Ana Isabel Prim Padilha

Identification of PDE5A-regulated genes in BRAF mutant melanoma cells

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UNIVERSIDADE DE COIMBRA

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Investigação Biomédica, realizada sob a orientação científica do Professor Doutor Richard Marais (The Institute of Cancer Research, University of London) e supervisão da Doutora Joana Correia (Faculdade de Medicina, Universidade de Coimbra).

2012

UNIVERSIDADE DE COIMBRA

Para ser grande, sê inteiro

Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive

Ricardo Reis

Acknowledgments

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Dreams do come true!

Table of contents

List of figures

List of tables

Abbreviations

%	per cent
$\Delta\Delta Ct$	delta delta cycle threshold
μl	microlitre
μm	micrometre
μM	micromolar
°C	Celsius
3D	three-dimensional
aa	amino acid
ACT B	actine beta
APS	ammonium persulfate
ATPase	adenosine triphosphatase
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	adenosine 3',5'- cyclic monophosphate
CD1	cyclin D1
CDKN2A	cyclin-dependent kinase inhibitor
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
CTL	control
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphat
E (aa)	glutamic acid
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic
ERK	extracellular signal-regulated kinase
et al.	*et alia*
FBS	fetal bovine serum

FDA	Food and Drug Administration
GAF	cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
gr (weight)	grammes
GTP	guanosine 5'-triphosphate
h	hour
H_2O	water
i.e.	*id est*
ICR	Institute of Cancer Research
IgG1	immunoblobulin G
K (aa)	lysine
l	litre
logFC	logarithmic fold change
MAPK	mitogen-activated protein kinase
MEK	mitogen activated protein kinase/extracellular-signal regulated kinase
mg	milligram
min	minute
ml	millilitre
mM	millimolar
MM-2	metalloproteinase 2
M-MLV	Moloney Murine Leukemia Virus
mRNA	messenger RNA
Na3VO4	sodium ortovanadate
NaCl	sodium chloride
NaF	sodium fluoride
ng	nanogramme
NP40	nonidet P40
NT	not treated
OPTIMEM	Reduced-Serum Medium
PBS	phosphate buffered saline
PDE	phosphodiesterase
pH	potential hydrogen
PKA	protein kinase A

PKG	protein kinase G
PLX4032	Plexicon 4032
PTEN	phosphatase and tensin homologue
qRT-PCR	Real time quantitative polymerase chain reaction
R (aa)	arginine
RAF	murine sarcoma viral (v-raf) oncogene homolog
RAS	rat sarcoma viral oncogene homolog
RBD	RAS binding domain
RGP	radial growth phase
RIN	RNA integrity number
RIPA	radioimmunoprecipitation assay
RNA	ribonucleic acid
rpm	revolutions per minute
SC	Scramble control
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
siRNA	small interfering RNA
TBS	tris buffered saline
TBS-T	tris buffered saline tween
TEMED	N,N,N',N'-tetramethyl-ethylenediamide
Tris-Cl	tris(hydroxymethyl)amino methane
TRPM1	melanocyte-specific gene melastatin 1
uPA	urokinase type plasminogen activator
V (aa)	valine
VGP	vertical growth phase
vs	*versus*
VSM	vascular smooth muscle
x	times

Abstract

Melanoma is the most aggressive type of skin cancer and the leading cause of death from skin disease. This disease shows a very poor prognosis when progressing to a metastatic stage. Our laboratory previously discovered a key role for the cGMP phosphodiesterase PDE5A in inhibiting melanoma cell invasion. We found that PDE5A was mostly downregulated via oncogenic BRAF in a panel of highly invasive human melanoma cell lines. Following those findings, our aim was to identify PDE5A regulated genes in BRAF mutant melanoma cells.

In the present study, two previously published A375M2 melanoma cell clones genetically modified to stably re-express PDE5A were analysed using gene expression microarrays and compared to parental control cells. We identified 24 downregulated and 587 upregulated genes in common between the two A375M2 stably re-expressing PDE5A clones compared to control cells. For further validation, from the list of downregulated genes, TFPI2 (Tissue factor pathway inhibitor 2) and SERPINB2 (Plasminogen activator inhibitor type 2) were selected. Among the upregulated genes, MUC15 (mucin 15, cell surface associated), SERPINA5 (serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5) and SERPINF1 (serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1) were chosen for further validation.

Our data show that PDE5A regulates expression of a large number of genes in melanoma cells. The identification of these differentially expressed genes will facilitate improved understanding of metastatic melanoma biology and could lead to the identification of new therapeutic targets that could inhibit metastatic dissemination.

Resumo

O melanoma é o tipo de cancro da pele mais agressivo, sendo a principal causa de morte por doenças da pele. Esta doença demonstra um pobre prognóstico quando progride para um estado metastático. O nosso laboratório descobriu previamente o papel chave da cGMP fosfodiesterase PDE5A na inibição da invasão celular. Foi descoberto que a PDE5A era maioritariamente regulada negativamente pelo oncogene BRAF num painel de linhas celulares de melanoma que demonstram uma grande capacidade de invasão. No seguimento destes factos, o nosso principal objetivo foi então identificar genes regulados pela PDE5A em linhas celulares que possuem BRAF mutado.

Neste estudo, dois clones da linha celular A375M2 geneticamente modificados de modo a expressar estavelmente a PDE5A foram analisados usando microarrays de forma a verificar a expressão genética e comparados com os controlos parentais. Foram identificados 24 genes regulados negativamente e 587 regulados positivamente em comum entre os dois clones que expressam PDE5A, em comparação com o controlo. Para futura validação, da lista dos 24 genes regulados negativamente, TFPI2 (*Tissue factor pathway inhibitor 2*) e SERPINB2 (*Plasminogen activator inhibitor type 2*) foram selecionados. Quanto aos genes positivamente regulados pela PDE5A, MUC15 (*mucin 15, cell surface associated*), SERPINA5 (*serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5*) e SERPINF1 (*serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1*) foram escolhidos para validação.

Os resultados demonstram que a fosfodiesterase PDE5A regula a expressão de um grande número de genes em células de melanoma. A identificação desses genes, expressos de forma diferencial, irá facilitar uma melhoria na compreensão da biologia do melanoma mestastático e poderá permitir a identificação de novos alvos terapêuticos que poderão inibir a metastização.

1. INTRODUCTION

Melanoma

Melanoma is the most lethal type of skin cancer and it is the leading cause of death from skin disease (Jemal et al., 2010). Local invasion and metastatic spread are responsible for the high morbidity and mortality in melanoma (Miller and Mihm, 2006). Although melanoma is the third most common skin cancer, accounting for only 10% of all skin cancers, malignant melanoma accounts for 65% of all skin cancer deaths (Cummins et al., 2006). There are four major types of cutaneous melanoma: superficial spreading melanoma (70% to 75%), nodular melanoma (20% to 25%), lentigo maligna melanoma (5% to 10%) and acral lentiginous melanoma (5%) (Saldanha et al., 2006). Rarely, melanomas appear in other parts of the body, including mouth, iris of the eye or retina, oesophagus, urinary tract and small intestine (Gaudi et al., 2011; Machado et al., 2011; Patti et al., 2012).

1.1. Cutaneous Malignant Melanoma

1.1.1. Biology

Melanoma arises from melanocytes (Gray-Schopfer et al., 2007). Melanocytes are specialised cells that are found predominantly in the skin and eyes, where they produce melanins, the pigments responsible for skin and hair colour (Gray-Schopfer et al., 2007). These cells are located in the basal layer of the epidermis (Figure 1.1), where they occur in a life-long stable ratio of 1:5 with basal keratinocytes (Slominski et al., 2004). The epidermal melanin unit of the human epidermis denotes the symbiotic relationship between one melanocyte and approximately 36 associated keratinocytes (Haass et al., 2005).
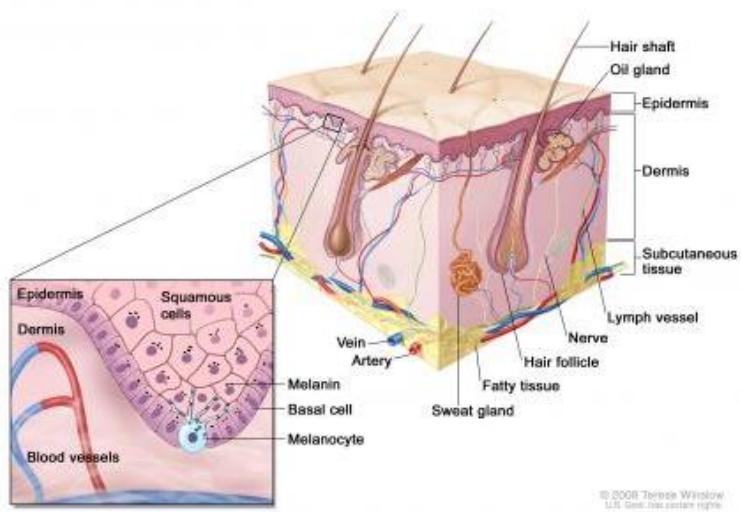
Figure 1.1: Anatomy of the skin showing the epidermis, dermis, and the subcutaneous tissue. Melanocytes are in layer of basal cells, at the deepest part of the epidermis (http://www.cancer.umn.edu/cancerinfo/NCI/CDR62713.html, accessed on 23-07-12).

Melanocyte growth is controlled by extracellular communication through paracrine growth factors with the surrounding keratinocytes, by intracellular communication through second messengers and signal transduction and by intercellular communication through cell–cell adhesion molecules, cell–matrix adhesion, and gap junctions. Under normal conditions, homeostasis determines whether a cell remains quiescent, proliferates, differentiates, or undergoes apoptosis. Dysregulation of the homeostasis may disturb the balance of the epidermal melanin unit and triggers the continuous proliferation of the melanocytes, which may lead to the development of melanoma (Haass et al., 2005).

1.1.2. Epidemiology

The incidence of malignant melanoma is continuously increasing worldwide in Caucasian populations (Jemal et al., 2010). The risk factors that contribute to the development of malignant melanoma are family history, the existence of multiple benign or atypical naevi, and a previous incidence. There are additional risk factors, including light skin, eyes and hair, exposure to ultraviolet radiation and weakened immune system due to disease or medication (Miller et al., 2006; Garbe et al., 2009; Volkovova et al., 2012). The increasing incidence of melanoma has been mainly

reported from industrial countries with white populations, with the highest incidence rates in Australia and the southern states of the United States (Figure 1.2) (Garbe and Leiter, 2009).
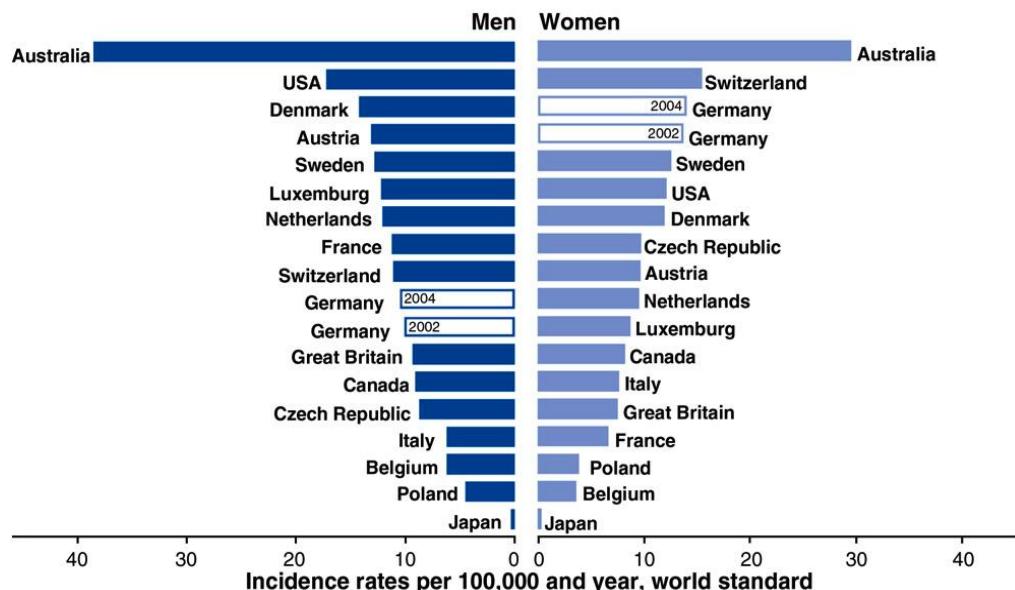
Figure 1.2: Age-standardised (world standard population) incidence rates from 17 countries worldwide for the year 2002. Here are represented the incidence rates per 100,000 and year, world standard (Taken from Garbe and Leiter, 2009).

Exposure to ultraviolet light increases skin pigmentation. This occurs in part through the action of the α-melanocyte-stimulating hormone (α-MSH) on its receptor, the melanocortin receptor 1 (MC1R) in melanocytes. When activated, MC1R stimulates intracellular signalling which increases the expression of the enzymes required for the production of melanin, a pigment that plays a critical role in protecting our skin from the damaging effects of ultraviolet radiation (Miller and Mihm, 2006). Light-skinned people often carry germ-line polymorphisms in the MC1R gene, increasing the susceptibility to melanoma (Miller and Mihm, 2006). Epidemiologic data also suggest that gender and genetics may influence the distribution of melanoma on the body surface and histopathologic characteristics of the lesion (Garbe and Leiter, 2009). Early diagnosis, followed by surgical resection is currently the only cure for melanoma (Gray-Schopfer et al., 2007), but patients with deep primary tumours or with tumours that have metastasised to regional lymph nodes frequently develop distant metastasis (Erdei and Torres, 2010). Metastatic

18

melanoma is largely refractory to existing therapies and has a very poor prognosis, with a median survival rate of 6 months and a 5-year survival rate of less than 5% (Gray-Schopfer et al., 2007).

1.1.3. Progression of Cutaneous Melanoma

The Clark model (Figure 1.3) describes the progression from normal melanocytes to malignant melanoma. This model is based on histopathological changes (Miller and Mihm, 2006).

Histopathological Appearance	Description	Histologic Features
Benign nevus	**Step 1** The first event is a proliferation of structurally normal melanocytes leading to the benign nevus. Clinically, these nevi present as flat or slightly raised lesions with either uniform coloration or a regular pattern of dot-like pigment in a tan or dark brown background. Histologically, such lesions have an increased number of nested melanocytes along the basal layer (arrows).	Proliferation of melanocytes Benign lesions
Dysplastic nevi	**Step 2** The next step is the development of aberrant growth. This may occur within a preexisting benign nevus or in a new location. Clinically such lesions may be asymmetric, have irregular borders, contain multiple colors, or have increasing diameters. Histologically, such lesions have random and discontiguous cytologic atypia (arrows).	Dysplastic cells Random atypia
Radial-growth phase	**Step 3** During the radial-growth phase, cells acquire the ability to proliferate intraepidermally. Clinically, they sometimes present as raised lesions. These lesions no longer display random atypia and instead show cytomorphologic cancer throughout the lesion. In addition to the intraepidermal cancer, the cells can penetrate the papillary dermis singly or in small nests but fail to form colonies in soft agar.	Intraepidermal growth Continuous atypia
Vertical-growth phase	**Step 4** Lesions that progress to the vertical-growth phase acquire the ability to invade the dermis and form an expansile nodule, widening the papillary dermis. The cells can also extend into the reticular dermis and fat, are capable of growth in soft agar, and have the capacity to form tumor nodules when implanted in nude mice.	Dermal invasion
Metastatic melanoma	**Step 5** The final step in the model is the successful spread of cells to other areas of the skin and other organs, where they can successfully proliferate and establish a metastatic focus. These cells can grow in soft agar and can form tumor nodules that may metastasize when implanted in nude mice.	Metastasis
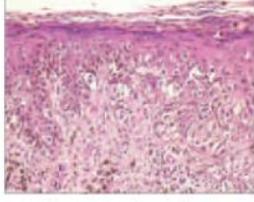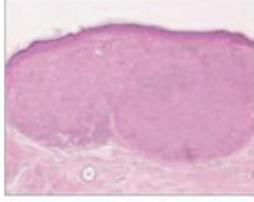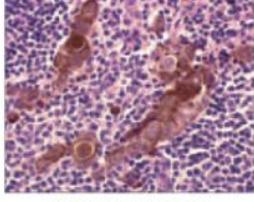
Figure 1.3: The Clark Model. Melanocytes progress through a series of steps toward malignant transformation. This model emphasises the histopathological changes that occur in the progression of melanoma (Taken from Miller and Mihm, 2006).

Progression from benign naevus to metastatic melanoma is characterised by the acquisition of step-by-step phenotypic differences and also by molecular changes (Figure 1.4) (Miller and Mihm, 2006). Naevus is the medical term for sharply-circumscribed and chronic lesions of the skin (http://www.merriam-webster.com/medlineplus/, accessed on 29-08-12). The development of benign naevus is the first phenotypic change in melanocytes. Usually naevi are benign lesions, however they can progress to radial-growth-phase (RGP) melanoma. RGP is an intra-epidermal lesion that can already involve some local microinvasion of the dermis (Miller and Mihm, 2006). In benign naevus, BRAF mutations and activation of the mitogen-activated protein kinase (MAPK) pathway have been described (Poynter et al., 2006; Uribe et al., 2006). The progression from dysplastic nevus to RGP is associated with loss of cyclin-dependent kinase inhibitor (CDKN2A) and phosphatase and tensin homologue (PTEN) pathways (Nogueira et al., 2010). Further progression of melanoma is related with decreased differentiation. The switch between RGP to VGP (vertical-growth phase) is also characterised by an increase in cyclin D1 (CD1) (Miller and Mihrn, 2006). The progression from VGP to metastatic melanoma is correlated to changes in the control of cell adhesion. Normally, cell adhesion controls cell migration and tissue organisation and disturbances in this mechanism contribute to tumour invasion. Those changes are due to loss of E-cadherin, increased expression of N-cadherin, αVb3 integrin and metalloproteinase 2 (MMP-2) (Mikesh et al., 2010). Further motility is promoted by changes in cytoskeletal organisation and altered contacts with extracellular matrix (ECM). Other changes include the loss of melanocyte-specific gene melastatin 1 (TRPM1) (Guo et al., 2012). The understanding of how these genes promote invasion of melanoma cells will allow the determination of molecular changes from RGP to VGP transition and subsequent motility of melanoma cells.
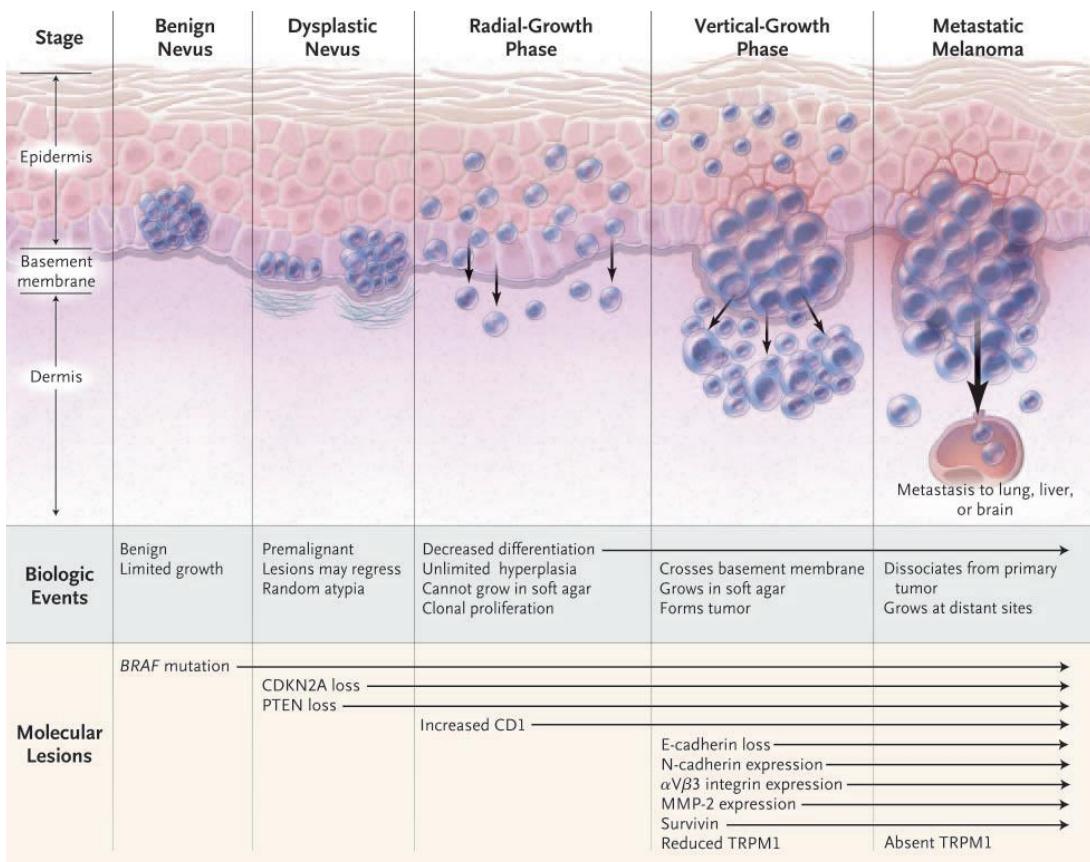
Figure 1.4: Biologic events and molecular changes in the progression of melanoma (Taken from Miller and Mihm, 2006).

Melanoma cells also interact with keratinocytes and fibroblasts. This interaction enables them to survive as well as to proliferate outside their normal epidermal location. Recent studies also confirmed that RGP cells are keratinocyte-dependent for survival (Soo et al., 2011). Clearly, improved understanding of how the driver oncogenes in melanoma promote invasion of melanoma cells will allow the determination of molecular changes from RGP to VGP transition and subsequent motility of melanoma cells.

1.2. MAPK Pathway - RAS/RAF/MEK/ERK

The RAF-MEK-ERK signal transduction pathway is a conserved RAS-activated protein kinase cascade that regulates cell growth, proliferation, and differentiation (McKay and Morrison, 2007). There are 3 *RAS* (*HRAS*, *KRAS* and *NRAS*) and 3 *RAF* (*ARAF*, *BRAF* and *CRAF*) genes in humans (Figure 1.5) (Gray-Schopfer et al., 2007). The three functional RAF proteins in humans, ARAF, BRAF, and CRAF are dependent on phosphorylation of the activation segment for activity. However, the details of their regulatory mechanisms differ because CRAF and ARAF require additional serine and tyrosine phosphorylation within the N region of the kinase domain for full activity, and BRAF has a much higher basal kinase activity than either ARAF or CRAF (Marais et al., 1997; Wan et al., 2004).
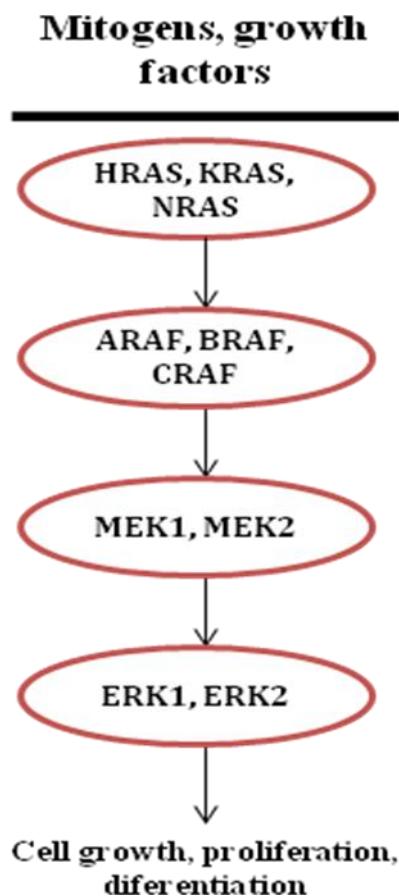
Figure 1.5: Schematic model of the MAPK signaling pathway (adapted from Rebocho, 2010).

In normal conditions, this pathway is activated in response to growth factor, cytokines and hormone receptors. The first protein activated is RAS, a small G protein; RAS activation is followed by activation of RAF. RAF activation is initiated by RAS-GTP association with the RAS binding domain (RBD) situated within the N-terminal regulatory region of the kinase. Concomitant conformational changes and recruitment to the cell membrane promote changes in RAF phosphorylation that combine to stimulate its serine/threonine kinase activity (Wellbrock et al., 2004; Mendoza et al., 2011). Then, RAF phosphorylates and activates another protein kinase called mitogen and extracellular signal-regulated protein kinase kinase (MEK). Activated MEK will then activate a third protein kinase called extracellular signal-regulated protein kinase (ERK) (Maurer et al., 2011; Mendoza et al., 2011). The response generated by ERK will depend on the signal received initially. Despite this simple appearance, this pathway is regulated by a complex network that ensure the appropriate signalling response.

This pathway is responsible for the regulation of proliferation, senescence, survival and differentiation in normal cells. However, in cancer this pathway is constitutively activated via oncogenic RAS or BRAF (Maurer et al., 2011), promoting survival and proliferation (Gray-Schopfer et al., 2007; Mendoza et al., 2011; McKay and Morrison, 2007).

1.2.1. BRAF mutation

BRAF is frequently mutated in different cancer types: 30% of papillary thyroid, 15% of colorectal, 30% of serous ovarian carcinoma and 45% of melanomas carry an activating mutation in the gene encoding BRAF (Davies et al., 2002; Gray-Schopfer et al., 2007; Flaherty et al., 2010).
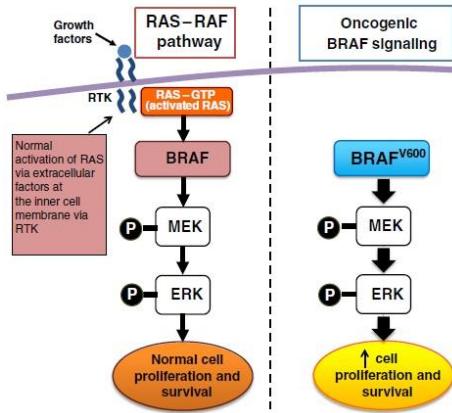
Figure 1.6: Schematic representation of MAPK activation under normal conditions or under oncogenic BRAF signaling (adapted from Ascierto et al., 2012).

90% of reported BRAF mutations result in a substitution of glutamic acid for valine at codon 600 (BRAF^{V600E}). This mutation constitutively activates BRAF and downstream MAPK pathway signalling, promoting an increased cell proliferation and survival (Figure 1.6.) (Flaherty et al., 2010). However, other activating mutations of BRAF are known in melanoma (e.g., BRAF^{V600K} and BRAF^{V600R}) (Figure 1.7) (Arkenau et al., 2011; Long et al., 2011; Catalogue of Somatic Mutations in Cancer (COSMIC) at http://www.sanger.ac.uk/cosmic).
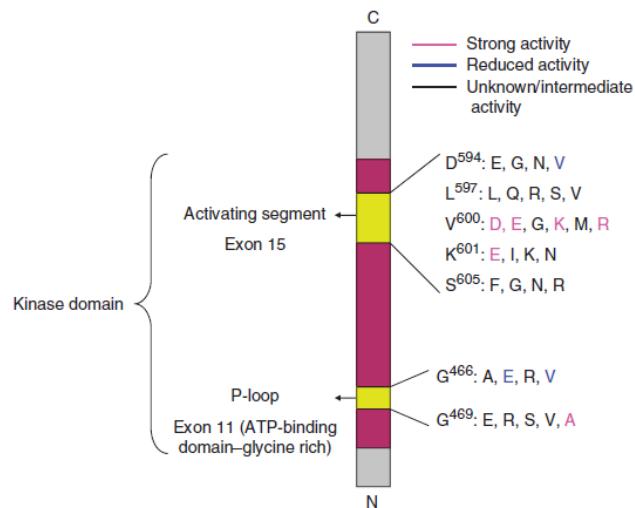
Figure 1.7: Common types of BRAF mutations in melanoma (Taken from Arkenau et al., 2011).

1.3. Targeted therapies in melanoma

Malignant melanoma is one of the most chemoresistant and aggressive human neoplasias (Mouawad et al., 2010). This is due to the genetic heterogeneity of this disease. Several strategies to overcome resistance to cytotoxic agents have been tested, including cytokines and vaccination (Mouawad et al., 2010). However, the identification of oncogenic BRAF as a therapeutic target in melanoma provided novel strategies for clinical management, not only of melanoma, but also other cancers harbouring mutations in this gene (Karasarides et al., 2004, Ribas et al., 2011).

The first generation of RAF inhibitors were designed to inhibit CRAF. Sorafenib was the first RAF inhibitor to enter the clinic. Sorafenib is a biaryl modified urea molecule that competes with ATP for binding to RAF (Lyons et al., 2001). However, phase III trials failed to demonstrate improvement in survival for patients with metastatic melanoma (Hauschild et al., 2009).

The most successful example of a targeted therapeutic drug in melanoma is vemurafenib, also known as PLX4032, RG7204, or RO5185436. Vemurafenib is a potent and specific inhibitor of BRAF^{V600E} activity, recently approved for the treatment of advanced melanoma (Chapman et al, 2011, Flaherty et al, 2010). Vemurafenib improves overall and progression-free survival in patients with previously untreated melanoma harbouring oncogenic BRAF, compared to dacarbazine (Chapman et al., 2011). Despite the high response rate to Vemurafenib, a percentage of patients treated with this drug develop resistance. The mechanisms related to resistance to BRAF inhibition are still under investigation (Johannessen et al., 2010; Wagle et al., 2011; Nazarian et al., 2010; Poulikakos et al., 2011). Dabrafenib, another inhibitor of oncogenic BRAF recently entered clinical trials; safety and tolerability of Dabrafenib were assessed in a phase I study in patients with melanoma, untreated brain metastases, and other solid tumours (Falchook et al., 2012).

A recently published MEK inhibitor (trametinib) phase III study report showed improved rates of progression-free and overall survival among patients who had metastatic melanoma with a BRAF^{V600E} or BRAF^{V600K} mutation as compared with chemotherapy (Flaherty et al., 2012).

Monoclonal antibody therapy with ipilimumab also improves overall and progression-free survival in patients with metastatic melanoma (Hodi et al., 2010).

Ipilimumab is a human monoclonal antibody that blocks cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), that is an immune check-point downregulating the pathways of T-cell activation (O'Day et al., 2007; Rovert and Ghiringhelli, 2009).

1.4. Cyclic Nucleotide Phosphodiesterases

Adenosine 3',5'- cyclic monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are second messengers that regulate many different cellular functions including phototransduction in retinal cells (Biel and Michalakis, 2009) and relaxation of the smooth muscle cells lining the veins (Morgado et al., 2012).   Cyclic nucleotide phosphodiesterases (PDEs) catalyse the degradation of cAMP and cGMP and therefore regulation of PDEs hydrolytic activity is important for modulation of cellular functions (Omori and Kotera, 2007).

Mammalian PDEs are categorised into 11 families (Figure 1.8) based on sequence homology, enzymatic properties, and sensitivity to inhibitors and composed of 21 genes (Omori and Kotera, 2007). PDEs protein family differ in tissue-expression patterns, gene regulation, enzymatic regulation by phosphorylation and regulatory proteins, subcellular localisation, and interaction with association proteins (Omori and Kotera, 2007; Conti and Beavo, 2007). Five of the 11 PDE families contain GAF (cGMP binding) domains (Figure 1.8). These families are PDE2, PDE5, PDE6, PDE10 and PDE11. The function of these domains is not clear, however they are thought to act as regulatory elements that bind nucleotides and other molecules (Conti and Beavo, 2007).
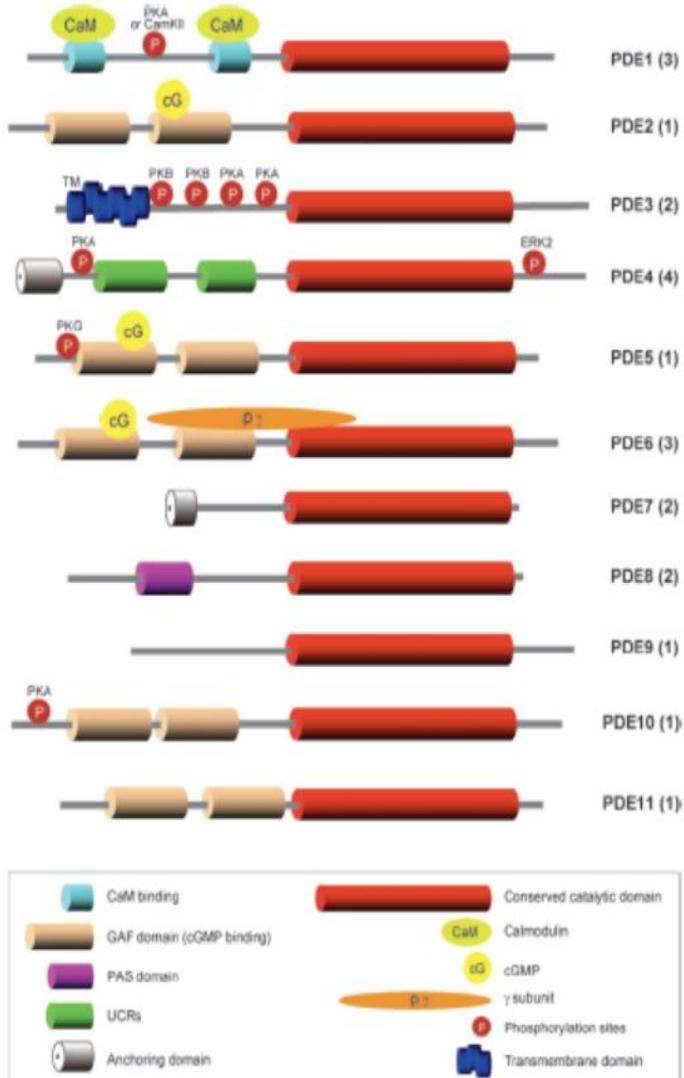
Figure 1.8: Representation of the 11 families of PDEs (Taken from Conti and Beavo, 2007).

1.4.1. PDE5A

PDE5A is a cGMP-specific phosphodiesterase. PDE5A gene, through alternative splicing, produces three proteins (PDE5A1, PDE5A3 and PDE5A3) that differ in their N-terminal region (Lugnier, 2006). PDE5 isoforms contain twin GAF domains (GAF A and GAF B) in the N-terminal half. cGMP binding to PDE5A GAF-A domain promotes PKG- and PKA-dependent phosphorylation in the N-terminal region. PDE5A phosphorylation not only activates the catalytic function but also increases cGMP binding affinity (Figure 1.9) (Conti and Beavo, 2007).
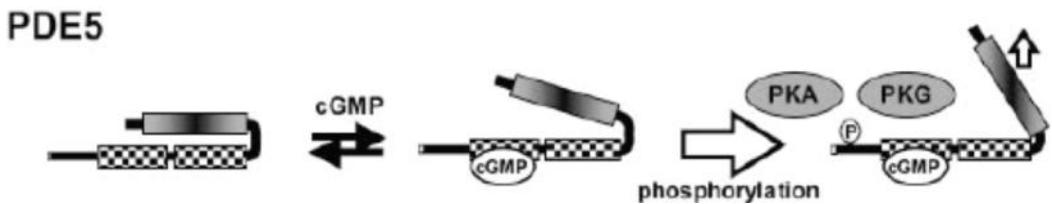
PDE5

Figure 1.9: Regulation of enzymatic activity in PDE5.

PDE5A proteins are ubiquitously expressed, however PDE5A3 has a restricted localisation, in the vascular smooth muscle (VSM) cells, while the other two proteins are ubiquitous (Lin et al., 2006). PDE5A is the therapeutic target of some drugs including sildenafil (Viagra), vardenafil (Levitra), and tadalafil (Cialis). These drugs are used to treat erectile dysfunction and pulmonary arterial hypertension (Ghofrani et al., 2006).

1.5. PDE5A and Invasion

A previous study from our lab showed that oncogenic BRAF downregulates the expression of a number of genes, including PDE5A (Packer et al, 2009). It had been previously demonstrated that oncogenic BRAF increases expression of the transcription factor BRN2 in melanoma cells (Goodall et al., 2004) and that BRN2 binds to the PDE5A promoter thus suppressing its transcription (Arozarena et al, 2011). PDE5A downregulation leads to an increase in cGMP levels. The higher levels of cGMP induce an increase in cytosolic Ca^{2+}, which triggers the phosphorylation of myosin light chain 2, inducing shape change and stimulating increased contractility therefore inducing invasion (Figure 1.10) (Houslay, 2011; Arozarena et al., 2011). We showed that downregulation of PDE5A results in increased melanoma cell invasion both *in vivo* and *in vitro*, establishing a link between PDE5A, cGMP and Ca^{2+} metabolism, and the regulation of melanoma cell invasion by oncogenic BRAF (Arozarena et al., 2011).
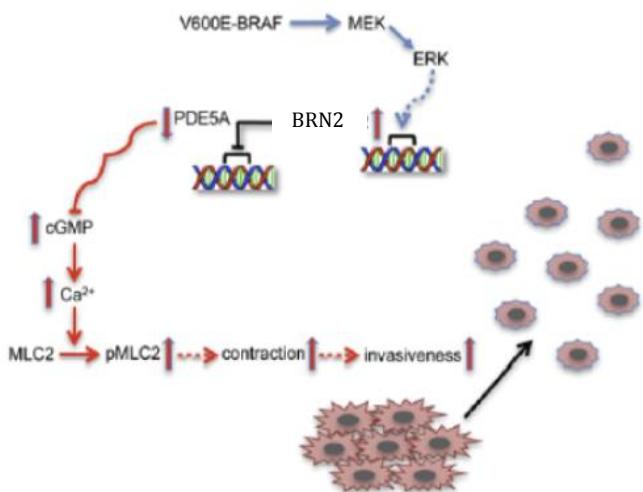
Figure 1.10: PDE5A downregulation in V600E-BRAF-Expressing melanoma cells promotes invasiveness (Adapted from Houslay, 2011).

1.6. Objectives

The aim of this project was to identify and then validate genes that are regulated by PDE5A in different BRAF mutant melanoma cell lines matched pairs that were genetically modified either to decrease PDE5A expression in PDE5A-expressing cells or to express PDE5A in cells where its expression was downregulated. For this purpose we used previously published cell lines generated in our lab, i.e. 501Mel shPDE5A stable clone vs empty vector control transfected cells and two A375M2 stably re-expressing PDE5A clones vs empty vector control transfected cells (Arozarena et al, 2011). RNA from these cells was analysed for gene expression using Affymetrix GeneChip® Human Exon 1.0ST arrays and the top hits of down and upregulated candidate genes were then validated by qRT-PCR and protein expression. Moreover, we generated new WM266.4 clones stably re-expressing PDE5A and performed siRNA for PDE5A in 501Mel as extra samples to be further analysed and compared to A375M2 and 501Mel previously generated stable clones.

2. MATERIALS AND METHODS

2.1. Cell biology

2.1.1. Cell culture

A375M2, WM266.4 and 501Mel cells (Table 2.1) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humified atmosphere at 37°C/10% CO_2. In addition, the antibiotics penicillin and streptomycin, were added to complete the medium. Cells were passaged every 3-4 days when confluent at dilutions of 1:5 to 1:10. As all the cell lines growth in an adherent way, they were washed once with Phosphate Buffer Saline (PBS) containing EDTA (1:500) to facilitate detachment from tissue culture flasks. After this, trypsin (1:5) was added to cells for 2-3 minutes. Detached cells were resuspended in fresh medium and replated into new flasks.

For culture in thick collagen layers, 1.5ml serum-free fibrillar bovine dermal collagen (2.3mg/ml) was diluted in DMEM5x and it was dispensed into 6 well tissue culture plates. The collagen was coagulated at 37°C/10% CO_2 (1h30min), then 1.5 x 10^5 cells were seeded in DMEM/10% serum for 24h.

Table 2.1: Cell lines an nomenclature

Name	Description	RAF mutational status
501Mel	**Human melanoma**	**BRAF V600E**
501Mel sh CTL	Control, high PDE5	BRAF V600E
501Mel sh4 PDE5A	shPDE5A clone 4, low PDE5A	BRAF V600E
A375M2	**Human melanoma**	**BRAF V600E**
A375M2 C66 CTL	Control, clone 66, low PDE5A	BRAF V600E
A375M2 C67 PDE5A	Stably re-expressing PDE5A clone 67, high PDE5A	BRAF V600E
A375M2 GFP PDE5A	Stably re-expressing PDE5A/GFP clone 67, high PDE5A	BRAF V600E
WM266.4	**Human melanoma**	**BRAF V600D**
WM266.4 PBP	Control, low PDE5	BRAF V600D
WM266.4 PDE5A C4	Stably re-expressing PDE5A clone 4, high PDE5A	BRAF V600D

2.1.2. WM266.4 DNA transfection

1×10^5 cells were seeded per well in a six-well plate and incubated overnight. On the following day, cells were transfected. 4µl of lipofectamine was diluted in 100µl of OPTIMEM (Invitrogen), and it was left at room temperature for 5 minutes. Then, 250 ng of DNA was diluted in 100µl of OPTIMEM. The lipofectamine plus OPTIMEM mix was added with the DNA plus OPTIMEM. This mixture was left 20 minutes at room temperature to allow the formation of the lipid-DNA complexes. Culture medium was removed from the cells, then cells were washed once with PBS and 800µl of OPTIMEM were added to each well. 200µl of the lipid-DNA mixture were then added to each well. After 6 hours of transfection, 1ml of medium supplemented with 20% FBS was added to each well in order to obtain a final 10% FBS medium. After 48 hours cells were placed into 10cm^2 petri dishes and incubated in 1µg/ml puromycin. The medium was refreshed weekly and after 2-3 weeks, single colonies were selected and expanded.

For stable PDE5A expression, cells were double transfected with pEF-PDE5A (250 ng/well) together with pBabepuro (25 ng/well). Parental control (PBP) was obtained with a single transfection with pBabepuro (25 ng/well) (Arozarena et al, 2001).

2.1.3. 501Mel siRNA transfection

1×10^5 cells were seeded per well in a six-well plate and incubated overnight. On the following day, cells were transfected. 6µl of lipofectamine were diluted in 100µl of OPTIMEM, and the mixture left at room temperature for 5 minutes. Then, 20µM of each RNA oligo (Table 2.2) were diluted in 100µl of OPTIMEM in order to obtain a final concentration of 20 nM in the well. The lipofectamine plus OPTIMEM mix was then added with the RNA oligo plus OPTIMEM mixture. This mixture was left 20 minutes at room temperature, to allow the formation of the lipid-RNA complexes. Culture medium was removed from the cells, then cells were washed once with PBS and 800µl of OPTIMEM were added to each well. 200µl of the lipid-RNA mixture were then added to each well. After 6 hours of transfection, 1ml of medium supplemented with 20% FBS was added to each well in order to obtain a final 10% FBS medium.

Table 2.2: Summary of siRNA oligonucleotides sequences.

Target	Name	DNA target sequence
None	Scramble control (SC)	5'-AAGUCCAUGGUGACAGGAGAC-3'
PDE5A	siRNA 1	5'-GAAGACAGCUCCAAUGACA-3'
PDE5A	siRNA 2	5'-GGAAACGGUGGGACAUUUA-3'

2.1.4. Invasion Assay

The 96-well plate (Perkin-Elmer black with clear bottom ViewPlates) was coated with 100 μl/well 0.2% low-fat BSA in DMEM without phenolred and was left at 4°C for an hour. BSA/DMEM was flicked off. 1 x 10^4 cells were resuspended in serum-free collagen mixture (2.3mg/ml collagen I, 5x DMEM and 1M NaOH) and seeded in the 96 well ViewPlates. Each sample was plated in six wells. Cells were sedimented at 1000 rpm for 5 minutes and incubated at 37°C/10% CO$_2$ for 1 hour to coagulate the collagen, then overlaid with 30 μl DMEM/10% FBS, to act as a chemo attractant. After 24 hours, cells were fixed with 4% formaldehyde and stained with Hoechst 33342 (Invitrogen). Plates were protected with a black sticker as the staining reagent, Hoechst, is sensitive to light. Confocal Z sections were collected at the bottom of the wells and at 40 μm with an INCELL analyzer. For each well, 9 pictures were taken at 0μm and 20 pictures were taken at 40μm. Nuclear staining was quantified with INCELL 3000 software with the Object Intensity module. The invasion index is calculated as cell number at the top divided by cell number at the bottom. The Student's t test was performed for fold-invasive index.

2.2. RNA extraction and microarray gene expression arrays

For RNA extraction, cells, that were plated on collagen, were disrupted with 500μl RLT buffer and the RNA was purified with RNeasy mini kits (QIAGEN) according to manufacturer's recommendations and quality assessed using a Bioanalyser 2100 (Agilent, Santa Clara, USA). RNA labelling, hybridisation to the Affymetrix (Santa Clara, USA) GeneChip® Human Exon 1.0ST arrays and

processing were performed by standard protocols at the Paterson Institute for Cancer Research, Manchester (http://bioinformatics.picr.man.ac.uk/mbcf/protocols.jsp).

Affymetrix CEL files were processed using Affymetrix Power Tools. Normalisation and summarisation were conducted using apt-probeset-summarize rma-sketch. Differential gene expression analysis was conducted using the BioConductor package limma (Smyth, 2005) including batch and RIN as covariates. Analysis was performed by Dr. Simon Furney, Signal Transduction Team (Marais laboratory).

2.3. DNA techniques

2.3.1. Quantitative Real-Time PCR

RNA extracted was reverse transcribed to generate cDNA using M-MLV Reverse Transcriptase (Sigma) according to the manufacturer's instructions. Quantitative real-time PCR was performed in a final volume of 10μl containing 2μl of diluted cDNA sample, 5μl of 2x Precision Mastermix (PrimerDesign) and 0.5μl of 20x TaqMan Gene Expression Assay probes (Table 2.3). All of the samples were prepared in triplicate, including a blank. Reactions were performed on an Applied Biosystems 7900HT Fast Real Time Machine (Applied Biosystems). Relative expression was calculated using Sequence Detection System software. Relative expression of mRNA was calculated using the ΔΔCt method and GAPDH or ACT B was used as internal control.

Table 2.3: qRT-PCR primers/probes.

Gene	Identification	Species	Source
PDE5A	Hs00153649_m1	Human	Applied biosystems
TFPI2	Hs00197918_m1	Human	Applied biosystems
SERPINB2	Hs01010736_m1	Human	Applied biosystems
MUC15	Hs00377336_m1	Human	Applied biosystems
SERPINF1	Hs01106934_m1	Human	Applied biosystems
SERPINA5	Hs00941459_m1	Human	Applied biosystems
GAPDH	Hs02758991_g1	Human	Applied biosystems
ACTB	Hs01060665_g1	Human	Applied biosystems

2.4. Biochemical Techniques

The composition of the buffers and gels described above, are listed in Table 2.4

2.4.1. SDS-PAGE

Cells were disrupted with 40μl-200μl of RIPA buffer according to the size of the cell pellet or number of plated cells. Cell lysates were then centrifuged for 20 minutes at 4°C at maximum speed. For conditioned medium, 1.5×10^6 cells were plated for each cell line. After 24 hours, medium was removed and cells were washed with PBS. Then, 12ml of DMEM medium without FBS and antibiotics was added. 24 hours later, medium and cells were collected separately. The conditioned medium was concentrated using Amicon ultra-4 centrifugal filters (Millipore), according manufacturer's recommendations.

Before the electrophoresis, samples were prepared in 4x Sample Buffer (Laemli buffer) and denatured for 5 minutes at 96°C. Samples were run in 1x Running Buffer for approximately 1h30 at 120 Volts. Resolving gels were prepared with 10% acrylamide and stacking gels with 5% acrylamide and were polymerised using 0.1% ammonium persulfate (APS) with 0.1% N,N,N',N'-tetramethyl-ethylenediamide (TEMED).

Table 2.4: Composition of gels and standard buffers.

Buffer/Gel	Description	Composition
RIPA Buffer	Protein extraction	50 mM Tris-Cl pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor, 5 mM NaF, 0.2 mM Na3VO4, water.
Sample Buffer	Protein sample buffer	250 mM Tris-Cl pH 6.8, 8% SDS, 40% glycerol, 8% betamercaptoethanol, 0.02% bromophenol blue.
Resolving Gel (10%)	Resolving gel	30% Polyacrylamide, 1.5M Tris-Cl pH 8.8, SDS 10%, 0.1% APS, 0.1% TEMED, H_2O.
Stacking Gel (5%)	Stacking gel	30% Polyacrylamide, 1M Tris-Cl pH 6.8, SDS 10%, 0.1% APS, 0.1% TEMED, H_2O.
Running Buffer	Protein gel electrophoresis	Protein Electrophoresis buffer 10x, 1% SDS, H_2O.
Transfer Buffer 10x	Western blotting	30gr Tris, 144gr Glycine, 1L H_2O.

Blocking Buffer	Blocking buffer	LI-COR blocking buffer (1:2), TBS (1:2).
Washing Buffer	TBS-T	25 mM Tris-Cl pH 8.0, 150 mM NaCl, 1% Triton X-100.

2.4.2. Immunoblotting

Before protein transfer, the Immobilon-FL membranes were activated with methanol. Proteins were transferred electrophoretically, using 1x Transfer Buffer, for 90 minutes at 100 Volts. After the transfer, the membranes were blocked with LI-COR blocking buffer diluted (1:2) in TBS for 40 minutes, while shaking. Then, the membranes were incubated with the primary antibody diluted (Table 2.5) in LI-COR blocking buffer (1:2), TBS 0.1% Tween (TBS-T) for 2 hours at room temperature or overnight at 4 °C. Membranes were then washed 3 times for 10 minutes, with TBS-T washing buffer and probed with the secondary antibody for approximately 50 minutes, protected from the light, while shaking. The secondary antibody was diluted in LI-COR blocking buffer (1:2), TBS-T 0.015% SDS. After this step, membranes were washed twice with TBS-T washing buffer; membranes were then washed with a final wash in 1x with TBS. Membranes were then scanned on an Odyssey Infrared Scanner (Li-COR Biosciences).

Table 2.5: Antibodies and dilutions.

Antigen	Antibody	Species	Dilution	Source
PDE5A	PDE5A (H-120)	Rabbit	1:200	Santa Cruz Biotechnology
SERPINF1	SERPINF1/PEDF (1C4)	Mouse	1:1000	LifeSpan Biosciences
ERK2	ERK2	Rabbit	1:2000	Santa Cruz Technology
Rabbit IgC	Rabbit IgG IRDye 800CW	Rabbit	1:10000	LI-COR Biosciences
Mouse IgC	Mouse IgG Alexa-Fluor 680	Mouse	1:10000	Invitrogen

2.5 Statistical analysis

Data presented were analysed by unpaired Student's t-test; p values less than 0.05 were accepted as statistically significant different compared to controls.

3. RESULTS

3.1. Melanoma cell lines characterisation

3.1.1. PDE5A mRNA and protein levels in previously generated stable clones

To confirm PDE5A expression in the A375M2 vector transfected control cells and PDE5A stably re-expressing clones and 501Mel vector control transfected cells and shPDE5A clone from Arozarena et al, (Arozarena et al., 2011), we measured both PDE5A mRNA and protein levels.

Using qRT-PCR, we confirmed that A375M2 C67 PDE5A (C67 PDE5A) and A375M2 GFP PDE5A (GFP PDE5A) have higher PDE5A gene expression levels compared to empty vector transfected control cells (C66 CTL). By contrast, the 501Mel cells stably transfected with shRNA for PDE5A (shPDE5A) showed lower PDE5A expression compared to the matched shRNA empty vector transfected control (shCTL) (Figure 3.1A). Protein extracts were then prepared from each cell line and PDE5A levels were assessed by immunoblot. We confirmed that PDE5A protein levels were higher in the two A375M2 PDE5A stably re-expressing clones compared to control cells (Figure 3.1B, lanes 1 to 3); by contrast, the 501Mel shPDE5A clone showed lower PDE5A protein levels compared to vector control cells (Figure 3.1B, lanes 4 and 5).

Since Arozarena and colleagues showed differences in the invasiveness of these melanoma cell lines in a 3D collagen based cell environment (Arozarena et al., 2011), RNA samples for further microarray gene expression analysis were extracted from cells plated on collagen. Relative gene expression was assessed in three independent sets of samples prepared in three different days for each cell line. Similarly to what observed in cells plated on plastic, an increase in the PDE5A mRNA levels was observed in the two A375M2 PDE5A re-expressing clones and a decrease was observed in 501Mel shPDE5A clone compared to matched controls when cells were plated on collagen (Figure 3.1C).
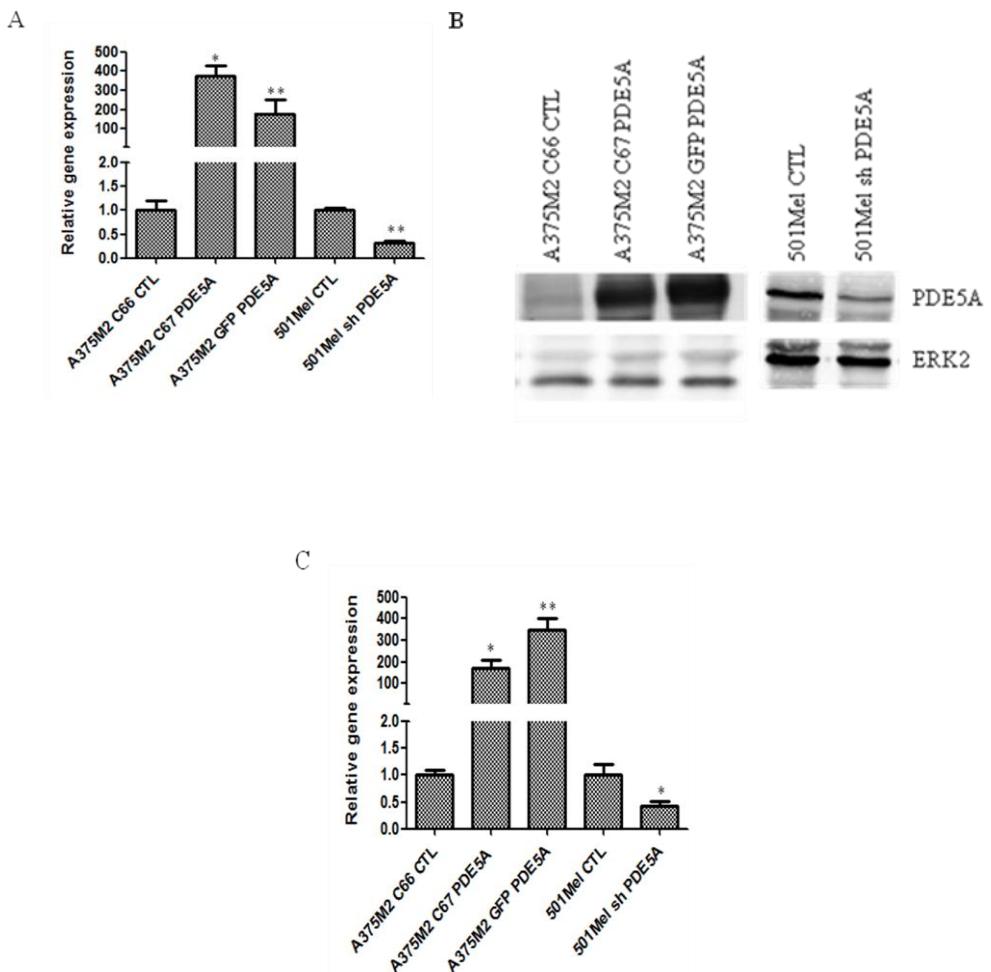
Figure 3.1: Melanoma cell lines characterisation. **A** Relative PDE5A gene expression of cells plated on plastic; A375M2 C66 CTL=vector control, A375M2 C67 PDE5A and A375M2 GFP PDE5A=stably re-expressing PDE5A clones, 501Mel shCTL=vector control, 501Mel shPDE5A=shPDE5A stable clone. **B** PDE5A protein levels of cells plated on plastic. ERK2 was measured as loading control. **C** Relative PDE5A gene expression of cells plated on collagen. Samples in **C** were used for further microarray gene expression analysis. Graphs are representative of three independent experiments. (*) p < 0.05 or (**) p<0.01.

3.2. 501Mel: PDE5A siRNA

In addition to the shPDE5A stably transfected 501Mel cells, we also transfected 501Mel cells with two small-interfering RNA (siRNA) probes. We observed downregulation of PDE5A mRNA and protein levels in cells transfected with both the siRNA probes (Oligo1 and Oligo2) compared to scrambled oligo control transfected cells (SC) (Figure 3.2A and B). All the RNA samples were then prepared from cells plated on collagen. We confirmed by qRT-PCR that PDE5A mRNA levels from 501Mel cells transfected directly on collagen with PDE5A Oligo1 and Oligo2 were decreased compared to control (Figure 3.2C). These samples were collected on three different days and RNA prepared for further microarray gene expression analysis.
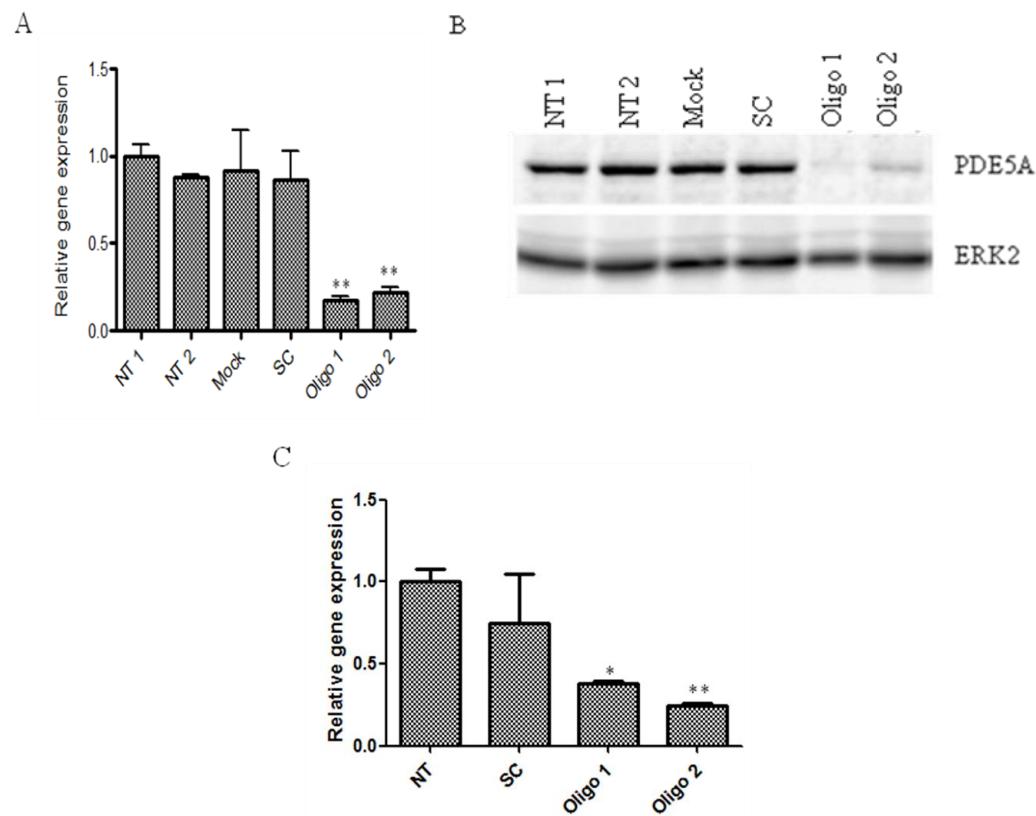
Figure 3.2: 501Mel transfected cells. **A** Relative PDE5A gene expression of the 501Mel cells transfected with siRNA on plastic: NT = not treated, Mock = lipofectamine only, SC = scrambled oligo transfected, Oligo 1 and Oligo 2 = PDE5A siRNA. **B** PDE5A protein levels from 501Mel cells transfected on plastic, ERK2 was measured as loading control. **C** Relative PDE5A gene expression of 501Mel cells transfected on collagen. Graphs are representative of three independent experiments. (*) p < 0.05 or (**) p<0.01.

3.3. PDE5A expression in WM266.4 cells

In addition to A375M2 stably re-expressing PDE5A clones, we also generated stable clones of WM266.4 re-expressing PDE5A. Five single cell clones were selected under 1 µg/ml puromycin selection as well as a pool of empty vector control (PBP) cells and a pool of stably re-expressing PDE5A cells. qRT-PCR (Figure 3.3A) and immunoblot (Figure 3.3B) were performed in order to verify the levels of PDE5A mRNA and protein, respectively. The pool of PDE5A stably transfected cells and two of the analysed clones (clone 1, C1 and clone 4, C4) showed upregulation of PDE5A mRNA levels. However, only the C4 clone showed an upregulation of PDE5A protein levels. This clone was therefore selected for further experiments. PDE5A mRNA upregulation in C4 was also confirmed by qRT-PCR in cells plated on collagen (Figure 3.3C).
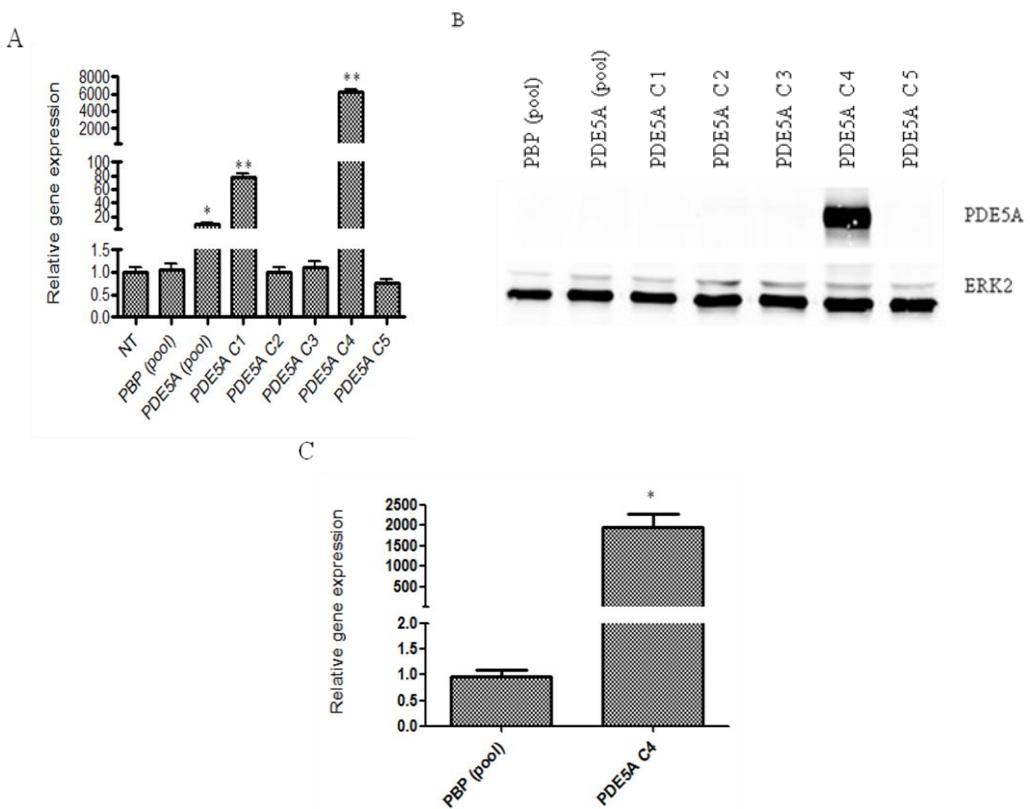
Figure 3.3: WM266.4 DNA transfection. **A** Relative PDE5A gene expression of not transfected cells (NT), PBP vector transfected cells (PBP (pool)), 5 different WM266.4 clones (C1 to 5) and one pool (PDE5A (pool)) obtained after stable transfection of cells plated on plastic. **B** PDE5A protein levels of WM266.4 cells plated on plastic, ERK2 was measured as loading control. **C** Relative gene expression of WM266.4 empty vector control (PBP) and PDE5A expressing clone 4 (PDE5A C4) from cells

plated on collagen. Graphs are representative of three independent experiments. (*) p < 0.05 or (**) p<0.01.

3.4. PDE5A suppresses melanoma cell invasion

Invasion abilities from A375M2 vector control and stably re- expressing PDE5A clones and 501Mel vector control and shPDE5A clone were previously reported by Arozarena and colleagues (Arozarena et al., 2011).

In order to characterise the new stably re- expressing WM266.4 PDE5A clone that we generated, we performed an invasion assay in collagen. The clone re-expressing PDE5A (WM266.4 PDE5A C4) showed significantly decreased invasion compared to empty vector control transfected cells (WM266.4 PBP) (Figure 3.4). This was in agreement to what was previously described by Arozarena and colleagues in WM266.4 cells (Arozarena et al., 2011).
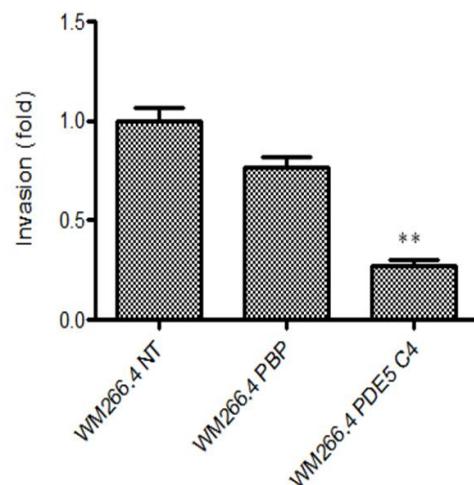
Figure 3.4: PDE5A suppresses melanoma cell invasion. Invasion as fold difference of non-transfected cells (WM266.4 NT) for empty vector control (WM266.4 PBP) and WM266.4 clone 4 engineered for stable expression of PDE5A. Graph is representative of three independent experiments. (**) p<0.01.

3.5. Gene expression microarray analysis

3.5.1. Quality control test: hierarchical clustering

RNA from three different sets of A375M2 C66 CTL (A), A375M2 C67 PDE5A (B), A375M2 GFP PDE5A (C), 501Mel CTL (D) and 501Mel shPDE5A (E) was extracted in three different days (1, 2, and 3) and further processed for gene expression microarray analysis.

A first quality control test was run by Dr. Simon Furney on the entire set of genes. A hierarchical clustering analysis confirmed that the two cell lines A375M2 (A, B and C) and 501Mel (E and D) clustered onto the two different branches of the tree and all the three independent replicates (1, 2, and 3) were correctly clustering together, suggesting a good reproducibility. Only two samples did not cluster correctly, i.e. D1 and E3 (Figure 3.5).
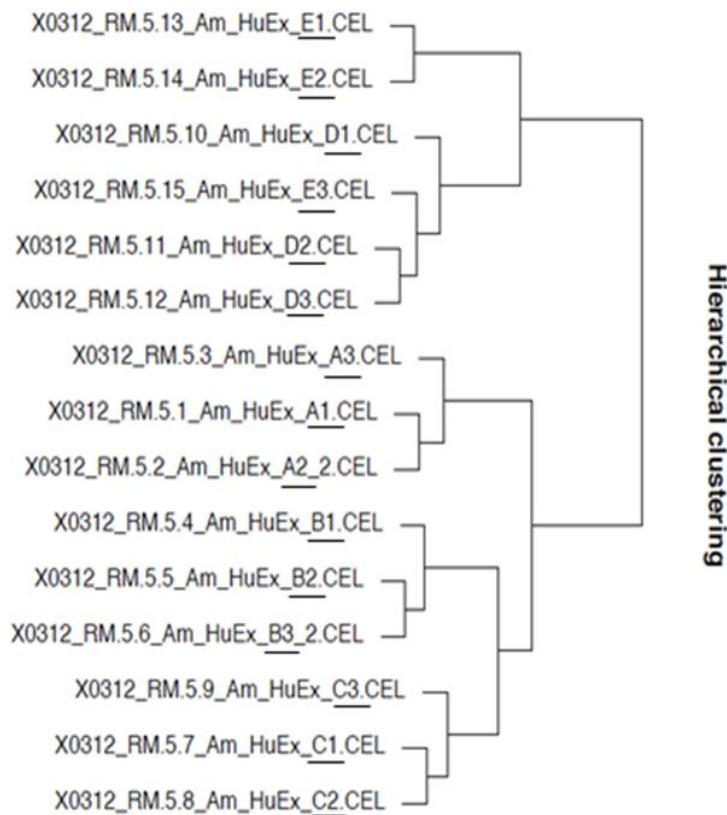
Figure 3.5: Quality control test: hierarchical clustering. Hierarchical clustering for the three different sets of the A375M2 (A1, 2, 3; B1, 2, 3; C1, 2, 3) and 501Mel (D1, 2, 3 and E1, 2, 3) cell lines.

3.5.2. 501Mel shPDE5A vs 501Mel control

We have shown here that PDE5A expression was significantly lower in 501Mel shPDE5A compared to 501Mel shCTL by qRT-PCR with a difference of at least two fold (Figure 3.1). Surprisingly, the gene expression analysis by microarray run by Dr. Simon Furney showed a much lower fold difference in the order of 1.1-1.2 (logFC = -0.113265676). Due to this low PDE5A gene expression differential we could not include these 501Mel sets of samples in our analysis. Moreover, the hierarchical clustering also showed that two samples (D1 and E3) were not clustering correctly. These data taken together indicated that those samples should be excluded from our gene expression analysis and further validation.

3.5.3. Downregulated genes

Analysis of the gene expression array data showed 24 downregulated candidate genes shared between the two A375M2 stably re-expressing PDE5A clones (C67 PDE5A and GFP PDE5A, cut off ≤ 1.5 fold decrease, LogFC ≤ -0.58) compared to vector control. The downregulated genes are listed in Table 3.1. Among these genes SERPINB2 and TFPI2 were selected for further validation as they have been previously described to play a role in invasion in melanoma or other cancers (Jang et al., 2010; Tan et al., 2010; Gessler et al., 2011).

Table 3.1: Downregulated genes in common between A375M2 C66 CTL and the two expressing PDE5A clones (C67 PDE5A and GFP PDE5A).

Gene	Designation	logFC
OR4N2	olfactory receptor, family 4, subfamily N, member 2	-5.258335963
MGST1	microsomal glutathione S-transferase 1	-3.527108108
TFPI2	tissue factor pathway inhibitor 2	-2.567368294
TCEAL6	transcription elongation factor A (SII)-like 6	-2.056143041
PASD1	PAS domain containing 1	-1.36721641
APOL1	apolipoprotein L, 1	-1.194623167
CHRM2	cholinergic receptor, muscarinic 2	-1.019436208
PARM1	prostate androgen-regulated mucin-like protein 1	-0.945836757
LOC283174	uncharacterized LOC283174	-0.892536182
NRIP3	nuclear receptor interacting protein 3	-0.775216216
IQCF1	IQ motif containing F1	-0.737780946
TMEM132D	transmembrane protein 132D	-0.732243666
LCE1D	late cornified envelope 1D	-0.732071867
OR52B4	olfactory receptor, family 52, subfamily B, member 4	-0.726581537
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	-0.700365389

MAGEA1	melanoma antigen family A, 1 (directs expression of antigen MZ2-E)	-0.687654012
MTRF1L	mitochondrial translational release factor 1-like	-0.67021826
ANKRD30BL	ankyrin repeat domain 30B-like	-0.647559307
PCDH11Y	protocadherin 11 Y-linked	-0.639371909
RPS19	ribosomal protein S19	-0.609937618
FAM26D	family with sequence similarity 26, member D	-0.599652508
TNFRSF10A	tumor necrosis factor receptor superfamily, member 10a	-0.593822872
RAB42	RAB42, member RAS oncogene family	-0.58869234
SLC14A1	solute carrier family 14 (urea transporter), member 1 (Kidd blood group)	-0.584647914

3.5.4. Upregulated genes

Analysis of the gene expression array data showed that more than 500 candidate genes shared between the two A375M2 stably re-expressing PDE5A clones were upregulated (cut off ≥ 1.5 fold increase, LogFC ≥ 0.58) compared to vector control cells. The top 30 upregulated genes are listed in Table 3.2, among these genes we selected MUC15 and SERPINF1 for further validation. In addition, we also selected SERPINA5 (LogFC = 0.73, not shown in Table 2) for further validation as it belongs to the SERPIN family. The role of the SERPIN and MUCIN proteins family in invasion has been previously described in melanoma and other cancers (Riker et al., 2008; Orgaz et al., 2009; Bijsmans et al., 2011).

Table 3.2: Top 30 of the up-regulated genes in common between A375M2 C66 CTL and the two expressing PDE5A clones (C67 PDE5A and GFP PDE5A).

Gene	Designation	LogFC
PDE5A	phosphodiesterase 5A, cGMP-specific	4.279452703
HIST1H3A	histone cluster 1, H3a	2.498262855
SERPINF1	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	2.370222551
LY96	lymphocyte antigen 96	2.324614907
TPMT	thiopurine S-methyltransferase	2.137499645
PCLO	piccolo (presynaptic cytomatrix protein)	1.975431512
CNIH3	cornichon homolog 3 (Drosophila)	1.796186351
FAM36A	family with sequence similarity 36, member A	1.788345676
TC2N	tandem C2 domains, nuclear	1.632316951
TOMM20	translocase of outer mitochondrial membrane 20 homolog (yeast)	1.530937914
TUBA1B	tubulin, alpha 1b	1.484268091
DECR1	2,4-dienoyl CoA reductase 1, mitochondrial	1.478867306
MAMDC2	MAM domain containing 2	1.473540025

HPGD	hydroxyprostaglandin dehydrogenase 15-(NAD)	1.457482796
SDHC	succinate dehydrogenase complex, subunit C, integral membrane protein, 15kDa	1.435840963
DNAH14	dynein, axonemal, heavy chain 14	1.405168066
GUF1	GTPase homolog (S. cerevisiae)	1.382840819
ARMCX1	armadillo repeat containing, X-linked 1	1.382389679
PCDH7	protocadherin 7	1.375470777
IVNS1ABP	influenza virus NS1A binding protein	1.350759882
MUC15	mucin 15, cell surface associated	1.326169789
LMBR1	limb region 1 homolog (mouse)	1.325073193
LRRCC1	leucine rich repeat and coiled-coil centrosomal protein 1	1.296911943
SEMA6A	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A	1.291305887
STK32A	serine/threonine kinase 32A	1.277024797
TIPRL	TIP41, TOR signaling pathway regulator-like (S. cerevisiae)	1.260517145
LYRM7	Lyrm7 homolog (mouse)	1.257578167
TAPT1	transmembrane anterior posterior transformation 1	1.253077432
FGFBP2	fibroblast growth factor binding protein 2	1.250691275
PPP2R5A	protein phosphatase 2, regulatory subunit B', alpha	1.243457475

3.6. Validation of down and upregulated genes using qRT-PCR and immunoblot

Some of the downregulated and upregulated genes were selected for further validation. Relative gene expression was measured in the two A375M2 stably re-expressing PDE5A clones and matched vector control, both from cells plated on collagen and plastic. Immunoblots for some of the genes were also performed, however further validation is necessary.

3.6.1. PDE5A expression downregulates TFPI2 and SERPINB2 gene expression in A375M2 and WM266.4 cells

We confirmed downregulation of TFPI2 and SERPINB2 gene expression levels in A375M2 and WM266.4 stably re-expressing PDE5A clones compared to their matched control (Figure 3.6).
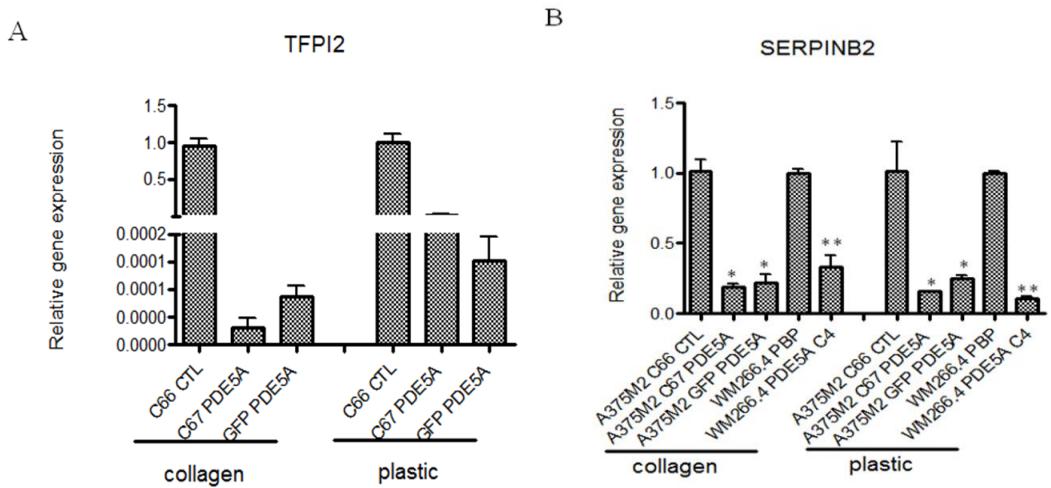
Figure 3.6: PDE5A expression downregulates TFPI2 and SERPINB2 gene expression. **A** Relative gene expression of TFPI2 in A375M2 cells, A375M2 C66 CTL=vector control, A375M2 C67 PDE5A and A375M2 GFP PDE5A=stably re-expressing PDE5A clones. **B** Relative gene expression of SERPINB2 in A375M2 and WM266.4; WM266.4 PBP=vector control, WM266.4 PDE5A C4= stably re-expressing PDE5A clone 4. Graphs are representative of three independent experiments. (*) $p < 0.05$ or (**) $p < 0.01$.

3.6.2. PDE5A expression upregulates MUC15, SERPINF1 and SERPINA5 gene expression in A375M2 cells

We next confirmed by qRT-PCR that MUC15, SERPINF1 and SERPINA5 were upregulated in A375M2 stably re-expressing PDE5A clones compared to vector control, both in collagen and plastic (Figure 3.7).
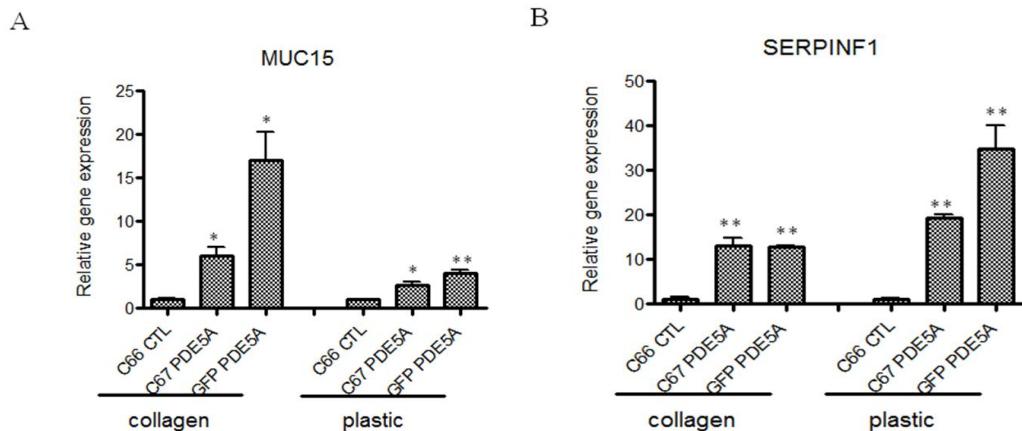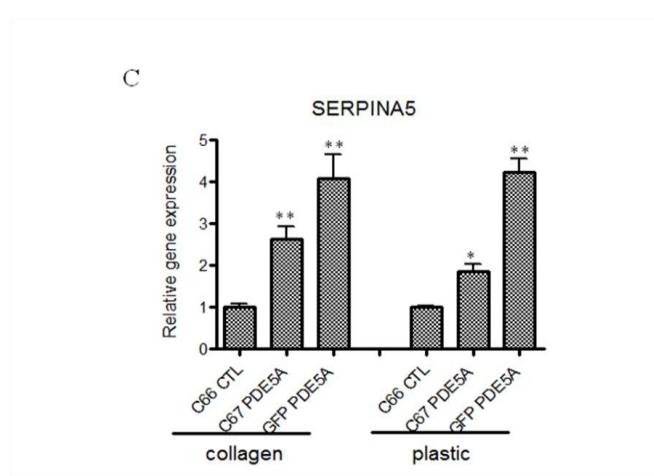
Figure 3.7: PDE5A expression upregulates MUC15, SERPINF1 and SERPINA5 gene expression. Relative gene expression of MUC15 (**A**), SERPINF1 (**B**) and SERPINA5 (**C**) in A375M2 cells; A375M2 C66 CTL=vector control, A375M2 C67 PDE5A and A375M2 GFP PDE5A=stably re-expressing PDE5A clones. Graphs are representative of three independent experiments. (*) $p < 0.05$ or (**) $p<0.01$.

3.6.3. PDE5A upregulates SERPINF1 protein levels in the conditioned medium of A375M2 cells.

In order to confirm that an increase in gene expression was followed by an increased protein production, we measured the levels of secreted SERPINF1 in the conditioned medium of A375M2 cells by immunoblot (Figure 3.8). As a result of increased SERPINF1 mRNA we observed an increase in secreted SERPINF1 protein in the conditioned medium from both A375M2 PDE5A re-expressing stable clones compared to control cells. However, more of the differentially expressed genes need to be further validated at the protein level and in different melanoma cell lines.
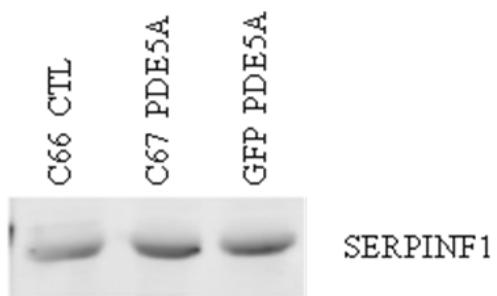
Figure 3.8: PDE5A upregulates SERPINF1 protein levels of A375M2 cells. Protein levels of the secreted protein SERPINF1 in A375M2 cells plated on plastic. A375M2 C66 CTL=vector control, A375M2 C67 PDE5A and A375M2 GFP PDE5A=stably re-expressing PDE5A clones. Representative blot of two independent experiments.

4. DISCUSSION/CONCLUSIONS

Melanoma is a heterogeneous disease that has a very poor prognosis when progressing to a metastatic stage (Gray-Schopfer et al., 2007). A previous study from our lab showed a key role for the cGMP phosphodiesterase PDE5A in inhibiting melanoma cell invasion (Arozarena et al., 2011). We found that PDE5A is downregulated via oncogenic BRAF in a panel of highly invasive human melanoma cell lines. Following those findings, our aim was to identify PDE5A regulated genes in BRAF mutant melanoma cells.

The first aim of the present study was to characterise the previously published cell lines (Arozarena et al., 2011) in order to confirm their PDE5A mRNA and protein levels. We confirmed that the two A375M2 PDE5A stably re-expressing clones, C67 PDE5A and GFP PDE5A express higher levels of both PDE5A mRNA and protein compared to control. We also confirmed lower PDE5A mRNA and protein levels in 501Mel clone transfected with an shRNA for PDE5A compared to control cells by qRT-PCR and immunoblot. However, gene expression microarray analysis showed a much lower difference in PDE5A gene expression between the control and the shRNA stable transfected 501Mel cells; for this reason these cells were excluded from the analysis.

For gene expression microarray analysis, cells were plated on collagen as previous studies have demonstrated that gene expression in cells growing in a 3D environment is closer to *in vivo* compared to monolayer cultured cells (Even-Ram and Yamada, 2005). Furthermore, in our previous study (Arozarena et al., 2011) many of the experiments were run in collagen rather than in plastic, however when we compared gene expression changes in collagen vs plastic we did not observe a significant difference.

Gene expression microarrays allowed us to identify 24 downregulated and 587 upregulated candidate genes shared between the two stably re-expressing PDE5A clones (C67 PDE5A and GFP PDE5A) compared to vector control transfected cells (C66 CTL). We selected TFPI2 (Tissue factor pathway inhibitor 2) and SERPINB2 (Plasminogen activator inhibitor type 2) among the downregulated and MUC15, SERPINA5 and SERPINF1 among the upregulated candidate genes for further validation.

TFPI2 is a Kunitz-type serine proteinase inhibitor that has been associated with cancer cell invasion, metastasis and angiogenesis (Sierko et al., 2007; Tanemura et al., 2009; Zhang et al., 2012). Tanemura and colleagues found that this gene was

hypermethylated in 5 of 17 (29%) metastatic melanoma lesions, but none of the primary tumours (Tanemura et al., 2009). Methylation of this gene induces its inactivation. Although dowregulation/inactivation of TFPI2 has been described in more invasive cancer phenotypes (Tan et al., 2010; Gessler et al., 2011), we found TFPI2 dowregulated in A375M2 stably re-expressing PDE5A clones that are less invasive compared to control cells. Therefore, we were interested in understanding if dowregulation of TFPI2 in melanoma cells with a lower invasion potential may be a mechanism to compensate the effect of PDE5A expression and possibly overcome it. In the present work we only confirmed TFPI2 gene expression by qRT-PCR but we did not further investigate its methylation status, thus more work is required to address our hypothesis.

The SERPIN family has been reported to play an important role in various types of cancer, such as renal, prostate, ovarian and breast (Asanuma et al., 2007; Bijsmans et al., 2011). SERPINB2, also known as PAI-2, is an efficient inhibitor of urokinase-type plasminogen activator (uPA), and it is involved in various biological processes including differentiation and tissue remodelling (Jang et al., 2010). We found this gene downregulated in A375M2 stably re-expressing PDE5A clones compared to control cells. Upregulation of the urokinase plasminogen activation (uPA) system leads to increased cancer invasion and metastasis. Plasminogen activator inhibitors type-1 (PAI-1/SERPINE1) and type-2 (PAI-2/SERPINB2) have similar uPA inhibitory properties yet PAI-1 promotes cell invasion by modulating cell adhesion and migration (Lobov and Ranson, 2011). Paradoxically, expression of PAI-2 is associated with increased survival in patients with breast cancer and other studies have highlighted key structural and functional differences between these serpins (Croucher et al., 2008). Since we found this gene upregulated in A375M2 stably re-expressing PDE5A clones our future aim is to better characterise its function in melanoma.

Mucins are heavily glycosylated proteins. These proteins are divided in two different classes, according to their cellular localisation: membrane-bound mucins and secretory mucins (Singh and Hollingsworth, 2006; Shyu et al., 2007). Mucins participate in various physiological functions, such as cell adhesion, migration and invasion (Riker et al., 2008; Yamada et al., 2011) and have been studied in various cancers (Huang et al., 2009; Nam et al., 2011). MUC15 is a membrane bound mucin, which levels were upregulated in A375M2 stably re-expressing PDE5A clones

compared to control cells. We investigated genes like MUC15 that are overexpressed in cells with a lower metastatic potential, such as A375M2 stably re-expressing PDE5A clones, because of their possible role in the decrease of invasiveness. Riker et al. (Riker et al., 2008) reported downregulation of MUC15 in metastatic melanoma in a study where they performed a gene expression profile of primary and metastatic melanoma samples supporting our hypothesis that mucin 15 may have a role in inhibiting invasion and metastasis.

Following gene microarray analysis we found two upregulated genes that belong to the serpin superfamily of serine protease inhibitors: serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5 (SERPINA5) and pigment epithelium derived factor (SERPINF1). SERPINA5 inactivates a variety of plasma proteases, including anticoagulant serine proteases, blood coagulation factors, fibrinolytic enzymes and the urokinase-type plasminogen activator (Bijsmans et al., 2011). This protein prevents the conversion of plasminogen to plasmin, inhibiting extracellular matrix degradation (Asanuma et al., 2007; Carriero et al., 2011). This is an important step to prevent cancer metastasis, as matrix degradation is one of the hallmarks of invasion (Carriero et al., 2011). Loss of SERPINA5 mRNA in serous carcinomas is associated with downstream activation of matrix metalloproteinase 9 (MMP-9), followed by extracellular matrix degradation (Sieben et al., 2005). We found that SERPINA5 expression was higher in A375M2 stably re-expressing PDE5A clones compared to control cells, so provide for the first time, a link between SERPINA5 and malignant melanoma. However further validation is necessary to confirm this association.

SERPINF1 (pigment epithelium-derived factor, PEDF) gene expression was upregulated in A375M2 stably re-expressing PDE5A clones compared to control cells therefore SERPINF1 was chosen for further validation. SERPINF1 is a secreted glycoprotein but with no demonstrable protease inhibitory activity (Orgaz et al., 2009; Subramanian et al., 2012). This protein plays a critical role in angiogenesis inhibition, proliferation and migration and has been described as an anti-tumour and anti-metastatic in melanoma (Abe et al., 2008; Orgaz et al., 2009). SERPINF1 expression is upregulated in normal skin melanocytes suggesting an inverse correlation between the levels of this gene and aggressiveness potential of cells (Orgaz et al., 2009; Garcia et al., 2004; Fernandez-Garcia et al., 2007). The loss of SERPINF1 enabled the switch

of melanoma cells from proliferative to an invasive phenotype (Orgaz et al., 2009) showing the important role of this gene in melanoma progression.

Our results are in accordance with previous findings, as SERPINF1 mRNA and protein levels are upregulated in the A375M2 stably re-expressing PDE5A clones that are less invasive compared to control cells. We can then conclude that high expression of SERPINF1 is associated with decreased invasion in A375M2 melanoma cells expressing PDE5A.

Our data indicate that PDE5A expression in A375M2 melanoma cells, that normally do not express this protein, triggers differential gene expression. These changes together with the anti-metastatic role of PDE5A itself in these genetically modified cells may contribute to their lower invasive potential. Among the list of differentially expressed candidate genes that we obtained, we identified genes that have a known role in cancer cell invasion and metastasis. These genes may lead to the identification of new potential targets for pharmacological modulation to inhibit the metastatic progression of melanoma.

Although more validation in other genetically modified melanoma cells such as WM266.4 stably re-expressing PDE5A clone and cross-validation in 501Mel shPDE5A or siPDE5A is needed, we postulate that our initial findings add new insight into the PDE5A regulated cell motility in melanoma.

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