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Managing Melanoma in the era of personalised therapy: more than one disease

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and meeting inspirational patients.
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"Risk more than others think is safe.
Care more than others think is wise.
Dream more than others think is practical.
Expect more than others think is possible."

Claude Bissell

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Abstract

Melanoma is the most aggressive form of skin cancer and its incidence has risen more than any other cancer in the United Kingdom. Surgery is the mainstay of treatment for localised and regional disease. Hitherto prognosis for metastatic melanoma has been poor, with a median life expectancy of less than 12 months. However, in the last decade there have been considerable molecular advances leading to greater understanding of tumour biology and molecular characterisation of tumours. These developments have highlighted the heterogeneous nature of melanoma and provided a basis for personalised therapy.

This thesis presents the clinical use of surgical (sentinel lymph node biopsy) or molecular (genetic mutation screening) techniques in Oxford to stratify melanoma patients into subgroups. The use of sentinel lymph node biopsy for patients with thick (greater than 4mm) primary tumours predicted overall and recurrence free survival. Screening for BRAF and NRAS mutation in patients with metastatic melanoma demonstrated associations between clinical and genetic characteristics, and differences in clinical outcomes. Identifying subgroups through genetic screening enables targeted therapy. This thesis further investigated the role of phosphatidylinositol-3-kinase (PI3K) inhibitors in melanoma cell lines as pro-senescence therapy. Inhibition of the PI3K axis showed an increased in DNA damage; a marker for senescence. The PI3K pathway was shown to regulate the anti-senescent factors TBX2 and TBX3. Evaluation of transcriptional activity indicated cooperation between the T-box and Forkhead factors, raising the prospect of combination therapy in metastatic melanoma.

The management of melanoma is evolving. Following substantial molecular advances, a new generation of targeted therapies have emerged, demonstrating clinical responses in patients that would have been inconceivable a decade ago with conventional therapies. In this progressive era of targeted therapies, it should not be assumed that all melanomas respond or progress in a similar manner. Therefore personalised investigations and therapies should be offered to melanoma patients in order to improve clinical outcomes.

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List of Abbreviations

AJCC	American Joint Committee on Cancer
ASCO	American Society for Clinical Oncology
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia-telangiectasia and Rad3-related
CDK	Cyclin-dependent kinases
CI	Confidence Interval
CLND	Completion Lymph Node Dissection
CP	Carboplatin and Paclitaxel
CSD	Chronically sun damaged
DDFS	Distant-disease free survival
DDR	DNA damage response
DFS	Disease free survival
DSB	Double strand breaks
DTIC	Dacarbazine
ERK	Extracellular-signal regulated Kinases
FDA	US Food and Drug Administration
FISH	Fluorescent in situ hybridization
FOXO	Forkhead family of transcription factors
GIST	Gastrointestinal stromal tumours
H&E	Haematoxylin and eosin
HR	Hazard Ratio
IL-2	Interleukin-2
MAPK	Mitogen-activated protein kinase
MDM2	Murine Double Mature
MEK	Mitogen-activated or Extracellular signal-related protein Kinases
mTOR	Mammalian target of rapamycin
OIS	Oncogene-induced senescence
OS	Overall survival
PDGF	Platelet derived growth factor
PDK1	Phosphoinositide-dependent protein kinase 1
PI3K	Phosphatidylinositol-3-kinase
PTEN	Phosphate and tensin homolog deleted on chromosome ten
PTK	Protein tyrosine kinases
Rb	Retinoblastoma
RFS	Recurrence-free survival
RTK	Receptor tyrosine kinase
SAHF	Senescence-associated heterochromatin foci
Saβgal	Senescence-associated β-galactosidase activity
SLN	Sentinel Lymph Node
SLNB	Sentinel Lymph Node Biopsy
UK	United Kingdom
UV	Ultra-violet
γH2AX	Histone H2AX
53BP1	p53-binding protein

Chapter 1:
Introduction to Thesis

1 Introduction

1.1 Incidence of melanoma

Melanoma is a cancer that develops from melanocytes, cells that produce the pigment melanin, found mainly in the skin. Exposure of the skin to ultraviolet radiation stimulates the process of melanogenesis, but can also trigger dysplastic change in melanocytes. In the developed world the incidence of melanoma is increasing. In the United Kingdom (UK), melanoma rates have risen more than any other cancer, with over 12,800 people every year being diagnosed and it has become the most common cancer in young adults [Uk, 2011].

1.2 The management of melanoma

Surgery continues to provide a cure for localised and regional disease. However once beyond surgery, metastatic melanoma has until recently been notoriously resistant to drug therapies and radiation. Evidence for patients with regional (stage III) disease demonstrates a 5-year survival of 39% [Balch et al., 2009]. Patients with distant metastases (stage IV) have a median life expectancy of less than 12 months. Pathological features of the primary tumour such as Breslow depth and ulceration form the basis of the staging of the disease (Table 1) Other patient characteristics such as age, gender and the anatomical site of the primary tumour have also been used to estimate prognosis [Shaw *et al.*, 1980].

The surgical technique of Sentinel Lymph Node Biopsy (SLNB) was introduced in 1992 as a minimally invasive tool to assess the microscopic spread of melanoma. The technique is established practice in the surgical management of breast cancer. Worldwide, the use of SLNB has risen in melanoma patients leading to the American Joint Committee on Cancer (AJCC) incorporating the status of the Sentinel Lymph Node (SLN) in the staging system for melanoma in 2009 (Table 1) [Balch *et al.*, 2009]. The use of SLNB has enabled further stratification of subgroups in the management of melanoma. However SLNB is not standard practice across all centres in the UK and its therapeutic effect is still under debate.

A.

Classification	Tumour depth
T1a T1b	≤1.00 mm, without ulceration and mitosis <1/mm^2 ≤1.00 mm, with ulceration or mitosis ≥1/mm^2
T2a T2b	1.01-2.00 mm without ulceration 1.01-2.00 mm with ulceration
T3a T3b	2.01-4.00 mm without ulceration 2.01-4.00 mm with ulceration
T4a T4b	>4.00 mm without ulceration >4.00 mm with ulceration
Classification	**Number of metastatic lymph nodes**
N0	0
N1a N1b	1 micrometastasis 1 macrometastasis
N2a N2b N2c	2-3 micrometastasis 2-3 macrometastasis 2-3 in transit metastases
N3	≥4 metastatic nodes
Classification	**Distant metastases**
M0	No distant metastases
M1a-1c	Distant skin, lung, visceral metastases

B.

Stage	Tumour	Node	Metastases
IA	T1a	N0	M0
IB	T1b T2a	N0 N0	M0 M0
IIA	T2b T3a	N0 N0	M0 M0
IIB	T3b T4a	N0 N0	M0 M0
IIC	T4b	N0	M0
IIIA	T1-4a	N1a N2a	M0
IIIB	T1-4b T1-4a	N1a N2a N1b N2b N2c	M0 M0
IIIC	T1-4b Any T	N1b N2bN2c N3	M0 M0
IV	Any T	Any N	M1

Table 1. Staging and classification of cutaneous melanoma.

A. Classification of melanoma based on Tumour (T), Node (N) and Metastases (M).

B. Staging of melanoma based on TNM classification
Adapted from the American Joint Committee on Cancer (AJCC) criteria 2009 [Balch *et al.*]

In the last decade there have been considerable advances in our understanding of melanoma biology, which have emphasised the heterogeneity of the disease. These advances have provided a better understanding of which groups are at high-risk of recurrence after surgery, and of some of the different genetic drivers and signalling pathways behind melanoma. Based on understanding these molecular mechanisms exciting new treatments have emerged, offering the prospect of improving outcomes for patients with metastatic disease, that has not been possible with conventional treatments. This chapter provides a background to these signalling pathways and their associated targeted therapies.

1.3 Key signalling pathways associated with melanoma

The targeting of mutated oncogenes has been a step change in cancer therapeutics. Two-thirds of patients with melanoma have activating mutations in the oncogenic protein kinases RAF, RAS and KIT, and inhibition of kinase activity has been associated with tumour shrinkage.

1.3.1 MAPK

The mitogen-activated protein kinase (MAPK) pathway is a key regulator in cell growth, to regulates proliferation and survival in many cancers (Figure 1) [Garnett *et al.*, 2004]. The MAPK pathway acts as a relay between the plasma membrane and nucleus and responds to extracellular signals that are cascaded downstream through a series of mediated events. The serine-threonine protein kinase RAF, is downstream of RAS, and has three isoforms; ARAF, BRAF and CRAF [Fecher *et al.*, 2008]. Activating mutations in the serine-threonine BRAF kinase were first described in 2002 and have been identified in the tumours of just under 50% of advanced melanoma patients. The two most commonly observed BRAF mutations, V600E and V600K, account for 95% of these mutations. Activated BRAF phosphorylates and activates MEK proteins, which then activate downstream MAP kinases. Most commonly a single nucleotide mutation (GTG to GAG) results in valine substituted by glutamate resulting in a V600E mutation. The other commonly reported mutation (V600K) results from two nucleotide mutations (GTG to AAG) where lysine replaces valine.

The interest in BRAF mutations arises from the oncogene addiction notion whereby a cancer cell's survival is dependent on an activated genetic or chromosomal abnormality irrespective of non-activated pathways in normal cells [Weinstein *et al.*, 2006]. The insight into BRAF mutations and their role in cancer was published a decade ago by the Sanger Institute [Davies *et al.*, 2002]. From this we gained that BRAF somatic missense mutations were noted in 66% of melanomas, with over 90% of these attributed to V600E codon. In the ten years that have followed, a stream of publications have reported the occurrence of BRAF V600E mutations and commented on its potential role in therapy.

One of the first attempts to target the MAPK pathway was with the multi-kinase inhibitor sorafenib (BAY 43-9006) [Flaherty, 2005]. As a monotherapy, sorafenib had limited clinical activity [Wilhelm *et al.*, 2004]. When combined with carboplatin and paclitaxel (CP) responses were seen in 30% of patients but there was no correlation with BRAF mutational status [Flaherty *et al.*, 2008]. In the subsequent phase III randomised placebo controlled trial of CP with or without sorafenib there was no effect on overall or progression free survival for the kinase inhibitor [Hauschild *et al.*, 2009]. This lack of activity is likely explained by sorafenib's lack of specificity for the BRAF protein and therefore it is a weak inhibitor of the activating BRAF mutation. Since these initial studies MAPK inhibitors have evolved and become more selective.

Recently two BRAF inhibitors PLX4032 and GSK2118436, now known as Vemurafenib and Dabrafenib, respectively have emerged to gain widespread clinical interest (Figure 1) [Flaherty *et al.*, 2010].

Vemurafenib (PLX4032, RG7204, RO5185426) selectively inhibits the V600E BRAF kinase, and in its phase I study (at full dose) showed a 69% response rate in patients whose tumour harboured the mutation [Flaherty *et al.*, 2010]. None of the melanoma patients with wild type BRAF responded to treatment. In a phase III trial involving 672 patients vemurafenib was compared with dacarbazine (DTIC) as first line treatment for patients with V600E BRAF melanoma [Chapman *et al.*, 2011]. At interim analysis the data and safety monitoring board determined that vemurafenib performed statistically significantly better than DTIC and

recommended that patients assigned to DTIC be allowed to cross over to vemurafenib. When first reported median progression free survival was 5.3 months on vemurafenib and 1.6 months on DTIC, for a hazard ratio of 0.26. In the subset of patients evaluable for response this too favoured vemurafenib (48% vs 5%), as did OS with hazard ration 0.37. Vemurafenib is not without adverse effects most commonly being; arthralgia (21%), rash (18%) and fatigue (13%). The latter adverse effect is also associated with DTIC therapy. A noteworthy finding was that 61 patients (18%) developed a cutaneous squamous cell carcinoma or keratoacanthoma, which required surgical excision. This latter adverse effect is not a substantial problem in patients with metastatic disease but this has implications if targeted therapy is to be considered in adjuvant treatment. Updated results for Vemurafenib were presented at the 2012 meeting of the American Society for Clinical Oncology (ASCO). Progression free survival on vemurafenib was 6.9 months, with hazard ratio 0.38, compared with DTIC. Median OS was 13.6 months on vemurafenib, as opposed to 9.7 months on DTIC (HR 0.70, censoring at cross-over or 0.76 without censoring), noting that a quarter of patients assigned chemotherapy crossed over to vemurafenib. The objective response rate for vemurafenib was reported as 57%, and 56% of patients remained alive at 12 months.

Another ATP-competitive BRAF inhibitor of clinical interest is GSK2118436 or Dabrafenib (GlaxoSmithKline). In the first-time dose escalation study 79% of patients with BRAF mutated melanoma showed response to therapy [Kefford, 2010]. In a phase II trial Dabrafenib showed clinical response in 50% of patients with V600E mutation and 13% in V600K. The six-month overall survival (OS) was 69% with a median progression free survival of 27 weeks [Trefzer, 2011]. Interestingly Dabrafenib was extended to ten patients with previously untreated asymptomatic brain metastases. Seven of these patients were evaluable for disease response and all showed response ranging from 20% to 100% reduction [Long, 2010, Trefzer, 2011]. A phase III trial versus DTIC is now in progress and open to recruitment.

The results of clinical trials with Vemurafenib and Dabrafenib have impressively demonstrated tumour regression through targeted therapy on selected genetic populations. However the OSof patients in this group, even after therapy, is still poor. What has become apparent is that after a certain period (median 5.3 months) melanoma acquires resistance to

BRAF inhibition and disease progresses. The mechanisms for this resistance are currently being explored and not fully understood. However two mechanisms have been proposed for activation of melanoma through the bypass of MAPK pathway from within and activation by parallel pathways.

Tumour progression may occur by activation of the MAPK pathway by alternative drivers within the cascade. It has been reported that an activating NRAS mutation can occur in the presence of BRAF mutation, hence up regulation of RAS or RTK pathways may modulate MAPK pathway dynamics. [Nazarian *et al.*, 2010]. Alternatively elevated levels of CRAF have been shown to desensitise BRAF mutant cells to inhibition [Montagut *et al.*, 2008]. By the formation of dimers, CRAF also suppresses BRAF and subsequently Extracellular-signal regulated Kinases (ERK) activation [Inamdar *et al.*, 2010]. The activation of COT demonstrated insensitivity to BRAF inhibotors in melanoma cell lines harbouring BRAF mutations [Johannessen *et al.*, 2010]. Furthermore increased COT expression was measured in tumour samples from patients showing resistance to BRAF inhibitor therapy. The role of parallel pathways involving IGF, AKT and ERK will be discussed later in this thesis.

Some initial studies have commented that BRAF inhibition through Vemurafenib may not be as selective as initially thought. For example ERK activation and cell proliferation maybe occurring in wild type BRAF cells. This may contribute to the squamoproliferative lesions arising from normal cells [Halaban *et al.*, 2010].

Downstream of RAF in the MAPK pathway is the Mitogen-activated or Extracellular signal-related protein Kinases (MEK). During cellular signalling as RAF travels from the cytoplasm to the cell membrane the new activated complex enables signal cascade by consecutive phosphorlylation through MEK1 and MEK2. This in turn activates ERK 1 and 2 which are able to enter the nucleus and interact with several transcription factors to promote cellular growth and differentiation [Russo *et al.*, 2009]. Interestingly isoforms of RAS and RAF are differentially expressed, whereas MEK isoforms occurring downstream are usually present and functionally active. Furthermore in melanocytes following activation by ERK proliferation

is induced, apoptosis is prevented, and in the absence of oncogenic alterations, the cell can enter senescence. Therefore given the high prevalence of BRAF or NRAS mutations in melanoma, their mutually exclusive nature and the common downstream interactions with MEK, inhibition of MEK is an attractive option for combination therapy [Goel *et al.*, 2006, Flaherty *et al.*, 2010].

Preclinical studies of the MEK inhibitor PD0325901, and its precursor CI-1040, showed direct inhibition of ERK in cell lines and reduced tumour growth in animal models [Solit *et al.*, 2006]. Clinically PD0325901 and CI-1040 showed some benefit in melanoma patients however the toxicity associated with the therapies prevented further development [Rinehart *et al.*, 2004, Lorusso *et al.*, 2005].

Selumetinib (AZD6244, ARRY-142886) had similarly unimpressive results in a randomised phase II multicentre study comparing it with temozolomide. The MEK inhibitor had a 12% objective response rate, which was unaffected by the BRAF or NRAS mutation status of the tumour [Kirkwood et al, 2011]. An issue for selumetinib may be its relatively short half life, meaning that with tolerable doses there is likely to be some period without MEK inhibition. Several other MEK inhibitors are in clinical development and have better pharmacokinetic profiles. Amongst these trametinib (GSK1120212) has reported results from a randomized trial comparing it with chemotherapy in patients with V600 mutant BRAF melanoma [Flaherty et al 2012]. In this study 322 patients were assigned 2:1 to trametinib or chemotherapy (DTIC or paclitaxel). The kinase inhibitor gave improved progression free (HR 0.45) and OS (HR 0.54) despite crossover to trametinib of 51 out of 108 patients assigned chemotherapy). Median progression free survival on trametinib was 4.8 months, suggesting that the problem of acquired resistance seen with BRAF inhibitors holds for drugs targeting MEK too.

A further attraction of MEK inhibition, since this targets wild type protein, is the possibility that this provides a means of treating NRAS mutant melanoma. In a phase 2 trial three out of 13 evaluable patients with NRAS mutations responded to MEK162. Insufficient data exist to judge whether single agent MEK inhibition is worth pursuing.

1.3.2 C-KIT

As the receptor for stem cell factor the C-KIT receptor tyrosine kinase (RTK) is important in the development of melanocytes. The association of intermittent, high grade ultra-violet (UV) exposure and skin cancer has been well documented [Gandini *et al.*, 2005, Von Thaler *et al.*, 2010]. In anatomical sites of low UV exposure such as palms of hands, soles of feet and mucous membranes melanoma still occurs. These acral or mucosal melanomas have a preponderance of activating C-KIT mutations [Ashida *et al.*, 2009]. Further examination of these tumours has shown amplification of C-KIT mutations in selected subtype of melanoma; mucosal (39%), acral (36%) and melanomas with chronically sun damaged (CSD) skin (28%) [Curtin *et al.*, 2006]. Subsequent data has suggested the actual rates to be lower than these reported figures. The specific C-KIT mutations identified in melanoma subtypes are also commonly reported in gastrointestinal stromal tumours (GIST) [Curtin *et al.*, 2006].

Imatinib (Gleevec, Novartis pharmaceuticals, Basel, Switzerland) is a small-molecule inhibitor that *in vitro* inhibits C-KIT RTK. It also extends its inhibition to platelet derived growth factor (PDGF) and protein tyrosine kinases (PTK) [Buchdunger *et al.*, 1996]. An initial small scale study with imatinib presented unremarkable results. Subsequent *in vitro* treatment of melanoma cells lines indicated no anti-proliferative effect of the drug [Ugurel *et al.*, 2005]. Despite these negative findings, dramatic tumour responses were reported in two melanoma patients following treatment with imatinib [Hodi *et al.*, 2008, Lutzky *et al.*, 2008]. Following this, trials have been conducted on the use of selective C-KIT inhibition with imatinib on melanoma patients harbouring C-KIT mutations or amplification with overall response rate of 23% [Guo *et al.*, 2011]. A further phase II multicentre trial using the same dose of imatinib observed durable responses in 4 of 25 evaluable patients, analysed for primary endpoint [Carvajal *et al.*, 2011]. Imatinib as a monotherapy has offered some initial, but not sustained benefit to selected patients. Melanoma, unlike GIST, comprises of multiple mutations that can occur synchronously. Different C-KIT mutations display variable sensitivities for inhibitors. There is certainly scope for combination therapies or multiple targeting of C-KIT. Two studies of nilotinib are now open: in the UK the NICAM trial is a single arm assessment

of the activity of the drug in patients with KIT mutated melanoma, and an international study randomises such patients between nilotinib and DTIC.

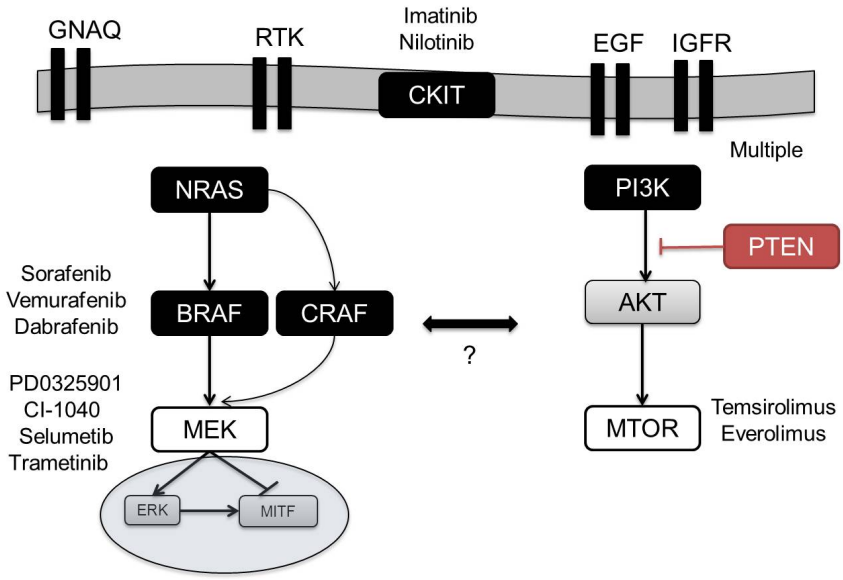


Figure 1. Signalling pathways and targeted therapies involved in melanoma

Illustration of the key proteins in the MAPK and PI3K pathways. Arrows represent activation, while bars represent inhibition. Activating mutation genes in melanoma (shown in black) are BRAF, NRAS, PI3K and CKIT. Inactivating gene (shown in red) is PTEN. Examples of targeted therapies against various effectors shown as free text beside the pathways. The potential bypass of BRAF inhibition shown via CRAF.

1.4 Aims of thesis

While surgery forms the mainstay of treatment for localised melanoma, metastatic melanoma remains resistant to current oncology therapies. For example until recently the only approved agents by the US Food and Drug Administration (FDA) for therapy in metastatic melanoma were DTIC and Interleukin-2 (IL-2). DTIC as a single agent chemotherapy for melanoma has response rates of approximately 10%, all of which are partial [Eggermont *et al.*, 2004]. Advances in molecular medicine have enabled a greater understanding of melanoma biology, genetic mutations and signalling pathways. The occurrence of a common BRAF V600E mutation in melanoma has defined a subpopulation within the cancer. Consequently, biological therapies have emerged targeting the BRAF protein and aiming to inhibit its activity. There is still a need for the identification of new and effective personalised therapeutic strategies for melanoma, as well as a means to monitor their activity [Davies *et al.*, 2011].

This research thesis aims to explore the surgical and molecular techniques of identifying subgroups in melanoma in a local setting. Clinical outcomes of these patients are discussed in the context of managing melanoma in the emerging and exciting era of personalised therapies.

Three main themes are incorporated into the respective chapters outlined below. Each chapter introduces a background to the research, a description of the methodology undertaken, presentation of the results followed by discussion and conclusions.

- To evaluate the prognostic value of a novel surgical technique (SLNB) in melanoma patients in Oxford.
- To describe the natural history of distinct subgroups in metastatic melanoma according to molecular classification, within an Oxford cohort.
- To understand the role of the PI3K pathway in melanoma cells in relation to senescence.

Chapter 2:
The prognostic value of Sentinel Lymph Node Biopsy in patients thick melanoma

2 Introduction

SLNB was introduced 20 years ago as a minimally invasive technique in patients with clinically negative lymph nodes to ascertain the spread of melanoma to the regional nodal basin [Morton *et al.*, 1992]. The SLNB technique combines pre-operative lymphatic mapping and intra-operative visualisation to identify a primary or sentinel node(s) that can be harvested. Subsequent pathological analysis aims to assess the presence of microscopic nodal metastases. The result of the SLNB influences the decision for further Completion Lymph Node Dissection (CLND) and eligibility for adjuvant therapy protocols.

2.1.1 Sentinel lymph node biopsy for melanoma

The increasing use of SLNB has generated many studies reporting the surgical success of nodal harvesting, pathological techniques to improve the determination of microscopic metastases, and evaluating the safety profile and limited morbidity of the technique [Thompson et al., 2004]. The AJCC has recognised the use of SLNB in the management of melanoma patients and incorporated it into their staging criteria [Balch et al., 2009]. In the 2010 UK guidelines for melanoma SLNB is recommended for clinically node-negative patients with ≥1 mm primary tumours [Marsden et al., 2010]. This recommendation is based on the data for intermediate (≥1.00 mm to ≤3.99 mm) Breslow depth tumours (Table 1). There were no data for patients with thick (≥4 mm) melanoma and therefore the use of SLNB in this group has not been specifically addressed.

In the two decades since SLNB was introduced two prominent prospective randomised controlled trials have reported on the impact of the SLN status on patient survival and disease progression [Morton *et al.*, 2006] [Mcmasters *et al.*, 2004]. Although the therapeutic impact of SLNB is unclear few still question the prognostic value of the technique [Thomas, 2009]. However the evidence to date has focused on patients with primary tumours of intermediate Breslow depth. Some centres do not employ the technique on patients with thicker tumours, on the basis that they have already been identified as having a poor prognosis.

2.1.2 Role of sentinel lymph node biopsy in thick melanoma

Patients with thick primary tumours have shown a propensity to progress to regional and visceral metastases and hence they represent a high-risk group [Perrott *et al.*, 2003]. The role of SLNB in patients with thin (<1.0 mm) or thick primary tumours has been the subject of debate and as yet there are no clear guidelines for these subgroups. Thin melanomas have been noted to yield a 5% positive rate at SLNB [Morton *et al.*, 2005]. In 2009 the AJCC recommended that in the presence of histological ulceration or high mitotic rate the staging should increase from IA to IB and that the patient be considered for SLNB (Table 1) [Balch *et al.*, 2009].

Breslow depth, ulceration and SLN status have been shown to have significant effects on prognosis for patients with primary tumours of intermediate thickness [Wiener *et al.*, 2010]. However the relationship of these factors is less defined in the thick melanoma subgroup. Furthermore, the current staging system discounts Breslow depth once a positive SLN is identified: patients are assigned to stage IIIA if there is no ulceration, or if present to stage IIIB (Table 1).

In the multidisciplinary management of melanoma an understanding of a patient's regional nodal basin is important to all clinicians. Given the implications for staging, further surgery and eligibility for adjuvant trials, patients with thick primary tumours warrant a more detailed analysis to assess the role of SLNB in this subgroup.

2.2 Aims of chapter

In order to evaluate the role of SLNB in thick melanoma my aim was to determine the prognostic value of SLNB in these patients in terms of overall OS and recurrence-free survival (RFS). In addition I aimed to report the characteristics of patients undergoing the technique and comment on the implications of the findings.

2.3 Methods

2.3.1 Patient group selection

Between June 1998 and June 2011 the Department of Plastic and Reconstructive Surgery performed SLNB on 945 melanoma patients. The clinical data, subsequent pathology reports and clinical correspondence were incorporated in a clinical database and prospectively maintained. From this database 136 patients were identified with a primary Breslow depth tumour of ≥4.0 mm (defined as thick melanoma, pT3 or pT4) who underwent SLNB in Oxford. Two patients where a SLN was not successfully harvested were excluded from statistical analyses.

2.3.2 Sentinel Lymph Node Biopsy technique

SLNB were performed based on a technique involving pre-operative lymphoscintography (using technectium 99 m sulphur colloid), an on-table injection of Patent Blue V dye (Laboratoire Guerbet, Aulnay-Sous-Bois, France) and intra-operative gamma probe detection with direct visualisation of the sentinel node(s)[i]. A standard 2.5% Patent Blue V was injected intra-dermally around the biopsy scar. The choice of dye used in the UK differs from that used in the United States which is Isosulfan Blue (Tyco Healthcare, Bedford, MA, USA). The SLN was identified intra-operatively using both a gamma probe and direct visualisation. The node was harvested using ligaclips to seal the afferent and efferent lymphatics, and the radioactive count measured ex vivo using the gamma probe. Echelon nodes were harvested if they had a count ≥ 10% of the sentinel node regardless of whether they were blue or not [Mcmasters *et al.*, 2004]. The background count of the lymph node basin was measured to ensure that no further 'hot' nodes remained.

Patients were offered a SLNB based on initial pathological findings which included a primary tumour with Breslow depth ≥1.0 mm. SLNB was offered to patients with Breslow depth <1.0mm in the presence of certain high risk pathological features such as Clarks level IV, V or ulceration. Patients who had clinical or radiological detectable distant disease or were not suitable for further therapy were not offered a SLNB. Post-operatively patients were followed

[i] The technique for SLNB was conducted by doctors based at the John Radcliffe Hospital, Oxford.

up in the outpatient setting at one week and then on a three monthly basis for the first three years. Thereafter followed up six monthly for two years, in total completing five years of standard follow up.

Patients gave their consent to the surgeon pre-operatively acknowledging the investigative nature of the SLNB, the potential benefits of the technique and being aware of subsequent surgical implications if a positive result was detected. Clinical data regarding the primary site and relevant pathological details were collected prospectively by means of a proforma. These data, in combination with pathological reports, clinical case notes and correspondence were incorporated on a specialist melanoma database and maintained prospectively. Melanoma follow up of patients was in accordance with national guidelines, every three months for the first three years, every six months for the following two years, therefore completing a five-year follow up. It should be noted follow up was conducted by a range of clinicians, including plastic surgeons, oncologists, dermatologists and general practitioners, and therefore the detection of disease relapse could be subject to potential bias. Furthermore, some patients with thick melanomas were participants in clinical trials and therefore may have undergone more frequent examination or radiological imaging for disease recurrence.

2.3.3 Pathological analysis of sentinel lymph nodes

Pathological analysis of sentinel nodes involved an initial bisection of the node along its hilum enabling processing of all nodal tissue[ii]. An initial slide was stained with haematoxylin and eosin (H&E) and 10 further unstained slides were produced (discarding 20-30 micron of tissue between each section). All slides were examined histologically, if melanoma cells were detected then immunohistochemistry (S100, HMB-45 and MelanA) was applied to the slides for confirmation. If no tumour was evident from the initial slide then alternate slides were stained. If no melanoma cells were detected in this series then a further 10 close step sections were cut and stained as before. It should be noted that pathological reporting and protocols did vary between the years 1998 and 2002. The introduction of standardised

[ii] Pathological analysis of the sentinel node was conducted by pathologists working at the John Radcliffe Hospital, Oxford.

reporting of melanoma pathology was recommended in 2002 by the European Organization Research and Treatment of Cancer (EORTC) Melanoma Group [Ruiter *et al.*, 2002]. The protocols in Oxford employed by the dermatopathologists are consistent with the change in practice as reported by the Sydney melanoma unit [Scolyer *et al.*, 2004].

2.3.4 Statistical methods

All statistical analyses were carried out using the SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). Patient and tumour characteristics were compared across patients with negative SLNB result and those with a positive SLNB result using a two-sample t-test or Wilcoxon rank sum test, depending on normality, for continuous covariates and the chi-squared test (or Fisher's exact test where appropriate) for categorical data. OS was calculated as the time between the date of primary diagnosis and date of death, whatever the cause. Patients still alive at the time of analysis were censored at the last known contact date. RFS was calculated as the time between the date of primary diagnosis, and following surgery, the date of first disease progression or date of death from melanoma, whichever occurred first. Disease progression was defined as local, in -transit or regional within a nodal basin of interest. Disease progression beyond the nodal basin was defined as distant recurrence and subgrouped to skin/soft tissue (M1a) or visceral (M1b or above) in line with the AJCC staging protocol [Balch *et al.*, 2009]. Survival curves were constructed using the Kaplan-Meier method and compared across characteristics using the log-rank chi-squared test. Hazard rates and 95% confidence intervals were calculated from a Cox regression analysis including the required covariates[iii]. A multivariate Cox proportional hazards regression model was fitted to identify important prognostic factors for OS and RFS. Significance was determined at the 5% level. The variables analysed were gender, age, SLNB result, primary melanoma site, Breslow depth and ulceration.

[iii] Advanced statistical models were performed with the acknowledgement of the clinical trials centre, University of Warwick.

2.4 Results

2.4.1 Patient characteristics

A total of 136 melanoma patients (82 men and 54 women) with thick primary melanomas gave consent for SLNB (Table 2). In two of these patients a sentinel node could not be identified intra-operatively and they have been excluded from further analysis. The median age at diagnosis of the study population was 59 years (range 20 to 93 years). The median Breslow depth for the Oxford cohort was 5.5 mm (range 4 .0 mm to 17.4 mm). Ulceration was noted in 60% of the primary tumours. The predominant histological subtype was nodular melanoma (44%). For all patients the anatomical distribution of the primary melanoma was most commonly noted in the trunk (33%), followed by lower limbs (28%), head and neck (16%) and upper limbs (13%). Eleven (8%) patients had subungual melanomas. Of the 134 patients in whom a sentinel node was successfully identified 71 were positive for microscopic nodal metastases. The primary tumour pathology and demographics were compared across groups based on the patients' SLNB result (Table 2). Only the presence of ulceration was significantly higher for the SLNB positive patients compared to the SLNB negative patients (69% vs. 49 respectively, $p = 0.03$).

Characteristics	All patients N (%)	Negative SLNB N (%)	Positive SLNB N (%)	P-value
Total	134	63 (47)	71 (53)	
Age (years)				0.44[a]
Mean (SD)	60 (16)	61 (16)	59 (15)	
Range	20 – 93	20 – 90	25 – 93	
Gender				0.98[b]
Male	81 (60)	38 (60)	43 (61)	
Female	53 (40)	25 (40)	28 (39)	
Primary site				0.75[c]
Trunk	45 (34)	19 (30)	26 (37)	
Lower extremities	37 (28)	16 (25)	21 (29)	
Upper extremities	16 (12)	9 (14)	7 (10)	
Head and Neck	22 (16)	13 (21)	9 (13)	
Subungual	11 (8)	5(8)	6 (8)	
Other	3 (2)	1(2)	2 (3)	
Breslow depth (mm)				0.23[d]
Median	5.5	5.3	5.8	
(Range)	(4 – 17.4)	(4.0 – 16.0)	(4.0 – 17.4)	
Ulceration				0.03 [c]
Present	80 (60)	31 (49)	49 (69)	
Absent	50 (37)	29 (46)	21 (30)	
Unknown	4 (3)	3 (5)	1 (1)	

Table 2. Characteristics of thick melanoma patients undergoing Sentinel Lymph Node Biopsy

SLNB; Sentinel Lymph Node Biopsy. Total 134 patients, percentages shown in brackets as indicated. P values calculated using [a] two-sample t-test; [b] Chi-squared test; [c] Fisher's exact test; [d] Wilcoxon rank sum test.

2.4.2 Overall survival

The actuarial median follow-up was 4.0 years (95% confidence interval (CI) 3.5 – 4.5 years). The median OS was 9.2 years (95% CI 3.2 – 11.3 years) with 54 deaths. Of the 71 patients with a positive SLNB, 58% had died in contrast to 21% of the 63 patients with a negative SLNB. OS differed significantly for the SLNB positive patients (median 2.7, 95% CI 1.8 – 3.6 years) compared to the SLNB negative group (median 10.9, 95% CI 9.3 – 10.9 years; $p < 0.0001$; Figure 2A). Furthermore the five-year OS for SLNB positive patients was 32% (95% CI 19 – 45%) in comparison to 78% (95% CI 63 - 87%) for node negative patients. The patients with ulcerated primary tumours had a significantly shorter OS than those with non-ulcerated tumours ($p=0.03$), with five-year OS of 44% (95% CI 31 – 56%) and 71% (54 – 83%), respectively. The combinations of SLNB status and the absence or presence of ulceration also had a significant impact on OS ($p < 0.0001$;Figure 2B). The five–year OS, for the two extreme groups by melanoma staging, was 87% (95% CI 65 - 96%) for stage IIA patients and 29% (95% CI 15 - 40%) for stage IIIB (Figure 3A). In a multivariate analysis, the most significant independent predictors of poorer OS in patients with thick melanomas were increasing age ($p = 0.03$; hazard ratio (HR) 1.02, 95% CI 1.00 - 1.04), increasing Breslow depth ($p = 0.03$; HR 1.12, 95% CI 1.01 – 1.25) and SLNB positive status ($p < 0.0001$; HR 4.60, 95% CI 2.22 – 9.52). It should be noted that the CI for age as a predictor is limited and, interestingly, ulceration was not noted as an independent significant predictor of OS.

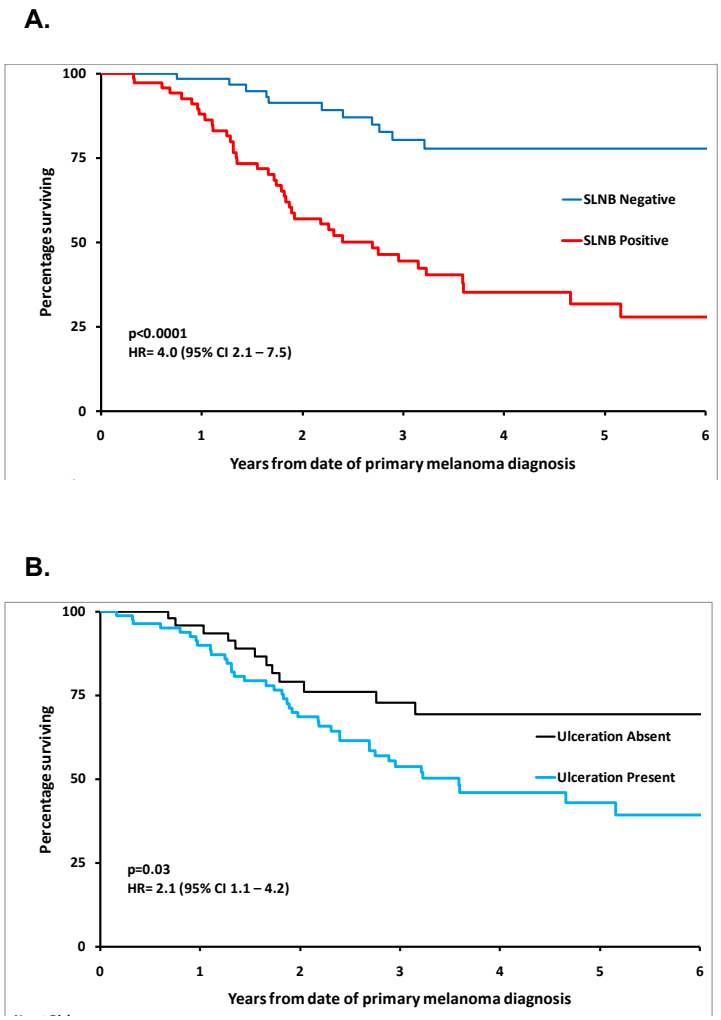


Figure 2. Overall survival for all patients undergoing sentinel lymph node biopsy.

A. Overall survival with positive and negative sentinel lymph node biopsy (SLNB) results. SLNB negative N = 63, SLNB positive N = 71. Calculated using Kaplan-Meier technique. Log-rank chi-squared test p < 0.0001. Cox regression analysis Hazard Ratio = 4.0 (95% confidence interval, 2.1 – 7.5).

B. Overall survival for all patients with pathological ulceration present and absent. Log-rank chi-squared test p=0.03. Cox regression analysis Hazard Ratio = 2.1 (95% confidence interval, 1.1 – 4.2).

2.4.3 Recurrence free survival

Disease recurrence occurred in 52 patients. The most common site of relapse was visceral (40%), followed by distant skin or subcutaneous (20%), regional nodal (18%) and local (10%). The median RFS was 3.2 years (95% CI 1.9 – 10.9). The RFS differed significantly between the SLNB positive and SLNB negative groups ($p < 0.0001$), with the five-year RFS of 27% (95% CI 15 - 40%) and 66% (95% CI 50 - 78%) respectively. The combinations of SLNB status and ulceration based on melanoma stage showed significant differences in RFS ($p = 0.0002$,Figure 3B), with, for example stage IIA with 79% 5-year RFS (95% CI 58 - 90%), whereas stage IIIB had 24% 5-year RFS (95% CI 11 – 39%). In a multivariate analysis, only patients with a positive SLNB result ($p<0.0001$, HR= 3.62; 95% CI 1.93-6.81) and larger Breslow depth ($p= 0.006$, HR=1.15; 95% CI 1.04-1.26) had a significantly higher risk of recurrence or death.

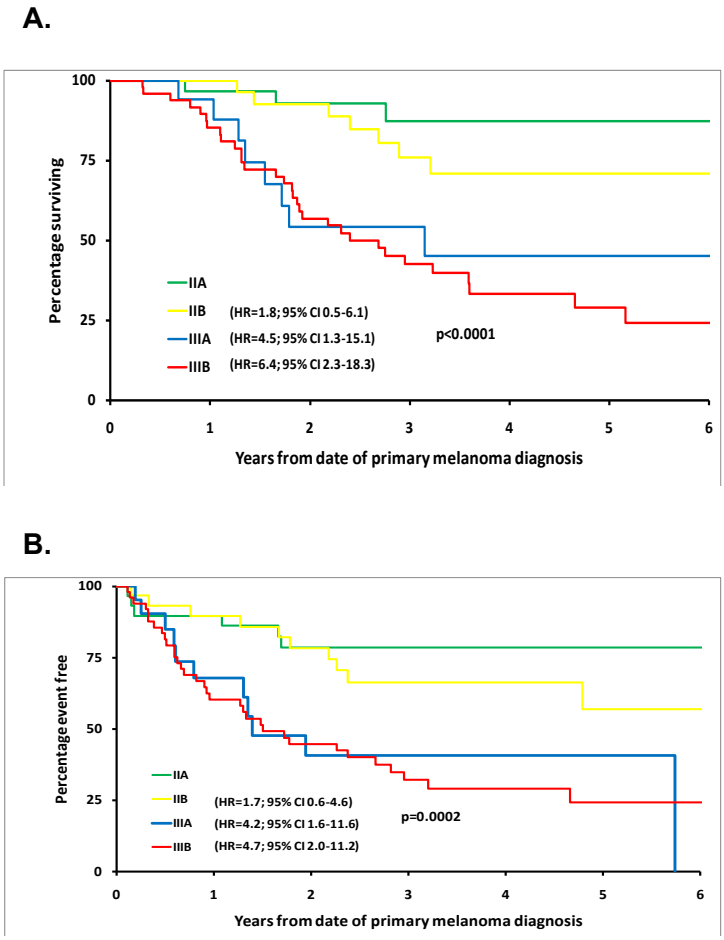


Figure 3. Overall survival for all patients grouped by melanoma stage.

A. Overall survival for all patients by stage.
SLNB; Sentinel Lymph Node Biopsy. Log-rank chi-squared test p < 0.0001. Cox regression analysis Hazard Ratio as shown for each group in comparison to baseline Stage IIA (SLNB negative Ulceration Absent). Staging based on 2009 American Joint Committee on Cancer. Survival curves calculated using Kaplan-Meier method.

B. Recurrence free survival analysis according to stage.
SLNB; Sentinel Lymph Node Biopsy. Log-rank chi-squared test p = 0.0002. Cox regression analysis Hazard Ratio as shown for each group in comparison to baseline Stage IIA (SLNB negative Ulceration Absent). Staging based on 2009 American Joint Committee on Cancer [Balch et al, 2009].

2.5 Discussion

2.5.1 Principal findings

These results represent the largest cohort of thick melanoma patients undergoing sentinel node biopsy for melanoma from a single UK centre. The patient characteristics were similar to other reported studies. Of the 134 patients who underwent successful SLNB, the node positive result is higher than that noted in other studies [Gajdos *et al.*, 2009]. In this subpopulation, after median follow up of 4 years, there were significantly ($p < 0.0001$) worse rates of OS and RFS in comparison to patients with a negative SLNB. Further examination by multivariate analysis demonstrated that SLNB result, Breslow depth and age were significant prognostic factors for OS, and only SLNB and Breslow depth were significant for RFS.

2.5.2 Sentinel Lymph Node Biopsy in thick melanoma

Since the introduction of SLNB in 1992, its use has become standard in the management of intermediate melanomas across North America [Mcmasters *et al.*, 2004, Morton *et al.*, 2005] and Australasia [Thompson *et al.*, 2004]. In the UK SLNB is still largely offered by specialist centres. The technique has been shown accurately to identify microscopic metastases in nodal basins, with minimal morbidity [Ross, 2010]. Randomised controlled trials have focused on intermediate thickness primary tumours and have excluded data for thick melanomas [Mcmasters *et al.*, 2004, Morton *et al.*, 2005, Gajdos *et al.*, 2009].

In 2000 Gershenwald et al first reported SLNB as a prognostic indicator in thick melanomas. Their study of 126 patients confirmed the status of the sentinel node as the most powerful predictor of OS [Gershenwald *et al.*, 1999]. In 2003 these findings were confirmed by Carlson and colleagues, who in addition to SLN status, established ulceration as a predictor for disease free survival (DFS) [Carlson *et al.*, 2003]. The largest cohort of thick melanoma patients undergoing SLNB was published by Gajdos *et al* where the study population of 227 confirmed the prognostic value for patients with a negative SLN (in the absence of ulceration) in comparison to the positive group, in terms of OS and distant-disease free survival (DDFS) [Gajdos *et al.*, 2009]. Further examination of the primary pathology showed that the presence

of ulceration, satellites and angiolymphatic invasion were significantly associated with a positive SLNB result. With a growing number of centres reporting their individual outcomes on thick melanoma, a meta-analysis of the prognostic role of SLNB in this subset of patients was published in 2011 [Rondelli *et al.*, 2011]. Nine retrospective observational studies published between 2000 and 2009 were included in the analysis; six were from North America, two from Europe and one from Australia. A total of 1,262 patients with a weighted mean Breslow depth of 4.4 mm were analysed; ulceration was present in 42% with a positive sentinel node found in 36%. The meta-analysis confirmed that the positivity rate of SLN increases with increasing primary tumour thickness. At 3-year follow up, the meta-analysis demonstrated an improved OS for patients with a negative (71%) compared to a positive SLN (49%).

2.5.3 Findings in relation to the literature

The principal findings are in keeping with the literature. It is important to acknowledge that the Oxford sample size is modest and the interpretation of findings should be in keeping with this. The median Breslow depth is in accordance with other studies (median range 5 mm to 6 mm). The high rate of ulceration of the cohort is greater than all but one of the nine studies included in the meta-analysis by Rondelli *et al*, which could clearly influence the nodal positivity rate [Rondelli *et al.*, 2011]. The influence of ulceration is highlighted by Cecchi *et al* who reported a relatively low median Breslow depth of 4.5 mm, yet with a high ulceration rate, generating a positive SLN rate of 37% [Cecchi *et al.*, 2007]. Not all UK centres offer SLNB and Oxford's referral patterns can be from outlying regions that may attract patients specifically seeking the investigation. Therefore an inherently higher risk population may contribute to the high positive rates of SLNB noted in Oxford. It is important to acknowledge that compared to specialist centres in the US, the UK has gradually adopted the SLNB into the management of melanoma. Consequently UK pathologists have benefited from the lessons learned of histological analysis by pioneering SLNB centres and adopted a more detailed analysis technique. This may contribute to the relatively higher rate of positive SLNB in this cohort. Future considerations may involve retrospectively evaluating the positive SLN

status from the first five years to the subsequent five years. However the number of cases would be low in numbers and therefore not achieving statistical significance.

2.5.4 Findings in relation to clinical practice

Patients with a Breslow depth greater than 4.0 mm have been grouped into a high-risk population with a collective poor outcome. The aforementioned findings challenge the notion that all patients with thick melanoma experience a similar disease progression. Based on certain factors, patients may be stratified into subgroups that have markedly different prognoses thus reflecting the diverse natural history of melanoma. Furthermore, the concept of thick melanomas (≥4.0 to ≤6.0 mm) being distinct biological variants of ultra thick melanomas (>6.0 mm) has been proposed; indeed a significant difference in OS and DFS has been reported between the two groups [Ferrone *et al.*, 2002, Meguerditchian *et al.*, 2011]. However, this may not add further to the clinical management of patients.

2.5.5 Findings in relation to the guidelines

The AJCC reported five-year OS for all melanoma patients staged IIB, IIC, IIIA and IIIB as 68%, 53%, 78% and 59% respectively. The number of patients in the Oxford cohort is much smaller compared to the AJCC, however this study focused on patients with thick melanomas who underwent SLNB testing. The five-year OS for patients in Oxford with thick melanoma undergoing SLNB based on staging groups IIB, IIC, IIIA and IIIB were 87%, 71%, 45% and 29% respectively. This represents a dramatic difference in OS for patients who were negative for SLNB. It should be noted that these subgroups are not equivalent as the AJCC categorises patients who have not undergone SLNB. The prognosis for patients in stage IIIA and in particular IIIB reflects the continued impact of tumour thickness in the presence of nodal disease.

2.6 Conclusions

Sentinel node biopsy is considered a safe, minimally invasive and effective technique, which when offered to suitable patients provides significant prognostic information [Rughani, 2011]. This research has demonstrated the prognostic value of sentinel node sampling, in terms of OS and RFS, for patients with thick primary tumours and adds to current data [Rondelli *et al.*, 2011]. By investigating this perceived high risk group, we can identify a subset that display a prognosis that is comparable to intermediate thickness groups. This in turn has implications for the future design of clinical trials involving adjuvant therapies. The findings from this study challenge the current UK guidelines to recognise and specifically address the use of sentinel node biopsy in patients with thick melanomas.

Chapter 3:
Melanoma is a heterogeneous disease in the Oxford cohort

3 Introduction

The incidence of melanoma in the UK is increasing [Uk, 2011]. The reasons for this are not fully understood but contributing factors include; increased sun exposure, lifestyle habits of greater travel, raised public awareness and improved diagnostic services. Investigative techniques such as SLNB and radiological imaging have also provided more information for staging and prognosis. Surgery continues to provide a cure for localised and regional disease. However, beyond surgery, current oncology therapies are relatively ineffective. Consequently there is a demand for novel and effective means to identify patients in order to offer tailored therapeutic strategies.

3.1 Aims of chapter

In the last decade there have been considerable advances in molecular characterisation of tumours. This has led to a greater understanding of melanoma biology and highlighted the heterogeneous nature of melanoma. Consequently there has been an exponential rise in the publications associated with basic science, translational medicine and clinical trials of melanoma therapeutics. Two major new treatment approaches have emerged; oncogene targeted and immune mediated.

The introduction to this thesis (chapter 1.3, page 14) provided an overview of the current research and clinical prospects for melanoma therapeutics in relation to targeted therapies. It is pertinent to appreciate the cellular and biological mechanisms that underlie these therapies and to consider the impact of different tumour biologies on clinical outcomes. This will describe the natural history of melanoma in an Oxford cohort, based on genetic subpopulations, and comment on the potential for personalised therapies in the clinical management of metastatic melanoma patients.

3.2 Methods

3.2.1 Patient selection

The study cohort was formed from a review of the patients referred to the Department of Oncology at the Churchill Hospital, Oxford between 1993 and 2006, presenting with cutaneous malignant melanoma. Further consecutive patients were prospectively recruited between in 2009 and 2011 as part of the Oxford Radcliffe Biobank, which included specific consent for genetic analysis. A total of 269 patients were identified with tumours suitable for genetic testing. The project was ethically approved and storage complaint with the Human Tissue Act 2004 (HTA licence 12217, OXREC 09/H0606/5). Older samples were identified from existing holdings and as agreed under the terms of ethical approval no retrospective consent was sought. Patient records were correlated with primary pathology reports, radiological reports and clinical correspondence. These were incorporated and maintained prospectively in a melanoma specific database.

3.2.2 Histological analysis

Histological cases were examined by a consultant dermatopathologist[iv] with the inclusion criteria that the H&E stained slide should show > 3mm^2 of viable, invasive and confluent tumour. Areas of tumour were then marked on the HE slide 1mm diameter cores of tissue were taken from these areas using the Beecher Instruments Manual Tissue Arrayer MTA-1 (Beecher Instruments Inc, WI, USA)

3.2.3 DNA extraction and genotyping

Genetic testing was undertaken at the Wellcome Trust Centre for Human Genetics Oxford[v]. DNA was extracted from cores using Qiagen DNEasy Blood and Tissue Kit (Qiagen, CA, USA) according to manufacturer's instructions with the addition of an incubation in Proteinase K for 48 hours at room temperature prior to the start of the protocol to compensate for the presence of paraffin in the cores.

[iv] I acknowledge the work of Dr Ruth Asher for the pathological analysis undertaken at the John Radcliffe Hospital, Oxford.
[v] I acknowledge the work of Professor Ian Tomlinson, Dr Mike Churchman and Mr Richard Lisle in the genetic testing of tumour samples.

Codon 600 of the BRAF gene was analysed using the TaqMan® SNP genotyping assay using the following: Total volume of 10 µl, using 20 ng (2 µl at 10 ng/µl), 5 µl of 2X Taqman Genotyping Mastermix (Lifetechnologies, UK), 2.75 µl 40X Taqman Assay mix (Lifetechnologies) and 2.75 µl molecular biology grade water (Sigma-Aldrich, UK) per sample. A standard sample (8 µl) of the reaction mixture was transferred to each well of a 96 well PCR plate (Starlab, Milton Keynes, UK) and cycled on a Tetrad thermal cycler (Bio-Rad). Following an initial denaturisation at 95°C for 15 minutes, then 50 cycles consisting of 10 seconds at 92 °C followed by 60 seconds at 60 °C. On completion of the reaction the end-point fluorescence was read using an ABI7900 and analysed using SDS v2.2. To confirm the BRAF genotype, each reaction was conducted in duplicate using the KASPar v4.0 genotyping system (Kbioscience, UK) with 2 µl DNA (10 ng/µl) and the reaction mixture according to the manufacturer's instructions. A standard sample (8 µl) of this mixture was transferred to each well of a 96 well plate and then placed onto a Tetrad thermal cycler (Bio-Rad) and cycled as follows: An initial denaturation/activation of 94°C for 15mins, followed by 10 cycles of 94 °C for 20 seconds and touchdown 65-57 °C for 60 seconds (-0.8 °C/cycle), followed by 36 cycles of 94 °C for 20 seconds and 57 °C for 60 seconds. Due to a wide range of polymorphisms that occur in the NRAS gene, codons 12,13,61 and 146 were analysed using the sequence method detailed above.

3.2.4 Clinical outcomes

Tumour samples were correlated with clinical patient details in the melanoma database. These cases were prospectively maintained with reference to primary pathology reports, radiological investigations and clinical records of most recent encounters. Statistical analysis was performed using the same protocol and software as described before (see page 27). The end date was recorded as the last known clinical encounter or date of death. OS and RFS were calculated as before. Survival curves were constructed using the Kaplan-Meier method and compared across characteristics using the log-rank chi-squared test. Hazard rates and 95% confidence intervals were calculated as previously described. The variables analysed in the multivariate cox-model were genetic status, age, primary melanoma site, Breslow depth and ulceration.

3.3 Results

3.3.1 Patient characteristics

A total of 269 patients were identified from genetic mutation analyses and correlated to clinical characteristics. Nineteen of these patients had incomplete clinical records and were excluded from further subgroup analyses. Subsequently 250 patients, comprising of 145 males and 105 females, underwent statistical analyses (Table 3). The median age at diagnosis for the cohort was 58 years (range 21 to 90 years). The median Breslow depth for the cohort was 2.8 mm (range 0.3 mm to 23.5 mm). The most common primary anatomical site was the upper and lower limbs (38%) followed by trunk (36%), head and neck (19%) and palms and soles (7%).

3.3.2 Mutational status

The patient cohort were grouped by genetic mutation status into the following: BRAF mutant (86 patients), *NRAS* mutant (75 patients), *BRAF* and NRAS mutants (23 patients), and BRAF and NRAS wildtype (66 patients). Patients with BRAF mutation were approximately 11 years younger than patients with a NRAS mutation, this difference was shown to be statistically significant (Table 3). Further analysis of the groups showed a statistical difference (p=0.007) in the anatomical distribution of the primary tumour (Figure 4). From these analyses we note that the majority of BRAF mutant patients have a primary melanoma on the trunk (47%) or limbs (39%) and none in the palms or soles. In contrast tumours with NRAS mutations were more evenly distributed across the body, with 11% present on the palms or soles. When both BRAF and NRAS mutations were found in the same tumour the most common site was on the limbs (60%).

Characteristic	Genetic status				P value
	BRAF mt	NRAS mt	BRAF & NRAS mt	BRAF & NRAS wt	
	N	N	N	N	
Number of patients	86	75	23	66	
Gender:					0.37[a]
Female	36	26	11	32	
Male	50	49	12	34	
Median age (years)	52	63	53	61	0.02[b]
(range)	(27 – 85)	(25 – 90)	(26 – 81)	(21 – 84)	
Ulceration present					0.22[a]
No	18	12	3	19	
Yes	22	28	4	18	
Breslow depth					0.93[c]
Median (mm)	3.0	2.6	2.2	2.8	
(range)	(0.3 – 12.6)	(0.6 – 10.0)	(0.7 – 8)	(0.9 – 23.5)	

Table 3. Patient characteristics grouped by genetic status.

Grouped by genetic status of BRAF or NRAS gene. Wildtype (wt), Mutation (mt). Total N = 250 patients. P values calculated using: [a] Chi-squared test; [b] Anova test; [c] Kruskal-Wallis test; [d] Wilcoxon rank sum test.

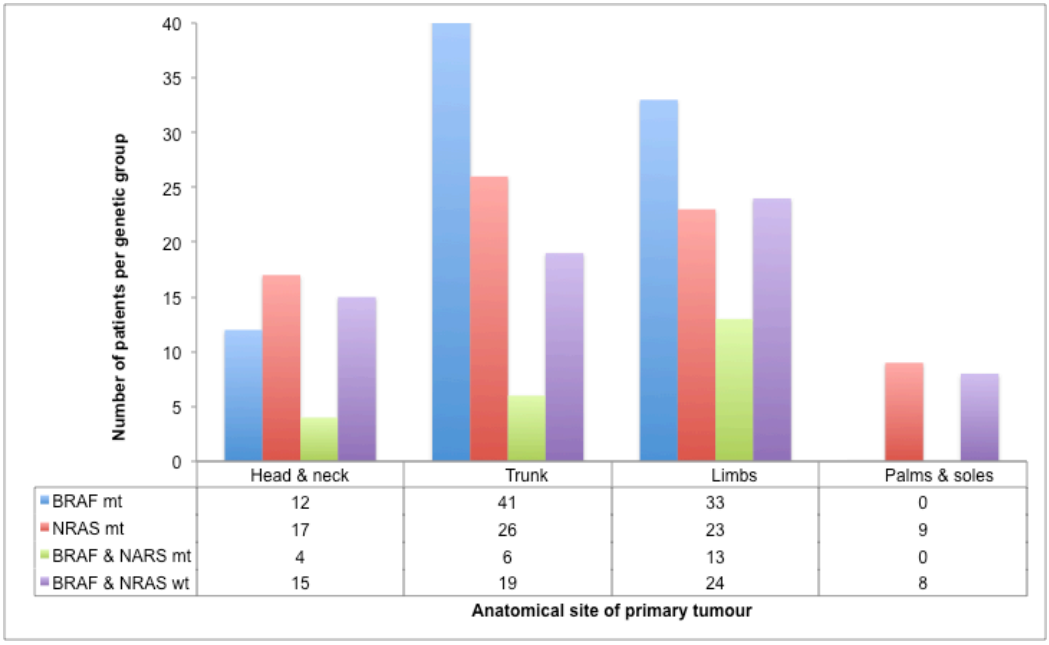


Figure 4. Anatomical distribution of primary tumours grouped by genetic status.

Number of patients based on mutational status of BRAF or NRAS gene. Wildtype (wt), Mutation (mt). Anatomical sites grouped as shown. Statistical difference between groups based on Chi-squared test p=0.007.

Comparison was made according to BRAF status: wildtype versus mutant (Table 4). There were no differences in gender. However, the BRAF mutant patients were significantly younger (p=0.002) than the wildtype patients with median ages at primary diagnosis of 62 years and 52 years, respectively. The proportion of patients whose tumours showed pathological ulceration was non-significantly higher in the widltype group compared to the mutant group.

When comparing the status of patients with NRAS wildtype and mutant tumours, there was no significant difference (p=0.15) in the median ages between the two groups (Table 4). There were a greater percentage of males noted in the NRAS mutant (62%) versus wildtype (55%) patients. The NRAS mutants (1:2.1) also demonstrated a higher ratio of pathological ulceration compared to the wildtype (1:1.1) patients. The anatomical distribution of the primary tumour was similar across the two groups.

Characteristic	Genetic status			
	BRAF wt	**BRAF mt**	**NRAS wt**	**NRAS mt**
Patients (n)	141	109	152	98
Gender ratio				
Female:Male	1:1.4	1:1.3	1:1.2	1:1.6
Median age (years) (range)	62 (21 – 90)	52 (26 – 85)	56 (21 – 85)	61 (25 – 90)
Ulceration ratio				
Absent:Present	1:1.5	1:1.2	1:1.1	1:2.1
Breslow depth Median (mm) (range)	2.7 (0.6 – 23.5)	2.9 (0.3 – 8.5)	2.9 (0.3 – 23.5)	2.6 (0.6 – 10.0)
Primary Site (%)				
Head & Neck	17	14	17	13
Trunk	30	40	36	32
Upper & Lower Limbs	33	40	35	39
Palms & Soles	11	0	4	10
Median RFS (years)	3.9	4.2	4.2	4.1
Median OS (years)	5.0	4.7	4.9	4.7

Table 4. Patient characteristics grouped by genetic status.

Grouped by genetic status of BRAF or NRAS gene. Wildtype (wt), Mutation (mt). Breslow depth for the primary tumour. RFS; Recurrence free survival, OS; Overall survival

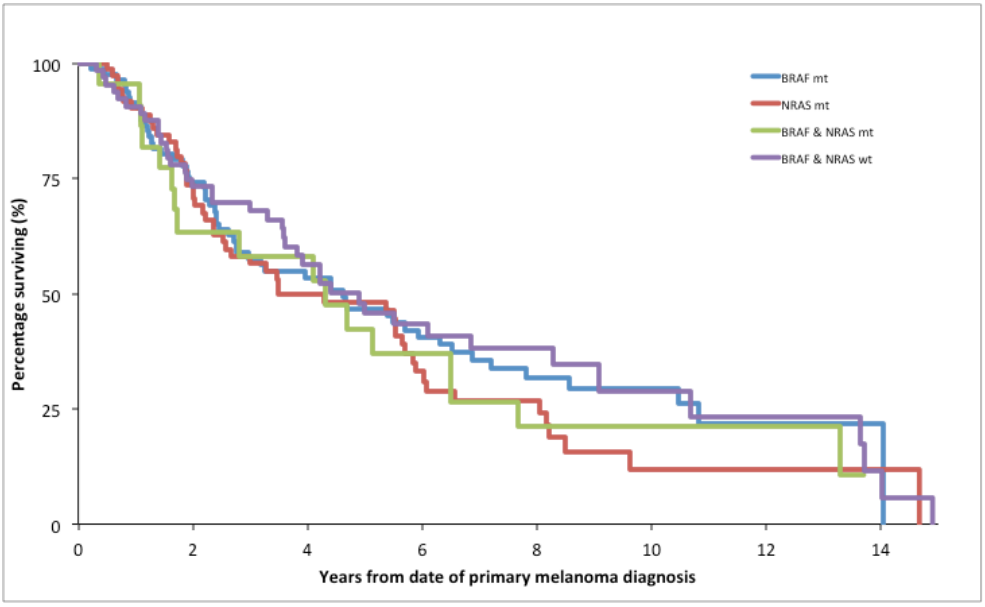
3.3.3 Overall survival

The median follow up for all patients was 38 months. There were no significant differences (p=0.73) between the median OS of BRAF mutant (4.7 years, range 2.7 to 5.8) and wildtype (5.0 years, range 3.1 to 6.3) patients. Similar median OS were also noted between NRAS mutant and wildtype patients (Table 4). Multivariate analyses showed the risk of death for the BRAF and NRAS mutant group to be significantly greater (p=0.01, HR 3.54) compared to baseline wildtype (Table 5). The multivariate model reported Breslow depth (0.04), ulceration (p=0.006) and primary anatomical site (p=0.01) as independent factors for survival.

3.3.4 Recurrence free survival

The median time in years for disease recurrence was similar across BRAF mutant and wildtype, and NRAS mutant and wildtype patients (Table 4). When comparing RFS for all patients based on their genetic status, there was an improved event free period for patients with BRAF and NRAS wildtype (Figure 5B). The multivariate analyses showed the BRAF and NRAS mutant group to be twice as likely for disease recurrence, compared to the BRAF and NRAS wildtype group (Table 5).

A.



B.

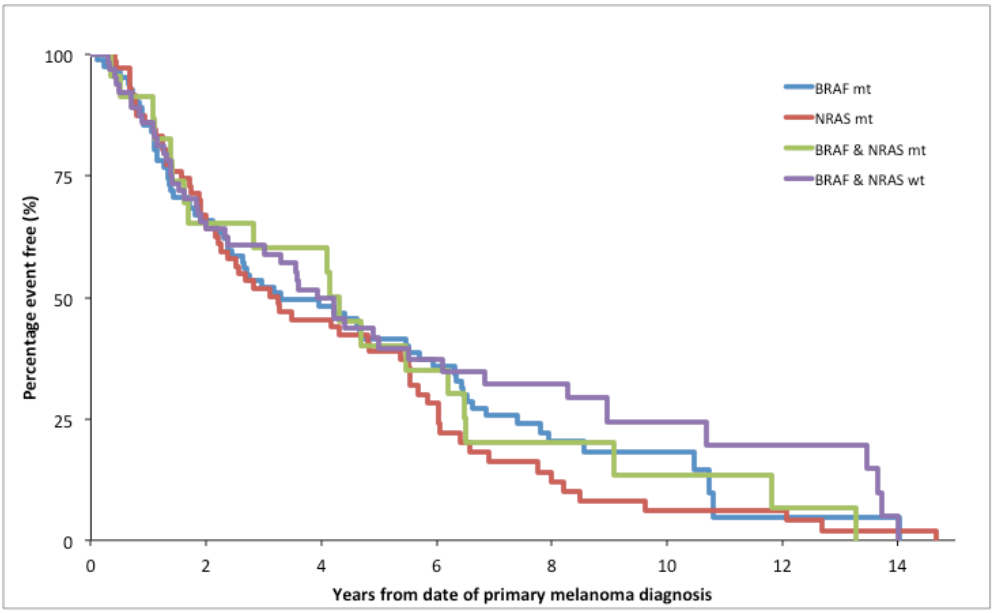


Figure 5. Clinical outcomes for all patients based on genetic status.

A. Overall survival for patients based on BRAF and NRAS mutations.
Wildtype (wt), Mutation (mt) Log-rank test p=0.02. Cox regression analysis Hazard Ratio (HR), 95% Confidence Interval (CI) using BRAF and NRAS mutant group as baseline. Based on Kaplan-Meier method.

B. Recurrence Free Survival for all patients based on genetic status.
Wildtype (wt), Mutation (mt). Log-rank test p=0.05. Cox regression analysis Hazard Ratio (HR), 95%. Based on Kaplan-Meier method.

	Univariate		Multivariate	
	HR (95% CI)	P value	HR (95% CI)	P value
Risk of death				
BRAF & NRAS wt	1.00			
BRAF mt	0.98 (0.66-1.46)	0.92	1.74 (0.87-3.51)	0.12
NRAS mt	1.14 (076-1.71)	0.52	1.36 (0.65-2.84)	0.42
BRAF & NRAS mt	1.17 (0.67-20.6)	0.58	3.54 (1.34-9.36)	0.01
Risk of recurrence				
BRAF & NRAS wt	1.00			
BRAF mt	1.09 (0.75-1.58)	0.64	1.81 (0.94-3.47)	0.07
NRAS mt	1.23 (0.84-1.79)	0.29	1.10 (0.56-2.16)	0.77
BRAF & NRAS mt	1.30 (0.77-2.19)	0.33	2.07 (0.80-5.35)	0.13

Table 5. The effect of mutational status on clinical outcomes.

Based on univariate and multivariate cox-regression models (n=250). Risk of death related to overall survival, risk of recurrence related to recurrence free survival (RFS), Hazard ratio (HR), Confidence interval (CI). Multivariate model incorporates Breslow depth, ulceration and anatomical site of primary tumours, which were all significant independent predictive factors.

3.4 Discussion

3.4.1 Principal findings

Examining the natural history of the Oxford cohort demonstrates that melanoma patients can be grouped into subsets according to genetic mutations. These groups indicate characteristics such as age and sites of primary melanoma can be associated with certain mutations in BRAF and NRAS. There were borderline differences in OS and RFS between the groups, with patients harbouring mutations in BRAF and NRAS showing poorer clinical outcomes. Interestingly, some patients were found to harbour mutations in both BRAF and NRAS, previously described to be mutually exclusive. Although several centres have recently reported findings that challenge the notion of mutual exclusivity between BRAF and NRAS.

3.4.2 Findings in relation to other studies

A study by the McArthur group prospectively assessed 249 patients for NRAS and BRAF mutations and evaluated the clinical outcomes and pathological features [Devitt *et al.*, 2011]. Of the 249 patients in this study 45% carried the BRAF mutation, 14% had NRAS mutations and 40% were widltype for both, none of these patients carried both mutations. Clinical features reported by the McArthur group for BRAF mutations include a younger age (less than 60 years) and non-chronic sun-damaged skin sites. Pathologically superficial spreading melanomas were more commonly associated with BRAF. Whereas NRAS mutated tumours were more prevalent with nodular subtype and a higher mitotic count (>1/mm^2). The clinical outcome relating to melanoma specific survival showed borderline significance (HR=2.51, 95% CI 0.99-6.36, p=0.05) for NRAS patients when comparing to wildtype, this did not dramatically change when assessed in the multivariate cox model.

A study to assess the clinical significance of BRAF mutations in metastatic melanoma patients in the United States reported a mutation rate of 44%, mainly occurring at BRAF V600E [Chang *et al.*, 2004]. Of note one patient was reported to have a mutation in both BRAF and NRAS. Overall, the study by Chang and colleagues showed no significant differences in age, gender, location of primary tumour or survival between melanomas with or without BRAF mutations.

An Australian study by Long et al assessing the frequency of BRAF mutation in metastatic melanoma patients found that 95 of the 197 patients had a BRAF mutation. Of these BRAF mutant patients 74% harboured the V600E mutation, but interestingly 20% were noted to have the V600K mutation [Long *et al.*, 2011]. When demographics were compared between the BRAF mutant and wildtype patients, the only significant factor was age. The patients with BRAF mutations were significantly younger (56 years) at diagnosis of primary melanoma than the wildtype patients (63 years). This confirms the observation made in the Oxford cohort of younger patients with BRAF mutated melanomas. Furthermore the majority of BRAF mutant Australian patients were noted to have a primary melanoma on the trunk (43%), this is in keeping with the rate of 40% noted in Oxford. A study of 302 primary cutaneous melanomas identified age below 55 years as the single most predictive factor for BRAF mutation [Viros *et al.*, 2008]. This preponderance for younger age with BRAF mutation is consistent with the notion that BRAF mutations occur earlier in life due to intermittent sun exposure rather than in later years on chronic sun damaged skin.

The study by Long *et al* calculated the interval from diagnosis to distant metastases for all patients and found no significant difference between the BRAF mutant and wildtype patients. In their multivariate model factors such as Breslow depth, ulceration, age and primary site were significant ($p<0.05$) and independent from mutational status. The Australian study was unable to report an unbiased OS rate due to the clinical use of a BRAF inhibitor for some patients. This represented a selection bias for patients receiving the novel BRAF inhibitor as phase I trials have shown a better overall survival, plus to participate in any trial these patients would have improved performance status or lack of brain metastases, which can obviously influence survival. Other studies have also reported BRAF mutant patients to be associated with a younger age, primary site as trunk and lack of chronic sun damage [Edlundh-Rose *et al.*, 2006, Hacker *et al.*, 2010].

3.4.3 Limitations of the study

It is important to note the limitations of the current research. A preferred study methodology would have been to prospectively conduct genetic analyses on all patients diagnosed with melanoma and follow their progress. This would have been a considerable financial commitment. The prospective nature of the study would have been beyond the time constraints of this research. The study sample of the Oxford cohort was relatively low, however compared to other worldwide studies the numbers in the distinct groups are comparable. Ideally patients in each group could be matched with a control group for age and gender, this may represent an avenue for further research.

3.4.4 New era of classifying melanoma

There appears to be a relationship between intermittent, rather than chronic, sun exposure and BRAF mutations. A study assessing sun exposure (by using solar elastosis) in primary melanomas showed that BRAF mutations occurred significantly more in sites with intermittent sun exposure (trunk) than chronic (limbs) or unexposed (palms or soles) sites [Maldonado *et al.*, 2003]. A landmark study also highlight the presence of oncogene mutations associated with chronic sun damaged and aimed to provide a further classification for melanomas [Curtin *et al.*, 2006]. From this a classification of melanoma phenotypes is emerging based on clinical, pathological and genetic factors. For example the BRAF phenotype would be associated with a younger patient, exposed to intermittent sun, with multiple naevi and primary sites involved the trunk [Scolyer *et al.*, 2011].

Most studies have not shown a difference in survival associated with BRAF studies. However, a study by Kumar et al, reported a longer DFS of median 7 months for cases with BRAF mutations then without, these findings were not statistically significant [Kumar *et al.*, 2003].

3.5 Conclusions

Molecular advances have provided a better understanding of cancer biology, signalling pathways and genetic subpopulations that exist in melanoma. This has led to a new generation of targeted therapies that have demonstrated clinical responses in patients that would have been inconceivable a decade ago with conventional therapies. However, enthusiasm for targeted therapies should be considered in the context of disease progression noted in long-term follow up. This has highlighted that targeting single pathways with mono-therapies has limitations and tumours can develop resistance. Currently there are several clinical prospects using combination therapies for patients with advanced melanoma.

While BRAF inhibitors have shown some improvement for patients eventually disease recurrence occurs. Subsets of BRAF patients experience higher rates of recurrence that maybe related to additional epimutations or cross talk with other signalling pathways. For example deletions in the phosphate and tensin homolog deleted on chromosome ten (PTEN) gene occur in up to 30% of melanomas, leading to increased activity of the PI3K pathway, which are associated with BRAF mutations but not NRAS mutations [Tsao *et al.*, 2000, Goel *et al.*, 2006]. Therefore further work is warranted to assess the interactions and influences of PTEN mutations or PI3K pathway, in BRAF mutated cases.

Clinically the challenge remains to safely deliver these therapies with tolerated adverse effects and monitor their efficacy. It is clear that patients require personalised therapy tailored to their genetic characteristics. Furthermore greater collaboration should be encouraged between doctors, scientists and the pharmaceutical industry to ensure that progress is maintained in the advancement of treatment approaches for metastatic melanoma.

Chapter 4:
Inhibition of PI3K pathway in relation to cellular senescence

4 Introduction

The PI3K axis is emerging as one of the key pathways in cancer as it is important for cell proliferation and survival [Vivanco *et al.*, 2002]. Activation of PI3K by RTK results in downstream activation of AKT, a serine-threonine kinase, that is recruited from the cytoplasm to the plasma membrane [Davies *et al.*, 2011]. Here, AKT is phosphorylated at two important residues Ser473 and Thr308 by mammalian target of rapamycin (mTOR) complex and phosphoinositide-dependent protein kinase 1 (PDK1), respectively. Activated AKT contributes to the pathways influence on cell proliferation and survival by downstream phosphorylation of the Forkhead family of transcription factors (FOXO) [Davies *et al.*, 2011]. The predominant AKT isoform, AKT3 is overexpressed in 60% of melanomas [Stahl *et al.*, 2004] and this overexpression shifts melanoma from radial to vertical growth phase [Govindarajan *et al.*, 2007].

PTEN negatively regulates the PI3K pathway and therefore inhibits AKT [Simpson *et al.*, 2001]. PTEN loss has been noted in 30% to 50% of melanomas [Birck *et al.*, 2000]. In addition somatic mutations have been demonstrated in 10% of melanomas [Guldberg *et al.*, 1997]. There is evidence that cooperativity exists between the MAPK and PI3K pathways [Smalley *et al.*, 2006]. Animal models have demonstrated the rapid progression of melanoma when activating BRAF and inactivating PTEN mutations occur in parallel [Dankort *et al.*, 2009]. Based on these findings there is prospect for combination therapies targeting the MAPK and PI3K pathways.

In a melanoma mouse model, the combination of a PI3K inhibitor (LY294002) and MEK inhibitor (UO126), resulted in increased apoptosis, reduced angiogenesis and inhibited tumour growth [Bedogni *et al.*, 2004]. PI3K modulation can occur through direct inhibition or focus downstream on AKT or mTOR. The evidence for using PI3K inhibitors with clinical response in melanoma patients is limited. Beyond melanoma, combination therapy with the PI3K inhibitor (GDC0941) enhances antitumor effects, increases apoptosis and inhibits AKT in breast and thyroid cancers [Salphati *et al.*, 2010].

4.1.1 Cellular mechanisms in melanoma

The cell cycle is characterised by four successive phases; G1, synthesis, G2 and mitosis. This sequence is regulated by cyclin-dependent kinases (CDKs) that phosphorylate two important gatekeepers for cellular progression; p53 and Retinoblastoma (Rb). The CDK Inhibitor 2A (*CDKN2A*) locus, situated on chromosome 9p21, encodes the tumour suppressor proteins, $p16^{INK4A}$ and $p14^{ARF}$ [Cannon-Albright *et al.*, 1994]. The $p16^{INK4A}$ protein halts cell cycle progression at the G1 phase by inhibiting the activity of CDK4 that normally phosphorylates Rb. When Rb undergoes phosphorylation it releases the transcription factor E2F that enables S phase progression. The p53 protein responds to DNA damage to enhance DNA repair or induce apoptosis at the G1 phase and is one of the most important regulators of cell cycle arrest and senescence. The $p14^{ARF}$ protein binds and inactivates the human-analogue to Murine Double Mature (MDM2) protein, which is a negative regulator of p53, and therefore acts to stabilise p53 in this model [Sharpless *et al.*, 2003].

4.1.2 Senescence

The term senescence arises from the latin word, *senex*, which essentially means old age. Cellular senescence was originally described in fibroblasts when they were noted to lose the ability to divide and yet remain viable [Hayflick *et al.*, 1961]. This observation was related to cell cycle arrest where an irreversible G0/G1-like status occurs and a cell in culture exhausts its replicative potential [Hayflick, 1965; 1974]. Based on these early studies senescence can also be termed cellular aging or replicative senescence. Unlike quiescence, altering the environment or providing additional nutrients to the cell cannot reverse senescence.

Senescent cells in culture display a characteristic flattened morphology that express senescence-associated β-galactosidase activity (Saβgal) and form visible DAPI-stainable senescence-associated heterochromatin foci (SAHF) [Narita et al., 2004; 2005]. The expression of activated Ras and BRAF in primary cells can lead to a phenotype almost indistinguishable from replicative senescence including the expression of Saβgal and formation of SAHF [Lanigan *et al.*, 2011]. The silencing of the INK4A/ARF pathways in melanoma enables cells to bypass senescence that represents a critical event in melanoma

progression. No marker alone is specific for senescence [Campisi *et al.*, 2007, Itahana *et al.*, 2007]. A senescence phenotype is characterised by a combination of growth arrest, apoptosis resistance and altered gene expression. Telomere dysfunction represents a mechanism for initiating senescence; a shortening in the telomere sequence leads to dysfunction and triggers a DNA damage response (DDR). Telomeres are distinct DNA-protein sections located at the ends of chromosomes [Campisi *et al.*, 2001]. These telomeric ends act as a protective mechanism to genomic DNA.

4.1.3 DNA damage response as a marker of senescence

DDR is a cascade of events that aim to prevent the propagation of defective genetic sequences and maintain integrity by initiating DNA repair [Shiloh, 2006]. DNA damage can result from multiple stimuli that can be grouped into endogenous (oxidative stress) or exogenous (ultraviolet light or radiation). These stimuli lead to damage of the complex DNA structure and produce single or double strand breaks (DSB). The accumulation of DSB triggers the DDR [D'adda Di Fagagna, 2008]. Single and double stranded breaks in the DNA are detected respectively by specific protein kinases: ataxia-telangiectasia and Rad3-related (ATR) or ataxia-telangiectasia mutated (ATM). As ATM is recruited to the site of DSB an immediate phosphorylation of histone H2AX (γH2AX) occurs which acts in a positive feedback loop and results in local accumulation of ATM and γH2AX. This mechanism propagates γH2AX along the chromatin and is key to the DDR. Key mediators in this process are the downstream kinases checkpoint-1 (CHK1), checkpoint-2 (CHK2) and p53-binding protein (53BP1) [Herbig *et al.*, 2004, Chen *et al.*, 2007]. The localisation of these proteins in response to dysfunctional telomeres and DSBs leads to foci that can be used to monitor DDR.

The significance of the DDR relates to the aforementioned p53-p21 pathway as activation of DNA damage and the subsequent response mediates p53 activation. This determines the ultimate fate of the cell to either apoptosis, or temporary (quiescence) or permanent (senescence) cell cycle arrest (Chen *et al.*, 2007.). The exact reason for which route a cell takes is not fully known and this varies depending on the cell or species [Shay *et al.*, 2005].

However the presence of DNA-damage foci representing DDR can be used as a marker for cellular senescence in cells not exposed to an external DNA damaging agent.

4.1.4 T-box factors and senescence

The *Brachyury* or T protein is the founding member of the family of transcriptional regulators now known as T-box factors. Studies on the T-box proteins have shown a characteristic DNA-binding domain of approximately 200 amino acids [Smith, 1997]. T-box factors have been identified in many species ranging from humans to zebrafish. With respect to cancer it is the mammalian T-box factors (TBX2 and TBX3) that are of interest (Table 6). The TBX2 gene, which is located on 17q23, is mutated in ovarian tumours [Law *et al.*, 1995], overexpressed in BRCA1 and BRCA2 breast cancers [Sinclair *et al.*, 2002] and in pancreatic cell lines [Mahlamaki *et al.*, 2002]. The TBX3 gene, located on 12q24, is mutated in BRCA1 breast tumours [Fan *et al.*, 2004]. In artificial systems using fibroblasts the TBX2 and TBX3 proteins cooperate with active RAS to promote growth [Carlson *et al.*, 2002]. In melanocytes TBX2 and TBX3 are expressed at low levels in but strikingly are overexpressed in multiple melanoma cell lines [Lu *et al.*, 2010]. TBX2 was first shown to be expressed in the murine melanoma cell line B16 [Carreira *et al.*, 2000]. This work was extended into human cell lines by Vance and colleagues demonstrating that TBX2 was overexpressed in 501mel cells [Vance *et al.*, 2005]. Furthermore by silencing TBX2 and also using a dominant-negative form, they observed senescence in melanoma cells lines. Using a combination of DNA binding and transfection assays Prince et al, showed that TBX2 can bind and repress the p21 promoter *in vitro* and *in vivo* [Prince *et al.*, 2004]. Additionally TBX2 can specifically associate with active Rb protein and these mutations in the TBX2 gene that disrupt interaction with Rb affect the target selectivity [Vance *et al.*, 2010]. With respect to senescence, TBX2 and TBX3 regulate the tumour suppressor gene p14ARF [Brummelkamp *et al.*, 2002, Lingbeek *et al.*, 2002]. Consequently TBX2 and TBX3 bypass senescence and are regarded as anti-senescent factors (Figure 6). These studies have enabled a greater understanding of the role that T-box factors play in oncogenesis and the regulation of senescence through the pathways of p53 and p21.

	Cancer				
	Breast	**Pancreas**	**Melanoma**	**Ovarian**	**Liver**
TBX2	50-80%	56-60%	63%	-	-
TBX3	70-90%	-	57%	69%	79-87%

Table 6. TBX2 and TBX3 are expressed in many human cancers

Displays levels of expression (shown in percentages) for TBX2 and TBX3 in the outline human cancers. Adapted from Lu et al 2010.

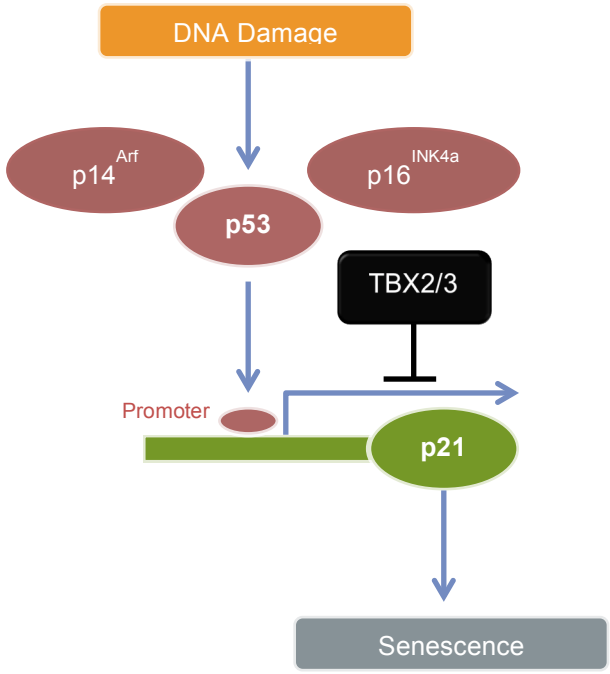


Figure 6. Repression of p21 by TBX2 and TBX3 bypasses senescence

In this model DNA damage activates the p53 pathway, cells can under apoptosis or temporary (quiescence) or permanent (senescence) growth arrest. Here the T-box factors inhibit p21 and bypass cell entry into senescence. Adapted from Prince et al 2004.

4.1.5 The FOXO factors and senescence

The FOXO proteins are a family of transcription factors. Originally identified in Drosophila by a mutant whose head structure resembled a fork. The key members of the FOXO family phosphorylated via the PI3K pathway and its downstream kinase AKT, are FOXO1 and FOXO3a [Brunet *et al.*, 1999]. This activity results in these FOXO proteins being exported from the nucleus and causing inhibition of dependent transcription, leading to cell cycle arrest or apoptosis [Nakamura *et al.*, 2000]. The role of FOXOs in cell cycle arrest has been linked to up-regulation of p27 that induces G1 arrest [Medema *et al.*, 2000]. In this manner FOXOs act as tumour suppressor proteins.

4.2 Aims of chapter

Given the role of the PI3K pathway in senescence bypass [Peeper, 2011], a key question that needs to be answered is does PI3K signalling promote expression of TBX2 and or TBX3 to bypass senescence. I aim to explore this notion by inhibiting the PI3K pathway and observing the downstream effects, in particular with respect to T-box factors. A further avenue to explore is that if T-box factors are regulated by PI3K inhibition, do they work alone or in co-operation with other factors. Proposed candidates are the FOXO family, and in particular FOXO1 and FOXO3, which are expressed in melanoma. The mechanistic role of these FOXO factors has not been fully explored in melanoma. Given the crucial role they play in cell cycle regulation does inhibiting the PI3K pathway regulate the levels of these FOXO factors.

4.3 Methods and Materials

4.3.1 Mammalian cell media

The standard protocol for cell cultures was applied for the human melanoma cell lines: 501mel and SKMel28. These underwent incubation at 37°C in humidified air with 5-10% CO2. Cells were routinely grown in plastic tissue culture plates (10cm2 cell culture plates, Corning Inc, NY) and flasks (75cm2 cell culture flasks, corning Inc, NY) in media comprising either DMEM (Dulbecco's modified Eagle medium; Lonza, Wokingham, UK) or RPMI-1640 (Roswell Park Memorial Institute medium; Invitrogen, Paisley, UK) supplemented with 10% foetal bovine serum (Lonza, Wokingham, UK) and 1% penicillin-streptomycin (to final concentration 100µg/ml penicillin, 100µg/ml streptomycin).

For cell passage, all media and solutions were prewarmed to 37°C. The tissue culture hood was disinfected with 70% IMS and allowed to dry. All required plates or flasks were transferred to the tissue culture hood thereafter. The previous media was aspirated by vacuum pump and cells were washed with Phosphate Buffered Saline (PBS; NaCl 8 g/l, KCl 0.2g/l, Na_2HPO_4 1.15 g/l, KH_2PO_4 0.2 g/l; Oxoid, Basingstoke, UK) and an appropriate volume of 0.05% trypsin-EDTA (Invitrogen, Paisley, UK) solution added. A short period of 30 to 60 seconds was allowed for the trypsin to be applied to the cells and appropriate media (containing serum) was added to counteract any remaining trypsin. The solution was then removed by vacuum pump and cells are counted by microscopy or automated cell counter (TC10 Automated cell counter, Bio-Rad laboratories, UK), prior to replating.

4.3.2 Transfection of DNA to mammalian cells

Mammalian cells identified for transfection were plated at approximately 50% confluency or and incubated overnight in the appropriate media. Transfection was achieved using the FuGENE 6 reagent (Roche Diagnostics Ltd, Burgess Hill, UK) at the recommended ratio of FuGENE 6:DNA 3:1 (µl/µg). The DNA component was based on increasing amounts of reporter plasmid but maintaining a standard total amount of DNA. The appropriate volume of FuGENE was added to the serum free media and kept at room temperature for 5 minutes. The predetermined DNA in each transfection was then added to this solution and left for a

further 25 minutes at room temperature to enable the formation of DNA-lipid complex. The mixture is then applied to the cells with fresh media and incubated under standard conditions. The cells are assessed at 24 hours (and up to 72 hours) post transfection for further analysis.

4.3.3 Luciferase assay system

The Luciferase Assay System (Promega, Southampton, UK) was used for reporter quantification in mammalian cells and is based on detecting the production of light from the chemical energy of luciferin oxidation. The firefly luciferase enzyme catalyses the oxidation reaction forming the product oxyluciferin, which emits a photon of light, measured by the Glomax Multi Detection System (Promega, Southampton, UK). The activity of the Firefly luciferase enables quantification of the genes of interest on the promoter sequence. Cells of interest were plated at 1.5x105 in 12 wells plates and incubated overnight. Transfection of DNA was as previously described. Preparation of the cell lysate for luciferase analysis is consistent with the manufacturers protocol. Cells were washed once with cold PBS, removed by vacuum pump and then lysed with 100µl passive lysis buffer (Promega, Southampton, UK). From this 20µl was added to a 96 well plate and mixed with 50µl of Luciferase Assay Reagent (Promega, Southampton, UK). DNA expression vectors of TBX2, TBX3, FOXO1 and FOXO3 were used for transfection into cells (Figure 7). Luciferase promoters of TBX2 and TBX3 were co-transfected and used to assess activity for each plasmid alone or in combination. A total of 400ng DNA per sample was maintained using CMV plasmid. A control of CMV on the promoter was used to assess the efficiency of the luciferase assay. Subsequent samples were compared to the fold change to the control. Experiments were performed in triplicate and repeat three times.

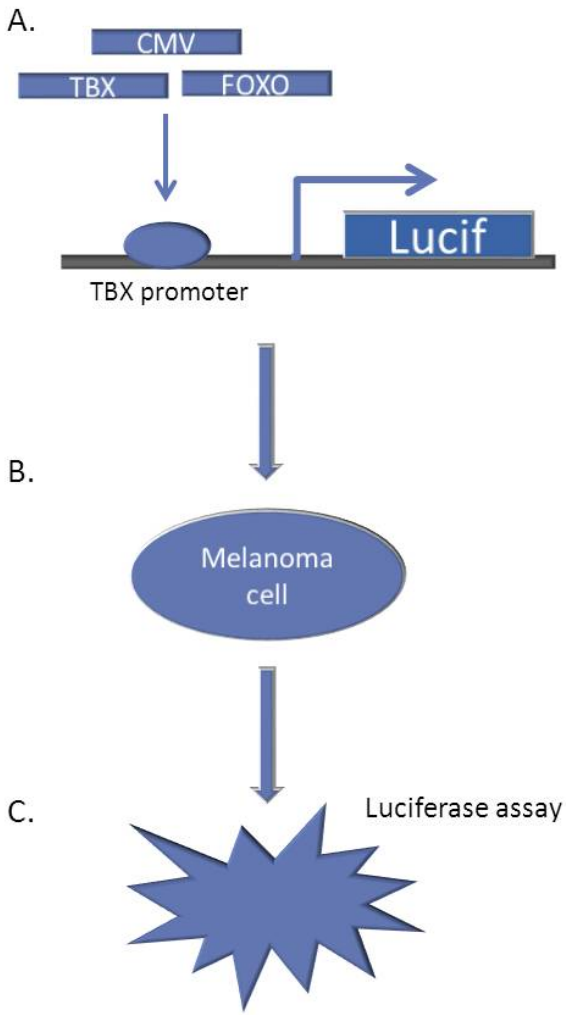


Figure 7. Method for luciferase reporter assay.

A. Expression vector of TBX2, TBX3, FOXO1, FOXO3 are transfected with TBX2 or TBX3 promoter (with luciferase reporter). CMV plasmid is used to maintain standard total of DNA.

B. DNA plasmids are transfected into melanoma cells (plated prior, ready for transfection) using FUGENE transfection method with fresh cell culture media. These are incubated for 48 hours at 37^{o}, 10% CO_2.

C. Samples are extracted and plated in 96 wells. Luciferase re assay reagent is then added and light emitted detected by Glomax multi-detection system.

4.3.4 Bacterial media

Bacterial selection was performed on single colonies on solid LB (Luria broth – tryptone 10g/l, NaCl 10g/l, yeast extract 5g/l, Tris 1.5g/l; Fisher, Loughborough, UK) agar (1.5%) plates which were prepared by melting, allowing the agar to cool to 50-55°C then pouring into Petri dishes. These were stored at 4°C. The plates were air dried and prewarmed at 37°C prior to use.

Liquid cultures of bacterial strains utilised L-broth as the growth medium and were maintained at 37°C in a rotary incubator (200 rpm). For *Escherichia coli* strains carrying plasmids of interest, the most common antibiotic used was ampicillin (50-100µg/ml), which was added prior to inoculation of bacterial material.

4.3.5 Immunofluorescence

Cells of interest were plated at a density of $1.5x10^5$ in 12 well cell culture plates. The wells contained a 16mm cover glass slip (VWR International, Pennsylvania, USA) in 1 ml of the appropriate cell culture media. This was incubated overnight in standard conditions. Wells underwent treatment PI3K inhibitor (LY294022 or GDC0941). Control wells included no treatment, exposure to ultraviolet light or application of DMSO. All treatments were conducted over a 24-hour timecourse.

In between each of the following steps the glass cover slips were washed with PBS up to three times. After aspiration of the culture media cell were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature. Subsequently cells were permeabilised with Triton X-100 (0.2%) for 5 minutes at room temperature, and blocked with either BSA (3%) or dry milk (5%) solution for 60 minutes at room temperature. The glass cover slips were then transferred to a humidified container and incubated with the primary antibody overnight at 4^o. For DNA damage assessment the primary antibodies used were anti-phospho-Histone H2AX (Ser139, monoclonal, clone JBW301, Merck Millipore, MA, USA) and p53-binding protein antibody (53BP1, clone 4937, Cell Signaling Technology, Beverly, MA, USA). Appropriate secondary antibodies were applied for 60 minutes at room temperature. The glass cover slips were mounted with VECTASHIELD® mounting medium containing DAPI (Vector

Laboratories Inc, Burlingame, CA, USA) on to microscope slides (Superfrost plus, VWR International, Pennsylvania, USA). These were allowed to air dry at room temperature and then stored at 4^{o} protected from light.

4.3.6 Real Time PCR

This method enables amplification and quantification of a particular DNA sequence from the gene of interest. Equal quantities of RNA (200 ng – 2 ug) from the desired experimental conditions were extracted and retrotranscribed to cDNA. Gene specific primers were designed across intron spanning regions to reduce co-amplification of any contaminating genomic DNA using either Primer 3 as before, or the Roche Universal Probe Library (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000). Samples were loaded to a Rotor Gene 100 disc (Qiagen, Crawley, UK), by a Corbett robot (model: CAS-1200, Qiagen) using liquid sensing filter tips (Qiagen) and running on Corbett Robotics 4 software. The mix was comprised of 6.5 ul 2x SensiMix SYBR (Quantace Bioline, London, UK), 2 ul of appropriate mixed forward and reverse gene specific primers (at 10 pmol/ul/primer) and 5.5 ul of cDNA diluted to an appropriate volume to complete all desired reactions with 0.1% DEPC water to give a total volume of 15 ul. Each sample was run in triplicate within the experiment. SYBR Green binds to the double stranded DNA produced by the PCR reaction and fluoresces, which is detected within the Light Cycler (model: 2-Plex HRM, Corbett/Qiagen) used to perform the PCR. The RotorGene 6000 software provides a real time report of the detected fluorescence with reference to the cycle number on a logarithmic scale. Once a threshold is set (usually 0.8 units), then a Ct value is given i.e. the cycle number at which the sample crossed the selected threshold. This enabled the fold difference in expression to be calculated between the gene of interest and the housekeeping gene chosen for the experiment (GAPDH). Melt curve analysis was used to ensure only one gene product for each pair of primers and minimal or no primer dimer formation i.e. only one peak visible for each pair in the melt profile.

4.4 Results

4.4.1 PI3K inhibition causes DNA damage; a marker of senescence

DDR has been associated with a state of senescence in cells. In preliminary experiments I aimed to induce DNA damage in cells and assess their response by monitoring key factors. Cells underwent immunofluorescence for key markers of DDR γH2AX and 53BP1. Cells were exposed to a range of DNA-damage-inducing agents such as DTIC, 5-Fluorouracil (5FU) and UV radiation. After 24 hours treatment with DTIC and 5FU melanoma cells showed increased signal for γH2AX (Figure 8A and 8B). Control of untreated cells are shown in Figure 8C. To assess the patterns of DNA damage a time-course experiment over 48 hours was conducted on the same cell line with UV radiation. The results showed that the γH2AX signal increased over the exposure to UV radiation with increased foci present in the nuclei of cells, peaking at 24 hours and then diminishing by 48 hours (Figure 8C). The 53BP1 foci were noted much earlier at 12 hours and were maintained at 24 hours and then once again reduced by 48 hours. The immunofluorescence methods were able to detect markers for DNA damage in the current cell line. Subsequent experiments focused on assessing the effect of PI3K inhibition on melanoma cells.

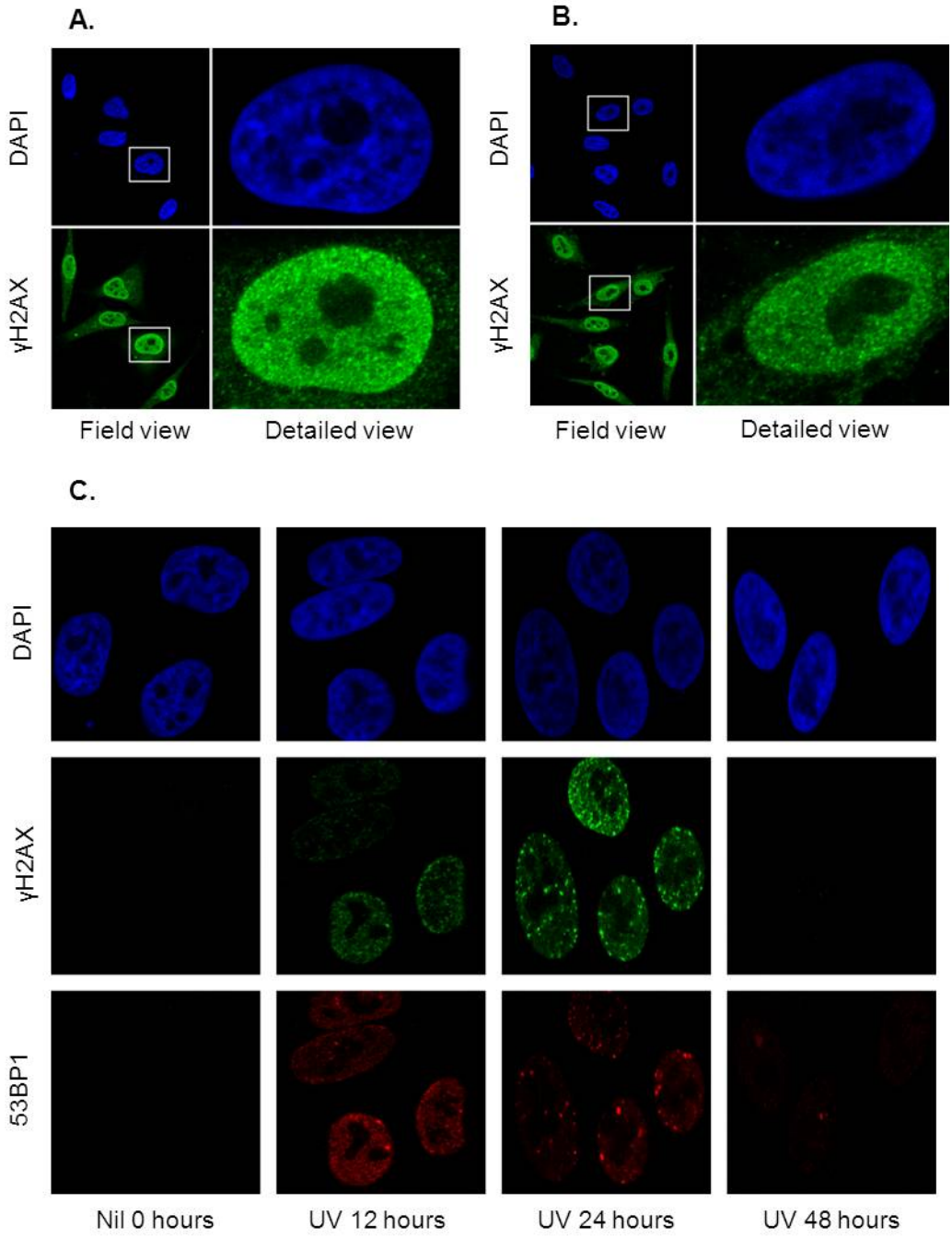


Figure 8. H2AX is a marker of DNA damage in melanoma cells

A. Immunofluorescence of 501 mel cells treated with dacarbazine (500μg/mL, DTIC) at 24 hour time course. Stained with anti-phospho histone H2AX (1:100). Images captured with Zeiss 710 confocal microscope, field view and detailed nuclear view shown.

B. Immunofluorescence of 501 mel cells treated with 5-fluorouracil (0.5μM, 5FU) at 24 hour time course. Stained with anti-phospho histone H2AX (1:100). Images captured with Zeiss 710 confocal microscope, field view and detailed nuclear view shown.

C. Immunofluorescence of 501 mel cells treated with ultraviolet radiation (24J/m2, UVC 254nM) over 48 hour time course, compared to no treatment at baseline timepoint (nil). Stained with anti-phospho histone H2AX (1:100) and anti-53BP1 (1:100). Images captured with Zeiss 710 confocal microscope, field view and detailed nuclear view shown.

Preliminary experiments aimed to assess the impact of the inhibitors on cell morphology and numbers. Cells were treated with the PI3K inhibitor (LY294002) over a 24-hour time course and compared to cells with no treatment or treatment with a control (DMSO). The effects of the PI3K inhibitor were noted by one hour in the cells, as they developed an altered morphology in comparison to the control conditions. By 24 hours the cells treated with the PI3K inhibitor appeared to change shape, forming a more rounded appearance, reflecting a reduction in cell growth and proliferation (Figure 9A). To exclude any cell death samples were assessed for cell viability (differentiated by trypan blue staining). The results showed that there were no significant differences in the proportion of live cells with increasing PI3K inhibitor concentrations in comparison to the control (Figure 9B).

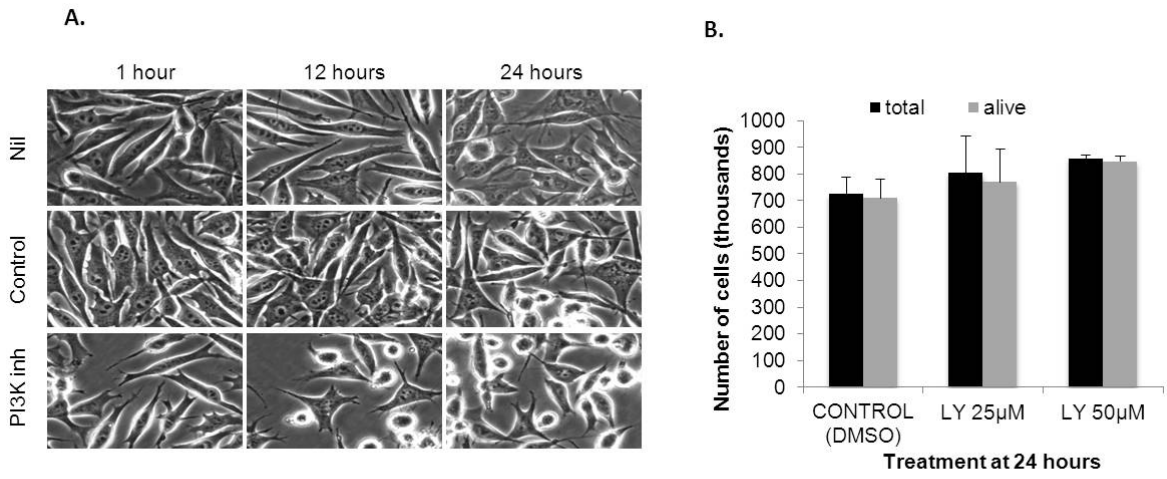


Figure 9. PI3K inhibition alters melanoma cell morphology without apoptosis.

A. Melanoma cells become more rounded in appearance after treatment with PI3K inhibitor.
Light microscopy images of 501 mel cells at x20 magnification. Treatments conducted over 24 hour timecourse. Nil represents no treatment. DMSO used as control. PI3K inhibitor used LY294002 at 50μM.

B. There is no significant cell death from PI3K inhibitor treatment.
Shows the number of alive and dead 501 mel cells detected after undergoing treatment (as before), being stained with trypan blue and examined with cell counter (TC-10, Bio-Rad laboratories, UK). Samples analysed at three focal points and repeated three times. LY indicates PI3K inhibitor LY294002 at specified doses. P value (p=0.78) calculated using t-test.

The 501mel cell morphology was noted to have changed with PI3K inhibition, however this observation was only at fixed time points. To gain an appreciation for cell migration a wound healing experiment with an extended time-lapse was performed. This experiment aimed to measure the amount of time for a monolayer of confluence cells to repair a wound. The repair of the wound is due to cell migration and proliferation. The 501mel cells were plated as per protocol and a standard size wound made in all wells. Cells were treated with DMSO or PI3K inhibitor (GDC0941) and compared to untreated cells as a control. Three sample points were captured for each treatment at 15-minute intervals up to 72 hours, images of the cells are shown in Figure 10. The results were analysed for the first 24 hours (Figure 11A). This showed that by 24 hours the cells with no treatment were able to heal the wound by 90%. At the same time point cells treated with the control repaired the wound by 66%. For the PI3K inhibitor group the rate of wound healing was substantially reduced and at 24 hours only 26% of the wound had repaired (Figure 11A). Images from the time points revealed reduced cell migration for the PI3K treatment cells and a grossly flattened morphology, in comparison to the other treatment groups. To further analyse cell migration the time taken to achieve 50% wound healing was calculated for each treatment arm (Figure 11B). This revealed that cells treated with the PI3K inhibitor had a significantly greater period (mean 103 hours, $p=0.0003$) to achieve 50% healing in comparison to the control (mean 16 hours) and no treatment (mean 15 hours). Reviewing the data at a time point of 10 hours there is marked difference in the size of the wound for the cells undergoing PI3K inhibition in comparison to the control or untreated (Figure 11A). This observation reflects a period shorter than the doubling time of the cells used and therefore highlights the effect of the agent on the cells.

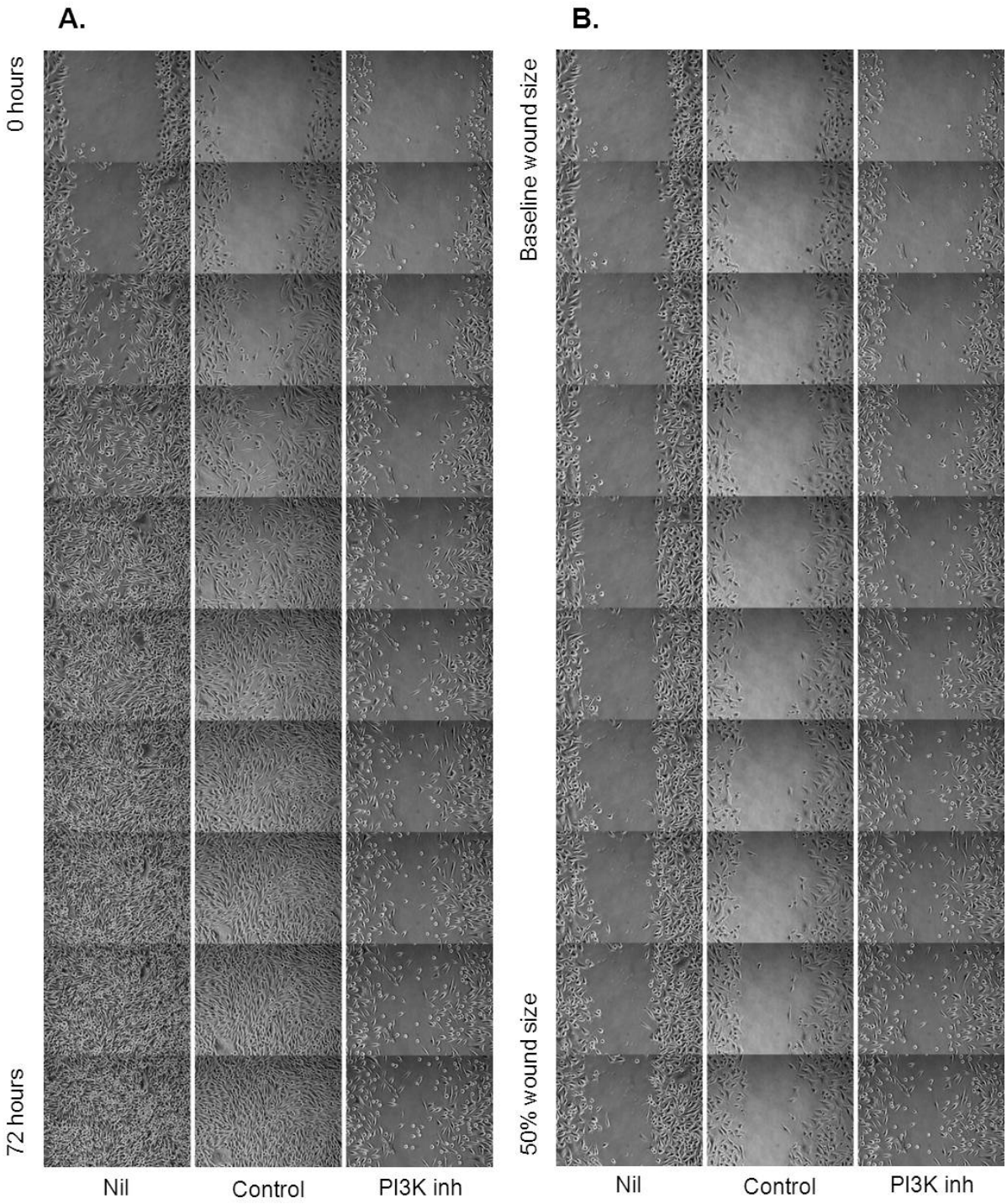


Figure 10. PI3K inhibition leads to reduced cell migration and prolonged wound healing.

A. Treatment of melanoma cells shows reduced wound healing and mobility of 501 mel cells in response to PI3K inhibitor (GDC0941 20μM), in comparison to no treatment (Nil) and exposure to DMSO (control).

B. Represents 50% wound size from baseline in response to treatments.
Images captured using confocal 510 Zeiss microscope (x10 magnification) at 15 minute intervals over 72 hours. Recordings conducted in triplicate.

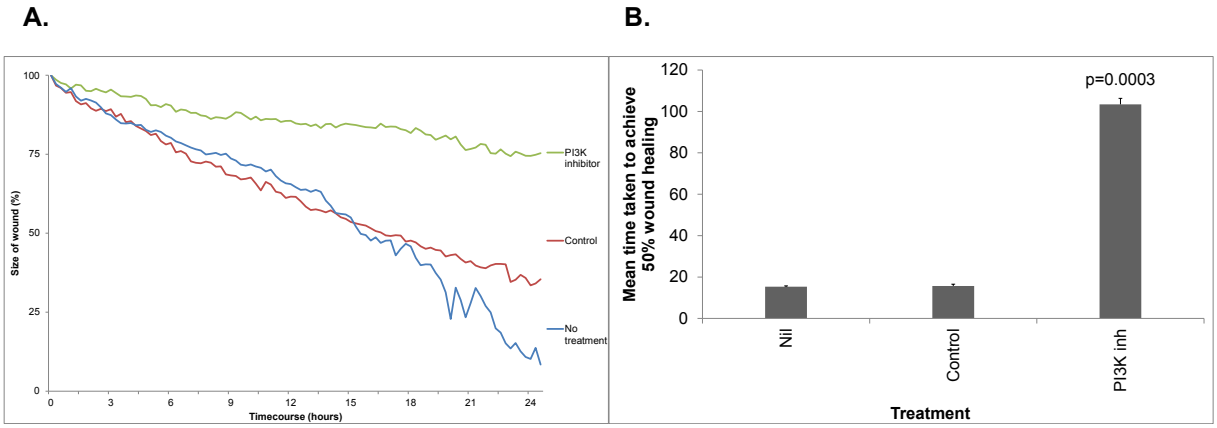


Figure 11. PI3K inhibition delays wound healing

A. Chart showing the percentage size of the wound over 24 hour time course based on different treatment conditions. 501 mel cells treated with PI3K inhibitor (GDC0941 20μM), in comparison to no treatment (Nil) and exposure to DMSO (control). Samples conducted in triplicate based on imaging from confocal time-lapse microscopy.

B. The mean time taken to achieve 50% wound healing from baseline based on treatment conditions. 501 mel cells treated with PI3K inhibitor (GDC0941 20μM), in comparison to no treatment (Nil) and exposure to DMSO (control). Samples conducted in triplicate based on imaging from confocal time-lapse microscopy. Error bars represent standard deviation. P value based on t-test.

As there was no major cell death from PI3K inhibitor exposure it was pertinent to assess senescence in cells, which can be indicated by levels of DDR. Therefore melanoma cells underwent treatment by a PI3K inhibitor and were assessed at 24 hours for the levels of DNA damage markers. The results showed a dramatic increase in the signal for γH2AX present in the nuclei of the PI3K inhibitor treated cells compared to the control samples (Figure 12A). Closer imaging of the cells exposed to PI3K inhibitor nuclei showed characteristic foci consistent with DNA damage. The signal intensity of 53BP1 was noted in both the control and treated cells, however closer examination of the nuclei showed more characteristic foci present in the cells exposed to the PI3K inhibitor (Figure 12A). This experiment was repeated in a different cell line (Skmel28) and again greater numbers of foci for γH2AX and 53BP1 were noted (Figure 12B).

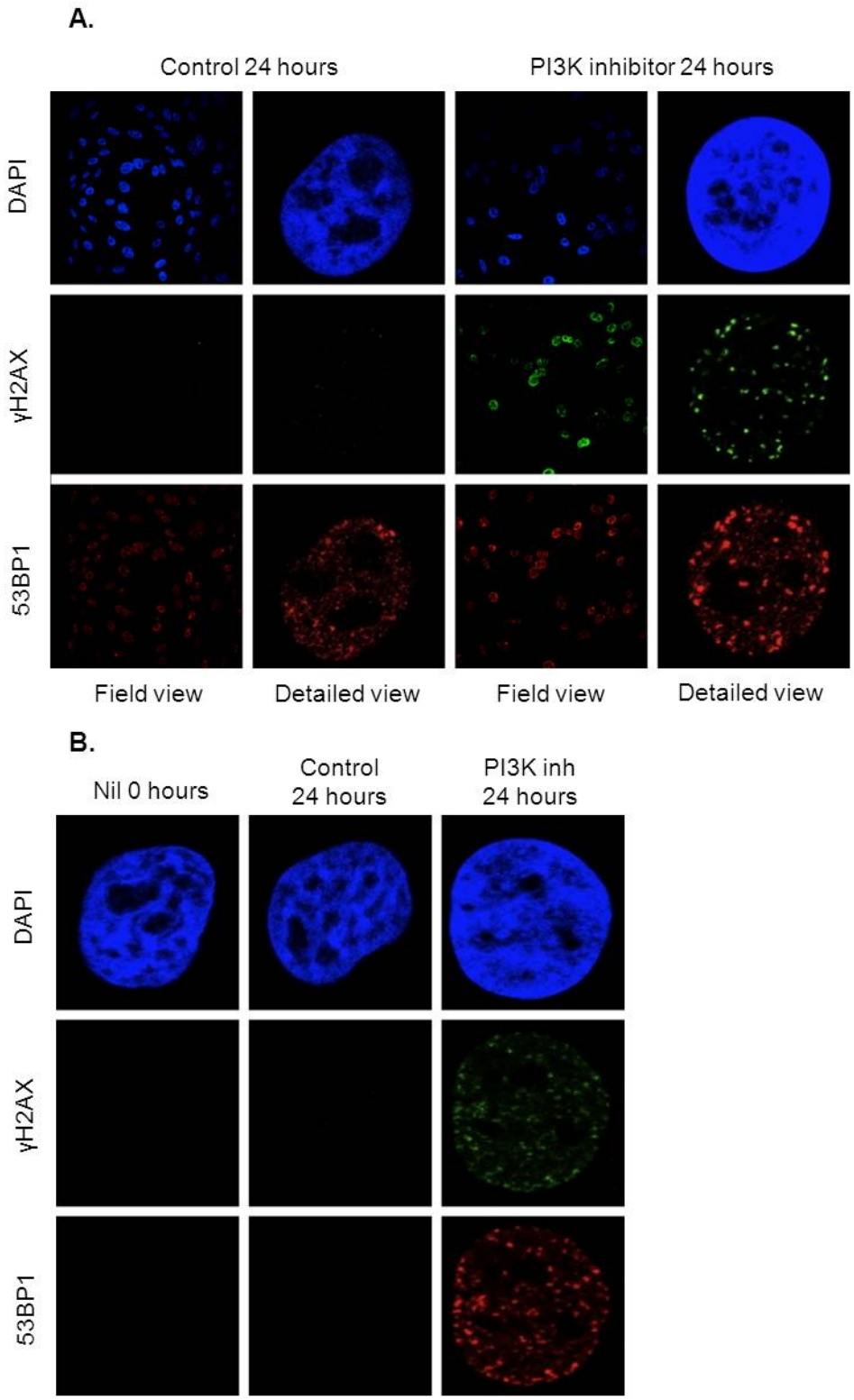


Figure 12. PI3K inhibition is associated with markers of DNA damage

A. Immunofluorescence of 501 mel cells treated with PI3K inhibitor (GDC0941, 20μM) over 24 hour time course, compared to treatment with DMSO (control). Stained with anti-phospho histone H2AX (1:100) and anti-53BP1 (1:100). Images captured with Zeiss 710 confocal microscope, field view and detailed nuclear view shown.

B. Immunofluorescence of skmel28 cells treated with PI3K inhibitor (GDC0941, 20μM) over 24 hour time course, compared to treatment with DMSO (control), and no treatment at time zero (Nil). Stained with anti-phospho histone H2AX (1:100) and anti-53BP1 (1:100). Images captured with Zeiss 710 confocal microscope detailed nuclear view shown.

4.4.2 PI3K inhibition down regulates T-box factors

Based on the marked signs of DNA damage noted by PI3K inhibition. It was important to assess the downstream effects of PI3K inhibition in the pathway, in particular to endogenous protein levels. A western blot for cells treated with LY294002 showed protein levels of TBX2 to be reduced by 12 hours and substantially reduced by 24 hours (Figure 13A). This was following effective inhibition of the PI3K signalling indicated by the reduction in downstream phosphoS6 (pS6) levels. ERK signal was probed for in the western blot as a loading control (Figure 13A). As an additional control, MITF levels were also proved. Since MITF is down regulated in relation to senescence [Giuliano *et al.*, 2010]. Reviewing the western blot it is interesting to note that the relative levels of MITF do not reduce over time from exposure to PI3K inhibitor (Figure 13A). This finding indicates that the DNA damage noted by the accumulation of γH2AX foci is unlikely to be related to MITF but to other factors such as TBX2. Subsequent analysis by quantitative real time PCR of the cell samples confirmed the reduction in relative expression of TBX2 mRNA and this was significantly ($p<0.0001$) repressed by 12 hours and remained so at 24 hours (Figure 13B). The significant decrease in TBX2 mRNA indicates that TBX2 expression is inhibited at the levels of mRNA expression. Therefore this required further investigation of the TBX2, and TBX3, promoter in relation to expression to itself and other transcription factors.

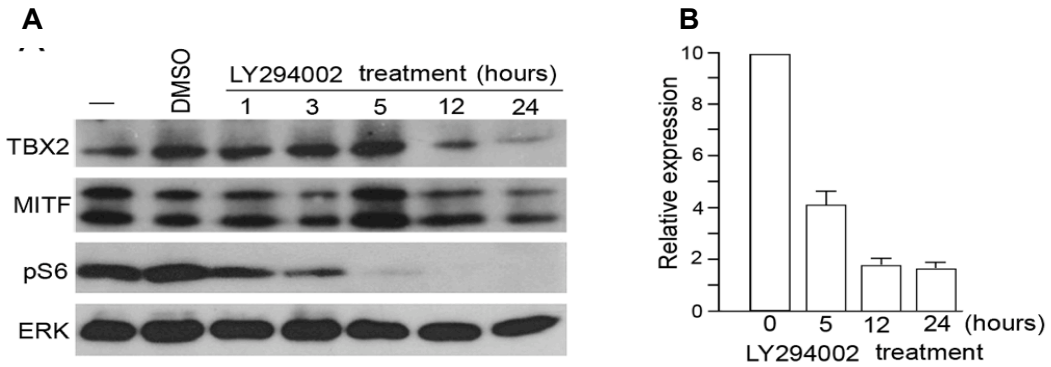


Figure 13. PI3K signalling represses TBX2 expression

A. Western blot following 24-hour time course treatment with PI3K inhibitor (LY294002, 50μ M), compared with controls of no treatment (shown as -) and DMSO. Western blot probed with anti-TBX2 (mouse monoclonal, AS32, 1:1000), anti-MITF (mouse monoclonal, 1:3000), anti-phosphoS6 (rabbit monoclonal, S235/236, 1:2000, Cell signalling). ERK2 (rabbit polyclonal, 1:5000, Santa Cruz) used as loading control.

B. Quantitative real time PCR data showing relative fold change for TBX2 expression in comparison to control for treatment with PI3K inhibitor (LY294002). Based on three samples repeated and controlled with GAPDH. P value calculated using t-test; $p<0.0001$.

4.4.3 T-box and FOXO factors act in cooperation

The results so far indicate that inhibition of PI3K lead to increased DDR and decreasing TBX2. The question remains how does the PI3K pathway mechanistically regulate TBX2? One possible candidate is FOXO1 and in the first instance it was important to ascertain if FOXO1 was regulated by PI3K. Staining with antibodies targeted against FOXO1 was conducted on melanoma cells and captured with immunofluorescence (Figure 14A). Subsequent imaging aimed to identify the localisation of FOXO1 within melanoma cells, whether these were in the cytoplasm or nucleus (Figure 14B). Examining the higher magnification images (Figure 14B) it is apparent that there is nuclear accumulation of FOXO1 in response to PI3K inhibition.

To assess if FOXOs regulate T-box factors transfection of expression vectors (TBX2, TBX3, FOXO1 and FOXO3) into melanoma cells (501mel) was conducted as per protocol. These transfected cells were assessed for activity against a TBX2 or TBX3 luciferase promoter using the Luciferase assay system.

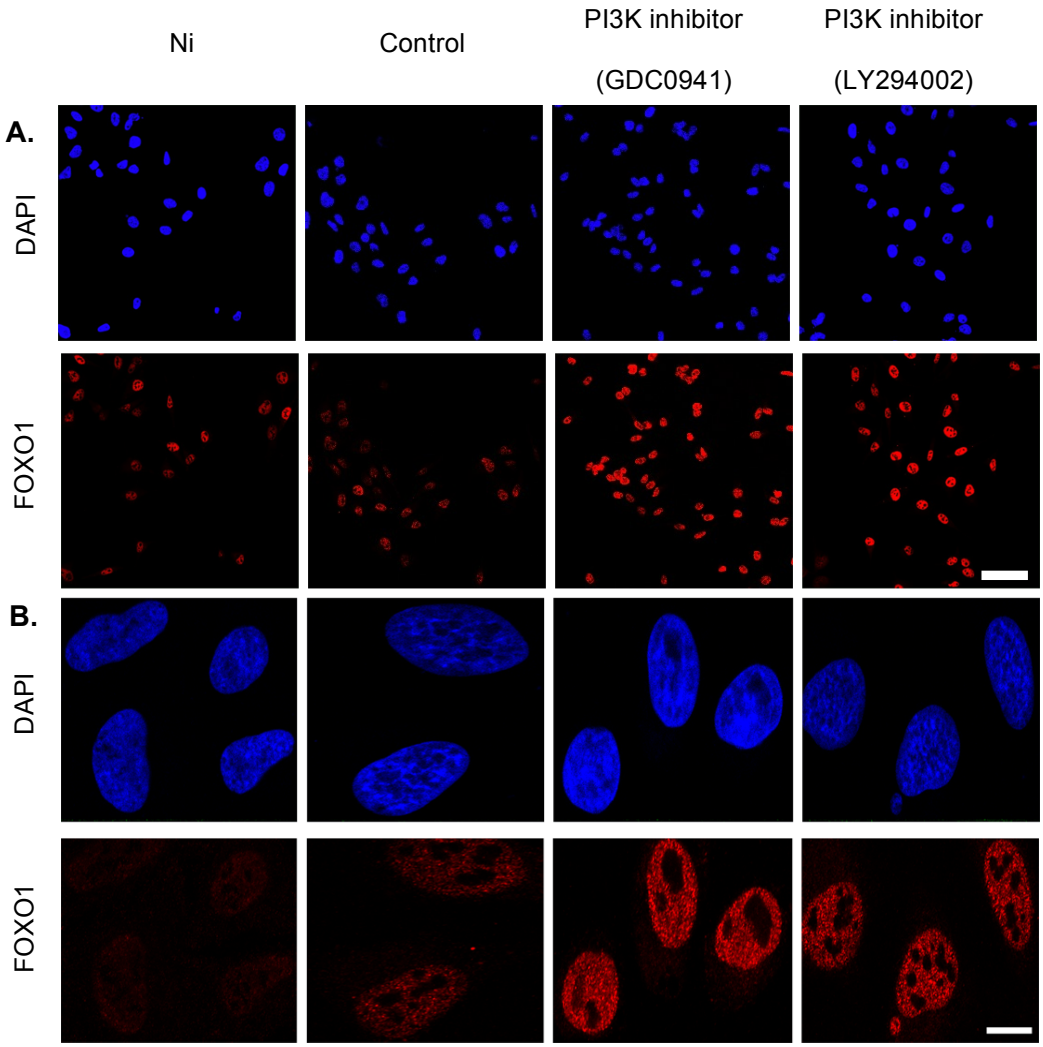


Figure 14. PI3K inhibition leads to nuclear accumulation of FOXO1

A. Field view of immunofluorescence of 501 mel cells treated with PI3K inhibitors (GDC0941, 20μM and LY294002 50μM) and over 24 hour time course, compared to treatment with DMSO (control). Baseline cells with no treatment also shown as control (nil). Samples stained with DAPI and anti-FOXO1 (rabbit monoclonal, 1:100, Cell Signalling). Images captured with Zeiss 710 confocal microscope, scale bar shown as 15μm

B. High magnification of immunofluorescence of 501 mel cells treated with PI3K inhibitors (GDC0941, 20μM and LY294002 50μM) and over 24 hour time course, compared to treatment with DMSO (control). Baseline cells with no treatment also shown as control (nil). Samples stained with DAPI and anti-FOXO1 (rabbit monoclonal, 1:100, Cell Signalling). Images captured with Zeiss 710 confocal microscope, scale bar shown as 15μm

Initial experiments were conducted to assess the role of FOXO1 and FOXO3a on the TBX2 and TBX3 luciferase promoters. Increasing amounts (50ng and 100ng) of expression vectors for FOXO1 and FOXO3a showed repression on the TBX2 promoter (Figure 15). TBX2 promoter activity was modestly reduced by FOXO1 (30%) and FOXO3 (27%) at 50ng. However at a greater DNA concentration of 100ng, both FOXO1 (40%, p=0.001) and FOXO3 (62%, p=0.003) showed significant repression on the TBX2 promoter (Figure 15A and Figure 15C). On the TBX3 promoter the addition of FOXO1 50ng did not demonstrate a significant reduction in activity (Figure 15B). However increasing the FOXO1 expression from 50ng to 100ng showed a significant reduction by 53% (p=0.002) (Figure 15B). The equivalent expression of FOXO3 on the TBX3 promoter confirmed reduction of 91% (p=0.0001) in comparison to the control (Figure 15D). Dominant-negative expression vectors of FOXO3 were also assessed on TBX2 and TBX3 promoters, the results showed a significant increase ($p<0.0001$) in transcriptional activity on the respective promoters (Figure 15E and Figure 15F).

Transcriptional activity was also measured for expression vectors of TBX2 and TBX3 on their respective promoters. Transfection of increasing TBX2 plasmids (50ng and 100ng) with the TBX2 promoter showed a reduction in activity of 57% (p=0.006) and 80% (p=0.00004), respectively (Figure 16A). This trend in repression was noted with the same conditions for the TBX3 plasmid on the TBX3 promoter, but with greater reductions of 97% for TBX3 100ng (Figure 16B). Based on these experiments there was an indication that TBX2 and TBX3 repressed their own promoters.

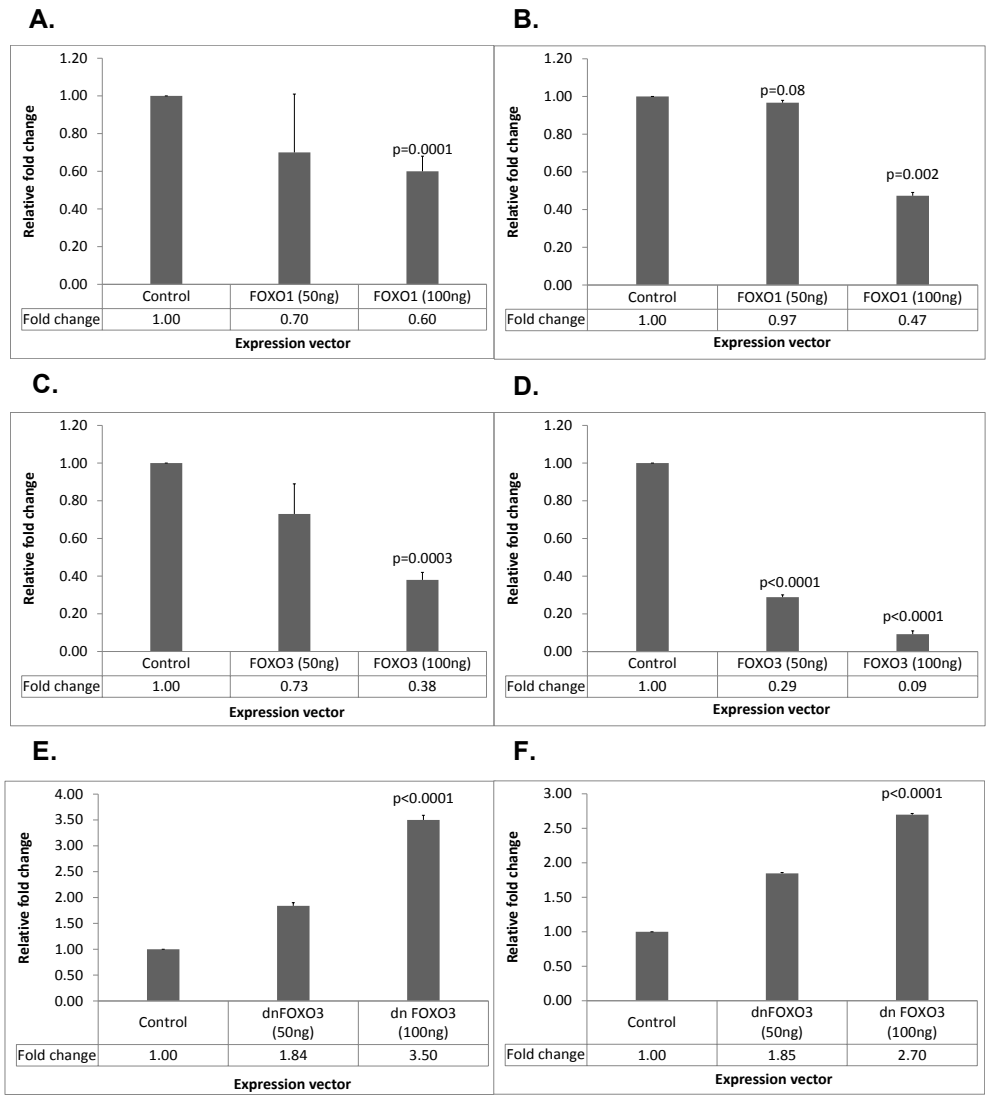


Figure 15. FOXO1 and FOXO3a repress transcriptional activity of TBX2 and TBX3 promoters.

A. FOXO1 on TBX2 promoter
B. FOXO1 on TBX3 promoter
C. FOXO3 on TBX2 promoter
D. FOXO3 on TBX3 promoter
E. dnFOXO3 on TBX2 promoter
F. dnFOXO3 on TBX3 promoter.

Expression vectors of the respective factors (amounts shown in ng) transfected with luciferase promoter reporters into 501 mel cells. Graphs showing relative fold changes in luciferase reporter activity. Total 400ng DNA maintained. Experiment conducted in triplicated and repeated independently. Error bars indicate standard deviation, p values based on t-test.

Acting alone TBX2, TBX3, FOXO1 and FOXO3 have shown repression on both TBX2 and TBX3 promoters. However combining the T-box factors with the FOXOs on these promoters would enable an assessment of their dual action. With respect to the TBX2 promoter FOXO1 and FOXO3 expression vectors alone did not show a significant repression (Figure 17A). However the combination of TBX2 and FOXO1 showed a significant ($p=0.00003$) repression by 95% (Figure 17A). This significant reduction in activity was also noted with TBX2 and FOXO3 (99%, $p<0.00001$).

With regards to the TBX3 promoter we have previously noted that TBX3 alone causes a significant repression on its own promoter (Figure 17B). FOXO1 acting alone did not cause any significant repression but FOXO3 alone was noted to have a reduction in activity of 71% (Figure 17B). The combination of TBX3 with FOXO1 and FOXO3 led to a significant reduction in activity by 98% and 99% respectively (Figure 17B).

Collectively the results indicate tat FOXO factors may cooperate with T-box proteins to impose transcriptional repression in there target genes and that then may contribute to the increased DDR in PI3K cells.

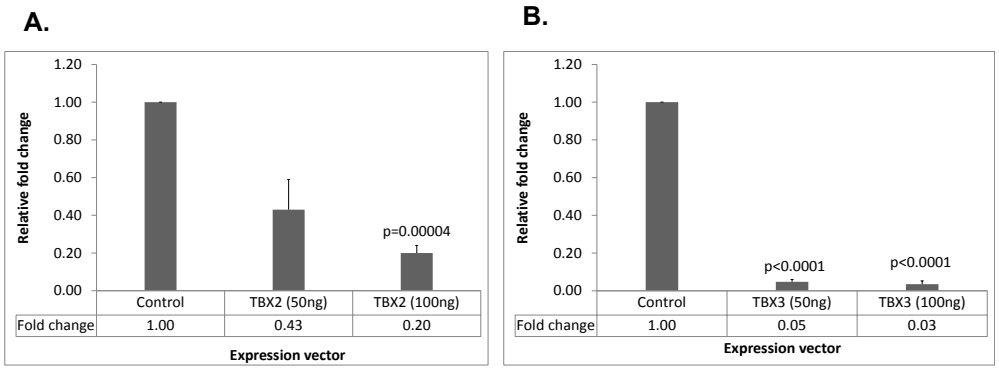


Figure 16. TBX2 and TBX3 repress transcriptional activity of TBX2 and TBX3 promoters.

A. TBX2 on TBX2 promoter **B.** TBX3 on TBX3 promoter.

Expression vectors of the respective factors (amounts shown in ng) transfected with luciferase promoter reporters into 501 mel cells. Graphs showing relative fold changes in luciferase reporter activity. Total 400ng DNA maintained. Experiment conducted in triplicated and repeated independently. Error bars indicate standard deviation, p values based on t-test.

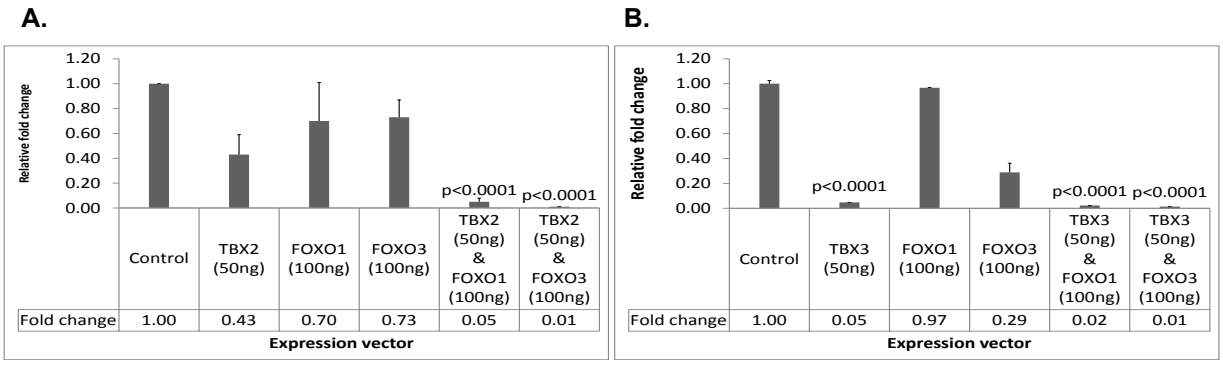


Figure 17. TBX and FOXO factors working synergistically onTBX2 and TBX3 promoter

A. TBX2, FOXO1 and FOXO3a on TBX2 promoter **B.** TBX2, FOXO1 and FOXO3a on TBX2 promoter.

Expression vectors of the respective factors (amounts shown in ng) transfected alone or in combination with luciferase promoter reporters into 501 mel cells. Graphs showing relative fold changes in luciferase reporter activity. Total 400ng DNA maintained. Experiment conducted in triplicated and repeated independently. Error bars indicate standard deviation, p values based on t-test.

4.5 Discussion

The PI3K pathway has emerged as a key regulator of cellular proliferation in cancer and in particular melanoma. This chapter has focused on inhibiting the PI3K pathway with pharmacological inhibitors and assessing the downstream effects.

4.5.1 PI3K inhibition in relation to DNA damage and senescence

The ability of a cell to choose between growth, select apoptosis or impose a cell cycle arrest is dependent upon the p53-p21 axis, which is influenced by DNA damage [Chen *et al.*, 2007]. The DDR cascade is an integral part of this process and its activity can be assessed by measuring the localisation of H2AX and 53BP1 proteins [Herbig *et al.*, 2004]. A range of DNA damage inducing agents showed increased staining in two markers of DDR. Initial experiments with PI3K inhibitors observed a change in melanoma cell morphology and reduced cell migration relating to the wound-healing assay. In mammalian cells lines initial experiments demonstrated DNA damage in response to DTIC, 5FU and UV radiation (Figure 8). In particular, increased foci and intensity for γH2AX were associated with cells exposed to PI3K inhibitors (Figure 12), indicating the accumulation of DNA damage by inhibiting the PI3K axis. The high levels of γH2AX are likely to represent DNA replication associated damage. PI3K inhibitors such as Wortmannin and LY294002 have been shown to induce DNA damage in other cell types such as acute myeloid leukaemia cells, and enhance the cytotoxic nature of chemotherapy agents [Skladanowski *et al.*, 2007]. More recently the use of dual PI3K and PARP inhibition revealed increased DNA damage and down-regulation of BRCA1/2 in breast cancer xenografts [Ibrahim *et al.*, 2012]. Therefore PI3K inhibition can induce DNA damage not only in melanoma cells but in other cancer cell types.

4.5.2 PI3K signalling in relation to T-box and FOXO factors

Melanomas have been noted to harbour mutations in PTEN and elevated AKT levels [Tsao *et al.*, 2000]. Deletions in PTEN lead to increased PI3K activity and activation of downstream proteins such as AKT. Here I have shown that pharmacological inhibition of the PI3K pathway represses TBX2 at the RNA and protein levels (Figure 13), highlighting the role of TBX2 (and TBX3) in melanoma cells. Consistent with other studies [Hoogaars *et al.*, 2008] I

have shown TBX2, and to a certain degree TBX3, to be over expressed in 501mel cells. Since depletion of TBX3 via its capacity to repress E-cadherin expression, and to a lesser extent of TBX2, has been shown to decrease melanoma invasiveness in vitro [Rodriguez *et al.*, 2008] this suggests these T-box factors both inhibit senescence and enhance invasiveness. The results suggest that increased invasiveness via PI3K signalling in PTEN-null mouse melanoma models maybe mediated in part via its regulation of TBX2 and TBX3 expression and function.

The final experiments in this research explored the mechanism between T-box factors and proliferation or senescence. We identified the regulation of TBX by the PI3K pathway, and highlight the likely involvement of FOXO1 and FOXO3a. Immunofluorescence staining of melanoma cells after PI3K inhibition showed increased nuclear accumulation of FOXO1, potentially contributing to invasiveness (Figure 14). Since it is known that FOXO proteins are retained in the cytoplasm in response to PI3K signalling, it is possible that any cytoplasmic FOXO1 in the melanoma cells is rapidly degraded but is stabilised on PI3K inhibition and subsequently accumulates in the nucleus. Beyond cell cycle regulation FOXOs influence DNA repair, apoptosis, oxidative stress resistance and glucose metabolism [Accili *et al.*, 2004]. FOXOs have been shown to have important roles in cancer. FOXO1 is down regulated in endometrial and ovarian cancer [Guttilla *et al.*, 2009]. In breast cancer over expression of HER2 leads to regulation FOXO1 via the PI3K pathway [Wu *et al.*, 2010]. Furthermore inhibition of the PI3K axis in pancreatic cancer cells caused activation of FOXO1 and FOXO3 leading to cell cycle arrest [Roy *et al.*, 2010]. A study by Tenbaum *et al* used PI3K and AKT inhibition in colon cancer and by immunostaining showed nuclear accumulation of FOXO3a [Tenbaum *et al.*, 2012]. The combination of high levels of β-catenin and activated FOXO3a promoted an invasive role rather than a tumour suppressor.

Subsequently the relationship of FOXO1 and FOXO3a was investigated by assessing the transcriptional activity of the TBX2 and TBX3 promoters. The promoters of TBX2 and TBX3 were repressed individually by each FOXO factor (Figure 15) and also by each T-box factor alone. Importantly, combinations of FOXO1 and FOXO3a with TBX2 showed significantly enhanced repression in their respective promoter activity (Figure 17). A similar enhancement

of repression with TBX3 was not observed, primarily because TBX3 exhibited maximal repression at the lowest expression levels. However, it seems possible that cooperative repression between TBX3 and the FOXO factors may also occur in vivo. These luciferase assay reporter experiments indicate that there is synergy between T-box and FOXO factors at a transcriptional level and that this may in part explain the regulation of TBX2 and TBX3 expression by the PI3K pathway.

Overall these findings contribute to explaining the anti-senescence role of PI3K in tumourgenesis and provide a potential mechanistic model (Figure 18). The exact mechanisms linking DNA damage, senescence and TBX regulation are yet to be fully explained. It is clear the pharmacological agents such as LY294002 and GDC0941 regulate the PI3K pathway and affect the regulation of proteins such as FOXO1 and TBX2. PI3K Inhibition leads to nuclear accumulation of FOXO1 and FOXO1, most likely in cooperation with TBX2, and possibly TBX3, represses the expression of TBX2 and TBX3 at their respective promoters.

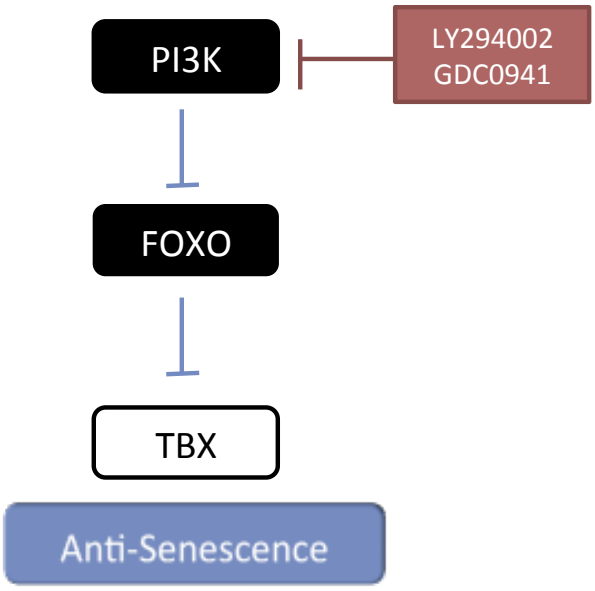


Figure 18. Mechanistic model for PI3K signalling

Diagrammatic representation of pharmacological PI3K inhibitors (LY294002 and GDC0941) used in melanoma cell lines showing repression of TBX factors (in particular TBX2). The proposed regulation with FOXO (in particular FOXO1 and FOXO3a) is consistent with transcriptional promoter activity. Overall cellular signalling indicates a halting of tumour progression and driving cells into a state of senescence.

4.5.3 Findings in relation to senescence

The DNA damage associated with PI3K inhibition may be linked to the DDR as a marker for senescence. Telomere dysfunction or attrition has been proposed as a key mechanism in cell senescence-associated cycle arrest [Bastian, 2003]. This would correlate with clinical observations that congenital naevi tend to demonstrate initial proliferation and are diffuse and cover larger body surface areas. In contrast, naevi acquired later in life tend to be more discrete and contained to smaller discrete areas. However studies using (FISH) to detect differences in telomere length did not demonstrate any significant changes between congenital and spitz naevi [Miracco *et al.*, 2002]. These findings challenge the notion that senescence always results from an accumulation of telomere attrition.

Pigmented naevi are a very common clinical finding and often represent benign lesions of cutaneous melanocytes. However, they frequently contain V600E BRAF mutations [Michaloglou *et al.*, 2005]. These melanocytic naevi initially undergo oncogene-activated growth and then cease to proliferate for many years demonstrating a growth arrest state. *In vivo* and *in vitro* studies on melanocytes overexpressing $BRAF^{V600E}$ have demonstrated an induction of $p16^{INK4a}$, confirmed senescence and growth arrest [Michaloglou *et al.*, 2008]. Indeed senescence represents a major barrier to cancer progression in general. In T-cell lymphomas senescence is related to RAS activation, which induces $p16^{INK4a}$ to maintain Rb in an hypo-phosphorylated state that inhibits S-phase progression [Serrano *et al.*, 1997]. A transgenic animal study showed senescence was dependent on p53 expression and protected against tumourgenesis [Braig *et al.*, 2005]. In prostate cancer PTEN and p53 are commonly inactivated tumour suppressor genes [Vogelstein *et al.*, 2000]. Chen and colleagues showed that acute PTEN inactivation regulates growth *in vivo* and *in vitro* through p53 dependent cellular senescence pathways [Chen *et al.*, 2005]. These findings support a model for senescence as a barrier to cancer progression. Moreover induction of senescence prevents cells entering apoptosis or malignant transformation and is driven by activating (NRAS, BRAF) or inactivating (PTEN) oncogenes.

Oncogene-induced senescence (OIS) has been proposed as a key tumour suppressive mechanism [Mooi *et al.*, 2006]. Mutations in the MAPK pathway such as $BRAF^{V600E}$ have been targeted by drugs such as Vemurafenib with aforementioned success [Fecher *et al.*, 2008]. While there have been exciting initial clinical responses to BRAF inhibitors, disease progression has occurred in a large cohort of patients. The reasons for melanoma recurrence have been discussed and mechanisms driving tumourgenesis have been proposed such as via CRAF and the PTEN/PI3K axis. PTEN mutations have been found in patients who have failed to show dramatic responses to BRAF inhibitors [Birck *et al.*, 2000]. This has raised the notion of the PI3K axis as an escape mechanism for tumour progression in melanoma. While NRAS and BRAF mutations have been proposed to be mutually exclusive, this may not be applicable to PTEN and BRAF mutations. In addition, preclinical studies have shown potential for combination therapies of BRAF and PI3K inhibitors in melanoma models [Bedogni *et al.*, 2004, Salphati *et al.*, 2010].

A study by Vredeveld *et al* using independent human cell and animal models showed that depleting PTEN abrogates BRAF induced senescence [Vredeveld *et al.*, 2012], and that pharmacological inhibition of the PI3K pathway in melanoma cells suppressed proliferation and eliminated subpopulations that were resistant to targeted BRAF inhibition. The authors used a combined inhibitor targeting mTOR and PI3K and occasionally, but not consistently, observed Saβgal activity. However, no mechanism explaining how PI3K signalling might suppress senescence was characterised. Our results, presented here, provide a mechanistic link between PI3K signalling and the well-characterised anti-senescence factors TBX2 and TBX3. Moreover In keeping with the Vredeveld study, we also observed a reduction in cellular migration of melanoma cells, by wound healing, following specific PI3K inhibition (Figures 9, 10 and 11).

4.5.4 Future considerations

Given the limited time for experimental work in this thesis there are notable shortcomings, but there are also many further questions to be answered and avenues of research to be explored. Future considerations in continuing this research would incorporate different

markers for senescence in melanoma cells for example Saβgal and SAHF. This research provides a basis for further defining the regulation of TBX by the PI3K pathway. This could be explored by incorporating siRNA for TBX2 or TBX3 in melanoma cells and probing them for senescence. Moreover would knockout of TBX2 or TBX3 in animal models abrogate tumourgenesis? Since we demonstrated the influence of FOXO factors on the TBX promoter, the next question raised would be whether these factors physically interacted with one another. This could be investigated by using GST pull down assays to determine physical binding and interaction between FOXO1 and TBX2. The role of p53 in senescence is yet to be fully understood. For example is p53 required for cellular senescence and does senescence occur in p53 null cell lines? By using cell lines with intact and null p53 mutations and assessing their cellular migration, markers for DNA damage and senescence could add to the role of p53 in melanoma. Beyond melanoma does the PI3K pathway influence invasiveness in other cancers, and if so, would pharmacological inhibition of PI3K lead influence senescence. These considerations would aim to further this research and build upon the current findings.

4.6 Conclusions

The role of the PI3K pathways and its downstream proteins is emerging as a new avenue for research, especially in BRAF and PTEN mutated tumours and cell lines. The research conducted in this thesis adds to the growing field of mechanisms underlying melanoma signalling pathways. Pharmacological inhibition with specific drugs has been demonstrated. The roles of T-box and FOXO factors at cellular and transcriptional levels have been highlighted. Recent therapies have targeted specific mutations in an attempt to halt tumour progression by apoptosis. However, because melanoma usually has wildtype p53 it may be vulnerable to pro-senescence therapy and this represents an attractive alternative strategy for what is currently an intractable disease. Establishing mechanisms for tumour progression in specific cell lines is not fully clear. Therefore given the heterogeneous nature of melanoma there is a need for combination therapy targeting multiple or simultaneous pathways. These therapies may not need to wholly down-regulate their targets, as this would be less toxic to the patient and provide better-tolerated treatments. Therefore a key advantage of pro-senescence therapy in melanoma is that it targets activating oncogenes that are not present in normal tissue, hence targeting invasive cells.

As striking pace of research continues into further establishing the role of pathways and proteins in melanoma therapy, I await the further emergence of molecular signalling pathway models that can be incorporated into clinical trials.

Chapter 5:
Summary

5 Thesis summary

In the last decade there have been substantial advances in molecular sciences. These have provided a far greater understanding of melanoma biology and influenced the management of melanoma. As therapeutic advances evolve, clinicians should be seeking to offer more personalised therapies to melanoma patients. In the progressive climate of targeted therapies for genetic subgroups, it should not be assumed that all melanomas respond or progress in a similar manner. Melanoma patients can be stratified by SLNB or genetic status. These represent distinct subgroups within the cancer with varying clinical outcomes. The last two years have seen unprecedented success in identifying promising new treatments for melanoma. However, patients with metastatic melanoma are still dying within one or two years of their diagnosis. The kinase inhibitors benefit only a sub-population but do so rapidly and reliably. The acquired resistance to targeted therapies highlights the need for more effective combination regimens in the near future, with pro-senescence therapy a valid option. The key to success will be identifying mechanisms that drive or slow down tumourgenesis by further understanding cancer biology and signalling pathways.

While certain genes and pathways have been targeted, not one single factor has been identified as the key to tumour progression. The recent advances in bioinformatics, genetic testing and sequencing are key to comprehend this complex and multifactorial cancer. The role of immunotherapy, which has been beyond the scope of this research, provides further avenues for melanoma therapy.

The treatment of melanoma will no doubt undergo more changes in coming years. The excitement generated by a decade of basic science, clinical trials and the new era personalised medicine reflects the dynamic nature of the research as well as the need for an effective treatment against this deadly cancer.

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