRNA compared to over-expression, resulting in a large relative change in expression. Importantly, overexpressing any of the microRNAs does not affect expression of any of the other microRNAs being investigated (figure 4.3-a). Compared to control, overexpression of miR-142-3p leads to a slight increase in the invasive ability of the cells while overexpression of miR-34a-5p leads to a very slight decrease in invasion. Importantly, overexpression of miR-148a-3p resulted in a substantial attenuation of invasion which was statistically significant (figures 4.3-b&c).

4.2.3 Effect of p53 depletion in K-RAS<sup>G12D</sup>;p53<sup>+/−</sup> and K-RAS<sup>G12D</sup>;p53<sup>R172H/+</sup> mouse PDAC cells

Having shown that it is possible to attenuate the invasive potential of the mutant p53 expressing cells by overexpressing miR-148a-3p, it may be assumed that directly depleting mutant p53 in these cells, would also reduce their invasive capacity. To test this hypothesis, p53 was depleted via siRNA in the K-RAS<sup>G12D</sup>;p53<sup>R172H/+</sup> cells and the effect on the invasive potential of the cells was investigated. The data shows that depletion of p53, in mutant p53<sup>R172H</sup> expressing cells (figure 4.4-a), led to a reduction in the percentage of invading cells, from 5% to 3%, although the results are not statistically significant (figure 4.4-b&c). As loss of wild type p53 has been shown to
Figure 4-4: Reverse trans-well invasion assay investigating depletion of p53 in mutant p53<sup>R172H</sup> expressing cells. The K-RAS<sup>G12D</sup>;p53<sup>R172H/+</sup> cells were transfected with an siRNA against p53 and their invasive potential was investigated using reverse trans-well migration assays. (a) A representative western blot showing depletion of p53 with β-actin as a loading control. (b) Each column is representative of the z-stacks investigated in this assay. Cells were considered as being invasive if they had detectable fluorescent signal beyond 30μm. (c) Each condition was investigated using 3 biological repeats, each consisting of two trans-wells with four z-stack images being taken for each transwell. An average of the six trans-wells was reported as the average percentage of invading cells, with the error bars representing the standard deviation between the six transwells. Statistical analysis: A two sample, two tailed, unpaired t-test was used to compare the six repeats from a the control transfected cells to the p53 siRNA transfected cells, with n.s representing no significant difference, p<0.05 (*), p<0.01 (**) and p<0.005 (***) (c). Knockdown of p53 was assessed using western blotting for p53 and β-actin.
Figure 4-5: Reverse trans-well invasion assay investigating depletion of p53 in p53 hemizygous cells. The K-RAS<sup>G12D</sup>,p53<sup>−/−</sup> cells were transfected with an siRNA against p53 and their invasive potential was investigated using reverse trans-well migration assays. (a) A representative RT-qPCR showing depletion of p53 mRNA. The ΔΔCT method was used to calculate a fold change between the ctrl siRNA group and the p53 siRNA group using β-actin as a normalisation gene. (b) Each column is representative of the z-stack images used in this assay. Cells were considered as being invasive if they had detectable fluorescent signal beyond 30μm. (c) Each condition was investigated using three independent experimental repeats, each consisting of two transwells with four z-stack images being taken for each transwell. An average invasion of each trans-wells was calculated as the average fluorescence past 30μm for the four z-stack images. An average invasion calculated from the six transwells was reported as the average invasion for each condition, with the error bars representing the standard deviation between the six transwells for each condition. Statistical analysis: A two sample, two tailed, unpaired t-test was used to compare the six repeats from a the control transfected cells to the p53 siRNA transfected cells, with n.s representing no significant difference, p<0.05 (*), p<0.01 (**) and p<0.005 (***)
promote invasion (Wang, Wang et al. 2009), siRNA mediated depletion of p53 in the p53 hemizygous cells was carried out, and the effect on invasion observed. Due to the low expression of p53 in these cells, it was not possible to show p53 depletion at the protein level. Instead, RT-qPCR was used to show that p53 had been sufficiently knocked down (figure 4.5-a). The data shows that depletion of p53 in p53 hemizygous cells leads to a statistically significant increase in invasion (figure 4.5-b&c). The degree of change in invasion is modest, with 0.04% of cells invading in the control group and 0.07% of cells invading when p53 is knocked down. However, this is still almost a 2 fold increase, and given that metastasis only requires a single cell to establish secondary tumour sites, it may still have some biological significance.

4.2.4 In-silico microRNA target prediction using a number of online resources
Having identified miR-148a-3p as being downregulated by mutant p53R172H which promotes an invasive phenotype, it is important to identify the mRNA targets which are causing this effect. While miR-142-3p and miR-34a-5p were not able to modulate invasion, it is possible that they are involved in some other aspect of PDAC progression, so their mRNA targets will also be investigated.

A large number of online resources are available for prediction of microRNA:mRNA interactions. This study used StarBase (http://starbase.sysu.edu.cn) which collates predictions from five well known online resources (Targetscan, Pictar, RNA22, PITA and miRanda). Putative mRNA targets of mouse miR-142-3p, miR-148a-3p and miR-34a-5p were identified using the mouse mm10 genome build. Predicted targets were only considered if at least three of the prediction programmes agreed that an MRE for the microRNA in question was present in the 3’ UTR of the target. For each microRNA, ten mRNAs with predicted MREs were selected, and primer pairs designed for RT-qPCR (see Table 2.2 for primer details, and methods section 2.2.5 for details on Sybr green RT-qPCR). These primers were then utilised to identify the presence of these target mRNAs in AGO2 immunoprecipitations (AGO2 IPs) and RT-qPCR following overexpression of the microRNAs of interest.
4.2.5 Endogenous AGO2 IP optimisation
As each microRNA is only ~22nts in length, and the seed sequence responsible for mRNA target interactions is only 8nts in length, each microRNA has hundreds of predicted interaction sites within the 3’ UTRs of gene. Realistically, only a fraction of these putative interaction sites will be functional MREs. Having identified a number of putative targets for miR-142-3p, miR-148a-3p and miR-34a-5p, it was necessary to establish which, if any of them are *bona-fide* targets of the microRNAs. In order to identify *bona-fide* targets of the microRNAs in question, AGO2 immunoprecipitations were carried out (see methods section 2.4.2 for details). In order to optimise the procedure, miR-142-3p was selected as it was downregulated by the greatest degree in the mutant p53<sup>R172H</sup> expressing tissue samples (Figure 3.4 and 3.5) and cell lines (figure 3.7 and 3.9). As microRNAs interact with mRNAs through Watson-Crick base-pairing, 2’-O-Mes against a particular microRNA will block microRNA:mRNA interactions, and prevent association of *bona-fide* targets of the microRNA with the RISC. Immunoprecipitation of AGO2 followed by analysis of associated mRNA abundance, will allow identification of true microRNA:mRNA interactions. This will also result in the microRNA-targeted mRNA being upregulated in total cellular abundance, due to reduced microRNA-mediated mRNA degradation.

U6, an abundant small RNA involved in splicing, should not be associated with the RISC and was used as a negative control to show that any enrichment of mRNAs in the AGO2 IPs were specific. β-actin and GAPDH were also used as negative controls, as they are abundant mRNAs which are *bona-fide* microRNA targets (Sikand, Singh et al. 2012), but are not known to be, or are predicted to be, targets of any of the microRNAs being investigated in this study.
Figure 4-6 First AGO2 immunoprecipitation (IP) to assess predicted miR-142-3p mRNA target. (a) Protein expression analysis investigating AGO2 and β-actin were carried out on the inputs and supernatants, while only AGO2 was investigated for the IPs. The inputs and supernatants are relative to 10% of the amount of protein used for the IPs. Taqman RT-qPCR was carried out for miR-142-3p (b), U6 (c), β-actin and GAPDH (d) as controls. A number of predicted miR-142-3p targets (e) were investigated using RT-qPCR. Calculations of all RT-qPCR for inputs was carried out using the ΔΔC\text{T} method with microRNAs being normalised to U6 and mRNAs being normalised to the geometric mean of β-actin and GAPDH, with the fold change being relative to the cells transfected with a control 2'-O-Me. All input graphs are blue. All RT-qPCR for IP samples was quantified using the ΔC\text{T} method with fold changes being relative to the control AGO2 IP levels. All IP graphs are red.
Western blotting for the initial optimisation experiment confirmed that AGO2 had been successfully immunoprecipitated from the lysate, with the supernatant post IP having a marked reduction in AGO2 (figure 4.6-a). The western blot of the input showed there was no significant change in AGO2 expression following inhibition of miR-142-3p. Taqman RT-qPCR was used to assess the amount of total and immunoprecipitated miR-142-3p and showed a ~2 fold reduction in RISC associated miR-142-3p, and a slight reduction in total levels of miR-142-3p. It also showed a very large enrichment in miR-142-3p levels in the AGO2 IP compared to the IgG control IPs (figure 4.6-b). Additionally, while there was some fluctuation in U6 levels, there was no enrichment in the AGO2 IPs compared to the IgG IPs (figure 4.6-c). As U6 should not be specifically immunoprecipitated with AGO2 or the IgG control, the variability is due to background noise and should not be of consequence. Both β-actin and GAPDH showed small and insignificant changes in both total levels and RISC incorporation. Unfortunately, the enrichment of both β-actin and GAPDH in the AGO2 IPs compared to the IgG IPs, was only ~4 fold (figure 4.6-d). This is highly suggestive of high background binding to the beads. Three mRNAs with predicted miR-142-3p target sites in their 3’ UTR: Integrin αV (ITGAV), Adenylate Cyclase 9 (ADCY9) and Transforming Growth Factor β Receptor 1 (TGFβR1), were then investigated and also found to have poor enrichment in the RISC (figure 4.6-e). Given the high background levels, this experiment required further optimisation.

The 2\textsuperscript{nd} attempt at optimising the endogenous AGO2 IPs included a more stringent wash step (increased from simply pipetting the beads in lysis buffer, to 3 minutes of end over end rotation at 4\textdegree C) following incubation with lysates, in order to improve the signal to noise ratio. The western blot for the inputs suggests that there was an increase in total AGO2 protein expression in the cells following inhibition of miR-142-3p (figure 4.7-a). However, none of the other experiments, inhibiting or
Figure 4-7 AGO2 IP to assess predicted miR-142-3p mRNA target RISC association following miR-142-3p 2'-O-Me treatment. This AGO2 IP implemented a more stringent wash step than was carried out previously. (a) Western blot for AGO2 and β-actin were carried out on the inputs and supernatants, while only AGO2 was investigated for the IPs. The inputs and supernatants are relative to 10% of the amount of protein used for the IPs. (b) Taqman q-PCR was carried out for miR-142-3p to ensure that there was a reduction in RISC associated miR-142-3p as well as to show a marked enrichment of the AGO2 associated microRNA compared to the IgG control. (c) U6 and sno202 are small RNAs which should not be differentially expressed by inhibition of miR-142-3p and should not be RISC incorporated. (d) B-actin and GAPDH are both bona-fide targets of microRNAs but not of miR-142-3p so act as negative controls in this experiment. (e) A number of predicted mRNA targets of miR-142-3p were investigated to assess any change in RISC association when miR-142-3p is unable to bind due to interactions with a 2'-O-Me against miR-142-3p. All q-PCR for inputs was carried out using the ΔΔCT method with microRNAs being normalised to U6 and mRNAs being normalised to the geometric mean of B-actin and GAPDH. All q-PCR for IP samples was not normalised to a control gene and was quantified using the ΔCT method with fold changes being relative to the control AGO2 IP levels. All blue graphs represent inputs while all red graphs represent immunoprecipitations.
overexpressing miR-142 (figures 4.6, 4.8, 4.9 and 4.10), showed any change in total AGO2 levels, suggesting it may be an artefact in this specific experiment. The loading control (β-actin) for the AGO2 lane in the input was also increased in comparison to the control lane, so could go some way to explain the anomaly (figure 4.7-a). The increased stringency of the wash improved the clarity of the IP lanes on a western blot, while also improving the enrichment of the β-actin and GAPDH, in the RT-qPCR (figure 4.7-d), as well as the putative miR-142-3p targets (figure 4.7-e). Due to the improvement in enrichment and clarity of the western blot, the increased stringency of wash steps was implemented for all further IP experiments. There was no change in miR-142-3p expression in the total lysate but a ~2 fold reduction in the AGO2 IPs. Additionally, there was a marked enrichment of miR-142-3p in the AGO2 IPs compared to the IgG IPs (figure 4.7-b). In this experiment, U6 was behaving as if it was specifically RISC associated, which it should not be, suggesting that something had failed in the IgG IPs, making then unsuitable as controls and making analysis of RISC association in this IP impossible (figure 4.7-c). In order to check this result, another small RNA which should not be RISC associated, sno202, was assayed and showed the same result (figure 4.7-c). A selection of 6 predicted targets of miR-142-3p: ADCY9, ITGAV, Lysine Acetyltransferase 7 (MYST2), Ras-Related C3 botulinum toxin substrate 1 (RAC1), TGFβR1 and Vesicle-Associated Membrane Protein 3 (VAMP3) were also investigated. Unfortunately, none of these showed significant change in AGO2 association or total cellular abundance (Figure 4.7-e). As the predicted miR-142-3p targets had similar degree of enrichment to U6 and sno202, they are unlikely to be trustworthy results. Due to these issues, the experiment was not taken any further.

The 3rd attempt at optimising the AGO2 immunoprecipitations implemented overexpression of miR-142, rather than inhibition. This change in protocol was attempted in order to check that the 2’-O-Me’s used in the previous experiments were not the cause of the unexpected experimental results. The western blot of the immunoprecipitations showed that AGO2 was specifically immunoprecipitated with the AGO2 antibody coated beads, with none present in the control IgG beads. The input showed no significant difference in total AGO2 protein levels in the cells following miR-142 overexpression (figure 4.8-a). Taqman RT-qPCR for miR-142-3p
Figure 4-8 AGO2 IP to assess predicted miR-142-3p mRNA target RISC association following overexpression of miR-142-3p. (a) Western blots for AGO2 and β-actin were carried out on the inputs, while only AGO2 was investigated for the IPs. The inputs are relative to 10% of the amount of protein used for the IPs. (b) Taqman q-PCR was carried out for miR-142-3p to ensure that there was an increase in RISC associated miR-142-3p as well as to show a marked enrichment of the AGO2 associated microRNA compared to the IgG control. (c) U6 is a small RNA which should not be differentially expressed by inhibition of miR-142-3p and should not be RISC incorporated. (d) B-actin and GAPDH are both bona-fide targets of microRNAs but not of miR-142-3p so act as negative controls in this experiment. (e) A number of predicted mRNA targets of miR-142-3p were investigated to assess any change in RISC association when miR-142-3p is overexpressed. All q-PCR for inputs was carried out using the ΔΔCT method with microRNAs being normalised to U6 and mRNAs being normalised to the geometric mean of B-actin and GAPDH. All q-PCR for IP samples was not normalised to a control gene and was quantified using the ΔCT method with fold changes being relative to the control AGO2 IP levels. All inputs are represented by blue graphs and all immunoprecipitations are represented by red graphs.
showed a large increase in miR-142-3p expression in the total and immunoprecipitated samples which were transfected with the miR-142 expression vector (figure 4.8-b). U6 was not significantly changing in the total or Immunoprecipitated samples and showed no enrichment in the AGO2 immunoprecipitated samples over the IgG (figure 4.8-c). Both β-actin and GAPDH showed no significant change in expression in the inputs or AGO2 IP samples when miR-142 is overexpressed, though they did show significant enrichment in the AGO2 IP compared to the IgG IPs (figure 4.8-d). A selection of predicted miR-142-3p targets: Acyl-CoA Synthetase Long-chain 4 (ACSL4), Absent, Small or Homeotic 1 - Like (ASH1L), ITGAV, TGFβR1, VAMP3 and ZEB1, were investigated, but none show the reduction in RISC incorporation which would be expected from bona-fide miR-142-3p target (figure 4.8-e). ITGAV and ACSL4 show a modest reduction of expression in the inputs as would be expected of a bona-fide target (figure 4.8-e) but without the change in RISC incorporation, the results are inconclusive.

### 4.2.6 FLAG tagged AGO2 immunoprecipitation optimisation

As investigation into microRNA targets using endogenous immunoprecipitations had not yielded any meaningful results, immunoprecipitation of transiently transfected FLAG tagged AGO2 was attempted. As FLAG peptide may be used to elute the FLAGAGO2 and RISC components following the IP, rather than relying on boiling the beads, a much cleaner sample can be obtained which should yield more accurate results, and remove any issues brought about by background binding of RNAs to the beads. Cells were transfected with the 2′-O-Me against miR-142-3p and 24 hours later were transfected with a FLAG-AGO2 expressing construct and allowed to express for a further 24 hours, before being immunoprecipitated using FLAG conjugated magnetic beads (please see section 2.4.3 for details). The western blot of the input shows that overexpression of FLAG-AGO2 was not significantly different between conditions. The supernatant showed that the majority of FLAG-AGO2 had been successfully immunoprecipitated from the sample, while the IP lanes showed roughly equal amounts of FLAG-AGO2 in the immunoprecipitations (figure 5.9-a). The RT-qPCR showed that miR-142-3p was slightly elevated in the total RNA level but was
a

AG02/FLAG

* GAPDH

10% input | Immunoprecipitations | 10% supernatants

b

miR-142-3p inputs

Fold Change

miR-142-3p IPs

Fold Change

C

B-actin inputs

Fold Change

B-actin IPs

Fold Change

GAPDH inputs

Fold Change

GAPDH IPs

Fold Change
Figure 4-9 FLAG tagged AGO2 immunoprecipitation (IP) to assess predicted miR-142-3p mRNA target RISC association following miR-142-3p 2’-O-Me treatment. This FLAG IP implemented M2 conjugated magnetic beads against FLAG (see section 2.4.3 for details) (a) Western blot for FLAG and β-actin were carried out on the inputs and supernatants, while only FLAG was investigated for the IPs. The inputs and supernatants are relative to 10% of the amount of protein used for the IPs. (b) Taqman q-PCR was carried out for miR-142-3p to ensure that there was a reduction in RISC associated miR-142-3p. (c) β-actin and GAPDH are both bona-fide targets of microRNAs but not of miR-142-3p so act as negative controls in this experiment. (d) A number of predicted mRNA targets of miR-142-3p were investigated to assess any change in RISC association when miR-142-3p is unable to bind due to interactions with a 2’-O-Me against miR-142-3p. All q-PCR for inputs was carried out using the ΔΔCT method with microRNAs being normalised to U6 and mRNAs being normalised to the geometric mean of B-actin and GAPDH. All q-PCR for IP samples was not normalised to a control gene and was quantified using the ΔCT method with fold changes being relative to the control AGO2 IP levels. All inputs are represented by blue graphs while all immunoprecipitations are represented by red graphs.
downregulated by roughly 2 fold in the RISC (figure 5.9-b). The negative controls, β-actin and GAPDH were downregulated in both the inputs and FLAG/AGO2 immunoprecipitations (figure 5.9-c). A selection of five predicted miR-142-3p targets: ACSL4, HSP70, ITGAV, TGFβR1 and Utrophin (UTRN), were also investigated, but no change in total abundance was observed (figure 5.9-d). While they were all downregulated to some degree, as would be expected of a bona-fide miR-142-3p target, as the negative control follows a similar pattern, it would seem there was no difference between conditions.

4.2.7 RT-qPCR of predicted targets of miR-142-3p, miR-148a-3p and miR-34a-5p
As the use of both endogenous and FLAG tagged AGO2 IPs had failed to identify any targets of miR-142-3p, a different approach was taken. Instead, overexpression of the microRNAs of interest was carried out, and any changes in the abundance of predicted mRNA targets were investigated. Total RNA for this experiment came from the K-RAS<sup>G12D</sup>;p53<sup>R172H/+</sup> cells which were transfected with microRNA overexpression vectors, to be used in the invasion assays (see section 4.2.2). As I have shown that the cells which were transfected with an expression vector for miR-148a had an attenuated invasive capacity, any change in mRNAs may be involved in this attenuation of invasion. miR-142-3p was overexpressed by an average of ~550 fold (figure 4.3-a). Of the ten predicted targets, only TGFβR1 changed in expression, however, it was upregulated by overexpression of miR-142-3p, rather than downregulated, as would have been expected (figure 4.10). It is possible that miR-142-3p does not affect TGFβR1 directly but may target a negative regulator of TGFBR1. ADCY9 was downregulated but due to high degree of variability between results, the average fold change was not statistically significant.

MiR-148a-3p was overexpressed by an average of ~14 fold (figure 4.3-a). Of the 10 predicted targets of miR-148a-3p only Matrix metalloproteinase 15 (MMP15) was downregulated to a statistically significant degree (figure 4.11). While all MMPs are involved in remodelling the extra cellular matrix (ECM), most are secreted into the ECM being cleaved by extracellular proteases. However, MMP15 is different, in that it
Figure 4-10: Overexpression of miR-142. Putative targets of miR-142-3p were investigated using q-PCR. Cells were transfected with an expression vector for miR-142 and the relative quantity of each predicted target was calculated. The data were normalised to the geometric mean of the CT values for β-actin and GAPDH using the ΔΔCT method. Fold changes are the average relative quantity of each mRNA from 3 repeat experiments. The error bars represent the maximum relative quantity calculated using the standard deviation of the ΔCT values for each group (see methods for details). Statistical significance was established using a two sample, two tailed, paired t-test comparing the ΔCT values for each group with n.s representing no significant change, p<0.05 (*), p<0.01 (**) and p<0.005 (***)
Figure 4-11: Overexpression of miR-148a Putative targets of miR-148a-3p were investigated using q-PCR. Cells were transfected with an expression vector for miR-148a and the relative quantity of each predicted target was calculated. The data were normalised to the geometric mean of the CT values for β-actin and GAPDH using the ΔΔCT method. Fold changes are the average relative quantity of each mRNA from 3 repeat experiments. The error bars represent the maximum relative quantity calculated using the standard deviation of the ΔCT values for each group (see methods for details). Statistical significance was established using a two sample, two tailed, paired t-test comparing the ΔCT values for each group with n.s representing no significant change, p<0.05 (*), p<0.01 (**) and p<0.005 (***).
Figure 4-12: Overexpression of miR-34a. Putative targets of miR-34a-5p were investigated using q-PCR. Cells were transfected with an expression vector for miR-34a and the relative quantity of each predicted target was calculated. The data were normalised to the geometric mean of the CT values for β-actin and GAPDH using the ΔΔCT method. Fold changes are the average relative quantity of each mRNA from 3 repeat experiments. The error bars represent the maximum relative quantity calculated using the standard deviation of the ΔCT values for each group (see methods for details). Statistical significance was established using a two sample, two tailed, paired t-test comparing the ΔCT values for each group with n.s representing no significant change, p<0.05 (*), p<0.01 (**), and p<0.005 (***)

![Graphs showing fold change for various targets](image-url)
is a member of the membrane bound MMPs which includes: MMPs, 14, 15, 16, 17, 24 and 25. B-cell Lymphoma 2 like 11 (BIM) was found to be significantly upregulated following overexpression of miR-148a-3p (figure 4.11). This may be due to miR-148a-3p targeting a negative regulator of BIM, allowing BIM expression to increase.

MiR-34a-5p was overexpressed by an average of ~5 fold (figure 4.3-a). Unfortunately, only six of the ten predicted targets of miR-34a-5p gave acceptable melt curves when optimised. None of the mRNAs were significantly altered in expression following overexpression of miR-34a-5p, suggesting none are bona-fide targets of miR-34a in this system (figure 4.12).

It is important to note that only mRNA expression was investigated, so changes in protein expression cannot be directly inferred. As the cells are only overexpressing the microRNA for 24 hours prior to RNA expression analysis, it is possible that the mRNAs of the predicted targets are not degraded, while the protein expression may have changed. Without a panel of antibodies, or the use of another technique such as Stable Isotope Labelling by Amino acids in Cell culture (SILAC) mass spectrometry, it is not possible to look at changes in protein expression.

4.3 Discussion
In Chapter 3 I presented data which identified three microRNAs which were consistently downregulated by mutant p53R172H (figures 3.4, 3.5, 3.7 and 3.9). As mutant p53 has been shown to promote an invasive phenotype, potentially by inhibiting Dicer function (Muller, Trinidad et al. 2013) and presumably inhibiting microRNA processing, I postulated that loss of either miR-142-3p, miR-148a-3p or miR-34a-5p could be responsible for the invasive phenotype associated with mutant p53R172H expression. In order to assess the microRNAs’ impact on invasion, the microRNAs were inhibited using 2’-O-Mes in p53 hemizygous cells and overexpressed in mutant p53R172H expressing cells, before reverse trans-well invasion assays were used to investigate the invasive potential of the cells. Only inhibition of miR-148a-3p was able to significantly increase the invasive potential of p53 hemizygous cells (figure 4.2), and decrease invasion in mutant p53R172H expressing cells when overexpressed
MiR-148a-3p has been implicated in invasion in a number of tumour types, including ovarian (Wen, Zhao et al. 2015) and hepatocellular carcinoma (Zhang, Liu 2014). While downregulation of miR-148a is well documented in PDAC, to the best of my knowledge, miR-148a-3p has not previously been reported as being associated with invasiveness in PDAC. Additionally, an association between miR-148a-3p and mutant p53\textsuperscript{R172H} has not previously been shown.

It is important to note that the overexpression vectors used in these experiments will result in the expression of both the 3p and 5p arms of a particular microRNA. For example when cells are transfected with the miR-148 expression vector, both miR-148a-3p and miR-148a-5p will be expressed. This study did not investigate the effect of miR-148a-5p and cannot rule out the possibility that it is responsible for the reduction of invasion in the mutant p53\textsuperscript{R172H} expressing cells. However, given that inhibition of miR-148a-3p was able to induce invasion in the p53 hemizygous cells (figure 4.2), it is not beyond reason to suggest that miR-148a-3p is responsible for the decrease in invasion observed when miR-148a is overexpressed (figure 4.3).

This study found that altering miR-34a-5p expression had no significant effect on the invasive potential of pancreatic cancer cell lines (figures 4.2 & 4.3). However, a previous study was able to show that exogenous expression of miR-34a in a mouse model of PDAC reduces invasion and promotes apoptosis (Ji, Hao et al. 2009). Importantly, the Ji study was overexpressing the entire miR-34 family, rather than just miR-34a, which may suggest a synergistic effect, requiring high expression of all miR-34 family members is necessary to attenuate invasion. While no change in miR-34b or miR-34c expression was observed in the primary tissue microarrays (see supplemental figure 3 for full microarray results) it is possible that these genes are downregulated in the primary cell lines used in the invasion experiment however, this has not been addressed in this study.

This current study also found no change in invasion associated with modulation of miR-142-3p (figures 4.2 and 4.3). Interestingly, miR-142-3p has been associated with inhibition of invasion in hepatocellular carcinoma (Wu, Cai et al. 2011), cervical cancer...
(Deng, Zhang et al. 2015) and osteosarcoma (Xu, Wang et al. 2014), potentially suggesting a cell type specific role for miR-142-3p in inhibiting invasion.

While neither miR-142-3p nor miR-34a-5p were found to influence the invasive potential of cells in this study, it does not mean that they are not important in PDAC progression and metastasis. Invasion is not the only mechanism which may influence metastasis. It may be that miR-142-3p and miR-34a-5p are involved in another mechanism, such as evading the immune system, or allowing the cells to survive in the vascular environment through which they must travel to form secondary tumour sites. Unfortunately, without an animal model, these questions cannot be answered directly.

Having shown that inhibition of miR-148a-3p is able to reduce the invasive potential of mutant p53R172H expressing PDAC cells, it was expected that directly depleting mutant p53R172H would be able to inhibit invasion in these cells. Interestingly, only a slight reduction in invasion was observed following depletion of p53 in mutant p53R172H expressing cells (figure 4.4). Mutations which interrupt wild type p53 function have been shown to promote invasion. A 2009 study showed that MDM2 is able to target SLUG for degradation (Wang, Wang et al. 2009). Loss of p53 leads to reduced MDM2, and therefore increased expression of SLUG, subsequent transcriptional repression of E-cadherin and an invasive phenotype. A later study found that MDM2 is also able to target SNAIL for degradation in a similar manner in HCC (Lim, Kim et al. 2010). An interesting previous study showed that wild type p53 leads to inhibition of MET signalling, partly through p53-dependent expression of miR-34, as well as direct, transcriptionally inhibitory, binding of p53 to MET promoter, in primary Ovarian Surface Epithelial cells (OSE) (Hwang, Matoso et al. 2011).

As inhibition of miR-148a-3p decreases the invasive potential of mutant p53R172H expressing cells (figure 4.3), while depletion of mutant p53R172H itself has a much smaller effect (figure 4.4), it suggests a vital role in invasion for miR-148a-3p. It is possible that directly inhibiting p53 is not a viable therapeutic approach. It may be that increasing expression of miR-148a-3p is a more suitable therapeutic possibility. It would be interesting to investigate the expression of miR-148a-3p following depletion...
of mutant p53<sup>R172H</sup> in the K-RAS<sup>G12D</sup>;p53<sup>R172H/+</sup> cells. If its expression does not sufficiently increase in the 72 hours in which the invasion assay occurs, it may be insufficient to alter the invasive potential of the cells. While a previous study found that siRNA targeting of mutant p53 has an effect on cell cycle and apoptosis (Zhu, Yang et al. 2013), to my knowledge, none have found a change in invasion.

Having found that at least one of the microRNAs identified as being dysregulated by mutant p53<sup>R172H</sup> is able to inhibit invasion, it was important to identify bona-fide mRNA targets which may promote invasion. As miR-142-3p was the most downregulated of the microRNAs, it was used to optimise AGO2 Immunoprecipitations, with the aim of identifying mRNA targets. All of the AGO2 IPs carried out for this study suffered from significant technical issues, and while some of these were addressed by protocol modifications or repeating the experiment, the technique was not sufficiently optimised to yield any reliable results (figures 4.6, 4.7, 4.8 and 4.9). It is possible that these types of experiments are not suitable for establishing microRNA interactions. If mRNAs are targeted by a number of microRNAs, it is possible that any change in RISC incorporation may be very small due to compensatory regulation by other microRNAs which target the same mRNA. Additionally, the 2′-O-Mes are only present in the cells for 24 hours prior to lysis. Therefore if the majority of RISC associated miR-142-3p is already bound to mRNAs, there may not be time for sufficient competition between 2′-O-Me binding and mRNA binding. Also, using this technique, it is not possible to assess whether microRNA:mRNA interactions are occurring within the cell or post lysis. The IP step takes 4 hours, in which time free AGO2 bound microRNAs may bind to mRNAs, which they would not under normal physiological conditions. It is also possible that AGO2 bound microRNA dissociate from mRNAs during the IP, and potentially become associated with different mRNA transcripts. These issues may be solved by using a cross linking technique prior to the IP, which should maintain any microRNA:mRNA interactions post lysis.

Two AGO2 immunoprecipitation experiments were attempted where miR-142-3p was overexpressed rather than inhibited. The hypothesis was that the 2′-O-Me may be becoming detached from the microRNA post lysis, allowing the microRNA to bind
mRNA during the immunoprecipitation step. Another possibility is that competition for miR-142-3p binding is increased following lysis potentially due to the loss of cellular localisation of mRNAs. By overexpressing the microRNA it was possible to see an increase in RISC associated miR-142-3p, which should lead to a reduction in miR-142-3p mRNA target abundance and potentially an increase in RISC associated miR-142-3p mRNA targets. This hypothesis does depend on the rate of processing of the microRNA following transfection. The cells were lysed 24 hours post transfection, and the vector must translocate to the nucleus, be transcribed, and the primary microRNA transcript processed into a mature microRNA before it can become RISC associated. This could mean an increased level of mature miR-142-3p was only present in the RISC for a short time prior to the IP and that mRNA degradation may not have occurred.

An important possibility is that none of the predicted mRNAs are bona-fide targets of miR-142-3p. However, a number of the predicted miR-142-3p targets had previously been validated as being true targets in other models. RAC1 has been shown to be a miR-142-3p target in Hepatocellular carcinoma (Wu, Cai et al. 2011), and arteriosclerosis obliterans (Liu, Li et al. 2014). ADCY9 has also previously been shown to be a validated target of miR-142-3p (Huang, Zhao et al. 2008).

While this current study was unable to successfully implement this assay, numerous studies have implemented this technique with success to identify microRNA targets (Karginov, Conaco et al. 2007, Thomson, Bracken et al. 2011), therefore it may be an issue with miR-142-3p itself, especially given its low abundance in these cells. It may have been useful to attempt the same experiment using a 2′-O-Me or overexpression of miR-148a-3p or miR-34a-5p, to ensure that it wasn’t a microRNA specific issue. Since, the experiment is very time consuming and expensive, other options for investigating microRNA:mRNA interactions were attempted instead.

MMP15 was identified as being significantly downregulated when miR-148a is overexpressed (figure 5.11). Upregulation of MMP15 has been linked to invasion and metastasis in Ovarian carcinoma (Lin, Xu et al. 2013) and increased tumour grade in breast cancer (Benson, Babu et al. 2013). Of interest to this current study, MMP15 has been shown to be upregulated in pancreatic tumour tissue, compared to both
healthy pancreatic tissue and chronic pancreatitis (Ellenrieder, Alber et al. 2000). The same study also showed correlation between high MMP15 expression and increased invasion in in-vitro assays. As well as being linked to invasion, MMP15 has also been shown to have anti-apoptotic functions (Abraham, Schafer et al. 2005). Unfortunately, it has not been possible to find an antibody against MMP15 which works in the mouse cells being used in this investigation, so no validation of this interaction has been possible so far.

Unexpectedly, BIM is found to be significantly upregulated following overexpression of miR-148a-3p in mutant p53 expressing cells. As the direction of change is opposite to what would be expected of a bona-fide miR-148a-3p target, it is possible that miR-148a-3p targets a negative regulator of BIM.

4.4 Summary

- Loss of p53 is able to promote invasion in pancreatic cancer cells with a background K-RAS mutation.
- siRNA mediated Depletion of mutant p53\(^{R172H}\) does not significantly affect the invasive potential of cells.
- Inhibition of miR-148a-3p causes a modest increase in invasion.
- Overexpression of miR-148a-3p significantly reduces invasion.
- MMP15 may be a target of miR-148a-3p.
Chapter 5  Investigation into the mechanisms of microRNA dysregulation, and tissue localisation of microRNAs in PDAC.

5.1 Introduction
Chapters 3 and 4 have characterised three microRNAs which are downregulated in tumours (figure 3.4 and 3.5) and cell lines (figure 3.7 and 3.9) expressing mutant p53<sup>R172H</sup>. Importantly, the 2'-O-Me mediated inhibition of one of these microRNAs, miR-148a-3p, has the capacity to induce an invasive phenotype in cells which are normally non-invasive (figure 4.2). Additionally, overexpression of miR-148a is able to attenuate the invasive ability of mutant p53<sup>R172H</sup> expressing cells (figure 4.3). As p53 is a well described transcription factor, it may be assumed that mutant p53<sup>R172H</sup> may perturb expression of microRNAs through a loss of wild type p53 function, and by exerting its dominant negative abilities. However, previous studies have shown that mutant p53 has gain of function characteristics which promote metastasis, a phenotype not observed following loss of p53 alone (see introduction section 1.3.5.2 for details). A number of hypotheses have been postulated to explain the mechanism behind this gain of function. A previous study showed that mutant p53 is able to interfere with Drosha interactions with p68, leading to reduced expression of a subset of microRNAs (Suzuki, Yamagata et al. 2009). Another found that that mutant p53 is able to inhibit p63 transcriptional function leading to attenuation of Dicer expression (Muller, Trinidad et al. 2013). This would presumably lead to reduced microRNA processing and downregulation of one or a number of microRNAs, as is seen in non-functional Dicer mutants (Cummins, He et al. 2006). It is possible that mutant p53<sup>R172H</sup> expression, through inhibition of p63 and Dicer, leads to loss of the microRNAs identified in this study. The involvement of p63 and p73 will be investigated in this chapter. Additionally expression of the microRNA processing components Drosha, Dicer and AGO2 will be investigated.
One of the microRNAs identified in this study, miR-142, has been shown to undergo RNA editing, which inhibits its processing through inhibition of Drosha binding (Yang, Chendrimada et al. 2005) (see section 1.5 for details on RNA editing). This chapter will investigate the mRNA expression of components of the RNA editing machinery, ADAR1 and ADAR2 in mutant p53<sup>R172H</sup> expressing tissues and cell lines.

As outlined in Chapter 3, a large number of the microRNA genes identified in this study have been shown to be hypermethylated in cancers, including PDAC, suggesting a possible role for DNMT1 in mutant p53’s gain of function (see sections 3.3 for details). Interestingly, DNMT1 has been shown to be a target of miR-148a-3p in other systems (Xu, Jiang et al. 2012, Zhan, Fang et al. 2015). Given these findings the role of dysregulated methylation will be investigated further in this chapter.

Finally, this chapter will investigate the localisation of microRNA expression within FFPE tissues (see methods section 2.2.7 for details on in-situ hybridisation). This is clearly an important factor in understanding the impact of microRNA expression. A microRNA which is expressed specifically within the tumour mass, is likely to be of great interest. It is possible that changes in microRNA expression may occur within healthy regions of tissue, in cells and structures outside of the tumour mass. While the change in expression of these microRNAs may still be important, it may be suggestive of alternate mechanisms of action. For example, changes in microRNA expression in connective tissue or blood vessels may contribute to invasion and metastasis, possibly by making the area more hospitable for invading tumour cells, while changes in microRNA expression within the tumour mass may induce changes in the tumour cells themselves.

### 5.2 Results

#### 5.2.1 Expression of the miR-34 family, miR-142-3p and miR-148a-3p following ectopic expression of p53 family members.

Mutant p53 has both dominant negative and gain of function characteristics (see section 1.3.5.2 for details). A number of previous publications have shown that some of the gain of function may be due to mutant p53 exerting dominant negative effects
on other members of the p53 family, p63 and p73 (see introduction section 1.3.5.2 for details). If mutant p53$^{R172H}$ were regulating microRNA expression through dominant negative inhibition of p63 or p73, it would be assumed that the microRNAs would be transcriptional targets of p63 or p73. To address this hypothesis, microRNA expression was investigated following ectopic expression of members of the p53 family (wild type p53, mutant p53$^{R175H}$, TAp63, ΔNp63, TAp73 and ΔNp73) in the p53 null cell lines: H1299 and SAOS. The TA isoforms are the functionally active members of the family while the ΔN isoforms are dominant negative, though recent data suggest more distinct roles for these isoforms (see section 1.3.5.2 for details).

Western blots showed that each member of the p53 family is only present at detectable levels (p63 and p73 are often expressed at very low levels, undetectable via western blot) when it is overexpressed via plasmid transfection in both H1299 and SAOS cells (figures 5.1-a and 5.2-a). There are slight differences in expression of the wild type p53 protein compared to the mutant p53$^{R172H}$ protein. This is likely due to mutant p53$^{R172H}$ not trans-activating MDM2 to promote p53 ubiquitination and subsequent degradation, which allows mutant p53 to accumulate in cells (Oren, Rotter 2010).

MiR-34a-5p is a well-documented transcriptional target of wild type p53 (see section 1.4.9 for details). Previously, this study has shown that miR-34a-5p is significantly downregulated in tumour tissues (figure 3.4 and 3.5) and cell lines (figure 3.7 and 3.9) which express mutant p53$^{R172H}$. Therefore the expression of miR-34a-5p following transfection of p53 family members was investigated. Here I show that miR-34a-5p expression is induced by all functionally active members of the p53 family (wild type p53, TAp63 and TAp73), while no change in expression is observed upon transfection of transcriptionally inactive family members: mutant p53$^{R175H}$, ΔNp63 or ΔNp73, in either SAOS or H1299 cells (figures 5.1 and 5.2).

Previous publications have shown that all miR-34 family members are p53-dependent, have similar expression profiles, and are all involved in DNA damage response (Hermeking 2010, He, He et al. 2007, Bommer, Gerin et al. 2007). However, the work presented here showed that only miR-34a expression, not that of miR-34b or miR-34c, was found to be inhibited in mutant p53$^{R172H}$ expressing tumour tissues (see
Figure 5-1: Analysis of microRNA expression in H1299 cells following transient ectopic expression of p53 family members. The NSCLC cell line H1299 was transfected with 1µg of either an empty vector control or one expressing wild type p53; the dominant negative/gain of function p53R175H mutant; full length TAp63, the truncated, dominant negative ΔNp63; full length TAp73 or the truncated, dominant negative ΔNp73. The vector backbone used for all transfections was pCDNA3.1. All plasmids were a gift from Dr Patricia Muller. (a) Protein expression of wild type p53, mutant p53R175H, p63, p73, as well as β-actin which was used as a loading control. 40µg of total cell lysate was loaded for each condition. (b) q-PCR was carried out for miR-142-3p, miR-148a-3p, miR-34a, miR-34b and miR-34c using Taqman RT-qPCR microRNA assays. The fold change represents the change in expression of each microRNA comparing the p53 family member expressing cells to those transfected with an empty vector. Statistical analysis: two-samples, two-tailed, paired t-test comparing the ΔCT values of each set of p53 family expressing cells to those of the empty vector control, with n.s representing no significance, * p<0.05, ** p<0.01 and *** p<0.005.
Figure 5-2: Analysis of microRNA expression in SAOS cells following transient ectopic expression of p53 family members. The osteosarcoma cell line SAOS was transfected with 1µg of either an empty vector control or one expressing wild type p53; the dominant negative/gain of function p53R175H mutant; full length TAp63, the truncated, dominant negative ΔNp63; full length TAp73 or the truncated, dominant negative ΔNp73. The vector backbone used for all transfections was pCDNA3.1. All plasmids were a gift from Dr Patricia Muller. (a) Expression of wild type p53, mutant p53R175H, p63, p73, as well as β-actin which was used as a loading control. 40µg of total cell lysate was loaded for each condition. (b) qPCR was carried out for miR-142-3p, miR-148a-3p, miR-34a, miR-34b and miR-34c using Taqman RT-qPCR microRNA assays. The fold change represents the change in expression of each microRNA comparing the p53 family member expressing cells to those transfected with an empty vector. Statistical analysis: two-samples, two-tailed, paired t-test comparing the ΔCT values of each set of p53 family expressing cells to those of the empty vector control, with n.s representing no significance, * p<0.05, ** p<0.01 and *** p<0.005.
figure 3.4, 3.5 and supplemental figure 3 for full microarray results). This does not correlate with previously published data which shows that the miR-34 family are all regulated by the same mechanisms (Bommer, Gerin et al. 2007, Hermeking 2010, He, He et al. 2007). Given this unexpected result, the expression of miR-34b and miR-34c were also investigated following ectopic expression of p53 family members in the p53 null cell lines H1299 and SAOS. In H1299 cells, miR-34a was induced following ectopic expression of wild type p53, TAp63 and TAp73; while miR-34b and miR-34c were not (figure 5.1). In SAOS cells, the whole miR-34 family were induced by ectopic expression of p53, TAp63 and TAp73 and did not change following ectopic expression of p53\textsuperscript{R175H}, ΔNp63 and ΔNp73 (figure 5.2). This suggests cell specific regulation of miR-34a, from miR-34b and miR-34c. Additionally, it shows that the whole miR-34 family can be regulated by all transcriptionally active p53 family members in a p53 null environment. As both p63 and p73 are able to induce the expression of miR-34a, it is possible that mutant p53\textsuperscript{R175H} inhibits miR-34a-5p expression by directly interfering with the transcriptional activities of TAp63 or TAp73, though this has not been tested in this study. Additionally, no change in expression of miR-142-3p or miR-148a-3p is observed following ectopic expression of any p53 family members, in either SAOS or H1299 cells (figures 5.1 and 5.2), suggesting that dominant negative regulation of p63 and p73 is not responsible for all mutant p53\textsuperscript{R175H} induced microRNA dysregulation.

No inhibition of miR-142-3p, miR-148a-3p or miR-34a-5p expression was observed when mutant p53\textsuperscript{R175H} was ectopically expressed in either H1299 or SAOS cells (figure 5.1 and 5.2). This may be due to the observed inhibition of expression of these microRNAs being specific to PDAC, perhaps requiring mutation of K-RAS. Alternatively, it may be due to the short time frame in which these experiments took place. The mutations in the mouse model were introduced using a PDX/Cre LOXp system. As PDX1 is expressed from early embryogenesis (Ahlgren, Jonsson et al. 1996), mutant p53\textsuperscript{R172H} would have been expressed in the cells for a long time, while cells which have been transiently transfected with a mutant p53 construct were lysed 48 hours post transfection, which is perhaps insufficient time for the negative regulation of the microRNAs to occur. This may mean the changes in microRNA
expression are not a transient effect, but require sustained expression of mutant p53^{R172H}.

### 5.2.2 DNA damage can induce miR-34a-5p in a p53-dependent and -independent manner in H1299 cells

As the miR-34 family has been shown to be induced by p53 following DNA damage, further investigation into their regulation was carried out following DNA damage in the presence of wild type or mutant p53^{R175H} (see introduction section 1.4.9 for details on the miR-34 family). Thanks to a kind gift from Dr Patricia Muller, this study was able to utilise three H1299 cell lines which have a doxycycline sensitive promoter for either wild type p53, mutant p53^{R175H} or a control which contains the promoter but no coding sequence. These cells were treated with the DNA damaging agent doxorubicin, with or without stimulation with doxycycline, in order to investigate microRNA expression following DNA damage independent of p53, or in the presence of wild type, or mutant p53^{R175H}. Cells were first treated with doxycycline (2μg/ml) or vehicle control for 24 hours, before treatment with either doxorubicin (1.2μM) or a vehicle control for a further 24 hours. The doxycycline was refreshed for the second 24 hour period to ensure continued expression from the doxycycline sensitive promoter. The western blots clearly show no change in total ATM protein expression but a dramatic increase in its phosphorylated form following DNA damage, as well as induction of p53 and P-p53(Ser15), confirming activation of the DNA damage response pathway (Bakkenist, Kastan 2003, Siliciano, Canman et al. 1997) (figure 5.3-a). To determine if apoptosis has been induced, Western blot analysis was performed to examine the integrity of PARP, a well-known target of the caspase cascade, which is cleaved upon induction of apoptosis (Lazebnik, Kaufmann et al. 1994). No detection of the 89kDa cleavage fragment of PARP was detected, suggesting the level of DNA damage had not caused an apoptotic response (figure 5.3-a). Demonstrating that mutant p53^{R175H} does not induce wild type p53 transcriptional targets, p21 is only present in the p53 wild type expressing cells (figure 5.3-a). As shown previously, miR-34a is dramatically induced by expression of p53, while miR-34b and miR-34c are not induced by p53 (figure 5.3-b). The experiment also demonstrates that combined p53 expression and
DNA damage induced miR-34a to a greater extent than DNA damage or p53 induction alone (figure 5.3). A ~3 fold induction of all members of the miR-34 family was observed.

**Figure 5-3:** Expression of miR-34 family following DNA damage. Cell lines which had been transfected and selected to stably express either wild type or mutant (p53^{R175H}) p53 from a doxycycline sensitive promoter, with the addition of a negative control with no open reading frame following the doxycycline sensitive promoter, were provided by Dr Patricia Muller. Each cell line was treated with either 2μg/ml doxycycline or an equal volume of DMSO vehicle as a control. After 24 hours, cells were treated with either 1.2μM doxorubicin or a H2O control for a further 24 hours. (a) Protein expression analysis shows total ATM and P-ATM, as well as total p53 and P-p53(ser15) as controls for DNA damage. p21 is a control for downstream p53 signalling. In order to show that the treatments are not causing excess apoptosis, total PARP was blotted for. β-actin was used as a loading control. (b) RT-qPCR was carried out using Taqman microRNA assays for miR-34a, miR-34b and miR-34c. The reported fold changes are compared to non-induced, non-DNA damaged control for each cell line, as an average of three experimental repeats. Statistical tests implemented two-samples, two-tailed, paired t-tests using the ΔCT values for the non-induced, non-DNA damaged compared to the ΔCT values for the test condition with p<0.05 (*), p<0.01 (**) and p<0.005 (**).
observed following DNA damage, independent of p53 expression (figure 5.3-b), suggesting a p53-independent mechanism for induction of these microRNAs. The upregulation of miR-34a, but not miR-34b or miR-34c, following ectopic expression of wild type p53, provides evidence to the hypothesis that miR-34a may be regulated independently from miR-34b and miR-34c.

5.2.3 Mutant p53R172H does not consistently affect members of the microRNA processing machinery in PDAC

Since both p63 and p73 can induce expression of miR-34a-5p (figures 5.1 and 5.2), it is possible that the loss of miR-34a-5p observed when mutant p53R172H is expressed, is due to the dominant negative function of mutant p53R172H on TAp63 or TAp73. However, as no change in miR-142-3p or miR-148a-3p expression is observed following ectopic expression of TAp63 or TAp73 in either SAOS or H1299 cells (figures 5.1 and 5.2), the dominant negative function of mutant p53R172H on TAp63 or TAp73 cannot explain why the expression of all of the microRNAs investigated in this study are inhibited upon expression of mutant p53R172H. Given this, more global causes of microRNA dysregulation were investigated. As previous studies have shown that both Drosha (Suzuki, Yamagata et al. 2009) and Dicer (Muller, Trinidad et al. 2013) are inhibited by mutant p53 expression, the status of these genes, as well as AGO2, were investigated in both the tumour tissue and cell lines. These experiments show that AGO2 mRNA and protein are not changed in mutant p53R172H expressing cell lines (figure 5.4-a and b), while AGO2 mRNA is upregulated in the mutant p53R172H expressing mouse tissue (figure 5.4-c). It is unclear how upregulation of AGO2 would cause inhibition of microRNA expression, though perhaps AGO2 is a target of one of the microRNAs found to be downregulated in this study. This hypothesis has not been investigated in this study.

This study has also found that Drosha protein and mRNA is induced in the mutant p53R172H expressing cell lines (figure 5.4 a and b), while Drosha mRNA expression was not significantly changed in the tissues (figure 5.4-c). It is unclear why upregulation of Drosha would cause a loss in microRNA expression, indeed it would be more likely to increase the processing of primary microRNAs. Drosha has been shown to be a
transcriptional target of c-MYC (Wang, Zhao et al. 2013), a gene regulated by miR-34a (Christoffersen, Shalgi et al. 2009, Yamamura, Saini et al. 2012). Upregulation of

Figure 5-4: Expression of microRNA processing components. Western blots and q-PCR were used to determine any changes in expression of the major components of the microRNA processing machinery, Drosha, Dicer and AGO2. (a) Representative western blots of AGO2, Drosha and Dicer with β-actin as a loading control. This blot is representative of 3 repeat experiments. (b) RT-qPCR was carried out using gene specific primers for AGO2, Drosha and Dicer using RNA from the same experiment as the protein expression analysis. The ΔΔCT method was used to normalise the date with β-actin used as the normalisation gene. The fold change represents the change in expression between each test condition and that of the K-RAS\textsuperscript{G12D},p53\textsuperscript{+/-}. The error bars represent the minimum and maximum relative quantities, calculated using the standard deviation between three biological repeats (please see methods section 2.2.6 for details). Statistical analysis: a two-sample, two-tailed, paired t-test was used to compare the ΔΔCT values of a test condition to those of the K-RAS\textsuperscript{G12D},p53\textsuperscript{+/-} condition, with p<0.05 (*), p<0.01 (**) and p<0.005 (***). (c) RT-qPCR was carried out on the tissues used for the microRNA profiling experiments in Chapter 3. On the box and whisker plots, the upper and lower band represent the highest and lowest value, respectively. The top and base of the box represent the 75th and 25th percentile respectively and the middle bar is the median value. Each dot represents a single sample. Statistical analysis: a two-sample, two-tailed, unpaired t-test was used to compare the ΔΔCT values of a the K-RAS\textsuperscript{G12D},p53\textsuperscript{+/-} to the K-RAS\textsuperscript{G12D},p53\textsuperscript{R172H/+} samples, with p<0.05 (*), p<0.01 (**) and p<0.005 (***).
Drosha may therefore be a consequence of increased c-MYC expression, an event common in cancer development (Dang 2012, Meyer, Penn 2008), possibly through loss of miR-34a. This hypothesis has not been tested in this study.

Dicer protein and mRNA expression were found to be higher in p53 hemizygous cells compared to wild type homozygous and wild type null cells, though there was no difference between p53 hemizygous and mutant p53^{R172H} expressing primary cells (figure 5.4-a and b). There was also no difference in Dicer mRNA between p53 hemizygous and mutant p53^{R172H} expressing tissues (figure 5.4-c).

These data suggest no consistent difference in expression of any of the investigated microRNA processing components when their expression in both tissue and cell lines are considered.

5.2.4 Mutant p53^{R172H} does not affect expression of members of the RNA editing machinery
As no consistent change of expression was observed for Drosha, Dicer or AGO2 in the tissue and cell lines, a different mechanism which could affect microRNA expression was investigated. Some microRNAs, including miR-142-3p, have been shown to undergo editing, which affects their processing (See section 1.5 for details). As miR-142-3p is one of the microRNAs identified in this current study as being downregulated by mutant p53^{R172H}, investigation into the mRNA expression of ADAR1 and ADAR2 was carried out. No significant change in mRNA abundance of ADAR1 and ADAR2 was found between the p53 hemizygous and mutant p53^{R172H} expressing tissue samples (figure 5.5-a), however, significant differences were observed between cell lines (figure 5.5-b). ADAR1 expression was found to be higher in p53 null cells, compared to other cell lines. ADAR2, on the other hand, was found to be significantly upregulated in p53 hemizygous, p53 null and mutant p53^{R172H} expressing cells compared to the p53 wild type cells. As there is a negative correlation between p53 and ADAR2 mRNA expression, this may suggest that ADAR2 is negatively regulated by p53. The expression of ADAR1 is upregulated, with ADAR2 is downregulated in p53 null cells with ectopic expression of mutant p53^{R172H} (figure 5.5-c), which may suggest
Figure 5-5: mRNA expression of RNA editing components. RT-qPCR was used to investigate the relative levels of ADAR1 and ADAR2 using gene specific primers (see materials and methods section 2.2.5 For details. All RT-qPCR was analysed using the \( \Delta \Delta C_T \) method, using \( \beta \)-actin as a normalisation gene. (a) RT-qPCR for ADAR1&2 in primary tissue samples. Fold change is relative to the K-RAS\(^{G12D},p53^+/−\);p53\(^+/−\) samples. On the box and whisker plots, the upper and lower band represent the highest and lowest value respectively. The top and base of the box represent the 75th and 25th percentile respectively and the middle bar is the median value. Each dot represents a single sample. A two sample, two tailed, unpaired t-test \( \Delta C_T \) values from each group was used to calculate statistical significance. (b) RT-qPCR for ADAR1&2 in primary cell lines. Fold change is relative to the K-RAS\(^{G12D},p53^+/−\) condition with error bars representing the minimum and maximum relative quantities (see section 2.2.6 for details). A two sample, two tailed, paired t-test \( \Delta C_T \) values from each group was used to calculate statistical significance. (c) RT-qPCR for ADAR1&2 in p53 null cells with ectopic expression of mutant p53\(^{R172H}\). Fold change is relative to the empty vector transfected cells, with error bars representing the minimum and maximum relative quantities (see materials and methods for details). A two sample, two tailed, paired t-test \( \Delta C_T \) values from each group was used to calculate statistical significance. Statistical significance is represented as * (<0.05) ** (<0.01) and *** (<0.005).
a compensatory mechanism between the ADAR proteins. As no consistent change was observed in tissues and cells lines, investigation into ADAR genes was not continued.

5.2.5 The DNMT family inhibitor, 5-aza-2-deoxycytidine, increases expression of miR-142-3p, but not miR-148a-3p or miR-34a-5p.
As no consistent change in ADAR mRNA expression was observed, another potential mechanism of microRNA regulation was investigated. DNA methylation is frequently shown to be dysregulated in cancer, and has been linked to numerous deleterious biological processes in tumour progression, including metastasis (see section 1.8 for details). MiR-142-3p (Abdul Razak, Baba et al. 2014) and miR-34a-5p (Cui, Zhao et al. 2014) have both been shown to be hypermethylated in tumours. Additionally, miR-148a-3p has also been shown to be hypermethylated in tumours, as well as directly targeting one of the best studied proteins involved in methylation, DNMT1 (Xu, Jiang et al. 2012, Zhan, Fang et al. 2015). In order to investigate any change in microRNA expression which could be due to hypermethylation, cell lines were treated with the DNMT family inhibitor, 5-aza-2′-deoxycytidine (5-aza-DC). This drug is a cytidine analogue, which is incorporated into de-novo synthesised DNA. When DNMT proteins attempt to methylate CpGs containing 5-aza-DC, they are cross-linked to the DNA, sequestering them and inhibiting further methylation (Christman 2002). Each cell line was treated with 1μM 5-aza-DC for 24 hours before microRNA expression analysis was carried out. Only miR-142-3p expression was induced following treatment, with miR-148a-3p and miR-34a-5p unaffected by the drug (figure 5.6). This was unexpected as both miR-148a-3p (Zhu, Xia et al. 2011) and miR-34a-5p (Siemens, Neumann et al. 2013, Zhu, Xia et al. 2011) are known to be hypermethylated in cancer. This experiment was carried out using a low dose of 5-aza-DC for only 24 hours. As miR-142-3p is expressed at such a low endogenous level, a small increase in expression may correspond to a large relative change in expression. If miR-148a-3p and miR-34a-5p are expressed at higher endogenous levels, the “relative” change from treated to untreated may be much smaller. Alternatively, miR-142-3p may be actively
Figure 5-6: Expression of miR-142-3p, miR-148a-3p and miR-34a-5p following treatment with 5-aza-deoxycytidine. Each cell line was treated with 1µm 5-aza-deoxycytidine (5-aza-Dc) or an equal volume of a vehicle control (DMSO) for 24h. The fold change represents the average relative quantity of each microRNA of three experimental repeats. The relative quantity of each microRNA was normalised to U6 levels using the ΔΔCT method. The error bars represent the maximum relative quantity calculated using the standard deviation of the ΔCT values for each condition (see section 2.2.6 for details). Statistical significance was calculated using a two sample, two tailed, paired t-test comparing the ΔCT values for each condition with n.s representing no significant difference, p<0.05 (*), p<0.01 (**) and p<0.005 (***)
de-methylated under normal conditions, therefore as soon as DNMT proteins are sequestered, miR-142-3p is able to express to high levels again.

5.2.6 Mutant p53R172H and p53 dosage affect DNMT1 mRNA and protein expression
DNMT1 is responsible for the maintenance of methylation following mitosis, while DNMT3a and DNMT3b are responsible to de-novo methylation events (see introduction section 1.8 for details). Additionally, DNMT1 has previously been shown to be involved in cancer progression, including through interactions with miR-148a-3p (Xu, Jiang et al. 2012). As DNMT1 has previous links to cancer, its mRNA and protein expression were investigated. DNMT1 mRNA was shown to be significantly induced in mutant p53R172H expressing tissues, cell lines and p53 null cells with ectopic expression of mutant p53R172H (figure 5.7-a i,ii,iii). This study also shows that DNMT1 protein expression is induced in p53 hemizygous, p53 null and mutant p53R172H expressing cells, compared to p53 wild type homozygous cells (figure 5.7-b). Additionally, a modest increase in DNMT1 protein and mRNA is observed in p53 null cells ectopically expressing mutant p53R172H (figure 5.7-a iii and 5.7-c). This posits the possibility that mutant p53R172H is able to induce DNMT1 expression.

5.2.7 DNMT1 depletion rescues miR-142-3p expression, but has no effect on miR-148a-3p or miR-34a-5p expression.
Having shown that miR-142-3p is induced following treatment with a methylation inhibitor (figure 5.6), and that DNMT1 is induced in tissues and cell lines which express mutant p53R172H (figure 5.7-a,b and c), the next step was to investigate the impact of DNMT1 depletion on microRNA expression. This was achieved by transfecting cells with an siRNA against DNMT1, or a control siRNA, for 48 hours prior to cell harvest. Western blot analysis shows sufficient depletion of DNMT1 in each cell line (figure 5.8-a). As observed with the 5-aza-DC treatment, miR-142-3p was significantly induced when DNMT1 was depleted (figure 5.8-b). Interestingly, DNMT1 depletion in p53 hemizygous, p53 null and mutant p53R172H expressing cells restored miR-142-3p to
Figure 5-7: DNMT1 expression in primary tissues, cell lines and p53 null cells with ectopic expression of mutant p53R172H.  

(a) RT-qPCR investigating DNMT1 mRNA abundance in primary tissues (i). Fold change is relative to the K-RASG12D;p53+/− samples, with the top and bottom bands on the box plots represent the highest and lowest value respectively, the roof and base of the box representing the 75 an 25 percentiles respectively and the central line representing the median value. Statistical analysis: a two sample, two tailed, unpaired t-test was used to compare the ΔCT values from each condition.  

(b) A representative western blot showing protein expression of DNMT1 and β-actin in primary cell lines.  

(c) A representative western blot showing DNMT1 and β-actin expression in p53 null cells with stable ectopic expression of mutant p53R172H.
Figure 5-8: Expression of miR-142-3p, miR-148a-3p and miR-34a-5p following depletion of DNMT1. Each cell line was treated with 20nM DNMT1 siRNA or a control siRNA for 48h. (a) A representative western blot showing the knockdown of DNMT1 protein levels following transfection, with β-actin as a loading control. (b) The fold change represents the average relative quantity of each microRNA of three experimental repeats. The relative quantity of each microRNA was normalised to U6 levels using the ΔΔCT method. The error bars represent the maximum relative quantity calculated using the standard deviation of the ΔCT values for each condition (see section 2.2.6 for details). Statistical analysis: two sample, two tailed, paired t-test comparing the ΔCT values for each condition, with n.s representing no significant difference, p<0.05 (*), p<0.01 (**) and p<0.005 (***).
those observed in the p53 wild type cells. The expression of miR-148a-3p and miR-34a-5p are not affected by siRNA depletion of DNMT1 (figure 5.8-b).

5-aza-DC treatments resulted in a greater induction of miR-142-3p than DNMT1 depletion. This is likely due to the fact that 5-aza-DC is an inhibitor of all DNMT proteins, so should inhibit all cytidine methylation events, while depletion of DNMT1 will only inhibit DNMT1 methylation events. Additionally, transfection efficiency must be taken into account in the siRNA depletion experiment, while drug treatment is likely to affect the vast majority, if not all of the cells.

5.2.8 Initial microRNA in-situ hybridisation optimisation
This study has identified and characterised three microRNAs which are downregulated by mutant p53<sup>R172H</sup>. As the tumour tissue may contain a variety of structures including, tumour stroma, healthy tissue and connective tissue, changes in microRNA expression may be occurring in any of these regions. While these changes may be biologically significant no matter where the change is occurring, understanding the location of the microRNAs may help elucidate how the changes are affecting tumour progression. For example, a change in expression of a microRNA in a blood vessel, may make the vessel more susceptible to tumour cell infiltration, while microRNA expression in ductal cells may be involved in directly affecting the invasive ability of the tumour cells. In order to investigate the tissue localisation of microRNAs, in-situ hybridisation was carried out on FFPE tissue section from the mouse model used in microRNA profiling (See section 2.2.7 for details on In-situ hybridisation).

It is vitally important to ensure that sufficient proteinase K treatment is used in order to reveal the epitopes for this experiment. This was achieved by testing a variety of concentrations of proteinase K and probing for snRNA U6 (U6). U6 is a nuclear RNA expressed in all cell types. It is the RNA component of a small nuclear ribonucleoprotein (snRNP) and a critical component of the spliceosome (Berget, Robberson 1986). The first optimisation of proteinase K treatment, followed the concentrations suggested by the manufacturer, 10μg/ml, 20μg/ml and 30μg/ml, did not show significant staining of U6 in any areas of the tissue, though there was some,
Figure 5-9  Initial proteinase K optimisation. The first attempt at optimising the proteinase k treatment with 10ug/ml (a), 20ug/ml (b) and 30ug/ml (c) of proteinase K, using sequential tissue sections and an LNA probe against U6. U6 would be expected to stain the nucleolus of the cells, though with this level of proteinase K treatment, very little staining was observed. In all cases there is some light staining of the nuclei of cells in the tumour stroma but none in the connective tissues or the acinar cells. Sections are counterstained with a nuclear fast red so the nuclei can be seen. All main images were taken at 5X magnification with an expanded image outlined with a dotted line. Please see methods section 2.2.7 For details on experimental procedure. There section come from a mouse with the K-RAS $^{\text{G12D R172H/p53}}$ genotype.
Figure 5-10 High proteinase K treatment optimisation. The second attempt at optimising the proteinase k treatment with 100ug/ml (a) and 200ug/ml (b) of proteinase K, an LNA probe against U6. U6 would be expected to stain the nucleolus and with this increased proteinase K treatment, staining of the nuclei in blue is clearly observed. In all cases, there is staining of the nuclei of cells in the tumour stroma, connective tissues and the acinar cells, which is stronger in the 200μg/ml sections. Sections are counterstained with a nuclear fast red so the nuclei can be seen, but this is clearly obscured by the blue staining from the alkaline phosphate/NBT-BCIP. All main images were taken at 5X magnification with an expanded image outlined with a dotted line. Please see methods section 2.2.7 For details on experimental procedure. This section came from a mouse with the K-RAS<sup>G12D</sup>;p53<sup>R172H/+</sup> genotype.
sporadic staining in some of the tumour stroma (figure 5.9). It would be expected that U6 should produce very heavy staining given its high expression in cells. As this was not observed, it suggested that either, a more stringent proteinase K treatment was required, or that the RNA in the tissue sections is too degraded to carry out *in-situ* hybridisation.

For the second optimisation experiment, the proteinase K concentration was increased to 100μg/ml and 200μg/ml. This dramatically improved the signal for U6 in nuclei, suggesting that the increase in proteinase K treatment, rather than the RNA integrity, was responsible for the poor signal in the first optimisation (figure 5.10). When proteinase K was used at 100μg/ml, staining for U6 was well defined in the tumour stroma and connective tissue, although the healthy acinar cells had quite poor signal. By increasing the proteinase K treatment to 200μg/ml, strong signal was observed in the tumour stroma, connective tissue and the acinar cells. This was vital as it allows a comparison to be made between healthy tissue and tumour tissue within the same section. This degree of proteinase K treatment is 10X higher than was recommended by the manufacturer. However, the signal was considerably better defined and the tissue integrity had not been compromised. This may be due to the inherent dense fibrotic nature of PDAC. It is possible that the amount of ECM components is so high that a much more stringent proteinase K treatment is required to reveal the epitopes. A proteinase K treatment at 200μg/ml was used for all subsequent experiments.

5.2.9 Investigation into the localisation of microRNAs using FFPE pancreatic tissue.

Following establishment of a proteinase K treatment protocol, it was essential to look at localisation of a highly expressed microRNA to demonstrate that it was possible to visualise RNA molecules other than nuclear RNAs using this technique. miR-21 is a microRNA which is found to be upregulated in a large number of cancer types including: NSCLC (Ma, Liu et al. 2012), breast (Yan, Huang et al. 2008), bladder (Tao, Lu et al. 2011) and colorectal (Asangani, Rasheed et al. 2007), among others. Moreover, it is frequently found to be upregulated in pancreatic cancer
Figure 5-11 Expression of miR-21 in pancreatic tissue of PDAC suffering mice. Assessment of miR-21-5p expression in mouse pancreatic tissue. Proteinase k treatment was carried out using 200ug/ml of proteinase K, on sequential tissue sections and an LNA probe against miR-21-5p (a-i and b-i) or a control LNA probe (a-ii and b-ii). In all cases, there is staining of cells in the tumour tissue but none of the connective tissues. In these images, no acinar cells are visible. Sections are counterstained with a nuclear fast red so the nuclei can be seen. All images were taken at 5X magnification with an expanded image outlined in a dotted line. Please see methods section 2.2.7 for details on experimental procedure. The sections came from mice with either the K-RAS<sup>GR12D</sup>;p53<sup>R172H/+</sup> genotype (a) or the K-RAS<sup>GR12D</sup>;p53<sup>−/+</sup> genotype (b).
a-i

Hepatocytes

Blood vessel

a-ii

Hepatocytes

Blood vessel
Figure 5-12 Expression of miR-21 in the liver and spleen of mutant p53R172H expressing mice. Assessment of miR-21-5p expression in liver (a) and spleen (b). Proteinase k treatment was carried out using 200ug/ml on sequential tissue sections and an LNA probe against miR-21-5p (a-I and b-i) or a control LNA probe (a-ii and bii). In these sections no staining is observed for miR-21 in either the liver or the spleen. Sections are counterstained with a nuclear fast red so the nuclei can be seen. All images were taken at 5X magnification with an expanded image outlined in a dotted line. Please see methods section 2.2.7 For details on experimental procedure. This section came from a mouse with the K-RASG12D;p53R172H/+ genotype.
Figure 5-13 In-situ hybridisation for miR-21 in sections from mice with the K-RAS$^{G12D;p53^{+/+}}$ genotype. Assessment of miR-21-5p expression in mouse pancreatic tissue. Proteinase k treatment was carried out using 200μg/ml on sequential tissue sections before an LNA probe against miR-21-5p (a) or a control LNA probe (b). In these sections, staining is only observed in the possible PanIn lesion labelled. No staining is observed in the acinar cells or connective tissue. Sections are counterstained with a nuclear fast red so the nuclei can be seen. All images were taken at 5X magnification with an expanded image outlined with a dotted line. Please see methods section 2.2.7 For details on experimental procedure. This section came from a mouse with the K-RAS$^{G12D;p53^{+/+}}$ genotype.
Lymphoid aggregate

Acinar cells

Connective tissue

Tumour stroma

Lymphoid aggregate

Acinar cells

Connective tissue

Tumour stroma
b-i
Tumour stroma
Lymphoid aggregate
Connective tissue

b-ii
Tumour stroma
Lymphoid aggregate
Connective tissue
c-i

Tumour stroma

Lymphoid aggregate

\[\text{Image}
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c-ii

Tumour stroma

Lymphoid aggregate

\[\text{Image}
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Figure 5.14 In-situ hybridisation for miR-142-3p. Assessment of miR-142-3p expression in lymph nodes (a, b and c) and the spleen (d). Proteinase k treatment was carried out using 200µg/ml of proteinase K, on sequential tissue sections and an LNA probe against miR-142-3p (a-i, b-i, c-i and d-i) or a control LNA probe (a-ii, b-ii, c-ii and d-ii). In these sections staining is observed for miR-142-3p in the lymph nodes (a, b and c), the spleen (d) and in some connective tissue (a). Sections are counterstained with a nuclear fast red so the nuclei can be seen. Images a and b were captured at 5X magnification with an expanded image outlined in a dotted line. Please see methods section 2.2.7 for details on experimental procedure. This section came from a mouse with the K-RAS$^{G12D}$;p53$^{R172/+}$ genotype.
As this gene is expected to be expressed at high levels in pancreatic cancer, a probe targeting miR-21 was used to further optimise the in-situ hybridisation experiments. It is important to note that no difference in miR-21 expression was observed between p53 hemizygous and mutant p53R172H expressing primary tissues, and that it was expressed at high levels in both (see supplemental figure 3 for full microarray results).

In tissues from mice with the K-RAS$^{G12D}$;p53$^{R172H/+}$ and K-RAS$^{G12D};p53^{+/-}$ genotypes, the signal for miR-21 was highly specific and associated only within the tumour tissue (figure 5.11). Importantly, there was no staining for miR-21 in the liver or spleen (figure 5.12) of the animals (liver and spleen tissue were often present on the same sections as the pancreatic tissue) suggesting that upregulation of miR-21 was specific to the tumours within the pancreas. The staining for miR-21 in the tissue from a mouse with only the K-RAS$^{G12D}$ mutation but wild type p53 expression, showed only minimal staining in what appeared to be an early PanIN lesion (figure 5.13).

As it was possible to get specific staining for microRNAs within the tissue, the expression and location of miR-142-3p was investigated. As expression of miR-142-3p has been shown to be inhibited in tissues from mice with the p53$^{R172H}$ mutation, it was expected that the healthy areas of the tissue would express miR-142-3p, while the damaged, tumour tissue would not. Unexpectedly, strong, specific signal was observed in lymphoid aggregates and white pulp of the spleen, with some weaker signal in the connective tissues surrounding the tumour and none in the healthy acinar cells (figure 5.14).

5.3 Discussion
The aim of this chapter was the identify mechanisms which cause the dysregulation of the microRNAs identified in chapter 3. As the dominant negative effect of mutant p53 is not sufficient to explain its ability to induce metastasis, or the dysregulation of the majority of microRNAs identified in this study, a number of other mechanisms were investigated.
Ectopic expression of p53, TAp63 and TAp73 was only able to induce expression of miR-34a-5p, leaving miR-142-3p and miR-148a-3p unchanged, in both SAOS and H1299 cells, while the dominant negative mutant p53$^{R175H}$, ΔNp63 and ΔNp73 were not able to induce expression of any of the microRNAs investigated (figures 5.1 and 5.2). The interplay between p53 and the miR-34 family is broadly studied. Here I present data which shows that ectopic expression of p53, in p53 null cells led to induction of miR-34a-5p (figures 5.1 and 5.2). This is in line with numerous previous studies showing a functional link between p53 and the miR-34 family (Tarasov, Jung et al. 2007, Tazawa, Tsuchiya et al. 2007, Raver-Shapira, Marciano et al. 2007, He, He et al. 2007, Bommer, Gerin et al. 2007, Chang, Wentzel et al. 2007). I also present data which shows that TAp63 and TAp73 were able to induce miR-34a-5p expression in both H1299 and SAOS cells, while ΔNp63 and ΔNp73 are not (figure 5.1 and 5.2). While this may not be surprising given the functional redundancy between p53 family members, very little data exist to suggest TAp63 or TAp73 induce expression of miR-34a-5p. A seemingly conflicting study showed that p63 was able to inhibit miR-34a and miR-34c transcription in keratinocytes (Antonini, Russo et al. 2010). However, this study focused on the dominant negative ΔNp63 isoform, rather than the transcriptionally active TAp63 isoform. The publication shows that the dominant negative ΔNp63 isoform represses miR-34a and miR-34c, which could suggest that the functional TAp63 isoform induces both microRNAs, though the authors do not address this possibility (Antonini, Russo et al. 2010). To my knowledge, a positive association between TAp63 and miR-34a expression has not previously been shown. Another study found that TAp73 can induce miR-34a in neurons (Agostini, Tucci et al. 2011). However no evidence of p73 regulating miR-34a expression in non-neuronal cells exists to date. Previous studies have implicated mutant p53’s gain of function, in its ability to inhibit TAp63 and TAp73 (Stindt, Muller et al. 2014). As both TAp63 and TAp73 are able to induce miR-34a expression, it is possible that the loss of miR-34a-5p observed in this study was due to mutant p53$^{R172H}$ exerting a dominant negative effect on TAp63 or TAp73. However, given that no change in miR-142-3p or miR-148a-3p expression was observed following ectopic expression of p53 family members, this hypothesis cannot explain all of the microRNA expression changes seen when mutant p53$^{R172H}$ is expressed. This means that either, there are other mechanisms governing
mutant p53^{R172H} mediated microRNA dysregulation, or that this hypothesis cannot explain all mutant p53^{R172H} mediated microRNA dysregulation.

A large number of publications have shown that all the miR-34 family members are regulated by the same mechanisms (Hermeking 2010, He, He et al. 2007). However, data I present here suggest that in some cases, miR-34a was regulated independently of miR-34b and miR-34c (figures 5.1, 5.2 and 5.3). I have shown that ectopic expression of either TAp63 or TAp73 was able to induce expression of miR-34a, miR-34b and miR-34c in SAOS cells, but only miR-34a in H1299 cells (figures 5.1 and 5.2). This not only showed that miR-34a may be regulated independently of miR-34b and miR-34c, but also that there are p53-independent mechanisms regulating the expression of the miR-34 family. I present further data to reinforce this, as the entire miR-34 family was induced following DNA damage in a p53-independent manner, while only miR-34a was induced following expression of p53 without DNA damage (figure 5.3). It is possible that p53-independent induction of the miR-34 family following DNA damage may be occurring through either TAp63 or TAp73, though this hypothesis was not tested in this study. While the majority previous publications have shown that the miR-34 family are regulated by p53 alone, a few publications have also shown p53-independent regulation of miR-34. In human leukaemia K562 cells an alternative promoter which produces a longer primary miR-34a transcript was identified which is sensitive to phorbol ester and independent of p53 (Navarro, Gutman et al. 2009). Induction of miR-34a independently of p53 during oncogene-induced senescence has also been observed (Christoffersen, Shalgi et al. 2009). In the neuronal study mentioned previously, where TAp73 was shown to induce miR-34a expression, no change was observed in miR-34b or miR-34c expression (Agostini, Tucci et al. 2011), further suggesting independent regulation of these microRNAs. The genomic loci of miR-34b/miR-34c has been shown to be hypermethylated in some conditions, including lung cancer (Wang, Chen et al. 2014), which may explain why TAp63 and TAp73 were unable to induce their expression in the transfected H1299 cells (figure 5.1). However data I present shows that miR-34b and miR-34c were both induced by DNA damage in the doxycycline inducible H1299 cells (figure 5.3). Given that the cells transfected with the plasmids encoding p53 family members were the
same parental line as the one used to produce the doxycycline inducible cells used in the DNA damage experiment, it would suggest that the primary miR-34b/miR-34c transcript was not hypermethylated in these cells, unless this happened after the establishment of stable doxycycline responsive cells, possibly during selection. This could be tested further with the use of methylation state PCR or bi-sulphite sequencing, but has not been investigated in this study. To my knowledge, induction of miR-34a, miR-34b and miR-34c expression following DNA damage, independent of p53 has not previously been shown.

Drosha, Dicer and AGO2 are all vital components of the microRNA processing machinery (see section 1.4.3 for details). Given that Chapter 3 showed that 78% of microRNAs which were dysregulated by mutant p53<sup>R172H</sup> expression, were downregulated (figure 3.4), it may be assumed that mutant p53<sup>R172H</sup>-mediated microRNA dysregulation may be due to its effects on components of the microRNA processing machinery. While previous studies have shown that both Drosha (Suzuki, Yamagata et al. 2009) and Dicer (Muller, Trinidad et al. 2013) are involved in mutant p53 mediated microRNA dysregulation, this current study did not observe a significant and consistent change in the expression of these genes due to mutant p53<sup>R172H</sup> expression (figure 5.4). The study which found that mutant p53 can interfere with Drosha and p68 interactions, found that a subset of microRNAs was downregulated, including: miR-143, miR-16 and miR-206 (Suzuki, Yamagata et al. 2009). It is important to note that none of these microRNAs were found to be dysregulated in the tissues analysed in this study (see supplemental figure 3 for full microarray results), suggesting that this mechanism is not present in the PDAC model system. The previous study which found Dicer to be dysregulated by mutant p53, employed a number of cell lines including, H1299, HT29, MDA MB 231, and A431 cells, none of which are derived from PDAC (Muller, Trinidad et al. 2013). As the data I present here suggests that loss of Dicer was not observed in mutant p53<sup>R172H</sup> expressing tumour tissues or primary cell lines (figure 5.4), an independent mechanism may be influencing microRNA expression in PDAC. Additionally, the Muller et al. study was investigating the role of the p53<sup>R273H</sup> mutant, which is a contact mutant rather than
the p53\textsuperscript{R175H} (p53\textsuperscript{R172H} in the mouse model used in this current study) which is a structural mutant, suggesting possible distinct roles for these different p53 mutants.

RNA editing has been shown to impact microRNA expression through inhibiting interactions between the microRNA processing machinery and the primary and precursor microRNAs (Yang, Chendrimada et al. 2005, Luciano, Mirsky et al. 2004, Kawahara, Zinshteyn et al. 2007). ADARs are involved with RNA editing of adenosine to inosine (see section 1.5 for details) The data presented here suggests no change in ADAR1 mRNA abundance following mutant p53\textsuperscript{R172H} expression, while a significant induction was observed in p53 null cells, when compared to wild type p53 expressing cells (figure 5.5). Conversely, ADAR2 mRNA was induced in cells deficient p53 or when mutant p53\textsuperscript{R172H} was expressed (figure 5.5). A previous study identified a p53 binding element within 5Kb of ADAR2 using ChIP (Bandele, Wang et al. 2010). The data presented here shows upregulation of ADAR2 in all cell lines without homozygous expression of wild type p53 (figure 5.5). This may suggest a transcriptionally repressive role for p53 in ADAR2 expression. While significant changes were observed in ADAR2 expression in the primary cell lines, no significant difference was observed in the tissues. While previous studies have identified miR-142 as being a target of RNA editing (Yang, Chendrimada et al. 2005), none of the other microRNAs investigated in this study have previously been shown to be edited. Additionally, none of the other microRNA shown to be edited in other studies, such as: miR-22 (Luciano, Mirsky et al. 2004), miR-151 (Kawahara, Zinshteyn et al. 2007) or miR-223 (Tomaselli, Galeano et al. 2015), were identified as differentially expressed by mutant p53\textsuperscript{R172H} in this current study (see supplemental figure 3 for full microarray results). Given these results, this study did not focus of ADAR1 or ADAR2 any further.

Hypermethylation is known to occur in numerous types of cancer and has been linked to deleterious mechanisms such as invasion and metastasis (see sections 1.2.3 and 1.8 for details). The data in this study showed that the DNMT1 transcript was upregulated by mutant p53\textsuperscript{R172H} (figure 5.7). Previous studies have shown that a complex consisting of p53 and Specificity Protein 1 (SP1) is able to transcriptionally repress DNMT1 in lung cancer. The same study found that in a panel of 102 lung cancer patients, mutant p53 expression correlated with increased DNMT1 expression (Lin,
Wu et al. 2010). While this may explain the observed increase in DNMT1 mRNA in tissues and cells lines expressing mutant p53R172H, it does not explain why there is no loss of DNMT1 mRNA in the cell lines which are hemizygous or null for p53. The present study has also shown that DNMT1 protein expression was induced in all cell lines with loss or mutation of p53 (figure 5.7). This may suggest a role for p53 in regulating the stability of DNMT1 protein. Alternatively, loss of p53 may lead to downregulation of a microRNA which regulates DNMT, leading to an increase in DNMT1 protein expression.

As I had shown that DNMT1 expression is affected by p53 dosage and mutation, its impact on microRNA expression was investigated. These experiments demonstrated that the DNMT inhibitor 5-aza-DC can induce expression of miR-142-3p, but not miR-148a-3p or miR-34a-5p (figure 5.6). The data also showed that siRNA mediated depletion of DNMT1 led to miR-142-3p expression returning to the level observed in p53 homozygous wild type cells, though no change in miR-34a or miR-148a-3p expression was observed (figure 5.8). The induction of miR-142-3p expression following treatment with 5-aza-DC was much larger than when DNMT1 is specifically depleted. This is likely due to 5-aza-DC targeting all member of the DNMT family, while the siRNA targeted only DNMT1. Additionally, the siRNA is unlikely to completely ablate DNMT1 expression in all cells, as this is highly dependent on transfection efficiency. 5-aza-DC led to very high expression of miR-142-3p, while siRNA against DNMT1 rescued miR-142-3p expression to that seen in p53 wild type expressing cells. This may suggest that only DNMT1 was dysregulated, rather than other members of the DNMT family. Expression of other DNMT proteins was not investigated in this study.

A number of previous studies have identified miR-142-3p as a target of hypermethylation (Abdul Razak, Baba et al. 2014, Andreopoulos, Andreopoulos et al. 2012, Chiou, Chien et al. 2013), which is consistent with the data we present in this study (figures 5.6 and 5.8). Interestingly, miR-148a-3p has been shown to be hypermethylated in cancer types such as glioma (Li, Chowdhury et al. 2014), gastric (Zhu, Xia et al. 2011) and pancreatic cancer (Zhan, Fang et al. 2015). The Zhan study showed an interplay between DNMT1 hypermethylating miR-148a, and miR-148a-3p
harming DNMT1. Furthermore, miR-34a-5p has also been shown to be hypermethylated in cancer (Lodygin, Tarasov et al. 2014, Chim, Wan et al. 2011, Siemens, Neumann et al. 2013), though no evidence of this was found in this study (figures 5.6 and 5.8). This could suggest that the methylation patterns differ between cell types or tumours.

The data presented here implemented both siRNA depletion of DNMT1 as well as inhibition via 5-aza-DC treatment, but no change in miR-148a-3p or miR-34a-5p expression was observed following either of these treatments (figures 5.6 and 5.8). It is possible that a longer course of treatment would lead to changes in miR-148a-3p and miR-34a-5p expression. It is also possible that under normal conditions miR-142-3p may be lightly methylated or that there is an active de-methylating mechanism. As a result miR-142-3p upregulation after short treatments with 5-aza-DC or DNMT1 depletion would be more pronounced. Alternatively, it may be that due to miR-142-3p’s extremely low abundance in these cells, a small change in transcription would lead to a large relative increase in miR-142-3p expression. While this may be the case for miR-142-3p, the greater endogenous expression of miR-148a-3p and miR-34a-5p may require a longer 5-aza-DC treatment to a detectable change in relative abundance.

It is important to note that while upregulation of miR-142-3p expression following 5-aza-DC treatment and siRNA mediated depletion of DNMT1, are highly suggestive of hypermethylation, they do not show it directly. Further experiments such as methylation state PCR or bi-sulphite sequencing would be required to test this hypothesis further.

The *in-situ* hybridisation experiments carried out in this study showed some unexpected results. Optimisation of the procedure involved probing for miR-21 in the tissues to show that microRNAs were detectable (figure 5.11). While miR-21 was not of interest to this study as no change in expression between p53 hemizygosity and p53 mutation was observed (see supplemental figure 3 for full microarray results), it is clearly very highly expressed in regions of the tumour within the tissue (figure 5.11). The staining for miR-21 showed very strong staining for miR-21 in the tissues from
mice with the K-RAS$^{G12D;p53^{+/+}}$ and K-RAS$^{G12D;p53^{R172H/+}}$ genotypes. While the microRNA microarrays did not identify a change in miR-21 expression between K-RAS$^{G12D;p53^{+/+}}$ and K-RAS$^{G12D;p53^{R172H/+}}$ tissues, it is still possible that miR-21 is highly expressed in both these tissues due to other mutations, such as the initiating mutation to K-RAS. Indeed, K-RAS mutations have been shown to drive miR-21 expression (Talotta, Cimmino et al. 2009, Hatley, Patrick et al. 2010). Some staining for miR-21 was observed in what may be a PanIN lesion, while no staining was seen within the healthy regions of tissue on the slides, including healthy acinar regions (figure 5.13), liver and spleen (figure 5.12). This reinforces the idea that K-RAS mutations are upregulating miR-21, initiating early lesion formation before loss or mutation of p53 is able to further drive miR-21 expression and fuel cancer progression. This would also explain why the microarrays did not show any significant change in miR-21 expression (see supplemental figure 3 for full microarray results). This may suggest a role for miR-21 in PDAC, as has been shown in numerous previous publications (Moriyama, Ohuchida et al. 2009, Sicard, Gayral et al. 2013).

Investigation into the expression of miR-142-3p found that all staining for this microRNA is in regions associated with the immune system (figure 5.14). The white pulp of the spleen and lymphoid aggregates are regions with a very high density of a variety of immune cells (Liao, von der Weid, P Y 2015, Elmore 2006, Cesta 2006). No specific staining was observed in pancreatic acinar cells or the tumour stroma, while some slight staining in the connective tissue seemed likely to be infiltrating leukocytes (figure 5.14-a). This could suggest that the loss of miR-142-3p expression observed was due to a reduction in the number of immune cells in the tumour. This hypothesis seems unlikely however, as a large immune response is a frequent occurrence in PDAC (Sideras, Braat et al. 2014). Additionally, as the cell lines also show loss of miR-142-3p, it would suggest that the change is occurring within the tumour cells themselves rather than in other cell types within the tumour mass (figure 3.7). This is not unexpected as the mutation to p53 is induced by PDX1/cre, which should be expressed specifically in the pancreatic cells, rather than the immune cells. It is probable that the technique is simply not sensitive enough to detect miR-142-3p in these tissues.
It would perhaps have been beneficial to have investigated the tissue localisation of miR-148a-3p and miR-34a-5p, but due to limitations in the number of sections available and the high cost of this experiment, localisation of those microRNAs was not investigated in this study.

5.4 Summary

- The miR-34 family are regulated by p53, TAp63 and TAp73.
- miR-34a is regulated independently of miR-34b and miR-34c in some cell types.
- The miR-34 family is induced in a p53-independent manner following DNA damage.
- DNMT1 is transcriptionally upregulated in mutant p53\textsuperscript{R172H} expressing tumours and cell lines.
- miR-142-3p is likely to be hypermethylated due to upregulation of DNMT1.
- miR-21 is highly expressed in pancreatic tumours, irrespective of mutation of p53.
Chapter 6 Conclusions

PDAC remains one of the most aggressive of human malignancies, with an incidence rate closely approaching the mortality rate. The main reason for this is the difficulty in diagnosis, resulting in the majority of PDACs being diagnosed only once the disease has metastasised (see section 1.3.1 for details). Current treatments for metastatic PDAC have poor clinical outcomes, with little hope of remission. For this reason, novel therapeutic strategies, aimed at treating metastatic disease are critical to reduce PDAC related morbidity and mortality. In order to develop drugs which specifically target metastasis, it is vitally important to understand the molecular events which result in this deleterious process.

Mutations to the tumour suppressor gene p53 are frequently observed in PDAC, with the majority resulting in a function protein with both dominant negative and gain of function characteristics (see section 1.3.5.2). A vitally important previous study showed that the p53^{R172H} mutation, which is the focus of this study, is able to induce a high incidence of metastasis in a mouse model of PDAC (Morton, Timpson et al. 2010). A number of studies have found that the gain of function associated with the p53^{R172H} mutant, may be due to inhibition of other p53 family members, p63 and p73 (see section 1.3.5.2 for details). Most notably, a very important study showed that mutant p53 may inhibit p63 function, leading to loss of Dicer expression, and subsequent dysregulation of microRNA expression (Muller, Trinidad et al. 2013). Additionally, loss of Dicer expression, and therefore presumably dysregulation of microRNAs, has been linked to invasion and metastasis in other cancer models (Martello, Rosato et al. 2010, Muller, Trinidad et al. 2013, Su, Chakravarti et al. 2010). These data strongly suggest an important role for microRNAs in suppression of metastasis, and posit the possibility that mutant p53^{R172H} expression in PDAC may induce metastasis, by dysregulating microRNA expression.

The aims of this study were to identify which microRNAs were dysregulated due to mutant p53 expression and to investigate how their dysregulation may impact invasion and metastasis. This chapter will discuss how the data I present in this thesis elucidates these questions.
6.1 Mutant p53\textsuperscript{R172H} inhibits expression of miR-148a-3p, which promotes invasion in PDAC

In Chapter 3 I was able to show that mutant p53\textsuperscript{R172H} is able to inhibit miR-148a-3p expression in tissues (figure 3.4 and 3.5), primary cell lines (figure 3.7) and p53 null cells which ectopically express mutant p53\textsuperscript{R172H} (figure 3.9). Following that, in Chapter 4 I was able to show that inhibition of miR-148a-3p is able to promote invasion in minimally invasive cells (figure 4.2), and ectopic expression of miR-148a is able to inhibit invasion in mutant p53\textsuperscript{R172H} expressing invasive cells (figure 4.3). These data strongly suggest that mutant p53\textsuperscript{R172H} may regulate invasion in PDAC through inhibiting the expression of miR148a-3p. This is in line with evidence suggesting that miR-148a-3p is able to regulate invasion in other cancer models (Zhang, Liu 2014, Wen, Zhao et al. 2015, Zheng, Liang et al. 2011). Moreover, miR-148a-3p expression has been shown to get progressively lower as pancreatic cancer develops, culminating with very low expression in invasive PDACs (Hanoun, Delpu et al. 2010). It has also been shown to be able to delineate pancreatitis, PanINs and PDAC with the greatest loss observed in PDAC (Xue, Abou Tayoun et al. 2013).

It is important to note that changes in invasion observed in \textit{in-vitro} experiments may not accurately recapitulate what occurs in animals, so it will be important to investigate the role of miR-148a-3p expression on invasion \textit{in-vivo}. Additionally, while invasion is an important aspect of metastasis, it is not a direct readout of metastatic ability. Validation of the role of miR-148a-3p in metastasis will also be required, which may be achieved through further manipulation of the animal models used in this study. It would be very interesting to knock out miR-148a-3p in the K-RAS\textsuperscript{G12D};p53\textsuperscript{-/-} mouse model and investigate the incidence of metastasis. If miR-148a-3p knockout were able to promote metastasis in this model, it would lend strong evidence to the hypothesis that mutant p53\textsuperscript{R172H} dependent metastasis occurs due to miR-148a-3p downregulation.

Having identified that loss of miR-148a-3p is able to promote invasion in PDAC, it was important to investigate the mRNAs which this microRNA targets. Here I was able to show that overexpression of miR-148a led to a significant decrease in MMP15 mRNA
expression (figure 4.11). This is very interesting, as MMP15 is a membrane bound metalloproteinase, which is able to remodel the ECM (Page-McCaw, Ewald et al. 2007, Lu, Takai et al. 2011), potentially increasing the invasive ability of cells. Unfortunately, it was not possible to find an antibody which could detect mouse MMP15, making direct analysis of miR-148a-3p’s ability to translationally regulate MMP15 impossible. There are other experiments which could be used to show that miR-148a-3p is able to regulate MMP15, such as luciferase reporter constructs, or SILAC mass spectrometry. However, they have not been carried out in this study, though may represent possible new avenues of investigation. It may also be interesting to investigate the effect of MMP15 depletion on the invasive ability of the cells used in this investigation. While this would not show any correlation between miR-148a-3p and MMP15, it would show whether MMP15 is able to promote invasion in this model of PDAC. Unfortunately, due to time constraints, these experiments have not yet been carried out.

Unfortunately, this study was not able to identify the mechanism which is regulating miR-148a-3p expression in this model. The use of methylation inhibitors (figure 5.6) and direct depletion of DNMT1 (figure 5.8) had no effect on miR-148a-3p expression. Additionally, ectopic expression of p53 family members, p63 and p73, did not affect miR-148a-3p expression (figures 5.1 and 5.2), suggesting that mutant p53R172H is not repressing miR-148a-3p expression through dominant negative regulation of p63 or p73. It would be very interesting to identify and investigate other mechanisms which may be regulating miR-148a-3p expression. A previous study found that miR-148a expression is driven by the WNT pathway in medulloblastoma (Yogi, Sridhar et al. 2015). They also showed that high miR-148a expression inhibits invasion and metastasis. Additionally, NF-κβ has been shown to induce miR-148a, which targets Quaking (QKI) to promote TGFβ signalling in glioblastoma (Wang, Pan et al. 2015). Mutant p53 has been shown to form complexes with SMAD proteins, (signal transduction effectors of TGFβ signalling) to promote invasion and metastasis through inhibition of p63 function in breast cancer (Adorno, Cordenonsi et al. 2009). This current study found no evidence that p63 is able to transactivate miR-148a expression which would suggest that mutant p53/SMAD/p63 complexes should not affect miR-
148a-3p expression. However, it is possible that mutant p53/SMAD complexes have functions other than those observed in the Adorno study, which may regulate miR-148a-3p expression. It would be very worthwhile to investigate the effect of modulation of these two pathways in this model of PDAC to investigate the effect on miR-148a-3p expression.

While follow up in-vivo experiments will be critical in directly associating miR-148a-3p loss of expression with metastasis, the fact that its loss promotes invasion in minimally invasive cells (figure 4.2) and its overexpression is able to attenuate the invasive ability of cells (figure 4.3), it may still make it an attractive therapeutic target. Targeting microRNA-based therapeutics to the majority of organs, including the pancreas, has not yet been fully realised (see section 1.4.12 for details), though new possibilities may arise in the future. A vital problem with RNA based therapeutic being directed at the pancreas is the high endogenous expression of RNases (Dastgheib, Irajie et al. 2014), which may degrade the therapeutic. With this in mind, targeting the cause of microRNA dysregulation, the mRNA targets of dysregulated microRNAs or even the protein products of mRNAs which are targeted by microRNAs may be a more suitable approach. Additionally, identification of the mechanism of miR-148a-3p downregulation and identification of bona-fide targets of miR-148a-3p would provide additional avenues for therapeutic intervention. However, a recent study concluded that overexpression of miR-148a was unsuitable for therapeutic intervention in PDAC (Delpu, Lulka et al. 2013). This study implemented a number of pancreatic cell lines and showed that overexpression of miR-148a had no effect on cell proliferation or chemosensitivity. In contrast to the data I present here, the Delpu study showed that overexpression of miR-148a-3p had no effect on the invasive ability of Mia-PaCa-2 cells. It is important to note that MIA-PaCa-2 cells express the p53R248W mutation which is a contact mutant, rather than the p53R172H, which is a structural mutant (see section 1.3.5.2 for details). Interestingly, the p53R248W mutation has been shown to have no impact on invasion in lung cancer (Yoshikawa, Hamada et al. 2010), possibly suggesting that MIA-PaCa-2 cells have achieved an invasive phenotype independent of p53 mutation. This may mean that microRNAs which are downregulated by mutant p53R172H expression may not be involved in invasion in MIA-PaCa-2 cells. This may also
mean that therapeutic modulation of microRNAs may require tailoring to tumours which express specific mutations to p53.

6.2 Mutant p53\textsuperscript{R172H} may acquire some gain of function abilities through dominant negative regulation of TAp63 and TAp73.

In Chapter 5 I was able to show that ectopic expression of either TAp63 or TAp73 was able to transactivate miR-34a-5p expression in H1299 and SAOS cells (figures 5.1 and 5.2). This finding could suggest that mutant p53\textsuperscript{R172H} is able to regulate miR-34a-5p expression through dominant negative inhibition of the transcriptional activity of TAp63 or TAp73. However, TAp63 and TAp73 were both shown to have no ability to regulate either miR-142-3p or miR-148a-3p, suggesting that this mechanism cannot explain all of the changes in microRNA expression which are associated with mutant p53\textsuperscript{R172H} in this model. While other studies have shown that mutant p53 is able to regulate Dicer expression through inhibition of p63 transcriptional activity (Muller, Trinidad et al. 2013), no change in Dicer expression was observed in this current study (figure 5.5). As previously mentioned, this may be due to diverse functions of different p53 mutations (see section 5.4 for details).

Importantly, transactivation of miR-34a-5p by TAp63 and TAp73 has not previously been shown, and present potential avenues of therapeutic intervention. While no effect on invasion was observed following miR-34a-5p modulation (figures 4.2 and 4.3), it is still possible that loss of miR-34a-5p is involved in another aspect of metastasis in PDAC. In order to answer this question, the use of animal models would be required. By deleting miR-34a in the K-RAS\textsuperscript{G12D};p53\textsuperscript{+/-} mouse model and investigating the incidence of metastasis, this question could be more thoroughly investigated. If miR-34a-5p was shown to be involved in pancreatic tumorigenesis, it may be an attractive therapeutic target. This posits the possibility that miR-34a-5p expression may be restored by increasing p63 or p73 expression. A previous study found that a drug named RETRA is able to destabilise mutant p53 interactions with p73, which leads to restoration of p73 transcriptional activity (Kravchenko, Ilyinskaya et al. 2008). Vitally, RETRA does not have any non-specific activity in cells which do not express mutant p53, making this drug highly selective. While this drug has so far...
only been used in cell culture and xenograft experiments (Kravchenko, Ilyinskaya et al. 2008), it presents an exciting opportunity for treating mutant p53 expressing tumours. It may be beneficial to investigate microRNA expression profiles of the cell lines implemented in this current study following treatment with RETRA. Given that this could be a simple RT-qPCR experiment, which could generate a very interesting result, this may be a good place to begin follow up to this study.

6.3 Mutant p53<sup>R172H</sup> may acquire gain of function through regulation of methylation through induction of DNMT1

The mechanism by which mutant p53<sup>R172H</sup> is able to acquire its gain of function properties is not fully understood. A number of studies have suggested that it may be through dominant negative regulation of p63 or p73 (see section 1.3.5.2 for details). However, this study found that miR-142-3p and miR-148a-3p expression were not affected by ectopic expression of p63 or p73 in SAOS and H1299 cells (figures 5.1 and 5.2). This suggests that mutant p53<sup>R172H</sup> must be affecting another mechanism to alter expression of these microRNAs. In Chapter 5 I was able to show that treatment with the methylation inhibitor 5-aza-DC (figure 5.6) and siRNA mediated depletion of DNMT1 (figure 5.8) were able to induce expression of miR-142-3p. Remarkably, siRNA mediated depletion of DNMT1 in mutant p53<sup>R172H</sup> expressing cells, was able to rescue miR-142-3p expression back to what was seen in a cell line with a K-RAS mutation but wild type p53 (figure 5.8). While the response of miR-142-3p to these treatments is highly suggestive of hypermethylation, further experiments such as methylation state PCR or bi-sulphite sequencing will be required to validate this hypothesis. However, a number of other studies (Abdul Razak, Baba et al. 2014, Chiou, Chien et al. 2013) have found miR-142-3p to be methylated, so it is not beyond reason to suggest that may be happening in PDAC as well.

As well as the response observed in miR-142-3p following inhibition of methylation, I was able to show that DNMT1 mRNA was upregulated in mutant p53<sup>R172H</sup> expressing tissues, cell lines and p53 null cells which ectopically express mutant p53<sup>R172H</sup> (figure 5.7-a). This would suggest that mutant p53<sup>R172H</sup> expression was able to transcriptionally induce, or possibly stabilise, DNMT1 mRNA. This has not previously
been observed and may be an alternate mechanism by which mutant p53<sup>R172H</sup> is able to affect the expression of genes which are not wild type p53 transcriptional targets and attain its gain of function ability. The transcription factors specificity protein 1&3 (SP1&3) have been shown to promote transcription of DNMT1 (Kishikawa, Murata et al. 2002). Interestingly, DNMT1 is transcriptionally repressed by a complex formed of wild type p53 and SP1, which directly binds to the promoter of DNMT1 (Lin, Wu et al. 2010). This complex has been shown to be inhibited by mutant p53, which leads to an upregulation of DNMT1 in lung cancer patients. It is possible that this same mechanism is inducing DNMT1 transcription in this PDAC model, leading to increased transcription of DNMT1 and subsequent hypermethylation of a subset of microRNAs. This study has not generated enough data to firmly state that this is occurring in this model. It is possible that mutant p53<sup>R172H</sup> stabilises DNMT1 mRNA, which offers another avenue for further investigation.

It is interesting to note that 78% of the microRNAs which were found to be dysregulated in the mouse tissue samples were downregulated (figure 3.1). Of these, 71% are previously known targets of methylation (see section 3.3 for details). It is tempting to speculate that some of these microRNAs may also be hypermethylated in this model of PDAC. While it would require further experiments to establish this link, if it proved true, it would add weight to the hypothesis that mutant p53<sup>R172H</sup> gets some of its gain of function through hypermethylation of genes, and would highlight DNMT1 as an attractive therapeutic target. It would perhaps be interesting to investigate the expression of other microRNAs which were found to be downregulated in the primary tissues following 5-aza-DC treatment and siRNA mediated depletion of DNMT1, in order to investigate the effect of inhibition of methylation on these microRNAs. A further experimental approach could be to use microRNA microarray of the primary cell lines used in this investigation following DNMT1 depletion, and compare the changing microRNAs to the result in figure 3.4. This could offer strong evidence in support of the hypothesis that mutant p53<sup>R172H</sup> achieves its gain of function ability through DNMT1 activity.

While this study was unable to link miR-142-3p to invasion, it is possible that this microRNA is involved in another step in metastasis in PDAC. In order to address this
question it would be necessary to implement in-vivo experiments, possibly deleting miR-142-3p in the K-RAS^{G12D};p53^{-/-} mice and observing any change in the incidence of metastasis. Given that depletion of DNMT1 is sufficient to rescue miR-142-3p expression, therapeutic options which target DNMT1 may be of value in PDAC.

Demethylating drugs have been available for some time but have variable therapeutic efficacy. 5-aza-DC has previously been used for cancer therapy but had sporadic efficacy and high toxicity (Jüttermann, Li et al. 1994). More recent clinical studies have shown some optimistic results using low doses of 5-aza-DC in haematological malignancies and myelodysplastic syndrome (Wijermans, Lübbert et al. 2000, Issa, Garcia-Manero et al. 2004, Kantarjian, Issa et al. 2006, Lübbert, Suciu et al. 2011, Kantarjian, O'Brien et al. 2007), suggesting that this drug may have therapeutic use. It is important to note that 5-aza-DC is an inhibitor of all DNMT proteins, and while this current study showed that treatment with of 5-aza-DC led to upregulation of miR-142-3p (figure 5.6), it upregulated it to a very high degree, while specific depletion of DNMT1 rescued miR-142-3p expression back to those seen in cells which express wild type p53 (figure 5.8). This may suggest that therapeutics exclusively targeting DNMT1 may be a more suitable therapeutic approach. Additionally, previous in-vitro studies have shown that of 5-aza-DC treatment promotes expression of both tumour suppressor genes and oncogenes, and promotes invasion and metastasis, while specific ablation of DNMT1 promotes expression of tumour suppressor genes alone, which do not promote metastasis (Chik, Szyf 2011). This highlights the need for drugs which target specific proteins involved in methylation. A number of drugs have been developed to target DNMT1, however, most show little to no therapeutic value, with the majority not affecting methylation at all (Gros, Fahy et al. 2012).

Currently, the lack of efficacious de-methylating drugs makes direct targeting of DNMT1 difficult. It is possible that in the future such compounds will exist and may have efficacy in treatment of PDAC.
6.4 The miR-34 family transcriptional regulation

Here I have shown that while expression of all members of the miR-34 family are induced following DNA damage, only miR-34a-5p is induced further in the presence of wild type p53 in H1299 cells. Additionally, only miR-34a-5p expression is induced due to ectopic expression of p53 without DNA damage (figure 5.3). This is contrary to a previous study which found that miR-34c-5p is the predominant family member responding to DNA damage, which regulates c-MYC translation to regulate cell cycle progression in MEF cells (Cannell, Kong et al. 2010). Another study found that DNA damage induced miR-34a and miR-34b in WI38 human fibroblast cell lines, which could be abolished using an shRNA targeting p53 (Bommer, Gerin et al. 2007). Additionally they showed DNA damage was able to induce miR-34a and miR-34b expression in HCT116 cells with normal p53 expression, but was not in HCT116 cells engineered null for p53. This paints a complicated picture, with p53 being necessary for induction of all miR-34 family members in some instances (Bommer, Gerin et al. 2007), while not requiring it at all in others (figure 5.3). Additionally, some studies find that miR-34c is the family member which responds to DNA damage via p38 MAPK signalling (Cannell, Kong et al. 2010) with other models finding miR-34a-5p is the predominant family member which responds to DNA damage (figure 5.3).

It is possible that different types of DNA damage cause different miR-34 family members to be induced. The Boomer study used doxorubicin to induce DNA damage in WI38 cells, as did I in this current study, while the Cannell study utilised etoposide. As both of these drugs are topoisomerase II inhibitors (Ross, Bradley 1981, Hande 1998), it is unclear why they would have independent responses from the miR-34 family. The Boomer study also used Ionising Radiation (IR) to induce DNA damage in HCT116 cells, resulted in induction of miR-34a and miR-34b which was lost in the p53 null HCT116 cells. The fact that Boomer demonstrated two independent forms of DNA damage were both able to produce the same effect in these two cell lines may suggest that the method of DNA damage is not the deciding factor on which miR-34 family member is induced.

This current study was also able to show that ectopic expression of TAp63 and TAp73 is able to induce the whole miR-34 family in SAOS cells (figure 5.2), while only inducing
miR-34a in H1299 cells (figure 5.1). It is intriguing to speculate that in a p53 null environment, TAp63 or TAp73 are able to induce expression of the miR-34 family in some cell lines following DNA damage. This cannot be addressed from data in this study, though repeating the DNA damage experiment following depletion of p63, p73 or both, may lead to interesting results.

All of the studies mentioned above used either cell lines which were null of p53 or transiently inhibited p53. It is possible that the different cell lines have each evolved independent compensatory mechanisms to cope with loss of p53, with some favouring expression of specific members of the miR-34 family. Given the array of conflicting results, it is likely to require many more experimental models to fully understand how the miR-34 family is regulated, with and without p53.


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Supplemental figures

Supplemental figure 1: The script used for the AgiMicroRNA plugin for R used to analyse the Agilent microRNA microarray results. R script is in black and a brief summary of the command is in blue below.

```r
>library(AgiMicroRna)
 Runs the AgiMicroRna programme.

>targets.micro=readTargets(infile="targets.txt",verbose=TRUE)
 Creates an object called targets.micro which contains data of which experimental condition each sample belongs to.

>dd.micro=readMicroRnaAFE(targets.micro,verbose=TRUE)
 Creates an object called dd.micro which contains the array data produced by Agilent feature extraction software.

>cvArray(dd.micro, "MeanSignal", targets.micro, verbose=TRUE)
 Calculates a coefficient of variation for each probe from the repeat probes for each microRNA.

>ddTGS.rma=rmaMicroRna(dd.micro, normalize=TRUE, background=TRUE)
 Background corrects each array and carries out a between array normalisation using the quantile method, before calculating the average signal for each microRNA.

>ddPROC=filterMicroRna(ddTGS.rma, dd.micro, control=TRUE, IsGeneDetected=TRUE, wellaboveNEG=FALSE, limIsGeneDetected=75, limNEG=25, makePLOT=FALSE, targets.micro, verbose=TRUE, writeout=FALSE)
 Filters out probes which are undetected.

>esetPROC=esetMicroRna(ddPROC, targets.micro, makePLOT=FALSE, verbose=TRUE)
 Creates an expression set object from the processed data which is used for statistical analysis.

>writeEset(esetPROC, ddPROC, targets.micro, verbose=TRUE)
 Writes the expression set object to an output file.

>levels.treatment=levels(factor(targets.micro$Treatment))treatment=factor(as.character(targets.micro$Treatment), levels=levels.treatment)
 Arranges the samples according to the treatment column of the targets file (a tab delimited file used to assign each sample to a condition).

design=model.matrix(~ -1 + treatment)
 Creates an object which contains the arranged samples.

>fit=lmFit(esetPROC,design)
 Uses LIMMA to fit a linear model to the data.

>CM=cbind(R172H_vs_Hemi=c(-1,1))
```
Specifies the design matrix for the comparison, essentially deciding which condition is the control and which is the test.

> fit2 = contrasts.fit(fit, CM)
Uses LIMMA to estimate differential expression using the design matrix.

> fit2 = eBayes(fit2)
Uses LIMMA to carry out statistical analysis.

> fit2 = basicLimma(esetPROC, design, CM, verbose=TRUE)
Enters log2 fold change and statistical data into the output file produced previously.

> DE = getDecideTests(fit2, DEmethod="separate", MTestmethod="BH", PVcut=0.10, verbose=TRUE)
significantMicroRna(esetPROC, ddPROC, targets.micro, fit2, CM, DE, DEmethod="separate", MTestmethod="none", PVcut=0.10, Mcut=0, verbose=TRUE)
Selects statistical cut off values for the data and plots a scatterplot.
Supplementary figure 2: The next 20 pages contain the full quality control outputs from Agilent feature extraction software for each of the samples used in this analysis. The K-RAS$^{G12D};p53^{+/-}$ samples are in the following order:

- 051152
- 06552
- 9840
- 10494
- 22407

Followed by the K-RAS$^{G12D};p53^{R172H/+}$ samples in the following order:

- 120994
- 120582
- 10579
- 38180
- 10580
## QC Report - Agilent Technologies: miRNA

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### Spot Finding of the Four Corners of the Array

Grid Normal

Feature | Local Background
---|---
Non Uniform | 3 | 0
Population | 1925 | 0

### Spatial Distribution of All Outliers on the Array

384 rows x 164 columns

### Net Signal Statistics

**Non-Control probes:**

- # Saturated Features: 0
- 99% of Sig. Distrib.: 3967
- 50% of Sig. Distrib.: 49
- 1% of Sig. Distrib.: 38

**Negative Control Stats**

- Average Net Signals: 47.82
- StdDev Net Signals: 3.42
- Average BG Sub Signal: 0.61
- StdDev BG Sub Signal: 3.26

### Histogram of Signals Plot

- # Features (NonCtrl) with BGSigSubSignal < 0: 17498 (Green)
Sample 051152
QC Report - Agilent Technologies: miRNA

Date: Tuesday, October 01, 2013 - 13:50
Image: GSE3703552_253543010366_S01 [1,3]
Protocol: miRNA_107_Sep09 (Read Only)
User Name: scanner
PE Version: 10.7.3.1
Sample (red/green): Saturated Value: 778718

Spot Finding of the Four Corners of the Array

Grid Normal

Feature Local Background
Non Uniform 0 0
Population 1911 47

Spatial Distribution of All Outliers on the Array
384 rows x 164 columns

Net Signal Statistics

Non-Control probes:

- Saturated Features: 0
- 99% of Sig. Distrib.: 5087
- 50% of Sig. Distrib.: 55
- 1% of Sig. Distrib.: 42

Negative Control Stats:

- Average Net Signals: 51.22
- StDev Net Signals: 3.53
- Average BG Sub Signal: -0.07
- StDev BG Sub Signal: 3.60

Histogram of Signals Plot

- Features (NonCtrl) with BGSubSignal < 0: 15704 (Green)
Sample 65552

Foreground Surface Fit

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Reproducibility: %CV for Replicated Probes
- Median %CV Signal (inliers)
- Non-Control probes

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Spatial Distribution of Median Signals for each Row

Spatial Distribution of Median Signals for each Column

Evaluation Metrics for miRNA_QCMT_Sep09:

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<th>Value</th>
<th>Excellent</th>
<th>Good</th>
<th>Evaluate</th>
</tr>
</thead>
<tbody>
<tr>
<td>IsGoodGrid</td>
<td>1.00</td>
<td>&gt;1</td>
<td>NA</td>
<td>&lt;1</td>
</tr>
<tr>
<td>AddErrorEstimateGreen</td>
<td>4.54</td>
<td>&lt;5</td>
<td>5 to 12</td>
<td>&gt;12</td>
</tr>
<tr>
<td>AnyColorPercentFreeRpmQC</td>
<td>4.01</td>
<td>&lt;8</td>
<td>8 to 15</td>
<td>&gt;15</td>
</tr>
<tr>
<td>gRoundChrmCentrCtVIRGSu...</td>
<td>7.17</td>
<td>0 to 10</td>
<td>10 to 15</td>
<td>&lt;8 or &gt;15</td>
</tr>
<tr>
<td>gTotalSignal75pcile</td>
<td>18.06</td>
<td>&gt;2.59</td>
<td>&lt;2.50</td>
<td></td>
</tr>
<tr>
<td>LabelingSpike-IndSignal</td>
<td>2.89</td>
<td>&gt;2.59</td>
<td>&lt;2.50</td>
<td></td>
</tr>
<tr>
<td>HydSpike-IndSignal</td>
<td>3.55</td>
<td>&gt;2.59</td>
<td>&lt;2.50</td>
<td></td>
</tr>
<tr>
<td>StringencySpike-IndRatio</td>
<td>0.69</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

 Emblem: Excellent, Good, Evaluate
QC Report - Agilent Technologies: miRNA

Sample 9840

Net Signal Statistics

Non-Control probes:

- # Saturated Features: 0
- 99% of Sig. Distrib.: 4411
- 50% of Sig. Distrib.: 55
- 1% of Sig. Distrib.: 43

Negative Control Stats

- Average Net Signals: 52.09
- StdDev Net Signals: 3.18
- Average BG Sub Signal: -0.36
- StdDev BG Sub Signal: 3.20

Histogram of Signals Plot

# Features (NonCtrl) with BGSubSignal < 0: 17423 (Green)

Spatial Distribution of All Outliers on the Array

- 384 rows x 164 columns
- # FeatureNonUnif = 0 (0.00%)
- # Feature Population Outliers = 2039 (4.28%)
- BG Nonuniform
- BG Population
- Green Feature Population
- Green Feature NonUniform
**Sample 9840**

**Foreground Surface Fit**

<table>
<thead>
<tr>
<th>Metric</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMS_Fit</td>
<td>0.88</td>
</tr>
<tr>
<td>RMS_Resid</td>
<td>4.53</td>
</tr>
<tr>
<td>Avg_Fit</td>
<td>59.39</td>
</tr>
</tbody>
</table>

**Reproducibility: %CV for Replicated Probes**

- Median %CV Signal (outliers)
- Non-Control probes

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>BGSubSignal</td>
<td>6.89</td>
</tr>
<tr>
<td>ProcessedSignal</td>
<td>6.99</td>
</tr>
</tbody>
</table>

**Reproducibility: %CV for Replicated Probes (plot)**

**Evaluation Metrics for miRNA_QCMT_Sep09**:  
**Excellent (4)** ; **Good (2)**

**Metric Name** | **Value** | **Excellent** | **Good** | **Evaluate**
--- | --- | --- | --- | ---
IndGoodGrd     | 1.00 | >1 | NA | <1
AddErrorEstimateGreen | 4.53 | <5 | 5 to 12 | >12
AnyColorPrmSeqPrmCL | 4.28 | <8 | 8 to 15 | >15
g3tInChrdPrntxGtBGSub | 6.89 | 0 to 10 | 10 to 15 | <0 or >15
gTotalSignal/gSpike | 16.35 |     |     |     
LabelingSpike-InSignal | 3.15 | >2.59 | <2.50 |
HydSpike-InSignal | 3.77 | >2.59 | <2.50 |
StringencySpike-InRatio | 0.63 |     |     |     |

★ Excellent ★ Good ★ Evaluate
QC Report - Agilent Technologies: miRNA

Date: Tuesday, October 01, 2013 - 13:51
Grid: Grid

Image: US83703553_253543010366_S01 [2.4]
BG Method: BG Method

Protocol: miRNA_107_Sep09 (Read Only)
Background Detrend: Background Detrend

User Name: scanner
Multiplicative Detrend: False

PE Version: 10.7.3.1
Additive Error: 4

Sample (red/green): Saturation Value

Net Signal Statistics
Non-Control probes:

- # Saturated Features: 0
- 99% of Sig. Distrib.: 5682
- 50% of Sig. Distrib.: 50
- 1% of Sig. Distrib.: 38

Negative Control Stats

- Average Net Signals: 48.66
- StdDev Net Signals: 3.38
- Average BG Sub Signal: 1.66
- StdDev BG Sub Signal: 3.18

Spatial Distribution of All Outliers on the Array
384 rows x 164 columns

# Feature NonUniform = 2 (0.00%)
# Feature Population Outliers = 1895 (3.98%)

Histogram of Signals Plot

# Features (NonCtrl) with BGSubSignal < 0: 16321 (Green)
Sample 10494

Foreground Surface Fit

<table>
<thead>
<tr>
<th>Metric</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMS_Fit</td>
<td>1.67</td>
</tr>
<tr>
<td>RMS_Resid</td>
<td>3.66</td>
</tr>
<tr>
<td>Avg_Fit</td>
<td>53.97</td>
</tr>
</tbody>
</table>

Reproducibility: %CV for Replicated Probes

| BGSubSignal  | 7.17  |
| ProcessedSignal | 7.17  |

Reproducibility: %CV for Replicated Probes(plot)

Evaluation Metrics for miRNA_QCMT_Sep09:

<table>
<thead>
<tr>
<th>Metric Name</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>InGoodGnd</td>
<td>1.00</td>
</tr>
<tr>
<td>AdjErrorEstimateGreen</td>
<td>3.66</td>
</tr>
<tr>
<td>AnyColorPntFeetPmppOL</td>
<td>3.98</td>
</tr>
<tr>
<td>gilenCtrlMedPntCtrlBGSub</td>
<td>7.17</td>
</tr>
<tr>
<td>gTotalSignal/Specifie</td>
<td>17.07</td>
</tr>
<tr>
<td>LabelingSpike-InSignal</td>
<td>3.18</td>
</tr>
<tr>
<td>HydSpike-InSignal</td>
<td>3.80</td>
</tr>
<tr>
<td>StringencySpike-InRatio</td>
<td>0.66</td>
</tr>
</tbody>
</table>

- Excellent (4) ; Good (2) ; Evaluate
## Sample 22407

### QC Report - Agilent Technologies: miRNA

**Date:** Tuesday, October 01, 2013 - 13:43  
**Grid:** 035430_D_F_20111226

**Image:** US83703553_253543010365_S01 [1_4]  
**BG Method:** No Background

**Protocol:** miRNA_107_Sep09 (Read Only)  
**Background Detrend:** On (FeatNCRange, LoPass)

**User Name:** scanner  
**Multiplicative Detrend:** False

**PE Version:** 10.7.3.1  
**Additive Error:** 4

**Sample (red/green):** Saturation Value  
**saturation Value:** 778697

### Spot Finding of the Four Corners of the Array

- **Grid Normal**
- **Feature**
- **Local Background**

### Net Signal Statistics

#### Non-Control probes:

<table>
<thead>
<tr>
<th>Metric</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td># Saturated Features</td>
<td>0</td>
</tr>
<tr>
<td>99% of Sig. Distrib.</td>
<td>6858</td>
</tr>
<tr>
<td>50% of Sig. Distrib.</td>
<td>52</td>
</tr>
<tr>
<td>1% of Sig. Distrib.</td>
<td>42</td>
</tr>
</tbody>
</table>

#### Negative Control Stats

<table>
<thead>
<tr>
<th>Metric</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Net Signals</td>
<td>52.72</td>
</tr>
<tr>
<td>StdDev Net Signals</td>
<td>3.30</td>
</tr>
<tr>
<td>Average BG Sub Signal</td>
<td>2.24</td>
</tr>
<tr>
<td>StdDev BG Sub Signal</td>
<td>3.23</td>
</tr>
</tbody>
</table>

### Spatial Distribution of All Outliers on the Array

**384 rows x 164 columns**

- **Non Uniform: 0**
- **Population: 1885**

### # Feature NonUnif = 0 (0.00%)  
# Feature Population Outliers = 1885 (3.96%)

**BG NonUnif**  
**BG Population**  
**Green Feature Population**  
**Green Feature NonUniform**

### Histogram of Signals Plot

- **# Features (NonCtrl) with BGSubSignal < 0: 17708 (Green)**
Evaluation Metrics for miRNA_QCMT_Sep09:

<table>
<thead>
<tr>
<th>Metric Name</th>
<th>Value</th>
<th>Excellent</th>
<th>Good</th>
<th>Evaluate</th>
</tr>
</thead>
<tbody>
<tr>
<td>InOut sincerely</td>
<td>&gt;1</td>
<td>NA</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>AddErrorEstimateGreen</td>
<td>3.95</td>
<td>&lt;5</td>
<td>5 to 12</td>
<td>&gt;12</td>
</tr>
<tr>
<td>AnyColorProcessFlagProcCL</td>
<td>3.95</td>
<td>&lt;8</td>
<td>8 to 15</td>
<td>&gt;15</td>
</tr>
<tr>
<td>gImsCV or MedianProcCV</td>
<td>6.46</td>
<td>0 to 10</td>
<td>10 to 15</td>
<td>&lt;0 or &gt;15</td>
</tr>
<tr>
<td>pTotalSignal7Spike</td>
<td>12.84</td>
<td>&lt;2.59</td>
<td>&lt;2.59</td>
<td>&lt;2.59</td>
</tr>
<tr>
<td>LabelingSpike-InSignal</td>
<td>3.17</td>
<td>&gt;2.59</td>
<td>&lt;2.59</td>
<td>&lt;2.59</td>
</tr>
<tr>
<td>HydSpike-InSignal</td>
<td>3.49</td>
<td>&gt;2.59</td>
<td>&lt;2.59</td>
<td>&lt;2.59</td>
</tr>
<tr>
<td>StringencySpike-InRatio</td>
<td>0.89</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
QC Report - Agilent Technologies: miRNA

Date: Tuesday, October 01, 2013 - 13:43
Image: US5703553_253543010365_S01 [1_1]
Protocol: miRNA_107_Sep09 (Read Only)
User Name: scanner
PE Version: 10.7.3.1
Sample (red/green): Saturation Value

Net Signal Statistics
Non-Control probes:
- # Saturated Features: 0
- 99% of Sig. Distrib.: 2521
- 50% of Sig. Distrib.: 57
- 1% of Sig. Distrib.: 45

Negative Control Stats
- Average Net Signals: 57.30
- StDev Net Signals: 4.15
- Average BG Sub Signal: 2.36
- StDev BG Sub Signal: 3.62

Histogram of Signals Plot
- # Features (NonCtrl) with BGSubSignal < 0: 18415 (Green)
### Foreground Surface Fit

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMS_Fit</td>
<td>1.99</td>
</tr>
<tr>
<td>RMS_Resid</td>
<td>3.88</td>
</tr>
<tr>
<td>Avg_Fit</td>
<td>62.63</td>
</tr>
</tbody>
</table>

**Reproducibility: %CV for Replicated Probes**
- Median %CV Signal (outliers)
- Non-Control probes

<table>
<thead>
<tr>
<th></th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BGSubSignal</td>
<td>7.19</td>
</tr>
<tr>
<td>ProcessedSignal</td>
<td>7.19</td>
</tr>
</tbody>
</table>

**Reproducibility: %CV for Replicated Probes (plot)**

### Spatial Distribution of Median Signals for each Row

![Graph showing spatial distribution of median signals for each row.]

**Spatial Distribution of Median Signals for each Column**

![Graph showing spatial distribution of median signals for each column.]

### Evaluation Metrics for miRNA_QCMT_Sep09:

**Excellent (4) • Good (2)**

<table>
<thead>
<tr>
<th>Metric Name</th>
<th>Value</th>
<th>Excellent</th>
<th>Good</th>
<th>Evaluate</th>
</tr>
</thead>
<tbody>
<tr>
<td>InGoodGnd</td>
<td>1.00</td>
<td>&gt;4</td>
<td>NA</td>
<td>&lt;1</td>
</tr>
<tr>
<td>AddErrorEstimateGreen</td>
<td>3.88</td>
<td>&lt;5</td>
<td>5-12</td>
<td>&gt;12</td>
</tr>
<tr>
<td>AnyColrProtrErrPopCol</td>
<td>3.79</td>
<td>&lt;8</td>
<td>8-15</td>
<td>&gt;15</td>
</tr>
<tr>
<td>gBlankChrmProtrCvBGSub</td>
<td>7.19</td>
<td>0-10</td>
<td>10-15</td>
<td>&lt;0 or &gt;15</td>
</tr>
<tr>
<td>gTotalSignalSpike</td>
<td>5.73</td>
<td>&gt;2.50</td>
<td>&lt;2.50</td>
<td></td>
</tr>
<tr>
<td>LabelingSpike-InSignal</td>
<td>2.96</td>
<td>&gt;2.50</td>
<td>&lt;2.50</td>
<td></td>
</tr>
<tr>
<td>HydSpike-InSignal</td>
<td>3.68</td>
<td>&gt;2.50</td>
<td>&lt;2.50</td>
<td></td>
</tr>
<tr>
<td>StringencySpike-InRatio</td>
<td>0.90</td>
<td>&gt;2.50</td>
<td>&lt;2.50</td>
<td></td>
</tr>
</tbody>
</table>

* Excellent • Good • Evaluate
QC Report - Agilent Technologies: miRNA

Date: Tuesday, October 01, 2013 - 13:51
Image: US83703553_253543010366_S01_2_3
Protocol: miRNA_107_Sep09 (Read Only)
User Name: scanner
PE Version: 10.7.3.1
Sample (red/green): Saturation Value

Grid Normal
Feature
Local Background

Net Signal Statistics
Non-Control probes:

- # Saturated Features: 0
- 99% of Sig. Distrib.: 3450
- 50% of Sig. Distrib.: 51
- 1% of Sig. Distrib.: 40

Negative Control Stats:

- Average Net Signals: 49.82
- StdDev Net Signals: 3.35
- Average BG Sub Signal: 1.18
- StdDev BG Sub Signal: 3.31

Spatial Distribution of All Outliers on the Array
384 rows x 164 columns

- # Feature NonUniform: 1 (0.00%)
- # Feature Population Outliers: 1938 (4.07%)

Histogram of Signals Plot

# Features (NonCtrl w/ BGSubSignal < 0: 17858 (Green)
**QC Report - Agilent Technologies: miRNA**

**Date**  
Tuesday, October 01, 2013 - 13:43

**Image**  
US83703553_253543010365_S01 [2_2]

**Protocol**  
mRNA_107_Sep09 (Read Only)

**User Name**  
scanner

**FE Version**  
10.7.3.1

**Sample (red/green)**  
Saturation Value

### Spot Finding of the Four Corners of the Array

<table>
<thead>
<tr>
<th>Feature</th>
<th>Local Background</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non Uniform</td>
<td>1</td>
</tr>
<tr>
<td>Population</td>
<td>1946</td>
</tr>
</tbody>
</table>

### Spatial Distribution of All Outliers on the Array

384 rows x 164 columns

### Net Signal Statistics

#### Non-Control probes:

<table>
<thead>
<tr>
<th>Feature</th>
<th>99% of Sig. Distrib.</th>
<th>50% of Sig. Distrib.</th>
<th>1% of Sig. Distrib.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated Features</td>
<td>0</td>
<td>2984</td>
<td>53</td>
</tr>
</tbody>
</table>

#### Negative Control Stats

<table>
<thead>
<tr>
<th>Feature</th>
<th>Average Net Signals</th>
<th>StdDev Net Signals</th>
<th>Average BG Sub Signal</th>
<th>StdDev BG Sub Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>50.71</td>
<td>3.42</td>
<td>0.67</td>
<td>3.13</td>
<td></td>
</tr>
</tbody>
</table>

### Histogram of Signals Plot

- Number of Points: 480
- Log of BG SubSignal
- # Features (NonCtrl) with BGSubSignal < 0: 16186 (Green)
Sample 10579

Foreground Surface Fit

<table>
<thead>
<tr>
<th>Metric</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMS_Fit</td>
<td>1.51</td>
</tr>
<tr>
<td>RMS_Resid</td>
<td>4.25</td>
</tr>
<tr>
<td>Avg_Fit</td>
<td>57.71</td>
</tr>
</tbody>
</table>

Reproducibility: %CV for Replicated Probes

<table>
<thead>
<tr>
<th>Metric</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BGSubSignal</td>
<td>8.55</td>
</tr>
<tr>
<td>ProcessedSignal</td>
<td>8.55</td>
</tr>
</tbody>
</table>

Spatial Distribution of Median Signals for each Row

Spatial Distribution of Median Signals for each Column

Evaluation Metrics for miRNA_QCMT_Sep09:

<table>
<thead>
<tr>
<th>Metric Name</th>
<th>Value</th>
<th>Excellent</th>
<th>Good</th>
<th>Evaluate</th>
</tr>
</thead>
<tbody>
<tr>
<td>IsGoodGrid</td>
<td>1.00</td>
<td>&gt;1</td>
<td>NA</td>
<td>&lt;1</td>
</tr>
<tr>
<td>AddErrorEstimateGreen</td>
<td>4.25</td>
<td>&lt;5</td>
<td>5 to 12</td>
<td>&gt;12</td>
</tr>
<tr>
<td>AnyColorProofPctFreePepnCl</td>
<td>4.99</td>
<td>&lt;8</td>
<td>8 to 15</td>
<td>&gt;15</td>
</tr>
<tr>
<td>gUnCovPctMedPctCVRG5G5u</td>
<td>8.55</td>
<td>0 to 10</td>
<td>10 to 15</td>
<td>&lt;8 or &gt;15</td>
</tr>
<tr>
<td>gTotalSignal75picrile</td>
<td>14.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LabelingSpike-IrSsignal</td>
<td>2.99</td>
<td>&gt;2.59</td>
<td>&lt;2.50</td>
<td></td>
</tr>
<tr>
<td>HydSpike-IrSsignal</td>
<td>3.40</td>
<td>&gt;2.59</td>
<td>&lt;2.50</td>
<td></td>
</tr>
<tr>
<td>StringencySpike-IntRatio</td>
<td>0.90</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

✨ Excellent ✨ Good ✨ Evaluate
QC Report - Agilent Technologies: miRNA

Date: Tuesday, October 01, 2013 - 13:50
Grid: 035430_D_F_20111226
Image: US8793553_25345010366_S01_2_1
BG Method: No Background
Protocol: miRNA_107_Sep09 (Read Only)
Background Detrend: On(FeatNCRange, LoPass)
User Name: scanner
Multiplicative Detrend: False
PE Version: 10.7.3.1
Additive Error: 5
Sample(red/green): Saturation Value 778718

Spot Finding of the Four Corners of the Array

Net Signal Statistics
Non-Control probes:
- # Saturated Features: 0
- 99% of Sig. Distrib.: 6635
- 50% of Sig. Distrib.: 53
- 1% of Sig. Distrib.: 41

Negative Control Stats
- Average Net Signals: 51.00
- StdDev Net Signals: 3.50
- Average BG Sub Signal: 0.45
- StdDev BG Sub Signal: 3.41

Spatial Distribution of All Outliers on the Array
384 rows x 164 columns

# FeatureNonUnif = 1(10.00%)
# Feature Population Outliers = 2218(4.66%)
BG Nonuniform
BG Population
Green FeaturePopulation
Green Feature NonUniform

Histogram of Signals Plot
- # Features (NonCtrl) with BGSigSignal < 0: 17098 (Green)
Sample 38180

<table>
<thead>
<tr>
<th>Metric Name</th>
<th>Value</th>
<th>Excellent</th>
<th>Good</th>
<th>Evaluate</th>
</tr>
</thead>
<tbody>
<tr>
<td>InGoodGnd</td>
<td>1.00</td>
<td>&gt;1</td>
<td>NA</td>
<td>&lt;1</td>
</tr>
<tr>
<td>AdjErrorEstimateGnd</td>
<td>4.73</td>
<td>&lt;5</td>
<td>5 to 12</td>
<td>&gt;12</td>
</tr>
<tr>
<td>AnyColorPromPromProCl</td>
<td>4.66</td>
<td>&lt;8</td>
<td>8 to 15</td>
<td>&gt;15</td>
</tr>
<tr>
<td>qTotalSignalQSpecie</td>
<td>12.91</td>
<td>0 to 10</td>
<td>10 to 15</td>
<td>&lt;0 or &gt;15</td>
</tr>
<tr>
<td>LabelingSpike-InSignal</td>
<td>3.24</td>
<td>&gt;2.59</td>
<td>&lt;2.59</td>
<td></td>
</tr>
<tr>
<td>HydSpike-InSignal</td>
<td>3.81</td>
<td>&gt;2.59</td>
<td>&lt;2.50</td>
<td></td>
</tr>
<tr>
<td>StringencySpike-InRatio</td>
<td>0.62</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
QC Report - Agilent Technologies: miRNA

Date: Tuesday, October 01, 2013 - 13:43
Image: US83703553_253543010365_S01_2_4
Protocol: miRNA_107_Sep09 (Read Only)
User Name: scanner
PE Version: 10.7.3.1
Sample (red/green): Saturation Value

Grid Normal

Feature Local Background
Non Uniform 0 0
Population 1900 0

Spatial Distribution of All Outliers on the Array
384 rows x 164 columns

# Feature NonUnif = 0 (0.00%)
# Feature Population Outliers = 1900 (3.99%)

Net Signal Statistics

Non-Control probes:

- # Saturated Features: 0
- 99% of Sig. Distrib.: 3897
- 50% of Sig. Distrib.: 48
- 1% of Sig. Distrib.: 37

Negative Control Stats:

Average Net Signals: 47.50
StdDev Net Signals: 3.51
Average BG Sub Signal: 1.90
StdDev BG Sub Signal: 3.16

Histogram of Signals Plot

# Features (NonCtrl) with BGSubSignal < 0: 16573 (Green)
Sample 10580

Foreground Surface Fit

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Reproducibility: %CV for Replicated Probes

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Supplemental figure 3: The results of the microRNA microarrays for every detected gene.

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