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**MOLECULAR MECHANISMS OF RESISTANCE TO THERAPEUTIC MEK
INHIBITION IN BRAF^{V600E} CANCER**

BY

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SUBMITTED FOR THE DEGREE OF

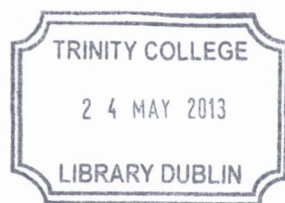
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Emma Dorris

January 2013

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I. Summary

Aberrant activation of the mitogen activated protein kinase (MAPK) pathway is common in solid tumours. This hierarchical pathway consists of a three-tiered kinase cascade of BRAF, MAPK kinase (MEK1/2) and the extracellular signal-regulated kinase (ERK1/2). Extracellular ligands activate the pathway by binding to receptors on the cell surface that signal through the small GTPase RAS to recruit and activate BRAF, which initiates a kinase cascade through MEK1/2 and ERK1/2 to activate downstream transcription factors involved in a range of biochemical processes including differentiation, proliferation, growth and survival. Mutations in BRAF occur at an estimated rate of 50-80% melanoma and 45% in thyroid carcinoma. The most common BRAF mutation is a single base pair change, T1799A, that results in a valine to glutamic acid change at position 600 in the BRAF polypeptide (BRAF^{V600E}). This causes a conformational change in the BRAF protein leading to constitutive activation and oncogene addiction to the MAPK pathway.

Our knowledge of molecular pathways and how they go awry in cancer is being employed to rationally design molecularly targeted therapeutics with the aim of personalised cancer therapy. The goal of personalised cancer therapy is to elucidate how to use cancer therapeutics in the most effective way by correctly targeting patients that are most likely to respond to a specific therapy. In order to successfully achieve this goal, it is not enough merely to know that a treatment works; rather, the mechanisms behind how it works, and how cancer cells can gain resistance to it, must be elucidated so that the correct treatment can be anticipated for the individual patient based on their underlying genotype and cancer phenotype. Selective inhibitors of the MAPK pathway are under intense development as anticancer drugs. Small molecule kinase inhibitors (SMKI) of BRAF and MEK have shown promising results in clinical trials for the treatment of BRAF^{V600E} malignant melanoma and other BRAF^{V600E} cancers.

In this thesis the molecular pathology of resistance to therapeutic MEK inhibition in BRAF^{V600E} melanoma and thyroid cells was investigated. A combination of gene expression, protein expression and small RNA-Seq next generation sequencing (NGS) of microRNAs (miRNAs) was used to identify gene networks involved in resistance. Chapter 1 introduces BRAF^{V600E}; its role in melanoma, colorectal and thyroid cancers; and the development of molecularly targeted therapeutics against it (Cantwell-Dorris et al, 2011). Chapter 2 outlines the methods used to approach the task of elucidating molecular mechanisms of resistance and includes techniques such as: BRAFT1799A allelic discrimination assays; drug treatment optimisation assays; Real-time PCR for gene and miRNA expression; Western blot, confocal microscopy and protein ligation technology; and, for the first time in Ireland, miRNA expression profiling using small

RNA next generation sequencing (small RNA-Seq) on the Applied Biosystems SOLiD™ 4 NGS platform.

Chapter 3 demonstrates how a specific miRNA might demonstrate utility as a diagnostic biomarker. *MIR141* was previously identified by our group as a potential biomarker candidate using microarray expression profiling (Aherne *et al.*, 2008). In this study we show that *MIR141* expression can discriminate Hashimoto Thyroiditis from classic papillary thyroid carcinoma (cPTC) and control (no evidence of malignancy) samples of formalin fixed paraffin embedded (FFPE) (Dorris *et al.*, 2012).

Chapter 4 demonstrates the importance of optimisation and the heterogenic sensitivity of molecular therapy to different cancer types. Within cell line type, the BRAF^{V600E/V600E} cell lines displayed greater sensitivity to the MEK inhibitor PD0325901, as previously reported (Solit *et al.*, 2006; Lebeouf *et al.*, 2008). A potential folly was avoided through genotyping all cell lines for BRAFT1799A, as a cell line previously reported in the literature as a BRAF wild-type (BRAF^{WT}) was found to be heterozygous for T1799A (Xing *et al.*, 2011). This mutation was identified in both the commercially available cell line and also in the cell line supplied directly by the originator.

The thesis culminates in the interrogation of the molecular mechanisms contributing to MEKi resistance in BRAF^{V600E/V600E} thyroid and melanoma cell lines. The molecular pathobiology of resistance to MEK inhibition, including an associated gene signature of MAPK pathway members and stemness-associated markers, was determined in MEKi resistant cells. The related miRNA clusters *MIR302* and *MIR520* were identified in association with resistance to MEK inhibition in BRAF^{V600E} cells. These signatures were not found in BRAF^{WT} cell lines. Viewing cancer through a prism of genetic interactions via gene ontology and integrative analysis of the miRNA profiling data with the gene and protein expression data, identified regulation of the cellular differentiation/self renewal axis as a key player in MEKi resistance. Whilst the massively parallel sequencing and genetic profiling performed in this thesis allows the generation of informed hypotheses; future directions of this work should include thorough investigation of the molecular mechanisms elucidated in this study through the use of functional genomics towards the miRNAs and key members of the gene signatures identified.

II. Statement of Novelty

The major focus of this thesis was to investigate a role for microRNAs in the process of emerging resistance to MEK inhibition and as potential biomarkers. Global microRNA expression profiling of naïve and resistant cells was performed via next generation sequencing (NGS). To the author's knowledge, this is the first time that small RNA sequencing of matched resistant and naïve samples of MEK-inhibitor treated samples has been used to elucidate molecular mechanisms of resistance to small molecule kinase inhibitors of MEK in BRAF^{V600E} mutated thyroid and melanoma cell lines. The method of NGS outlined in this thesis is the first time this sequencing technology has been used in Ireland. Through a series of gene, microRNA and protein expression profiling, differential patterns of resistance were identified between BRAF^{V600E} melanoma and thyroid cell lines. The underlying cause of these differential patterns has yet to be fully elucidated. However, this thesis provides a basis for the investigation of the cellular differentiation/self-renewal access and its role in resistance to MEK inhibition.

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IV. Publications

DORRIS, E., SMYTH, P., O'LEARY, J. & SHEILS, O. 2012.

MIR141

expression differentiates Hashimoto Thyroiditis from PTC and benign thyrocytes in Irish archival thyroid tissues. *Frontiers in Thyroid Endocrinology*, In Press.

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Networked T cell

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V. Abbreviations

4-IPBA	4-Iodophenylboronic Acid
AB	Applied Biosystems MagMax gDNA Extraction Kit
AMP	Adenosine Monophosphate
APS	Ammonium Persulfate
ASCO	American Society of Clinical Oncology
ATCC	American Tissue Culture Collection
ATP	Adenosine Triphosphate
BRIM-3	BRAF in Melanoma Phase 3 Study
BSA	Bovine Serum Albumin
CDK	Cyclin Dependent Kinase
cDNA	Complementary DNA
CIMP	CpG Island Methylation Phenotype
COSMIC	Catalogue of Somatic Mutations in Cancer
cPTC	Classic Papillary Thyroid Carcinoma
CRC	Colorectal Cancer
CSC	Cancer Stem Cell
CSD	Chronic Sun Damaged
DAPI	4',6-diamidino-2-phenylindole
dsDNA	Double Stranded DNA
DTT	Dithiothreitol
ECACC	European Collection of Cell Cultures
ECM	Extracellular Matrix
EMEM	Eagle's Minimum Essential Media
ePCR	Emulsion PCR
ESC	Embryonic Stem Cell
ESCC	Embryonic Stem Cell-Specific Cell Cycle-Regulating
FBS	Foetal Bovine Serum
FFPE	Formalin Fixed Paraffin Embedded
FTC	Follicular Thyroid Carcinoma
fvPTC	Follicular Variant PTC
gDNA	Genomic DNA

GO	Gene Ontology
HRP	Horse Radish Peroxidase
HT	Hashimoto Thyroiditis
ICS	Instrument Control Software
iPS	Induced Pluripotent Stem (iPS)
LCM	Laser Capture Microdissection
LSCM	Laser Scanning Confocal Microscope
MAPK	Mitogen-Activated Protein Kinase
MEKi	MEK Inhibitor
MEKK	MAPK Kinase
Mg	Magnesium
MGB	Minor Groove Binding
miRNA	MicroRNA
MMR	Mismatch Repair
MN	Macherey Nagel NucleoSpin gDNA Extraction Kit
mRNA	Messenger RNA
MSI	Microsatellite Instability
MSI-H	MSI High
MSKCC	Memorial Sloan Kettering Cancer Centre
MSP	Manufacturer's Supplied Protocol
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NFQ	Non Fluorescent Quencher
NGS	Next Generation Sequencing
NKT	Natural Killer T Cells
NPC	No Protein Control
NTC	No Template Control
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PASR	Promoter Associated Small RNA
PBS	Phosphate Buffered Saline
piRNA	PIWI-Interacting RNA
piRNP	PIWI Ribonucleoprotein Complex
PLT	Protein Ligation Technology
PROMPT	Promoter Upstream Transcript

PTC	Papillary Thyroid Carcinoma
PVDF	Polyvinylidene Difluoride
QAP	Qiagen AllPrep Nucleic Acid Extraction Kit
QDN	Qiagen DNeasy gDNA Extraction Kit
RECIST	Response Evaluation Criteria in Solid Tumors
RIN	RNA Integrity Number
RISC	RNA-Induced Silencing Complex
RNAi	RNA Interference
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
RQ	Relative Quantitation
SAPK	Stress-Activated Protein Kinase Signalling Pathway
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of the Mean
siRNA	Short Interfering RNA
SMKI	Small Molecule Kinase Inhibitor
snoRNA	Small Nucleolar RNA
snoRNP	Small Nucleolar Ribonucleoprotein Complex
SNP	Single Nucleotide Polymorphism
SOP	Standard Operating Protocol
TBE	Tris/Borate/EDTA
TBS-T	Tris-Buffered Saline and Tween 20
TE	Tris EDTA
TEMED	Tetramethylethylenediamine
tiRNA	Transcription Initiation RNA
TPO	Thyroid Peroxidase
TSSa-RNA	Transcriptional Start Site-Associated RNA
UVR	Ultraviolet Radiation
WFA	Work Flow Analysis

VI. Gene Symbols

ACTIVIN/ACVR	activin A receptor
AKT	v-akt murine thymoma viral oncogene homolog
ALDH1A1	aldehyde dehydrogenase 1 family, member A1
APC2	adenomatosis polyposis coli 2
ARAF	v-raf murine sarcoma 3611 viral oncogene homolog
BCR-ABL	breakpoint cluster region/c-abl oncogene 1, non-receptor tyrosine kinase chimera
BMP	bone morphogenetic protein
BRAF	v-raf murine sarcoma viral oncogene homolog B1
CCL2	chemokine (C-C motif) ligand 2
CCND1	cyclin D1
CDH1	cadherin 1, type 1, E-cadherin (epithelial)
CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)
CKS1	CDC28 protein kinase regulatory
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa
DICER	dicer 1, ribonuclease type III
DROSHA	drosha, ribonuclease type III
E2F	E2F transcription factor
EGFR	epidermal growth factor receptor
ELK1	ELK1, member of ETS oncogene family
EXPORTIN/XPOT	exportin, tRNA (nuclear export receptor for tRNAs)
FGFR	fibroblast growth factor receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSK3	glycogen synthase kinase 3
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
IGF1R	insulin-like growth factor 1 receptor
IKK	inhibitor of kappa light polypeptide gene enhancer in B-cells
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LSP1	lymphocyte-specific protein 1
MAP2K1	mitogen-activated protein kinase kinase 1
MAP2K2	mitogen-activated protein kinase kinase 2

MAP3K8/COT	mitogen-activated protein kinase kinase kinase 8
MAPK1	mitogen-activated protein kinase 1
MAPK3	mitogen-activated protein kinase 3
MC1R	melanocortin 1 receptor (alpha melanocyte stimulating hormone receptor)
MIR141	microRNA 141
MIR200A	microRNA 200a
MIR302	microRNA 302
MIR517	microRNA 517
MIR520	microRNA 520
MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)
MSH	mutS homolog, colon cancer, nonpolyposis type 1 (E. coli)
MTOR	mechanistic target of rapamycin (serine/threonine kinase)
NANOG	Nanog homeobox
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NODAL	nodal homolog (mouse)
NOTCH	NOTCH
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog
PAK2	p21 protein (Cdc42/Rac)-activated kinase 2
PDGFRB	platelet-derived growth factor receptor, beta polypeptide
PIK3CA	phosphoinositide-3-kinase, catalytic, alpha polypeptide
POU5F1	POU class 5 homeobox 1
PP2A/PPP2R4	protein phosphatase 2A activator, regulatory subunit 4
PTEN	phosphatase and tensin homolog
RAF1/CRAF	v-raf-1 murine leukemia viral oncogene homolog 1
RB1	retinoblastoma 1
RET1/PTC	ret proto-oncogene/papillary thyroid carcinoma chimera
RNU6B	RNA, U6 small nuclear 2
SKP2	S-phase kinase-associated protein 2, E3 ubiquitin protein ligase
SMAD2	SMAD family member 2
SOX2	SRY (sex determining region Y)-box 2
TGFB	transforming growth factor, beta
TGFBR1	transforming growth factor, beta receptor 1
TNF	tumor necrosis factor

TP53	tumor protein p53
TPO	thyroid peroxidase
TSHR	thyroid stimulating hormone receptor
TSHR	thyroid stimulating hormone receptor
VEGF	vascular endothelial growth factor A
WNT5A	wingless-type MMTV integration site family, member 5A

Chapter 1: Introduction¹

¹ Cantwell-Dorris ER, O’Leary JJ and Sheils O. *BRAFV600E: Implications for carcinogenesis and molecular therapy* (2011). *Molecular Cancer Therapeutics* 10(3):385-394

1.1 Introduction

Cancer is a group of diseases characterised by uncontrolled growth of abnormal cells in the body. Cancers can originate in almost any organ. Cancerous cells divide in an uncontrolled, relatively autonomous fashion which leads to a progressive increase in the number of dividing cells resulting in a mass of growing tissue, or tumour. In a tumour, cell division is uncoupled from cell differentiation and, as cells accumulate, the normal organisation and function of the tissue becomes disrupted. A malignant tumour has developed the ability to invade surrounding tissues and has the potential to spread from its origin to distant sites, making it a potentially life threatening disease.

Cancer treatment varies based on the type of cancer and its stage. The stage of a cancer refers to how much it has grown and whether the tumour has spread from its original location. If the cancer is confined to one location and has not spread, the most common treatment approach is surgery to cure the cancer. If surgery cannot remove all of the cancer, the options for treatment include radiation, chemotherapy, or both. Some cancers require a combination of surgery, radiation, and chemotherapy.

Traditional chemotherapy was first introduced into clinical practice at the end of the Second World War when nitrogen mustard was used to treat a patient with advanced malignant lymphoma. After an initial regression of the disease, a second course of therapy was given at a reduced dose due to the toxicity of the treatment, with an associated lesser therapeutic effect. By the time the third treatment was given, the tumour no longer responded to the chemotherapeutic agent (Goodman *et al.*, 1946). Since then, chemotherapy has been a main therapeutic strategies in cancer treatment but resistance to chemotherapy has continued to be a problem.

Two main groups of factors contribute to the development of drug resistance to traditional chemotherapy. The first group includes pharmacological and physiological factors such as drug metabolism, excretion, inadequate access of the drug to the tumour, inadequate infusion rate and inadequate route of delivery. The second group includes cell- or tissue-specific factors. For example, drug analogues of the chemotherapy DNA precursor agents such as 5-fluorouracil and cytosine arabinoside require metabolic activation, and resistance can arise from modification of these activation pathways. Altered topoisomerase I and II activity prevents drugs such as doxorubicin, etoposide and camptothecin from binding the topoisomerase–DNA complex, allowing the broken strands to be repaired. Many anticancer drugs, such as platinum compounds and alkylating agents, cause direct damage to the structural integrity of DNA, and

resistance to these compounds results from activation of DNA repair systems (Raguz and Yague, 2008).

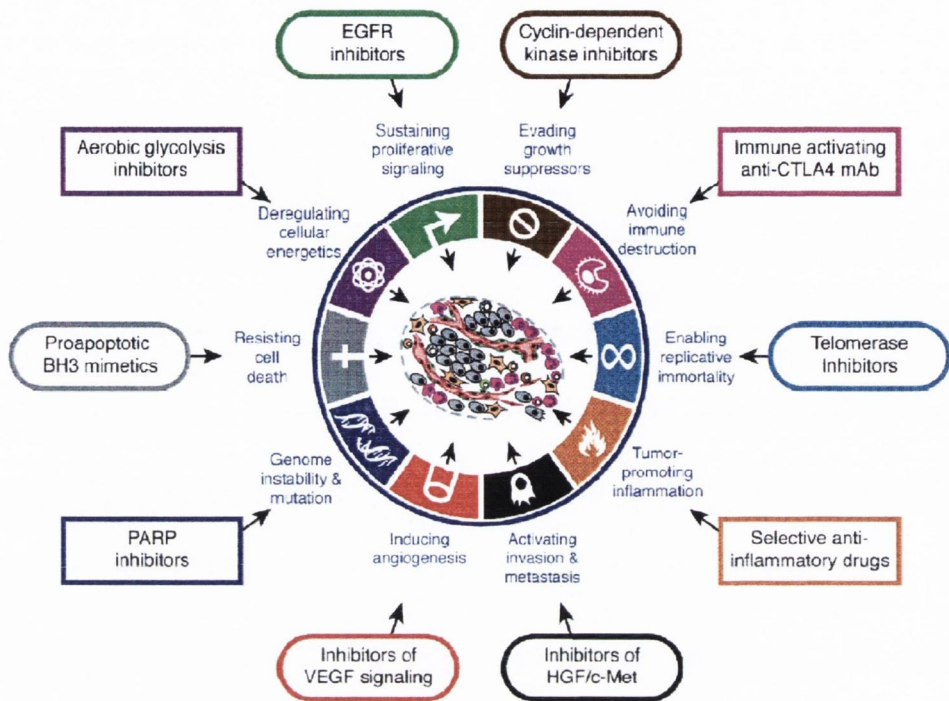
Traditional chemotherapeutic strategies have used a variety of drugs and hormonal agents that interfere with the basic machinery of the cell. Subsequent improved understanding of the molecular alterations present in cancer cells has enabled the development of targeted therapies for some forms of cancer. Resistance appears not only to traditional chemotherapy but also to targeted therapies. Targeted cancer therapies are drugs or other substances that block the growth and spread of cancer by interfering with specific molecules involved in tumor growth and progression. 1.1 Figure 1 illustrates targeted therapy of tumour characteristics.

This thesis focuses on targeted therapy using small molecule kinase inhibitors (SMKI). Several decades of research has revealed crucial roles in tumourigenesis for many of the predicted 518 human protein kinases, which have been found to have an impact on cell proliferation, survival and migration (Janne *et al.*, 2009). The vast majority of kinase inhibitors target the ATP-binding site, but a growing number of non-ATP competitive kinase inhibitors target unique allosteric sites. Kinase inhibitors achieve specificity by either directly targeting points that are structurally divergent in kinases or by exploiting differences in the conformational flexibility of the ATP binding site.

Mutationally activated kinases constitute drug-sensitizing genotypes in multiple different disease settings, which highlights the strict dependency on these activated kinases in many cancers. The term ‘oncogene addiction’ was first used by Weinstein (2002) to describe the unexpected dependency of tumour cells on the sustained activity of a single oncogene or oncogene-driven pathway, despite the probable accumulation of numerous oncogenic lesions. Although the precise molecular mechanisms underlying oncogene addiction remain unclear, the potential therapeutic opportunity presented by this phenomenon is substantial. BRAF^{V600E} mutated cancers display oncogenic addiction to the BRAF kinase oncogene and addiction to the extracellular signaling related kinase (ERK) pathway. This provides an opportunity for pharmacologic intervention with selective kinase inhibitors in these cancers, as discussed in section 1.2.

It is becoming increasingly clear that the effective use of selective kinase inhibitors as cancer therapeutics will require the identification of patients most likely to respond, based on specific tumour and germline genomic features. One of the major factors limiting the long-term efficacy of selective kinase inhibitors is drug resistance. There are two fundamental forms of drug resistance: *de novo* resistance, which refers to the failure of an agent to elicit any

detectable response to initial treatment; and acquired resistance, which refers to the progression of a tumour that had previously responded to treatment, despite continued



1.1 Figure 1 Therapeutic targeting of neoplastic characteristics.

Drugs that interfere with each of the acquired capabilities necessary for tumor growth and progression have been developed and are in clinical trials or approved for clinical use in treating certain forms of human cancer. Additionally, investigational drugs are being developed to target each of the enabling characteristics of cancer.

Image from Hanahan and Weinberg, 2011.

administration of the inhibitor. Accumulating evidence suggests that similar molecular mechanisms can underlie both forms of resistance, with the most significant distinction being the timing of the detection of resistance during the course of therapy (Janne *et al.*, 2009).

Evolutionary adaptation has been proposed as a mechanism that promotes drug resistance during cancer therapy (Gerlinger and Swanton, 2010) . The evolution of resistance to cytotoxic and targeted anti-cancer drugs arises according to Darwinian models. Genomic instability and high intra-tumour genetic heterogeneity are major accelerators of this

evolutionary process. Clinical implications and strategies that may prevent the evolution of resistance or target the origins of genetic heterogeneity are key areas to further the understanding of determinants and mechanisms of the evolution of drug resistance. Darwinian evolution results from the complex interactions of heritable phenotypes with multiple environmental and microenvironmental influences that need to be considered in the drug resistance process. The current models used to investigate cancer offer significant hurdles to achieve this.

In vitro cell line models are the most commonly used models of cancer. Cell lines are reference models in the field with the establishment of a complete genetic and proteinic profile of many commonly used lines. Cell lines allow an excellent control of all parameters of an experiment, and allow good quantifications. In vitro models are also simple and easy to handle. Furthermore, they are representative of a human system which is of utmost importance for molecularly targeted therapeutics, which are highly context dependent. The use of cell lines has many technical advantages including: the complete control of environmental conditions and standardised culture conditions; the reproducibility of results between experiments and laboratories; maintaining cells in culture is much less costly than working on animal models as animal models are expensive by themselves, but the care of animals and the staff necessary to a good work in an animal house are the main drain of resources. Conversely, the medium and the staff time required to grow cells are cheaper; Cryopreservation enables long-term conservation of the same strain and can theoretically permit the use of these cell lines indefinitely.

The major disadvantage of cell lines is their simplicity and limited representativeness. The relevance of cellular models is controversial since their over-simplicity implies difficulties in extrapolating results from the cell line to the tumour in humans and thus raises the question of their representativeness. Indeed, cell lines are homogeneous, theoretically consisting only of a single cell type due to the way they are established. Culture conditions eliminate some types of cells present in the original tumour, and cell lines in culture do not undergo the influence of nervous and hormonal regulatory systems active in vivo. These particularities reduce the similarity with the primary tumour.

An alternative to cell lines is the cultures of cancerous tissues that preserve the tumour architecture and the cell diversity of a tumour. However, this model suffers of limited reproducibility and cannot be easily maintained for a long time. Co-culture systems may offer an alternative with reproducible long term culture systems, and offer the possibility to study the relations between different types of cells in a tumour, but this model suffers from the same

controversies as cell lines as it mainly relies on their use. 3-dimensional systems allow the mimicking of the tumour architecture and microenvironment, but very few cell lines are able to form spheroids under specific conditions, although this technology is rapidly improving.

Xenografts are obtained by injecting cancer cells, usually derived from established cell lines, into a living organism. They are called xenografts because the injected cells are of human origin but are introduced into an animal organism. Xenografts appear to be good alternative models as they enable to take into account the tumour structure, its microenvironment, the role of the metabolism and they preserve the cell diversity of the tumour. But as other models, they also have drawbacks principally due to the metabolic and physiological differences existing between human and rodents, and to the fact that the role of the immune system against tumour is not taken into account with the immunodeficient rodent models used for xenografts. No cancer model is sufficient by itself and able to perfectly mimic the intricacies of human cancer. However it clearly appears that all these models are essential to accumulate data and information to elucidate mechanisms of cancer and the resistance to cancer therapy.

1.2 BRAF^{V600E}: Implications for carcinogenesis and molecular therapy.

Review

BRAF^{V600E}: Implications for Carcinogenesis and Molecular Therapy

Emma R. Cantwell-Dorris, John J. O'Leary, and Orla M. Sheils

Abstract

The mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway is frequently mutated in human cancer. This pathway consists of a small GTP protein of the RAS family that is activated in response to extracellular signaling to recruit a member of the RAF kinase family to the cell membrane. Active RAF signals through MAP/ERK kinase to activate ERK and its downstream effectors to regulate a wide range of biological activities including cell differentiation, proliferation, senescence, and survival. Mutations in the v-raf murine sarcoma viral oncogenes homolog B1 (BRAF) isoform of the RAF kinase or KRAS isoform of the RAS protein are found as activating mutations in approximately 30% of all human cancers. The BRAF pathway has become a target of interest for molecular therapy, with promising results emerging from clinical trials. Here, the role of the most common BRAF mutation BRAF^{V600E} in human carcinogenesis is investigated through a review of the literature, with specific focus on its role in melanoma, colorectal, and thyroid cancers and its potential as a therapeutic target. *Mol Cancer Ther*; 10(3): 385-94. ©2011 AACR.

Introduction

The mitogen-activated protein kinase (MAPK) kinase (MEKK)/extracellular signal-regulated kinase (ERK) pathway is a conserved kinase cascade involved in the regulation of cell proliferation, differentiation, and survival in response to extracellular signaling. The most potent activator of MAP/ERK kinase (MEK) is the v-raf murine sarcoma viral oncogenes homolog B1 (BRAF). The RAS/BRAF/MEK/ERK pathway is mutated in an estimated 30% of all cancers (1), with mutations in the *braf* gene found in approximately 7% of cancers (2). The predominant mutation in the *braf* gene involves a thymidine to adenosine transversion at nucleotide 1,799, accounting for greater than 90% of the observed mutations in *braf* (2). This results in an activating mutation due to the substitution of valine with glutamic acid at amino acid (aa) 600. Significant progress has been made in understanding the carcinogenic role of the BRAF^{V600E} mutation (3). BRAF^{V600E} is an attractive target for molecular therapy. Inhibitors targeted against BRAF and its primary downstream target MEK are producing interesting results in early-phase clinical trials. In this article, the molecular aspects of BRAF^{V600E}

and the consequences of this activating mutation are reviewed. The carcinogenic effect of BRAF^{V600E} is discussed through its effect on 3 cancers types with the highest incidence of *braf* mutation: melanoma, colorectal, and thyroid cancers. Furthermore, the clinical application of molecular inhibitors targeting BRAF^{V600E} and its downstream effector MEK is reviewed.

The Role of BRAF in the MAPK/ERK Pathway

BRAF is a member of the RAF family of serine/threonine protein kinases. This family consists of 3 kinases, ARAF, CRAF (RAF-1), and BRAF, of which the latter has the highest basal kinase. BRAF functions to regulate the MAPK/ERK pathway, a pathway that is conserved in all eukaryotes (4). The RAS/RAF/MEK/ERK pathway acts as a signal transducer between the extracellular environment and the nucleus. Extracellular signals such as hormones, cytokines, and various growth factors interact with their receptors to activate the small G-proteins of the RAS family. Active RAS acts via adaptor proteins to activate and recruit RAF proteins to the cell membrane where they are activated (1). Active BRAF signals through MEK to activate ERK, which, in turn, activates downstream transcription factors to induce a range of biochemical processes including cell differentiation, proliferation, growth, and apoptosis.

BRAF is the most potent activator of MEK. Active RAS induces conformational changes in RAF that allows its recruitment to the cell membrane, promoting changes in the phosphorylation status and triggering its kinase activity. Unlike BRAF, ARAF and CRAF require an additional phosphorylation in the N-region of their

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kinase domain for full activation (5), likely contributing to the predominance of the BRAF isoform in the activation of MEK.

BRAF phosphorylates and activates MEK1/2, which initiate a kinase cascade that acts through ERK1/2 to signal for ligand- and cell-specific responses. MEK1/2 contain proline-rich segments in the carboxy-terminal domains. These segments are not found in other MEK family members (4). It is postulated that these segments are required for MEK activation by RAF, supported by the observation that deletion of the proline insert from MEK-1 impairs its activation by RAF in transfected cells (6). RAF/MEK coupling is required for the downstream phosphorylation of ERK1/2. Regulation of this pathway is crucial for the maintenance of homeostasis in response

to extracellular signaling. It has been shown that hyperactivation of this pathway can induce cell-cycle arrest whereas aberrant regulation of the pathway can initiate tumorigenesis (2).

The *braf* Oncogene

More than 40 different mutations have been identified in the *braf* gene in human cancer (Fig. 1). Ninety percent of *braf* mutations are accounted for by a thymine to adenine single-base change at position 1,799. This missense mutation, located in exon 15, results in a change at residue 600 that substitutes glutamine for valine (V600E; ref. 2). BRAF^{V600E} can gain 500-fold increased activation, stimulating the constitutive activation of MEK/ERK

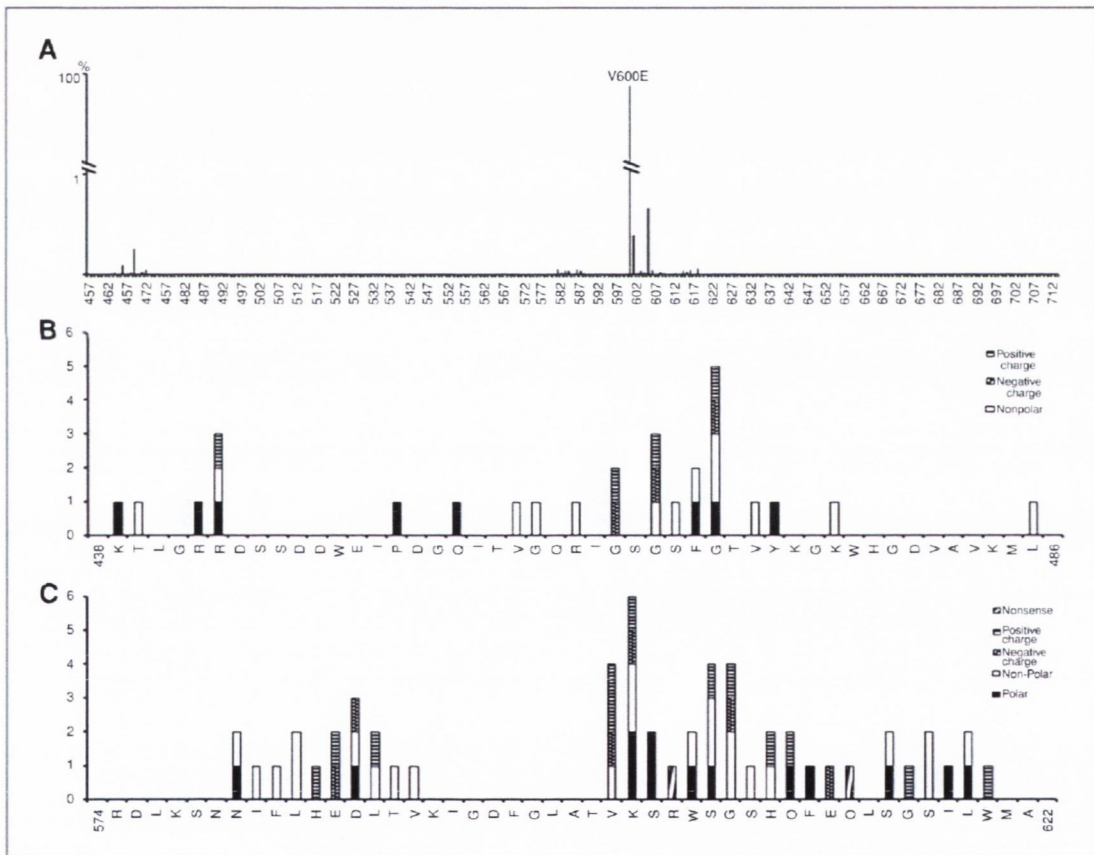


Figure 1. Somatic mutations of the *braf* gene identified in human tumor samples. A, the V600E mutation accounts for greater than 95% of the somatic mutations described in human tumor samples. The graph illustrates the locations and relative frequency of somatic mutations within the kinase domain of the *braf* gene identified in human tumor samples ($n = 11,860$). B, somatic mutations cluster close to the P-loop of the N-terminus. The graph illustrates the amino acid substitutions (grouped on the basis of side-chain properties) identified as arising from somatic mutations within the region aa438–486. C, somatic mutations cluster close to the activating segment of the kinase domain. The graph illustrates the amino acid substitutions (grouped on the basis of side-chain properties) identified as arising from somatic mutations within the region aa574–622. Note: The mutation data was obtained from the Sanger Institute Catalogue of Somatic Mutations in Cancer web site (7).

signaling in tumor cells. Furthermore, it allows activation of this signaling cascade in the absence of any extracellular stimuli, allowing the cell to become self-sufficient in growth signals within this pathway. The structure of the BRAF protein gives insight into how this constitutive activation is initiated.

The BRAF kinase domain has characteristic bilobal architecture, with small and large lobes separated by a catalytic cleft. In the inactive BRAF conformation, the conserved aspartate, phenylalanine, and glycine (DFG) motif found in the activation segment displays a flipped conformation that orients the region of activation toward the P-loop of the N-lobe. This brings the residues' glycine-rich segments G595-V600 near to the G463-V470, which allows hydrophobic interactions to be formed between these segments. This results in a conformational orientation that renders the catalytic cleft inaccessible. In this inactive conformation, the residues required for phosphotransfer reactions are aligned but the ATP and peptide substrate recognition segments are partially disorganized. Thus, all that is required for transition to an active state is a change in position of the DFG motif/activation segment (3). Phosphorylation of the activation segment results in the destabilization of the hydrophobic interactions between the activation segment and the P-loop, resulting in a flip of the DFG segment to its active state, aligning the ATP and peptide recognition segments, and allowing access to the catalytic cleft.

Activating mutations tend to cluster to the glycine-rich loop and activation segments (Fig. 1B and C) and are often located in residues that normally stabilize the interactions between these regions (1). The BRAF^{V600E} mutation replaces V600 valine, a medium-sized hydrophobic side chain that interacts with F467 phenylalanine of the P-loop, with E600 glutamine, a larger, charged side chain. V600 is located in the activation segment close to the DFG motif (3). V600E substitution disrupts the hydrophobic interaction, destabilizing the conformation that maintains the inactive orientation of the DFG motif, resulting in the restitution of DFG motif to its active state and thereby restoring the activation segment to its active orientation. This phosphomimetic mutation renders BRAF^{V600E} in a constitutively active state (1, 3).

BRAF Mutation in Carcinogenesis

BRAF has been shown to be mutated in a wide range of cancers including 40% to 70% of malignant melanomas, an average of 45% of papillary thyroid cancer, and 10% of colorectal cancers (CRC) and has also been identified in ovarian, breast, and lung cancers (2, 8, 9). Germline mutations in *braf* and *craf* are associated with LEOPARD syndrome, a developmental disorder with an increased incidence of multiple granular cell tumors (10). Mutations in *braf* commonly occur in the same cancer types that harbor *ras* mutations. Mutations in *braf* and *ras* generally occur in a mutually exclusive fashion, suggesting that aberrant regulation of the RAS/

BRAF/MEK/ERK pathway may be the pathogenesis of these tumor types (2), which can be achieved at different levels of the pathway.

BRAF Mutation in Melanoma

Melanoma arises from melanocytes, the specialized pigment cells found in the epidermis, meninges, inner ear, and eye. The RAS/RAF/MEK/ERK pathway is frequently mutated in melanoma, with *ras* mutations, predominantly of the *nras* isoform, found in 15% to 30% and *braf* mutations found in up to 70% of melanomas (2, 8). BRAF^{V600E} is the most frequent (>90%) BRAF mutation in melanoma (2). It is associated with nonchronic sun-induced damage (non-CSD) melanomas, indicating a different pathology of disease than that by the CSD-induced p53 loss of function progression to disease associated with long-term UV radiation (UVR; ref. 11). BRAF^{V600E} is associated with low UVR dose, younger presentation, and melanocortin-1 receptor (MC1R) variants (12). The relationship between *braf*-mutated melanoma and sun exposure is complex. Mutations in *braf* occur at a very low rate in melanomas located at mucosal lining and areas of low sun exposure such as the palms and the soles of the feet, indicating that sun exposure is required for the development of *braf*-mutated disease (13). However, *braf* mutation has a very low frequency in CSD melanoma associated with long-term sun exposure (12). Thus, the link between UVR and *braf* mutation does not seem to be straightforward.

The *mc1r* gene is a key determinant of pigmentation. It is highly polymorphic in humans, and specific variants are linked to the distinctive red hair, pale skin, and freckles phenotype, the same phenotype that is associated with increased melanoma risk in the Celtic population (14, 15). MC1R variants have been shown to be associated with *braf* mutation in non-CSD melanomas in both Italian and American populations (12, 16). MC1R is a G-protein-coupled receptor, and its variant isoforms can affect signaling through this pathway. MC1R binds α -melanocyte-stimulating hormone (α -MSH), a crucial regulator of melanocyte homeostasis. In response to UVB radiation, α -MSH binds MC1R signaling for increased proliferation and melanogenesis. The binding of α -MSH to MC1R upregulates cyclic AMP that results in a signaling cascade that acts through BRAF to signal to ERK. ERK activation induces proliferation or differentiation, depending on microenvironment-specific signals. α -MSH signaling is also extremely important for the reduction of free radical formation in response to UV and is involved in inhibition of UV-induced apoptosis (17).

Variant isoforms of MC1R display reduced function of the receptor due to an inadequate ability to respond to their ligands, such as α -MSH (17, 18). Wild-type MC1R signals for the production of eumelanin over pheomelanin in response to UVR. However, variant isoforms of MC1R are unable to signal for this switch in melanin

production, which can lead to an accumulation of pheomelanin. Pheomelanin can increase the production of free radical in response to UVR and thus can be regarded as a carcinogen. It is unclear whether the association between MC1R variants and BRAF is due to a direct relationship or is caused indirectly via the accumulation of pheomelanin, which can increase oxidative stress and thus induce DNA instability.

Human melanocytes are dependent on adhesion to the extracellular matrix (ECM) for efficient activation of ERK1/2 (19). ERK1/2 signaling is required for cell-cycle progression through the G₁/S phase, as both the upregulation of cyclin D1 and the downregulation of cyclin-dependent kinase inhibitors (cdk) p27kip1 are ERK1/2 dependent. To progress through the G₁/S phase, growth factors and integrin-mediated signaling from the ECM are required to induce hyperphosphorylation of the retinoblastoma protein via the downregulation of cdk inhibitors and the expression of D-type cyclins. This allows the derepression of the E2F transcription factor, thereby inducing its binding to promoter sites of genes involved in S-phase entry. It has been shown that loss of adhesion in melanocytes impairs the growth factor-mediated ERK1/2 activation, including the activation of cyclin D1 (19).

BRAF^{V600E} mutation in melanoma cells results in the activation of ERK1/2 without the need for signaling from the ECM. Constitutive activation of BRAF results in the upregulation of cyclin D1 in the absence of extracellular signaling. The cdk inhibitor p27kip1 is downregulated in mutant BRAF melanoma via the derepression of its transcription but also via the upregulation of the proteasomal proteins involved in the p27kip1 protein degradation. The S-phase kinase-associated protein 2 (SKP2) and its cofactor Cdc kinase subunit 1 (CKS1) are members of an ubiquitin-dependent proteasomal ligase complex involved in the degradation of p27kip1. SKP2 is dependent on adhesion in melanocytes and has been inversely correlated to p27kip1 levels (20). Knockdown studies of BRAF or cyclin D1 have shown a decrease in SKP2 and CKS1. Regulation of these proteasomal proteins involved in p27kip1 degradation is dependent on the expression of BRAF and cyclin D1. Thus, BRAF mutation subverts the adhesion and growth factor requirements for ERK1/2 signaling in melanoma cells, thereby allowing unregulated progression through the G₁/S checkpoint.

It has been shown that BRAF is an upstream signaling component of a principal signaling pathway in melanocytes (21). This is a melanocyte-specific pathway that regulates differentiation and proliferation, which is reliant on BRAF activation. The high frequency of *braf* mutation in melanoma relative to other cancers may be partly explained by the role of BRAF in melanocyte-specific biology (2).

BRAF Mutation in CRC

CRC is highly prevalent within the Western world and is 1 of 4 most prevalent cancer types worldwide (22). The most prevalent CRC type follows the Kinzler-Vogelstein

model, whereby a stepwise accumulation of mutations involving the adenomatous polyposis coli gene *p53* and members of the β -catenin signaling pathway (23, 24) leads to adenocarcinoma development. However, an alternative serrated pathway to CRC has recently been highlighted, involving mutations of RAS/RAF/MEK/ERK pathway and microsatellite instability (MSI; refs. 25, 26). This sporadic CRC frequently contains activating mutations in *kras* and *braf* (51% and 10% respectively; ref. 25). These mutations are found in a mutually exclusive fashion (2, 24, 25) and *kras* mutations tend to display a more aggressive phenotype and less favorable clinical outcome (27, 28). BRAF^{V600E} mutation is associated with CRC tumors that also display deficiency in mismatch repair (MMR). The prevalence of *braf* mutation in MMR-deficient tumors has been shown to be 3-fold greater than in MMR-proficient tumors, whereas *kras* mutation was equally prevalent (25).

braf mutation is tightly associated with a CpG island methylation phenotype (CIMP; ref. 29), which is characterized by methylation of the MMR gene *mlh1* and associated high MSI (MSI-H). MHL1 is involved in the MMR system, in which it is part of a protein complex that introduces single strand breaks close to the mismatch, thereby creating new entry points for exonucleases. It is also implicated in DNA damage signaling that can lead to the induction of cell-cycle arrest or apoptosis in response to major DNA damage. *mlh1* has a large CpG island within its promoter region. Methylation of a small proximal region (region C) close to its transcriptional start site has been correlated with loss of gene expression (30). The association between *braf* mutation and CIMP is unclear. However, it has been shown that *braf* mutations are also found in the precursor lesions (31–33), indicating that it is an early event in the development of these tumors.

The mutant *braf* CIMP tumors tend to exhibit a serrated morphology. This serrated morphology has been postulated to be as a result of the inhibition of apoptosis (34). Mutant BRAF can act as a potent inhibitor of apoptosis. Erhardt (35) showed that overexpression of BRAF can inhibit apoptosis via postmitochondrial regulation of apoptosis. Acting through MEK, overexpression of BRAF works at the level of cytosolic caspase activation to inhibit cytochrome *c*-induced apoptosis. BRAF mutations in colorectal tissue have also been associated with significant decreases in the rates of apoptosis in tumor cells (36). Thus, it has been postulated that BRAF mutation introduces a microenvironment resistant to apoptosis, which allows extensive DNA damage, such as that found in CIMP tumors, to be tolerated (37).

The high correlation of BRAF and CIMP (29) suggests that the association is causal. Forced overexpression of BRAF^{V600E} induced the methylation of the promoter region of *mlh1* in a colon cell line (37). DNA methyltransferase and DNA methylation can be regulated via the RAS/RAF/MEK/ERK pathway (38). This has led to the hypothesis that BRAF mutation induces a microenvironment of apoptosis resistance and predisposition to

promoter hypermethylation, as exemplified by the high rate of *mlh1* promoter methylation. This leads to a state permissive to MSI-H due to MMR deficiency, which gives rise to the CIMP CRC (37). This hypothesis explains the progression of the serrated pathway but has yet to be validated.

BRAF Mutation in Thyroid Cancer

Thyroid cancer is the most common endocrine malignancy and is the most rapidly increasing cancer type amongst women. Up to 90% of all thyroid cancers are papillary thyroid cancer (PTC; ref. 39), of which an average of 45% display mutant *braf* (9). Rearrangements of *ret* are found in up to 30% of PTC (*ret*/PTC; ref. 40) in a mutually exclusive fashion with *braf* and *ras* (41). RET is a tyrosine kinase receptor not usually expressed in thyroid follicular cells, but the chimeric RET/PTC is driven by the promoter of the partner gene, of which more than 10 genes have been identified. RET/PTC signals through the RAS/RAF/MEK/ERK pathway, indicating that it is overactivation of the pathway itself that is required for tumor pathogenesis in PTC.

RET/PTC is common among radiation-induced tumors and sporadic tumors in juveniles (42), whereas BRAF^{V600E} is found at a higher rate in adults. BRAF^{V600E} has also been associated with more aggressive characteristics (42), which has also been shown in animal models (43). BRAF has been shown to be necessary for RET/PTC-induced activation on the ERK pathway and its regulation of downstream effectors relevant to tumor pathogenesis (43).

BRAF^{V600E} is associated with silencing of multiple thyroid-specific iodine-metabolizing genes (43, 44). Iodine is important in the synthesis of thyroid hormones. Inorganic iodine is actively transported into the thyroid cells and then, in turn, it is transported into follicular cells where it is oxidized and incorporated into thyroid hormones. BRAF^{V600E} has been associated with the decreased gene expression of several of the genes involved in this process (43, 45, 46). Thyroid DNA methylation of the promoter regions of iodine-metabolizing genes have been linked with BRAF^{V600E} PTC but not with RET/PTC rearrangement-induced PTC. The function of these genes could be restored in a thyroid cell model carrying BRAF^{V600E} via the inhibition of MEK (43, 44). As previously discussed, DNA methyltransferase is regulated by the BRAF pathway (38). Promoter methylation seems to play a substantial role in the silencing of iodine-metabolizing genes in BRAF^{V600E} PTC (43, 44). It has been postulated that the decreased expression of iodine metabolism-associated genes may explain the more aggressive phenotype exhibited in BRAF^{V600E} PTC.

BRAF as a Therapeutic Target

BRAF-mutated tumors have been correlated with poor response to traditional chemotherapy and poor prognosis

in melanoma, thyroid, and colon cancers (43, 47). Targeted therapies are of great interest for these cancer types and the elucidation of the structure and functions of the BRAF kinase is the subject of a lot of ongoing research. The approach of targeting oncogenic kinases has been successful in the treatment of cancers, with activating mutations in the kinase gene that drives their progression (48). Imatinib (Gleevec; Novartis) is the original success story for small-molecule kinase inhibitors. Imatinib is used in the treatment of chronic myeloid leukemia, which is driven by a characteristic mutant fusion protein Bcr-Abl, resulting in constitutive activation of the Abl kinase. Imatinib effectively blocks the Abl kinase producing an effective clinical response that can be correlated to the presence of the activating kinase mutation.

BRAF-specific inhibitors such as GDC-0879 and PLX4720 effectively block BRAF^{V600E} and thus block BRAF^{V600E}-induced ERK activation (Fig. 2). PLX4720 was designed according to the atomic structure of BRAF^{V600E} and as a result has a 10-fold increased potency for BRAF^{V600E} over the wild-type kinase (49). These first-generation BRAF inhibitors were found to be effective in preclinical trials but underwhelmed in clinical trials. The emergence of second-generation inhibitors, such as the PLX4720 analogue PLX4032, is proving much better in clinical trials. The details of a phase I trial for PLX4032 announced an 80% response rate (as determined by complete or partial tumor regression) among patients with BRAF^{V600E} mutations whereas patients without the mutation did not respond (50). This 80% response rate is the highest response rate to date for a melanoma drug. This was a phase I trial and therefore is based on a small number of patients. It does however provide proof of concept for small BRAF^{V600E} inhibitors and provides hope for their use in the clinic.

Increasing research of BRAF-specific inhibitors has highlighted several mechanisms of interest that may negate their use in the clinic. It has been shown that mutant BRAF cell lines can acquire resistance toward BRAF-specific inhibitors through an increase in CRAF expression (51). In the presence of oncogenic or growth factor-activated RAS, BRAF-selective inhibitors can induce BRAF binding to CRAF. This can lead to transactivation of CRAF and consequent elevation of MEK and ERK signaling (Fig. 2C; ref. 52). This results in the cell regaining autonomy and hyperactivation of the MEK/ERK pathway through CRAF in a RAS-dependent manner (53). The genotyping of tumors is therefore critical to ensure not only that an activating mutation is present in *braf* but also that activating mutations of *ras* are not present. This reactivation of the pathway can occur with chemical or genetic (short hairpin RNA inhibition) inhibition of BRAF but does not seem to occur with pan-RAF drugs.

Pan-RAF drugs (e.g., sorafenib and XL281) target all the RAF isoforms. It has been shown in mice and human xenographs carrying BRAF^{V600E} that pan-RAF inhibitors hyperactivate CRAF through the inhibition of BRAF but

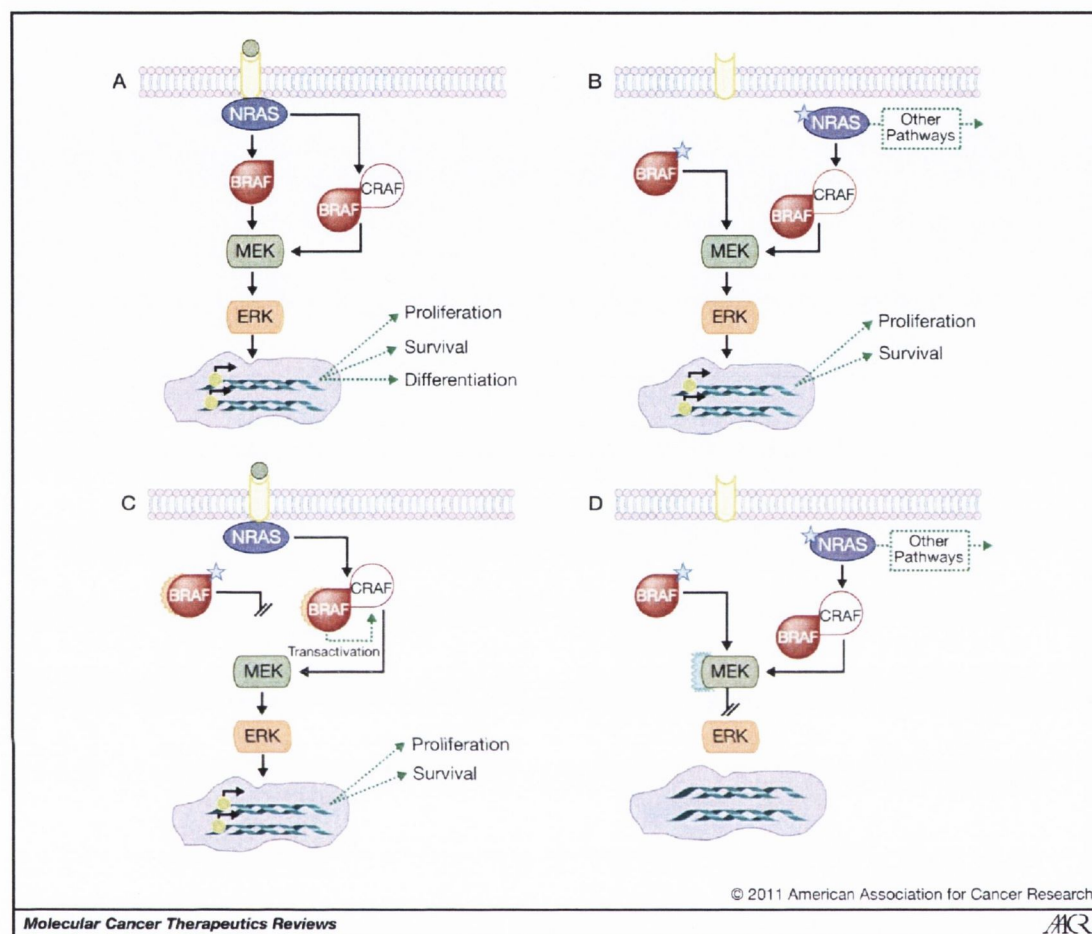


Figure 2. Targeted therapy in the BRAF pathway. A, the BRAF/MEK/ERK pathway. Extracellular signaling acts through RAS to activate BRAF, which signals through MEK to ERK culminating in activation of transcription factors involved in cell fate determination. B, mutations in the pathway lead to constitutive activation of the pathway. Activating mutation in BRAF^{V600E} activates the MEK/ERK pathway in the absence of extracellular signaling. Activating mutations in RAS can stimulate MEK/ERK signaling through CRAF in the absence of extracellular signaling. BRAF and RAS mutations occur in a mutually exclusive fashion. (Note: Whereas BRAF relies on the MEK/ERK pathway for signaling, mutant RAS can act through a number of diverse pathways.) C, BRAF-specific inhibitors prevent BRAF signaling. Small-molecule BRAF-specific inhibitors block the kinase activity of BRAF, preventing it from activating its downstream targets. However, in the cells with wild-type BRAF or activating mutations in RAS, BRAF inhibition can result in the transactivation of CRAF, which can signal to MEK, resulting in reactivation of the pathway and acquired resistance to BRAF-specific inhibitors. D, targeting downstream of BRAF may circumvent the pathway reactivation associated with BRAF inhibition. Targeting MEK may be useful in tumors with activating BRAF or RAS mutations due to its strategic position in the pathway. However, the reliance of BRAF on the MEK/ERK pathway makes mutant BRAF tumors more sensitive to MEK inhibition than mutant RAS tumors, which can signal through multiple pathways.

do not activate the MEK/ERK pathway because they concurrently inhibit CRAF. Thus, a major distinction between BRAF-specific and pan-RAF drugs is that in the presence of increased RAS expression, melanoma cell lines treated with BRAF-selective drugs result in pathway reactivation but this is less likely to occur with pan-RAF drug treatment as the CRAF isoform is also inhibited. However, the increase in CRAF expression in response to pan-RAF drugs can mediate acquired resistance to the drugs in mutant BRAF melanoma cells lines (51). Inter-

estingly, separate phase I clinical trials with BRAF-specific inhibitors both noted that 10% to 15% of patients treated with BRAF inhibitors developed squamous cell carcinoma, 22% of which harbored RAS mutations (50, 54). It has been postulated that BRAF-specific inhibitors may act as tumor promoters in premalignant skin cells with underlying RAS mutations (53). The release of results from phase II and III trials will aid in elucidating the role of BRAF in these subtle pathway regulations.

Inflammation is also a potential factor that needs to be accounted for the successful use of BRAF inhibitors. TNF- α is a pleiotropic cytokine that has been shown to block apoptosis in melanoma cells when BRAF signaling is inhibited (55). Macrophage infiltration has been correlated with tumor stage, progression, and angiogenesis in human malignant melanoma. Activated macrophages are strong secretors of TNF- α . The effect of TNF- α on melanoma cells depends on the context in which it acts. Epithelial malignancies have been associated with chronically produced TNF- α , whereas it can be tumor destructive in mice if high doses of TNF- α are administered therapeutically (56). Autocrine production of TNF- α in malignant melanoma has been associated with resistance to the cytolytic effects of TNF- α , a response that could have clinical implications (57). Melanoma cells treated with BRAF^{V600E} inhibitors resulted in both cytostatic and cytotoxic effects. Subsequent treatment with TNF- α resulted in apoptosis being blocked and recovery of the oncogenic cells to reenter the cell cycle (55). This effect was TNF- α specific and mediated by NF- κ B signaling. NF- κ B depletion blocks the ability of TNF- α to rescue BRAF-inhibited cells. The relations between the BRAF and NF- κ B signaling pathways is not fully understood, nor is the interaction that leads to the specific rescue that allows TNF- α to override the effect of BRAF inhibition. However, the observation that TNF- α can rescue cells undergoing BRAF inhibition may be important if the microenvironment has a high degree of macrophage infiltration.

Targeting MEK in Mutant BRAF Tumors

The effectiveness of a targeted agent depends on where in the pathway the activating mutant occurs and what other pathways the aberrant protein is channeling. As outlined earlier, targeting BRAF can lead to pathway reactivation under certain genetic conditions. Thus, it may be of greater benefit to target downstream in the BRAF pathway. Because of the strategic position of the MEK1/2 kinases in the RAS/RAF/MEK/ERK pathway, it is a very promising target for new drug development (Fig. 2D). Although MEK is downstream of both RAS and RAF, Solit and colleagues showed that *braf* mutation, but not *ras* mutation, could predict sensitivity to MEK inhibition, indicating that *braf* mutations are more reliant on MEK/ERK signaling than *ras* mutants. The reliance of BRAF on the MEK/ERK pathway seems to make it more sensitive to MEK inhibition (58). Preclinical trials have shown that small-molecule MEK1/2 kinase inhibitors can significantly reduce the proliferation of a variety of cancer cell lines via the induction of apoptosis and cell-cycle arrest (58, 59). Thus, MEK seems to be a logical target for targeted therapy.

The first small-molecule MEK inhibitor to enter clinical trials was CI1040, which is highly selective for MEK1/2. This molecule exerts its effect by binding a hydrophobic pocket adjacent to the Mg-ATP binding site of the kinase.

This results in the active site of the kinase becoming locked into a catalytically inactive conformation. The binding pocket is located in a region of low-sequence homology to other kinases, resulting in a high selectivity and noncompetitive kinetics of inhibition (60). However, it was found in phase II trials that CI1040 had poor pharmacodynamics and its development was stopped.

The second-generation MEK inhibitor PD0325901 is a structural analogue of CI1040. This MEK inhibitor had 100-fold greater activity than that of CI1040 and preclinical trials showed its ability to inhibit the growth of human tumor xenographs bearing mutant *braf* (58, 61). Phase I clinical trials seemed promising, but phase II trials displayed increased toxicity, more severe than that of CI1040. PD0325901 development was terminated because of these unacceptable toxicities (62, 63).

A second-generation inhibitor that is showing very promising results in clinical trial is the benzimidazole derivative AZD6244. This small-molecule inhibitor is selective for MEK1/2 by a mechanism that is not fully elucidated but occurs in a manner noncompetitive for ATP. Phase I trials produced very promising results, with one patient with malignant melanoma showing 70% tumor shrinkage (64). A phase II trial comparing AZD6244 to the standard treatment temozolomide in melanoma showed a survival advantage favoring AZD6244 in the subset of tumors with *braf* mutation, although there was no difference in the total progression-free survival between treatments. In this trial, the AZD6244 group had 6 confirmed responses, of which 5 carried mutant *braf* (65). In another phase I trial of AZD6244-based combination therapy, a positive trend was identified between *braf* mutation and clinical benefit (66). No such trend was identified for *nras* mutations. In this small study, all responders [as defined by RECIST (Response Evaluation Criteria in Solid Tumors) criteria] carried *braf* mutation and none of the subjects with *braf* mutation had early disease progression. There are currently more than 30 clinical trials ongoing (www.clinicaltrials.gov), involving AZD6244 in a variety of tumor types.

MEK inhibitors are very promising as targeted therapies in tumors with aberrant RAS/RAF/MEK/ERK pathway activation. MEK inhibitors are highly selective for their targets and those with improved pharmacodynamics are under development. Determining whether BRAF and/or MEK are a clinically viable level in the pathway to target will become clear as results from the ongoing and future trials are released. It is likely that any targeted therapies will be susceptible to the development of resistance due to secondary mutations in kinase domains that reduce drug binding while preserving catalytic function, activation of redundant signaling pathways, or upregulation or activation of a ligand or regulator of an alternative signaling pathway (67). Thus, it is likely that MEK inhibition, or indeed BRAF inhibition, will be less important as a single chemotherapeutic agent than it will be as a combination therapy. To this effect,

Merck and AstraZeneca announced a collaboration to test AZD6244 and the AKT inhibitor [phosphoinositide 3-kinase (PI3K) pathway] MK-2206 in 2009. It has been shown in preclinical trials for MEK inhibitors that PI3K activation can significantly decrease the response of *KRAS* mutants to MEK1/2 inhibition (68). Thereby, targeting both pathways should reduce the ability of the tumor to gain resistance. This unusual collaboration between pharmaceutical companies is more evidence of the expectation for the usefulness of these molecules in combination chemotherapy.

Conclusions

Combination therapy is likely to be the most effective management plan for the treatment of *braf*-mutated tumors. Many *BRAF*-specific inhibitors display a cytostatic response inducing senescence and are susceptible to acquired resistance (69, 70). Therefore, combination with traditional chemotherapeutic agents seems to be more effective than either treatment alone. Multiple signaling pathways are involved in tumor progression. *BRAF* mutation may be the activating mutation, but mutations subsequently acquired may also need to be targeted for successful treatment (71). For example, combined therapy blocking both the ERK and the PI3K pathways has been shown to be significantly more effective than blocking either pathway alone in a mouse model of melanoma (72). The strategy for treating mutant *braf* tumors will also rely on the tumor stage. Early tumor stage may be susceptible to *BRAF*-specific inhibition alone. However, as tumor stage progresses, as does the accumulation of mutations. Therefore, multiple pathways will need to be targeted in later-stage tumors (73).

Genotyping of tumors is of high importance when using molecular therapy. The *braf* mutation can predict sensitivity of a tumor to *BRAF* inhibition and to downstream MEK inhibition (58, 69). Correct use of molecular therapy is dependent on tumor genotyping not only for the identification of mutations that are susceptible to therapy but also for mutations that may confound therapeutic use of the inhibitor. The genotyping of tumors is also extremely important for clinical trials to ensure that the correct cohort is being assessed for drug response and to control for any underlying mutations that would confound results.

In summary, recent clinical trial results indicate that targeted therapy for mutant *BRAF* is a promising strategy (50, 54, 74). Both mutant *BRAF*^{V600E} and its downstream target MEK seem to be effective targets for tumors with activating *braf* mutations. However, acquired resistance through subsequent reactivation of the pathway or activation of redundant pathways is a major issue that must be overcome before these inhibitors will be ready for routine use in the clinic.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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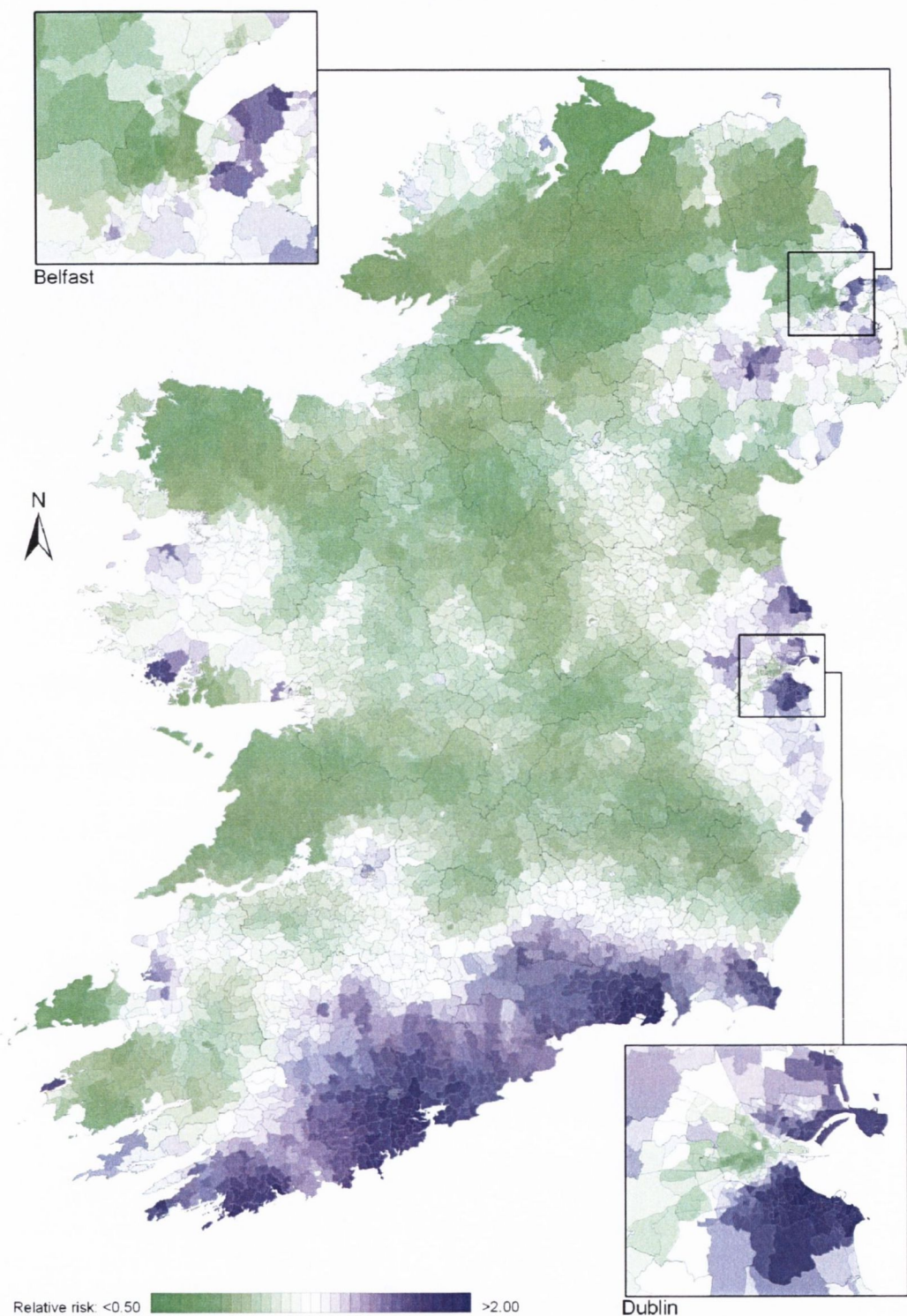
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1.3 Project Rationale

Malignant melanoma is the 7th most common cancer in Ireland, accounting for approximately 4.1% of all new cases reported in 2007 (National Cancer Registry of Ireland). Incidence of malignant melanoma is approximately equal between male and females with a cumulative risk to age 74 of 0.9% and 1.1%, respectively. There is a relationship between age and malignant melanoma incidence, with a relatively high incidence in the younger adult: 31% and 36% for male and females compared with the overall cancer average of 11% of diagnoses were patients under the age of 50 (incidence between 2008-2010). Worldwide, in patients under 40 years of age, the incidence of melanoma is second only to leukemia in men and breast cancer in women (Siegel *et al.*, 2012). Malignant melanoma has seen a sharp increase in Ireland with an average 5% increase per annum in the number of new cases diagnosed between 1995-2007. Melanoma risk is associated with higher socio-economic status, most likely due to greater recreational sun exposure among more affluent groups. There is a largely unexplained geographical patterns for melanoma in Ireland, even when adjusted for socio-economic and population density factors. This pattern is similar for both sexes (1.3 figure 1)

Thyroid cancer is a fast increasing malignancy, especially in females, with the incidence rate rising 3-fold in females and 2-fold in males since the mid 1990s. Thyroid cancer, however, has a small disease burden, accounting for just 0.9% of invasive cancers registered between 2006-2010 (National Cancer Registry of Ireland). Unlike melanoma, the lifetime risk of diagnosis of thyroid cancer is 2.5 times higher in females than males. Similar to melanoma, age is associated with thyroid cancer incidence. Between 1994-2010, over half of all thyroid diagnoses in women (55%) were in the under 50 age group compared to 35% of males. Both thyroid cancer and malignant melanoma show a bias towards increased incidence in women aged 35 to 50 (1.3 figure 2). The majority of thyroid cancers are classic papillary subtypes (1.3 figure 3). The five year relative survival has improved for both sexes since 1994, with the most recent estimates of 94% for females and 88% for males. This relative five year survival increases to nearly 100% for papillary and follicular subtypes diagnosed since 2005.

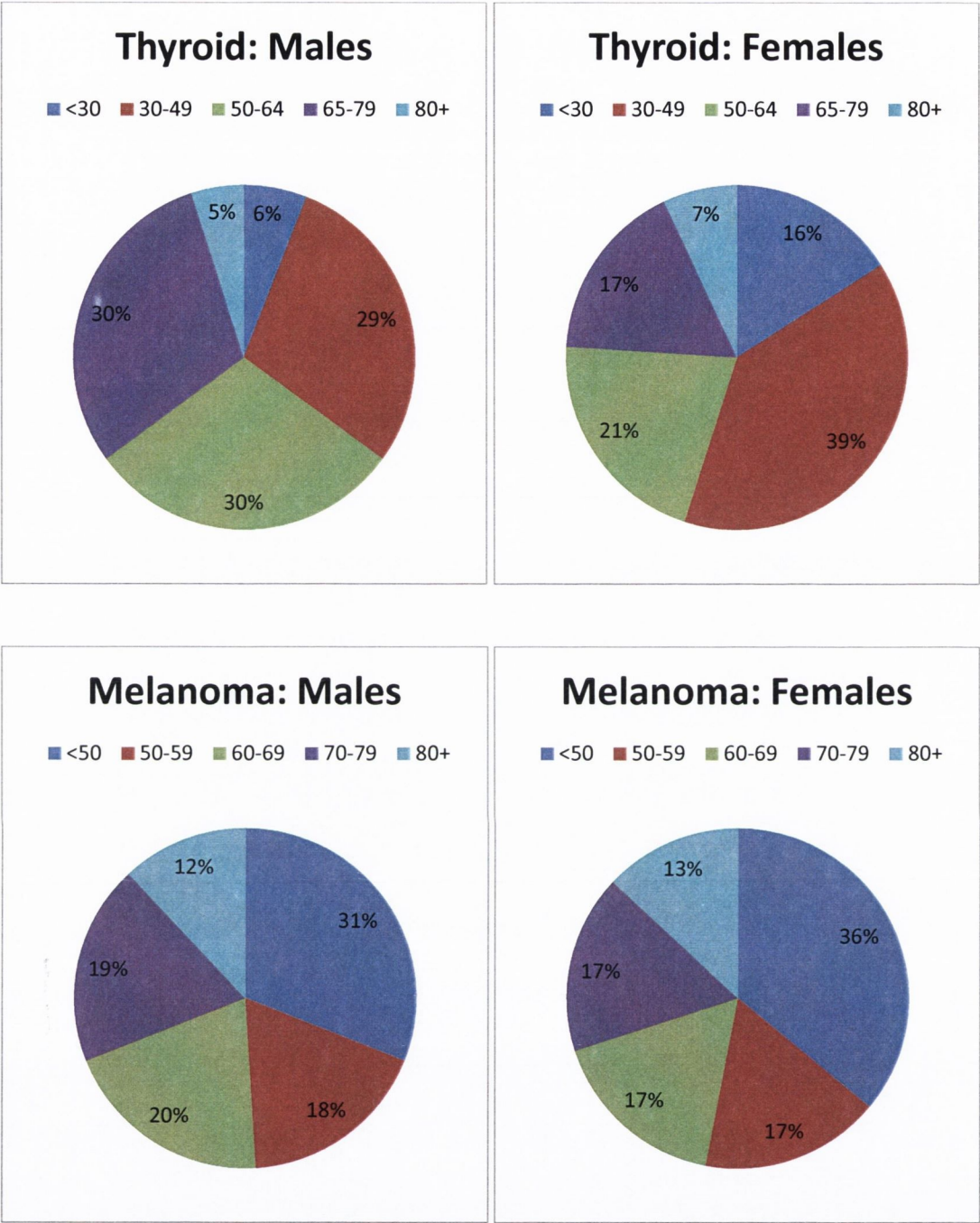
Both melanoma and thyroid cancers have a high incidence of BRAF^{V600E} mutation, with an estimated incidence of 50-80% of melanoma and 45% of papillary thyroid carcinomas, the most common type of thyroid cancer (Cantwell-Dorris *et al.*, 2011). Prior to 2010, no systemic therapy had been shown to improve overall survival among patients with metastatic melanoma (Flaherty *et al.*, 2012). Recently however, molecularly targeted therapy of the BRAF pathway has been associated with improved survival amongst patients with BRAF^{V600E}



1.3 Figure 1 Smoothed relative risks of melanoma of the skin for both sexes.

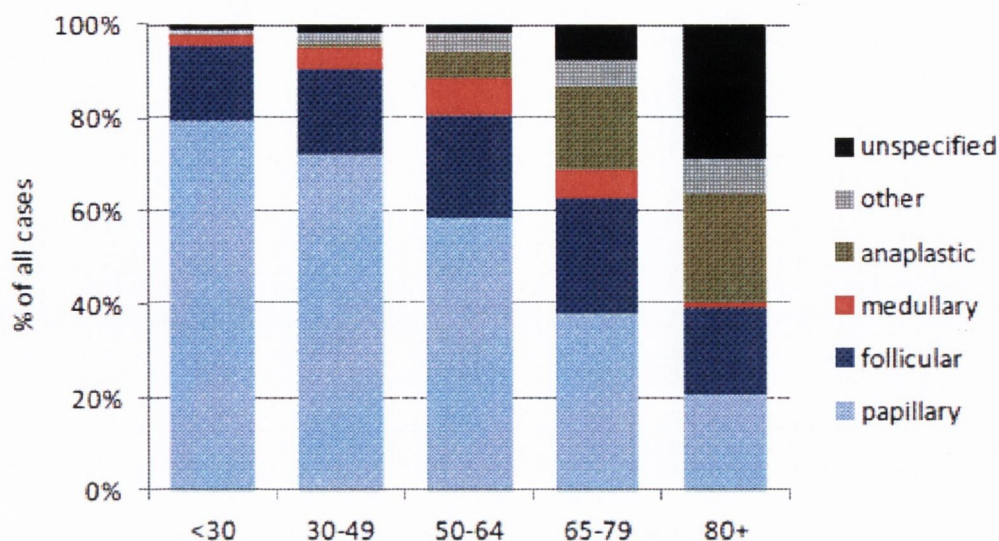
Areas with a higher relative risk are dark purple (relative risk >2.00) whereas those with a lower relative risk are dark green (relative risk <0.50).

Graph from National Cancer Registry of Ireland All Ireland Cancer Atlas



1.3 Figure 2 Age distribution of cases of thyroid (1994-2010) and melanoma (1995-2007) cancer in Ireland

Graphs created using statistics from the National Cancer Registry of Ireland



1.3 Figure 3 Variation in thyroid cancer histological subtypes by age group, 1994-2010

Graphs from the National Cancer Registry of Ireland's Cancer Trends Series *Cancers of the Thyroid* 2012.

melanoma (Flaherty *et al.*, 2012; Chapman *et al.*, 2011). BRAF is an upstream component of the growth promoting mitogen-activated protein kinase (MAPK) pathway. Cells containing mutant BRAF become oncogene-addicted and are dependent on MAPK signaling for their growth and survival. Treatment with inhibitors targeted against BRAF or its downstream effectors MEK1/2 have both demonstrated improved progression-free and overall survival compared to chemotherapy (Chapman *et al.*, 2011; Flaherty *et al.*, 2012). Unlike therapy using the BRAF inhibitor PLX4032, treatment with the MEK1/2 inhibitor was not associated with cutaneous squamous-cell carcinomas (Su *et al.*, 2012; Flaherty *et al.*, 2012; Chapman *et al.*, 2011). However, consistent with the pattern of inhibitors of other oncogenic kinases, such as the inhibition of ABL by imatinib for the treatment of chronic myelogenous leukemia (CML), the clinical benefit of these molecular therapies was limited by the emergence of drug resistance (Poulikakos and Solit, 2011; Wagle *et al.*, 2011; Gambacorti-Passerini *et al.*, 2003).

A motivation for studying the mechanisms of resistance is the potential for said studies to inform rational therapeutic strategies for tumours that demonstrate intrinsic or acquired resistance. The goal of personalised cancer therapy is to elucidate how to use cancer therapeutics in the most effective way by correctly targeting patients that are most likely to

respond to a specific therapy. In order to successfully achieve this goal, it is not enough merely to know that a molecular treatment works, the mechanisms behind how it works and how cells can overcome these mechanisms must be elucidated so that the correct combination and adaptive treatments can be anticipated for the individual patient based on their underlying genotype and cancer phenotype (Turner and Reis-Filho, 2012; Garnett *et al.*, 2012). Targeting of specific genetic changes in defined patient subsets has been successful, however, a poorly explained range of responses to appropriately selected therapies is often still observed in patients (Garnett *et al.*, 2012; Chapman *et al.*, 2011). An improved understanding of the adaptive response of cancer cells to MAPK pathway inhibition may thus aid in the identification of those patients most likely to respond to targeted pathway inhibitors and provide a rational basis for tailored combination strategies.

In this thesis, we investigated the diagnostic utility of a specific microRNA identified in a previous genetic profiling study of thyroid cancer (Aherne *et al.*, 2008) to translate into a clinically useful biomarker in an Irish population (Chapter 3). We then employed cell line models of melanoma and thyroid carcinoma, with and without the BRAF^{V600E} activating mutation, to identify mechanisms of resistance to the MEK inhibitor PD0325901. Cells were optimised for treatment with the MEK inhibitor PD0325901 (Chapter 4) and resistant cells harvested. Cells were treated in parallel under identical conditions with media alone to provide matched naive controls. Resistant and naive samples were assayed for relative gene and protein expression of key members of the MAPK pathway (MEK1, MEK2, ERK1, ERK2 and ELK1) and stemness-markers (NANOG, OCT4, ALDH1A1, β -catenin, and E-cadherin). Next generation sequencing was used for the global microRNA expression profiling of naive and resistant cells (Chapter 5). Bioinformatic tools were then employed for an integrative analysis of miRNA and gene expression data in the context of gene regulatory networks.

1.4 Overall Project Aims

The main aims of this project are:

1. To investigate if specific microRNAs identified as potential biomarkers in cell line studies can translate into clinically relevant biomarkers of disease.
2. To investigate if MAPK pathway signalling occurs in cell lines that have gained resistance to MEK inhibition.
3. To correlate MEK inhibitor-resistance-associated gene, microRNA and protein expression profiles with BRAF^{V600E} mutational status and organ of origin (thyroid/skin).
4. To investigate a role for stem cells or stemness-characteristics in the resistance of cell lines to MEK inhibition.
5. To employ hypothesis-neutral global profiling of microRNAs to identify possible mechanisms of resistance to MEK inhibition.
6. To use integrative analyses of gene, microRNA and protein expression profiles in combination with *in silico* analyses to identify molecular mechanisms of resistance to MEK inhibition.

Chapter 2: Methods

2.1 Archival Tissue and Laser Capture Microdissection

2.1.1 Ethical Approval

Ethical approval in accordance to the Helsinki Declaration was obtained for this study from the St. James's and Federation of Dublin Voluntary Hospitals Ethics Committee.

Thyroid tumours were selected from the archival formalin fixed paraffin embedded (FFPE) tissue between the years 1980 and 2009 from St. James's Hospital (Dublin, Ireland). Slides had previously been reviewed by a histopathologist and diagnoses confirmed according to the World Health Organisation classification guidelines.

2.1.2 FFPE Sample Preparation

The formalin-fixed paraffin-embedded (FFPE) samples were cut using a HM325 rotary microtome (MSC, Dublin, Ireland) to 7µm, mounted on slides and dried on a heating block to remove excess moisture. The slides were deparaffinised and stained with haematoxylin and eosin (standard operating protocol (SOP) can be found in appendix I).

2.1.3 Laser Capture Microdissection

Laser capture microdissection (LCM) was used to specifically isolate cells of the desired histology from slide mounted FFPE sections (SOP for Arcturus® LCM system (Life Technology, USA) is found in appendix I). The appropriate follicular epithelial cells were computationally selected and laser-captured (infrared 810nm) onto CapSure® Macro LCM caps (MDS Analytical Technologies, CA, USA). Following microdissection the transfer film containing the captured cells was peeled off the CapSure® macro LCM cap and placed into a sterile DNA LoBind tube (Eppendorf AG, Hamburg, Germany) to maximise downstream recovery.

2.2 Cell Culture

2.2.1 Cell Lines

The cutaneous malignant melanoma cell lines SK-MEL-28 and SK-MEL-31 (ATCC number HTB-72 and HTB-73) and the acral malignant melanoma cell line SK-Mel-2 (HTB-68) were obtained from the American Tissue Culture Collection (ATCC). The Nthy-ori 3-1 normal adult thyroid tissue transfected with the SV40 large T gene (ECACC number 90011609) and the undifferentiated thyroid carcinoma 8505C thyroid cell line (ECACC number 94090184) were obtained from European Collection of Cell Culture (ECACC).

2.2.2 Maintenance of Cell Lines

For SK-Mel-28, SK-Mel-2 and 8505C cell lines were maintained in Eagle's Minimum Essential Medium (EMEM) made to complete growth medium by the addition of fetal bovine serum (FBS) to a final concentration of 10% (cEMEM). For SK-Mel-31 the EMEM made complete with the addition of 15% FBS (cEMEM^{PLUS}). For Nthy-Ori 3-1, the base medium for these cell lines is Roswell Park Memorial Institute (RPMI)-1640 made to complete growth medium by the addition of 10% FBS. All media was supplemented with penicillin-streptomycin at 100units/100mg per ml.

Cultures were maintained in a humidified 37°C/5% CO₂ incubator. To subculture adherent cells the medium was aspirated, the monolayer was washed thrice with sterile 1X phosphate buffered saline pH 7.4 (PBS) then 0.25% trypsin/EDTA solution (1ml/25cm²) was added and incubated at 37°C until cell detachment (~12 mins). Upon detechment cells were resuspended in the appropriate medium and transfered to fresh culture flasks. Cells were monitored frequently for morphology, confluence, growth characteristics and for signs of gross infection using a Brightfield microscope with a 10X objective lens.

2.2.3 Cell Proliferation Assays (MTT™ Assay)

In a microwell 96-well flat-bottomed tissue culture plate (Nunc, Germany), 100µl/well of cells adjusted to the desired seeding density was added, using 4 technical replicates per sample. A blank standard containing media only was included to measure background. To minimize the evaporation effect, the edge wells were not used experimentally and 100µl/well media was added to all redundant wells and the plate was incubated for 24 hours at 37°C with 5% CO₂. After 24 hours, 10µl/well of MTT labeling reagent (Roche, Germany) was added to each sample well and controls. The plate was incubated for 4 hours at 37°C with 5% CO₂. 100µl/well MTT Solubilisation Solution (Roche, Germany) was added and incubated overnight at 37°C with 5% CO₂. The plate was analysed spectrophotometrically in an endpoint assay with the test filter wave length set to 570nm and the reference filter set to a wavelength of 650nm (SOP in appendix I). The blank control was used to measure background noise and was subtracted from all optical density (OD) readings. The OD readings were averaged across replicates and graphed against a logarithmic x-axis of seeding density (cells/ml).

2.2.4 Generation of Drug Dosage Response Curves (MTT™ Assay)

The above assay was employed to create drug dosage curves. The assay was seeded at a predetermined density. The MEK inhibitor PD0325901 (Pfizer, USA) was dissolved in DMSO at 10mM and stored at -20°C as per manufacturer's recommendations. Serial dilutions of the drug in media were prepared and 100µl/well added. A vehicle control was included with serial dilutions of DMSO alone (no drug) in media and 100µl/well added. The assays were then performed in the same manner as above with varying incubation periods prior to addition of the labeling reagent. The OD readings were plotted against a logarithmic x-axis of drug dosage (nM) and regression analysis was used to determine the IC₅₀ and IC₉₀ drug dosage. Paired Student's T-Tests and multivariate analysis were used to analyse vehicle control effect and optimal treatment lengths.

2.3 Nucleic Acid Extraction and Quantitation

2.3.1 Genomic DNA Extraction

A study of four commercially available kits for the extraction of genomic DNA (gDNA) from cell lines was performed: Applied Biosystems MagMax™ Multi Sample DNA kit; Qiagen DNeasy Blood and Tissue Kit™; Qiagen AllPrep DNA/RNA Mini Kit™; and the Macherey Nagel NucleoSpin Tissue Mini Kit™. The full manufacturers' supplied protocols are in appendix I.

2.3.2 RNA Extraction

Two RNA extraction kits were used. For extraction of RNA from FFPE tissues, the RecoverAll™ total nucleic acid isolation kit optimized for FFPE samples (Ambion, USA) was employed, whereas for extraction from cell lines the NucleoSpin™ miRNA extraction kit (Macherey Nagel) was used. The full manufacturers' supplied protocols are in appendix I.

2.3.3 Nucleic Acid Quantitation

Nucleic acids were quantitated by two methods: spectrophotometrically using the NanoDrop 2000 and fluorometrically via Quant-iT Broad Range DNA and RNA assays in conjunction with the Qubit fluorometer (SOP in appendix I).

2.3.4 Reverse Transcription of RNA to cDNA

The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) was used to convert total RNA to single stranded cDNA. The full manufacturer's supplied protocol is supplied in appendix I. Briefly, the kit reagents (10X reverse transcription buffer, 25X dNTP mixture (100mM), 10X random primers and Multiscribe® reverse transcriptase (50U/μl)) were prepared into a 2X mastermix in nuclease-free water. RNA samples were diluted to 10ng/μl in nuclease-free water and then combined 1:1 with the 2X mastermix to prepare a 1X mixture, with a final concentration of 2.5U/μl Multiscribe enzyme in a final volume of 20μl/sample. Samples were briefly centrifuged and the reverse transcription was run in a thermal cycler under the following conditions: 25°C x 10 mins; 37°C x 120 mins; 85°C x 5 mins; 4°C∞.

2.3.5 Reverse Transcription of miRNA to cDNA

MicroRNA-specific reverse transcription was performed using the primers supplied as part of the TaqMan® MicroRNA Assays. The reverse transcription reaction used the following components from the High Capacity cDNA Reverse Transcription Kit: 10X reverse transcription buffer, 25X dNTP mixture (100mM) and Multiscribe® reverse transcriptase (50U/μl) and a mastermix was prepared to 4.7X. For each 15μl reaction, 3μl of 5X primer and 5μl of RNA (1-10ng/μl) was added to 7μl mastermix and briefly centrifuged. The reverse transcription reaction was run in a thermal cycler under the following conditions: 16°C x 30 mins, 42°C x 3 mins, 85°C x 5 mins, 4°C∞.

2.4 Real-Time TaqMan® PCR

2.4.1 TaqMan® Gene Expression Assays

Predesigned RT-PCR TaqMan® assays were obtained for protein-coding transcripts (2.6 Table 1). The manufacturer's supplied protocol can be found in appendix I. Briefly, 20X TaqMan assay was mixed with 2X Gene Expression Mastermix and cDNA template (1-2µl/20µl reaction) to 1X in nuclease-free water and 20µl/well added to a MicroAmp® Fast Optical 96-well plates. The target and endogenous control for each sample was included on the same 96-well plate. No template controls (NTC), using water instead of cDNA template, were included for each assay on each plate as a negative control. All samples were assayed in triplicate. Plates were sealed with MicroAmp® Optical Adhesive film, briefly centrifuged and the RT-PCR run on a 7500HT Fast RT-PCR instrument under the following conditions: 50°C x 2 mins, 95°C x 10 mins and 40 cycles of: 95°C x 15 secs, 60°C x 60 secs using a standard ramp rate.

2.4.2 TaqMan® MicroRNA Expression Assays

The TaqMan® MicroRNA Expression Assays are performed as per the Gene Expression Assays with the following exceptions: 2X Universal PCR Master Mix with No AmpErase UNG was used and a minimum 1/15 dilution of the reverse transcription product was used.

2.4.3 TaqMan® SNP Genotyping Assays and Allelic Discrimination Assay

A 40X custom TaqMan SNP Genotyping Assay (5' CAT GAA GAC CTC ACA GTA AAA ATA GGT GAT 3' [BRAF-F], 5' GGA TCC AGA CAA CTG TTC AAA CTG A 3' [BRAF-R], VIC-5' CCA TCG AGA TTT CAC TGT AG 3' [BRAF-P^{WT}], and FAM-5' CCA TCG AGA TTT CTC TGT AG 3' [BRAF-P^{MUT}]) was mixed with 2X TaqMan Gene Expression Mastermix and gDNA template in nuclease-free water to a final concentration of 1X with 5ng gDNA per reaction. NTC, Homozygous wild-type, homozygous mutants and heterozygous controls were included in triplicate in each plate at 5ng/reaction. Q-PCR endpoint assay was run on a 7900 Prism® RT-PCR machine (Applied Biosystems, USA) (2.4 Figure 1) with cycling parameters: 50°C x 2 mins, 95°C x 10 mins and 40 cycles of: 92°C x 15 secs, 60°C x 60 secs. A post-read allelic discrimination assay (SOP appendix I) was run and the results were autocalled using a 95% confidence interval (2.4 Figure 1).

2.4 Table 1: TaqMan® Gene Expression Assays

Gene Symbol	Gene Name	TaqMan ID	Assay	Notes
CDKN1B	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	Hs01597588_m1		Endogenous Control
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Hs03929097_g1		Endogenous Control
CCL2	chemokine (C-C motif) ligand 2	Hs00234140_m1		
MAP2K1	mitogen-activated protein kinase 1	Hs00983247_g1		AKA MEK1
MAP2K2	mitogen-activated protein kinase 2	Hs00360961_m1		AKA MEK2
MAPK3	mitogen-activated protein kinase 3	Hs00385075_m1		AKA ERK1
MAPK1	mitogen-activated protein kinase 1	Hs01046830_m1		AKA ERK2
ELK1	ELK1, member of ETS oncogene family	Hs00901849_g1		
POU5F1	POU class 5 homeobox 1	Hs00999632_g1		AKA OCT4A
NANOG	Nanog homeobox	Hs02387400_g1		
ALDH1A1	aldehyde dehydrogenase 1 family, member A1	Hs00946916_m1		
TGFBR1	transforming growth factor, beta receptor 1	Hs00610320_m1		
LSP1	lymphocyte-specific protein 1	Hs00158886_m1		
TSHR	thyroid stimulating hormone receptor	Hs01053846_m1		

CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	Hs00355049_m1	
CDH1	cadherin 1, type 1, E-cadherin (epithelial)	Hs01023894_m1	
RET1/PTC	ret proto-oncogene/papillary thyroid carcinoma chimera	Custom	Primer Seq: TTA CCA TCG AGG ATC CAA
MIR141	microRNA 141	002145	
RNU6B	RNA, U6 small nuclear 2	001093	Endogenous Control

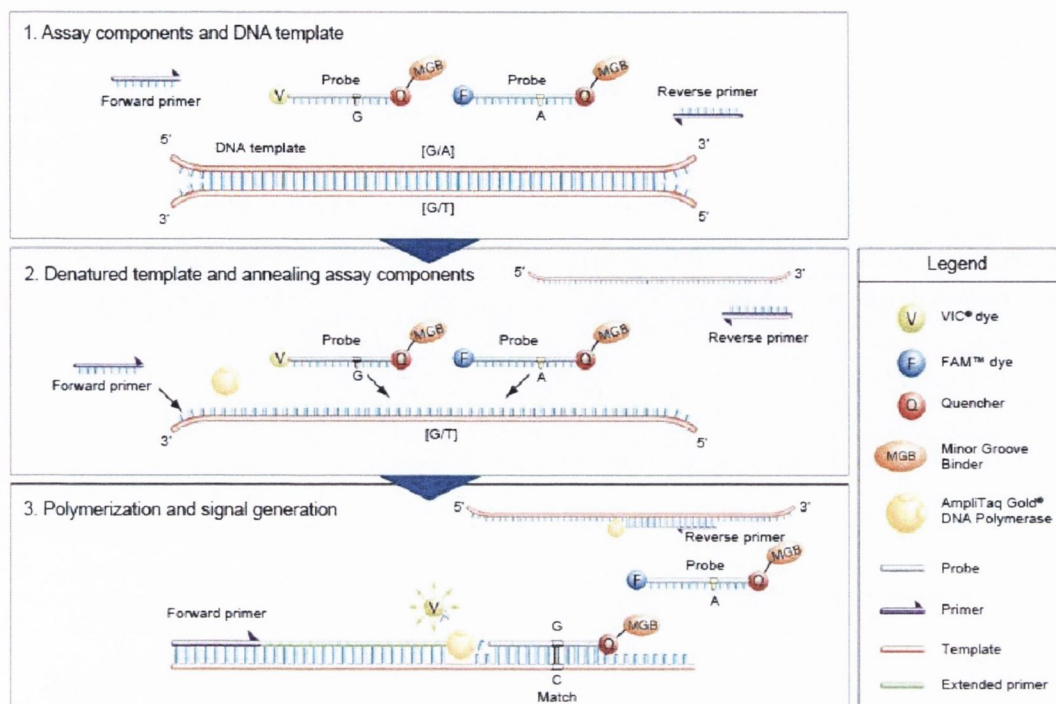


Image from TaqMan® SNP Genotyping Assays Product Bulletin (Life Technologies, USA)

2.5 Protein Analysis

2.5.1 Preparation of Whole Cell Lysates

Three protein preparation methods were used dependent on cell count and downstream application. As per the TaqMan® Protein Assay protocol (appendix I), cell pellets were washed in ice-cold 1X PBS then resuspended in either Applied Biosystems™ Protein Expression Sample Preparation Kit lysis buffer (cell count $<5 \times 10^4$) or Protein Quant Sample Lysis Kit (cell count $>5 \times 10^4$) with 1X Calbiochem® Phosphatase Inhibitor Cocktail Set I and II (Merck, Germany).

For Western Blot analysis, cell pellets were washed in 1X PBS then lysed directly into SDS-PAGE sample buffer (TRIS-HCL pH6.8 50mM, 10% glycerol, 2% SDS, 0.001% bromophenol blue and 100mM dithiothreitol (DTT)). Samples were vortexed for 3 minutes at high speed to resuspend then sonicated for 5 seconds at amplitude 12. Samples were then denatured at 98°C for 5 minutes.

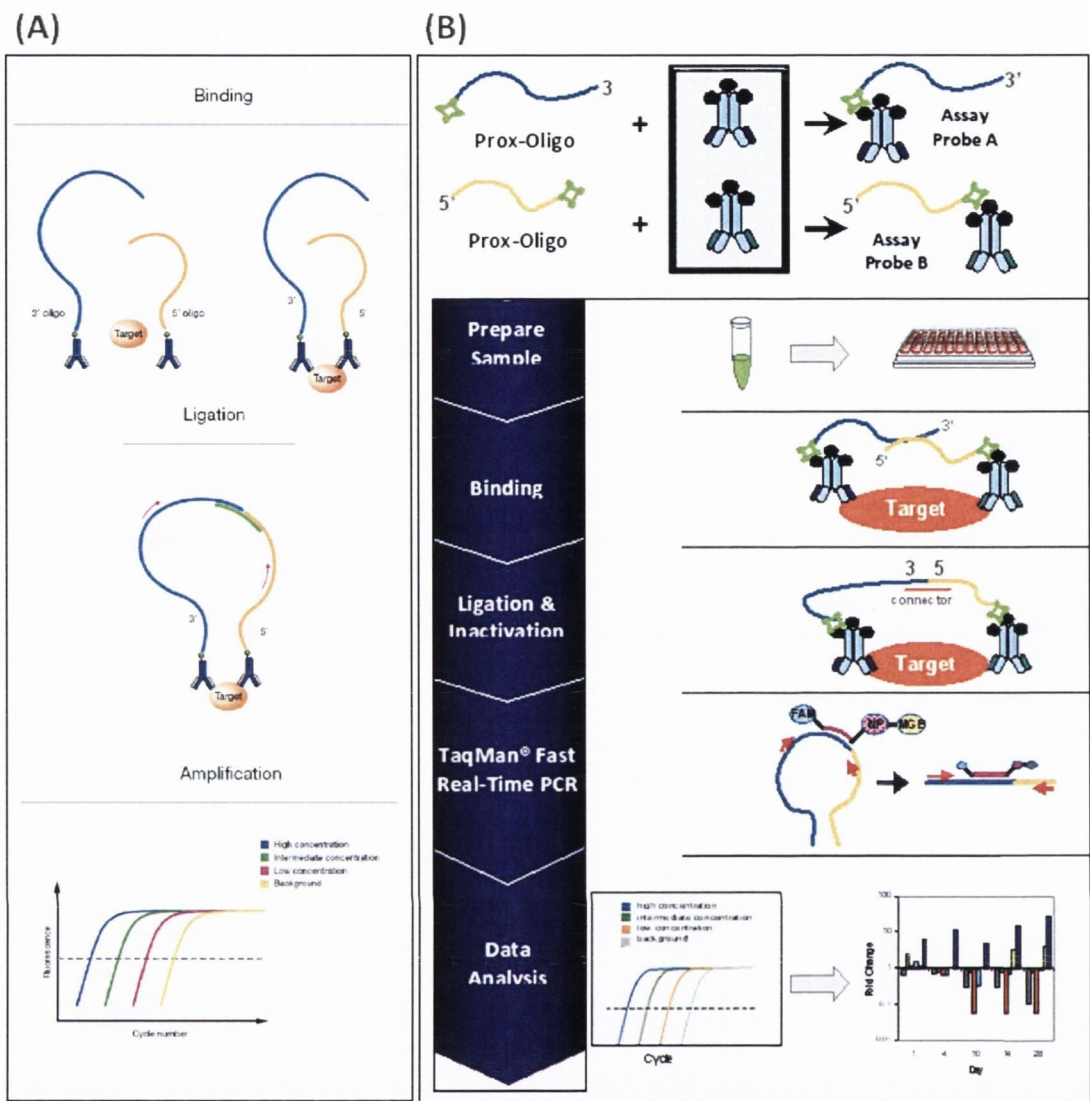
2.5.2 Protein Quantitation

Three methods of protein quantitation were used: cell count using a standard haemocytometer, which is not equivalent to protein quantitation but was used as a mechanism of standardisation (appendix I General Cell Culture SOPs), BCA Protein Assay (Pierce, USA. SOP Appendix I) or TaqMan® RNase P Detection Assay (Appendix I, in TaqMan Protein Assay Protocol Appendix B). RNase P detection assay estimates the cell equivalent concentration of a lysate through its gDNA concentration. The cell concentration was calculated by regression analysis of an RNase P standard curve prepared from kit supplied Human Genomic Control DNA data. For human diploid cells 6.6 pg of gDNA is equivalent to one cell and 1 ng of gDNA is equivalent to 151.5 cells.

2.5.3 TaqMan® Protein Assays

The full manufacturer's supplied protocol for TaqMan Protein Assays is found in appendix I and is illustrated in 2.5 figure 2(b). Briefly: cell lysates and a NTERA2 control cell lysate were serially diluted ½ from an input concentration of 500 cells/µl in lysate dilution buffer in a 96 well plate

on ice (plate A). The assay probe solution was prepared on ice by diluting probe pairs in probe dilution buffer. Assay probe solution was added to each lysate well including a no protein control (NPC) well. 2µl lysate from each dilution on plate A was then transferred to each of triplicate wells on a fresh 96 well plate on ice (plate B). Plate B was sealed, centrifuged briefly and placed in a thermal cycler at 37°C for 60 minutes to allow binding of the probes. Within 15 minutes, 96µl of fresh DNA ligase solution was added to each well, the plate was resealed and briefly centrifuged followed by incubation in a thermal cycler at 37°C for 10 minutes to permit ligation. The plate was placed on ice and 2µl protease solution was added per well. A fresh seal was added and the plate was placed in a thermal cycler at 37°C x 10 minutes; 95°C x 5 minutes; 4°C∞ to terminate ligation and subsequently terminate the protease. A fresh 96-well optical Fast PCR plate (plate C) was placed on ice, a TaqMan™ Universal PCR mastermix prepared and 11µl added per well. 9µl of the protease-treated ligation product from each well of plate B was transferred to each corresponding well of the plate C. The plate was sealed and briefly centrifuged then immediately run on a 7500 Fast PCR system at 95°C x 5 seconds; 40 cycles of (95° x 3 seconds, 60° x 30 seconds). Data was analysed using the 7500 Fast SDS software version 2 and Microsoft® Excel®.



2.5 Figure 1: Proximity Ligation Technology applied to TaqMan Protein Assay.

(A) The basis for PLT: binding, ligation and amplification. (B) Workflow for the protein TaqMan Assay, adapted from the manufacturer's supplied protocol.

2.5.4 Western Blot Protein Analysis

Full SOPs can be found in appendix I.

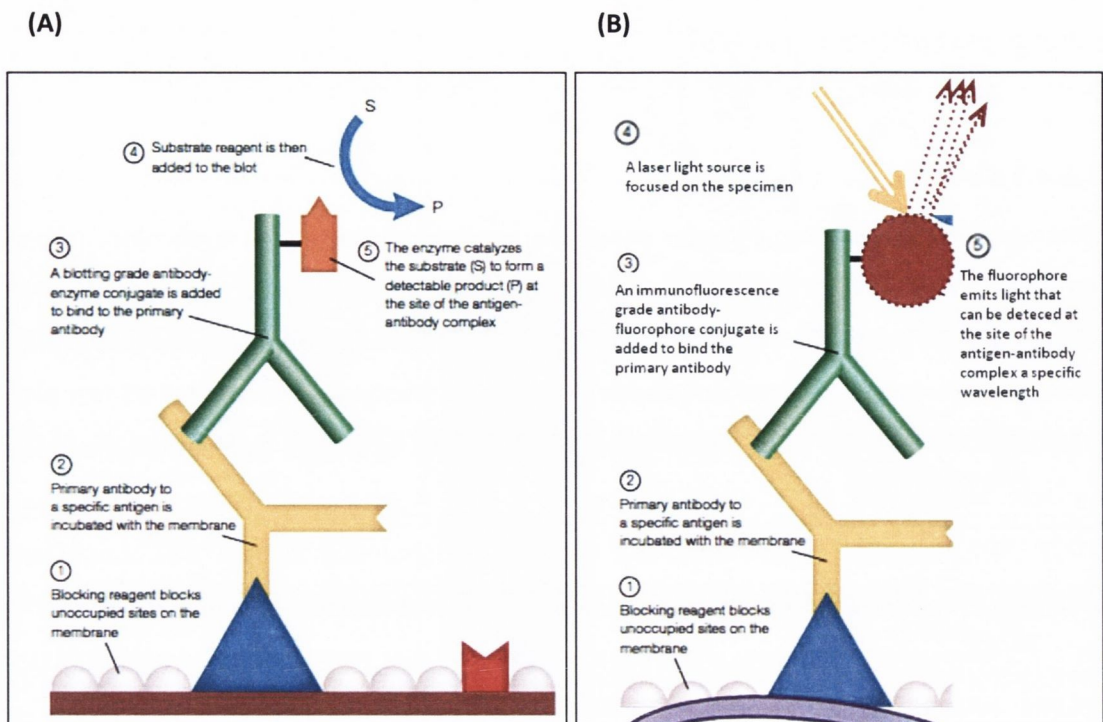
2.5.4.1 SDS-PAGE

Electrophoresis was performed under denaturing conditions using a 10% acrylamide resolving gel (10% acrylamide, 25% 1.5M Tris hydrochloride pH'd to 8.8 using hydrochloric acid (Tris-HCL), 0.1% SDS, 0.1% ammonium persulfate (APS) and 0.04% tetramethylethylenediamine (TEMED) dissolved in water) with a 5% acrylamide stacking gel (5% acrylamide, 12.6% TRIS-HCL pH6.8, 0.1% SDS, 0.1% APS and 0.1% TEMED dissolved in water). In an Atto standard electrophoresis rig (Atto, Japan) using 1X SDS-PAGE running buffer (25mM Tris base (TRIZMA), 250mM glycine, 0.001% SDS in water). Equal amounts of protein were denatured in 1X SDS-PAGE sample buffer (TRIS-HCL pH6.8 50mM, 10% glycerol, 2% SDS, 0.001% bromophenol blue and 100mM DTT) at 98°C x 5 mins and loaded alongside a broad range molecular marker (PageRuler™ broad range protein ladder Thermo Scientific: 5-250kDa). Electrophoresis was run at 300 volts and 100mAmps for approximately 90 minutes to separate proteins.

Following electrophoresis a PVDF membrane was activated by soaking in methanol for 90 secs and placed on top of filter paper soaked in Western blot transfer buffer (48mM TRIZMA, 39mM glycine, 0.04% SDS and 20% methanol). The resolving gel was removed from the cassette and placed on top of the activated PVDF membrane and filter paper soaked in Western blot transfer buffer was placed on top. The sandwich was soaked in Western blot transfer buffer and placed within an Atto Transfer System (Atto, Japan). Any excess transfer buffer was removed from the bottom of the rig and proteins were electrophoretically blotted at 100 volts/100 mAmps per gel x 1 hour.

2.5.4.3 Western Immunoblotting

Following transfer, membranes were blocked for non-specific binding for 1 hour at room temperature in 5% Marvel® milk powder in TBS-T. Membranes were probed with primary antibodies diluted (1/300 – 1/5000) in 5% BSA with 0.05% sodium azide in TBS-T overnight at 4°C with gentle agitation. Blots were washed 3X in TBS-T and incubated with a HRP-conjugated species-specific anti-IgG secondary antibody (1/5000 in 5% BSA in TBS-T) for 2 hours at room temperature with gentle agitation. Blots were washed 3X with TBS-T and incubated for 1 min



2.5 Figure 2: Immunoblotting (A) and immunofluorescence (B) for detection of protein.

A primary antibody raised against a specific epitope of the protein-of-interest is incubated with the membrane. Any unbound antibody is washed away. A secondary antibody-conjugate binds the primary antibody. Any unbound antibody is washed away.

(A) The conjugated enzyme's substrate is added and cleaved to produce a detectable product at the site of the antigen-antibody complex.

(B) A light source is applied to the sample exciting the conjugated fluorophore which can then be detected at a specific wavelength.

Image adapted from www.bio-rad.com

in HRP-development solution(0.018% hydrogen peroxide, 0.5% 250mM luminol, 2.2% 90mM 4-iodophenylboronic acid (4-IPBA) in Tris-HCL pH 8.8) away from light. Blots were exposed to autoradiography film in the dark and machine processed (Kodak®, USA) (2.5 Figure 2).

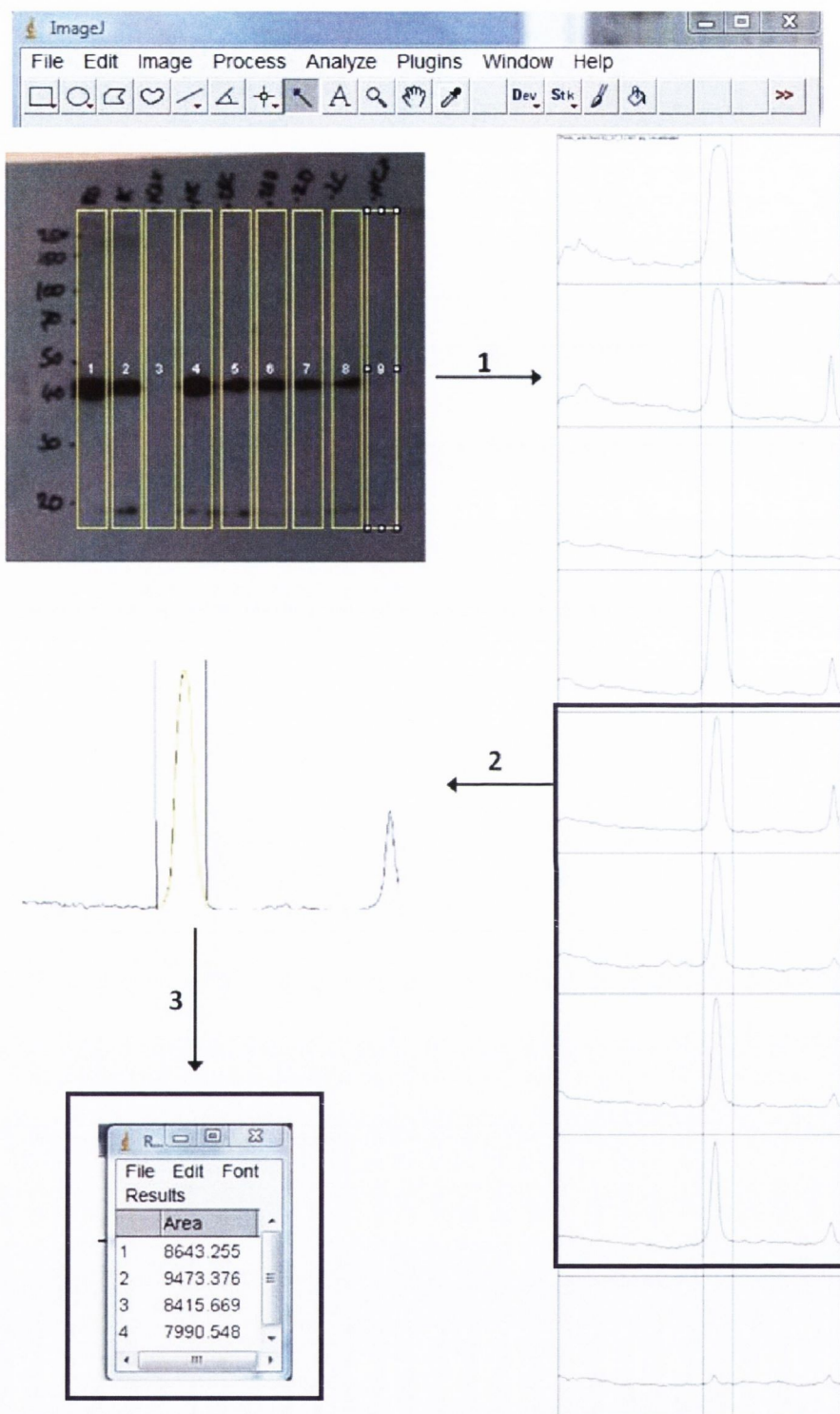
2.5.4.4 Densitometry

ImageJ software (rsbweb.nih.gov/ij/download.html) was used to measure the intensity of bands within Western Blot autoradiographs (2.5 Figure 3). Band intensity was measured for target proteins and positive control (actin). Intensity values were analysed using Microsoft Excel® and the relative density of peaks were calculated by normalisation to the actin control as follows:

$$\frac{Intensity\ target\ lane\ X}{Intensity\ actin\ lane\ X}$$

2.5 Table 1: Antibodies used in Protein Analysis

Antibody	Supplier	Catalogue #	Notes
Rabbit anti-Total ERK1/2	AbCam	Ab17942	Primary
Mouse anti-PhosphoERK 1/2	AbCam	Ab50011	Primary
Rabbit anti-ALDH1A1	Sigma	HPA002123	Primary
Rabbit anti-NANOG	AbCam	Ab21624	Primary
Rabbit anti-POU5F1	Cell Signalling	C52G3	Primary
Rabbit anti-ACTIN	Sigma	A2066	Primary
HRP-Anti-Rabbit IgG	Cell Signaling	7074S	Secondary (Western)
HRP-Anti-Mouse IgG	Cell Signalling	7076S	Secondary (Western)
Dylight-549 goat anti-mouse IgG	AbCam	Ab96880	Secondary (Confocal)
Alexa-488 goat anti-rabbit IgG	Invitrogen	A11008	Secondary (Confocal)



2.5 Figure 3: Densitometry using ImageJ Software.

The autoradiograph is opened in ImageJ and the sample lanes defined. The bands are plotted as intensity peaks (1). The peak representing the band of interest is selected (2). The intensity of that peak is calculated (3).

2.5.5 Confocal Microscopy

Cells were grown and treated in Lab-Tek™ 8-well chamber slides (Nunc, Germany) then fixed in 4% paraformaldehyde solution (150µl/well) and incubated at 37°C for 30 minutes. To permeabilise cells, 150µl/well 0.5% Triton X solution was added and incubated at room temperature for 10 minutes. Chambers were washed once with 1X PBS and stained for actin with 150µl/well Phalloidin-TRITC (Sigma, USA) incubated for 40mins at room temperature, away from light. Slides were washed 3 times with 1XPBS. Primary antibodies were diluted (1/1000 – 1/10,000) as per manufacturer's recommendation in 1% BSA blocking buffer and 150µl/well primary antibody was added and incubated overnight at 4°C with gentle agitation. Cells were washed 2X with PBS. 150µl/well secondary antibodies conjugated to fluorescent probes were diluted (1/3000) in 1% BSA blocking buffer. Slides were incubated at room temperature for 2 hours with gentle agitation. Slides were washed 3X with PBS. Chambers and any remaining polymer resin were removed from the slides. Two drops of Fluroshield™ with DAPI (Sigma, USA) was added to each slide. A coverslip (#1.5) was added sealed with a clear nail varnish top coat. Slides were mounted, inverted, onto a Zeiss 510 Meta Laser Scanning Confocal Microscope (LSCM) and imaged using a 63X oil emersion objective lens.

2.6 Next Generation Sequencing of Small RNAs on the SOLiD™ 4 Platform

2.6 Figure 1

2.6.1 Sample Preparation

Total RNA was diluted to 50ng/μl in water and 1μl/well was analysed using the Nano 6000 kit (Agilent, USA) and the Small RNA Kit (Agilent, USA) on the Bioanalyser 2100 (Agilent, USA) to determine the RNA integrity number (RIN) and the percentage of miRNA in the total RNA sample. Samples were diluted in nuclease-free water such that the RNA sample was less than 1μg/μl with miRNA content between 5-100ng/μl.

2.6.2 Library Preparation

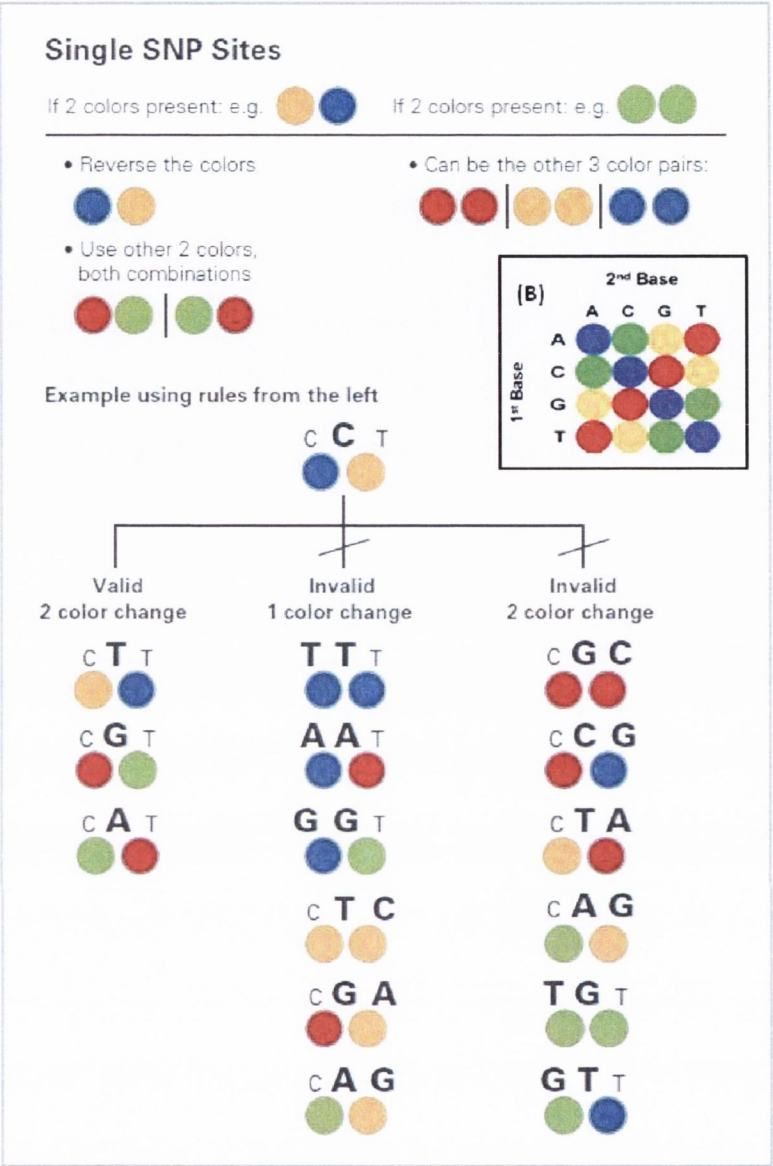
See 2.6 Figure 2.

2.6.2.1 Hybridisation, Ligation and Reverse Transcription

SOLiD adapter mix was added to hybridisation solution and RNA template at a ratio of 2:3:3, and run in a thermal cycler at 65°C x 10 mins, 16°C x 5 mins. The ligation enzyme (2μl/sample) and 2X ligation buffer (10μl/sample) was added to the hybridised sample and mixed well. The ligation reaction was run in a thermal cycler with the heated lid turned off at 16°C x 16 hours. To each ligated sample, 19μl of the reverse transcription mastermix (4μl 10X RT buffer, 2μl 2.5mM dNTP, 2μl SOLiD RT primer in 11μl water) was added. The solutions were mixed then briefly centrifuged and placed at 70°C x 5 mins, then snap cooled on ice. To each reaction, 1μl ArrayScript™ RT enzyme was added and the sample briefly and gently vortexed. Samples were centrifuged briefly then incubated at 42°C for 30 mins to allow the reverse transcription reaction to convert the adaptor-ligated RNA into cDNA. The RT reaction was purified using the MinElute® PCR Purification (Qiagen, USA).

2.6.2.2 Size selection of cDNA

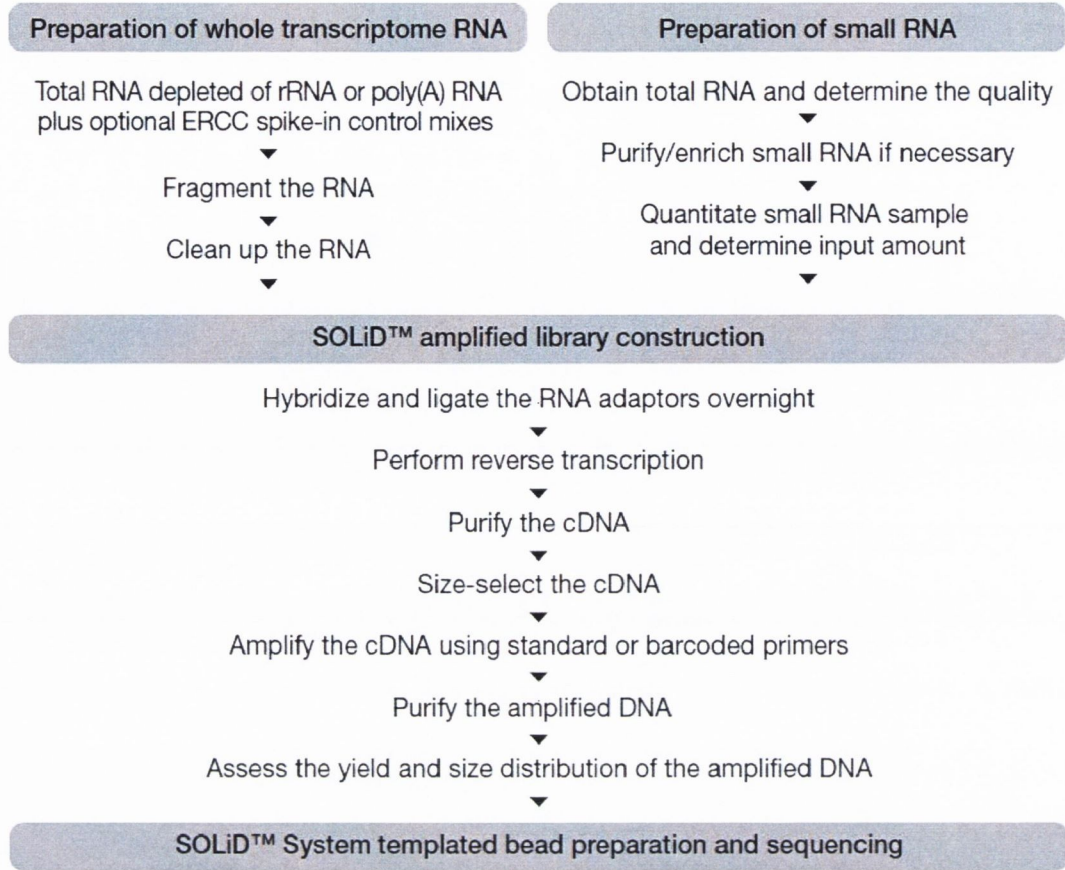
The cDNA samples were size selected using electrophoresis. 5μl cDNA sample was mixed with 5μl Novex 2X TBE-Urea Sample Buffer (Invitrogen, USA). A 40ng/μl 10bp DNA ladder (Invitrogen, USA) was mixed 1:1 in Novex 2X TBE-Urea Sample Buffer (Invitrogen, USA). Samples and ladder were heat denatured at 95°C x 3 mins then snap cooled on ice. A Novex 10% TBE-Urea gel was locked into position in an X-Cell Sure-Lock™ Mini-Cell (Invitrogen, USA) which was



2.6 Figure 1: Principle of two-base encoding.

Ligation-based sequencing chemistry enables two adjacent bases to be interrogated per reaction. The dual encoding of the two-base reaction is read in colour space. The colour space code is inset (B). A true SNP results in a valid 2 colour change as each base is read twice, as part of a duo with the adjacent left nucleotide and the adjacent right nucleotide. A random sequencing error, however, results in a single colour change or in an invalid 2 colour change.

Image adapted from the Applied Biosystems SOLiD™ System Accuracy Application Fact Sheet.



2.6 Figure 2: Workflow for SOLiD™ Small RNA library preparation.

The creation of small RNA libraries is based on size selection. Total RNA is ligated to adaptors and reverse transcribed to cDNA. cDNA is run on a 10% agarose gel and the desired size is cut from the gel and amplified using barcoded 3' primers directly from the gel. Amplified cDNA is analysed for quality and yield before progressing to sequencing.

Image from Applied Biosystems (USA) SOLiD Total™ RNA-Seq Kit Product Bulletin.

filled with 1X TBE running buffer (Invitrogen, USA). 10µl of ladder or sample was added per well and electrophoresis was run at 180V for approximately 45 mins. The gel was incubated in the staining solution (0.01% solution of SYBR Gold® nucleic acid stain (Invitrogen, USA) in 1X TBE running buffer) for 10 mins with gentle agitation at room temperature. Gels were visualised on a UV transilluminator and the gel band between 60-80nts was carefully and quickly excised for each sample with minimal exposure to UV. Each excised band was then cut vertically into 4 equal sections. The two middle sections of the gel per sample were each transferred into a 0.2ml PCR tube and the remaining gel slices were stored at -20°C.

2.6.2.3 In gel PCR and barcoding

The cDNA was amplified using a 5' primer that introduces a 5' sequence that is required for downstream binding to microbeads for ePCR (P1 adaptor) and a 3' Primer that incorporates a barcode to allow multiplexing of sample libraries and a universal adaptor at the 5' end required for downstream processes (P2 adaptor). For each gel slice a 100µl PCR mastermix was added (10µl 10X PCR buffer, 8µl 2.5mM dNTP, 2µl 5' Primer, 1.2µl AmpliTaq® DNA polymerase, 2µl barcoded 3' Primer in 76.8µl water). The PCR reaction was run in a thermal cycler as follows: 95°C x 5 mins; 18 cycles of 95°C x 30 secs, 62°C x 30 secs, 72°C x 30 secs; and 72°C x 7 mins. The PCR reaction was purified using the PureLink® PCR Micro Kit (Invitrogen, USA).

2.6.2.4 Prepare the Multiplexed Library

The Agilent DNA 1000 kit and Chip was used to assess the cDNA library on the 2100 Bioanalyser (SOP in appendix I). A post-read smear analysis was used to determine the quality of the library. The proportion of miRNA in relation to the total library was calculated. Barcoded libraries were calculated in nM:

$$\frac{(\text{Library concentration (ng/}\mu\text{l)} \times 10^6)}{(\text{Average base pair size} \times 650)}$$

Barcoded libraries were pooled by diluting each library to the lowest library's nM concentration and then combining in equal quantities to obtain a multiplexed library.

2.6.3 Library Quantitation

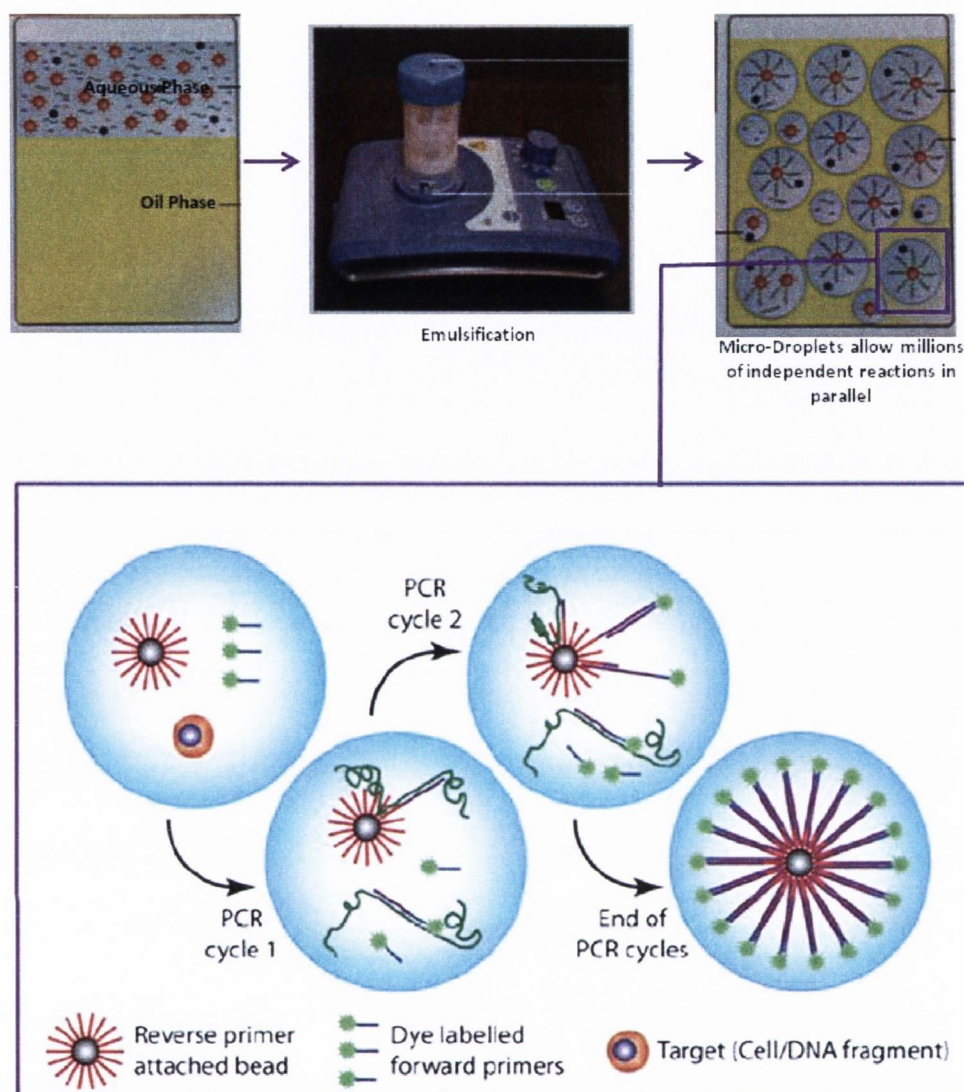
Quantitative RT-PCR was performed using the SOLiD™ Library TaqMan® Quantitation kit (Applied Biosystems, USA). The manufacturer's supplied protocol is in appendix I. Briefly, the multiplexed library was quantified via Qubit (section 2.3). The library was then diluted 1/100, 1/1000 and 1/10,000 to prepare the input sample within range of the standard curve for library quantitation. Kit-supplied library standards were diluted to 100pM (standard 1) and serially diluted 1/10 to 0.01pM (standard 5). Per well, 10µl SOLiD Library qPCR mix, 1µl TaqMan assay (Ac00010015_a1), 0.04µl 25µM ROX reference dye and 3.96µl nuclease-free water was added. 5µl of qPCR library standard (1-5), sample or water (NTC) was added per well. All wells were performed in triplicate. A fast RT-PCR was run using the 7500HT Fast RT-PCR Thermal Cycler as follows: 95°C x 20s; 40 cycles of 95°C x 3secs, 60°C x 30 secs. A standard curve was generated and the absolute quantitation of samples determined via regression analysis. Library concentrations were calculated in pM and diluted to 500pM in 1X TE buffer to proceed to emulsion PCR (ePCR).

2.6.4 Templated Bead Preparation

The full-scale templated bead protocol (appendix I) was followed for 0.4pM and 0.8pM libraries, with 4 ePCRs per concentration. To determine whether 0.4pM or 0.8pM input is optimal a workflow analysis (WFA) sequencing run was performed. The manufacturer's supplied SOLiD™ 4 System Templated Bead Preparation Guide is in appendix I. The steps are briefly described below.

2.6.4.1 Full scale emulsion PCR

The oil phase for ePCR was prepared by gently adding 4.5% Emulsion stabilizer 1 to 1% Emulsion Stabilizer 2, then adding 94.5% emulsion oil and vortexing to a uniform consistency. The oil phase is then degassed prior to the dispensation of 9mls oil phase to an ePCR tube. The aqueous phase was prepared to a final concentration of 1X PCR buffer, 14mM dNTP mix, 25mM Magnesium Chloride, 40nM ePCR primer 1, 3µM ePCR primer 2, 0.4pM or 0.8pM small RNA library template, 0.54U/µl AmpliTaq Gold DNA polymerase in nuclease free water. The



2.6 Figure 3: Principles of emulsion PCR (ePCR).

An aqueous phase containing template and reagents is emulsified to create millions of uniform droplets that act as independent micro-reactors. The hydrophobicity of the oil phase surrounding the droplets can prevent the contact between the surface of the reactor walls and the sample by confining the sample in droplets. Each functional PCR droplet contains a bead covalently labelled with the reverse primer, labelled forward primer, and a single target copy. Subsequent steps of PCR generate labelled double-stranded product on the bead surface. Any single bead bears thousands of single-stranded copies of the same PCR product, whereas different beads bear the products of different compartmentalized PCR reactions.

Image adapted from Zhu et al, 2012

aqueous phase per ePCR is prepared in a total volume of 5600µl: 5440µl PCR reagents and 160µl prepared and sonicated SOLiD™ P1 DNA Beads (2.6 Figure 3).

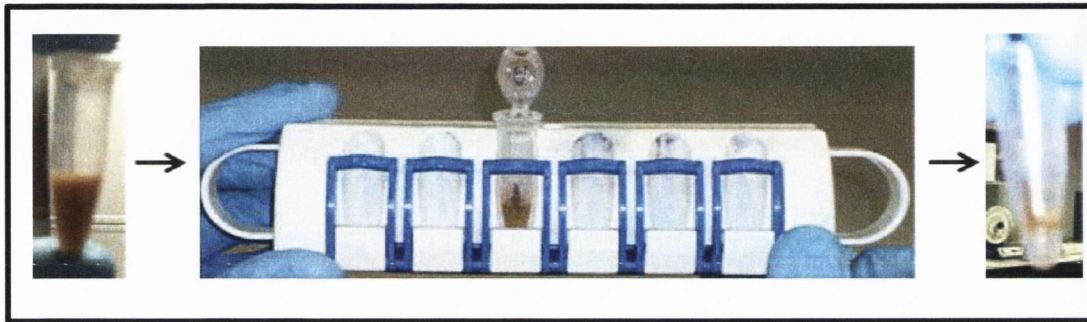
2.6.4.2 Breaking the emulsion and bead washing

The oil phase was placed onto a fixed-setting Ultra-Turrax® tube drive (IKA, China) and engaged in spinning. An X-Strem® Pipettor (Eppendorf, Germany) was used to add 5.6ml aqueous phase to the spinning oil phase and spun for 5 mins to create the emulsion. A Repeater® Plus Pippettor (Eppendorf, Germany) was used to pipette 150µl emulsion per well of a 96-well MicroAmp PCR plate and sealed with MicroAmp clear adhesive film. ePCR was run on a 9700 thermal cycler using the following conditions: 95°C x 5 mins; 40 cycles of 93°C x 15 secs, 62°C x 30 secs, 72°C x 75 secs; 72°C x 7 mins; and 4°C ∞ with reaction volume set to 50µl with a ramp speed of 9600. Following the ePCR run, the bottom of the PCR plates were checked for broken emulsions, which appear as brown/amber specks. If more than 3-wells were broken, the ePCR was repeated.

An emulsion collection tray was attached to the PCR plate, inverted and centrifuged at 550g x 2 mins at room temperature with a high acceleration and low deceleration centrifuge setting. The PCR plate was carefully removed from the collection tube and, in a fume hood, 10ml 2-butanol was added to the collection tray using a 10ml serological pipette. The solution was mixed via pipetting until homogenous then transferred to a 50 ml tube. Another 6mls 2-butanol was added to the collection tube to wash the tray and collect any remaining emulsion then transferred to the 50 ml tube. The tube was vortexed then centrifuged at 2000g x 5 mins to pellet the beads. Supernatant was discarded and the tube inverted onto a paper towel to maximise oil removal.

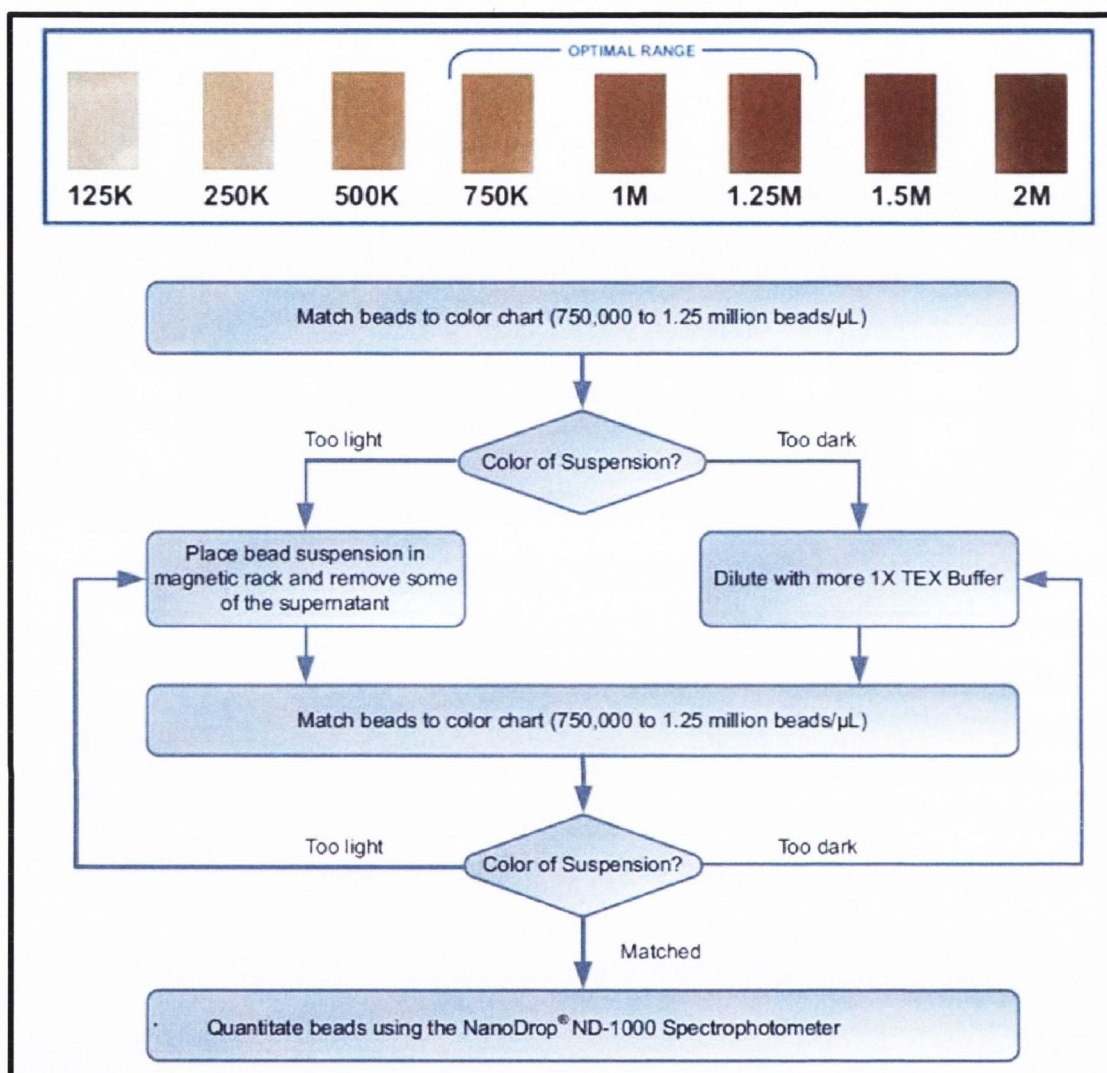
Pellets were resuspended in 600µl 1X bead wash buffer, incubated for 2 mins then transferred to a LoBind 1.5ml tube. The 50ml tube was rinsed with a further 600µl 1X bead wash buffer and added to the 1.5ml tube. The tube was vortexed and centrifuged at 21,000g x 1 min. The oil phase was removed as much as possible without disturbing the aqueous layer. The remaining supernatant was carefully removed, the pellet resuspended in 1X bead wash buffer, vortexed then pulse-spun and transferred to a fresh tube. The bottom of the original tube was rinsed with 1X bead buffer and transferred to the new tube. A further 1ml 1X bead wash buffer was added to the new tube and vortexed to mix. The tube was centrifuged 21,000g x 1 min and supernatant discarded. Beads were resuspended in 200µl 1X TEX buffer and placed on a

magnetic rack. When solution cleared, supernatant was removed. Samples were removed from the magnetic rack and 200µl 1X TEX buffer was added (2.6 figure 4).



2.6 Figure 4: Magnetic Bead-Based Washing of ePCR Products.

ePCR amplified templated beads were resuspended in buffer. Samples were placed on a magnet rack and beads adhered to the tube adjacent to the magnetic and the solution cleared. The clear supernatant was removed leaving the washed templated bead sample.



2.6 Figure 5: SOLiD Bead Concentration Chart and Workflow

2.6.4.3 Quantitation of SOLiD™ Beads

Surplus sonicated templated beads were counted using a haemocytometer. Beads were diluted in 1X TEX buffer to 6 standard concentrations ranging from $2 \times 10^5/\mu\text{l}$ – $1.2 \times 10^6/\mu\text{l}$. The standards were read 3 times each on the nanodrop spectrophotometer 1000 under the cell culture tab using 2 μl per read. A standard curve was generated and used to measure all future bead concentrations. The ePCR amplified templated bead sample was sonicated to declump the sample and a 1ml dilution of 1:10 beads:1X TEX buffer sample was prepared. The colour of the sample was then compared to the SOLiD Bead Concentration Chart (2.6 Figure 5) and diluted, if required, to within the 7.5×10^5 to 1.25×10^6 range. The beads were then quantified in triplicate on the nanodrop, the results averaged and the concentration calculated from the standard curve. The diluted and undiluted beads were recombined.

2.6.4.4 SOLiD™ Templated Bead Enrichment

Beads were placed onto a magnetic rack until the solution cleared and the supernatant was discarded. Beads were resuspended in 300 μl Denaturing Buffer Solution (10% denaturant, 90% Denaturing Buffer), incubated for one minute, placed on the magnetic rack for one minute and the supernatant discarded. This was repeated twice. Beads were washed in 1X TEX buffer three times, then resuspended in 150 μl 1X TEX buffer, transferred to a 500 μl LoBind tube and sonicated to declump the sample.

Prepared enrichment beads in 150 μl 1X low salt binding buffer (per ePCR) were added to the templated beads. Beads were vortexed, pulse-spun, sonicated, pulse spun then incubated at 61°C for 15mins, with a vortex and pulse spin every 5 mins. The beads were snap-cooled on ice for two mins then gently layered onto 60% glycerol in a 1.5ml LoBind tube and centrifuged at 21,000g x 3 mins. The templated beads were separated onto the top layer and untemplated beads are sedimented on the bottom of the tube. The top layer was carefully removed to a 2ml LoBind tube containing 1ml 1X TEX buffer. The tube was filled to 2mls with 1X TEX buffer and vortexed then centrifuged at 21,000g x 1 min and the beads pelleted. Supernatant was removed and beads were resuspended in 400 μl 1X TEX buffer.

2.6.4.5 Isolation of P2-Enriched Beads

Samples were centrifuged at 21,000g x 1 min, supernatant discarded and resuspended in denaturing buffer solution, placed in a magnetic rack x 1 min and the supernatant discarded, this was repeated until the supernatant appeared clear. Beads were washed in 1X TEX buffer

three times, then resuspended in 200µl 1X TEX buffer and transferred to a 1.5ml LoBind tube. The 2 ml tube was rinsed with 200µl 1X TEX buffer, which was added to the new 1.5ml tube. Beads were sonicated to declump the sample and placed in a magnetic rack until the supernatant cleared and was discarded. Beads were resuspended in 1X TEX buffer and the magnetic rack wash step was repeated. The beads were resuspended in 400µl 1X TEX buffer.

2.6.4.6 Modification of the 3' End

Sonicated beads were isolated in a magnetic rack and resuspended in 1X terminal transferase reaction buffer (terminal transferase buffer, cobalt chloride and water) and transferred to a fresh 1.5ml tube. Beads were magnetically isolated and again resuspended in 100µl terminal transferase reaction buffer, this was repeated once. Beads were isolated magnetically and resuspended in 178µl 1X terminal transferase reaction buffer and 20µl 1mM Bead Linker Solution. Beads were sonicated and 2µl terminal transferase (20U/µl) was added. Samples were incubated on a tube rotator for 2 hours at 37°C, gently rotating. Samples were placed on the magnetic rack for 1 minute and the supernatant discarded. The beads were washed in 1X TEX before being resuspended in 400µl 1X TEX.

2.6.4.7 Quantitation of the Modified SOLiD Beads

Beads are quantified as per section 2.6.4.3

2.6.5 SOLiD™4 Next Generation Sequencing Run

The manufacturer's supplied protocol for SOLiD sequencing can be found in appendix I and is described briefly below.

2.6.5.1 Workflow Analysis (WFA)

The WFA determined the optimal library concentration and the bead enrichment efficiency. WFA determined whether the 0.4pM or 0.8pM library should be used and the fraction of successfully amplified beads as a proportion of the total beads. This was then be used to determine the optimal input amount of beads for the full sequencing run. To perform the WFA, each library was deposited at a density of 1.5×10^7 beads per quadrant of a sequencing slide and the subjected to a single SOLiD™ ligation cycle.

2.6.5.1.1 Preparation of SOLiD™ XD Slides

1.5×10^7 declumped beads were transferred to a fresh 1.5 ml LoBind tube and stored and isolated on a magnetic rack. Supernatant was discarded and beads resuspended in 400µl SOLiD™ XD Deposition Buffer. Beads were placed on the magnetic rack and isolated, supernatant was discarded. This was repeated twice. SOLiD™ XD Slides were brought to room temperature. Beads were resuspended in the correct volume of SOLiD™ XD Slide Deposition buffer dependent upon slide carrier. The slide carrier was prepared and the XD slide assembled into the deposition chamber. Beads were sonicated twice to declump and immediately pipetted into the entry porthole of the deposition chamber and a piece of adhesive tape was placed over the porthole. Deposition chambers were centrifuged at 167g x 5 mins and incubated at 37°C for 1 hour. The slide carrier was removed from the deposition chamber and gently washed twice with slide storage buffer. The carrier was loaded onto the cleaned, prepared flowcell and secured into place. The flowcell was locked into the scan position, the instrument doors closed and the flowcell loaded with buffer to prevent it from drying out.

2.6.5.1.2 WFA sequencing run

The manufacturer's supplied protocol (appendix I) provides the computational steps required to set up an WFA run using the SOLiD Instrument Control Software (ICS) using a 4-spot WFA mask, with each spot representing a well and each well containing templated beads from a

The diagram illustrates the sequencing process for a cDNA library. The top part shows a linear sequence: 5' (Type 1) - P1 adapter - cDNA insert - Internal Adapter - Barcode 1 - P2 adapter. Below this, five primers (BC Primer A-E) are shown, each paired with a 'Bridge' and a 'Barcode' (represented by two yellow dots). The bottom part is a detailed sequencing grid. The grid has columns for 'Read Position' (0-30) and 'Template Sequence' (5'-3'). It lists five primers: 1. Universal seq primer 10-20, 2. Universal seq primer 10-51, 3. Universal seq primer 10-20, 4. Universal seq primer 10-20, and 5. Universal seq primer 10-40. The grid shows the sequence of bases (A, C, G, T) for each primer across the read positions. A red circle highlights the 'Bridge Index' column for primer 2, and a red box highlights the 'Barcode' column for primer 5. A legend at the bottom indicates the 'Index Code' for each primer.

Ligate

Visualise

Cleave

Every 5th Base Interrogated

78

single ePCR. The focal range was detected and confirmed by visualising the beads. The WFA run was started by selecting the start run tab for the appropriate flowcell. The WFA run was monitored as per the manufacturer's supplied protocol. The library concentration (0.4pM or 0.8pM) with the higher titration matrix was used for full sequencing (2.6 Figure 6). The volume of beads deposited was calculated based on WFA results as follows:

- (1) Calculate the P2 positive (P2⁺) beads/μl in each ePCR sample (each mask/well).

$$\frac{(P2\text{positive per panel [from WFA]} \times 426 \text{ panels})}{\text{Input volume of beads used for WFA run } (\mu\text{l})}$$

- (2) For a single well slide, deposition of 708 million beads is optimal. Approximately 20% of beads are lost in the washing and deposition preparation steps. Hence the input volume of bead solution is calculated as follows:

$$\frac{708 \text{ million}}{P2 \text{ positive beads}/\mu\text{l}} \times 120\%$$

2.6.5.2 SOLiD Sequencing Run

The steps for the sequencing are the same as those for WFA, using a single well deposition slide and adjusting the bead volume as per 2.6.5.1.2. The ICS is used to set-up a multiplex sequencing run as barcoded libraries were used. During this first step, barcodes were matched to libraries. The focal plane is detected as before and a sequencing run is performed using barcode tag (BC Tag MM10) primers. This initial step differs only by the strip reagents used and the selected sequencing run on the ICS. When this initial run was finished and saved, the strip reagents were changed (Fragment library 35bp read (F3 tag MM35) primers) and instrument reagents were checked to ensure sufficient quantities remain. The focal plane was again detected and the full sequencing run set up and run using the ICS. The run was monitored as per the manufacturer's supplied protocol (appendix I). Primary analysis of the sequencing run was performed using the ICS software as per protocol (appendix I). Following assessment of sequencing run success, full computational analysis of the SOLiD NGS sequencing data was analysed by Dr. Gordon Blackshields, a bioinformatician within the SOLiD™ Sequencing Group of the Department of Histopathology, TCD.

Chapter 3: Association of microRNA-141 and Follicular Thyroid Cancer in an Irish Archival Population²

² Dorris, ER, Smyth PA, O’Leary JJ and Sheils, O. *MIR141 expression differentiates Hashimoto Thyroiditis from PTC and benign thyrocytes in Irish archival thyroid tissues*. *Frontiers in Thyroid Endocrinology*. 2012 (3)102

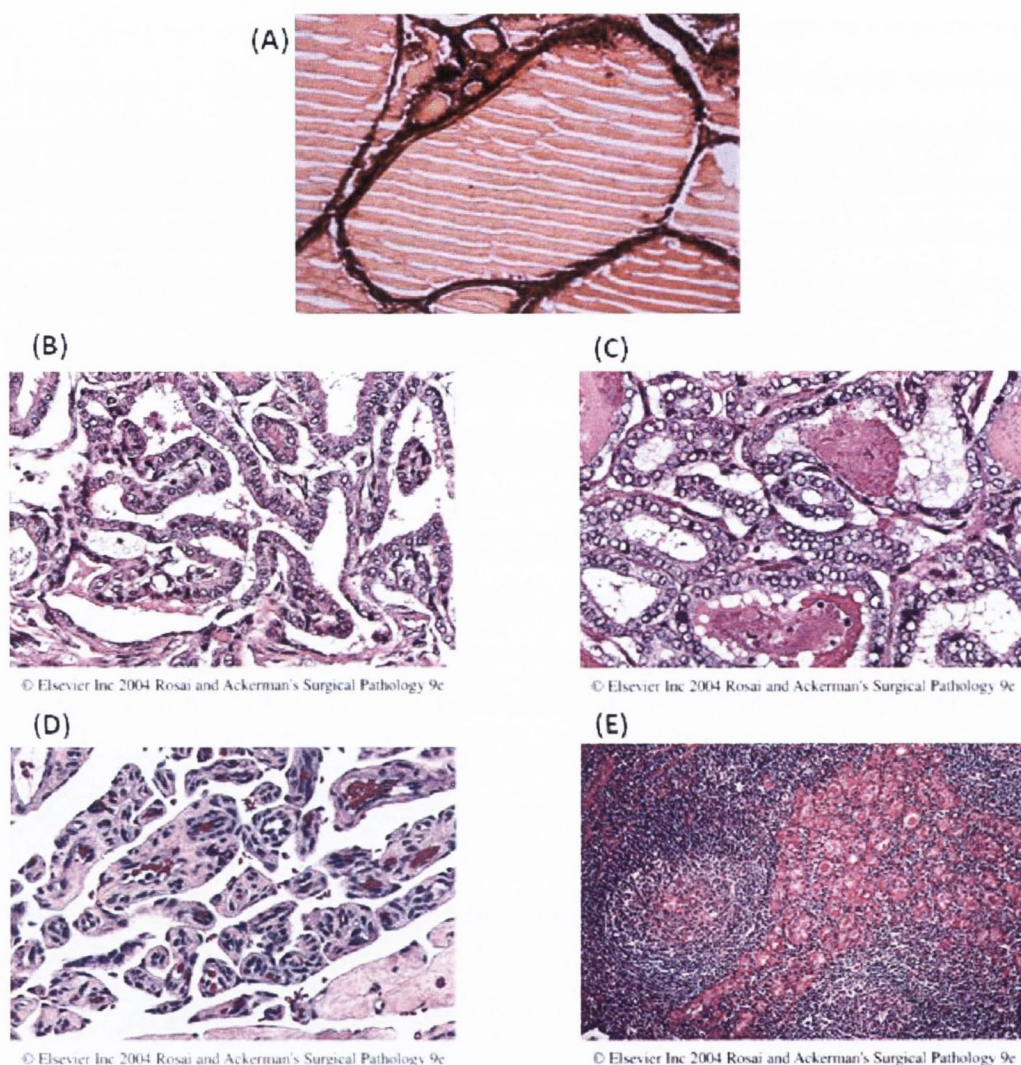
3.1 Introduction

Profiling of miRNA dysregulation is a potential diagnostic tool (Marini *et al.*, 2011, Braun *et al.*, 2011). Many microRNAs display exquisite tissue specificity that may aid in determining the tumour tissue of origin. It has been found that miRNA classification was more accurate than messenger RNA (mRNA) classification in determining tissue-of-origin in poorly differentiated tumours (Rosenfeld *et al.*, 2008). An estimated 50% of miRNAs are located at genomic regions linked to cancer. Nearly every type of tumour analysed by miRNA profiling has shown significantly altered miRNA profiles compared to normal counterparts (Calin and Croce 2006).

Carcinomas of the thyroid are the most common endocrine malignancy (Parkin *et al.*, 2005, 2010). The most common thyroid carcinoma is the well-differentiated classic PTC (cPTC), which accounts for an estimated 80% of thyroid cancers. cPTC is typically easily recognised microscopically due to its characteristic papillae and optically clear nuclei. The papillae are frequently associated with the formation of follicles, the ratio of which vary greatly (Rosai 2004). However, other subtypes of thyroid carcinoma can be more difficult to diagnose. The follicular variant of PTC (fvPTC) composes entirely or almost entirely of follicles. The diagnosis of fvPTC is primarily based on nuclear alterations associated with cPTC. Its invasiveness and incidence of nodal metastases also aid diagnoses. Follicular carcinoma of the thyroid (FTC) is usually a solitary neoplasm with a microscopic appearance varying from well-formed follicles to solid growth patterns (Rosai 2004). Hence, accurate diagnosis of certain thyroid carcinomas, in particular the differential diagnoses of fvPTC and FTC may be problematic. Given the difficulties in differentiating between subtypes, and with a view to understanding the underlying pathology, thyroid cancer research is ongoing with a view to identifying more robust discriminators of disease and prognosis.

Hashimoto thyroiditis (HT) is a chronic autoimmune disorder characterised by lymphocytic inflammation of the thyroid. This infiltration leads to apoptosis of thyrocytes and hypothyroidism. HT is the most common form of thyroiditis, with a strong prevalence for women over the age of 40 (Rosai, 2004). The triggers for this autoimmune response appear to be both humoral and cellular, with a complex etiology involving both genetic and environmental factors (Eschler *et al.*, 2011). HT is associated with an increased incidence of PTC. However, the relationship between HT and PTC remains controversial, with some arguing that the increased rate of screening in HT patients confounds the association between the two. However, RET/PTC chromosomal translocations, which were originally hailed to be a unique biomarker of PTC, have been identified in greater than 95% of HT tissue samples, 84% of which had no evidence of

malignancy (Sheils *et al.* 2000a). This evidence implies a shared molecular etiology between HT and PTC.



3.1 Figure 1: Pathology of thyroid cohorts.

(A) Normal thyroid tissue. Colloid-filled follicles are surrounded by a single layer of epithelial cells (B) cPTC, characterised by complex branching papillae. (C) fvPTC, typical overlapping optically clear nuclei. (D) FTC, this FTC shows a papillary configuration. (E) HT, with a vast lymphocytic infiltrate forming a prominent germinal centre.

(A) Control sample captured under a 10X objective lens. (B)- (E) from *Rosai and Ackerman's Surgical Pathology 9th Edition* (Rosai, 2004).

3.2 Project Rationale and Objectives

Investigation into miRNA deregulation in PTC has identified aberrant miRNA expression profiles in PTCs compared to normal thyroid (Pallante *et al.*, 2010; Aherne, 2008; Visone *et al.*, 2007; He *et al.* 2005; Cahill *et al.* 2006; Pallante *et al.* 2006). Our laboratory previously identified a miRNA signature profile for a cPTC cell line that displays significant upregulation of *MIR141* and *MIR200a* (Cahill *et al.* 2006). In the present study, an investigation into the expression of *MIR141* in a series of archival thyroid malignancies (cPTC, fvPTC, FTC, HT, or control (no evidence of disease or goitre tissues)) was performed, with the objective of elucidating the differential gene expression of *MIR141* across subtypes of follicular thyroid neoplasia. Each cohort had a minimum of 20 validated samples surgically excised within the period 1980 – 2009.

The potential mRNA targets for miR141 and its family member miR200a were identified using

miRNA	target	prediction	software
miR141	CCL2	Yes	miRGen

 (<http://www.diana.pcbi.upenn.edu/miRGen/v3/miRGen.html>) (Megraw *et al.* 2007). Chemokine (C-C motif) ligand 2 (*CCL2*) was identified as one of the predicted proteins that intersected both *MIR141* and *MIR200a*. *CCL2* was chosen for further study due to its established role in recruiting leukocytes and its known association with malignant transformation and promotion of tumour establishment and spread in breast tumour cells (Soria and Ben-Baruch 2008). Thyroid samples were analysed for *CCL2* gene expression with an aim to correlate its expression across subtypes with *MIR141* expression.

Multiple members of the TGF β pathway, including TGF β R1, were recently validated as direct targets of *MIR141* (Senanayake *et al.*, 2012, Braun *et al.*, 2010). This pathway is involved in signalling for the cytokine IL-2, which has a well documented association with HT (Weijl *et al.*, 1993). As this pathway is exquisitely involved in T-cell autoimmunity and has previously been linked with HT, a subset of the cPTC and HT cohort were analysed for *TGF β R1* gene expression with the objective of generating pilot data to determine if further investigation into the association of *MIR141*, the TGF β signalling pathway and HT is warranted.

As previously outlined in chapter 1, a major goal of this thesis is the identification of miRNAs associated with cancer drug resistance. The current study was undertaken to highlight the potential importance of miRNAs in cancer and also to illustrate a direct relevance of their study to the Irish population. By undertaking this study, the relevance of downstream profiling of miRNAs (chapter 5) can be better understood in a clinical context.

3.3 Results

3.3.1 Laser Capture Microdissection

Of the 140 thyroid tissues in the cohort, laser capture micro-dissection of 24 tissues did not capture either sufficient quantity or sufficient quality of cells to extract detectable levels of RNA. Of the remaining 116 tissues, 111 (95.69%) expressed the endogenous control, *RNU6B*, which was selected as the internal normalisation control for miRNA quantitation. Of the 111 samples expressing the endogenous control, 104 samples also expressed *MIR141*. This cohort of samples expressing both *RNU6B* and *MIR141* consisted of 20 cPTC samples, 20 fvPTC samples, 19 FTC samples, 21 HT samples and 24 control samples.

3.3.2 Characterisation of mRNA from HT samples

HT samples used in this study consisted of thyrocytes isolated from adjacent areas of lymphocytic infiltrate. As a negative control, a mixed population including lymphocytes was captured for a representative sub-cohort of samples. To confirm the purity of the LCM harvested cell populations (thyrocyte and lymphocyte), HT tissues were assayed for thyrocyte- and lymphocyte- specific markers (*TSHR* and *LSP1* respectively). mRNA extracted from thyrocytes harvested from HT tissues was compared to mRNA extracted from interspersed/adjacent areas of lymphocytic infiltrate captured separately but from the same 7µm sections. As expected, *TSHR* was significantly upregulated in each isolated thyrocyte population compared to each parallel sample containing lymphocytic infiltrate. Conversely, *LSP1* was detected in all of the harvested lymphocyte samples but was only detected in a single thyrocyte sample, albeit displaying significant downregulation compared to its paired lymphocyte extract (3.3 figure 1).

3.3.3 Expression of *MIR141* in thyroid tissues

A comparison of relative gene expression across the thyroid cohorts identifies an association between *MIR141* expression and malignant thyroid phenotypes. Analysis of variance (one-way ANOVA) was performed on log transformed data between cohorts and identified statistical significance between populations ($p=0.01$, $F=5.40$). Residual analysis on log transformed data confirmed normally distributed populations for each cohort ($p<0.005$). Expression of *MIR141* was significantly downregulated ($p<0.05$) in HT samples compared to both control and cPTC samples (3.3 figure 2). Analysis of variance also demonstrates that the follicular variant of PTC

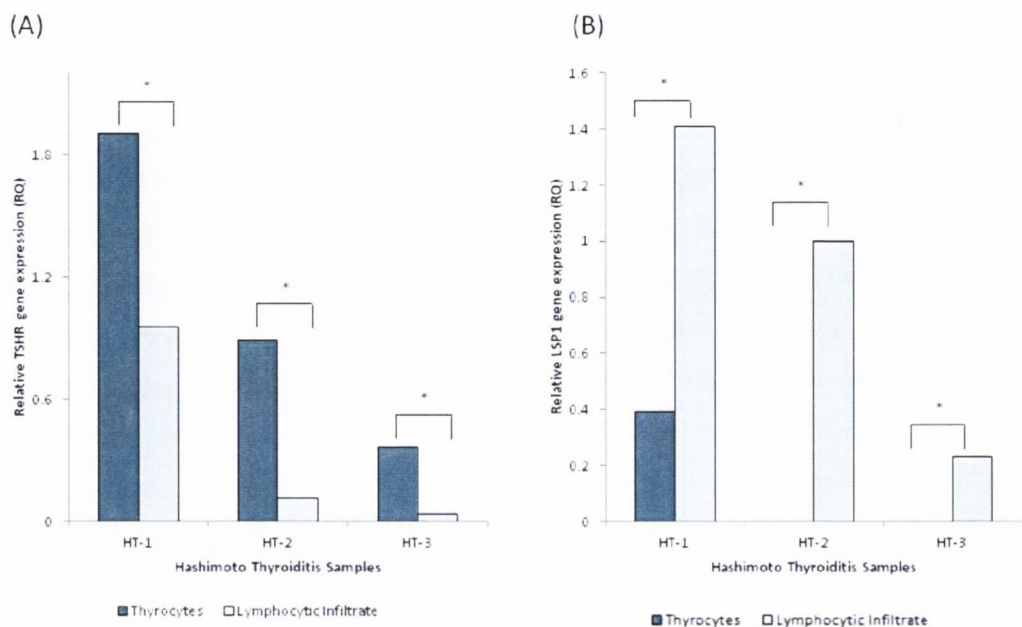
displays a statistically significant ($p<0.05$) pattern of downregulation of *MIR141* expression compared to both control and cPTC samples.

3.3.4 Expression of *CCL2* in thyroid tissues

A subset of 40 thyroid tissues were assayed for *CCL2* gene expression. One cPTC sample did not express the endogenous control (*CDKN1B*) and was excluded from further analysis. 3/9 (33%) of cPTCs, 5/9 (55.5%) of fvPTCs, 2/6 (33%) of FTC, 4/6 (66.6%) of HT and 5/9 (55.5%) of control thyrocytes had detectable levels of *CCL2* and *CDKN1B*. Normal probability plot of residuals for log transformed data confirmed normally distributed populations for each cohort. One-way ANOVA was performed on log transformed data between cohorts and identified statistical significance between populations ($p=0.044$, $F=3.26$). Tukey's Pairwise Comparison (95% simultaneous confidence intervals) identified statistically significant downregulation of *CCL2* gene expression in FTC compared to fvPTC cohorts (3.3 figure 3). However, as noted above, only 2 samples expressed *CCL2* in the FTC cohort. No statistically significant correlation was observed between *MIR141* and *CCL2* gene expression across cohorts.

3.3.5 *TGFBR1* is expressed in cPTC but not in HT thyrocytes

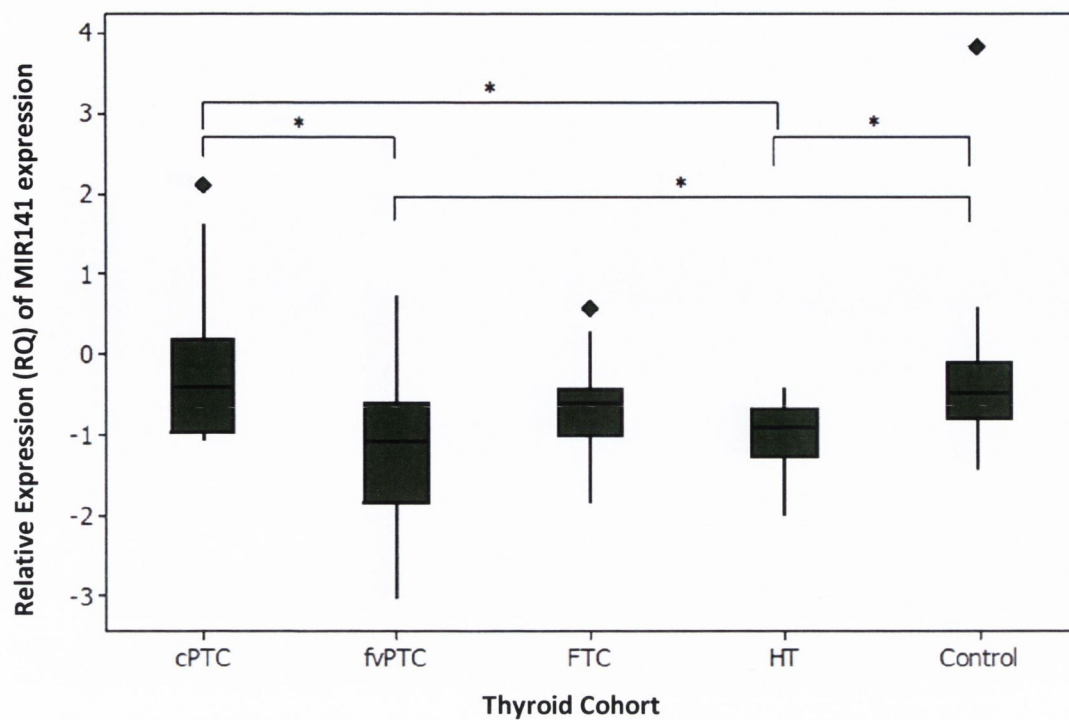
A subset of HT and cPTC samples ($n=3$ /cohort) were assayed for the expression of *TGFBR1*, a member of the TGF β pathway and known target of *MIR141* (Braun *et al.*, 2010). Expression of *TGFBR1* was detected in the cPTC samples but not in HT thyrocytes. Paired HT lymphocytes ($n=3$) were also assayed for *TGFBR1* and expression was detectable in these samples. As such, the cPTC subset ($n=3$) were assayed for *LSP1* gene expression to confirm that the *TGFBR1* expression detected was not due to lymphocyte contamination. *LSP1* gene expression was not detectable in cPTC samples confirming that *TGFBR1* expression was not due to lymphocyte contamination. Although the cohort number is low ($n=3$), this preliminary data supports future investigation of the role of *TGFBR1* in thyroid malignancies.



3.3 Figure 1: Assessment of purity within LCM harvested HT populations.

LCM was used to isolate a thyrocyte specific population from HT sections (n=3). Samples were assayed for thyrocyte- and lymphocyte- specific markers (*THSR* and *LSP1* respectively). As expected, *THSR* was significantly upregulated in each isolated thyrocyte population compared to each parallel sample containing lymphocytic infiltrate (A). Conversely, *LSP1* was detected in all of the harvested lymphocyte samples but was only detected in a single thyrocyte sample, albeit displaying significant downregulation compared to its paired lymphocyte extract (B).

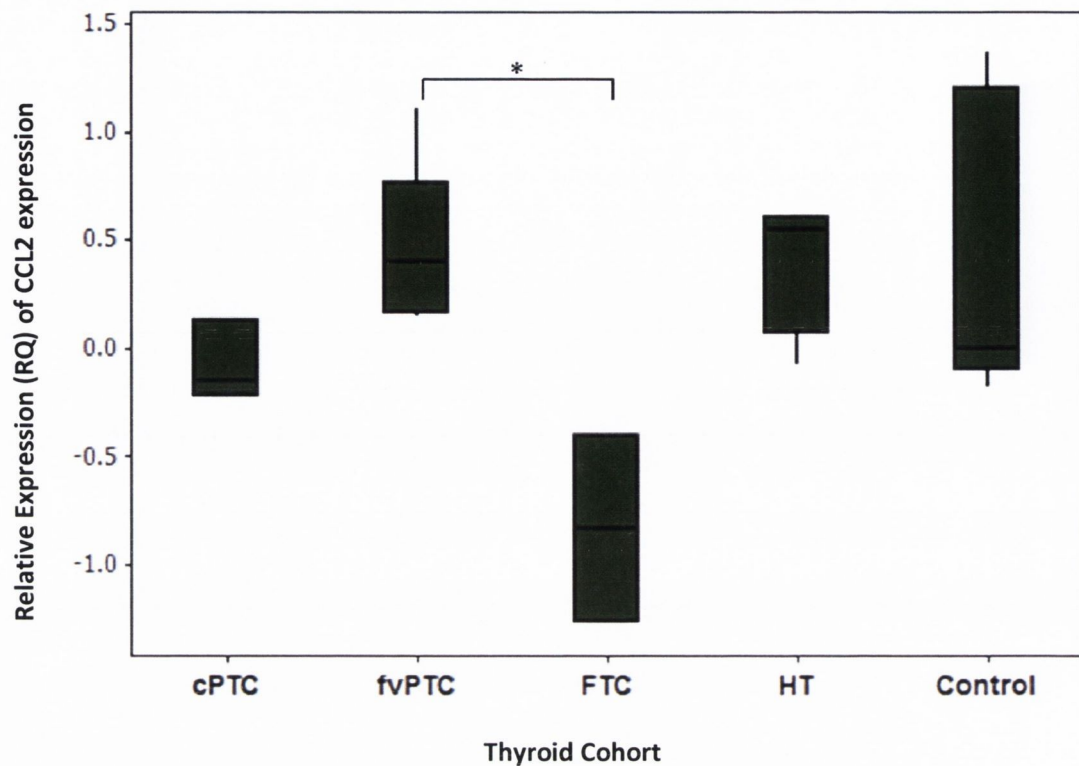
* $p < 0.05$ between gene expression of isolated thyrocytes –v- lymphocyte containing samples



3.3 Figure 2: Boxplot of relative quantitation (RQ) of change in expression of *MIR141*.

The expression of *MIR141* is statistically significantly down-regulated in HT tissues compared to both control and cPTC tissues. The fvPTC tissues also display a statistically significant down-regulation of *MIR141* in comparison to both control and cPTC tissues.

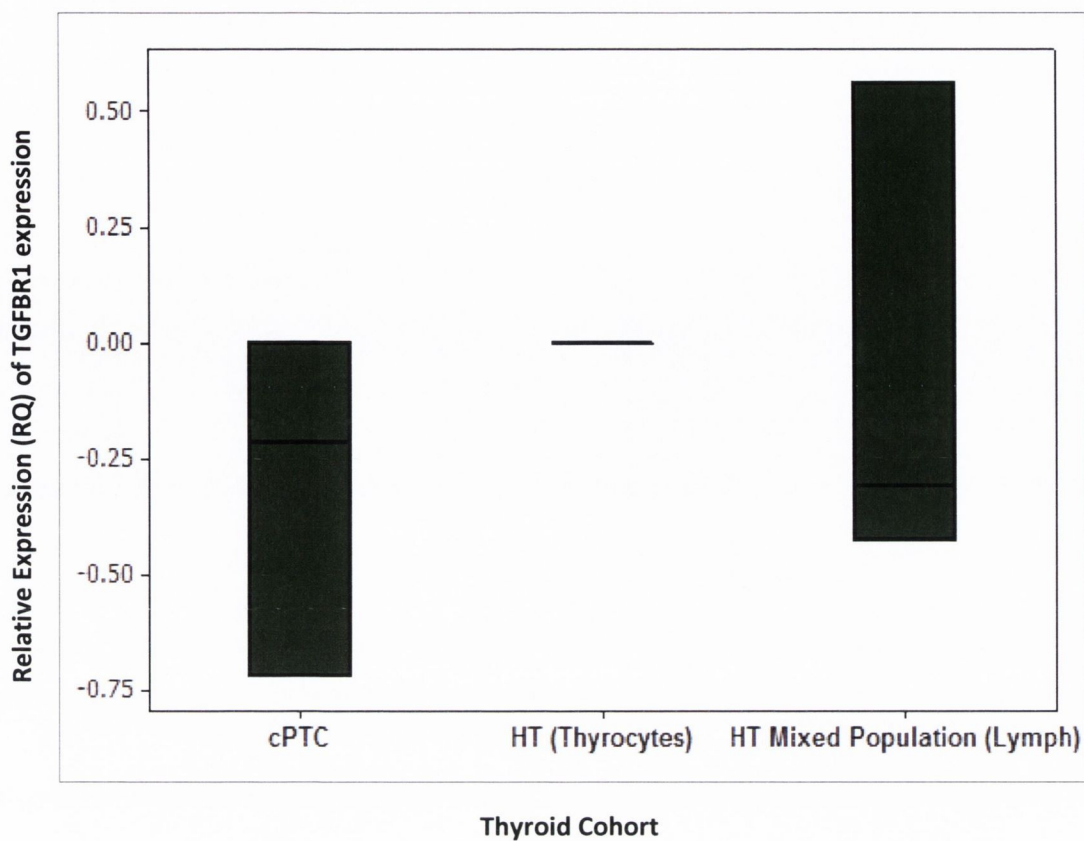
* indicates statistical significance ($p < 0.05$) between groups as identified by ANOVA using Tukey's Pairwise Comparison, ♦ indicates outliers.



3.3 Figure 3: Boxplot of relative quantitation (RQ) of change in expression of *CCL2*.

The expression of *CCL2* is statistically significantly down-regulated in FTC tissues compared to fvPTC.

* indicates statistical significance ($p < 0.05$) between groups as identified by ANOVA using Tukey's Pairwise Comparison.



3.3 Figure 4: Boxplot of relative quantitation (RQ) of change in expression of *TGFBR1* across thyroid cohorts.

TGFBR1 was detected in cPTC samples but not in HT samples. Negative control mixed populations containing lymphocytes were isolated from adjacent regions of the same HT sections and also expressed *TGFBR1*.

n=3 samples per cohort

3.4 Discussion

Inflammation and thyroid cancer are strongly linked. PTCs have a penchant to attract a florid of lymphocytic infiltrate. However, they can persist and metastasize in the presence of this infiltrate. Normal inflammation is self-limiting because the production of anti-inflammatory and pro-inflammatory cytokines is tightly regulated. During normal inflammation, blocking apoptosis provides time for DNA repair and the production of reactive oxygen species (ROS) fights against viral threat. However, the failure of mechanisms required for resolving the inflammatory response or the persistence of the initiating factors can result in chronic inflammation. ROS are mutagens. The blocking of apoptosis in cells that are gaining oncogenic mutations can lead to tumour progression. Thus chronic inflammation can act as both an initiator (DNA damage and growth signals) and as a promoter (blocking apoptosis and stimulating cell proliferation), which over time can induce oncogenesis.

FFPE tissue samples were used in this study. While FFPE preserves tissue for morphological analysis, the effect of formalin fixation on nucleic acids can hamper molecular analysis as nucleic acids become cross-linked during fixation. There are no standardised guidelines for processing FFPE tissues (Blow 2007), and as such there can be great variation in the time between ligation of circulation and fixation. Nucleic acids suffer damage through fixation. The greater the time prior to fixation, the greater the exposure to nucleases and modifying enzymes, which are up-regulated during ischaemia. Nucleic acids are often irreversibly damaged and can become increasingly fragmented during prolonged storage (Bergemeister, 2011). Mature miRNAs, however, are approximately 22 nucleotides in length. Due to this small size they tend not to suffer from the fragmentation effect. Thus, FFPE tissues tend to yield miRNA more effectively than mRNA. It has previously been demonstrated that FFPE thyroid tissues are a suitable resource for miRNA expression analysis (Tetzlaff *et al.*, 2007, Chen *et al.*, 2008, Sheu *et al.*, 2010). The method used for miRNA extraction in this study has previously been demonstrated to yield miRNA with expression levels that positively correlate with expression levels from snap frozen tissue (Li *et al.*, 2007).

3.4.1 MIR141 Expression Differentiates HT from cPTC and Benign Thyrocytes

MiRNAs have emerged as key regulators of the immune system, displaying unique expression profiles in cells of the adaptive and innate immune system (O'Connell *et al.*, 2010). Aberrant expression of miRNAs has been documented in a range of diseases associated with the immune system, including cancer and autoimmunity (Calin *et al.*, 2002; Xiao *et al.*, 2008). MiRNA signatures that can distinguish thyroid malignancies in fine needle aspiration, FFPE and fresh surgical samples have been identified (Shen *et al.*, 2012, Vriens *et al.*, 2012, Sheu *et al.*, 2010, Nikiforova *et al.*, 2008, Chen *et al.*, 2008, Tetzlaff *et al.*, 2007, Pallante *et al.*, 2006, He *et al.*, 2005). It has been demonstrated that *MIR141* is up-regulated in a cell line model of ret/PTC-1 associated papillary thyroid cancer (PTC) (Cahill *et al.* 2006). In the current study, we investigated if the pattern of *MIR141* expression would translate to clinical specimens. In fact, the pattern of upregulated *MIR141* was not observed in the study sample. This may be due to (a) variation between *in-vitro* and *in-vivo* expression or (b) the low rate of ret/PTC variants in the samples analysed (Table 1).

In this study a significantly decreased expression of *MIR141* was identified in thyrocytes isolated from HT tissues compared to both control and cPTC samples. This is of particular interest as it has been postulated that HT represents a circumstance in which thyrocytes are in a state between normal and neoplastic (Sheils *et al.*, 2000b). Histologically, HT can present with the occurrence of nuclear clearing and overlapping cells associated with PTC, but without evidence of malignancy (Rosai, 2004). On a molecular level, previous studies (Sheils *et al.*, 2000a, Wirtschafter *et al.*, 1997, Rhoden *et al.*, 2006) detected that up to 95% of HT samples displayed evidence of ret/PTC-1 activation, including HT samples that had no evidence of malignancy. The ret/PTC chromosomal rearrangement was previously considered to be a hallmark of PTC. However, evidence of their presence in HT samples indicates a more complex role of this genetic alteration. Furthermore; the occurrence of non-specific lymphocytic infiltrate in PTC tissues has been positively associated with the presence of RET/PTC-1 expression (Sheils *et al.*, 2000b). This has lead to the postulation that antigenic alterations induced by genetic alterations such as the activation of CRET are responsible for eliciting an immune response within the thyroid tissue. In HT, it is feasible that the presence of genetic alterations such as c-ret elicit an immune response to curtail the development of PTC; a subset of patients may be successful in this regard but with background damage of autoimmune destruction of thyroid tissue. However, the relationship between HT and PTC remains a highly controversial issue.

3.4.2 *CCL2* Expression Differentiates FTC from fvPTC

The expression of *CCL2* was detected using TaqMan® RT real-time PCR. All five tissue types had detectable levels of *CCL2*. No significant correlation was seen between *MIR141* expression and *CCL2* expression. Thus, this study does not support the prediction of the miRNA-target software. Targets of mature miRNA are extremely difficult to predict accurately due to their small size (~22nt) and incomplete-complementarity binding of targets. Computational predictions are usually based on assumptions regarding secondary structure, folding energetics and evolutionary sequence conservation (Doran and Strauss 2007). These assumptions are difficult to apply to a molecule of only 22nt. Programmes for miRNA targets are still relatively in their infancy. It is proposed that, similar to previous protein prediction and gene prediction algorithms, the prediction software will improve as more accurate annotation of the miRNA biology is elucidated (Sethupathy *et al.* 2006). The roles of miRNA-3'UTR-interacting proteins and alternative polyadenylation need to be better understood to improve the parameters of target prediction. The 3'UTR of coding sequences is also poorly characterised relative to the 5'UTR. Better and more precise annotation of these sequences should significantly improve target prediction software (Sethupathy *et al.* 2006).

The *CCL2* gene expression assay detected the significant down-regulation of *CCL2* expression in FTC cells compared to fvPTC cells. This is a particularly interesting observation as fvPTC and FTC cells are extremely difficult to distinguish microscopically. The follicular variant of PTC composes entirely or almost entirely of follicles (3.4 Figure 1 (B)). The diagnosis of fvPTC is primarily based on nuclear alterations associated with cPTC. Its invasiveness and incidence of nodal metastases also aid diagnoses. Follicular carcinoma of the thyroid is a rare, usually solitary neoplasm (3.4 Figure 1 (C)). The problem in diagnosing FTC arises because its microscopic appearance is extremely variable, ranging from well-formed follicles to solid growth patterns (Rosai 2004). The majority of tumours have poorly differentiated features for immunohistochemical analysis. Hence, accurate diagnosis of FTCs is a major clinical problem. In this study a possible biomarker for FTC has been identified. The significant down-regulation of *CCL2* in FTC in comparison to fvPTC may have potential as a clinical biomarker. However, the cohort in the *CCL2* gene expression study is quite small (FTC n = 6, fvPTC n = 9). Therefore, the sample population will need to be expanded to observe if the trend remains significant. If this trend holds true, it may have significant implications in the clinical diagnosis of FTC.

3.4.3 MIR141 is Associated with the TGF β Signalling Pathway

MIR141 has been confirmed to directly target a number of genes. Multiple members of the TGF β pathway have been identified to be direct targets of MIR141 (Senanayake *et al.*, 2012, Braun *et al.*, 2010). Braun *et al.* (2010) demonstrated direct targeting of transforming growth factor, beta receptor 1 (TGFB1) and SMAD family member 2 (SMAD 2). Both TGFB1 and SMAD2 are negatively regulated by MIR141 and are key members of the TGF β signalling pathway. The TGF β pathway is involved in cytokine signaling and can act to signal for interleukin-2 (IL-2), a cytokine involved in T cell proliferation. T cells play a crucial role in the development of HT as they secrete inflammatory cytokines in reaction to auto-antigens; primarily thyroid stimulating hormone receptor (TSHR) and thyroid peroxidase (TPO). IL-2 is essential for T-cell determination of self from non-self. IL-2R β /IL-2 knock-out mice developed a marked increase in T-cell mediated autoimmunity and deregulated T-cell proliferation (Sadlack *et al.*, 1995). IL-2 is used as treatment for certain cancers and has been associated with a demonstrated increased risk of HT (Weijl *et al.*, 1993). Thus, the association of MIR141 with the regulation of this pathway may be reflected in its down-regulation in HT epithelium. The relative expression of *TGFB1* was assayed in a subset of samples (n=3) in this study. *TGFB1* was found to be expressed in cPTC samples but not in isolated HT thyrocytes. *MIR141* expression is upregulated in these cPTC samples compared to the isolated HT thyrocyte samples. This initial data supports the argument for further investigation into the association of MIR141, the TGF β signalling pathway and HT.

3.4.4 LCM Reduces Intratumour Confounding Factors

Thyroid carcinomas frequently present with multiple phenotypic tumour-foci within a single thyroid gland. Direct experimental analysis of these tissues will therefore reflect the predominant cell type within the tissue which may not be the cell of interest nor be biologically relevant to the neoplasm under investigation. MiRNA profiles have been found to distinguish tumour types within a single thyroid biopsy (Aherne *et al.*, 2008). This highlights the requirement to extract a pure population of the specific tumour cells in order to identify and accurately define the molecular processes in pathogenic lesions. LCM provides a nondamaging microdissection process which allows isolation of pure populations while preserving the integrity of the captured material (Lui, 2010). In this study, LCM was utilised to specifically select the cell-types of interest and therefore reduce the confounding factors associated with whole section

analysis. This enforces the accuracy of the associations identified between MIR141 and malignant thyroid phenotypes.

3.5 Conclusions

HT epithelium can be distinguished from neoplastic follicular thyroid epithelium isolated from cPTC and control epithelium based on the relative expression of *MIR141*. It is postulated that the downregulation of this miRNA may be due, at least in part, to its involvement in regulating the TGF β signalling pathway. The MIR200 family, including *MIR141*, have been demonstrated to directly regulate members of this pathway as part of a regulatory feedback loop. The preliminary data presented here in combination with data from the literature (Senanayake *et al.*, 2012, Braun *et al.*, 2010) leads this postulation to warrant further investigation. The TGF β pathway is exquisitely involved in T-cell autoimmunity and has been linked with HT for some time, as treatment of cancer patients with IL-2, a downstream target of the pathway, resulted in increased incidence of HT (Weijl *et al.*, 1993). Further investigation of *MIR141* and its regulatory networks may aid in elucidating the molecular mechanisms that result in the fundamental physiological differences between these malignant thyroid phenotypes.

3.4 Table 1: Mutational Status of *RET/PTC-1* and *RET/PTC-3* for PTC samples expressing *MIR141*

PTC Variant	Year of Biopsy	RET/PTC Mutation	PTC Variant	Year of Biopsy	RET/PTC Mutation
Classic	1980	Unknown	Follicular variant	1986	Negative
Classic	1985	Unknown	Follicular variant	1987	Unknown
Classic	1987	Unknown	Follicular variant	1989	Negative
Classic	1994	RET/PTC-1	Follicular variant	1991	Negative
Classic	1998	Negative	Follicular variant	1991	Negative
Classic	1998	RET/PTC-1	Follicular variant	1992	RET/PTC-1
Classic	2002	Negative	Follicular variant	1992	Unknown
Classic	2002	Negative	Follicular variant	1992	Unknown
Classic	2002	Negative	Follicular variant	1992	Negative
Classic	2003	Negative	Follicular variant	1993	RET/PTC-3
Classic	2004	Negative	Follicular variant	1996	Unknown
Classic	2004	Negative	Follicular variant	2001	Negative
Classic	2004	Negative	Follicular variant	2003	Negative
Classic	2004	Negative	Follicular variant	2003	Negative
Classic	2004	Negative	Follicular variant	2004	Negative
Classic	2004	Negative	Follicular variant	2004	Negative
Classic	2005	Negative	Follicular variant	2004	Negative
Classic	2005	Negative	Follicular variant	2004	Negative
Classic	2005	Negative	Follicular variant	2005	Negative
Classic	2005	Negative	Follicular variant	2005	Negative

Chapter 4: Optimisation of Small Molecule MEK Inhibition Assays

4.1 Introduction

There are an estimated 518 kinases encoded in the human genome. Phosphotransfer cascades mediated by kinases are involved in virtually every signal transduction process. This suggests that inhibition of kinase cascades should elicit a real physiological response. As such, small molecule kinase inhibitors (SMKI) are intensely pursued for the treatment of cancer and many other diseases (Janne *et al.*, 2009). There are three main classes of kinase targets: class I targets are impervious to normal regulatory mechanisms due to mutation or translocation (e.g. BRAF^{V600E}); class II targets are non-oncogenic targets located in key signalling pathway and are preferentially required for the proliferation or survival of the tumour cell (e.g. MEK1/2); class III targets are required for tumour maintenance or formation and are located in related pathways or surrounding tissues (e.g. angiogenesis-related kinases).

BRAF mutated tumors have been correlated with poor response to traditional chemotherapy and poor prognosis in malignant melanoma and thyroid carcinoma (Houben *et al.*, 2004, Tang *et al.*, 2010). Targeted therapies are of great interest for these cancer types as the approach of targeting oncogenic kinases has been successful in the treatment of cancers with activating mutations in the kinase gene that drives their progression (Sawyers, 2004). BRAF^{V600E} mutated cancers display an oncogenic addiction to the MAPK pathway.

Molecular targeting of BRAF^{V600E}, as exemplified by the BRAF^{V600E} SMKI PLX-4032, has displayed some success for the treatment of malignant melanoma. The landmark BRAF in melanoma phase 3 study (BRIM-3) randomised 675 untreated malignant melanoma patients to either PLX-4032 (Vemurafenib, Plexxicon/Roche) or the standard of care, dacarbazine. The study was terminated early due to an obvious overall survival benefit with PLX-4032 treatment (Chapman *et al.*, 2011). The overall response rate for PLX-4032 was 48% compared with a 5% response rate for dacarbazine. Although this was an excellent response for malignant melanoma, the median progression free survival was approximately 7 months (Hersey, 2011) because drug resistance invariably occurs and patients relapse (4.1 figure 1).

PLX-4032 is an elegant example of both the promise of SMKI and the challenge of resistance to targeted therapeutics. Multiple mechanisms of resistance to SMKI-BRAF have been identified (4.1 figure 2) including: acquisition of secondary mutations such as NRAS^{Q61K} (Nazarian *et al.*, 2010); upregulation or activation of alternative RAF isoforms (Montagut *et al.*, 2008); splice variants of BRAF such as BRAFp61, an inhibitor refractory BRAF truncation (Poulikakos *et al.*, 2011); and increased COT expression that drives resistance via reactivation of the pathway (Johannessen *et al.*, 2010). COT reactivates the pathway primarily through MEK- dependent mechanisms that do not require RAF signalling. Additionally, COT expression



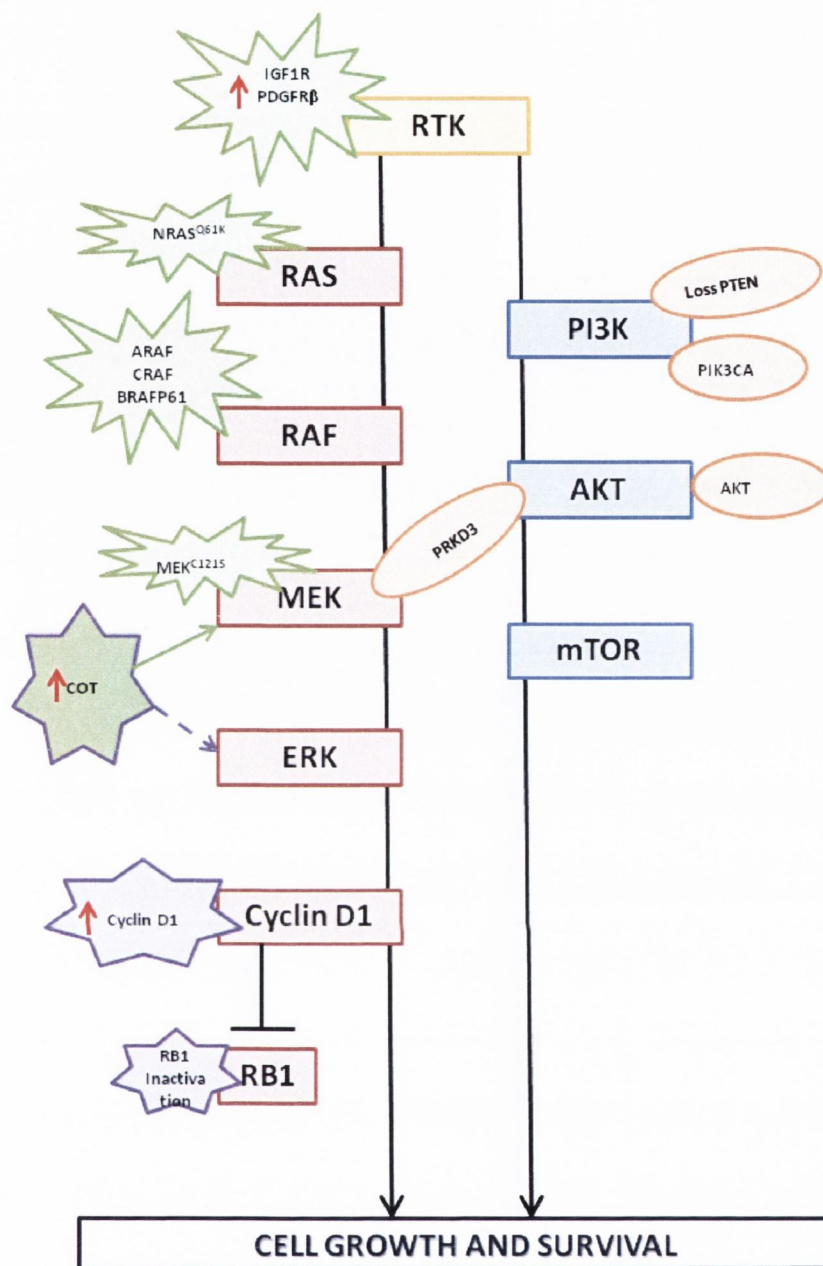
4.1 Figure 1: Clinical inhibitors molecularly targeting BRAF^{V600E} display excellent initial response but are frequently subject to acquired resistance and patient relapse in malignant melanoma.

A 38-year-old man with *BRAF* mutant melanoma and miliary, subcutaneous metastatic deposits. Photographs were taken (A) before initiation of PLX4032, (B) after 15 weeks of therapy with PLX4032, and (C) after relapse, after 23 weeks of therapy.

Images adapted from *Wagle et al, 2011*.

was associated with de novo resistance in BRAF^{V600E} cultured cell lines and has been implicated in acquired resistance in clinical melanoma cells and tissue obtained from relapsing patients following treatment with BRAF inhibitors (Johannessen *et al.*, 2010).

As outlined in 4.1 figure 2, targeting BRAF can lead to pathway reactivation under certain genetic conditions. Thus, it may be of greater benefit to target downstream in the BRAF pathway. Due to the strategic position of the MEK 1/2 kinases in the MAPK pathway it is a very promising target for drug development. Preclinical trials have demonstrated that small molecule MEK 1/2 kinase inhibitors can significantly reduce the proliferation of a variety of cancer cell lines via the induction of apoptosis and cell cycle arrest (Solit *et al.*, 2006). Thus MEK appears to be a logical target for targeted therapy and is being investigated as a potential therapy for BRAF^{V600E} cancers, both as a single agent and in combination with BRAF-inhibition (Smalley and McArthur, 2012; Flaherty *et al.*, 2011; Infante *et al.*, 2011; Villanueva *et al.*, 2010; Gopal *et al.*, 2010; Haass *et al.*, 2008).



4.1 Figure 2: Known mechanisms of BRAF inhibitor resistance

Long-term BRAF inhibition can lead to activation of the MAPK pathway through acquired (green) acquisition of: secondary NRAS^{Q61K} mutations; RAF isoform (ARAF, BRAF, CRAF) signal switching; increased IGF1R and PDGFR signalling; truncation of BRAF (BRAFP61), MEK1^{C121S} mutation; as well as increased COT expression, which has also been linked to intrinsic (purple) resistance. Intrinsic mechanisms of resistance have been associated with amplification of cyclin D1 or RB1 inactivation allowing cells to continue through the cell cycle. Loss of PTEN and genetic alterations in PIK3CA and AKT3 are associated with de novo resistance (orange) by increasing PI3K/AKT activity while PRKD3 activation can increase both MAPK and PI3K/AKT signalling.

Image adapted from Smalley et al, 2012

4.2 Project Rationale

The overall aim of this thesis is to investigate mechanisms of resistance to MEK inhibitors in cells initially sensitive to treatment, and to identify if these mechanisms are associated with BRAF mutational status (Chapter 5). Cancers display a state of genetic instability arising from multiple causes. Oncogene activation can directly result in genetic instability due to induction of replicative stress and increased production of reactive oxygen species (Bartkova *et al.*, 2005 & 2006). Different types of genetic instability vary in their effects on cancer genomes and ultimately on their response to treatment. MEK inhibitors are molecularly targeted therapies and as such their effectiveness can be highly context dependent. As such, optimization of treatment is required for each cell line as the background genotype and phenotype can impact response and cancer drug resistance (Turner and Reis-Filho, 2012; Ashworth *et al.*, 2011). The fidelity of BRAF^{V600E} status is an important aspect of this thesis and thus single nucleotide polymorphism (SNP) testing of cell lines for the BRAF^{T1799A} SNP that gives rise to the constitutively active BRAF^{V600E} phenotype is requisite.

4.3 Results

4.3.1 Silica membrane spin column-based gDNA extraction kits provide the most robust method for gDNA extraction from thyroid cell lines

gDNA was extracted from 3 thyroid cell lines grown to confluence, harvested via trypsinisation and aliquoted based on cell counts. The gDNA extraction kits had varied elution volumes, therefore results were calculated as total gDNA yield. However, some kits were tested on cells from a different passage. As cells from both passages were both processed using the NucleoSpin® Tissue Kit (MN) the total yields were transformed to their relative yield as a percentage of the MN total yield for each respective passage (4.3 Figure 1). The MN and DNeasy® Blood and Tissue Kit (QDN) kit are highly comparable in their total yields and process the highest yields of gDNA consistently across cell lines. The AllPrep® (QAP) and MagMax™ DNA Multi-Sample Kit (AB) kits tended to produce lower yields with increased variability across cell lines. The MN kit was used for future gDNA extraction.

4.3.2 Fluorometry is a more consistent method of gDNA quantitation than spectrometry

There were discrepancies between fluorometric (Quant-iT® dsDNA BR assay) and spectrometric (NanoDrop™ 2000) quantitation methods (4.3 figure 2). Total gDNA yields measured via NanoDrop™ were statistically significantly greater than those measured on Qubit™ (Student's paired t-test $p < 0.001$, 4.3 figure 2 (B)). The yields measured using the NanoDrop™ 2000 widely varied compared to the Qubit™ determined yields. The greatest fold differences were within the AB extracts with the NanoDrop™ platform reporting 1 - 9 fold increased relative yield (dependent on cell line) compared to Qubit™ yields. The a260/a280 ratios, as determined using the NanoDrop 2000™ platform, demonstrated that the MN and QDN kits processed the purest gDNA (4.3 Table 1). The Qubit™ platform was used for future nucleic acid quantitation.

4.3.3 Custom BRAF^{T1799A} SNP assay confirms BRAF mutational status in cell lines

A custom BRAF^{T1799A} SNP TaqMan® assay (Smyth *et al.*, 2005) identified mutational status and allelic discrimination of BRAF mutants in cell lines. Using a 95% confidence interval, all cell lines bar one were confirmed as carrying BRAF status expected from the catalogue of somatic mutations in cancer (COSMIC) database (Forbes *et al.*, 2011; 4.3 figure 3 and Table 2) and as previously reported in the literature (Smyth *et al.*, 2005; Xing *et al.*, 2011). However, malignant

melanoma cell line SK-Mel-31 was previously reported as carrying wild-type BRAF (Xing *et al.*, 2011 supplementary information Table 1

(<http://www.nature.com.elib.tcd.ie/onc/journal/v31/n4/extref/onc2011250x2.doc>)).

In this study SK-Mel-31 was found to be heterozygous for BRAF^{V600E}. As the corresponding author of that publication was based in the institute that SK-Mel-31 originated, a vial of early passage cells was kindly gifted. These cells, termed SK-Mel-31-MSK were grown, harvested and gDNA extracted as per the ATCC supplied SK-Mel-31. SK-Mel-31-MSK was also found to carry heterozygous BRAF^{V600E} (4.3 figure 3 (B)).

4.3.4 Optimisation of seeding density for MEK inhibition assays

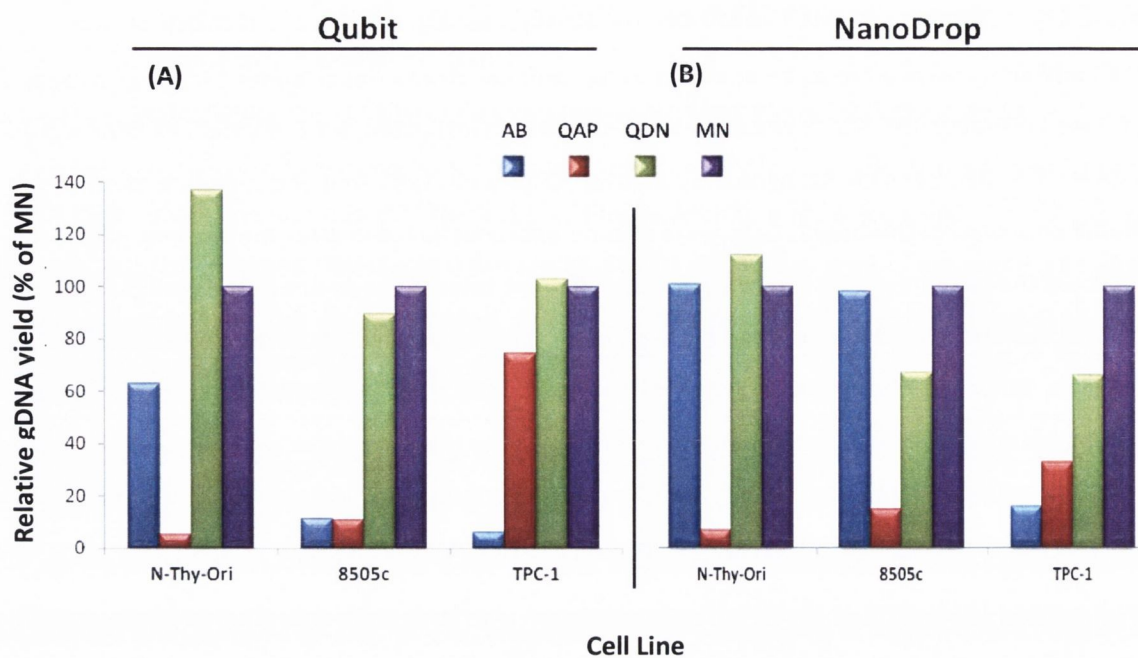
MTT growth proliferation assays were used to determine the appropriate seeding density for each cell lines. An 8-step dilution of cells ranging from 3×10^6 /ml to 1×10^3 /ml was seeded for 24 hrs. Each assay had quadruplicate technical replicates and was performed on 3 biological replicates (independent cell passages). The appropriate seeding density to enable both cytostatic and cytotoxic effects be accounted for downstream was determined to be 1×10^5 cells/ml for each thyroid cell line and 5×10^4 cells/ml for each melanoma cell line (4.3 figure 4). These seeding densities were used for all subsequent experiments.

4.3.5 Optimisation of MEK inhibitor dosage and treatment length

A 12-step 1/5 serial dilution in media of PD0325901 dosage from 2.5mM to 51.2pM was performed to assay for the optimal IC₅₀ PD0325901 dosage per cell line. As PD0325901 stock concentration is solubilised in DMSO, the equivalent serial dilutions in media of DMSO alone were also assayed to control for drug-vehicle effects. PD0325901 treatment was found to have cytotoxic effects independent from the vehicle control for all cell lines (paired Student's T-Test, statistical significance $p < 0.05$; 4.3 table 3) as exemplified by the effects on SK-Mel-2 (4.3 figure 5). Serial dilutions were exposed to cells for 24, 48, 72 or 96 hours (example SK-Mel-2 4.3 figure 6 (A)). All assays included media-only positive controls which were used to normalize data ($\frac{\text{Avg OD Test}}{\text{Avg OD Positive Control}}$). All assays used quadruplicate technical replicates and were performed on three biological (different cell passage) replicates. The optimal length of dosage was determined using Students paired T-Test between treatment times. Regression analysis was performed using Minitab® 16 statistical software and employed a 95% prediction interval to determine the IC₅₀ for each cell line (example SK-Mel-2, 4.3 figure 6 (B)). Optimal conditions for each cell line are summarized in 4.3 table 3.

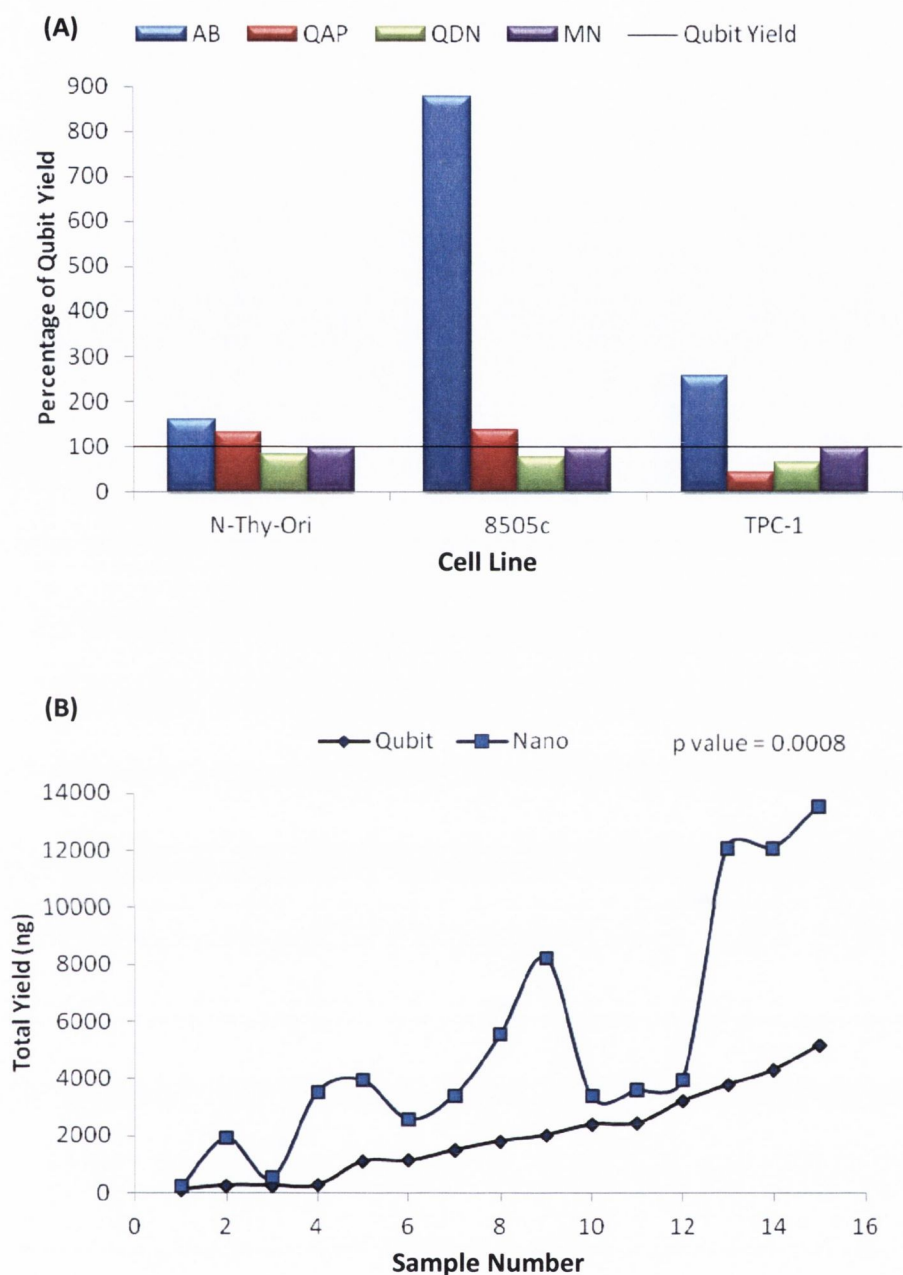
4.3.6 Optimisation of MEK inhibitor treatment assay in 8-well chamber slides

MEK inhibitor assays were to be analysed using confocal microscopy (chapter 5). The suitability of 8-well chamber slides as a vessel for the assay was investigated. Both thyroid cell lines were assayed for chamber slide fittingness by seeding 200µl/well (1×10^5 /ml) and exposure to a 72 hrs media-only control treatment. Cells were photographed before and after the washing steps. At 72 hours the cells grew with a dense nucleated pattern toward the centre of the well (4.3 figure 7). This nucleus of cells was washed off in some of the wells, especially in the 8505c (BRAF^{V600E}) cell line. However, the confluent layer of cell surrounding the nucleated centred remained, leaving plenty of cells available for imaging. Therefore, the chamber slides were deemed suitable vessels to carry out the MEK inhibition experiment of confocal microscopy. Images were taken without staining using Brightfield microscopy under a 10X objective lens.



4.3 Figure 1: Comparison of relative gDNA yields extracted from four commercially available kits.

Manufacturer's supplied protocols recommend different elution volumes between kits. Thus, total yield was calculated per kit. Total yields were then normalised to the MN total yield to reduce confounding factors. (A) fluorometric quantitation (B) spectrophotometric quantitation.



4.3 Figure 2: Evaluation of gDNA quantitation methods.

(A) Fold change difference between relative gDNA yields as assayed using NanoDrop™ 2.0 (coloured bars) compared to Qubit™ 1.0 (black line).

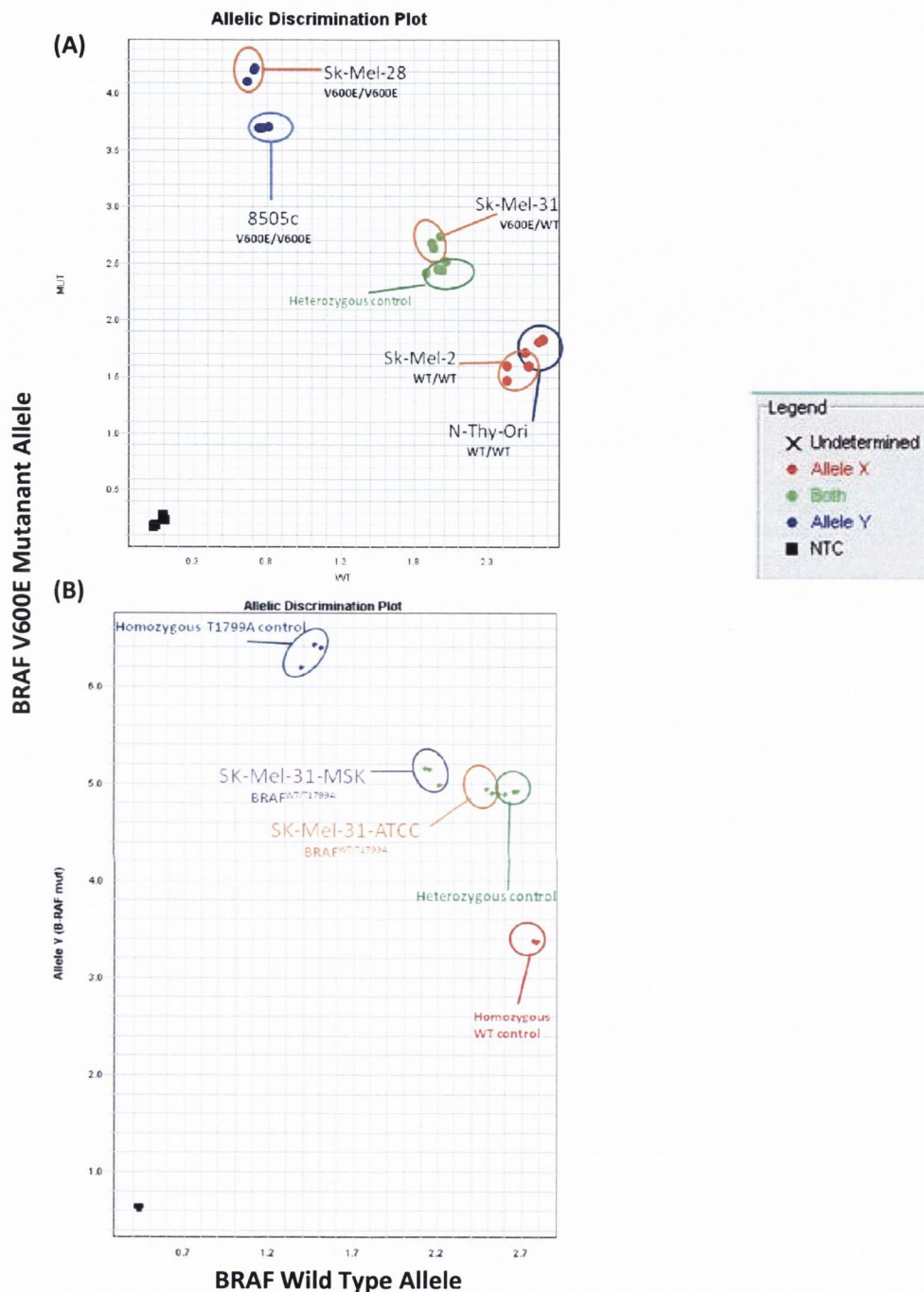
(B) Range of total gDNA yields (ng) by quantitation method. Total gDNA yields measured on NanoDrop™ are statistically significantly different compared to that measured on Qubit™. P-value determined by means of a Student's T-test.

4.3 Table 1: a260/a280 ratios of gDNA extracted from thyroid cell lines

	AB	QAP	QDN	MN
N-Thy-Ori	2.90	2.85	1.95	1.86
8505c	3.05	1.8	1.95	1.89
TPC-1	4.25	1.88	1.77	1.94

4.3 Table 2: BRAF^{T1799A} SNP Status

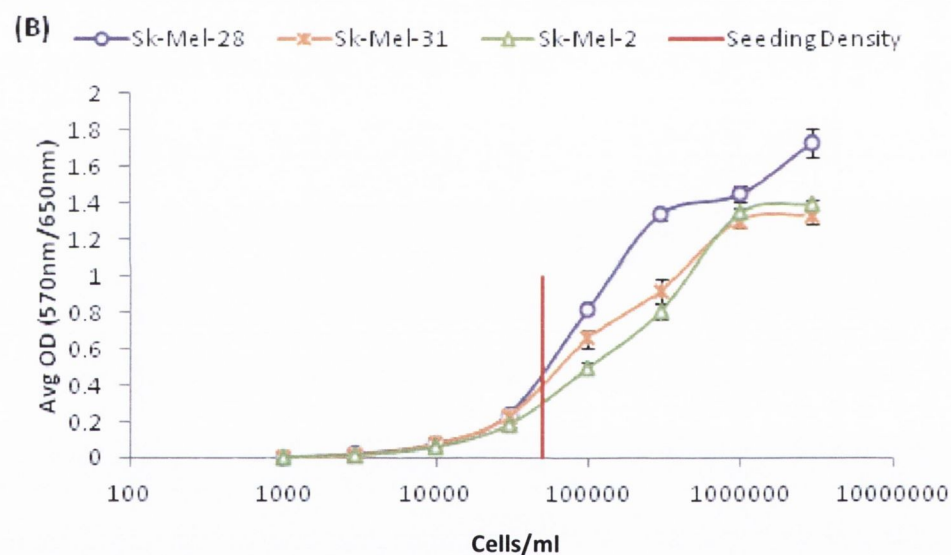
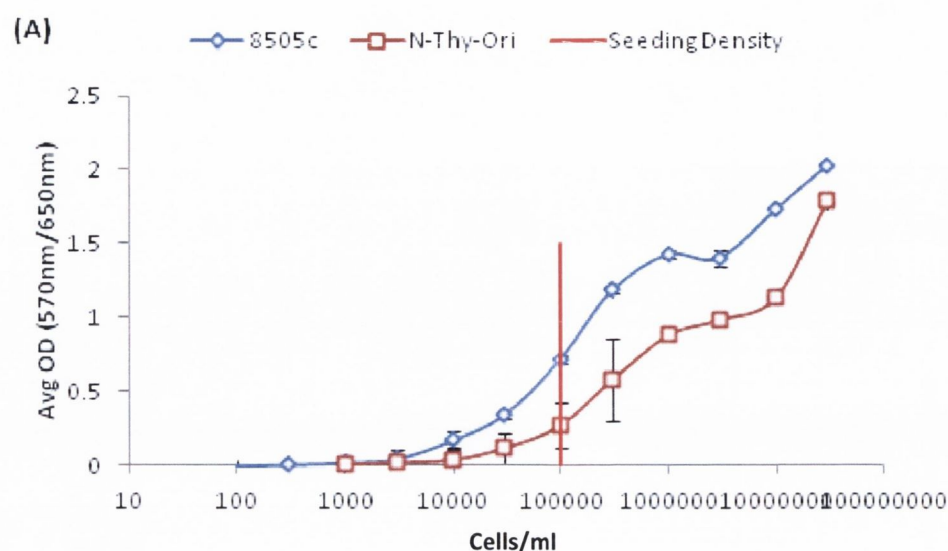
Cell Line	Origin	BRAF Status
8505C	Anaplastic Thyroid Carcinoma	T1799A/T1799A
NThy-Ori 3-1	Transformed Thyrocyte	WT/WT
SK-Mel-28	Cutaneous Malignant Melanoma	T1799A/T1799A
SK-Mel-31	Cutaneous Malignant Melanoma	T1799A/WT
SK-Mel-2	Acral Malignant Melanoma	WT/WT



4.3 Figure 3: BRAF^{T1799A} status of cell lines.

(A) Allelic discrimination plots for BRAF^{T1799A} SNP. All samples were auto-called for BRAF status (95% Confidence Interval)

(B) Allelic discrimination plots for BRAF^{T1799A} SNP for SK-Mel-31 cell lines commercially obtained from ATCC or gifted from the originators (MSK). Contrary to previous reports in the literature, SK-Mel-31 is heterozygous for T1799A.



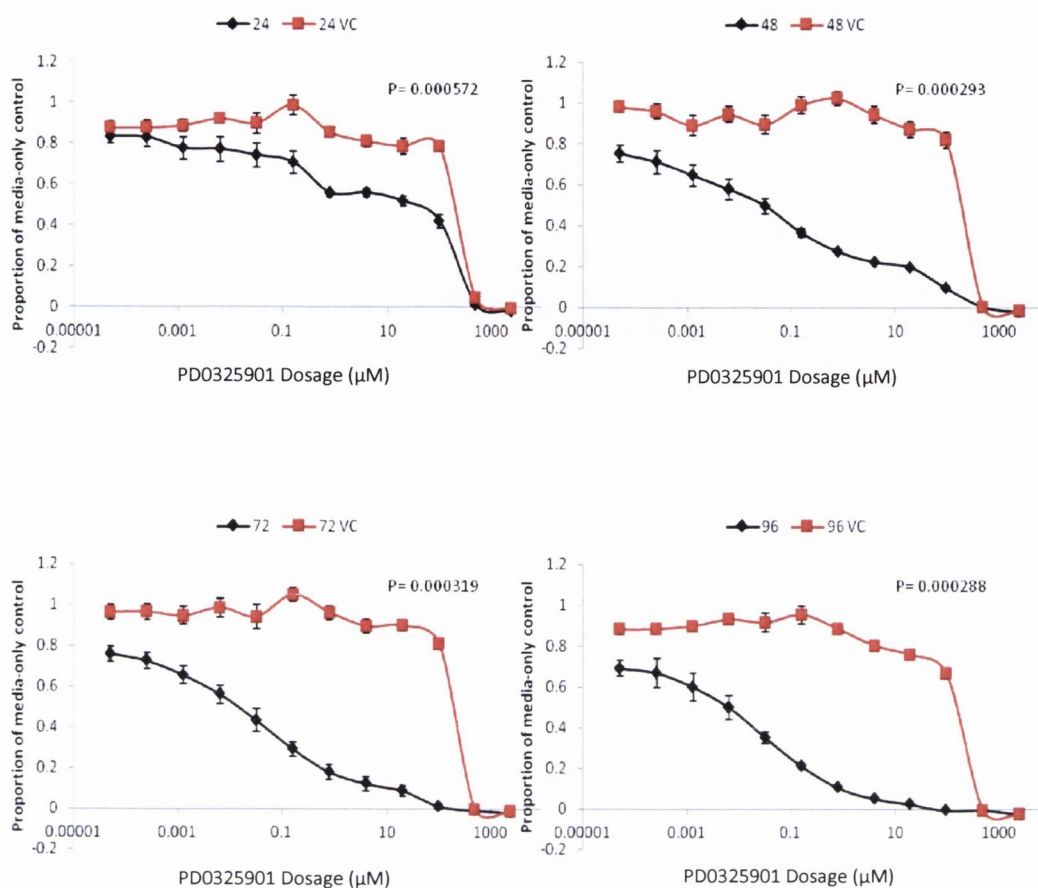
4.3 Figure 4: Optimisation of seeding density for MEK inhibition assays.

(A) A seeding density of 1×10^5 cells/ml (red line) was determined to be the optimal density for both thyroid cell lines

(B) 5×10^4 cells/ml (red line) was selected as the optimal seeding density for all three melanoma cell lines

n=12/datapoint

Error bars: +/- Standard error of the mean (SEM)

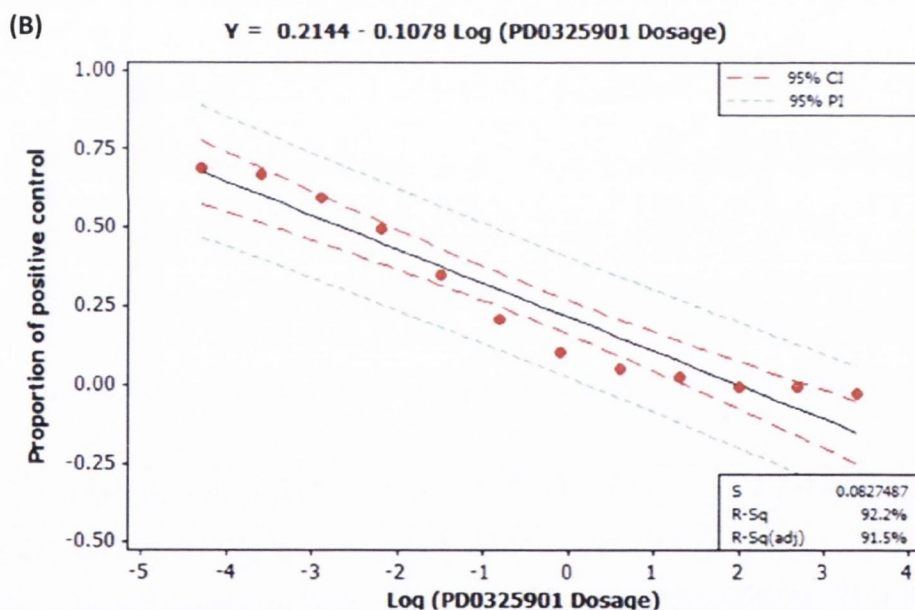
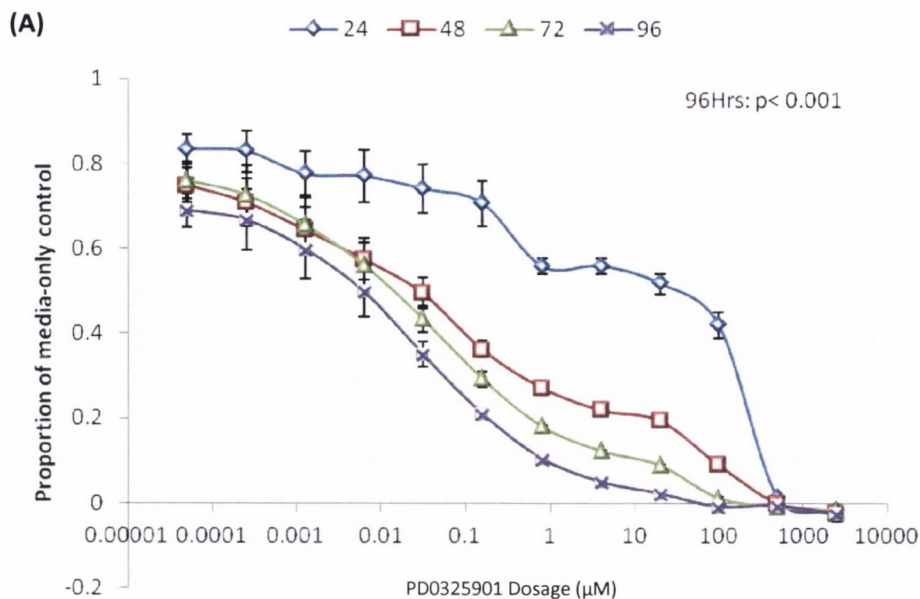


4.3 Figure 5: PD0325901 treatment effects are not due to the drug-vehicle.

The effect of PD0325901 (black) and the drug vehicle (DMSO)-alone (red) on SK-Mel-2 cell proliferation across 4 time-points was measured using a series of MTT assays with results normalised to the positive control. At the highest 2 dosages, there is an obvious vehicle-effect. However, this effect is quickly obliterated following 2 serial dilutions such that the effect of PD0325901 on cell proliferation is highly statistically significantly ($p < 0.001$) greater than that of the vehicle alone.

Error bars: $\pm$ SEM

n=12/datapoint



4.3 Figure 6: Optimisation of Drug Dosage and Length of Treatment

(A) SK-Mel-2 displayed the greatest sensitivity to PD0325901 following 96 hr treatment ($p < 0.001$)

(B) SK-Mel-2 drug-dose response curve (96 hr treatment).

Regression analysis (R-Sq (adj) 91.5%) identified an IC₅₀ of 2.2nM for SK-Mel-2 cell lines treated for 96 hours.

$Y = 0.5$ corresponds to 50% Inhibition of cell proliferation.

Error bars $\pm$ SEM

$n = 12/\text{datapoint}$

4.3 Table 3: Optimised PD0325901 MEK inhibitor treatment conditions by cell line.

Cell Line	Seeding Density	Length of Treatment (P<0.05)	MEKi (PD0325901) IC50 (Dose Curve R-Sq)	Versus Vehicle Control (DMSO)
NThy-Ori	1x10 ⁵ /ml	72Hrs	154.1µM (93.0%)	P<0.01
8505c	1x10 ⁵ /ml	72Hrs	126.7µM (97.8%)	P<0.01
SK-Mel-28	5x10 ⁴ /ml	48Hrs	1.28nM (86.0%)	P<0.01
SK-Mel-31	5x10 ⁴ /ml	96Hrs	3.76nM (88.2%)	P<0.01
SK-Mel-2	5x10 ⁴ /ml	96Hrs	2.24nM (92.2%)	P<0.01

(A) 8505C

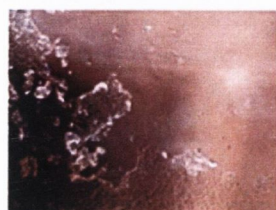
24 Hrs Post-
Seeding/Pre-
treatment



72 Hrs Post-
treatment
(cEMEM only)
Pre-Washing



72 Hrs Post-
treatment
(cEMEM only)
Post-Washing



(B) Nthy-Ori

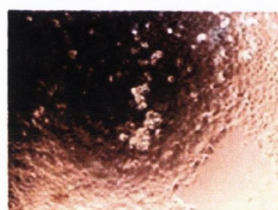
24 Hrs Post-
Seeding/Pre-
treatment



72 Hrs Post-
treatment
(cRPMI only)
Pre-Washing



72 Hrs Post-
treatment
(cRPMI only)
Post-Washing



4.3 Figure 7: Chamber slides are an appropriate vehicle to conduct MEK inhibition assays for confocal analysis

Confluent cells nucleated towards the centre of the well. Following the wash step, this nucleated centre was washed away in 8505C cells (A) or partially removed in NThy-Ori cells (B). However, an ample amount of cells remain for downstream confocal imaging and analysis.

Images taken under Brightfield microscopy using a 10X objective lens.

4.4 Discussion

4.4.1 gDNA Extraction

The MN and QDN kits achieved relatively consistent yields across both methods of quantitation (4.3 figure 1). These kits combine the binding properties of a silica-based membrane with simple microspin technology. DNA adsorbs to the silica membrane in the presence of high concentrations of chaotropic ions, which remove water from hydrated molecules in solution. Buffer conditions are designed to enable specific adsorption of DNA to the silica membrane and optimal removal of contaminants and enzyme inhibitors. The QAP Kit uses a column based purification method similar to that used for the QDN kit, but adapted to purify genomic DNA and total RNA simultaneously from a single biological sample by lysing and homogenising in a highly denaturing guanidine-isothiocyanate-containing buffer, to ensure isolation of intact DNA and RNA.

The AB kit uses magnetic beads which increases the available binding surface. This Kit employs mechanical disruption to rapidly release nucleic acid into a guanidinium thiocyanate-based solution that provides protection by inactivating nucleases. After lysis, samples are mixed with isopropanol, then combined with paramagnetic beads coated with a nucleic acid binding surface. The beads, with bound nucleic acid, are immobilized on magnets and washed to remove contaminants, then the nucleic acid is eluted using low-salt buffers. This kit produced the gDNA elutant with the most variable results dependent on quantitation methods. The NanoDrop reported results 1.16-8.78-fold greater than those reported by Qubit quantitation. However, this kit also reported unusually high a260/a280 ratios of between 2.9-4.25.

Due to the nitrogenous bases of DNA, DNA absorbs light in the ultraviolet range, at a wavelength of approximately 260 nanometres (nm). The spectrophotometer measures how much light is absorbed at the optimal wavelength. There is proportionality between how much of the compound is present and how much light is absorbed. The strength of the absorption is also a function of the molecular structure, which has been determined empirically for DNA and RNA and incorporated into the NanoDrop™ software to calculate the DNA or RNA concentration in a sample.

Proteins can contaminate the DNA eluent and also absorb in the ultraviolet range. Therefore, using A260 alone to calculate the concentration of DNA may give deceptively high results. The significance of protein contamination can be assessed using the A260:A280 ratio. The aromatic rings on tryptophan and tyrosine absorb at 280nm. If the absorbance of the sample at 260nm is more than 1.75 times the absorbance at 280nm, the DNA is pure

enough to proceed with downstream applications. If the ratio is greater than 2.1 in a DNA sample, it is likely highly contaminated with RNA. If the ratio is less than 1.75, the sample is badly contaminated with protein and may not behave well in subsequent experiments.

The a260/a280 ratios show that the MN and QDN kits processed the purest gDNA (4.3 Table 1) therefore the MN kit was used for all future gDNA extraction. The AB kit had unusually high a260/a280 ratios of between 2.9-4.25. The AB kit utilises a magnetic bead separation technology and these ratios are likely a result of bead contaminants leading to gross overestimation of yield. Unlike the Qubit platform, the NanoDrop™ quantitates via light absorbance and as such does not directly discriminate the target molecule. The Quant-iT™ Broad Range (BR) DNA Qubit assay utilises fluorophores that fluoresce upon binding their target molecules (double stranded DNA (dsDNA)), thereby avoiding interference from contaminants. The fluorescence signal is linear with the target molecule allowing the generation of a standard curve based on kit-supplied standards. This more selective method of quantitation reported a much more consistent range of gDNA concentrations compared to the NanoDrop (4.3 figure 2b). As such, the Qubit system was used for future gDNA quantitation.

4.4.2 BRAF^{V600E} Cell Line Status

Cell lines to be used in this study were selected based on BRAF^{V600E} status reported in the literature (Smyth *et al.*, 2005, Xing *et al.*, 2011) and in the COSMIC database (Forbes *et al.*, 2011). To confirm the mutational status an in-house developed custom TaqMan® SNP assay for T1799A, the SNP that confers the mutant V600E phenotype, was used to determine the allelic BRAF mutational status (Smyth *et al.*, 2005). BRAF status was as reported for all but one of the cell lines. SK-Mel-31 had been annotated as BRAF^{WT} in previous studies (Solit *et al.*, 2006; Xing *et al.*, 2011) however; in this study SK-Mel-31 was found to be heterozygous for the T1799A mutation. Those previous studies were performed using cells from the originators (Houghton and Chapman, Memorial Sloan Kettering Cancer Centre (MSKCC), USA) whereas the cells used in this study had been obtained commercially through ATCC. As such, a vial of early passage cells was gifted from the originators via Dr. David Solit, MSKCC. These cells (termed SK-Mel-31-MSK) were cultured and handled in the same manner as the ATCC cell line. Consistent with the commercially obtained cells, SK-MEL-31-MSK was also found to be heterozygous for the T1799A mutation³ (4.3 figure 3b).

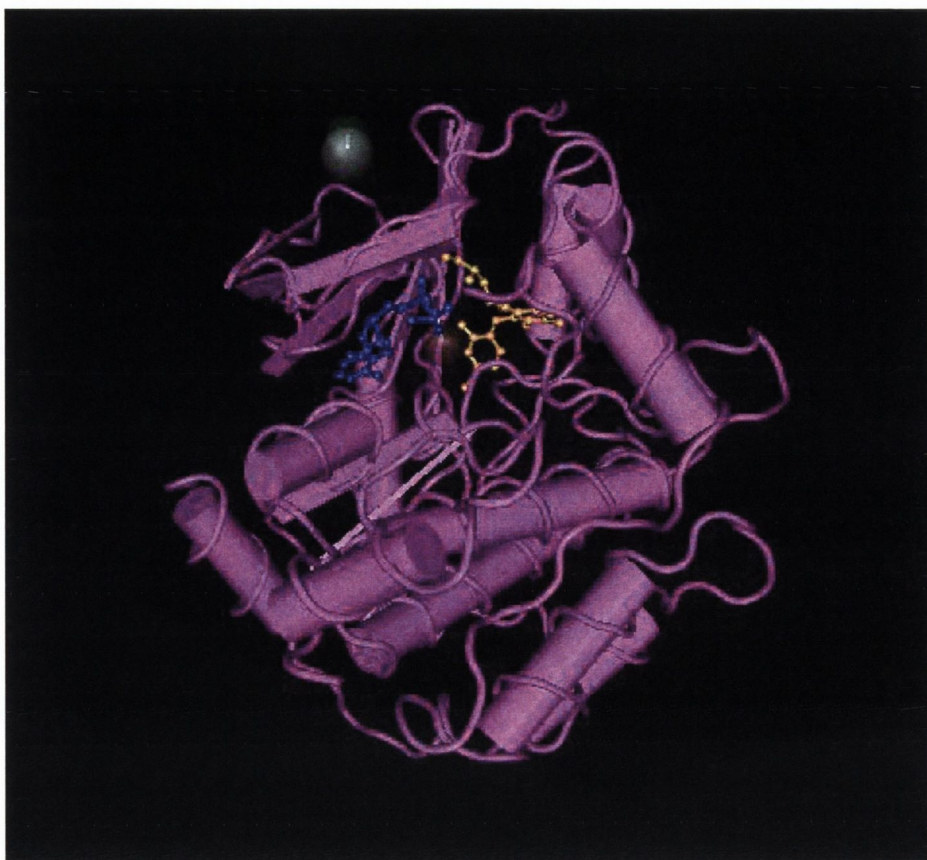
³ MSK were keep informed of these findings and the entry for SK-Mel-31 in COSMIC has recently been changed to reflect the presence of a BRAF^{V600E} mutation

4.4.3 Optimisation of PD0325901 MEK Inhibitor Treatment

The SMKI used in this study, PD0325901, is an allosteric inhibitor of MEK1 and MEK2. Allosteric inhibitors are non-ATP competitive; rather they bind to a site distinct to the enzyme active site. Allosteric sites typically function to bind a ligand that either positively or negatively regulates activity of the enzyme. These binding sites and their regulatory actions are often unique to that particular kinase, thus allosteric inhibitors exhibit the highest degree of kinase selectivity (Zhang *et al.*, 2009). The specificity of PD0325901 is further enhanced by the unusually specific interaction of MEK1/2 and its downstream target ERK1/2: MEK1/2 are the only confirmed activators of ERK1/2 and ERK1/2 are the only known substrates for MEK1/2 (English *et al.*, 2002).

MEK1/2 requires both the correct native tertiary fold of ERK1/2 and activating T-Loop residues to activate ERK1/2 via phosphorylation and will not phosphorylate ERK1/2 peptides nor denatured ERK1/2 (Seger *et al.*, 1992). PD0325901 induces a conformational change in MEK1 that locks the kinase in its inactive state (4.4 Figure 1). The precise mechanism of action has not been fully elucidated but the crystal structure strongly indicates that PD0325901 does not induce a structural change but rather promotes the stabilisation of the inactive conformation through a series of interactions with H-bonds, ADP and ATP, mimicking an important regulatory element of MEK (Fischmann *et al.*, 2009).

In BRAF mutated cancers, MEK is a second class kinase target; that is, inhibition of MEK results in a synthetic-lethal phenotype when paired with BRAF^{V600E}. Synthetic lethality refers to a gene interaction in which single gene defects are compatible with cell viability but the combination of gene effects results in cell death. Inhibition of the products of genes that are synthetic lethal to oncogenic mutations theoretically kill cells that harbour the oncogenic mutation whilst sparing normal cells. Hard synthetic lethality are the preferred targets of molecular cancer therapy. Hard synthetic lethal interactions are relatively unaffected by changes in additional genes, whereas soft synthetic lethality are readily rescued by alterations in other genes (Ashworth *et al.*, 2011). Elucidation of the mechanism of resistance of BRAF^{V600E} versus BRAF^{WT} cells to MEK inhibition should greatly aid in determining the extent of synthetic lethal interactions induced by PD0325901.



4.4 Figure 1: 3D molecular model of MEK1 bound to both ADP (blue) and the allosteric MEK inhibitor PD0325901 (yellow).

Modelled by the author using the NCI Molecular Modelling Database (MMDB; Madej et al, 2012) and the crystal structures deposited by Fischmann et al, (2009).

Cancers are highly heterogenic malignancies that develop through stochastic genetic and epigenetic events. Cancer is a dynamic state and over time genetic instability generates a plethora of alterations and diversity within a cancer. This genetic diversity has lead to dramatically different responses to targeted therapies dependent on tumour type. In some cancers targeted therapies have markedly improved survival whereas in others it has lead to only modest benefits (Turner and Reis-Filho, 2012). This has highlighted the importance of adapting targeted therapies for optimum delivery depending on the underlying genotype. Hence, the importance of optimising MEK inhibitor treatment for each cell line used in this study.

Regardless of BRAF status, thyroid cell lines had IC50 concentrations within the 125-155 μ M range whereas melanoma cell lines were exquisitely more sensitive with IC50 concentrations ranging between 1-4nM. The IC50 determined for melanoma cell lines were relatively consistent with that previously reported (Solit *et al.*, 2006) with the exception of SK-Mel-31. However, as previously outlined, in that study SK-Mel-31 was also deemed BRAF^{WT} and as such, it is highly unlikely that the SK-Mel-31 from that study (IC50 >100nM) is the equivalent cell line to the SK-Mel-31 cell line used in the current study (IC50 = 3.76nM). Differences in the methods used to obtain the IC50s between studies, such as the length of treatment prior to determination of the IC50 (the previous study specified as 3-5 days) likely accounts for the slight variation in IC50 for the remaining cell lines (SK-Mel-28 5.3nM compared to 1.28nM; SK-Mel-2 ~40nM compared to 2.2nM, Solit *et al.*, 2006 compared to the current study respectively.)

The IC50 for the 8505C cell line (126.7 μ M) was magnitude-fold greater than that previously reported in the literature (<5nM, Lebouef *et al.*, 2008). However, there are several differences in the method of obtaining the IC50 dosages between the studies. In that previous study there was no optimised treatment length reported and cells were treated over a 6-day period with media changes every two days. The method of growth curve analysis was via cell viability counts but there was no detail in terms of controls or replicates. In the current study cells were exposed to a single treatment and harvested at the time points 24, 48, 72 and 96 hours. All assay plates included controls treated with media-alone. Results from each 96-well plate were normalised to the media-only controls included on said plate. Four technical replicates were used and all assays were repeated across 3 biological replicates (independent cell passages). Therefore, confounding factors such as normal confluence effects and inter-assay variables were minimised. These differences may account for some of the variation observed between studies. To the best of the author's knowledge, an IC50 dosage for PD0325901 has not previously been reported for the NThy-Ori cell line.

PD0325901 is soluble in DMSO. As per recommended protocol and consistent with previous studies a stock concentration of PD0325901 was prepared at 10mM in DMSO and stored at -20°C (Xing *et al.*, 2011; Henderson *et al.*, 2010; Lebouef *et al.*, 2008; Solit *et al.*, 2006). For the serial dilutions, stock concentrations were diluted in media. As DMSO is in itself toxic to cells, a control was included for all assays to ensure the observed drug effect was not due to DMSO toxicity. As demonstrated in the SK-Mel-2 cell line (4.3 figure 5), the vehicle control is toxic at the first two dosages (equivalent to 25% and 5% DMSO), which is consistent across all cell lines. However, these dosage points are much higher than the IC50s for all cell lines and the

remaining range of DMSO dilutions are clearly distinct from the PD0325901 treated cells. Student's paired T-Tests confirm that the effect on cell proliferation is due to PD0325901 treatment and not an artefact of the vehicle control ($P < 0.05$ for all cell lines).

4.4.4 Translation of MEK inhibition Assay to a Chamber-Slide Format

Confocal microscopy was used downstream in the study (Chapter 5). Confocal microscopy can be used to visualise the native protein within its biological environment. In confocal microscopy, there is never a complete image of the specimen because at any instant only one point is observed; rather, an image builds up one pixel at a time. The ability of a confocal microscope to create sharp optical sections makes it possible to build 3D renditions of the specimen. Data gathered from a series of optical sections imaged at short and regular intervals along the optical axis are used to create the 3D reconstruction. Software combines the 2D images to create a 3D rendition of the target proteins within their environment (Semwogerere and Weeks, 2005).

Chamber slides are a commonly used and well suited for confocal microscopy. However, over-confluent cells can have a tendency to lift off chamber slides and be washed off during wash steps. To minimize this, cells are commonly grown on another platform and then transferred to the chamber slide for 24 hours prior to fixation. However, this method would not represent a direct correlation of the endpoint used for other analyses in the study. The preferred method to maintain consistency between assays was to perform the MEK inhibition assay in 8-well chamber slides and fix the cells at the same endpoint that they are harvested for other analyses. Hence, an analysis of the suitability of the chamber slides for this purpose was conducted. It was found that over confluent cells did tend to nucleate toward the centre of the wells and this area was more susceptible to removal during the wash steps, especially in the 8505C cell line (4.3 figure 7). However, an ample number of cells remained for imaging and this method was used for future confocal microscopy analysis.

4.5 Conclusions

Silica membrane spin-column based gDNA extraction is the preferred method of gDNA extraction from cell lines. Quantitation of gDNA is more consistent when measured via a method that directly discriminates the target molecule as demonstrated by the Qubit™ fluorometric system used in this study. Confirmation of BRAF^{V600E} status was a necessary endeavor for all cell lines to ensure the fidelity of the cell lines with information reported in the literature. Optimisation of the parameters required for MEK inhibition assays demonstrated that parameters clustered based on origin of the cell lines, with thyroid cell lines demonstrating PD0325901 IC50 levels between 120-155μM whereas melanoma cell lines were exquisitely more sensitive with an IC50 range between 1-4nM. The thyroid cell line carrying BRAF^{V600E} had a lower IC50 (127.6μM) than the BRAF^{WT} thyroid cell line (154.1μM) but all other parameters were consistent between thyroid cell lines. Similarly, the homozygous BRAF^{V600E} melanoma cell line had the lowest IC50 and this cell line also had the shortest treatment length (1.28nM, 48Hrs). The remaining melanoma cell lines shared the same optimal treatment length; however, the heterozygous melanoma cell line had a higher IC50 (3.76nM) than the homozygous BRAF^{WT} cell line (2.24nM). The latter cell line carries an NRAS^{Q61R} mutation which is also upstream of MEK and has previous been reported as displaying sensitivity to PD0325901 (Solit *et al.*, 2006).

Chapter 5: Molecular Mechanisms of Resistance to the MEK Inhibitor PD0325901

5.1 Introduction

The impact of modern molecular biology on our understanding of many cellular functions and how these processes go awry in cancer has been dramatic and inexorable. Since the Human Genome Project and the revolution of next generation sequencing our understanding of how genes and genomes work in health and disease has grown exponentially. The gene is no longer considered to be merely a region that encodes a polypeptide; rather it is a highly regulated and complex physiological unit (Gingeras *et al.*, 2007). It is now recognised that widespread transcription from much of the genome is common, producing an abundance of non-coding transcripts (Wu *et al.*, 2008). The developing insights of the complexity of nuclear organisation, genes, and genetic regulation have significant impact on our understanding of disease. Cancer genomes are being elucidated at a striking rate, as too are the underlying genetic principles of cancer progression. As the complexity of the evolution of cancer is unravelled the classical theories of genetic interactions in cancer progression and treatment are continuously being reassessed and expanded.

A cell's progression from normal to cancerous is driven by a biological imperative to survive, which perpetuates fundamental changes in the cell's behaviour (Hanahan and Weinberg, 2000). Mutations in DNA allow the acquisition of these traits, however, the mechanisms by which these mutations connect to transformative phenotypes remains largely unclear (Ashworth *et al.*, 2011). The tumour landscape is incredibly complex with cells estimated to carry 10^4 - 10^5 genetic alterations compared to germline cells. These genetic alterations largely arise due to exogenous mutagens such as UV radiation and endogenous reactions such as replication. It is likely that only a very small subset of these mutations are required for tumour formation (driver mutations) and that the majority of genetic alterations are merely collateral damage, or passenger mutations.

The simple theory of driver and passenger mutations in facilitating tumour formation only accounts for the genes in isolation. However, cellular systems do not arise from the independent actions of different genes; they are the result of complex networks of genetic interactions and extensive epistasis. These gene networks often provide functional buffering (genetic canalisation): the ability of complex molecular networks to buffer against the tendency of new alleles to impair fitness or viability. Functional buffering against the dysfunction of individual genes is common (Kamath *et al.*, 2003; Giaever *et al.*, 2002; Hughes *et al.*, 2000). Understanding this functional buffering is critical for targeted therapies, which aim to block individual pathways.

Targeted cancer therapy applies a stringent selective pressure upon the tumour cells. The molecular mechanisms of functional buffering that gives rise to synthetic viability are likely to be selected for and thus have the potential to give rise to resistance. This is illustrated by resistance to BRAF inhibitors (chapter 4, 4.1 figure 2). Following a strong initial response, the therapeutic effect of BRAF inhibitors appears to be abrogated by compensatory changes in the activity of other signalling pathways (Villanueva *et al.*, 2010; Nazarian *et al.*, 2010; Johannessen *et al.*, 2010). Functional buffering mechanisms within the cells give rise to synthetic viability between the drug target and the genes/mutations that lead to the compensatory mechanism (Ashworth *et al.*, 2011). Hence, the elucidation of compensatory mechanisms to molecularly targeted therapies will allow combinational therapies that simultaneously target both the primary target and buffering mechanisms. Therefore, identifying global networks in human cells is a major objective in molecular therapy. This is a technically challenging objective; however by viewing cancer as a network of genetic interactions a more in-depth understanding of cancer biology can be gained, allowing the development of better therapeutic approaches.

5.2 Project Rationale

MiRNAs are crucial regulators of gene expression that function by binding their mRNA targets and promoting their degradation or translational repression. The human genome is believed to encode approximately 1,000 miRNAs compared to an estimated 30,000 mRNAs (Pritchard *et al.*, 2012). A single miRNA can regulate hundreds of mRNAs and thus have extensive effects on gene expression networks. Variation in the expression of hundreds of mRNA can be captured in the expression patterns of a few miRNAs that regulate them. Profiling of miRNAs can therefore be particularly rich in biological information.

MiRNA expression profiling has identified a range of biological processes regulated by miRNAs, including the maintenance of tissue differentiation (Ren *et al.*, 2009; Alvarez-Garcia *et al.*, 2005; Wienholds *et al.*, 2005). Unique patterns of miRNAs have been identified in both human and mouse embryonic stem cells, reinforcing the role of miRNAs in maintaining stemness (Ren *et al.*, 2009; Morin *et al.*, 2008; Lakshmipathy *et al.*, 2007; Suh *et al.*, 2004; Chen *et al.*, 2007; Tang *et al.*, 2006; Houbaviy *et al.*, 2003).

As discussed in chapter 4, cancer is not heterogeneous. A mechanism for the phenotypic heterogeneity evident in cancer may be the existence of cancer stem cells, which have been associated with resistance to chemotherapies and radiation therapies (Li *et al.*, 2007; Liu *et al.*, 2006; Dean *et al.*, 2005). The original cancer stem cell model suggests that a minor subpopulation of cells (5-10%) with indefinite potential for self renewal exists within a tumour (Reya *et al.*, 2001). However, recent evidence suggests that stem-like cancer cells arise *de novo* from non-stem-like cells (Gupta *et al.*, 2011; Chaffer *et al.*, 2011). These concepts are not mutually exclusive and both may cooperate in tumour resistance and response to therapy.

In this study, human cell lines naïve or resistant to MEK inhibitor treatment were analysed for relative expression of key markers of the MAPK pathway (MEK1/2; ERK1/2; ELK1), stemness markers (ALDH1A1; NANOG; OCT4), and adhesion markers (β -catenin and E-cadherin). Naïve and resistant samples were then analysed via next generation sequencing (SOLiD™ 4 Platform, Applied Biosystems, USA) to detect global expression profiles of miRNAs (5.2 Table 1). Bioinformatic analyses were performed to identify validated gene targets of miRNAs displaying statistically significant differential regulation between naïve and resistant states. Bioinformatic analyses were then used for integrative analyses of miRNAs in the context of gene regulatory networks. Gene ontology (GO) analysis identified differentially regulated biological processes associated with MEK inhibitor resistance.

5.2 Table 1: Summary of cell line models

	Origin	BRAF Status	Optimised Treatment	Assayed for
8505C	Thyroid	V600E/V600E	72Hr; 126.7µM	Gene; Protein; miRNA
NThy-Ori	Thyroid	WT/WT	72Hr; 154.1µM	Gene; Protein; miRNA
SK-MEL-28	Skin	V600E/V600E	48Hr; 1.28nM	Gene; Protein; miRNA
SK-MEL-31	Skin	V600E/WT	96Hr; 3.76nM	Gene; miRNA
SK-MEL-2	Skin	WT/WT	96Hr; 2.24nM	Gene; Protein

5.3 Results

5.3.1 Gene expression of key members of the MAPK pathway are differentially expressed in BRAF^{V600E/V600E} MEKi-resistant cells

BRAF^{V600E} and BRAF^{WT} cell lines (8505C, NThy-Ori 3-1, SK-MEL-28, SK-MEL-31, SK-MEL-2) were treated under optimised conditions (chapter 4, 4.3 Table 3). Cells surviving MEKi treatment (resistant) and cells treated with media-alone (naïve) were harvested for total RNA and analysed for relative gene expression of the MAPK pathway members *MAP2K1* (MEK1), *MAP2K2* (MEK2), *MAPK3* (ERK1), *MAPK1* (ERK2) and *ELK1*. The assay was repeated with cells undergoing sustained exposure by treating cells with two sequential optimised treatments of either PD0325901 or media-alone. Relative gene expression of MEKi resistant cells were normalised to their paired media-only naïve controls:

$$\text{fold change expression of resistant cells} = \left(\frac{\text{Avg RQ Resistant (3 technical replicates)}}{\text{Avg RQ Paired Naïve (3 technical replicates)}} \right).$$

The average fold change of MEKi resistant expression relative to naïve expression was obtained across 2 biological replicates. A fold change of (+/-) 2-fold was considered statistically significant (5.3 figure 1; 5.3 table 1).

MEKi resistance did not induce significant differential expression in BRAF^{WT/WT} in either thyroid or melanoma cell lines. The thyroid cell line 8505C (BRAF^{V600E/V600E}) MEKi resistant cells had statistically significant downregulated expression of MAPK pathway members in both the optimised and sustained treatments (5.3 Table 1). MEKi resistant SK-MEL-28 melanoma cell line (BRAF^{V600E/V600E}) displayed a trend of upregulated gene expression, however this only reached significance for *ELK1* expression following the single optimised treatment. MEKi resistant SK-MEL-31 melanoma cell line (BRAF^{V600E/WT}) displayed statistically significant upregulated gene expression in all genes bar *MAPK3* following the optimised treatment (5.3 figure 1 (A)); this trend continued but did not reach significance following sustained treatment (5.3 figure 1 (B)).

5.3.2 Stemness-associated genes *NANOG* and *POU5F1* are upregulated in MEKi resistant in BRAF^{V600E} melanoma but not MEKi resistant BRAF^{V600E} thyroid cell lines

As per section 5.3.1, cells were assessed for the relative expression of the stemness-associated genes *ALDH1A1*, *NANOG* and *POU5F1* (OCT4A) and the adhesion associated genes *CDH1* (E-cadherin) and *CTNNB1* (β-catenin). Following a single optimised treatment, *POU5F1* and *NANOG* were statistically significantly upregulated in both SK-MEL-28 and SK-MEL-31 MEKi resistant

cells; with no statistically significant relative expression in either the BRAF^{WT} melanoma or thyroid resistant cells (5.3 figure 2(A); 5.3 table 1). Statistical significance was observed in the expression of adhesion genes in thyroid but not melanoma cell lines: in 8505C, *CDH1* was upregulated whereas *CTNNB1* was downregulated in MEKi resistant cells. In resistant NThy-Ori cells, *CDH1* was downregulated. The stemness-associated gene *ALDH1A1* was differentially regulated in the thyroid cells: in resistant 8505C it was significantly downregulated whereas in resistant NThy-Ori it was significantly upregulated.

Following sustained treatment, *ALDH1A1* remained significantly downregulated in 8505C. SK-MEL-28 resistant cells expressed significantly increased expression of *ALDH1A1*. *POU5F1* remained significantly upregulated in both resistant BRAF^{V600E} melanoma cells. *NANOG* remained significantly upregulated in resistant SK-MEL-31 cells; SK-MEL-28 resistant cells trended towards upregulation of *NANOG* expression but just failed to meet significance (1.9-fold upregulation). NThy-Ori resistant cells averaged across biological replicates as expressing significantly upregulated *NANOG* and *POU5F1*, however, the standard error of the mean (SEM) is very large for the relative expression of both of these genes (5.3 figure 2(B)). The adhesion genes were no longer significant for 8505C. There was no significant differential expression observed in any cell line for *CTNNB1*. *CDH1* remained significantly downregulated in NThy-Ori resistant cells and was upregulated in resistant SK-MEL-31 cells.

5.3.3 Altered gene expression between BRAF^{V600E/V600E} thyroid and melanoma cell lines is not due to additional drug burden caused by magnitude IC50 differences

The IC50 for 8505C was 126.7μM compared to 1.28nM for SK-MEL-28. To investigate if the difference in gene expression patterns witnessed in MEKi resistant cells is due to this magnitude difference, the IC90 (153.9μM, R-Sq 85%) for SK-MEL-28 was used to treat SK-MEL-28 cells as per section 5.3.1. The IC90 treated cells followed the same trend as the IC50 treated SK-MEL-28 cells (5.3 figure 3; 5.3 table 1). The IC90 MEKi resistant cells reached statistical significance in upregulation of all genes in both optimised and sustained samples bar *CDH1* (E-cadherin).

5.3.4 The stemness markers *NANOG* and *ALDH1A1* are differentially expressed at protein level in MEKi-resistant BRAF^{V600E} cells

Protein isolated from MEKi resistant and MEKi naïve samples were probed for the expression of stemness markers *NANOG*, *OCT4A*, and *ALDH1A1* for the homozygous BRAF^{V600E} and BRAF^{WT}

thyroid and melanoma cell lines. Samples were also probed for actin as an endogenous control and densitometry was calculated using imageJ software and normalisation to actin (Schneider *et al.*, 2012). A normalised fold change of (+/-) 1.5-fold between naïve and resistant samples was considered statistically significant (5.3 figure 4). NANOG was detected in SK-MEL-2, SK-MEL-28 and 8505C cell lines. The 8505C and SK-MEL-2 cell lines had significantly downregulated levels of NANOG in MEKi resistant cells. SK-MEL-28 had a trend towards an increased NANOG expression in MEKi resistant cells but failed to reach significance (1.3X). OCT4A was only detected in SK-MEL-28 cells and no difference in expression levels was evident between MEKi resistant and naïve samples. ALDH1A1 was identified in the BRAF^{V600E/V600E} samples only. No difference in protein expression was evident in 8505C cells; however ALDH1A1 was significantly upregulated in SK-MEL-28 MEKi resistant cells.

OCT4A and NANOG protein expression was also analysed using TaqMan™ Protein Assays (Applied Biosystems, USA). However, although SEM was excellent for all samples, the background levels for all assays were not significantly different from the protein samples and thus expression could not be determined (5.3 figure 5). Western blot analysis was only performed on one biological replicate per cell line.

5.3.5 Total ERK1/2 but not phosphoERK1/2 displays differentially regulated protein expression in MEKi resistant melanoma cell lines

Western blot analysis for total ERK1/2 (tERK1/2) and phosphorylated ERK1/2 (pERK1/2, which does not recognise the unphosphorylated forms of ERK1/2) was performed as per section 5.3.4. Total ERK1/2 was detected in all samples. No difference was detected in either thyroid cell line; however, in the melanoma cell lines MEKi resistance induced an upregulation of tERK1/2 in SK-MEL-28 and downregulation in SK-MEL-2 (5.3 figure 6(A)). pERK1/2 was detected by Western blot analysis in the melanoma cell lines only, with no significant difference observed between resistant and naïve samples (5.3 figure 6(B)). Western blot analysis was only performed on one biological replicate per cell line.

5.3.6 pERK1/2 localises to different areas of the cell following MEKi resistance in thyroid cell lines

Confocal analysis was used to detect tERK1/2 and pERK1/2 in the thyroid cell lines. Confocal settings such as intensity and gain were optimised by Dr. Victoria McEaney and samples were imaged using identical settings. No difference between treatments was observed for tERK1/2.

However, pERK1/2 polarised differently in the cell in resistant samples. Furthermore, the opposite polarisation was induced dependent upon BRAF status. In 8505C naïve cells, pERK1/2 is present in all three layers of the cell (apical, midsection & basolateral), however, following acquisition of MEKi resistance pERK1/2 staining is no longer present in the basolateral layer (5.3 figure 7). Conversely, in NThy-Ori naïve cells pERK1/2 staining is evident in apical and midsections but not in the basolateral layer; however, in MEKi resistant cells, pERK1/2 staining is detected in all three layers (5.3 figure 8).

5.3.7 Next generation sequencing and annotation of miRNAs

Global miRNA expression was analysed among MEKi resistant and MEKi naïve samples for 8505C; NThy-Ori; SK-MEL-28; and SK-MEL-31 cell lines on the SOLiD™ 4 NGS platform. Workflow analyses (WFA) indicated a 0.4pM barcoded library was optimal (5.3 figure 9), however, as an ePCR reaction failed during template bead preparation there was insufficient quantity of 0.4pM library and as such, the deposited library consisted of a mix of 0.4pM and 0.8pM libraries. 708 million P2+ (enriched) beads were required for deposition. Assuming a loss of 15% during the deposition procedure, 814 million P2+ beads were required. Using data from the WFA, 1.085×10^9 beads with an average 75% P2+ beads, were prepared and deposited. Following the sequencing run, the data was assessed and analysed by a bioinformatician. The sequencing run passed all primary analyses and was deemed successful. Colour space data was converted to 35bp reads and mapped to the reference genome by the in-house bioinformatician.

During the library construction for all samples, there was an error in the size selection which resulted in a small shift in the size of cDNA amplified (5.3 figure 10). The size range still covered the miRNA range but resulted in a greater amount of contaminants (degraded mRNA transcripts) in the sample, resulting in reduced miRNA coverage. Contaminants were bioinformatically removed and sequences were annotated based on their overlap with publicly available genome annotations of miRNAs, rRNAs, tRNAs, other small RNAs and genomic repeats (Appendix 3, Tables 1-8). The total reads and proportion of miRNAs per mapped sample is found in 5.3 table 2. It should be noted that these are only the annotated reads and do not include novel transcripts. MiRNAs were the most abundant class of annotated small RNA on average. As there was variability in the amount of total reads between samples, annotated reads were normalised to allow analysis of differential expression across samples (Li *et al.*, 2011).

$$\text{Coverage target miRNA} = \frac{\text{count miRNA target}}{\text{Total count of miRNAs in sample}}$$

MiRNAs shared between resistant and naïve samples were analysed for each cell line. To increase stringency, any miRNA with ≤ 3 reads were excluded and the relative fold change of resistant samples was calculated as follows:

$$\text{Fold Change} = \frac{\text{Coverage miRNA Resistant}}{\text{Coverage miRNA Naïve}}$$

A relative fold change of (+/-) 10-fold was considered statistically significant and were analysed further. MiRNAs uniquely identified in either resistant or naïve samples were considered significant if >10 reads were identified (Appendix 3, tables 9-12). Consistent with previous studies within the literature, more genes were downregulated than upregulated following MEKi treatment (Packer *et al.*, 2009).

5.3.8 Differential miRNA expression profiling

Differential expression of miRNAs associated with naïve and resistant samples was found between cell lines. *MIR143* was uniquely found significantly expressed in naïve melanoma cells (SK-MEL-28 and SK-MEL-31) but not thyroid cells. Over-expression of *MIR143* has been associated with increased resistance to radiotherapy by protecting cells from DNA damage induced killing (Lin *et al.*, 2011a). *MIR33* was identified in the NThy-Ori (BRAF^{WT/WT}) naïve cells only. *MIR33* is associated with virally induced head and neck cancers (Wald *et al.*, 2011), which is of interest as NThy-Ori is a virally transformed thyrocyte cell line. Perhaps the most interesting differential regulation is that of the *MIR302* and *MIR520* clusters between the BRAF^{V600E/V600E} melanoma and thyroid cell lines. The *MIR302* cluster is significantly relatively upregulated in naïve SK-MEL-28 samples, whereas the *MIR520* cluster is significantly upregulated in MEKi resistant samples in 8505C. These clusters are classified as embryonic stem cell-specific cell cycle-regulating (ESCC) miRNA families (Subramanyam *et al.*, 2011; Ren *et al.*, 2009) and, although located on different chromosomes, are often referred to as part of the *MIR302/373/374/520* family (Mazda *et al.*, 2011). The miRNAs of these clusters are functionally similar and share a 7-mer seed sequence; as such their targeted genes tend to overlap with greater than 90% of the predicted targets of the *MIR302* cluster also predicted as targets of the *MIR520* family (Ren *et al.*, 2009). Using miRWalk, with $p < 0.01$, to predict the target genes of the miRNAs from each cluster significant in this study (14 members of *MIR520*; 4 members of *MIR302*); an overlap of 36% between predicted targets of each cluster was identified.

5.3.9 Integrative analyses of miRNAs in the context of gene networks

MiRWalk: The database on predicted and validated microRNA targets (Dweep *et al.*, 2011) was used to identify validated mRNA targets of statistically significant miRNAs associated with naïve and resistant samples for each cell line (Appendix 3, tables 13-16). MiRWalk utilises 8 of the established miRNA prediction algorithms (RNA22, miRanda, miRDB, TargetScan, RNAhybrid, PITA, and Diana-microT) to identify validated miRNA targets (Betel *et al.*, 2010; Wang and El Naqa 2008; Wang 2008; Lewis *et al.*, 2005; Kruger and Rehmsmeier, 2006; Segal and Widom, 2009; Krek *et al.*, 2005; Maragkakis *et al.*, 2009). The computationally derived gene targets of the differentially expressed miRNAs were examined by Gene Ontology (GO) “biological processes” classifications (Appendix 3, tables 17-20). GOSTat analytical software was used to identify GO terms statistically overrepresented in each sample (naïve and resistant) for each cell line with significance set to $P < 0.01$ and multi-variant correction using Benjamini false discovery rate (Beissbarth and Speed, 2004). For each cell line, GO terms shared by both naïve and resistant groups of the cell line were filtered out. The remaining GO terms, which were uniquely overrepresented in either naïve or resistant samples, were compared between cell lines (5.3 table 3).

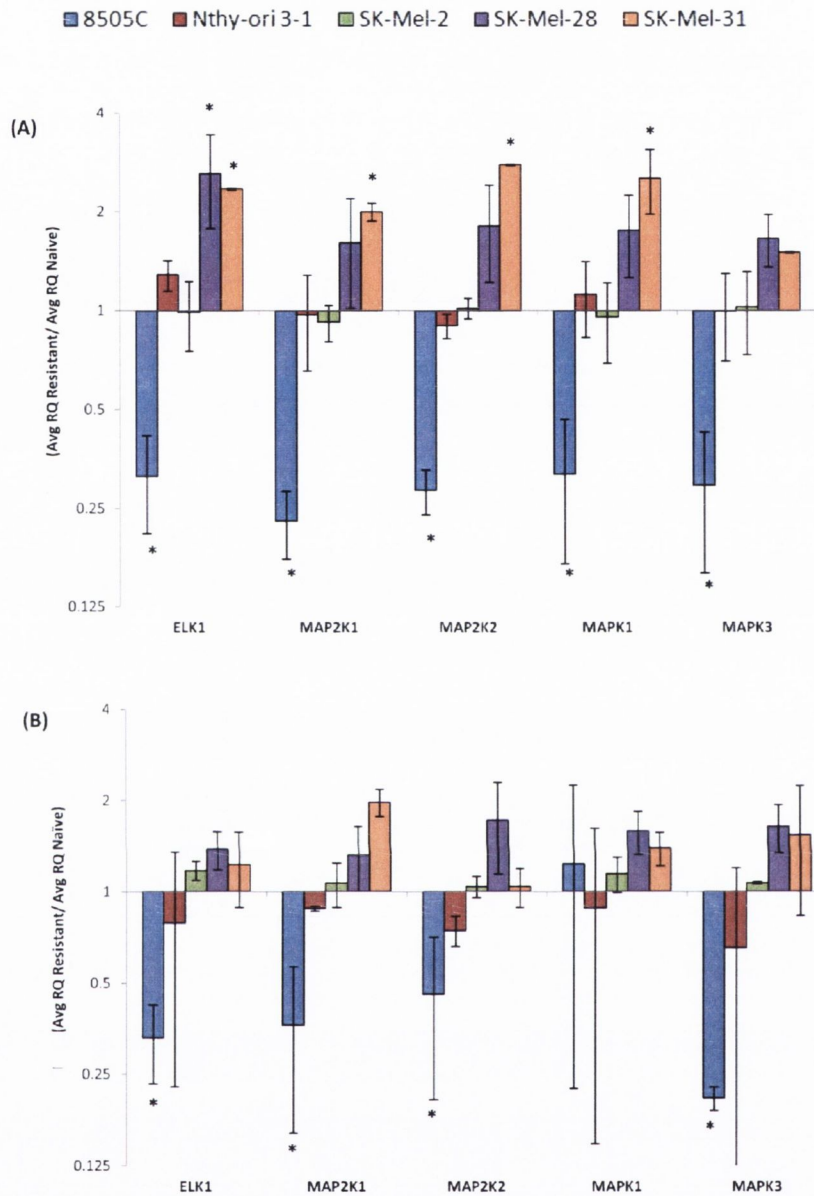
GO terms associated with the characteristics of each cell line were identified. For example, regulation of natural killer T (NKT) cells, which recognise lipid antigens, was associated with SK-Mel-28 only. SK-MEL-28 is CD1-negative but expresses high levels of melanoma-associated gangliosides (Wu *et al.*, 2003). Genes involved with thyroid gland development was associated with the resistant 8505C samples and genes involved in the response to UVB was associated with resistant SK-MEL-31 samples.

GO terms overrepresented in melanoma cell lines include: epidermis development, response to retinoic acid, response to vitamin A and response to vitamin D. Interestingly, and in line with stemness patterns seen in the gene expression data, cell dedifferentiation and re-entry into the mitotic cell cycle were also overrepresented in melanoma cell lines only (5.3 table 3). The terms associated with the BRAF^{WT/WT} cell line are predominantly the regulation of alternate signalling pathways including insulin-like growth factor signalling pathway, AKT (protein kinase B) signalling cascade and phosphatidylinositol-mediated signalling, whereas the only GO term significantly overrepresented in all of the BRAF^{MUT} cell lines was “neurotransmitter metabolic process”. Terms relating to the MAPK pathway were found in all samples and therefore filtered out during the GO term analysis.

GO terms statistically significantly ($P < 0.01$) associated with naïve samples in both homologous BRAF^{V600E} cell lines include those relating to the NFκβ pathway and the regulation of its import into the nucleus. Terms including (p=8505C¹; SK-MEL-28²): cytoplasmic sequestering of NFκβ (p=0.000466¹, p=0.000495²) and negative regulation of NFκβ import into the nucleus (p=0.000731¹; p=0.0000249²) are significantly overrepresented. More generally, negative regulation of import into the nucleus of transcription factors (p=0.00312¹; p=0.000991²) and protein (p=0.00312¹; p=0.000991²) and cytoplasmic sequestering of transcription factors (p=0.00224¹; p=0.00654²) and protein (p=0.00267¹; p=0.00866²) are also overrepresented in naïve samples.

5.3.10 GO terms are differentially regulated in BRAF^{V600E/V600E} melanoma and thyroid cell lines

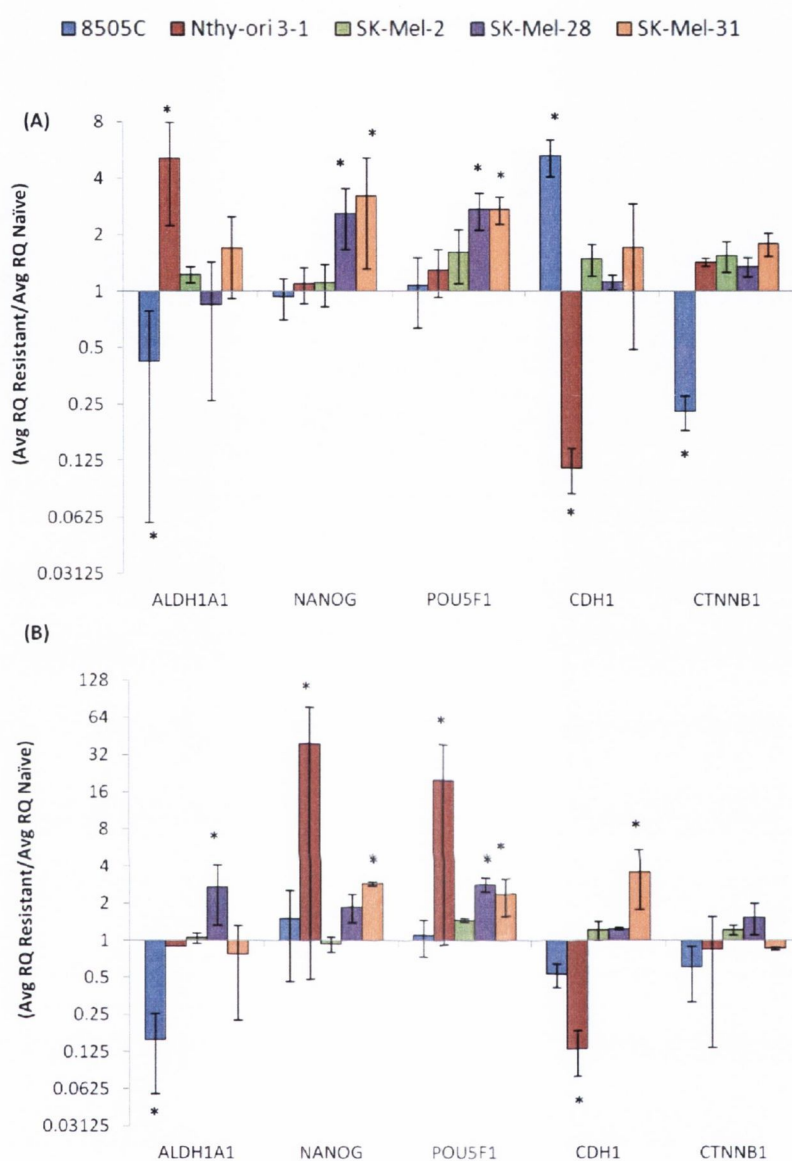
GO analysis between cell lines indicates that significantly overrepresented GO terms in SK-MEL-28 naïve samples are also frequently overrepresented in 8505C resistant samples (5.3 figure 11). A selection of GO terms (from Appendix 3 tables 17-20) divided by term-association are found in 5.3 table 4. In this table, the differential overrepresentation of both T-cell and B-cell associated GO Terms between BRAF^{V600E} thyroid and melanoma cells is clearly evident. Chromatin modification (p=0.000509¹; p=3.9x10⁻⁹²) and remodelling (p=0.00656¹; p=0.00212²) are overrepresented in resistant 8505C¹ and naïve SK-MEL-28². This pattern is again observed in mitochondrial-associated apoptosis terms and response to stress. This trend of differential regulation of processes between BRAF^{V600E} melanoma and thyroid samples is reminiscent of that seen in the gene expression analyses.



5.3 Figure 1: MEKi resistance induces differential gene expression of key members of the MAPK Pathway in BRAF^{V600E} thyroid and melanoma cell lines.

Cells surviving treatment under (A) optimised or (B) sustained conditions with PD0325901 (resistant) or media-alone (naïve) were analysed for gene expression. Fold change (RQ) expression in resistant samples was normalised to RQ of paired naïve samples (naïve samples normalised =1) and averaged across biological replicates.

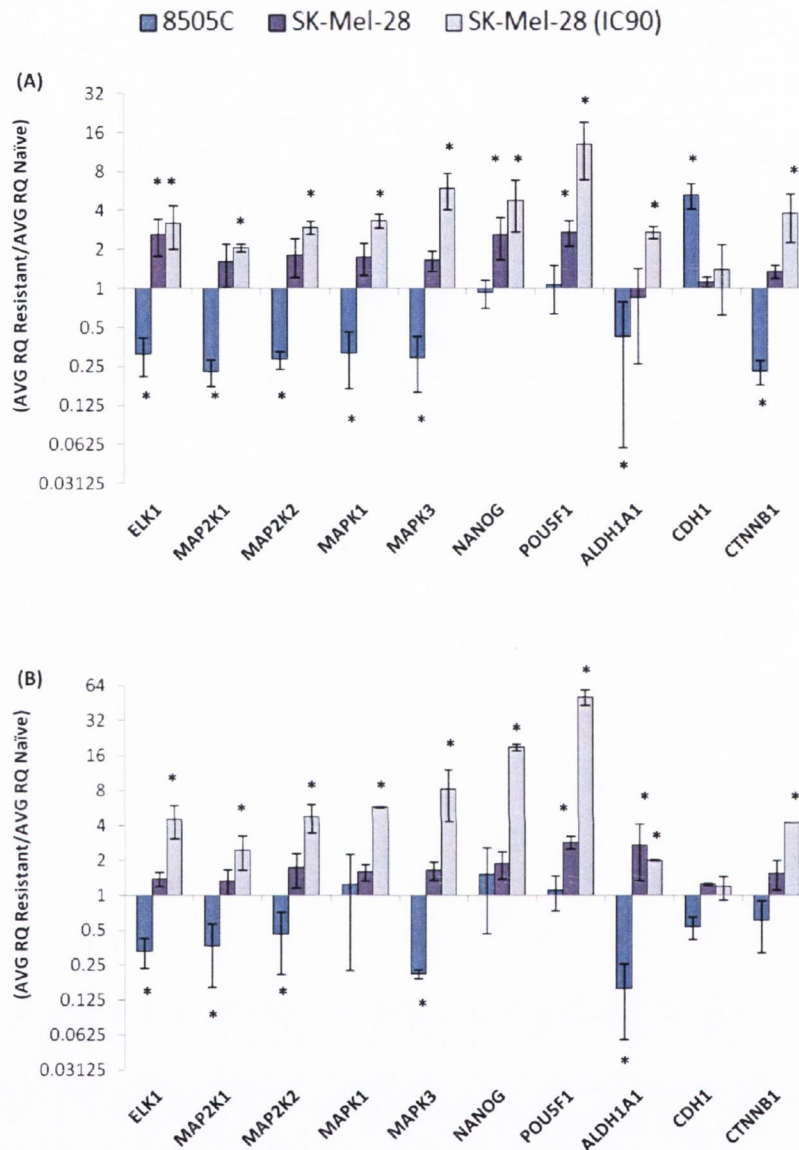
Statistical significance +/- 2-fold (*) difference between resistant samples (bars) and naïve samples (=1, x-axis). Error bars: SEM of n=6 observations/datapoint.



5.3 Figure 2: MEKi resistance induces upregulation of stemness-associated genes in BRAF^{V600E} melanoma cell lines.

Cells surviving treatment under (A) optimised or (B) sustained conditions with PD0325901 (resistant) or media-alone (naïve) were analysed for gene expression. RQ expression in resistant samples was normalised to RQ of paired naïve samples and averaged across biological replicates.

Statistical significance +/- 2-fold (*) difference between resistant samples (bars) and naïve samples (=1, x-axis). Error bars: SEM of n=6 observations/datapoint.



5.3 Figure 3: Differential expression between resistant BRAF^{V600E/V600E} melanoma and thyroid cells is not due to additional drug burden caused by magnitude PD0325901 IC50 differences.

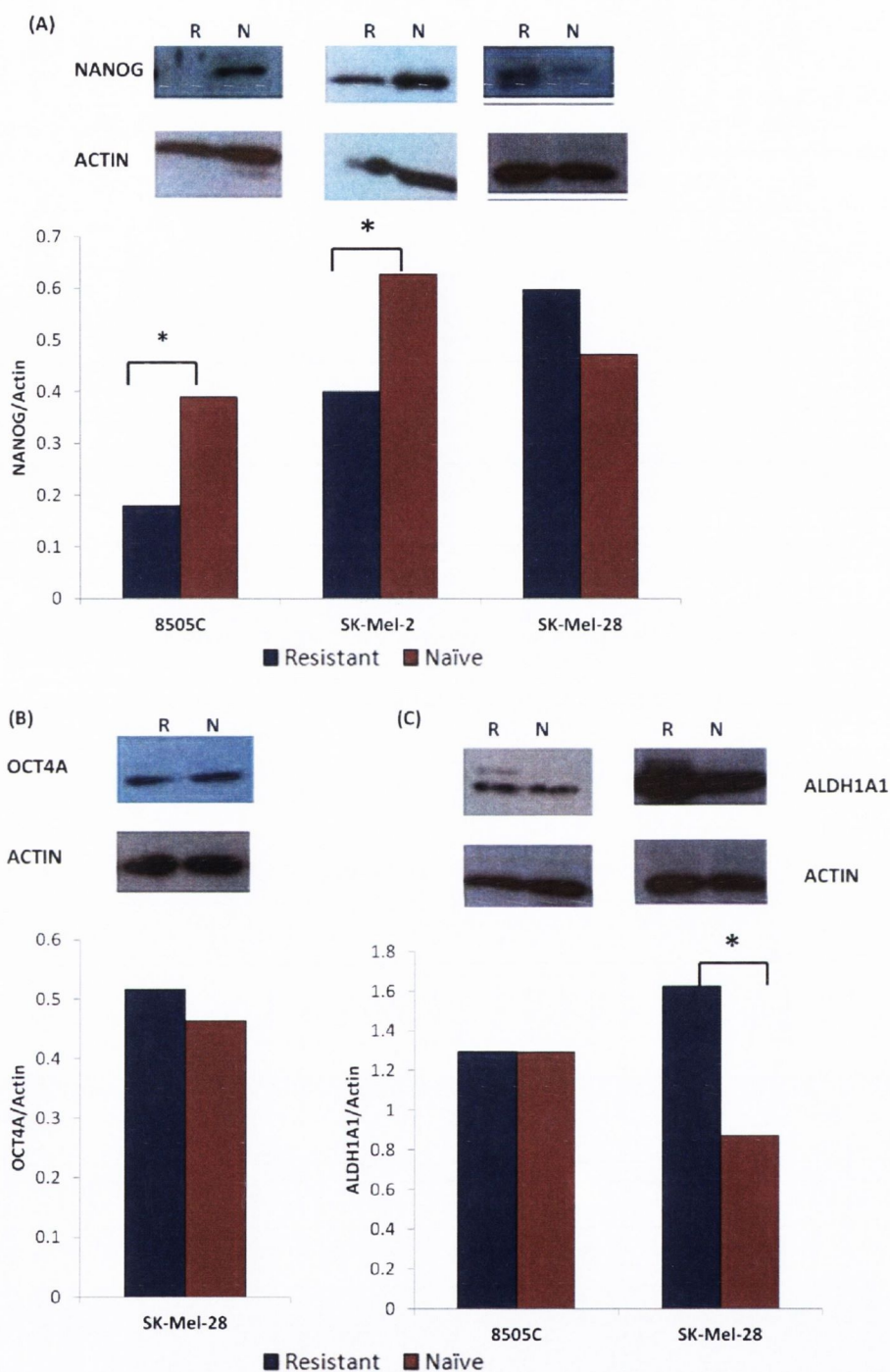
(A) Optimised and (B) Sustained MEK inhibition assays were performed on SK-MEL-28 cells using PD0325901 IC90 (153.9μM) as before. Results were compared to those obtained using IC50 for SK-MEL-28 and 8505C. The IC90 results followed the same pattern as the SK-MEL-28 IC50 results, with an exaggerated upregulation that reached significance for all genes bar CDH1.

Statistical significance +/- 2-fold (*) difference between resistant samples (bars) and naïve samples (=1, x-axis). Error bars: SEM of n=6 observations/datapoint.

5.3 Table 1: Gene expression results for all cell lines following a single MEKi treatment.

Resistant samples were normalised to their naïve control. Significant difference in gene expression was +/- 2-fold difference between resistant and naïve samples. All results are the average of n=6.

		BRAF T1799A/ T1799A	BRAF WT/WT	BRAF WT/WT	BRAF T1799A/ T1799A	BRAF T1799A/ WT	BRAF T1799A/ T1799A
		8505c	N-Thy-Ori	SK-Mel- 2	Sk-Mel- 28	Sk-Mel- 31	Sk-Mel-28 (IC90)
Pathway	Gene	Fold Change (RQ Avg Resistant/RQ Avg Naïve) *resistant samples associaed with Up- or Down-regulated gene expression					
MAPK	ELK1	0.312 *DOWN	1.284	0.989	2.609 *UP	2.344 *UP	3.179 *UP
MAPK	MAP2K1 (MEK1)	0.228 *DOWN	0.968	0.92	1.608	2.000 *UP	2.054 *UP
MAPK	MAP2K2 (MEK 2)	0.283 *DOWN	0.898	1.014	1.814	2.780 *UP	2.958 *UP
MAPK	MAPK1 (ERK2)	0.317 *DOWN	1.119	0.953	1.751	2.528 *UP	3.340 *UP
MAPK	MAPK3 (ERK1)	0.293 *DOWN	0.999	1.023	1.656	1.503	5.910 *UP
Stem	NANOG	0.933	1.097	1.106	2.596 *UP	3.224 *UP	4.797 *UP
Stem	POU5F1 (OCT4A)	1.071	1.295	1.614	2.719 *UP	2.731 *UP	13.058 *UP
Stem	ALDH1A1	0.422 *DOWN	5.11 *UP	1.228	0.845	1.702	2.725 *UP
Adhesion	CDH1 (E-Cad)	5.253 *UP	0.114 *DOWN	1.488	1.12	1.708	1.401
Adhesion	CTNNB (β-Cat)	0.229 *DOWN	1.425	1.544	1.347	1.784	3.817 *UP

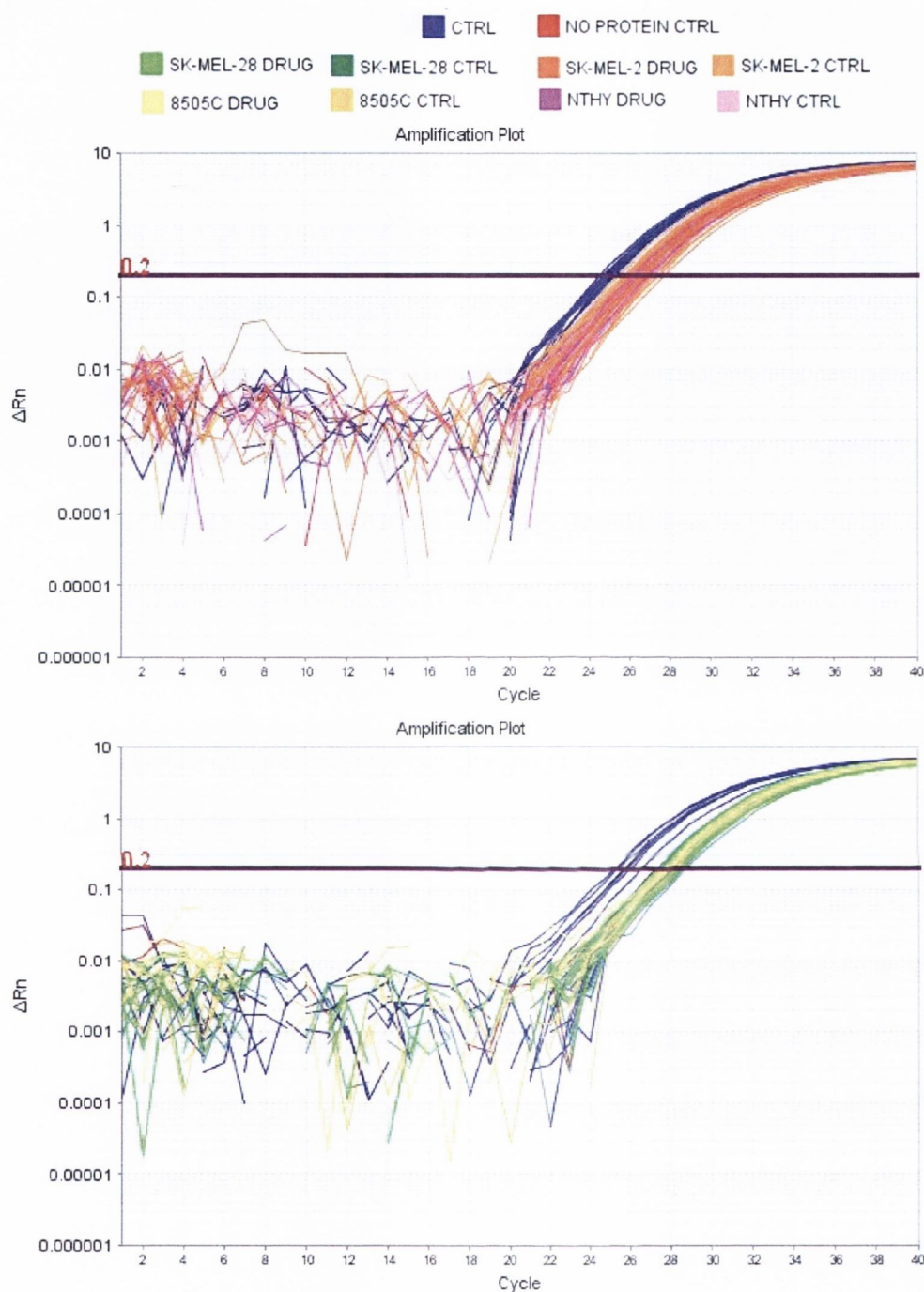


5.3 Figure 4: The stemness marker NANOG is downregulated in MEKi resistant 8505C and ALDH1A1 is upregulated in MEKi resistant SK-MEL-28 protein samples.

Western blot and autoradiography intensity analyses was performed on MEKi resistant and naïve samples to detect the stemness-associated markers: (A) NANOG, (B) OCT4A, (C) ALDH1A1. All samples were normalised to Actin.

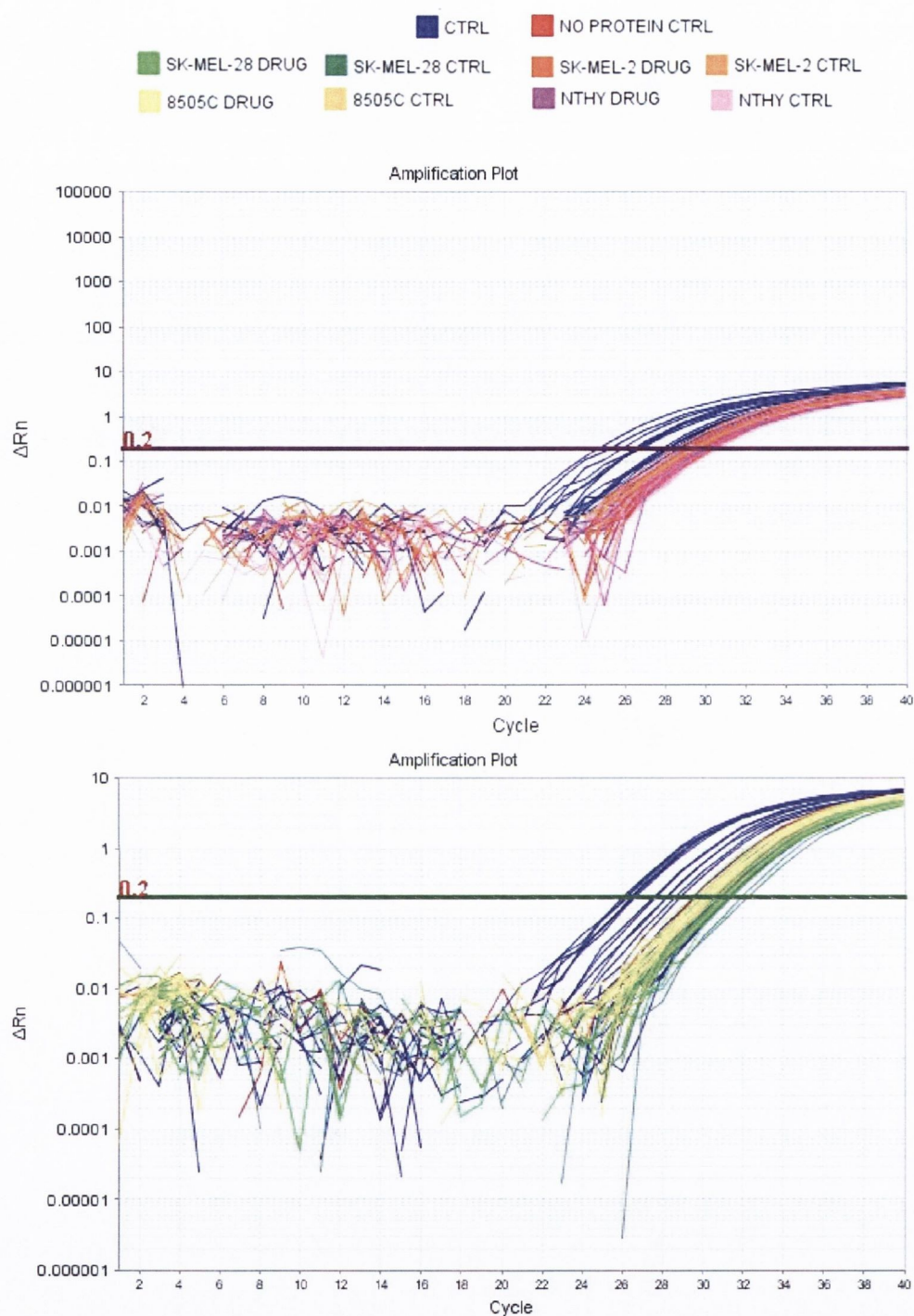
Statistical significance +/- 1.5-fold (*).

X-axis: Cell Line; Y-axis: Intensity target protein/Intensity Actin



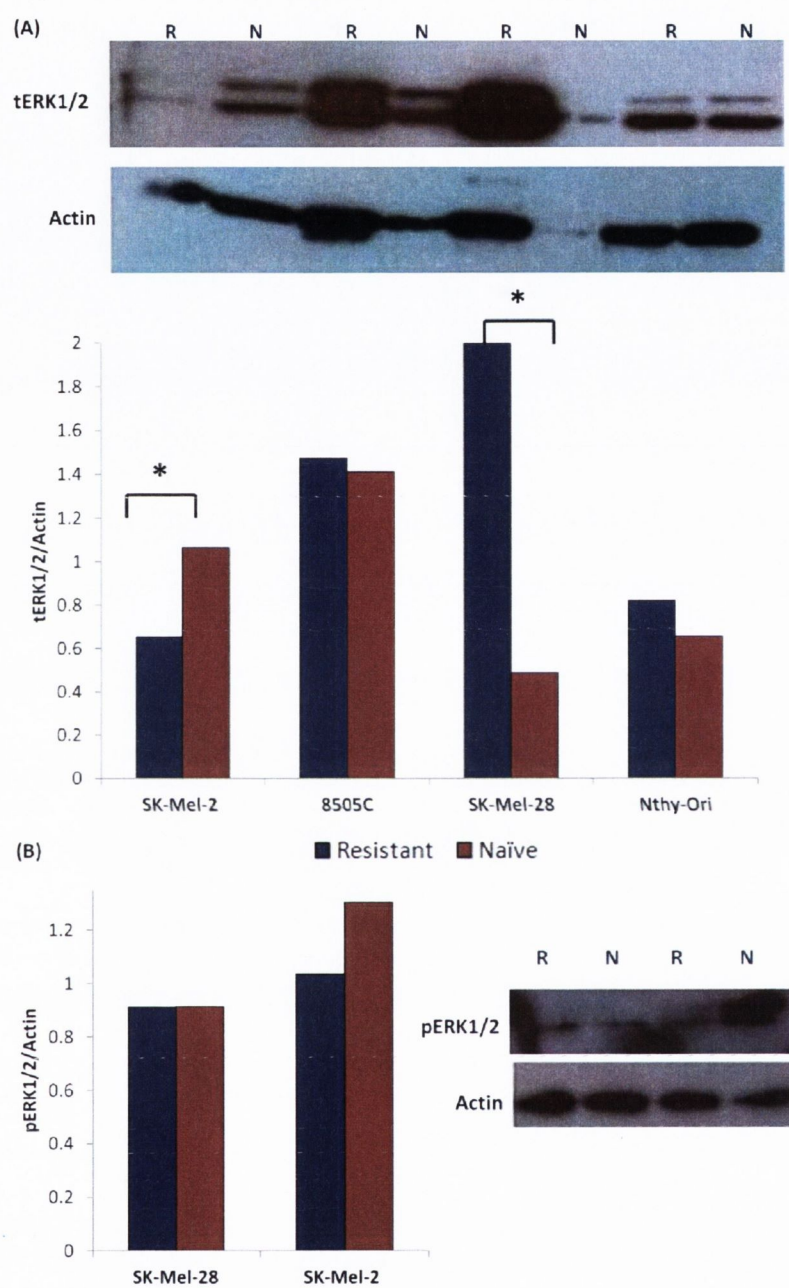
5.3 Figure 5 (A): Background levels are too high to determine relative protein expression using TaqMan™ Protein Assays

Preoptimised TaqMan™ Protein Assays for (A) NANOG were used to detect the relative expression of protein between samples. Technical replicates were highly consistent for all samples as evident by the signal ΔRn /cycle amplification plot. However, the background levels (lysate buffer only/no protein control (red)) did not cross the cycle threshold (CT) at a significantly different cycle compared to the control (blue). For successful assays a minimum 3 CT difference is required between target and no protein controls.



5.3 Figure 5 (B): Background levels are too high to determine relative protein expression using TaqMan™ Protein Assays

Preoptimised TaqMan™ Protein Assays for (B) OCT4A were used to detect the relative expression of protein between samples. Technical replicates were highly consistent for all samples as evident by the signal (ΔRn)/cycle amplification plot. However, the background levels (lysate buffer only/no protein control (red)) did not cross the cycle threshold (CT) at a significantly different cycle compared to the control (blue). For successful assays a minimum 3 CT difference is required between target and no protein controls.



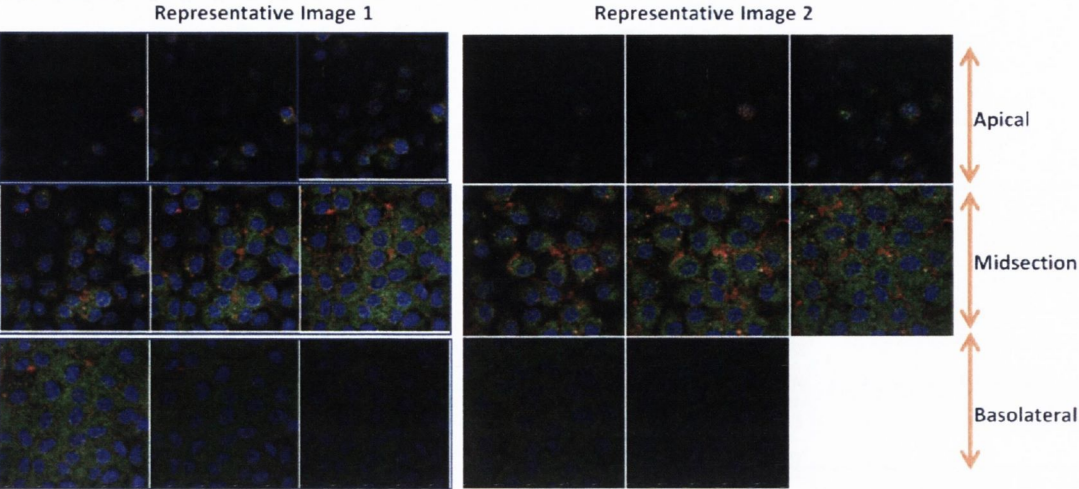
5.3 Figure 6: tERK1/2 but not pERK1/2 is differentially regulated in SK-MEL-28 and SK-MEL-2 MEKi resistant protein samples.

Western blot and autoradiography intensity analysis was performed for MEKi resistant and naïve samples to detect (A) tERK1/2 and (B) pERK1/2. All samples were normalised to Actin.

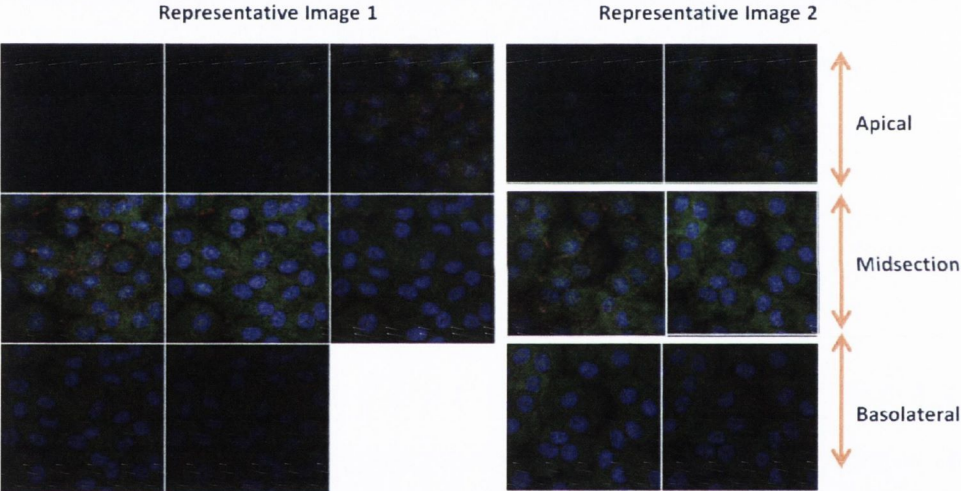
Statistical significance +/- 1.5-fold (*).

X-axis: Cell Line; Y-axis: Intensity target protein/Intensity Actin

(A) Z Stacks Naive:



(B) Z Stacks Resistant:

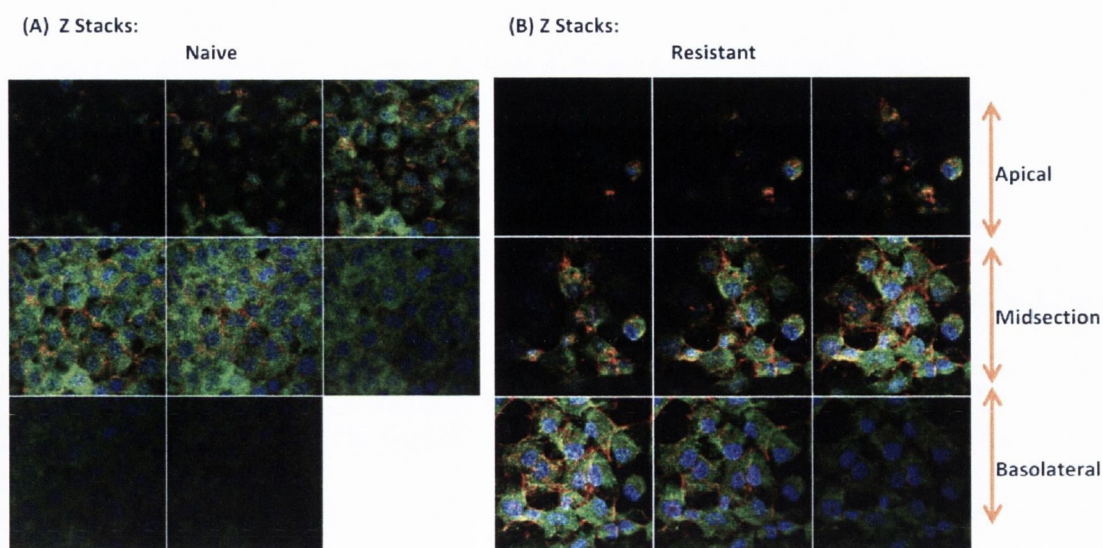


5.3 Figure 7: pERK1/2 was differentially polarised in 8505C MEKi resistant and naïve samples.

Confocal analysis for tERK1/2 detected no change between (A) Naïve and (B) Resistant 8505C samples. pERK1/2 was detected in all 3 layers of the cell in (A) naïve samples whereas it was only detected in the apical and midsection in (B) MEKi resistant samples.

tERK1/2: Green; pERK1/2: Red; Nucleus: Blue

Images taken on Zeiss Confocal LSM under 63X objective lens.

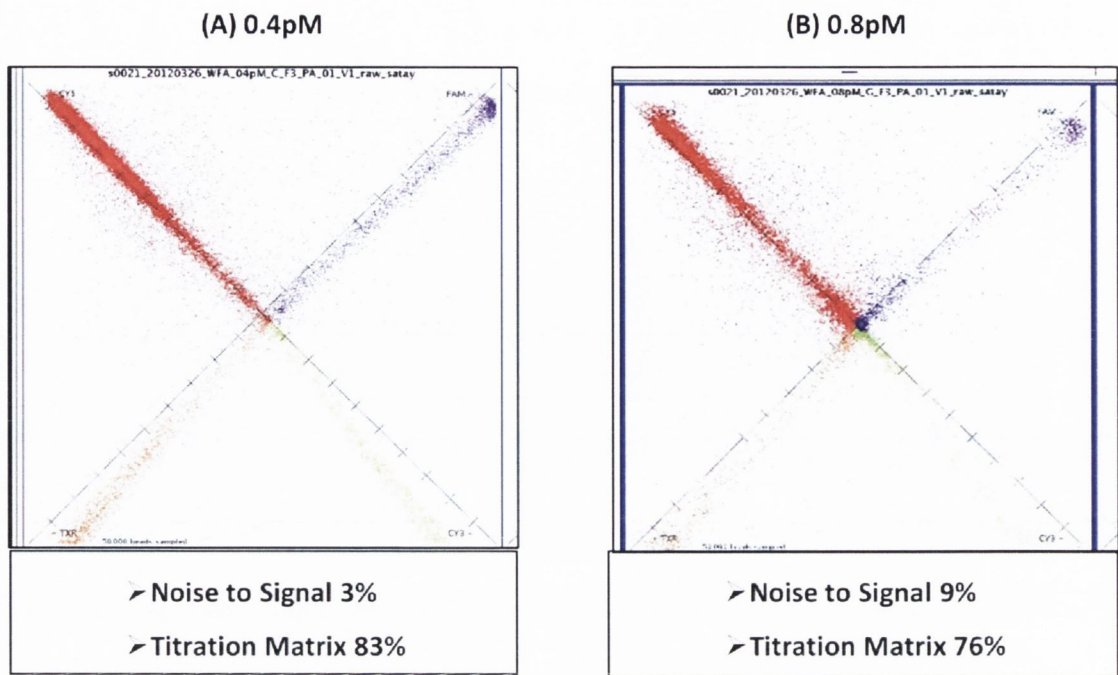


5.3 Figure 8: pERK1/2 was differentially polarised in NThy-Ori MEKi resistant and naive samples.

Confocal analysis for tERK1/2 detected no change between (A) Naïve and (B) Resistant NThy-Ori samples. pERK1/2 was only detected in the apical and midsection in (A) naïve samples whereas pERK1/2 was detected in all 3 layers in (B) MEKi resistant samples.

tERK1/2: Green; pERK1/2: Red; Nucleus: Blue

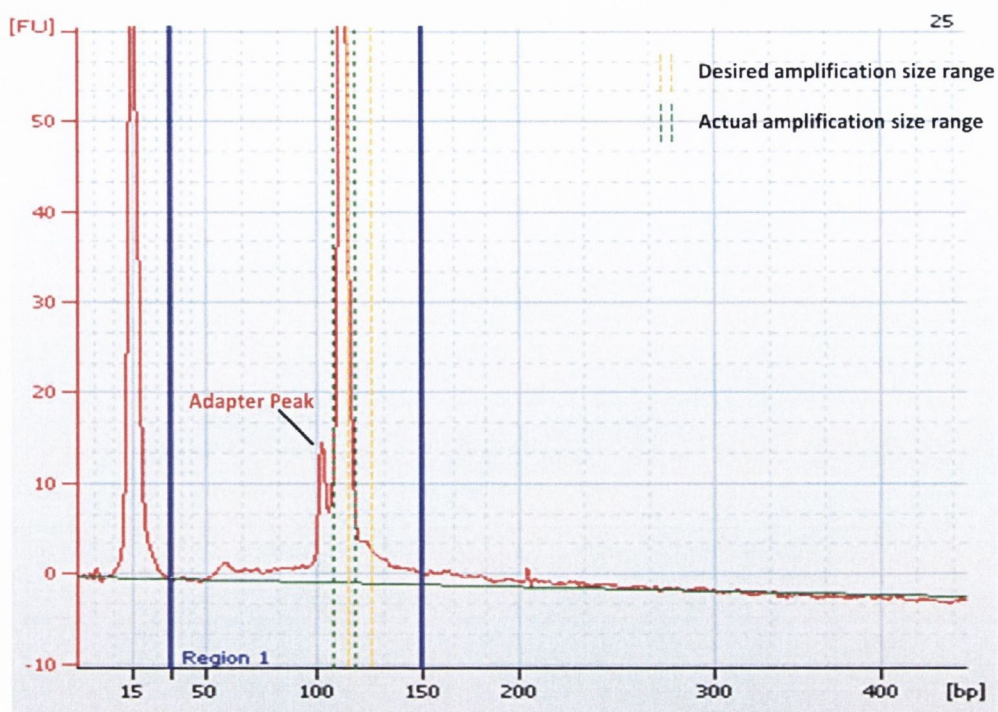
Images taken on Zeiss Confocal LSM under 63X objective lens.



5.3 Figure 9: Workflow Analysis (WFA) for SOLiD NGS

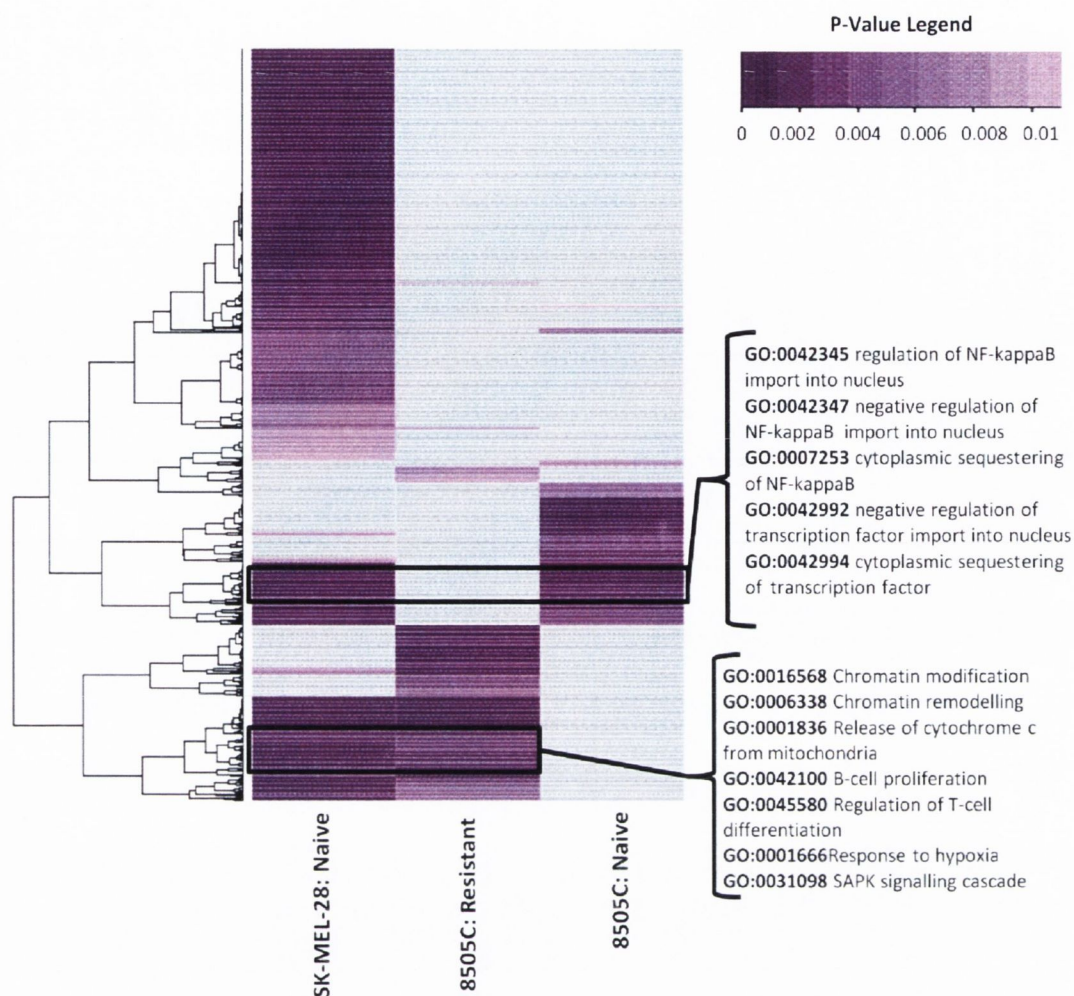
A WFA was performed for each successful ePCR for both the (A) 0.4pM and (B) 0.8pM barcoded sequencing library. Satay plots from a representative ePCR WFA for each library concentration is shown here. Satay plots demonstrate both bead spectral purity and signal intensity. Each point shows the spectral purity and intensity of a single sequenced template (bead). Desired beads are spectrally pure and thus monochromatic, found within a 9° angle window of the axis. The noise-to-signal ratio (N2S) is the ratio between the brightest and second brightest dye and is an index of polyclonal beads, but deposition can also influence N2S: if beads are not properly declumped prior to deposition they can increase noise. The titration matrix = (% $p2^+$ beads) $\times$ (% On axis (within 10°) beads).

Both libraries meet the criteria required for sequencing: >75% on-axisbeads and <25% N2S. A higher titration matrix is desired for optimal sequencing and therefore the 0.4pM library is preferred for this and future sequencing runs.



5.3 Figure 10: Error in cDNA size selection during library preparation resulted in amplified libraries covering a range of basepairs smaller than that recommended in the protocol.

This figure shows the Agilent Bioanalyzer electropherogram and smear analysis of the cDNA library for the 8505C MEKi-resistant library. The yellow bands indicate the recommended size range for small RNA sequencing; whereas the green bands indicate the actual size range amplified for this library. This shift is consistent across the cDNA libraries for all samples and still contains the miRNA fraction.



5.3 Figure 11: Pattern of GO terms significantly over-represented in homologous BRAF^{V600E} samples.

GOSat was used to identify overrepresented ($P < 0.01$) GO terms in the computationally determined validated mRNA targets of each sample (MirWalk). Overrepresented terms that were common to both naïve and resistant samples within each cell line were filtered out (Appendix 3 tables 17 and 19).

Purple: Overrepresented terms ($P < 0.01$). Purple darkens with significance from light purple ($p = 0.01$) to dark purple ($p = 0$)

Grey: Not significant/associated

X-axis: sample group; Y-axis: GO Terms

5.3 Table 2: The proportion of annotated miRNA reads per mapped sample sequenced by SOLiD™ NGS miRNA profiling (MiRNAs annotated using MiRBase).

	Total Mapped Reads	Total Mapped miRNA Reads	% miRNA Reads in Mapped Sample
8505C Resistant	270,788	242,857	89.7%
8505C Naïve	195,489	167,439	85.7%
NThy-Ori Resistant	104,861	19,559	18.7%
NThy-Ori Naïve	282,268	177,658	62.9%
SK-MEL-28 Resistant	8,684	2,750	31.7%
SK-MEL-28 Naïve	16,452	4,777	29.0%
SK-MEL-31 Resistant	365,747	116,335	32.0%
SK-MEL-31 Naïve	127,604	84,140	65.9%

5.3 Table 3: GO terms by BRAF status or tissue of origin (not exhaustive; from appendix 3, tables 17-20)

BRAF ^{WT/WT} Cell line Only (Nthy-Ori) Naïve-Associated
epidermal growth factor receptor signaling pathway
insulin-like growth factor receptor signaling pathway
phosphatidylinositol-mediated signaling
phospholipase C-activating G-protein coupled receptor signaling pathway
response to tumor cell
positive regulation of cyclin-dependent protein kinase activity
BRAF ^{MUT} Cell Lines Only (8505C/SK-MEL-28/SK-MEL-31) Naïve-Associated
neurotransmitter metabolic process
BRAF ^{V600E/V600E} Cell Lines Only (8505C/SK-MEL-28) Naïve-Associated
alcohol metabolic process
Notch signaling pathway
Melanoma Cell Lines Only (SK-MEL-28/SK-MEL-31) Naïve-Associated
response to retinoic acid
response to vitamin A
response to vitamin D
negative regulation of protein kinase B (AKT) signaling cascade
regulation of protein kinase B signaling cascade
cell dedifferentiation
re-entry into mitotic cell cycle

5.3 Table 4: Correlation of GO terms across cell lines by biological association (not exhaustive; from appendix 3 tables 17-20)

Go Terms statistically significantly associated with gene targets of miRNAs upregulated in resistant samples (MEKi) or Naïve samples by cell line as identified by GOSTat				
Cell Line	8505C	SK-MEL-28	SK-MEL-31	Nthy-Ori
GO Term	Thyroid V600E/V600E	Melanoma V600E/V600E	Melanoma V600E/WT	Thyroid WT/WT
Translation				
positive regulation of translation	MEKi	Naïve		
regulation of translation		Naïve		
post-translational protein modification		Naïve		
histidyl-tRNA aminoacylation		Naïve		
nuclear-transcribed mRNA catabolic process, deadenylation-dependent decay		Naïve		
Epigenetic				
chromatin modification	MEKi	Naïve		
chromatin remodeling	MEKi	Naïve		
DNA methylation		Naïve		
Methylation		Naïve		
histone modification		Naïve		
positive regulation of gene expression, epigenetic		Naïve		
negative regulation of gene expression, epigenetic			MEKi	
Mitochondria				
mitochondrial membrane organization	MEKi	Naïve		
release of cytochrome c from mitochondria	MEKi	Naïve		
mitochondrial transport		Naïve		
mitochondrion organization		Naïve		
regulation of mitochondrial membrane permeability		Naïve		
Apoptosis				
anti-apoptosis		Naïve	MEKi	
positive regulation of anti-apoptosis	MEKi			Naïve
regulation of anti-apoptosis	MEKi			

	8505C	SK-MEL-28	SK-MEL-31	Nthy-Ori
activation of cysteine-type endopeptidase activity involved in apoptotic process by cytochrome c			MEKi	
apoptotic nuclear change			MEKi	
cellular component disassembly involved in apoptotic process			MEKi	
negative regulation of anti-apoptosis		Naïve		
DNA damage response, signal transduction by p53 class mediator resulting in transcription of p21 class mediator		Naïve		
DNA damage response, signal transduction resulting in induction of apoptosis		Naïve		
induction of apoptosis via death domain receptors		Naïve		
apoptotic mitochondrial changes		Naïve		
Autophagy				
autophagic cell death			Naïve	
ER overload response			MEKi	
Pathways				
positive regulation of small GTPase mediated signal transduction		Naïve	MEKi	
regulation of transforming growth factor beta receptor signaling pathway		Naïve	MEKi	
positive regulation of Rac protein signal transduction	Naïve		MEKi	
positive regulation of JNK cascade	MEKi			Naïve
negative regulation of JAK-STAT cascade			MEKi	Naïve
insulin receptor signaling pathway	MEKi		MEKi	Naïve
insulin-like growth factor receptor signaling pathway				Naïve
negative regulation of insulin receptor signaling pathway			MEKi	
JNK cascade		Naïve		
regulation of JNK cascade				Naïve
phosphatidylinositol-mediated signaling				Naïve
protein kinase B signaling cascade				Naïve

	8505C	SK-MEL-28	SK-MEL-31	Nthy-Ori
phospholipase C-activating G-protein coupled receptor signaling pathway				Naïve
Rho protein signal transduction	Naïve			
vascular endothelial growth factor receptor signaling pathway	Naïve			
Notch signaling pathway	Naive	Naive		
NF-kappaB				
regulation of NF-kappaB import into nucleus	Naive	Naïve		
negative regulation of NF-kappaB import into nucleus	Naive	Naïve		
cytoplasmic sequestering of NF-kappaB	Naive	Naïve		
negative regulation of NF-kappaB transcription factor activity		Naïve		
regulation of I-kappaB kinase/NF-kappaB cascade		Naïve		
Epithelial				
endoderm development		Naïve		
endoderm formation		Naïve		
regulation of epithelial cell differentiation		Naïve		
epithelial cell differentiation		Naïve		
positive regulation of epithelial cell proliferation		Naïve		
epithelial to mesenchymal transition		Naïve		
Mesenchymal				
mesenchymal cell differentiation		Naïve	MEKi	
mesenchymal cell development		Naïve		
mesoderm development		Naïve		
mesodermal cell differentiation	MEKi			
mesodermal cell fate commitment	MEKi			
mesodermal cell fate determination	MEKi			
B Cell				
B cell proliferation	MEKi	Naïve		
positive regulation of B cell proliferation	MEKi	Naïve		

B cell differentiation		Naïve		
	8505C	SK-MEL-28	SK-MEL-31	Nthy-Ori
B cell mediated immunity		Naïve		
positive regulation of B cell activation	MEKi			
T Cell				
alpha-beta T cell activation	MEKi	Naïve		
alpha-beta T cell differentiation	MEKi	Naïve		
CD4-positive, alpha-beta T cell differentiation	MEKi	Naïve		
regulation of T cell differentiation	MEKi	Naïve		
T cell activation involved in immune response	MEKi	Naïve		
T cell differentiation involved in immune response	MEKi	Naïve		
positive regulation of T cell activation	MEKi	Naïve	MEKi	
negative regulation of T cell activation	Naïve			
NK T cell differentiation		Naïve		
positive regulation of NK T cell differentiation		Naïve		
regulation of T cell cytokine production		Naïve		
regulation of T cell mediated immunity		Naïve		
regulation of T-helper cell differentiation		Naïve		
T cell cytokine production		Naïve		
T cell mediated immunity		Naïve		
Response to Stress				
stress-activated protein kinase signaling cascade	MEKi	Naïve		
response to hypoxia	MEKi	Naïve		
cellular response to nutrient	MEKi	Naïve		
regulation of survival gene product expression		Naïve	MEKi	
response to oxidative stress		Naïve		
cellular response to stress			MEKi	
response to heat	MEKi			
Angiogenesis				
regulation of angiogenesis		Naïve	MEKi	
Angiogenesis		Naïve		
positive regulation of angiogenesis	MEKi			

	8505C	SK-MEL-28	SK-MEL-31	Nthy-Ori
Skin				
pigment biosynthetic process	MEKi	Naïve		
epidermis development		Naïve	MEKi	
Anagen	Naïve		MEKi	
developmental pigmentation		Naïve		
response to UV-B			MEKi	
Thyroid				
thyroid gland development	MEKi			

5.4 Discussion

BRAF^{V600E} is commonly mutated in both thyroid and melanoma cancers. Constitutive activation of BRAF^{V600E} results in oncogene addiction and dependency on the MAPK pathway (Cantwell-Dorris *et al.*, 2011). This allows targeting of the MEK1/2 kinase as a molecularly targeted therapy as the presence of BRAF^{V600E} combined with small molecule inhibition of MEK1/2 results in a synthetic lethal phenotype. MEK inhibitors are currently undergoing clinical trials for the treatment of both melanoma and thyroid carcinomas (<http://www.cancer.gov/clinicaltrials>). To date, MEK inhibitors have displayed promising but limited success as a single agent therapy in clinical trials (Flaherty *et al.*, 2012; Kirkwood *et al.*, 2012; Smalley and Flaherty, 2009; Adjei *et al.*, 2008). This highlights the need to better understand the interactions of MEK inhibition in BRAF^{V600E} cells to assist the design of new therapeutic strategies. To this end, MEK inhibition with the potent, selective inhibitor of MEK, PD0325901, was used in conjunction with gene expression analysis, protein analysis and NGS profiling of miRNA to investigate molecular mechanisms associated with resistance to MEK inhibition.

5.4.1 Next Generation Sequencing of Small RNAs on the SOLiD™ 4 Platform

The small RNA fraction of total RNA samples was subjected to hypothesis-neutral small RNA discovery using the Applied Biosystems SOLiD 4™ next generation sequencing platform (CA, USA). The SOLiD system is a ligation-based sequencing platform that enables massively parallel sequencing of clonally amplified DNA fragments linked to magnetic beads. The ligation-based chemistry is based on probe recognition rather than sequential addition, as found in polymerase-based technologies, thereby reducing the rate of sequencing error. Furthermore, the ligation-chemistry allows two-base encoding that discriminates measurement error versus true polymorphisms. Each probe interrogates two bases and via multiple rounds of ligation-based sequencing each base is interrogated twice by two different probes. This dual interrogation reduces sequencing error: for example, a true SNP will have a change at two adjacent positions in the sequence due to two-base encoding, whereas changes at a single position in the sequence are random errors, and can be filtered out using bioinformatic software.

The SOLiD platform uses emulsion PCR to generate clonally amplified DNA libraries in a technique termed BEAMing. BEAMing has four principal components: beads, emulsion, amplification and magnetics. In emulsion PCR (ePCR), a water-in-oil emulsion permits millions of noninteracting amplifications within a milliliter-scale volume (Tawfik *et al.*, 1998). In BEAMing,

the single-molecule emulsion PCR is performed by encapsulating a single primer-immobilized microbead with a single DNA template in individual droplets. Ideally, each aqueous droplet should encapsulate one microbead together with similar quantities of template and reagents to ensure reliable and efficient results. The DNA template is amplified on the microbead in the same droplet resulting in thousands of DNA copies coated on the bead. This prevents the possibility of the mixing of DNA products from different droplets after breaking the emulsion droplets, the use of microbeads maintains monoclonality of the droplets during downstream processing (Zhu *et al.*, 2012, Zhang *et al.*, 2012). The beads generated by ePCR have highly desirable characteristics: high signal density, geometric uniformity, strong feature separation, and a size that is small but still resolvable by inexpensive optics.

The SOLiD platform uses cyclic array multiplex methods. The sequencing platform leverages a single reagent volume to enzymatically manipulate millions of immobilized DNA features in parallel. Reads are built up over successive cycles of imaging-based data acquisition. This provides a hypothesis-neutral means of investigating the transcriptome and identification of both annotated and novel transcripts. A major advantage of this method of profiling compared to the likes of microarray profiling is that all transcript isoforms are detected, whether annotated or novel. This is of particular importance for downstream applications such as functional miRNA analysis as it allows optimised design of pre-miR and anti-miR/siRNA directly to the transcript-of-interest. Transfection with pre-miRs to overexpress miRNAs tend to have a much higher success rate than siRNA/anti-miR knockdown of miRNA. This difference is likely due to the construction of siRNA to the miRNA transcript annotated in the literature. However, the annotated miRNA frequently is not the predominant isoform and thus, RNA interference (RNAi) has a depleted effect. The small nature of mature miRNAs (~22nt) means even a slight variation between isoforms can have potentially dramatic effects. Hence, the importance of targeting the correct isoform for functional studies and the benefit of this method of miRNA profiling.

5.4.2 Resistance to MEK inhibition associated differential regulation of the MAPK pathway in BRAF^{V600E} thyroid and melanoma cells

Key members of the MAPK pathway were analysed on the mRNA and protein levels for their relative expression between paired resistant and naïve samples. Acquisition of resistance did not result statistically significant differential gene expression of any of the MAPK genes analysed in the BRAF^{WT/WT} thyroid or melanoma cell lines. However, in the BRAF^{V600E} thyroid cell lines, resistance to MEKi was associated with downregulation of all MAPK genes' expression;

conversely, BRAF^{V600E} melanoma samples trended towards upregulation in MAPK gene expression in resistant samples. This indicates that resistance to MEKi may be induced by activation of alternative pathway(s) in BRAF^{V600E} thyroid cells, whereas reactivation of the pathway may be a mechanism of resistance in BRAF^{V600E} melanoma cells.

The MAPK pathway member ERK1/2 was assayed on the protein level for both tERK1/2 and the activated form pERK1/2 in both naïve and resistant samples. Total ERK was detected in all samples and displayed no difference in expression between naïve and resistant samples in thyroid cell lines. However, tERK1/2 was differentially regulated in melanoma samples dependent on BRAF status: BRAF^{V600E/V600E} had statistically significant increased expression in resistant cells whereas tERK1/2 expression was decreased in the BRAF^{WT/WT} resistant sample. This pattern did not hold for active ERK, as neither melanoma cell line displayed any difference between pERK1/2 expression levels demonstrating that melanoma cells resistant to MEKi have an active MAPK pathway. In line with evidence from the gene expression studies and consistent with previous reports in the literature, this data indicates that reactivation of the MAPK pathway is associated with resistance to MEKi in melanoma cells (Johannessen *et al.*, 2010; Villaneuva *et al.*, 2010).

5.4.3 MEKi resistance in thyroid cell lines is associated with cytoskeleton rearrangement

pERK1/2 was not detected by Western blot analysis in the thyroid cell lines. However, confocal analysis using the same antibodies detected tERK1/2 and pERK1/2 in both thyroid cell lines. It is unclear why these antibodies worked for confocal analysis but not for immunoblotting. It has previously been reported that MEK inhibition induces downregulation of pERK1/2 within hours of treatment but that pERK1/2 levels quickly rebound within 24 hours (Leboeuf *et al.*, 2008). It is therefore particularly interesting that this study has identified a differential localisation of pERK1/2 within the cell following MEKi. The localisation of pERK1/2 in BRAF^{V600E} naïve samples reflects the localisation in BRAF^{WT} resistant samples and vice-versa. Subcellular localisation of ERK is now recognised as a physiologically relevant means of regulating ERK activity (Aplin *et al.*, 2001). Spatiotemporal regulation of active ERK has been associated with deregulated ERK signalling (Bharadwaj *et al.*, 2008). In response to extracellular signalling, ERK translocates to the nucleus and then relocates to the cytoplasm again. The relocation of nuclear ERK to the cytoplasm is mediated by a MEK-dependent active transport mechanism (Adachi *et al.*, 2000).

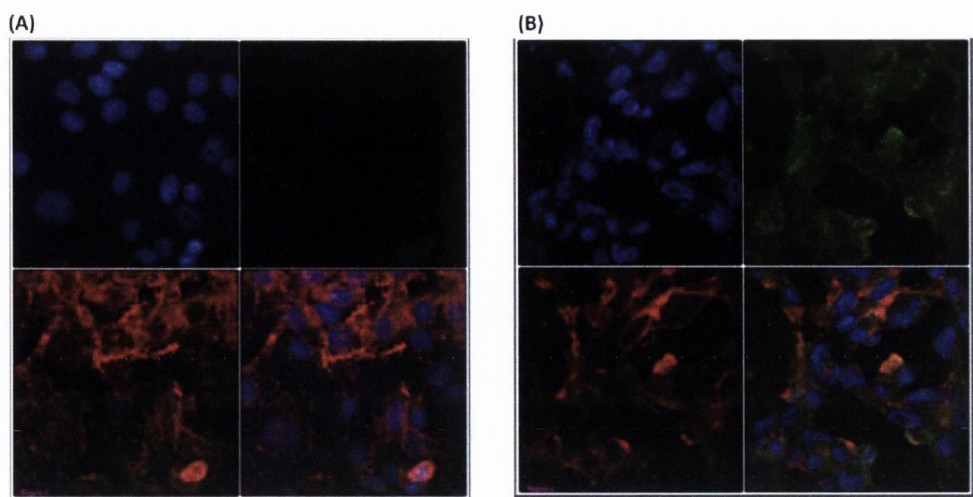
The basolateral layer of the cell is the area of the basal lamina, containing the extracellular matrix (ECM) in epithelial cells. Regulation of the MAPK pathway is closely linked with the ECM. ERK1/2 is anchored to the cytoskeleton by MEK1/2 (Adachi *et al.*, 2000). Association of ERK1/2 with the cytoskeleton is hypothesised to be involved in the transport of ERK1/2 to other organelles of the cell rather than the nucleus (Wortzel and Seger, 2011). For example, the scaffold protein IQGAP1 interacts with BRAF to recruit MEK1/2 and ERK1/2 to actin filaments, thereby influencing mitogenic, morphological and migratory cell behaviour. They can also direct ERK1/2 to influence cytoskeleton reorganisation and cell motility and is believed to be involved in the metastatic potential of BRAF^{V600E} cancers (White *et al.*, 2009; Ren *et al.*, 2007). Inhibition of MEK in lung cancer cell lines (BRAF^{WT}) has been demonstrated to inhibit invasion via cytoskeleton remodelling (Niu *et al.*, 2012).

MiRNA profiling was performed and validated targets of statistically significant miRNAs (significance associated with naïve or resistant samples) were analysed for overrepresentation of GO biological processes in each thyroid sample. Cytoskeleton organisation, microtubule cytoskeleton organisation, ECM organisation and actin filament polymerization were overrepresented in 8505C resistant samples ($p=2.28 \times 10^{-5}$, 0.00607, 0.00312, and 0.00785 respectively). Cell adhesion was highly overrepresented in 8505C resistant cells and in NThy-Ori naïve cells ($p=1.53 \times 10^{-8}$; 8.95×10^{-6}). Consistent with the differential regulation of these terms, expression of *CDH1* (E-cadherin), an integral mediator of cytoskeleton signalling and maintenance of tissue architecture in epithelial cells, is also differentially regulated between 8505C (upregulated) and NThy-Ori (downregulated) resistant cells (5.3 figure 2).

MEK inhibition has an evident differential effect on the MAPK pathway between thyroid cell lines, as evident by gene, protein and miRNA analysis. These differences could be due to BRAF status. However, the patterns of pERK1/2 localisation between thyroid cell lines may also be due to the anaplastic nature of 8505C compared to the differentiated phenotype of NThy-Ori. Further investigation into the role of cytoskeleton-related signalling and MEK inhibition in thyroid carcinomas is warranted. The thyroid cell line panel should be expanded to include a greater range of BRAF^{WT} and BRAF^{V600E} cell lines with varying states of cellular differentiation to determine if the polarised effects are associated with BRAF status, the state of cellular differentiation at the start of treatment, or both.

Recently, work within the research group has demonstrated that sustained MEK inhibition of 8505C cells induces the cells to express the thyroid stimulating hormone receptor (TSHR). Sustained treatment (10 days; 3 sequential IC50 treatments with treatment 3 lasting for

96 rather than 72 hours) induced the re-expression of TSHR (5.4 figure 1). This reexpression was associated with morphological changes indicative of differentiation: elongated flat shape with spindle formation and attachment to other cells and better demarcation of the cellular membrane. Furthermore, actin production and polymerization did not appear to be affected, but demonstrated a different organisation pattern than that seen in naïve 8505C cells. TSHR and its interactions with TSH are essential for normal thyrocyte function. However, in 8505C cells TSHR expression is near-obliterated due to the BRAF^{V600E} mutation (Franco *et al.*, 2011; Durante *et al.*, 2007; Liu *et al.*, 2007). Current treatment of thyroid cancers is based on surgical excision and radioiodine therapy. Loss of TSHR is associated with low to null radioiodine avidity. This data indicates that MEK inhibition prior to radioiodine therapy has the potential to significantly benefit treatment of BRAF^{V600E} carcinomas. Further studies including radioiodine uptake assays in correlation with MEK inhibitor assays should be performed.



5.4 Figure 1: Sustained treatment with the MEK inhibitor PD0325901 restores TSHR expression in the 8505C anaplastic thyroid carcinoma cell line.

Cells were treated for 10 days with (A) media-only or (B) PD0325901 and analysed via confocal analysis for TSHR expression. Following sustained treatment with PD0325901 cells displayed a differentiated phenotype and TSHR expression was restored.

Red: Actin stained with phalloidin tetramethylrhodamine B isothiocyanate.

Green: TSHR (Mouse anti-human TSHR detected with goat anti-mouse Alexa488 conjugate)

Blue: Nuclear staining with 4',6-diamidino-2-phenylindole (DAPI)

From: *Dorris ER, Alhashemi M, Smyth P, Dunne R, Flanagan M, McEneaney V, Dias A, O'Leary JJ and Sheils O (2012) MEK inhibition induces differential mechanisms of resistance in thyroid and melanoma BRAF^{V600E} cell lines. In Prep*

5.4.4 Resistance to MEKi is associated with stemness-markers in BRAF^{V600E} melanoma

Cancer stem cells (CSC) have been known to exist in melanoma populations for over 30 years (Pierce, 1977; Salmon *et al.*, 1980). Recent findings demonstrated that cancer stem cells can convert from a non-stem to a cancer stem cell-like state (Kumar *et al.*, 2012; Chaffer *et al.*, 2011; Gupta *et al.*, 2011). Tumourigenesis and somatic cell reprogramming share common mechanisms; the factors that drive reprogramming tend to be oncogenes or related to cellular transformation (Daley, 2008). The transcription factor OCT4A (POU5F1) is of particular importance for reprogramming of stem-cells and has been reported to function as a single agent to reprogramme adult neural cells to induced pluripotent stem (iPS) cells (Kim *et al.*, 2009). OCT4-mediated dedifferentiation has been demonstrated as a mechanism of acquired CSC phenotype (Kumar *et al.*, 2012). Gene expression analysis in this study identified an upregulation of *OCT4A* in MEK resistant cells in BRAF^{V600E} melanoma cell lines. Furthermore, the CSC markers *ALDH1A1* and *NANOG* were also upregulated in MEKi resistant BRAF^{V600E} melanoma and this upregulation was enhanced with a higher dosage of PD0325901 in SK-MEL-28 cells (5.3 figure 3). However, this pattern of upregulation was not reflected on the protein level. *ALDH1A1* was upregulated on the protein level in resistant SK-MEL-28, but no relative change in protein expression was observed for *NANOG* or *OCT4A* in SK-MEL-28, whereas *OCT4* and *ALDH1A1* protein was not detected in either naïve or resistant samples of SK-MEL-2. The differences observed between the gene and protein expression studies may be due to translational regulation by miRNAs.

MiRNAs have been associated with regulation and maintenance of a stem cell phenotype in recent studies of human ESCs (Ren *et al.*, 2009; Morin *et al.*, 2008). The regulatory hierarchy involved in regulation of OCT4-mediated reprogramming included direct regulation by the *MIR302* cluster of miRNAs. The *MIR302* cluster of miRNAs, found on chromosome 4q, was significantly upregulated in naïve samples compared to resistant samples of SK-MEL-28 and, to a lesser extent, SK-MEL-31 melanoma cell lines. The *MIR302* cluster has a demonstrated role not only in embryonic stem cells but also in the reprogramming of somatic cells to iPSC (Lin *et al.*, 2011b; Lin *et al.*, 2010). Recently, Fareh *et al.*, elegantly demonstrated a role for *MIR302* cluster in cancer stem cell maintenance by demonstrating a *MIR302* family-induced suppression of a stemness gene signature that disrupted the sonic hedgehog (SHH)-*NANOG* network in glioma cancer cells (Fareh *et al.*, 2012).

The molecular mechanisms by which the *MIR302* cluster functions is somewhat conflicted in the literature dependent upon its association with human or mouse ESC; iPSC; or CSC (Bourguignon *et al.*, 2012; Fareh *et al.*, 2012; Subramanyam *et al.*, 2011; Lin *et al.*, 2010; Lipchina *et al.*, 2011; Judson *et al.*, 2009; Ren *et al.*, 2009). The majority of embryonic stem cell studies are performed in mice, however, at least some of the regulatory mechanisms of *MIR302* in humans are fundamentally different to those in murine ESCs; the *MIR302* family promoted transition through G1-S and induced cell proliferation in mice, whereas the *MIR302* family repressed G1-S transition and reduced cell proliferation in human ESCs (Lin *et al.*, 2010). The *MIR302* family has also been associated with regulation of epigenetic remodelling involved with OCT4-mediated reprogramming of human somatic cells (Lin, 2011).

MiRNA profiling and gene ontology analysis identified significant overrepresentation of epigenetic terms in BRAF^{V600E/V600E} melanoma cells. Methylation (p=0.00495), histone modification (p=0.0022) and chromatin modification (p=3.9 x 10⁻⁹) were all overrepresented in naïve samples in SK-MEL-28. Furthermore, *MIR143* is found in naïve SK-MEL-28 and SK-MEL-31 samples only. This miRNA is also involved in epigenetic regulation by directly targeting DNA methyltransferases (Ng *et al.*, 2009). Both *MIR143* and the *MIR302* cluster target *SOX2*, a binding partner of OCT4 and are involved in OCT4-mediated reprogramming (Tay *et al.*, 2008).

Our study has provided ample evidence to warrant further investigation into the relationship between the *MIR302* family, OCT4-NANOG transcription network and resistance to MEK inhibition. Studies in mice using the 2i/LIF dual kinase inhibitor of MEK and glycogen synthase kinase 3 (GSK3), demonstrated that inhibition of MEK activated Nanog to enable somatic cell reprogramming (Theunissen *et al.*, 2011). Interestingly, in the Theunissen study, chemical inhibition of DNA methylation also induced Nanog to enhance reprogramming. The observation that Nanog can induce pluripotency in minimal conditions, including MEK inhibition, has yet to be demonstrated in humans. Considering the significantly increased expression of *NANOG* in MEKi treated BRAF^{V600E} melanoma observed in the current study, in combination with the differential regulation of OCT4-NANOG network-associated miRNAs, further investigation into the interaction of this network during MEKi resistance is merited.

5.4.5 Resistance to MEKi is associated with miRNA regulation of the NFκβ pathway in BRAF^{V600E/V600E} melanoma and thyroid cell lines

Analysis of overrepresented GO terms common to both SK-MEL-28 and 8505C identifies regulation of the NFκβ pathway in naïve samples of both cell lines. NFκβ is transcription factor that regulates many critical pathways including immune and inflammatory responses, cellular growth and apoptosis. The aberrant expression of the NFκβ signalling pathway has been associated with BRAF^{V600E} mutation in both melanoma and thyroid carcinoma (Madonna *et al.*, 2012; Liu and Xing, 2008). NFκβ is a major anti-apoptotic factor that functions by curbing the enzymatic activity of caspases. It is also involved in the promotion of cell growth through its action in enhancing cyclin D1 expression and progression through G1/S transition.

The NFκβ pathway has been implicated in resistance to BRAF inhibitors in both melanoma and thyroid cells. In BRAF^{V600E} melanoma, co-treatment of BRAF inhibitors with tumour necrosis factor α (TNF-α) blocked apoptosis induced by inhibition of the MAPK pathway through an NFκβ-pathway mediated mechanism (Gray-Schopfer *et al.*, 2007). In thyroid cells, combination therapy with PD0325901 and an inhibitor of the NFκβ pathway demonstrated a highly significant additive effect. Furthermore, cells non-sensitive to PD0325901 alone displayed dramatic synergistic inhibition by this combination therapy (Liu and Xing, 2008).

MiRNAs targeting the NFκβ genes *NFKB1/NFKB1A* can be found in naïve samples for all cell lines (appendix 3, tables 13-16). However, GO terms relating to the regulation of the NFκβ pathway are significantly overrepresented ($P < 0.01$) in naïve samples of the BRAF^{V600E/V600E} cell lines only. The presence of these terms in the naïve and not the resistant samples is consistent with previous association of NFκβ signalling in resistance to inhibition of the MAPK pathway (Madonna *et al.*, 2012; Pacifico and Leonardi, 2010; Liu and Xing, 2008; Gray-Schopfer *et al.*, 2007). This indicates that the NFκβ signalling pathway may be involved in resistance to MEK inhibition in a BRAF-specific manner and therapeutic co-targeting the MAPK and NFκβ pathways should be further investigated.

5.4.6 A central axiom of cellular differentiation/self renewal is differentially regulated in response to MEKi in thyroid and melanoma BRAF^{V600E/V600E} cell lines

A theme consistent throughout all analyses is the opposing responses between 8505C and SK-MEL-28 cells. This is clearly seen in the gene expression analysis (5.3 figures 1-3) where MEKi resistant samples demonstrated downregulation of both the MAPK pathway genes and the

stemness markers in 8505C resistant samples. Conversely, the SK-MEL-28 resistant samples trend towards upregulation in both sets of genes. Furthermore, β -catenin (*CTNNB1*), which has been demonstrated to maintain the undifferentiated state of human ESCs and induced pluripotent stem cells (Ding *et al.*, 2010), is down regulated in MEKi treated 8505C and upregulated in IC90 MEKi treated SK-MEL-28. It has previously been demonstrated that the MAPK pathway is downregulated upon differentiation (Armstrong *et al.*, 2006). Consistent with this is the observed downregulation of the MAPK pathway in resistant 8505C but not SK-MEL-28. The evidence on the protein level is not as clear-cut; however, although the stemness marker NANOG was downregulated in 8505C whereas the stemness marker ALDH1A1 was upregulated in SK-MEL-28 (5.3 figures 4 and 6). The polarised pattern was again reflected in the miRNA profiles for each cell line, most notably in regards to the *MIR302* and *MIR520* clusters. The *MIR302* cluster was upregulated in naïve relative to resistant SK-MEL-28 samples whereas the *MIR520* cluster was upregulated in MEKi resistant relative to naïve 8505C samples. The *MIR520* cluster located on chromosome 19 shares a consensus seed sequence (AAGUGC) with the *MIR302* cluster (chromosome 4) and their predicted target genes tend to overlap and they display functional similarity (Mazda *et al.*, 2011; Ren *et al.*, 2009).

Gene ontology analysis confirms differential inferred miRNA functions between SK-MEL-28 and 8505C. Many GO terms statistically significantly ($P < 0.01$, Benjamini false error rate correction) associated with naïve samples in SK-MEL-28 are significantly associated with resistant samples in 8505C. From 5.3 table 3, it can be seen that regulation of translation, chromatin modification and remodelling, response to hypoxia and nutrients and regulation of the stress-activated protein kinase signalling pathway (SAPK), a structurally similar but functionally distinct pathway to the MAPK pathway activated in response to stress, are differentially associated between naïve SK-MEL-28 and resistant 8505C samples.

The GO terms also demonstrate differential association with immune-system related terms. B cell proliferation and T cell activation, regulation and differentiation are all differentially regulated between 8505C resistant and SK-MEL-28 naïve samples. The state of differentiation of a cell has a significant effect on the immune response; as tumours dedifferentiate they can express surface antigens not usually associated with adult tissue and as such, reveal a potential avenue for immunotherapy. The differential associations with these GO terms supports the hypothesis that treatment with PD0325901 induces the anaplastic cell line 8505C into a more differentiated state, as supported by MEKi-induced reexpression of the TSHR receptor (5.4 figure 1); whereas SK-MEL-28 is induced into a more stem-like state. Thus, further investigation into

the mechanisms of the differential response to MEKi observed between the BRAF^{V600E} thyroid and melanoma cells is merited and an initial investigation into the role of the *MIR302/MIR520* clusters in this response should be performed.

5.5 Conclusions

In this study, we have identified a gene and miRNA signature differentially regulated by MEK inhibition in thyroid and melanoma BRAF^{V600E} cells. Evidence from the analysis of gene, protein and miRNA expression, has highlighted a central axiom associated with induced differentiation/dedifferentiation that is being alternatively regulated by MEKi in homozygous BRAF^{V600E} thyroid and melanoma cells. A MEKi-associated signature of MAPK and stemness-associated genes is downregulated in the resistant thyroid cell line 8505C but expression trends towards upregulation in the resistant melanoma BRAF^{V600E} cells. This signature is observed in neither thyroid nor melanoma BRAF^{WT/WT} cell lines. Furthermore, differential regulation of ESCC-associated miRNAs is also observed between the BRAF^{V600E/V600E} cell lines. The consensus seed sequence-sharing and functionally similar miRNA clusters *MIR302* and *MIR520* are associated with naïve SK-MEL-28 samples and resistant 8505C samples respectively. Whether this differential association is due to tissue specificity or the *ab initio* state of cellular differentiation of the cell models has yet to be elucidated. However, if the polarised effect induced by MEKi is due to the state of cellular differentiation at the start of treatment it could have significant consequences on the combinatorial treatment required for primary and metastatic lesions. Furthermore, consistent with previous reports, we have identified the NFκβ signalling pathway as a possible BRAF^{V600E/V600E} specific mechanism of resistance to MAPK inhibition.

Chapter 6: Discussion and Future Directions

6.1 Introduction

Up to 80% of malignant melanomas and 45% of thyroid cancer have activating mutations in the RAS/RAF/MEK/ERK pathway resulting in tumour growth and progression. These tumours initially respond well to targeted therapy of this pathway but resistance invariably occurs. For most advanced cancers response to targeted therapy is short-lived, owing to evolution of resistant populations under the selective pressure of therapy. The large-scale genomic alterations and consequent diversity of tumours impart a predictable emergence of resistance as a property of carcinogenesis itself. Although emergence of resistance seems inevitable, the proliferation of the resistant populations is not. Cancer cells cannot predict future evolutionary or environmental dynamics and only adapt to immediate selection forces. A fundamental advantage in designing new therapeutic strategies is the ability to anticipate, such that knowledge of tumour evolution can be employed to direct Darwinian processes for the prevention of outgrowth of resistant populations thereby improving patient outcomes. In this project we focused on the molecular pathology of BRAF^{V600E} mutant malignant melanoma and thyroid carcinoma in response to targeted therapy of the MAPK pathway and whether resistance is mediated by regulatory small RNAs. Elucidation of the identified resistance mechanisms will contribute to the arsenal of anticipatory strategy in the development of long-term therapeutic strategies.

In this thesis gene expression, protein, and miRNA analyses were performed to investigate the molecular pathology of MEK inhibitor resistance in BRAF^{V600E} and BRAF^{WT} melanoma and thyroid cell lines. In order to demonstrate the relevance of miRNA profiling in an Irish-specific context, Chapter 3 of this study investigated the association of a specific miRNA, *MIR141*, with different thyroid malignancies and identified its potential use as a diagnostic biomarker. The association of *MIR141* with thyroid tissues had been identified in a previous miRNA profiling experiment within the laboratory using microarray technology (Aherne *et al.*, 2008). This chapter demonstrated how data from profiling studies can be carried through to clinical samples and produce a clinically relevant effect, as demonstrated by the ability of differential *MIR141* expression to distinguish Hashimoto Thyroiditis from both cPTC and control thyrocytes. Chapter 4 discussed the optimisation of the MEK inhibition assays to be used in chapter 5. This chapter illustrated the necessity to genotype all cell lines for use in a study focused on a specific molecular target regardless of their documentation in the literature, however recent. A potential folly was averted by genotyping all cell lines for the BRAF^{T1799A} SNP, as SK-MEL-31 which had been documented as BRAF^{WT} (Xing *et al.*, 2011; Solit *et al.*, 2006), was in fact heterozygous for BRAF^{V600E}. BRAF mutational status is associated with response to MEK inhibition (Leboeuf *et al.*, 2008; Solit *et al.*, 2006). Due to the nature of small molecule

kinase inhibitors, the IC50 can vary widely depending upon the biological heterogeneity of the cell and its reliance upon the MAPK pathway. As expected from previous reports in the literature, the melanoma cell lines were exquisitely more sensitive to MEK inhibition than the thyroid cell lines. SK-MEL-28, carrying homozygous BRAF^{V600E}, had the lowest IC50 dosage and shortest treatment time, half the length of the optimal treatment time for both the heterozygous and homozygous BRAF^{WT} melanoma cell line. The homologous BRAF^{V600E} thyroid cell line demonstrated a higher IC50 than was expected from previous reports in the literature of BRAF^{V600E} thyroid sensitivity (Henderson *et al.*, 2010; Leboeuf *et al.*, 2008). However, the assays were repeated on three distinct biological replicates, with a technical replicates in quadruplicate and the R-Sq for the 8505C IC50 dosage response curve was 97.7%. A possible explanation for the relatively high IC50 could be the underlying state of differentiation: 8505C is an anaplastic cell line and as such may be less susceptible to MEK inhibition, reinforcing the requirement to carry out these optimisation experiments.

The thesis culminated in chapter 5, where the molecular pathology of MEKi resistance in BRAF^{V600E} thyroid and melanoma cell lines was elucidated through a series of gene and protein expression analyses, and next generation sequencing of miRNAs in cells surviving drug (resistant) or media (naïve) treatments. Integrative analysis from these experiments provides evidence for the role of pathways involved in cell fate/self renewal determination and the NFκβ signalling pathway in resistance to MEKi. The embryonic stem cell cell-cycle related (ESCC) miRNA clusters *MIR302* and *MIR520*, sometimes described as the miR-302/372/373/520 family (Mazda *et al.*, 2011), were identified as potential regulators of MEKi resistance.

6.2 The cellular differentiation axis and response to MEKi in BRAF^{V600E} carcinoma

In this study, treatment with MEKi induced opposite roles in BRAF^{V600E/V600E} melanoma and thyroid cell lines. Resistance to MEKi was associated with the promotion of differentiation in 8505C and enhanced stemness in SK-MEL-28. A signature of stemness-associated and MAPK genes were upregulated in the SK-MEL-28 cell line following MEKi whereas MAPK genes were significantly downregulated in 8505C MEKi resistant cell lines. The reinstatement of cell surface receptors and morphological changes associated with a differentiated phenotype following sustained MEKi, in combination with the downregulation of NANOG protein and the stemness genes *ALDH1A1* and *CTNNB1*, indicates that resistance to MEKi is associated with induced differentiation of the anaplastic 8505C cell line. Conversely, upregulation of stemness-associated

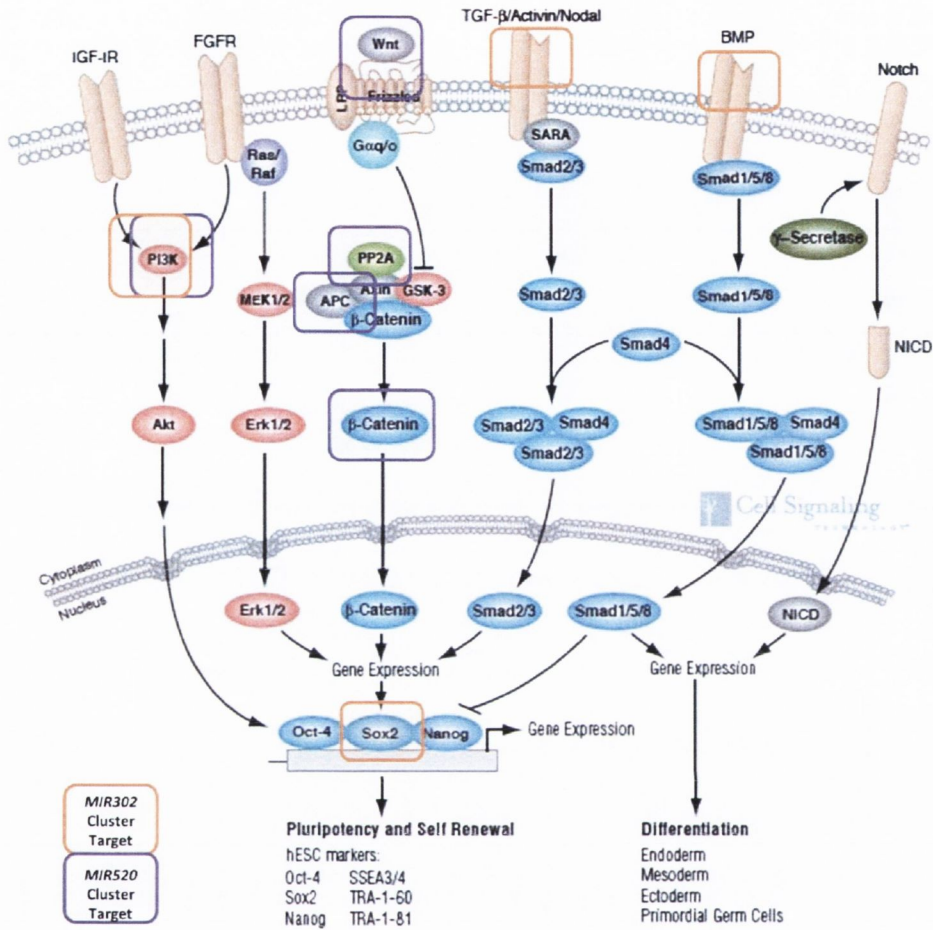
genes (*OCT4A*, *NANOG*, *ALDH1A1* and *CTNNB1*) and *ALDH1A1* protein following MEKi treatment indicates that an induced state of pluripotency is associated with MEKi resistance in SK-MEL-28. The reexpression of TSHR in 8505C cells treated with MEKi demonstrates the potential for pretreatment of low avidity thyroid cancers with MEKi followed by radioactive iodine therapy to improve the treatment of thyroid cancers with radiotherapy.

The pathways governing differentiation and pluripotency in ESCs control both lineage specification and regulation of pluripotency in a context specific manner. The canonical WNT pathway plays a major role in regulating pluripotency in both ESC and adult stem cells from various tissues (Sokol, 2011). Stage specific effects of the different WNT pathways produce complex and sometimes opposite outcomes, which allows the generation of embryonic cell diversity. How the balance between differentiation and pluripotency is achieved remains largely disputed. However, regulation of these pathways by miRNAs is one mechanism. In this study, the ESCC *MIR302* cluster was upregulated in naïve SK-MEL-28 samples whereas the related ESCC *MIR520* cluster was upregulated in resistant 8505C samples compared to naïve samples. The differential regulation of these clusters may be concerned with the differential regulation of the central axiom of differentiation between these cell lines.

Multiple members of the WNT signalling pathway are targeted by members of the *MIR520* cluster (6.2 figure 1). Key regulators of WNT signalling, including WNT genes (*WNT5A*), regulatory molecules such as the adenomatous polyposis coli (*APC2*) and protein phosphatase 2A activator, regulatory subunit 4 (*PPP2R4/PP2A*) and the crucial mediator of canonical WNT signalling β -catenin (*CTNNB1*) are regulated by the *MIR520* cluster (MIRWalk validated targets Dweep *et al.*, 2011). *MIR517* was greater than 10-fold upregulated in resistant compared to naïve samples of 8505C. In the same samples, the *MIR517* target *β -catenin* was significantly downregulated in resistant compared to naïve samples. Previous studies have shown that β -catenin can maintain the undifferentiated state of human ESC and iPS (Ding *et al.*, 2010). The mechanism by which β -catenin promotes both human and mouse ESCs to self-renew has been attributed to its effect on the OCT4/NANOG regulatory network (Yun *et al.*, 2012; Sokol 2011).

The MAPK, PI3K/AKT and TGF β pathways are also involved in the regulation of the differentiation/pluripotency axis through the regulation of the OCT4/NANOG network (6.2 figure 1). These pathways also interact with each other. The PI3K/AKT pathway has previously been demonstrated to inhibit the MAPK pathway dependent on the differentiation state of the cell (Rommel *et al.*, 1999). Crosstalk occurs between pathways by direct interaction of AKT mediated by its phosphorylation of RAF; which may switch the biological response from

ESC Pluripotency and Differentiation



6.2 Figure 1: Signalling pathways involved in ESC pluripotency and differentiation.

Multiple pathways converge on the OCT4/NANOG regulatory network to regulate cell fate and self-renewal. Multiple members of the pathway are targeted by the *MIR302* and *MIR520* clusters, with *MIR520* targets concentrated on the WNT signalling pathway and *MIR302* targets targeting TGFβ/BMP signalling. Both clusters target PI3K signalling but no validated targets in the MAPK have been identified for either cluster.

Orange box: *MIR302* target; Purple box: *MIR520* targets

Figure adapted from Cell Signalling Technologies (USA)

(<http://www.cellsignal.com/pathways/wnt-hedgehog.jsp>)

growth arrest to proliferation (Zimmermann and Moelling, 1999). Members of both *MIR520* and *MIR302* clusters target PI3K (*PIK3CA*). Transforming growth factor- β (TGF β)/bone morphogenic protein (BMP) signals through a complex of SMAD proteins in a context specific manner. Both mesenchymal and epithelial cells display canonical TGF β pathway signalling; however, this pathway induces growth inhibition in epithelial cells whereas mesenchymal cells are growth stimulated by TGF β (Hough *et al.*, 2012).

Crosstalk between TGF β and MAPK has been well documented (Blanchette *et al.*, 2001; Kretzschmar *et al.*, 1999; Kretzschmar *et al.*, 1997) but the mechanism by which this occurs is still largely unknown. One mechanism that describes cell-type dependent crosstalk involves TGF β activation of PI3K to activate the p21-activated kinase 2 (PAK2), which in turn activates CRAF leading to ERK activation (Hough *et al.*, 2012). This signalling mechanism was found in mesenchymal but not epithelial cells. TGF β 1 and BMP are targeted by *MIR302* whereas PAK is targeted by *MIR520*. These pathways all converge on OCT4/NANOG to determine cell fate or self-renewal. Interestingly, neither OCT4 nor NANOG have any predicted miRNA targets. However, SOX2, which forms a trimeric complex with OCT4 and NANOG, is targeted by the *MIR302* family.

The crosstalk between pathways involved in cell fate and self-renewal and the targeting of multiple levels of the hierarchical signalling by *MIR302* and *MIR520* provides ample circumstantial evidence for further investigation into the role of *MIR302*, *MIR520* and MEK inhibition in the regulation of the cellular differentiation axis in BRAF^{V600E/V600E} carcinoma. Taken in combination with the empirical evidence of differential gene and protein signatures identified in this study, we postulate that regulation of the axis of cellular differentiation plays an important role in the acquisition of resistance to MEK inhibition and deserves further investigation.

BRAF mutational status is important in treatment with MAPK inhibitors. However, the genomic background beyond BRAF has also been demonstrated to impact on response to MAPK inhibitor treatment in melanoma (Corcoran *et al.*, 2010, Wagle *et al.* 2011, Su *et al.* 2012). Therefore, the cell line models of melanoma and thyroid cancer used in this study should be expanded to represent multiple genomic states. The cell line panel should be screened for the identified profiles of stemness-associated genes and miRNAs in response to MEK inhibition to further validate the evidence-based effects identified in this thesis.

6.2.1 Future Work: Functional genomics of *MIR302* and *MIR520* clusters and response to MEKi

Functional genomics to investigate the impact of miRNA (*MIR302* and *MIR520*) modulation on the stem cell phenotype and resistance to therapy should be performed. Knockdown (RNA interference (RNAi)) and overexpression (mimic/transfection) assays should be employed for functional evaluation (Kuhn *et al.*, 2008). “Stemness” assays (proteomic and genomic characterisation of melanoma and thyroid cancer stemness-associated markers combined with drug survival assays) could be used to characterise the consequent biological response in a pluripotency/resistance context. Data from the NGS sequencing run should be employed to design optimal RNAi and miRNA mimics for the transfection assays based on the most abundant miRNA isoforms. Furthermore, considering *MIR302* and *MIR520* share a common consensus sequence, mutation assays of this sequence and their effect on *MIR302/MIR520* function should also be investigated.

6.2.2 Future Work: Functional genomics of *NANOG* and *OCT4A* and response to MEKi

The cellular differentiation/self-renewal axis converges on the OCT4/*NANOG* regulatory network. *NANOG* has been demonstrated to be able to overcome MEK inhibition and induce progression to pluripotency in mouse ESC (Theunissen *et al.*, 2011). Conditional in-vitro experiments for the key pluripotency factors OCT4 and *NANOG* should be performed. Chemical or RNAi mediated knockdown of *NANOG* and OCT4, with Western blotting to confirm knockdown, could be used in combination with a phenotypic-response assay based on response to MEK inhibitor treatment to demonstrate the effect of knockdown on cell fate decision and survival.

6.3 NFκβ and response to MEKi

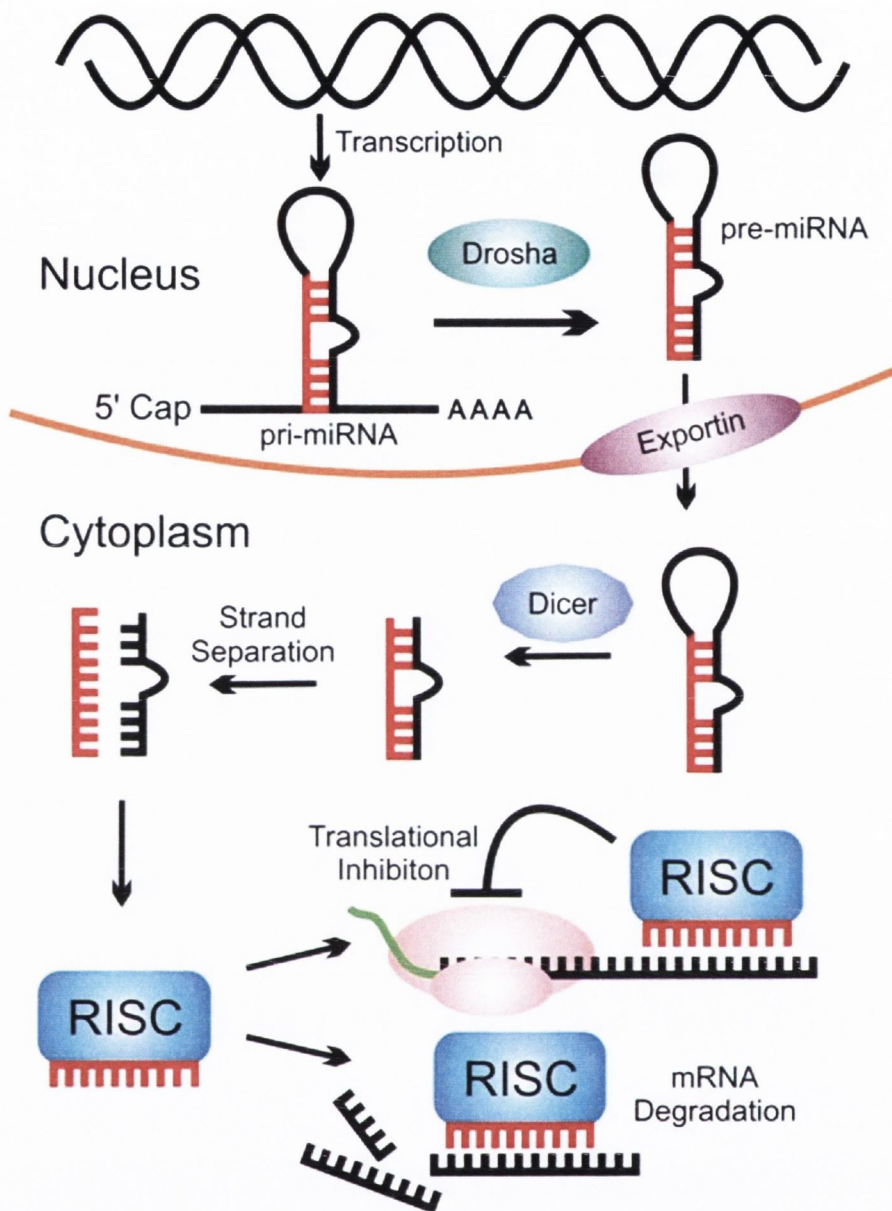
NFκβ and the inhibitor of NFκβ (IKK) proteins regulate many physiological processes in the cell. Disruption of NFκβ and IKK has been demonstrated to contribute to cancer. The NFκβ pathway has considerable crosstalk with other signalling pathways including the MAPK pathway (Perkins, 2007). This crosstalk is involved in cell fate decision-making processes and has also been implicated as a mechanism of resistance to BRAF inhibitors in malignant melanoma. The genes regulated by NFκβ include those controlling apoptosis, cell adhesion, proliferation, the innate-

and adaptive-immune responses, inflammation, the cellular-stress response and tissue remodelling (Hayden *et al.*, 2004; Pahl *et al.*, 1999). Overexpression of the MAPK pathway negatively regulates NFκβ transcriptional activity.

In NFκβ inactive cells, a subunit of NFκβ, p105, complexes with the MAPK-associated mitogen activated protein kinase kinase kinase 8 (MAP3K8 or COT). Activation of the NFκβ pathway results in degradation of p105 and the derepression of COT, which activates CRAF and can reinstate MAPK signalling. This COT-mediated reinstatement of MAPK signalling has been identified as a mechanism of resistance to BRAF inhibitors in melanoma cell lines and a subset of BRAF inhibitor treated relapsed tumour samples. Furthermore, ectopic expression of COT in combination with MEK inhibition resulted in increased survival, indicating a MEK-independent mechanism of ERK activation (Johannessen *et al.*, 2010). However, combination therapy of BRAF and MEK inhibitors potentiated the effect of ectopic COT expression.

MiRNAs targeting *NFKB1/NFKB1A* can be found in naïve samples for all cell lines, however GO terms relating to the regulation of the NFκβ pathway are significantly overrepresented ($P < 0.01$) in naïve samples of the BRAF^{V600E/V600E} cell lines only. The presence of these terms in the naïve and not the resistant samples is consistent with previous association of NFκβ signalling in resistance to inhibition of the MAPK pathway (Madonna *et al.*, 2012; Pacifico and Leonardi, 2010; Liu and Xing, 2008; Gray-Schopfer *et al.*, 2007). MAPK can participate in the activation of NFκβ in the cytoplasm as well as in the modulation of its transactivation potential in the nucleus (Dhawan and Richmond, 2002). This is reflected in the associated GO terms including negative regulation of NFκβ import into the nucleus and cytoplasmic sequestering of NFκβ which are found overrepresented in naïve samples only.

The association with the NFκβ pathway in the miRNA data and the recent association of NFκβ/COT in resistance to MAPK inhibition indicate a role for this pathway that warrants further investigation. Combined therapy targeting both the MAPK and NFκβ pathways may be a strategy for better treatment (section 6.6). It should be noted, however, that previous studies co-targeting PI3K in combination with NFκβ resulted in the inactivation of the secondary inhibitor. Curcumin, an NFκβ inhibitor, ablated the ability of the AKT inhibitor to suppress AKT phosphorylation. Hence, extensive investigation into the molecular mechanisms induced by synergistic treatment would be required.



6.4 Figure 1: Biogenesis of miRNAs.

MiRNA is transcribed as pri-miRNA: a stem loop structure with a 5' cap and polyadenylated tail by RNA polymerase II. Pri-miRNA is processed to pre-miRNA by DROSHA to produce the classic hairpin structure which is exported out of the nucleus. In the cytoplasm, they are further processed by a complex involving DICER to produce mature miRNA. Mature miRNA can then be incorporated in the RNA-induced silencing complex (RISC) whereby they carry out their function of targeted mRNA degradation.

Diagram from O'Connor et al, 2012 *Molecular Psychiatry* 17:359-376

6.4 Future Work: MiRNA profiling: identification of novel miRNA

The next generation sequencing (NGS) used in this thesis investigated the transcriptomic profile of small RNAs in samples naïve and resistant to MEK inhibition in melanoma and thyroid cell lines with different BRAF^{V600E} status. While we analysed previously annotated and identified miRNAs within the transcriptome of the cell line matrix central to this study, a wealth of data remains for novel miRNA discovery. A major advantage of using a hypothesis neutral method of massively parallel sequencing for miRNA profiling is the precise identification of miRNA sequences. The application of NGS using this method, termed RNA-Seq, and can readily distinguish miRNAs that differ by a single nucleotide and isomers of varying length (Pritchard *et al.*, 2012). A plethora of small RNAs of novel sequence may be found in RNA-Seq data; however, only a small number of these putative miRNAs may be authentic miRNAs (Chiang *et al.*, 2010).

Criteria have been established for the annotation of small RNA sequences as miRNAs including: mature sequence length of approximately 22nt; conservation across species (preferably); genomic origin that predicts a precursor RNA sequence that can fold into a duplex structure (6.4 figure1); identification of sequence reads that correspond to both 3' and 5' arms of the putative pre-miRNA hairpin (Pritchard *et al.*, 2012). Bioinformatic analysis of the sequence data can be used to predict these criteria; however, elaboration of the methods and algorithms employed in this bioinformatic analysis is beyond the scope of this thesis. Future work to identify novel miRNAs and the experimental validation of their targets could highlight new networks involved in resistance to MEK inhibition, increasing our understanding of the underlying biology and potentially providing secondary drug targets.

6.5 Future Work: Analysis of other small RNA species

The data from the small RNA fraction sequenced via RNA-Seq contains a plethora of expression information on all small non-coding RNAs found within the 10-40nt region. Research is only starting to highlight the nature and extent of involvement of other species of small non-coding RNAs in health and disease. Types of small to medium regulatory RNAs include: transcription initiation RNA (tiRNA, 17-18 nt); PIWI-interacting RNA (piRNA, 26-31nt); transcriptional start site-associated RNAs (TSSa-RNA, 20-90nt); promoter associated small RNA (PASR, 22-200nt); small nucleolar RNA (snoRNA, 60-300nt); and promoter upstream transcripts (PROMPTs, <200nt). These elements are emerging as key regulators of cellular homeostasis and their dysregulation

has been associated with cancer, as well as developmental, neurological and other diseases (Esteller, 2011).

The best defined of the non-miRNA small RNAs are the snoRNAs and piRNAs. The main documented function of snoRNAs is the methylation and pseudouridylation of ribosomal RNA, which is accomplished through a small nucleolar ribonucleoprotein (snoRNP) complex (Kiss-Laszlo *et al.*, 1996). Multiple studies have identified dysregulation of snoRNAs in cancer compared to control tissue (Liao *et al.*, 2010; Dong *et al.*, 2009; Dong *et al.*, 2008; Chang *et al.*, 2002). Translation is frequently perturbed in cancer and snoRNA likely contribute to this via their role in protein translation. However, recent observations that snoRNAs can undergo processing by DICER, but not DROSHA, to a mature miRNA like structure with miRNA-like functions (Ender *et al.*, 2008), may also explain their association with cancer. Future work to profile the snoRNA fractions and miRNA-like snoRNA may elucidate if MEKi resistance is also associated with regulation at the level of the protein synthesis machinery in the cell, as mediated by snoRNAs.

PiRNA are not generally expressed in adult tissues but can be aberrantly expressed in cancer. In germ cells, piRNA maintain genetic stability via epigenetic regulation (Siddiqi and Matushansky, 2012; Chu and Rana, 2007; Reik, 2007). PiRNA function to repress transposons during germ cell development by targeted silencing and epigenetic regulation via interaction with PIWI proteins to form the PIWI ribonucleoprotein (piRNP) complex. Tumours often present with an aberrantly stem-like epigenetic state (Van Vlerken *et al.*, 2012; Ohm *et al.*, 2007; Baylin *et al.*, 2001). Aberrant piRNA expression has been identified in a range of cancer types (Yan *et al.*, 2011; Park *et al.*, 2010; Cichocki *et al.*, 2010), albeit not always in association with PIWI proteins, which are required for piRNA activity. Ectopic expression of piRNA and PIWI proteins has been demonstrated to drive malignant tumour growth in model organisms but the mechanisms that drive this oncogenic effect are largely unknown, but likely due to global epigenetic dysregulation (Siddiqi and Mathushansky, 2012). The observation that PiRNA are not generally expressed in adult tissues but aberrantly expressed in cancer represents an interesting avenue as diagnostic biomarkers and future analysis of the RNA-Seq data for piRNAs will aid in expanding this area.

As the functional roles of small non-coding RNAs are elucidated, their differential expression between the samples investigated in this study can be put into context. Thus, investigation of differential expression patterns of annotated and novel small RNAs between MEKi resistant and naïve; BRAF^{WT} and BRAF^{V600E} would be a worthy endeavour. The initial investigation would be bioinformatically based, as outlined in section 6.4 and any follow-up functional genomics could be approached in a manner similar to that outlined in section 6.2.1.

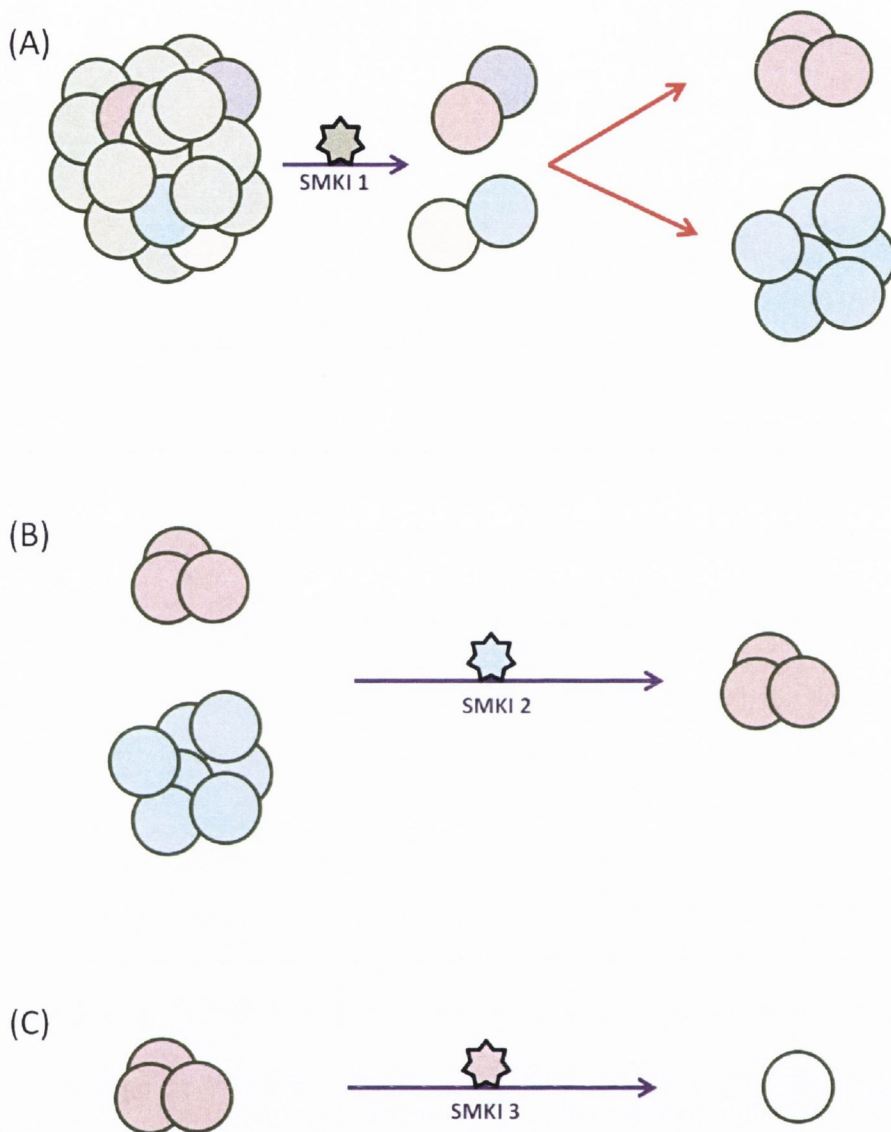
Most exciting is the potential for small RNAs to act as biomarkers of disease and response to treatment. Future work profiling piRNAs could identify a piRNA signature associated with MEK inhibition that could be used as clinical biomarkers of resistance. This would allow adaptive therapy to be used and aid in furthering the goal of personalised cancer therapy.

6.6 Future Direction: Towards personalised therapy

In this thesis, the molecular pathology in response to MEK inhibition has been described and several pathways highlighted in association to resistance. Recently, data from a phase I/II clinical trial of combination BRAF and MEK inhibitors in malignant melanoma was presented that identified an enhanced synergistic effect compared to the effect of either compound as a single agent (ASCO Annual conference Weber *et al.*, 2012). These results are promising and consistent with in-vitro studies (Greger *et al.*, 2012). However, *in vitro* studies using this combination therapy on cells following acquired resistance to single agent BRAF inhibition has demonstrated cross resistance (Gowrishankar *et al.*, 2012). It would be interesting to rerun the experiments outlined in chapters 4 and 5 for single agent BRAF inhibitor and combination BRAF and MEK inhibitors with a view to determining if the observed mechanisms of resistance arise due to inhibition of the individual kinases or if the effect is due to the inhibition of the pathway as a whole and thus inform us of preferred secondary drug targets.

Currently, 93 clinical trials involving small molecule inhibitors of MEK are registered with the clinical trials registry of the United States (www.clinicaltrials.gov). A large proportion of these trials are combination therapies with inhibitors of BRAF, AKT, mTOR (PI3K/AKT signalling pathway), PI3K, epidermal growth factor receptor (EGFR, upstream of the BRAF pathway) and vascular endothelial growth factor (VEGF, class III kinase target). The goal of personalised cancer therapy is to elucidate how to use cancer therapeutics in the most effective way by correctly targeting patients that are most likely to respond to a specific therapy. In order to successfully achieve this goal, it is not enough merely to know that a combination treatment works, the mechanisms behind how it works must be elucidated so that the correct combination treatment can be anticipated for the individual patient based on their underlying genotype and cancer phenotype (Garnett *et al.*, 2012).

To design rational combination therapies a deeper understanding than that currently available of driver and passenger mutation characteristics, and epistatic and non-epistatic interactions is required (Turner and Reis-Filho, 2012). If resistance mechanism present in cancers are thoroughly catalogued, personalised therapy for long-term remission could yet be



6.6 Figure 1: Personalised cancer therapy

(A) SMKI treatment specific to the dominant genotype kills most of the tumour but some cells are capable of functional buffering against SMKI-1. Resistant clones do not have equal survival fitness and only a subset will survive and proliferate.

(B) Biomarkers and rationale treatment design specific to the patient's underlying tumour genotype informs which therapy should be employed next. The second SMKI is targeted against the most clinically dangerous clone.

(C) Rationale design is used to determine the most effective therapy to target the remaining clones.

achieved. Resistance to targeted therapies is probably a convergent phenotype, that is, there are multiple ways for a cancer to become resistant to a given drug. Resistance incurs a cost to the cell. If mechanisms of resistance in a given tumour can be anticipated, sequential combination therapy could be employed, whereby the secondary therapy is employed after the primary therapy has induced a genetic bottleneck in the tumour (6.6 figure 1). To employ personalised therapy on a large scale and cost effective basis, a catalogue of resistance mechanisms and molecular biomarkers of resistance are requisite. By first elucidating the resistance mechanisms of individual therapies and identifying potential biomarkers of resistance, it will allow a more informed investigation of synergistic and cumulative effects of combination therapy.

6.7 Overall Conclusions

In this thesis, we have demonstrated the potential clinical utility of miRNAs in clinical diagnostics and the relevance of miRNAs in an Irish setting. We have highlighted the necessity for genetic profiling of models and the optimisation of drug treatment in the study of targeted therapies. The molecular pathology of resistance to MEK inhibition and an associated gene signature including the MAPK pathway members *MEK1/2*, *ERK1/2* and the stemness-associated genes *NANOG*, *POU5F1/OCT4* and *CTNNB1/β-catenin* was elucidated in MEKi resistant cells compared to naïve controls. The miRNA clusters *MIR302* and *MIR520* have also been identified in association with resistance to MEK inhibition in BRAF^{V600E} cells. These signatures were not identified in BRAF^{WT} cell lines. Our data show that many of the gene targets of the *MIR302* and *MIR520* are involved in pathways associated with the cellular differentiation/self renewal axis. Viewing cancer through a prism of genetic interactions can help us to better understand cancer biology and improve the development of new therapeutic approaches (Ashworth *et al.*, 2011). However, it is important to emphasise that while massively parallel sequencing and genetic profiling allows the generation of informed hypotheses; practical application requires a thorough investigation of the molecular mechanisms identified.

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