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PAX3 expression, protein modifications and downstream target gene profiling in melanocytes and melanoma cells

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***PAX3* Expression, Protein Modifications and Downstream Target Gene Profiling in Melanocytes and Melanoma Cells.**

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Abstract

PAX3 is a transcription factor. It plays a major role in the development of melanocytes in the embryo. As a result of alternative splicing, the gene gives rise to eight different transcripts which encode proteins that have differing structures and are therefore likely to activate different downstream target genes. The presence of post-translational modifications has also been shown to alter the functions of the proteins.

PAX3 regulates the maintenance of undifferentiated melanoblasts and mediates pathways involved in proliferation, migration and survival. It has been shown to be expressed in melanoblasts, adult melanocytes, naevi and in most melanoma cells. This implies that PAX3 may be involved in such regulatory pathways in all of these cell types including melanoma. Melanoma is a notoriously aggressive and drug resistant form of skin cancer. Research into the role of *PAX3* in melanoma could provide novel treatment options for targeted therapies aimed at *PAX3* or its regulatory pathways.

Therefore, in this study, the expression profile of *PAX3* alternate transcripts was compared in normal melanocytes and melanoma cells. Moreover, differences in post-translational modifications between these cell types were assessed, as were changes in downstream target genes. This research further clarifies the role of PAX3 in melanoma and details the differences in its role here, relative to that in melanocytes.

Declaration

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List of Abbreviations

Akt: also known as protein kinase B (PKB), serine/threonin protein kinase Akt

APC: antigen presenting cell

BCL2L1: BCL2-like protein 1 (also known as BCL-XL)

BRAF: v-raf murine sarcoma viral oncogene homologue B1

CCNA2: cyclin A2

CDK4: cyclin-dependent kinase 4

CDKN2A: cyclin-dependent kinase inhibitor 2A

cDNA: complementary deoxyribonucleic acid

CREB: cAMP (cyclic adenosine monophosphate) response element binding

CSPG4: chondroitin sulphate proteoglycan 4 (also known as MCSP or HMW-MAA)

CXCR4: CXC chemokine receptor 4

Dct: dopachrome tautomerase (also known as Trp-2- tyrosinase related protein 2)

DNA: deoxyribonucleic acid

DSHB: Developmental Studies Hybridoma Bank

EDTA: ethylenediaminetetraacetic acid

FBS: foetal bovine serum

GAPDH: glyceraldehydes-3-phosphate dehydrogenase

GSK3: glycogen synthase kinase 3

HES1: hairy and enhancer of split 1

Lef1: lymphoid enhancer binding factor 1

MCAM: melanoma cell adhesion molecule

MDM2: murine double minute 2

MITF: microphthalmia-associated transcription factor

mRNA: messenger ribonucleic acid

NES: nestin

Neurog2: neurogenin 2

NFKβ2: nuclear factor kappa beta 2

NGS: normal goat serum

NRAS: neuroblastoma RAS viral oncogene homologue

PAX3: paired box 3

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PDGF: platelet derived growth factor

PIP3: phosphatidylinositol (3,4,5)-triphosphate

PTEN: phosphatase and tensin homologue

qPCR: quantitative polymerase chain reaction

RNA: ribonucleic acid

RT-PCR: reverse transcription polymerase chain reaction

RT-qPCR: real time quantitative polymerase chain reaction

Shc: squalene-hopene cyclase

siRNA: small inhibitor ribonucleic acid

SIRT1: sirtuin 1

SOX9: SRY (sex-determining region Y) box 9

SOX10: SRY (sex-determining region Y) box 10

TAE: tris base, acetic acid and EDTA buffer

TCF: T cell factor

TGF-β: transforming growth factor beta

TPA: tissue plasminogen activator

TPD52: tumour protein 52

Trp-1: tyrosinase related protein 1 (also known as Tyrp1)

TX-TBS: Triton X-100 tris buffered saline

Tyr: tyrosinase

Wnt: wingless type MMTV integration site family

PAX3/Pax3 protein in humans and mice, respectively

PAX3*/*Pax3 gene in humans and mice, respectively

1. Introduction

Cutaneous Malignant Melanoma (from here on referred to as melanoma) is a skin cancer that arises from normal skin melanocytes. Studies have shown that the expression of the developmental gene, *PAX3*, is required for neural crest cells to commit to the melanocyte lineage and for proliferation. *PAX3* is expressed across all cells of the melanocytic lineage, including the cells of naevi and melanoma. Furthermore, PAX3 has been shown to differentially regulate downstream target genes in melanoma cells compared to melanocytes. Thus further research into the role of PAX3 in melanocytes and melanoma cells is required in order to assess whether PAX3 is involved in key cellular processes that drive melanomagenesis and whether these pathways differ from those in melanocytes.

2. Literature review

The most aggressive form of skin cancer is cutaneous melanoma (Chin et al., 2006). Melanoma is responsible for only 4% of skin cancers, but accounts for 80% of deaths from skin cancer (Lewis et al., 2005). Moreover, metastatic melanoma is highly resistant to most drug therapies, making it difficult to treat (Chen et al., 2006; Chin et al., 2006). Melanoma is the most common cancer in people aged 15-39 years and the third most common cancer in people aged 40 years and above and is more frequent in males than in females (Threlfall and Thompson, 2010). Over ten thousand new cases are diagnosed every year in Australia (Cancer Council Australia, 2012).

Melanoma is categorised clinically into Tumour, Node, Metastasis (TNM) stages 0, I, II, III and IV. During early stage melanoma (stages 0, I and II) the tumour is confined to the epidermal and dermal layers of the skin. At this point, the lesion can be excised from the skin. For patients with early stage melanoma, the 5-year survival rate is 95% (Balch et al., 2009). As the disease progresses the tumour cells migrate through the underlying layers of skin and can invade blood and lymph vessels, allowing the aberrant cells to travel throughout the body and establish secondary tumours. For patients with advanced stage metastatic melanoma the 5-year survival rate drops significantly to below 45% (Balch et al.,

2009). Currently, there are limited safe and effective treatment options available for metastatic melanoma (reviewed in Mackiewicz-Wysocka et al., 2013). This highlights the importance of further investigations into the mechanisms behind melanoma formation and progression so that an effective treatment focussed on inhibiting the pathways involved in disease progression can be developed.

The paired box 3 gene (*PAX3* in humans, *Pax3* in mice) plays a crucial role in specification, proliferation and migration of melanocytes in the embryo (Bang et al., 1997; Goulding et al., 1991; Hornyak et al., 2001). *PAX3* continues to be expressed in normal melanocytes of the adult epidermis and is also expressed in naevi and melanoma cells (He et al., 2010; Medic and Ziman, 2010). Exposure to ultraviolet (UV) radiation is known to be linked to melanoma development. Following UV exposure, *PAX3* is seen to be up-regulated in normal melanocytes, resulting in an increase in melanocyte proliferation (Dong et al., 2012; Yang et al., 2008). Since *PAX3* is commonly expressed in melanomas (Medic et al., 2011; Medic and Ziman, 2010; Plummer et al., 2008; Scholl et al., 2001), this raises the possibility that *PAX3* is involved in pathways that cause the transformation of melanocytes to malignant melanoma cells.

2.1 The development of melanoma

Normal skin contains neural crest derived melanocytes at the dermal-epidermal border and keratinocytes, the most abundant cell type in the epidermis. The number of keratinocytes is greater than that of melanocytes, usually in the ratio of one melanocyte for every 36 keratinocytes. The melanocytes in naevi (a benign melanocytic lesion), and in melanomas, however, far outnumber the keratinocytes (Hauschild et al., 2011; Hsu et al., 2002). Therefore, keratinocytes, which regulate the growth of melanocytes and maintain their correct location in the skin, have diminished control of melanocytes in these lesions. Melanocytes in naevi ultimately become quiescent and proliferation ceases (Li et al., 2003). These melanocytes do, however, have the potential to progress to radial growth phase melanoma tumours.

Melanoma can arise from naevi or occur spontaneously (*de novo*) (Weatherhead et al., 2006). The transformed cells proliferate and spread in a radial manner within the epidermis,

known as the radial growth phase. Subsequently, if left to continue proliferating, the melanoma cells enter the vertical growth phase, where melanoma cells invade the dermis (Crowson et al., 2006). Melanoma cells have a propensity to metastasise, which involves their migration through the dermis, entrance into blood and lymph vessels and dissemination to distant sites where secondary tumours can arise (Hsu et al., 2002).

In response to UV radiation, melanin is produced in melanocytes, a process termed melanogenesis. There are two types of melanin produced as a result of melanogenesis; eumelanin and pheomelanin. Eumelanin is a brown/black pigment and pheomelanin, a red/yellow pigment. Together, these pigments give the skin and hair their colour and protect the DNA of epidermal cells from UV radiation (Hearing, 2011).

During the process of melanogenesis, melanin is packaged into melanosomes that travel along the dendritic projections of the melanocytes to the keratinocytes. One melanocyte may serve up to 36 keratinocytes and this is known as an epidermal-melanin unit (Figure 1). The melanin forms a barrier around the keratinocyte nucleus, preventing UV radiation from damaging the DNA of dividing keratinocytes (Hsu et al., 2002; Yamaguchi et al., 2007). Exposure to excessive UV radiation is known to cause an increase in melanogenesis and contributes to darkening of the skin, or tanning (Tadokoro et al., 2005).

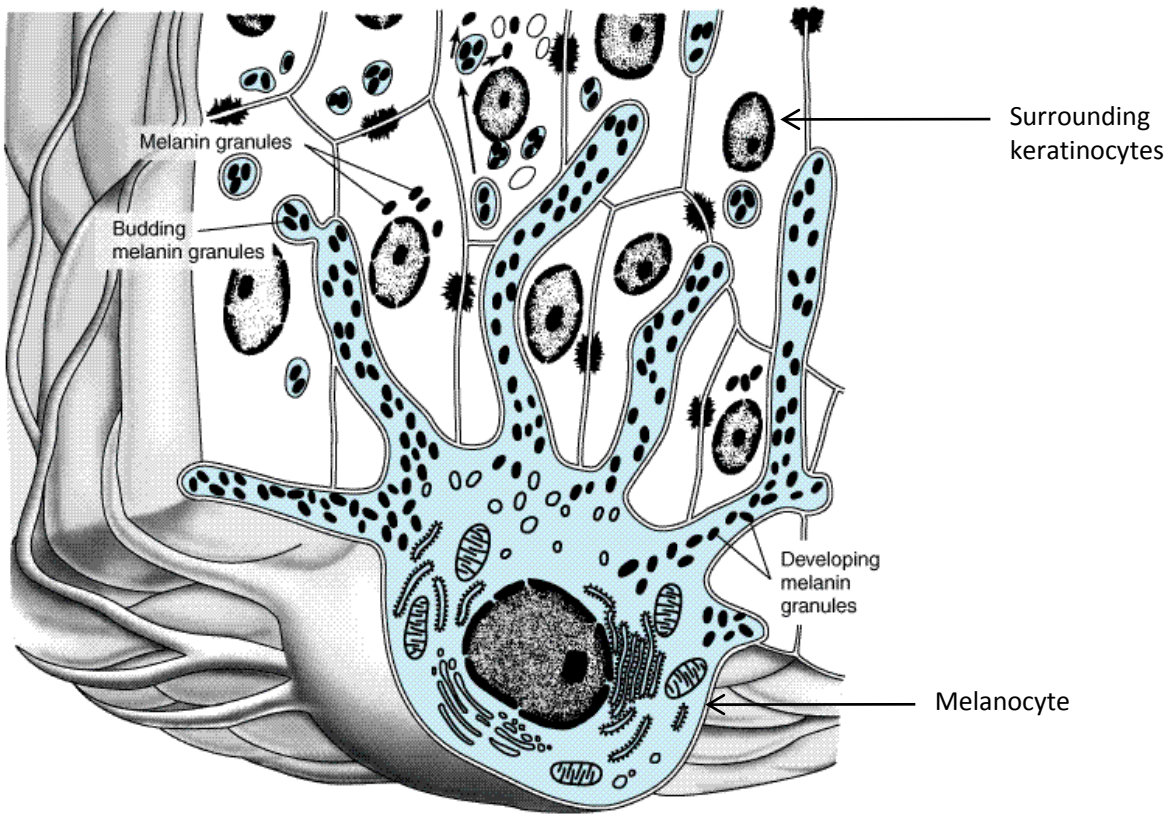

Figure 1: The epidermal melanocyte-keratinocyte unit. Note the long dendritic projections of the melanocyte that transport the melanin to surrounding keratinocytes. Adapted from Junqueira et al (1998).

2.2 Risk factors for melanoma

The risk factors associated with melanoma development can be divided into two categories; those that are environmental and those that are genetic (Cho and Chiang, 2010; Goodall et al., 2008). The main environmental risk factor associated with melanoma development is UV radiation exposure (Leiter and Garbe, 2008; MacKie, 2006). It has been shown that multiple intermittent sun exposures, particularly during childhood, increase the risk of melanoma occurring (Veierød et al., 2003). Since PAX3 is up-regulated in melanocytes that have been exposed to UV radiation (Yang et al., 2008), it is possible that PAX3 has a role in the transformation of melanocytes into melanoma cells.

Previous melanoma lesions, numerous benign or abnormal naevi and a family history of melanoma also lead to an increase in the risk of developing melanoma (Miller and Mihm, 2006). A person's phenotype, the colour of their hair and the number of naevi they have are indicators of their risk of developing melanoma. Other phenotypic traits, such as blue eyes, numerous freckles and red hair are also indicative of a risk of melanoma development due to inherited variants of the melanocortin receptor 1 (MC1R) (Miller and Mihm, 2006; Naysmith et al., 2004). The MC1R receptor responds to the binding of α-melanocyte-stimulating hormone (αMSH) by initiating a signalling cascade that causes an increase in expression of enzymes that are crucial to melanin production (Frändberg et al., 1998). Inherited variants of the MC1R receptor can reduce its activity, thus increasing susceptibility to UV exposure and melanoma (Kennedy et al., 2001).

People with a family history of melanoma due to inheritance of mutations in the familial melanoma genes cyclin-dependent kinase inhibitor 2A (*CDKN2A*) or cyclin-dependent kinase 4 (*CDK4*), both of which are tumour suppressor genes, also have an increased risk of developing melanoma (Miller and Mihm, 2006). Recently, a microphthalmia-associated transcription factor (MITF) variant (E318K) has also been implicated in predisposition to melanoma (Yokoyama et al., 2011). The role of PAX3 in melanoma development has not been determined to date, but in this thesis we aim to further clarify its role.

2.3 Treatment of melanoma

According to the Australian Cancer Network Melanoma Guidelines Revision Working Party (2008), TNM stage 0, I and II primary stage melanomas are treated by surgical removal. Metastatic melanoma lesions of TNM stages III and IV, characterised by lymph node and distant metastases respectively, are removed by surgery as a first line treatment (Australian Cancer Network Melanoma Guidelines Revision Working Party, 2008). For inoperable melanoma, treatment involves chemotherapeutic agents or, less commonly, radiotherapy (Sondak et al., 2001). Until 2011, chemotherapy drugs included temozolomide (an alkylating agent) or decarbazine (inhibits cell replication) (Middleton et al., 2000). Interleukin-2, an

immunological drug that influences T-cell growth, was also used for treatment (Atkins et al., 1999). These drugs, however, have very low success rates (less than 20%) (Agarwala, 2009).

Emerging new treatments now target the specific mutation associated with cancer development, making treatment more specific and effective (Amaria et al., 2011; Becker et al., 2006). For example, one of the most successful new drugs for melanoma treatment targets a mutation in the *BRAF* gene, a mutation found in greater than 50% of cases (Amaria et al., 2011). The *BRAF* inhibitor Vemurafenib (Roche, Plexxikon), for example, decreases the activation of the MAPK pathway by inhibiting the mutant *BRAF* gene (V600), hence decreasing cell proliferation (Amaria et al., 2011; Davies et al., 2002). In general however, patients develop resistance to these drugs after 6-9 months (Poulikakos and Rosen, 2011). Recent research into the development of resistance to Vemurafenib in melanoma cells suggests that *Stat3* and *PAX3* are activated by mutant Braf (Liu et al., 2013). Further research suggests that the Stat3-PAX3 signalling pathway could prove an effective target for surmounting the resistance to Vemurafenib in melanoma (Liu et al., 2013).

2.4 Introduction to *PAX3*

The *PAX3* gene is at the top of the hierarchy of genes that specify melanocytes. *PAX* genes are highly conserved and encode a family of transcription factor proteins having a unique DNA binding sequence, a 128 amino acid long paired domain (Bopp et al., 1986; Frigerio et al., 1986). There are nine members of the *PAX* gene family (*PAX1-9*) (Chalepakis et al., 1993) and these are classified into four groups according to whether they possess a full or partial homeodomain, a second conserved DNA binding domain and an octapeptide. *PAX3* belongs to group III of the *PAX* genes, i.e. those *PAX* genes characterised as having both a full

homeodomain and an octapeptide (Lang et al., 2007; Robson et al., 2006; Walther et al., 1991).

The proteins encoded by the *PAX* genes are involved in organogenesis (Chi and Epstein, 2002). One role of the PAX proteins is to maintain the multipotency of "stem" cells while they proliferate and migrate, until they terminally differentiate into lineage specific cells that make up several tissues. Mutations in *PAX* genes are known to result in the formation of mutant embryos with severe defects. Moreover, *PAX* genes are commonly expressed in tumours (Balling et al., 1988; Mansouri et al., 1999). Mutations in the *Pax3/PAX3* gene are known to produce *Splotch* mice and Waardenburg syndrome in humans (Baldwin et al., 1992; Henderson et al., 1997; Pingault et al., 2010).

The human *PAX3* gene is located at 2q35 and consists of 9 exons and 9 introns, as shown in Figure 2A. The encoded PAX3 protein is a transcription factor that binds to specific DNA target sequences on downstream target genes in order to regulate their transcription. The PAX3 protein contains highly conserved protein domains; a paired domain, a paired-type homeodomain, a conserved octapeptide and a C-terminal transactivation domain rich in proline, serine and threonine. The paired domain and homeo-DNA binding domains are encoded by exons 2 to 4 and exons 5 to 6, respectively, and while they are able to bind DNA independently, functionally they have an interdependent relationship (Parker et al., 2004; Strachan and Read, 1994; Underhill and Gros, 1997). The transactivation domain, which interacts with RNA polymerase to activate or repress transcription, is encoded by exons 7 and 8 (Apuzzo et al., 2004).

The human *PAX3* gene gives rise to eight different transcripts due to alternate splicing of the gene. These transcripts are *PAX3A*, *PAX3B*, *PAX3C*, *PAX3D*, *PAX3E*, *PAX3G*, *PAX3H* and *PAX3I* (also known as *PAX3DQ*-) (Figure 2B) (Barber et al., 1999). These mRNA transcripts produce proteins that differ in structure and thus function. The PAX3A and PAX3B isoforms are derived from exons one through four and encompass the beginning of intron 4, at which point the protein truncates. These two isoforms do not contain a C-terminal transactivation domain or a homeodomain (Tsukamoto et al., 1994). The *PAX3C* transcript includes exon 8 and 5 codons of the beginning of intron 8. *PAX3D* does not contain intron 8, but continues through exons 8 and 9 (Parker et al., 2004). *PAX3E* includes exons 8 through 9, with the 8th

and 9th introns spliced out. PAX3G is a truncated isoform of PAX3D, while PAX3H is a truncated isoform of PAX3E, both lacking exon 8. Neither PAX3G nor PAX3H contain the entire transactivation domain (Parker et al., 2004). *PAX3I* is almost identical to *PAX3D*; the only difference is the absence of a 5' terminal glutamine at the region encoded by the intron exon boundary at the beginning of exon 3. As shown by the difference in exons and introns encoding the varied isoforms of PAX3, it can be seen that the structure of the protein differs primarily due to the absence of parts or the whole of the transactivation domain and as a result, the different isoforms have been shown to have different functions (Wang et al., 2006).

In fact, Wang et al. (2006) showed that the different PAX3 isoforms have different effects on the behaviour of transfected melanocytes. PAX3A and PAX3B have a negative effect on melanocyte proliferation. PAX3B also reduces melanocyte migration and increases the rate of melanocyte apoptosis. PAX3C and PAX3D increase cell proliferation, migration, differentiation and survival. PAX3E reduces melanocyte proliferation, but increases apoptosis. PAX3G reduces melanocyte migration and PAX3H increases melanocyte survival, migration and proliferation. These results show that the PAX3 isoforms have differing effects on melanocytes and their expression levels and roles in the development of melanoma remain to be identified.

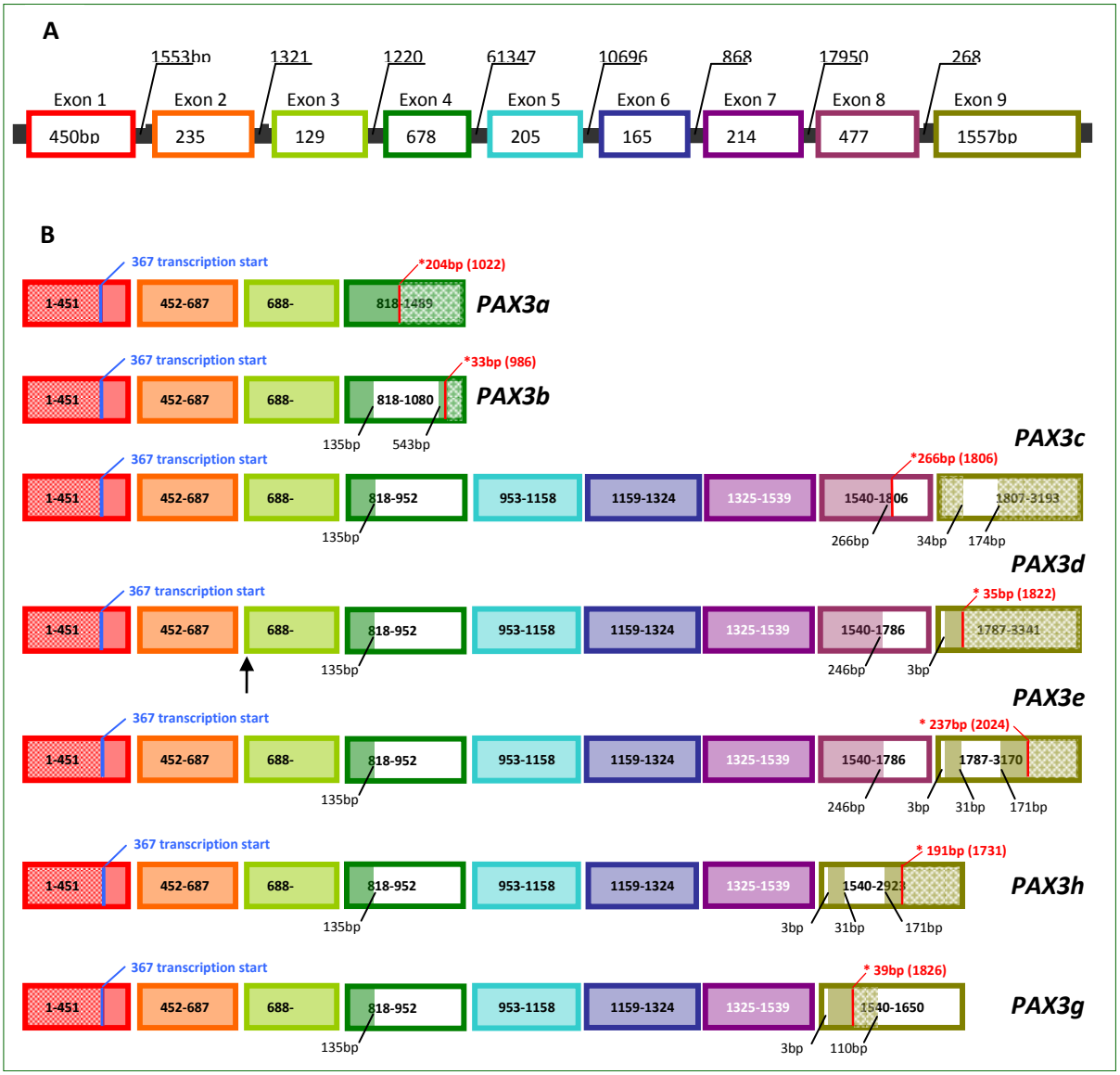

Figure 2: The entire *PAX3* gene and alternate transcripts. A) The entire *PAX3* gene encompassing exons 1 through 9. B) Eight alternate transcripts, of which seven are shown, are produced from the *PAX3* gene as a result of alternate splicing. The *PAX3i* transcript is not present in the image, but contains the same sequence as *PAX3d* with the absence of a CAG encoding a glutamine (Q) at the 5' end of exon 3 (indicated by the arrow). The numbers are indicative of base pairs and an asterisk (*) denotes the stop codon. Colours are coded to indicate the sequences retained in each alternative mRNA transcript. The transcripts essentially run from the transcription start site (blue line) to the stop site (red line), contributing to their differing lengths (Medic and Ziman, 2009).

Activation of PAX3

PAX3 is activated by two POU family transcription factors, BRN2 and Oct-1 (Berlin et al., 2012). Only phosphorylated BRN2 activates *PAX3* transcription, whereas *PAX3* transcription is repressed in the absence of BRN2 phosphorylation (Pruitt et al., 2004; Zhu and Pruitt, 2005). BRN2 also plays a role in the repression of the *MITF* promoter (Goodall et al., 2008). In this way, BRN2 regulates the migration and proliferation of cells of the melanogenic lineage through the activation of *PAX3* by phosphorylated BRN2 and the repression of *MITF* through non-phosphorylated BRN2 (Berlin et al., 2012). *BRN2* itself can be regulated by micro RNA, particularly miR-211. This regulation affects the potential of melanoma cells to invade underlying tissues (Boyle et al., 2011).

2.5 *PAX3* in melanocyte development, melanoblast maintenance and melanogenesis

During mouse embryonic development, *Pax3* is expressed in neural and neural crest cells as early as stage E8.5 (Figure 3) (Goulding et al., 1991). These *Pax3* expressing cells arise from the multipotent stem cells of the neural tube, neural crest and dermomyotome and give rise to neural cells, skeletal muscle cells and neural crest derived lineages, including pigment producing melanocytes (Sommer, 2011; Yanfeng et al., 2003).

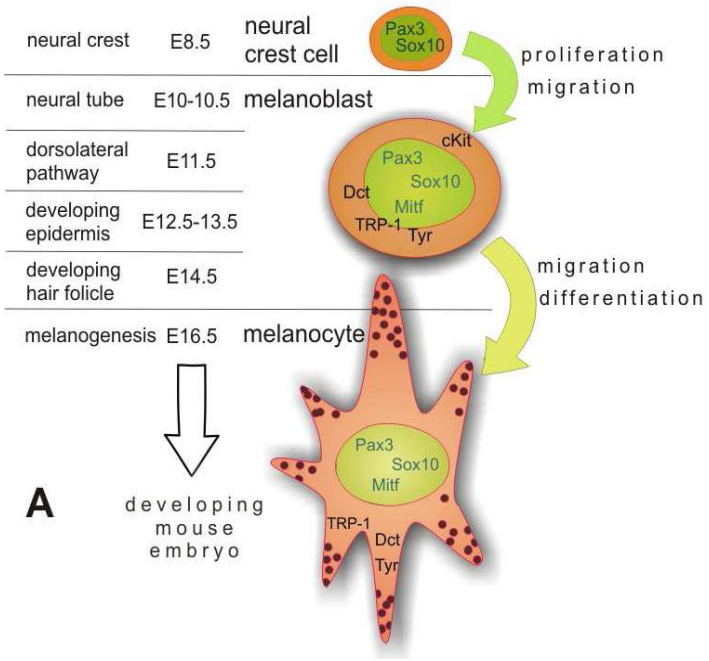

Figure 3: The expression of *Pax3* as early as stage E8.5 in mouse neural crest cells, committing them to the melanogenic lineage. PAX3 remains expressed throughout the development of melanocytes (Medic and Ziman, 2009).

The co-expression of SOX10 in a portion of the Pax3 positive neural crest cells designates their differentiation along the melanogenic lineage (Sommer, 2011). These neural crest cells then express markers indicative of melanoblasts; MITF, Kit and Dct (Hou et al., 2000; Steel et al., 1992), and they migrate toward the developing epidermis, where they terminally differentiate into melanocytes. At this stage, they express Tyr and Trp-1, which are genes involved in melanin production (Steel et al., 1992).

Not all melanoblasts, however, become fully differentiated functioning melanocytes simultaneously. Some of the melanoblasts remain as melanoblasts within the hair follicles in adult skin, where they function to replace melanocytes that have been lost during the hair replacement cycle (Nishimura et al., 2002). The PAX3 positive melanoblasts remain at the base of the arrector pili muscle in the hair follicle in a quiescent state until external factors cause them to proliferate, differentiate and migrate to take the place of lost melanocytes in the epidermis (Lang et al., 2005; Nishimura et al., 2002). The PAX3 protein is required to maintain these melanoblasts in an undifferentiated state, while they remain committed to the melanocyte cell lineage (Lang et al., 2005). Furthermore, *PAX3* continues to be

expressed in a proportion of epidermal melanocytes of adult skin, presumed to be fully differentiated, particularly those exposed to UV radiation (Yang et al., 2008).

2.6 *PAX3* in cell cycle inhibition and maintenance of stem cells

PAX3 directs the maintenance of an undifferentiated "stem" cell-like state in melanoblasts, by regulation of apoptosis and cell migration. *PTEN* and *p53* tumour suppressor genes (both direct targets of PAX3) are inhibited by the PAX3 protein, preventing them from activating factors that bring about apoptosis during the cell cycle, allowing "stem" cell survival (Furnari et al., 1998; Van Dyke, 2007). In addition, the *BCL2L1* gene is activated by PAX3 and prevents apoptosis by releasing factors that inhibit apoptotic enzymes (Cheng et al., 2001). Neural crest derived melanoblasts are thus maintained as undifferentiated cells during migration to the epidermis. PAX3 regulates migration by spatial and temporal activation of key migration genes *c-Met*, *NCAM*, *STX* and *TGF-β* (Epstein et al., 1996; Mayanil et al., 2000; Nakazaki et al., 2009).

The controversial "cancer stem cell theory" describes a population of cells within the tumour that, in contrast with stem cells, exhibit the ability to self-renew and propagate, in addition to having the potential for multidirectional differentiation, resisting apoptosis and having the ability to enable tumour development, growth and metastasis (Calabrese et al., 2004; Kucia and Ratajczak, 2006; Reya et al., 2001). Recently, melanoma has been the subject of debate in regards to the theory of cancer stem cells (Adams and Strasser, 2008; Klein et al., 2007; Monzani et al., 2007; Schatton et al., 2008; Shackleton et al., 2009). Furthermore, as described above, PAX3 has been shown to maintain the "stem cell-like" state of melanocyte precursors and is also expressed in melanoma tumour cells. Thus, there is the potential for PAX3 to contribute to melanoma development, growth and metastasis through the maintenance of melanoma cancer stem cells.

2.7 Downstream target genes of PAX3

As indicated above, *PAX3* plays an important role in the development of melanocytes, particularly in maintenance of undifferentiated melanoblasts and is the main regulatory gene involved in melanogenesis. Its ability to perform its various roles is achieved by regulation of a variety of downstream target genes.

Previous research conducted by Medic et al. (2011) showed that PAX3 differentially regulates downstream target genes in human epidermal melanocytes compared to metastatic melanoma cells (Figure 4). PAX3 has numerous downstream targets (Barber et al., 2002; Begum et al., 2008; Bondurand et al., 2000; Lang et al., 2005; Li et al., 2007; Margue et al., 2000; Nakazaki et al., 2008; Wang et al., 2007; Watanabe et al., 1998). Using a subset of 14 downstream target genes, based upon their association with processes likely to be important in melanoma progression, Medic et al. (2011) showed that the promoters of *SOX9* and *NES* (involved in cell differentiation), *TPD52* (cell proliferation), *PTEN* and *TGFβ1* (cell survival) as well as *MCAM* and *CSPG4* (cell migration) were all bound by PAX3 in melanoma cells, but to a lesser extent or not at all in melanocytes (Figure 4). Furthermore, the expression of these genes was found to be differentially upregulated in melanoma cells compared to neonatal melanocytes (Figure 5) (Medic et al., 2011). This fact, together with the fact that PAX3 is expressed by fewer cells in normal skin, naevi and primary melanoma cells compared to metastatic melanoma cells (Figure 6) (Medic and Ziman, 2010), implicates PAX3 and genes regulated by PAX3 in melanoma. The mechanisms underpinning this differential regulation and expression have not been investigated prior to this research.

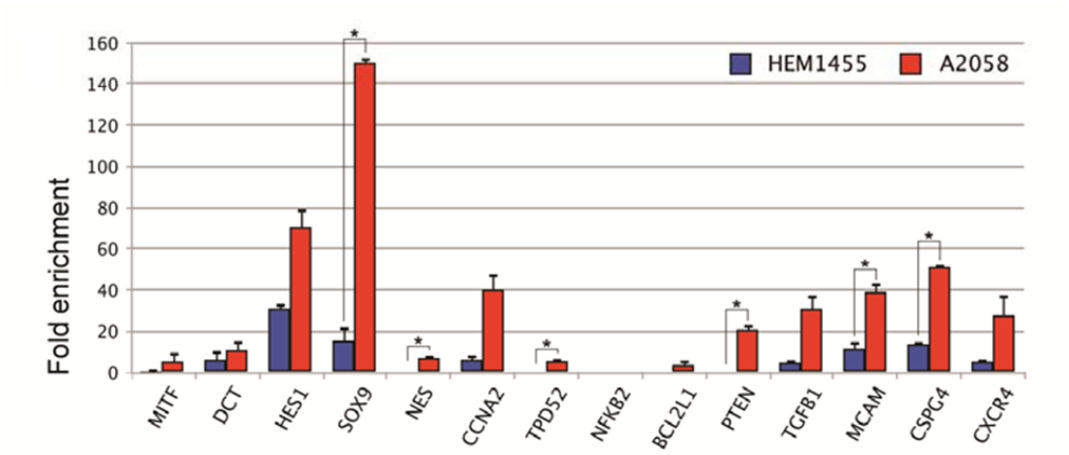

Figure 4: Differential binding by PAX3 to downstream target genes in neonatal human epidermal melanocytes (HEM1455) compared to metastatic melanoma cells (A2058), quantified by qPCR. Downstream genes involved in cell differentiation (*MITF*, *DCT*, *HES1*, *SOX9* and *NES*), cell proliferation (*CCNA2*, *TPD52* and *NFKβ2*), cell survival (*BCL2L1*, *PTEN* and *TGFβ1*) and cell migration (*MCAM*, *CSPG4* and *CXCR4*) were analysed. Enrichment in PAX3-IP was normalised to input DNA. The fold increase over normalised IgG-IP was then calculated (Medic et al., 2011).

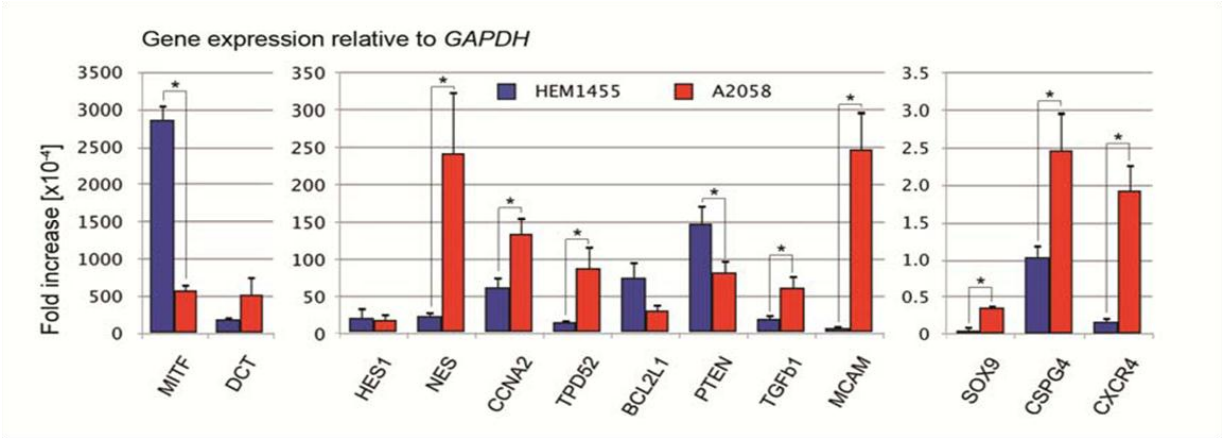

Figure 5: RT-qPCR analysis of relative expression of downstream target genes of PAX3. A correlation was found between the binding of PAX3 to each target gene (Figure 4) and target gene mRNA expression levels (above) (Medic et al., 2011).

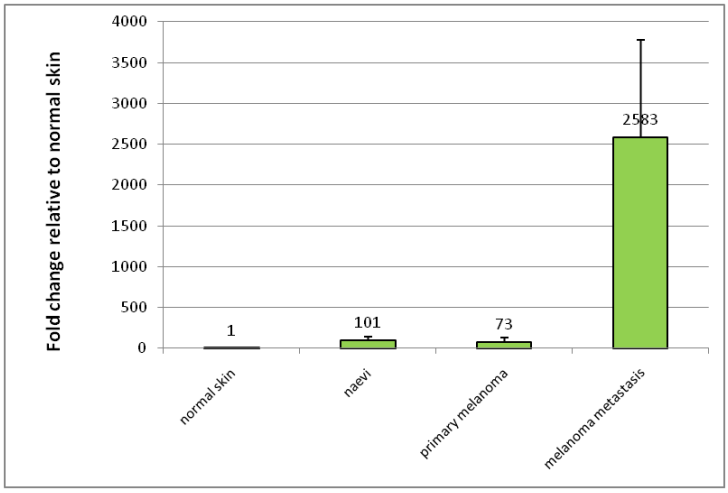

Figure 6: *PAX3* expression in naevi, primary melanoma and metastatic melanoma relative to that of normal skin. The number of cells expressing *PAX3* increases from melanocytes to melanoma cells (Medic and Ziman, 2010).

2.8 Downstream targets of PAX3 differentially regulated in melanocytes and melanoma cells

Microphthalmia associated transcription factor (*MITF)*

The key downstream target of PAX3 during melanogenesis, *MITF,* is involved in melanocyte differentiation, survival and proliferation. In normal melanocytes, the expression of *MITF* is activated by PAX3. Through binding with co-factors CREB and LEF1/β-catenin, PAX3 enhances the transcription of the *MITF* gene (Figure 7) (Watanabe et al., 1998). The transcription factor MITF is responsible for controlling the survival, proliferation and differentiation of melanocytes through activation of melanocytic target genes (Steingrimsson, 2006). PAX3 activates the expression of *MITF* in early neural crest cells leading to the specification of melanoblasts, their migration to the epidermis and to their proliferation and differentiation into melanocytes. MITF activates the melanogenic cascade via activation of various downstream targets, including Tyr, Trp-1 and Dct, enzymes responsible for the final steps of melanogenesis, as well as MelanA and gp100/Silv, which are structural components of melanosomes (Figure 7) (Yasumoto et al., 1997).

PAX3 binds the *MITF* promoter in melanocytes and melanoma cells, however in melanoma the tight regulation of *MITF* by PAX3 is lost and *MITF* is not regulated by PAX3 alone. In melanoma cells, *MITF* activation is also by Wnt3a signalling, which causes β-catenin and Lef1 transcription factors to bind to the promoter region of the *MITF* gene, allowing transcription to occur (Cook et al., 2005; Takeda et al., 2000b). This alteration in *MITF* regulation allows activation of proliferation pathways in melanoma cells (Cronin et al., 2009).

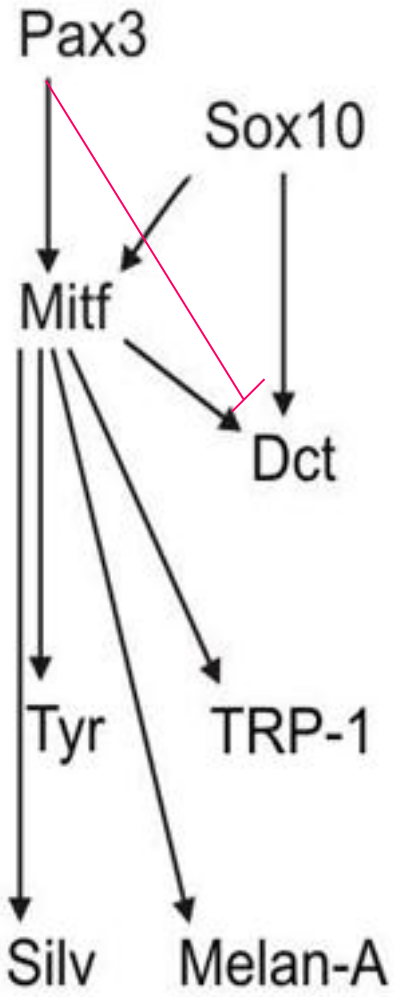

Figure 7: The role of Pax3 in melanogenesis. Pax3 activates *MITF*, which in turn activates *Silv*, *Tyr*, *Melan-A* and *Trp-1*. Pax3 and MITF compete for the promoter of *Dct* (Medic and Ziman, 2009).

Dopachrome tautomerase (*DCT*)

Dct is involved in differentiation of melanoblasts. During development PAX3 inhibits the expression of *Dct*, allowing the melanoblasts to remain in their undifferentiated cell state (Lang et al., 2005), allowing cells to proliferate, migrate and survive.

The Dct enzyme acts together with tyrosinase and Trp-1 during melanogenesis. Dct causes dopachrome to be converted to dehydroxyindole-2-carboxylic acid (DHICA)(Figure 8), which is a precursor of the pigment, eumelanin (Park et al., 2009). *Dct* is activated by MITF, but MITF competes with PAX3 to bind to the *Dct* promoter. In fact, PAX3 has a stronger affinity for the *Dct* promoter than MITF (Lang et al., 2005). The displacement of PAX3 by β-catenin, which is regulated by the Wnt signalling pathway, allows MITF to bind freely to the *Dct* promoter, resulting in *Dct* activation (Figure 9). The inhibition of *Dct* by PAX3 is important for the maintenance of melanoblasts during their proliferation and migration to the epidermis and the hair follicle. Without this inhibition, melanoblasts differentiate into melanocytes (Lang et al., 2005).

As indicated by Figure 4, *DCT* is bound by PAX3 in melanoma cells as well and while PAX3 up-regulation leads to *DCT* gene inhibition and maintenance of a stem cell state in melanoblasts, this regulation in melanoma cells (Medic et al., 2011) remains to be determined.

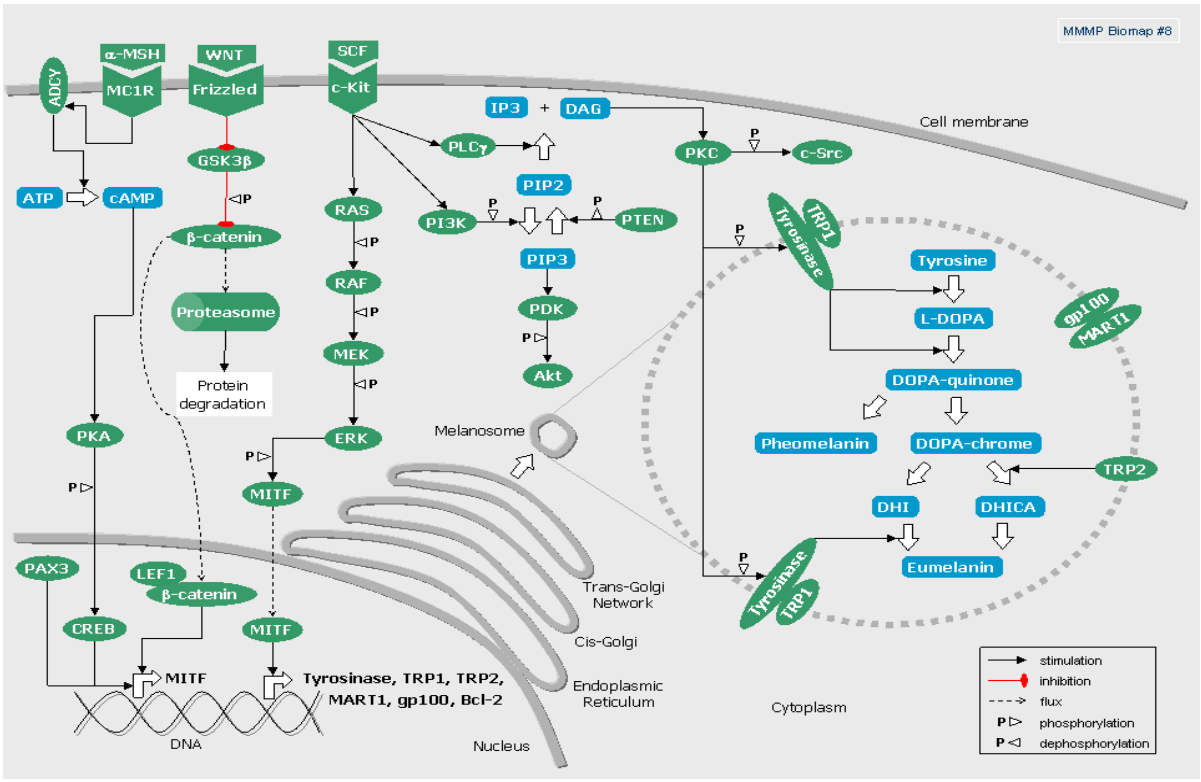

Figure 8: The genes involved in melanogenesis. Note that *PAX3* is the key regulatory gene for this process; without PAX3 the entire melanogenic cascade is not activated (Melanoma Molecular Map Project, 2011). Also note that in this image Dct is referred to as TRP2.

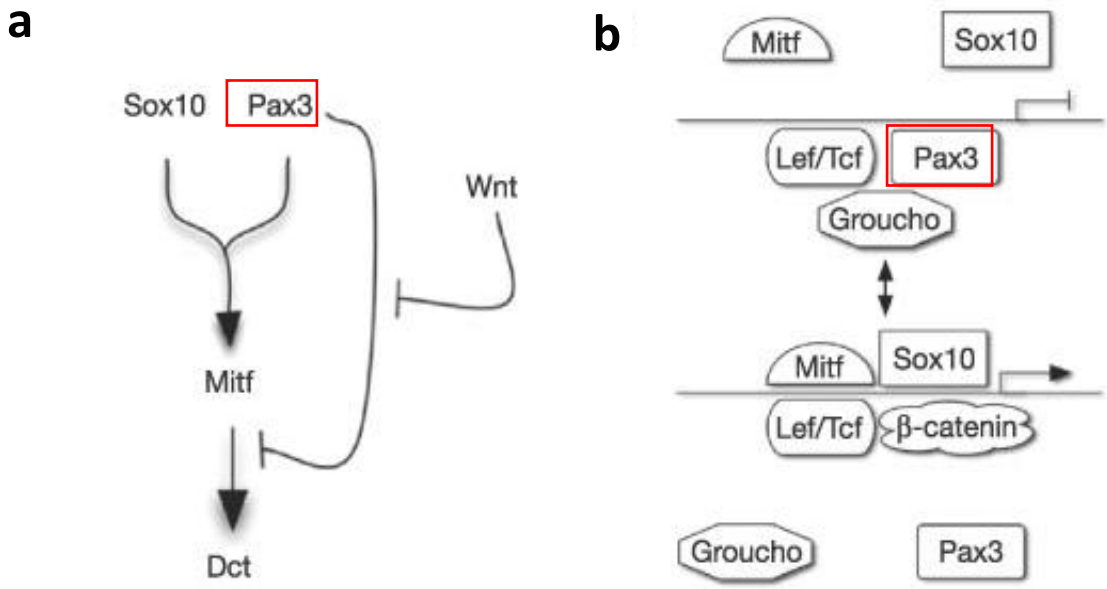

Figure 9: The interaction between Pax3, *Dct* and Wnt. Pax3 inhibits the *Dct* gene (a), whereas Wnt causes the inhibition of Pax3 through β-catenin, which displaces Pax3 and allows *Dct* transcription (b). Adapted from Lang et al. (2005).

Hairy and enhancer of split-1 (*HES1*)

HES1 is a basic helix-loop-helix (bHLH) gene encoding a transcription factor repressor (Kageyama et al., 2000). The primary role of HES1 is to inhibit apoptosis in melanoblasts, thereby playing a critical role in the survival of these melanocyte precursors (Moriyama et al., 2006). Additionally, HES1 is expressed in melanoma cells and has been shown to repress promoter activity of MAP2, a microtubule-associated protein, during melanoma progression, leading to cell survival (Bhat et al., 2006; Soltani et al., 2005).

SRY (Sex-determining region Y) box 9 (*SOX9*)

The *SOX9* gene belongs to the SOX family of transcription factors. The SOX9 protein is essential in development, aiding in sex determination and cartilage development (Nakazaki et al., 2008). More importantly, SOX9 plays a role in the differentiation of melanoblasts into melanocytes and the production of melanin during melanogenesis (Cook et al., 2005). Furthermore, *SOX9* transcription is regulated by PAX3 (Figure 4) and is upregulated after exposure to UV radiation by PAX3 (Passeron et al., 2007). *SOX9* is bound by PAX3 in melanoma cells at a significantly higher rate than in melanocyte cells, so it is speculated that the regulation of *SOX9* by PAX3 in melanoma cells may lead to their stem-like phenotype, particularly of the more metastatic phenotype (Medic et al., 2011).

Nestin (*NES*)

NES is an intermediate filament expressed predominantly in stem cells of neuroepethelial lineage. It has been shown to be expressed in stem cells that reside in hair follicles until these cells mature and *NES* expression ceases (Amoh et al., 2005). A study conducted by Ehrmann et al (2005) revealed that malignant melanoma tumours are positive for NES, whereas benign naevi were NES negative. Its importance here lies in the fact that the protein is highly expressed in proliferating progenitor and stem cells of neuroectodermal origin (Brychtova et al., 2006; Redies et al., 1991). The protein ceases to be produced when

these cells mature, however, it is re-expressed in tumour cells of neuroectodermal (such as melanoma) and mesenchymal lineage (Ehrmann et al., 2005); and in melanoma it is presumably regulated by PAX3 (Medic et al., 2011).

Cyclin A2 (*CCNA2*)

CCNA2 is a cell cycle regulator, required to allow cells to progress from the S phase to the M phase of the cell cycle (Flørenes et al., 2001; Pagano et al., 1992). In normal melanocytes, *CCNA2* is upregulated after transfection with PAX3 (Wang et al., 2007). Previous research of PAX3 and its downstream targets demonstrated a low enrichment of the promoter of *CCNA2* in melanocytes, signifying that PAX3 has the potential to regulate melanocyte proliferation through activation of *CCNA2* (Medic et al., 2011).

The binding of PAX3 to the *CCNA2* promoter has been shown to occur to a larger extent in melanoma cells compared to melanocytes (Medic et al., 2011). Furthermore, knockdown of *PAX3* in melanoma cells inhibits cell proliferation and arrests the cell cycle in both the S and G2/M phases (He et al., 2010). This shows that activation of *CCNA2* by PAX3 presumably contributes to proliferation of both melanocytes and melanoma cells.

Tumour protein D52 (*TPD52*)

TPD52 is involved in cell proliferation, exerting its effects on the cell cycle at the G2/M transition phase (Boutros et al., 2004). *TPD52* does not show enrichment when analysed by PAX3-IP in melanocytes, however, it does so in melanoma cells (Figure 4), which suggests that PAX3 regulates different pathways of proliferation in these two cell types (Medic et al., 2011). Furthermore, *TPD52* overexpression is associated with cell proliferation in cancer, including in melanoma (Roesch et al., 2007) and the ability of PAX3 to regulate proliferation of melanoma cells might be through the activation of *TPD52*.

BCL2-like protein (*BCL2L1*)

The *BCL2L1* gene regulates anti-apoptosis/cell survival during development when cells are undergoing proliferation (Margue et al., 2000). PAX3 activates the *BCL2L1* gene directly in melanoma cells (Strasser and Youle, 2008) but not in melanocytes (Medic et al., 2011), presumably reducing apoptosis and increasing cell survival (Margue et al., 2000).

Furthermore, it has been suggested that PAX3 and BCL2L1 are part of the same pathway that inhibits apoptosis - the viability of rhabdomyosarcoma cells decreased to a similar extent when treated with antisense oligonucleotides for *PAX3* or *BCL2L1* or a combination of both (Margue et al., 2000).

Phosphatase and tensin homolog (*PTEN*)

The *PTEN* gene is a tumour suppressor gene which is responsible for controlling the progression of the cell cycle by acting on the G1 phase checkpoint. Through $p27^{Kip1}$, a cell cycle inhibitor, PTEN antagonistically regulates the PI3K/AKT signalling pathway (Li and Sun, 1998; Zhou et al., 2003). It is responsible for binding to PIP3 and deactivating it via dephosphorylation. If PIP3 is not dephosphorylated it can cause the expression of its downstream target gene *Akt*, which is responsible for proliferation of cells, anti-apoptosis and the formation of tumours (Inoue-Narita et al., 2008). Down-regulation of PTEN sees an increase in activation of *Akt*, which increases phosphorylation of targets involved in regulating apoptosis (Cantley and Neel, 1999).

The PTEN protein also prevents growth, migration and invasion of cells via the Shc adaptor protein and PDGF (Gu et al., 1999). In primary melanoma, less than 15% of cases have a mutation in the *PTEN* gene and the loss of a PTEN allele at one locus (loss of heterozygosity) is shown in 30% to 50% of cases (Reifenberger et al., 1999). However, up to 91% of metastatic melanoma cases show a decrease or complete absence of PTEN protein expression (Whiteman et al., 2002). *PTEN* is bound by PAX3 in melanoma cells, but not in melanocytes (Figure 4, Medic and Ziman, 2011) and may be down-regulated by PAX3 in melanoma cells, resulting in a decrease in its function of PIP3 dephosphorylation and therefore a decrease in tumour suppressor function (Li et al., 2007; Medic et al., 2011). Essentially, if PAX3 levels rise, PTEN levels decrease and so does its apoptotic role (Li et al., 2007).

Transforming growth factor beta (*TGF-β*)

TGF-β is directly regulated by PAX3, but in turn represses *PAX3* transcription in a feedback loop (Yang et al., 2008). TGF-*β* plays a crucial role in cell-cell adhesion and migration by regulating genes responsible for modifying the cytoskeleton or adhesion molecules and

their receptors (Mayanil et al., 2006). TGF-β regulates these cellular properties by activating Wnt inhibitors, that bring about the alteration of the cytoskeleton and activation of cell adhesion molecules, increasing the metastatic potential of cells (Yang et al., 2008). Additionally, neural tube defects are apparent in *TGF-β* knock-out and Pax3-deficient *Splotch* mice, demonstrating that *TGF-β* is regulated by Pax3 and plays a role in the migration of precursor cells during development (Sanford et al., 1997). The *TGF-β* promoter is bound by PAX3 in melanoma cells to a greater extent than in melanocytes and the expression of *TGF-β* is also significantly higher in melanoma cells compared to melanocytes (Figures 4 and 5, Medic et al., 2011). The implication of an increase in TGF-β is that it could possibly contribute to melanoma cell migration and metastasis (Hoek et al., 2006; Medic et al., 2011; Yang et al., 2008).

Melanoma cell adhesion molecule (*MCAM*)

MCAM is a transmembrane glycoprotein and is a key factor in progression of melanoma from the radial growth phase to vertical growth phase (Johnson, 1999; Lehmann et al., 1987; Mills et al., 2002). The *MCAM* gene encodes a protein involved in cell migration (Xie et al., 1997). In general, *MCAM* is not expressed in normal adult epidermal melanocytes, but it is expressed in melanoblasts of the hair follicle, where it presumably regulates migration to the epidermis. Moreover, *MCAM* is directly regulated by PAX3 in melanoma cells to a significantly higher extent than in melanocytes (Medic et al., 2011). MCAM plays a key role in melanoma cell migration and adherence to endothelial cells, which also express MCAM (Figure 10). The activation of *MCAM* by PAX3 in melanoma cells is thought to play a role in melanoma cell migration and metastasis (Moh and Shen, 2009; Yoshioka et al., 2003).

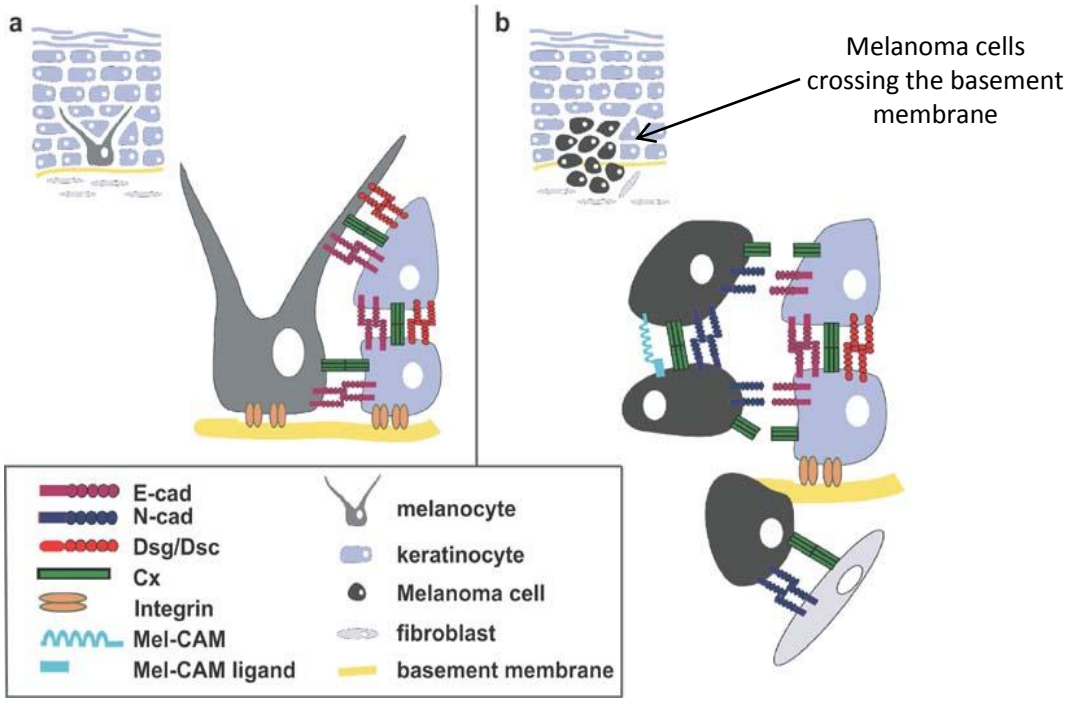

Figure 10: The interaction between keratinocytes and melanocytes (*a*) and keratinocytes and melanoma cells (*b*). The melanocytes are kept in place due to the attachment to keratinocytes via cadherin molecules. When the melanocytes transform and proliferate, they no longer attach to keratinocytes. Instead, they attach to each other through MCAM molecules. Adapted from Li et al (2003).

Chondroitin sulfate proteoglycan 4 (*CSPG4*)

The CSPG4 protein is a cell surface proteoglycan that has little to no expression in normal epidermal melanocytes, but has been associated with melanoma (Campoli et al., 2004). Increased levels of the proteoglycan CSPG4 have been found in benign and dysplastic naevi and in both primary and metastatic melanomas (Campoli et al., 2004). *CSPG4* contributes to the adhesion, motility and invasion of tumour cells (Yang et al., 2009). PAX3 binding to *CSPG4* is significantly increased in melanoma cells compared to melanocytes, and its mRNA levels are significantly increased in melanoma cells (Figures 4 and 5), suggesting that PAX3 may regulate *CSPG4*, contributing to melanoma cell migration and metastasis (Medic et al., 2011).

Chemokine (C-X-C motif) receptor 4 (*CXCR4*)

CXCR4 is a chemokine receptor that elicits cell migration (Robledo et al., 2001; Rollins, 1997). CXCR4 and its ligand, CXCL12, have been implicated in the migration and positioning of melanoblasts as well as in the metastatic progress of numerous neoplasms, including melanoma (Belmadani et al., 2009; Murphy, 2001; Robledo et al., 2001). Research conducted by Scala et al. (2005) showed a correlation between *CXCR4* expression and unfavourable prognosis in patients with malignant melanoma. PAX3 binds to *CXCR4* in melanoma cells and melanocytes, and there is an increase in *CXCR4* mRNA expression in melanoma cells compared to melanocytes (Figures 4 and 5) (Medic et al., 2011). This suggests that PAX3 regulation of *CXCR4* may result in melanoma cell migration and metastasis.

All of the aforementioned genes have a role in the proliferation, migration, survival, differentiation or melanogenic functions of either melanocytes or melanoma cells, or both, and are all regulated by PAX3, either by inhibition or activation. Several of these genes are differentially regulated in melanoma cells compared to melanocytes. The mechanism by which this differential regulation takes place is presumably due to an alteration in the *PAX3* gene or protein which affects the activation of its downstream targets. Such changes could include variation in PAX3 isoform profiles leading to activation or inhibition of different downstream target genes. Moreover, isoforms may have altered post-translational modifications, again altering binding to downstream target genes, potentially resulting in abnormal pathway activation in the cell.

2.9 Post-translational modifications of PAX3

Not only can genes be regulated by alternative splicing, but the proteins produced can also be modified and controlled through post-translational modifications. Three protein modifications that commonly occur in cells to regulate protein function are phosphorylation, acetylation and ubiquitination. These modifications are formed by the attachment of an organic group to specific amino acid residues, altering the three dimensional structure and thus the function of the protein.

Phosphorylation of the PAX3 protein, as shown in myogenic cells, results in attachment of a phosphate moiety to serine residues 201, 205 or 209 (Dietz et al., 2011; Miller et al., 2008).

Research conducted by Miller and Hollenbach (2007) on mouse primary myoblasts showed that in the first 24 hours of embryonic muscle cell differentiation the levels of Pax3 protein decreased, whereas the levels of *Pax3* mRNA did not. Labelling with [^{32}P]-orthophosphate demonstrated that the Pax3 protein was phosphorylated *in vivo* at serine residues 201, 205 and 209 (Miller et al., 2008). Moreover, the changing pattern of phosphorylation of the protein at these sites, resulted in the switching of proliferating myoblasts to differentiating myoblasts (Dietz et al., 2011).

Ichi et al. (2011) showed that the PAX3 protein can be acetylated at lysine (K) residues 437 and 475, and this dramatically affects neural development. Acetylated PAX3 upregulates *Neurog2* expression, resulting in differentiation of neuroblasts into neural cells. Moreover, acetylated PAX3 downregulates *Hes1,* thus inhibiting proliferation of these cells (Figure 11). When PAX3 is deacetylated by SIRT1 (a deacetylase enzyme), there is an up-regulation of *Hes1* and down-regulation of *Neurog2*, resulting in proliferation of undifferentiated cells. This shows that acetylation of the PAX3 protein can alter the regulation of downstream target genes and ultimately cell fate, switching cells from undifferentiated to differentiated cells (Ichi et al., 2011).

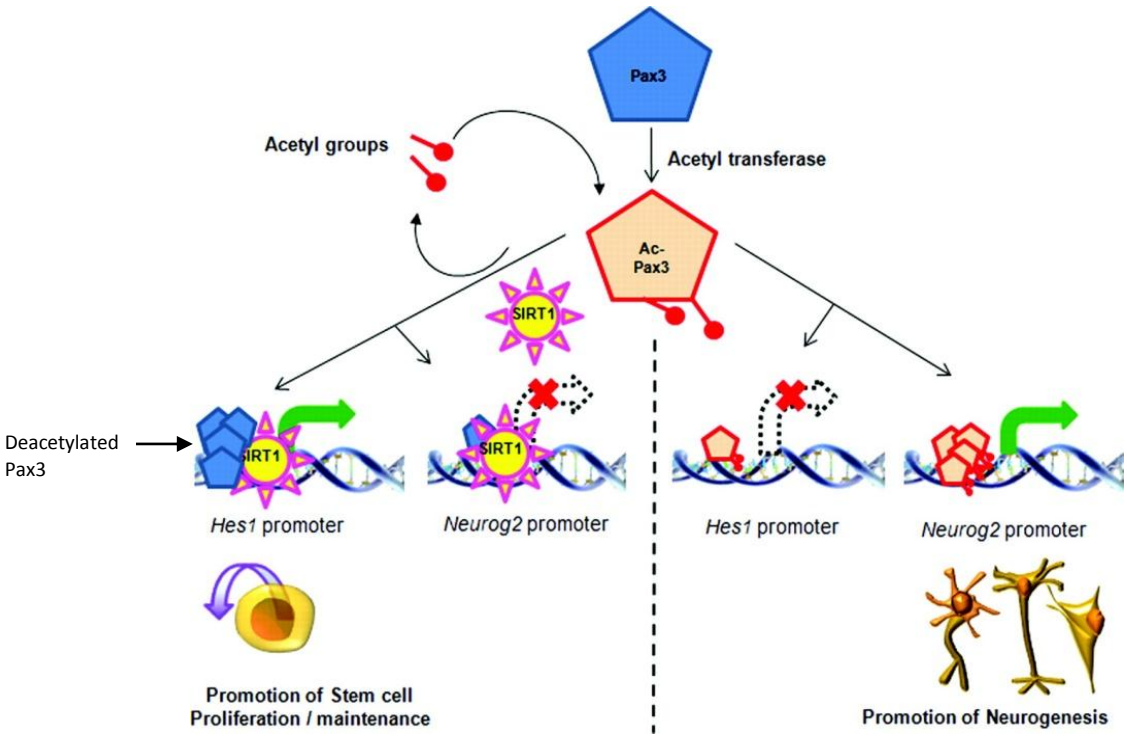

Figure 11: Acetylation of PAX3 promotes neurogenesis through the activation of *Neurog2*, while deacetylation of PAX3 by SIRT1 promotes stem cell proliferation and maintenance through the activation of *Hes1*. Adapted from Ichi et al. (2011).

Ubiquitination, the process by which a ubiquitin moiety is attached to a protein, causes it to have an altered function or be recognised by proteases and degraded. Ubiquitination at lysine residues involves attachment of a 76 amino acid ubiquitin molecule through the action of ubiquitin-activating enzyme (E1), cysteine enzyme (E2) and ubiquitin ligase (E3) (Figure 12) (Kaiser and Huang, 2005). Boutet et al. (2010; 2007) have shown that monoubiquitination occurs at the PAX3 protein during skeletal muscle development and this causes the PAX3 protein to be degraded by proteases. This degradation of PAX3 inhibits the protein from activating pathways that maintain undifferentiated myoblasts, thus allowing skeletal muscle cell differentiation.

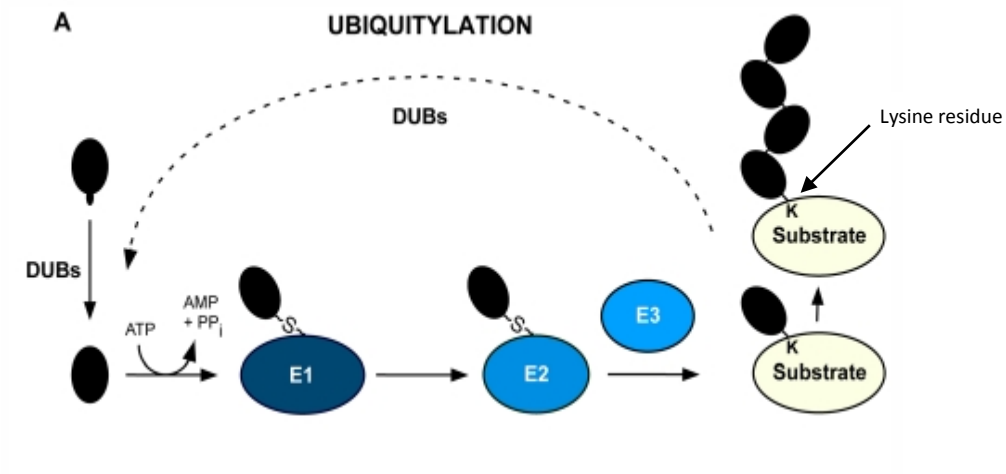

Figure 12: The process of ubiquitination: Enzymes attach ubiquitin moieties to proteins to mark them for degradation by proteases. Adapted from Boutet et al (2010; 2007).

Summary:

In this project we aimed to assess whether *PAX3*/PAX3 is differentially expressed and differentially modified post-translationally in human melanocyte cells and melanoma cell lines as this may affect its regulation of downstream target genes and possibly play a role in driving pathways that lead to transformation of cells.

3. Theoretical Framework

PAX3 is expressed in embryonic melanoblasts, melanoblasts in the hair follicle niche, adult melanocytes (epidermal and follicular) and in naevi and melanoma cells. It is responsible for regulating pathways involved in cell survival, differentiation, migration and proliferation. The exposure to UV radiation up-regulates the expression of *PAX3* in epidermal melanocytes. This upregulation of *PAX3* results in cells that can survive, proliferate and migrate. While these are characteristics of developing cells, they are also highly applicable to cancer cells. PAX3 has been shown to differentially regulate downstream target genes in melanoma cells compared to melanocytes. This gives us reason to believe that PAX3 is involved in pathways that lead to melanoma progression and therefore further investigation is required to examine whether any alterations in the *PAX3* gene, its transcript expression profile or protein modifications lead to alternate roles in melanoma cells compared to melanocytes, via differential regulation of downstream target genes.

The exact role of PAX3 in adult melanocytes and melanoma cells is not clear. It has been confirmed that PAX3 has an anti-apoptotic function in melanoma cells and it maintains an undifferentiated state in melanoblasts, but these might not be the only roles it plays in epidermal melanocytes and melanoma cells. The purpose of this investigation is to determine whether there are any differences between melanocytes and melanoma cells in the *PAX3* gene, its splice variants, its encoded protein isoforms or its downstream targets. Differences in *PAX3* transcripts or proteins between melanocytes and melanoma cells could be harnessed to provide strategies for therapeutic intervention.

4. Hypotheses

Previous research has shown PAX3 to differentially regulate downstream target genes in melanocytes and metastatic melanoma cells. We hypothesised that the differential regulation of downstream targets could be due to differential expression of *PAX3* alternate transcripts, or differences in post-translational modifications of the PAX3 protein between melanocytes and melanoma cells. Therefore, *PAX3* alternate transcript expression profiles and the presence of post-translational modifications, particularly acetylation, phosphorylation and ubiquitination, were investigated in normal human epidermal melanocytes and primary and metastatic melanoma cells. Furthermore, using *PAX3* silencing experiments, differential regulation of downstream target genes was confirmed in primary melanoma cells compared to normal melanocytes and metastatic melanoma cells.

5. Aims

The aims of this research were:

1. To confirm *PAX3* expression and compare levels of mRNA and protein between neonatal and adult melanocytes and primary and metastatic melanoma cells.
2. To compare *PAX3* transcript expression profiles between neonatal and adult melanocytes and primary and metastatic melanoma cells.
3. To compare post-translational modifications of PAX3 including phosphorylation, acetylation and ubiquitination, between neonatal and adult melanocytes and primary and metastatic melanoma cells.
4. To confirm that previously identified downstream target genes are differentially regulated in neonatal and adult melanocytes relative to melanoma cells lines after *PAX3* silencing *in vitro.*

6. Methods and Materials

6.1 Summary

PAX3 expression was analysed in four groups of cell types - neonatal and adult normal human epidermal melanocytes and primary and metastatic melanoma cell lines. Six primary and five metastatic melanoma cell lines were analysed for *PAX3* expression to confirm a similar expression pattern within groups of cell lines. Thereafter, one cell line from each of the primary and metastatic groups, in addition to neonatal and adult melanocytes, was used for further analysis throughout the remainder of this research. Cells were stained using immunocytochemical methods to confirm PAX3 expression. Furthermore, total RNA and total nuclear proteins were extracted from the cells and levels of *PAX3*/PAX3 expression were analysed by real time RT-qPCR and western blot, respectively. To assess *PAX3* alternate transcript expression profiles in these four cell types, RNAseq was used. To test for post-translational modifications of the PAX3 isoforms in the four cell types, immunoprecipitation and western blot were performed. *PAX3* silencing was used to confirm that PAX3 does in fact differentially regulate downstream targets in the four cell types. The outcomes of these experiments confirm a differential role of PAX3 in melanocytes and melanoma cells.

Ethics was granted by the Human Research Ethics Committee at Edith Cowan University (project number 8375).

6.2 Cell cultures

Human melanoma and melanocyte cell cultures were maintained as a monolayer at 37˚C, in 5% CO_2. Metastatic melanoma cell lines (A2058, M14, SKMEL2, SKMEL5 and UACC62) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) (Sigma Aldrich), L-glutamine (300µg/ml), penicillin (400 U/ml) and

streptomycin (50µg/ml). Primary melanoma cell lines (MM200, MM229, MM329, MM540 and MM622) were maintained in RPMI-1640 media supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin), whereas WM115 primary cell line was cultured in Eagle's minimum essentials media (EMEM) supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin). One representative cell line from the group of primary and metastatic cell lines was chosen for further investigation. Adult human epidermal melanocyte primary culture (NHEM-a (P), PromoCell) was maintained in Melanocyte Growth Media (PromoCell) and adult melanocyte primary culture (NHEM-a (I), Invitrogen) was maintained in 254 media (Invitrogen). Neonatal human epidermal melanocyte primary culture (NHEM-n, Promocell) was maintained in Melanocyte Growth Media (PromoCell).

The media was replaced every 48 hours until cells became 80-90% confluent. Cells at confluency were detached with trypsin/EDTA (Invitrogen). An equal volume of Trypsin Neutralising Solution (Invitrogen) was added to melanocyte cultures. Trypsin/EDTA used in melanoma cell line cultures was neutralised using respective media supplemented with FBS. The cells were then transferred to a falcon tube and centrifuged at 180xg for 7 minutes (for melanocytes) or 300xg for 5 minutes (for melanoma cells). The supernatant was discarded, new medium was added and cells were resuspended before being replated. Seeding density for melanocytes was 8.6×10^3 cells/cm^2, and for melanoma cells was 2.8×10^3 cells/cm^2.

6.3 RNA extraction

Total RNA was extracted from the cultured cells using the Isolate RNA mini Kit (Bioline) according to the manufacturer's instructions. Briefly, cells grown to 80% confluency were washed with PBS and detached from the flask with trypsin/EDTA. Up to 5×10^6 cells were transferred into a fresh 10ml centrifuge tube and pelleted by centrifugation at 300xg for 5 minutes (melanoma cells) or 180xg for 7 minutes (melanocytes). 450µl Lysis Solution was added to cells and mixed thoroughly. Homogenised lysate was placed into a shredding column which was centrifuged for 2 minutes at 12000rpm. Thereafter, 450µl of 70% ethanol was added to the flowthrough and the solution was transferred to a spin column and centrifuged at 12000rpm for 2 minutes. Following two washing steps, the RNA was eluted from the column in 40µl of RNase-free water and stored at -80°C until analysed further.

The integrity of the RNA was assessed using a Bioanalyser (Agilent 2100). An aliquot of filtered gel was prepared as per the manufacturer's instructions. Using the chip priming station, the gel was loaded onto the chip. 1µL of dye was added to the 12 sample wells and the ladder well. 1µL of each RNA sample and the ladder were loaded into their respective wells and the chip was vortexed for 1 minute. The chip was then subject to electrophoresis and the fluorescence assessed, from which the concentration of RNA was provided in ng/µL, as was the RNA integrity number (RIN) out of 10, showing the quality of the RNA. Only RNA with a RIN value above 7 was used.

6.4 Reverse transcription and qPCR

To determine the level of *PAX3* mRNA and its downstream targets in all cell lines, RNA was isolated and reverse transcribed from each cell line and subjected to real time qPCR using an iQ5 cycler (Bio-Rad). To do this, total RNA was reverse transcribed using the Omniscript RT kit (Qiagen), according to the instructions. Briefly, 500ng of total RNA (or 200ng for silencing experiments) was reverse transcribed in a 20µl reaction mixture containing: 1x RT Buffer, 0.5mM dNTP, 1µM Oligo-dT primer (Qiagen), 0.5 units/µL of RNase OUT Recombinant Ribonuclease Inhibitor, RNase-free water and RNA sample. The reaction was incubated for 1 hour at 37°C, followed by 5 minutes at 95°C and finally cooled to 4°C.

qPCR

1µl of template cDNA, 0.2µM primers and KAPA SYBR FAST qPCR Master Mix (KapaBiosystems) was used in a final reaction volume of 15µl. Primers used were as follows:

Table 1: Primers used in RT-qPCR analysis (Medic et al., 2011).

Gene	*Forward primer*	*Reverse primer*
GAPDH	5'-TTCTTTTGCGTCGCCAGCCGAG-3'	5'-GTGACCAGGCGCCCAATACGA-3'
PAX3	5'-AGCCGCATCCTGAGAAGTAA-3'	5'-CTTCATCTGATTGGGGTGCT-3'
MITF	5'-CGTCTCTCACTGGATTGGTG-3'	5'-CCGTTGGGCTTGCTGTATGT-3'
DCT	5'-CGACTCTGATTAGTCGGAACTCA-3'	5'-GGTGGTTGTAGTCATCCAAGC-3'
HES1	5'-AGAAAGATAGCTCGCGGCATTCCA-3'	5'-TTCCCCAGCACACTTGGGTCT-3'
SOX9	5'-AGCAAGACGCTGGGCAAGCTCTGG-3'	5'-CCCGTTCTTCACCGACTTCCTCCG-3'
NES	5'-GCGCACCTCAAGATGTCCCTCA-3'	5'-GGTGTTTGCAGCCGGGAGTTC-3'
CCNA2	5'-AGAGGCCGAAGACGAGACGG-3'	5'-TGAATGGTGAACGCAGGCTGTT-3'
TPD52	5'-ACATGGACCGCGGCGAGCAA-3'	5'-GTGGCACTGATCGTGGCAGCAAC-3'
BCL2L1	5'-CAGGTATTGGTGAGTCGGATCGC-3'	5'-GGCTCTCGGCTGCTGCATTGTT-3'
PTEN	5'-GCTGGAAAGGGACGAACTGGTGTA-3'	5'-CACATAGCGCCTCTGACTGGGAA-3'
TGFβ1	5'-GTCACCGGAGTTGTGCGGCA-3'	5'-GCAGTGGGCGCTAAGGCGAA-3'
MCAM	5'-GGGTACCCCATTCCTCAAGT-3'	5'-CTGGGACGACTGAATGTGG-3'
CSPG4	5'-GCCTTCACTGTCACTGTCCTGCCT-3'	5'-TCCTCAGACCCAGAGTCGCCGT-3'
CXCR4	5'-GCCTTCACTGTCACTGTCCTGCCT-3'	5'-AGTCCCCTGAGCCCATTTCCTCG-3'

Binding sites of the *PAX3* forward and reverse primers, as well as the expected length of the product, for each of the *PAX3* transcripts are detailed in Figure 13.

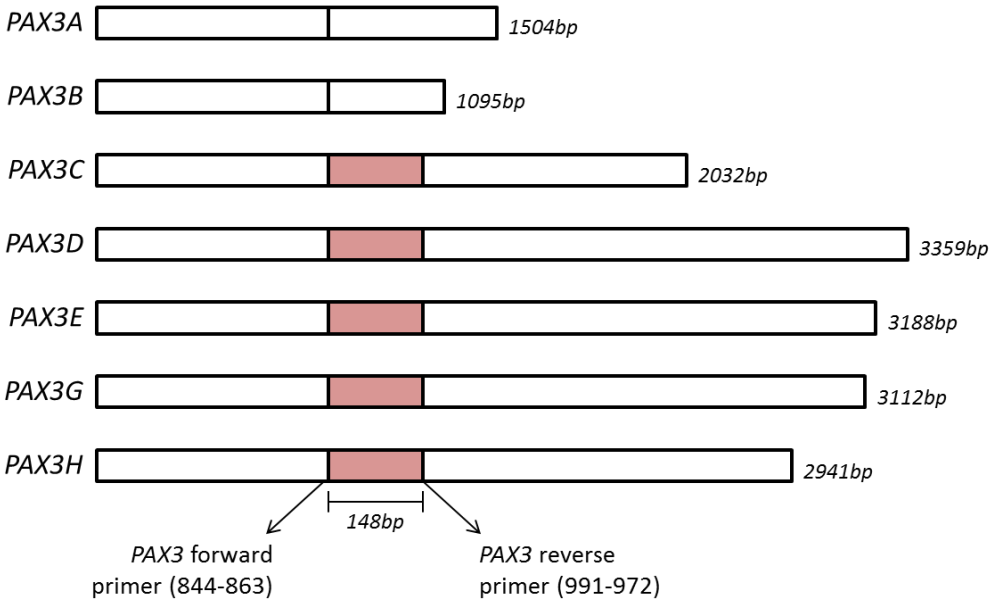

Figure 13: Binding sites for the *PAX3* forward and reverse primers on each of the PAX3 transcripts. The expected product size was 148bp. The reverse *PAX3* primer does not align to PAX3A or PAX3B.

Amplification was performed as follows: Briefly, samples were amplified with a pre-cycling hold at 95˚C for 3 seconds, followed by 40 cycles of a 2-step protocol (denaturation at 95˚C for 3 seconds, followed by annealing at 60˚C for 30 seconds). Data acquisition was performed at the annealing step of each cycle. Melt curve analysis to determine reaction specificity was included in the protocol with the following additional steps; 1 minute at 95°C, 1 minute at 55°C followed by 81 cycles with the temperature increasing from 55 by 0.5°C per cycle and data collected every 30 seconds. Products were initially analysed by 2% agarose-gel electrophoresis and ethidium bromide staining to confirm the production of a single amplicon of the correct size.

Each sample reaction was performed in triplicate, as biological replicates and included reagent controls (reagents without RNA or cDNA) and each Ct value was generated from a standard curve that was produced initially to confirm that each reaction had the same

efficiency. Reaction efficiency of between 90 and 100% and a correlation coefficient of over 0.99 was considered appropriate for each reaction. The threshold cycle value of the sample was determined from the standard curve. The mean Ct value of the triplicate for each sample reaction was used to calculate fold change over *GAPDH* (ΔCt).

Statistical analysis: Statistical analysis of changes in gene expression in the different cell lines was performed using a Kruskall-Wallis test. A Kruskall-Wallis test was performed as these data sets did not exhibit a normal distribution. The house-keeping gene, *GAPDH* was used to calculate the fold changes of expression levels. Each gene expression analysis was performed in triplicate and a mean was taken from these values. A Kruskall-Wallis test was used to compare the expression of each gene relative to that of *GAPDH*.

6.5 RNAseq

To assess *PAX3* alternate transcript expression profiles, next-generation sequencing for transcriptome analysis (or RNAseq) was used. Briefly, total RNA was extracted from all of the representative cell types as previously described (Section 6.3). Library construction and 100bp paired-end sequencing were performed by the Australian Genome Research Facility (AGRF) Services on Illumina HiSeq2000 systems using 1-10µg of total RNA per sample. Custom data alignment to the known *PAX3* sequences was performed and analysed for alternative transcript identification.

The Illumina HiSeq2000 method (Figure 14) reads up to 100 base pairs per read. Using the paired-end method enables the mRNA strand to be read from both directions, with 100 base pair reads from each end giving a reading of 200 base pairs from each strand resulting in the formation of short fragments of sequenced material. The short read fragments were then compiled together to form a library. These sequences were then compared to reference sequences for *PAX3* transcripts. Any alternative splicing was identified by analysing the junction reads of the sequences. RNAseq also allows the expression of each transcript to be quantified by calculating the amount of junction reads that correspond to exons of the reference sequence. Thus a data set of *PAX3* expression profiles for each alternate transcript, for each cell type, was constructed.

The sequence reads generated were cleaned and aligned against the human genome using the Tophat aligner. Raw gene counts that were mapped to known gene sequences in the human genome were used to calculate differential gene expression in the samples. The Cufflinks tool was used to assemble the transcripts and to check for novel transcript expression. Cuffmerge and cuffdiff were then used to first merge the compared samples and then analyse differential expression. Expression levels were then calculated as number of reads per kilobase of exon model per million mapped fragments.

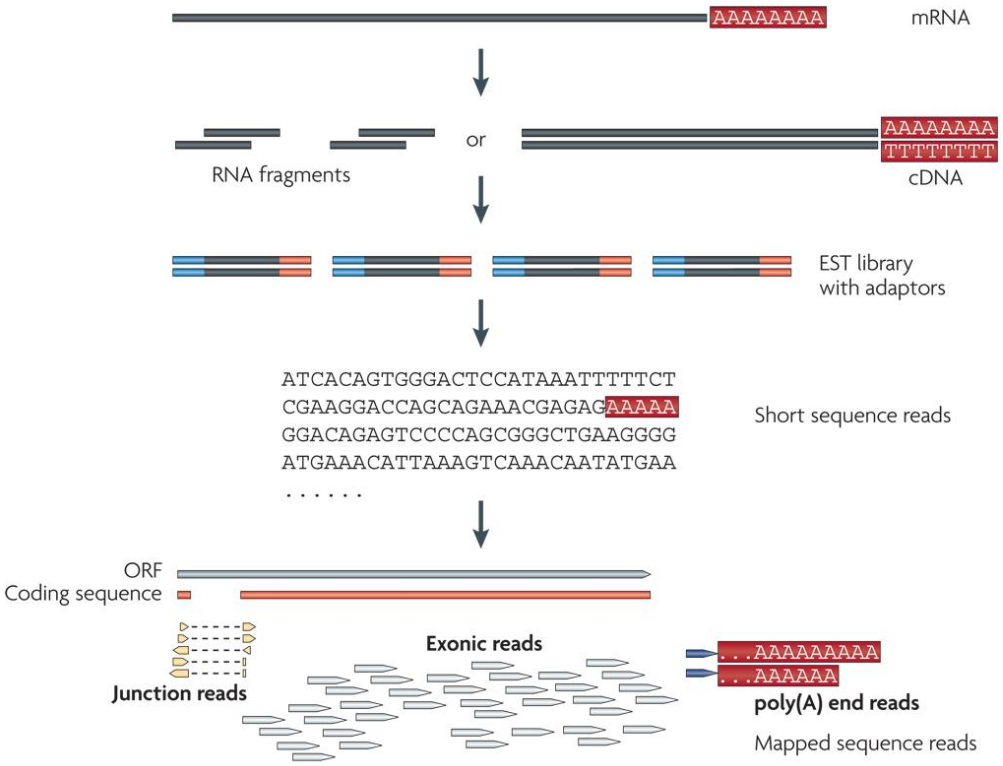

Figure 14: Illumina HiSeq 2000 protocol. Adapted from Gerstein et al. (2009).

6.6 Immunocytochemistry

First, to confirm PAX3 protein expression in the cell lines, immunocytochemistry was performed as previously described (Medic and Ziman, 2010, 2011). Briefly, $5x10^4$ cells per cover slip were grown for 24 hours. Cells were fixed in 4% paraformaldehyde and permeabilised for 15 minutes at room temperature with PBS containing 0.2% Triton-X100

(0.2% PBST), then blocked with 10% normal goat serum (NGS) in 0.2% PBST for 1 hour. Cells were stained overnight at 4˚C with mouse monoclonal PAX3 antibody (DSHB) at 1:50 dilution. Excess primary antibody was washed off by rinsing in PBS three times for 5 minutes. This was followed by incubation with anti-mouse IgG conjugated with Dylight-550 for 1 hour. All antibodies were diluted in 0.2% PBST containing 1% NGS. Cover slips were mounted onto microscope slides with Prolong Gold Antifade Reagent with DAPI (Invitrogen) in order to stain cell nuclei. Slides were viewed with an Olympus BX41 epi-fluorescent microscope. Controls with primary antibodies withheld were also included to ensure non-specific staining was not evident.

6.7 Western blot

To assess the level of expression of PAX3 proteins in the melanoma cell lines and primary melanocytes, western blot was performed as previously described (Medic et al., 2011). To extract total cellular proteins from cultured melanoma and melanocyte cells, cells were washed twice with ice-cold PBS and then scraped from the base of the flask into a solution of PBS containing protease inhibitor cocktail (PIC, 1x, Roche). Cells were transferred into individual falcon tubes and centrifuged at 300xg for 5 minutes. Cells were resuspended in 300µl cell lysis buffer (50mM pipes, 85mM KCl, 1% Nonidet P-40, 1x PIC, pH 8.0), briefly vortexed and incubated on ice for 30 minutes. Cell debris was centrifuged at 10000rpm for 5 minutes at 4˚C and the supernatant (containing cytosolic proteins) was collected and stored at -80˚C to later analyse by SDS-PAGE alongside the total nuclear protein in order to confirm the protein extraction procedure was effective in isolating the nuclear proteins of the cells. The cell pellet was then resuspended in 150µL of nuclear lysis buffer (50mM Tris, 10mM EDTA, 1% SDS, 1x PIC, pH 8.0), vortexed and incubated on ice for 30 minutes. The samples were then passed through a 27 gauge needle to aid lysis. The samples were centrifuged at 12000rpm for 10 minutes at 4˚C. The supernatant (containing nuclear proteins) was transferred to a new tube and stored at -80˚C for further analysis. The pellet (containing DNA) was discarded.

The concentrations of extracted proteins were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce) and absorbance was read using the Fluostar Optima plate reader.

Cell lysates were diluted to a standard of 20µg (10µg for silencing experiments) with TBS. Sample reducing agent (4x) and sample buffer (10x) were added to each sample, vortexed and heated at 70˚C for 10 minutes. The electrophoresis tank was filled with 1x NuPAGE MES SDS running buffer. Protein samples were loaded onto a NuPAGE Bis-Tris precast 10 well gel (Life Technologies) alongside 7µL Novex Sharp pre-stained protein standard (Life Technologies) and the gel was run at constant 400A for 2 hours.

Proteins were transferred from the gel onto nitrocellulose membrane (Biorad) as follows: A transfer sandwich was assembled under transfer buffer (0.02M Tris, 0.08M Glycine, 0.001M SDS, 20% methanol). The sandwich was placed into the plastic transfer cassette and then into the transfer tank. The internal and external sections were filled with transfer buffer and run at a constant 0.25A overnight at 4°C. After disassembly, the nitrocellulose membrane was cut for orientation of protein samples and the ladder marked on the membrane.

The Westernbreeze chemiluminescent detection kit (Life Technologies) was used for immunodetection of the proteins by western blot as per the instructions. The nitrocellulose membrane was immersed in blocking buffer (Life Technologies) for 30 minutes. The anti-PAX3 antibody (mouse monoclonal, DSHB) at working concentration 1:1000 (diluted in blocking buffer) was added to the nitrocellulose membrane and incubated overnight at 4˚C. The membrane was washed 4 times at room temperature using the wash buffer provided in the kit for 5 minutes on a rocking platform. Secondary IgG antibody linked to alkaline phosphatase, at a working concentration 1:2000, was applied to the nitrocellulose membrane and incubated for 30 minutes at room temperature on a rocking platform. The membrane was then washed 4 times, as previously described.

For the immunoprecipitation experiments proteins on western blots were analysed using the TrueBlot western blot kit (Rockland) as per the instructions. Membranes were washed with 0.05% TBS-Tween 20 and then blocked using the TrueBlot Blocker provided in the kit for 2 hours at room temperature. The membranes were washed again with TBS-Tween 20 and incubated with primary antibody in blocking buffer overnight at 4˚C. Primary antibodies

included anti-human PAX3 (rabbit polyclonal, 1:400, Abcam), anti-acetyl-lysine (rabbit polyclonal, 1:500, Abcam), anti-phospho-serine (rabbit polyclonal, 1:500, Abcam) and anti-ubiquitin (rabbit polyclonal, 1:500, Abcam). Membranes were then washed with TBS-Tween 20 and incubated with TrueBlot anti-rabbit IgG secondary antibody (1:1000, Rockland). Blots were then washed with TBS-Tween 20.

Probed membranes were visualised by chemiluminescence using the substrate provided in each kit (Westernbreeze and Trueblot). The substrate was mixed according to the kit instructions and then added to the nitrocellulose membrane and incubated for 5 minutes at room temperature. The nitrocellulose membrane was sandwiched between transparent overhead sheets and placed into a cassette. In the dark room, x-ray film (Kodak) was placed onto the nitrocellulose membrane and exposed for various times (30 seconds, 1 minute, 3 minutes, 5 minutes, 10 minutes and overnight). After exposure, the medical x-ray film was placed in developer solution for 2 minutes and agitated, washed with water and then placed in fixer solution for 2 minutes, washed (water) and air dried.

Membranes were then stripped using Restore Western Blot Stripping Buffer (Thermo Scientific) for 15 minutes and re-probed for β-actin by incubating with anti-actin antibody (rabbit polyclonal, Abcam, 1:1000) overnight at 4˚C. The western blot was performed using the anti-rabbit Westernbreeze kit (Invitrogen) and viewed by chemiluminescence as previously described.

The levels of expression of each protein were assessed by densitometry using the GS-800 Calibrated Densitometer (Biorad), which compares the density of the individual protein bands in each lane to that of β-actin in the same lane. Each sample was run as a biological replicate.

6.8 Post-translational modifications of PAX3 proteins (phosphorylation, acetylation, and ubiquitination)

Immunoprecipitation and western blotting were performed to assess the phosphorylation, acetylation and ubiquitination status of PAX3 in all cell types, as previously described (Ichi et

al., 2011). Briefly, cultured cells were lysed and nuclear proteins extracted as described above in section 6.7. PAX3 proteins were immunoprecipitated with mouse monoclonal anti-PAX3 antibody (DSHB, 10µg/ml), which recognises the C-terminus region of the protein, or rabbit polyclonal (Abcam, 2.5µg/ml) or goat polyclonal (Abcam, 2.5µg/ml) antibody, which both recognise the common N-terminus of each of the PAX3 isoforms. The antibody/antigen complex was immunoprecipitated using protein A (for mouse and rabbit antibodies) or G (for goat antibody) magnetic Dynabeads (Life Technologies) or anti-IgG (for rabbit antibody) agarose beads (Rockland). Both direct and indirect methods of immunoprecipitation were performed to determine the most optimal procedure. The direct method involved incubating the immunoprecipitating antibody (mouse monoclonal anti-PAX3 (DSHB), rabbit polyclonal anti-PAX3 (Abcam) or goat polyclonal anti-PAX3 (Abcam)) with the appropriate beads (protein A or G magnetic Dynabeads (Life Technologies), rabbit anti-IgG agarose beads (Rockland)) for 30 minutes at room temperature. For immunoprecipitation using protein A or G magnetic beads, the antibodies were cross-linked to the beads using BS^3 cross-linking agent (Thermo Scientific) for 15 minutes at room temperature before incubating the bead/antibody complex with the protein sample. The indirect method, using the same antibodies and beads, included incubating the antibody with the protein first, then incubating the antibody/protein complex with the beads.

Beads were washed in 250µL 1xPBS and centrifuged at 2500xg for 2 minutes (centrifugation was for agarose beads only). Proteins were eluted from the bead/antibody complex by heating samples at 70˚C for 10 minutes with 20µL glycine elution buffer (glycine buffer was used for magnetic beads only), sample reducing agent (1x) and sample buffer (1x) and then the mixture was run on a NuPAGE Bis-Tris precast gel. The following antibodies were used for western blotting: anti-PAX3 (mouse monoclonal, DSHB, 1:1000), anti-phosphoserine (rabbit polyclonal, Abcam, 2.5µg/ml), anti-acetyl-lysine (rabbit polyclonal, Abcam, 2.5µg/ml) and anti-ubiquitin (rabbit polyclonal, Abcam, 2.5µg/ml). Similarly, immunoprecipitation was performed with the anti-phosphoserine, anti-acetyl-lysine and anti-ubiquitin antibodies and anti-PAX3 (mouse monoclonal, DSHB, 1:1000) was used to probe the membrane (or precipitated proteins). Table 2 outlines important information regarding the binding sites and suppliers of the antibodies used throughout the western blot and immunoprecipitation procedures.

Table 2: The amino acid epitopes and catalogue numbers for the antibodies used in western blot and immunoprecipitation procedures throughout this project.

Antibody	Supplier	Catalogue Number	Binding site/s	PAX3 transcripts recognised by antibody
Anti-human PAX3 (mouse), monoclonal	DSHB	N/A	Amino acids 298-481, C-terminal region	PAX3C-PAX3E
Anti-human PAX3 (rabbit), polyclonal	Abcam	ab50193	Amino acids 181-230, N-terminal region	PAX3C-PAX3H
Anti-human PAX3 (goat), polyclonal	Abcam	ab15717	Amino acids 2-12, N-terminal region	PAX3A-PAX3H
Anti-acetyl lysine (rabbit), polyclonal	Millipore	AB3879	Acetylated lysine residues on proteins	N/A
Anti-phosphoserine (rabbit), polyclonal	Millipore	AB1603	Phosphoserine, peptidylphosphoserine, serine-phosphorylated proteins	N/A
Anti-ubiquitin (rabbit), polyclonal	Millipore	07-375	Ubiquitin and ubiquitinated proteins	N/A

6.9 *PAX3* silencing and analysis of PAX3 downstream targets in melanoma cell lines and primary melanocytes

To assess whether PAX3 does indeed regulate the selected downstream target genes, *PAX3* was silenced using siRNA and RT-qPCR was performed using the primers provided in Table 1. The genes studied (Table 1) were chosen based on data showing them all to be regulated by PAX3, as well as being expressed in melanocytes and melanoma cells.

Knock-down of all *PAX3* transcripts was accomplished using pre-designed *PAX3*-specific Silencer Select siRNA (s10059 and s224172, Ambion), and Lipofectamine 2000 Transfection Reagent (Life Technologies) following the manufacturer's recommendations. Briefly, $1x10^5$ (for melanoma cells) or $2x10^5$ (for melanocytes) cells were seeded in a six-well plate 24 hours prior to transfection with 10nM of *PAX3*-specific siRNA (s10059 and s224172 individually, or in combination), or negative control siRNA (Negative Control #1 siRNA, Ambion) using RNAiMAX Transfection Reagent (Ambion). Each silencing experiment also included positive control, transfection with Silence Select GAPDH siRNA (Ambion), as well as non-treated cells. Efficient *PAX3* knock-down was confirmed after 24, 48 and 72 hours post-transfection by RT-qPCR and western blot as previously described (section 6.7). The changes in the expression of previously identified PAX3 targets, following its knock-down, were then analysed by RT-qPCR and quantified by calculating the fold change (ΔΔCt) of the gene, first relative to 18S (ΔCt) and then to the negative control siRNA (ΔΔCt). A threshold of greater than 70% knockdown of *GAPDH* and *PAX3* using siRNA was considered a valid knockdown (Hsieh et al., 2004; Krueger et al., 2007). Each silencing experiment was performed in duplicate using biological replicates.

7. Results

A panel of five metastatic and six primary melanoma cell lines, one neonatal and two adult melanocyte cultures were analysed for PAX3 expression. The levels of *PAX3* mRNA and PAX3 protein were assessed in each cell line using RT-qPCR, immunocytochemistry and western blot. One representative cell line from the primary and metastatic melanoma groups was chosen for further analysis of *PAX3* transcript expression profiles and for analysis of PAX3 post-translational modifications and downstream targets. To identify differences in *PAX3* expression between melanocytes and melanoma cells that would explain the previously observed differences in regulation of downstream target genes, RNAseq was used to compare expression profiles of *PAX3* transcripts, whereas immunoprecipitation was performed to test for differences in post-translational modifications of the PAX3 protein isoforms. Finally, *PAX3* silencing was used to confirm differences in the regulation of known downstream target genes between primary and metastatic melanoma cells and neonatal and adult melanocytes.

7.1 Clarifying consistency of *PAX3* expression within groups of cell lines

7.1.1 PAX3 expression analysed by immunocytochemistry

Figure 15 shows the expression of PAX3 in a panel of primary and metastatic melanoma cell lines and in neonatal and adult primary melanocyte cell cultures. Mouse anti-PAX3 (DSHB, 1:50) primary antibody followed by anti-mouse IgG-550 (Dylight, 1:500) secondary antibody were used to stain PAX3 protein. Nuclei were stained with DAPI to confirm nuclear localisation of PAX3 in all cell types.

Immunocytochemistry results show that PAX3 is expressed in all of the melanoma cell lines and both neonatal and adult melanocytes (Figure 15). Furthermore, 90-95% of cells were PAX3 positive in all cell types.

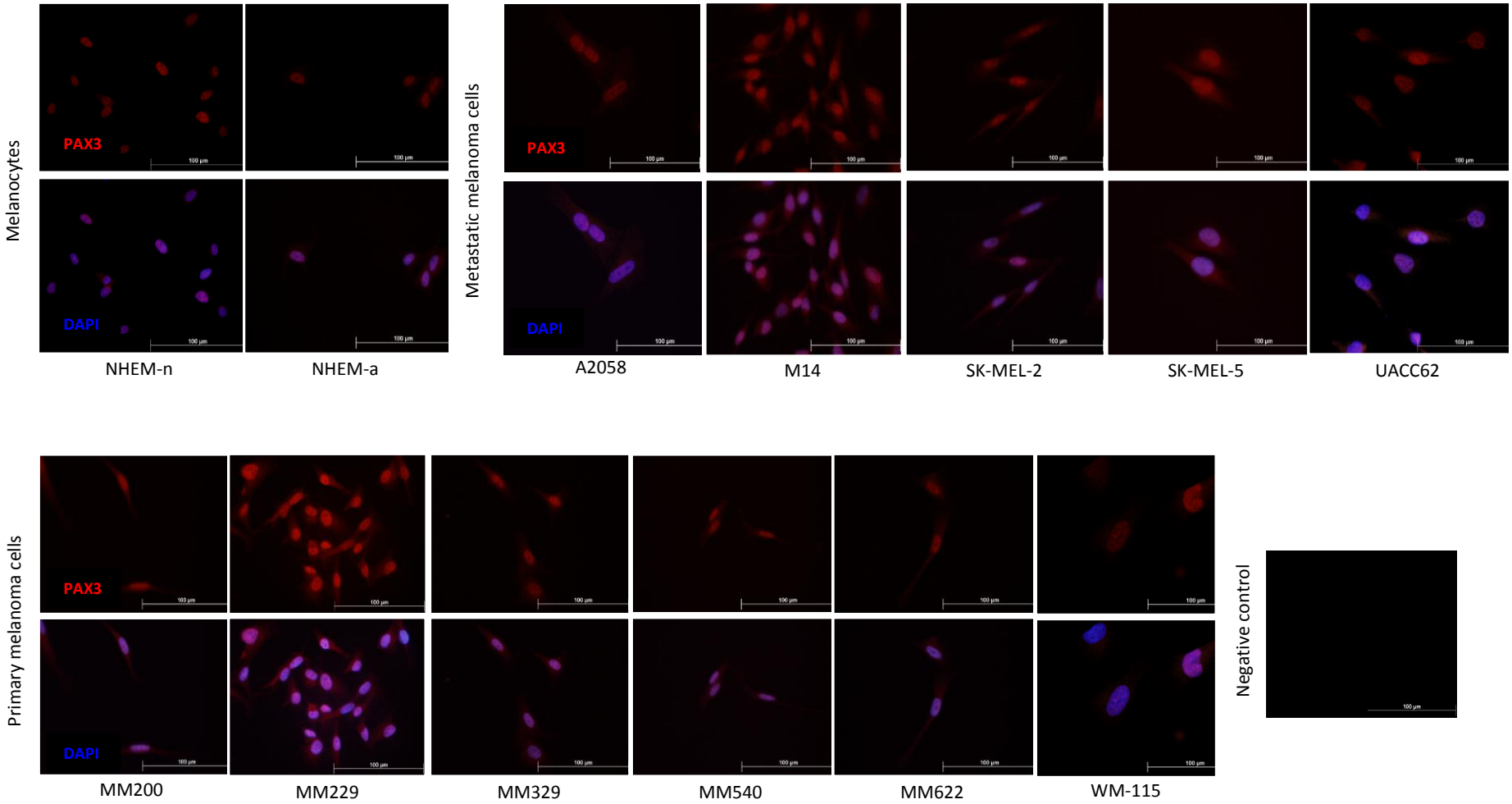

Figure 15: Immunocytochemistry of neonatal and adult human epidermal melanocytes, five metastatic melanoma cell lines and six primary melanoma cell lines. Nuclear PAX3 protein expression (red) is observed in all melanoma cell lines and primary melanocytes. Cell nuclei are stained with DAPI (blue). The scale measures 100µM. A negative control was included with each experimental run and proved negative in each instance.

7.1.2 *PAX3* expression analysed by Reverse-Transcriptase Polymerase Chain Reaction (RT-qPCR)

In order to quantify *PAX3* mRNA expression, RT-qPCR was performed in all primary and metastatic melanoma cell lines and neonatal and adult primary melanocytes (Figure 16).

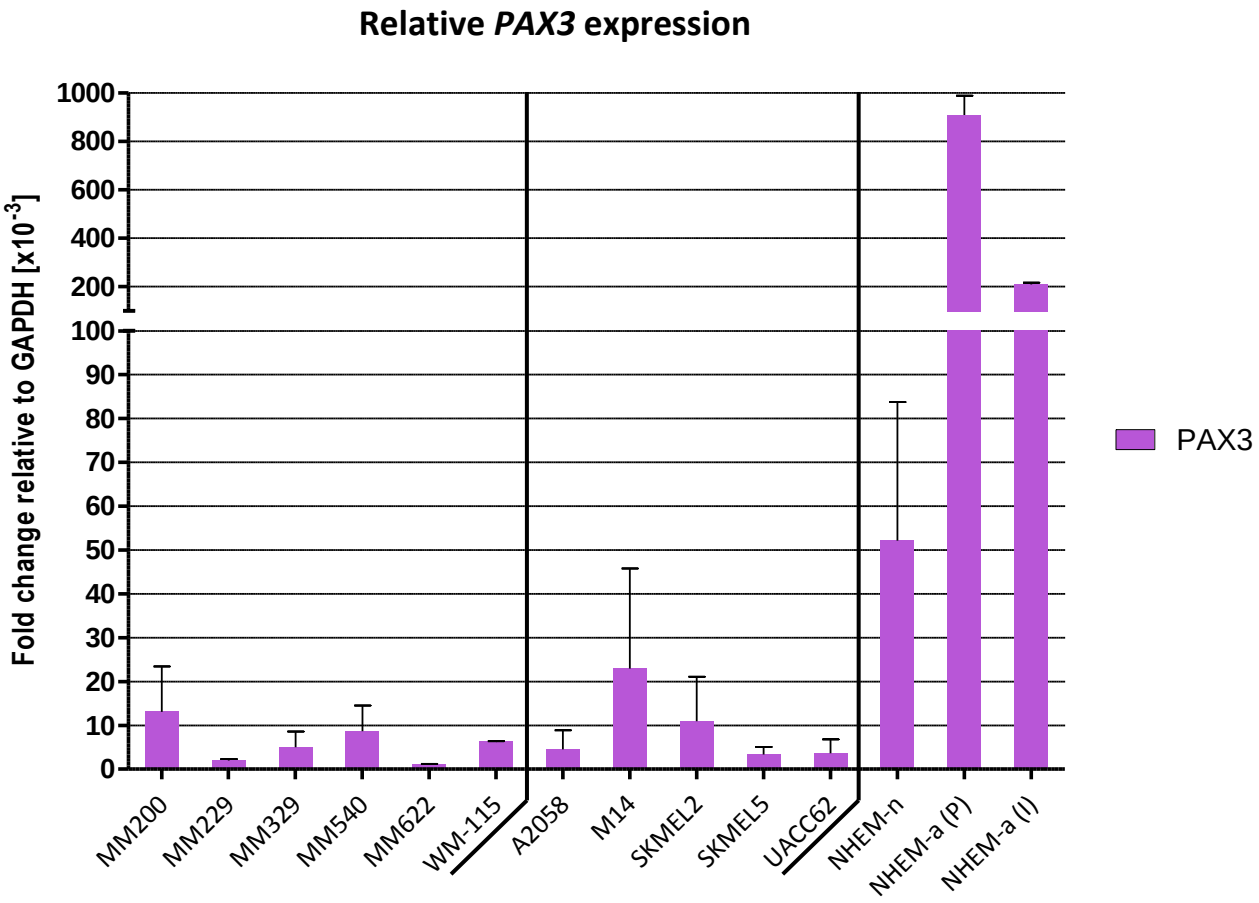

Figure 16: RT-qPCR was performed on six primary and five metastatic cell lines and neonatal and adult normal human epidermal melanocytes. The relative level of expression of *PAX3* compared to housekeeping gene *GAPDH* was calculated by the ΔΔCt method. All PCRs were performed in triplicate for each of two biological replicates and average values were used to calculate fold change over *GAPDH* for each sample. All samples were run in biological duplicates and the average fold change values are calculated and presented in the graph. The error bars represent the standard deviation of the biological replicates.

Figure 16 represents the relative expression of *PAX3* for each of the primary melanoma cell lines (MM200, MM229, MM329, MM540, MM622 and WM115), metastatic melanoma cell lines (A2058, M14, SKMEL2, SKMEL5 and UACC62) neonatal human epidermal melanocytes (NHEM-n) and adult human epidermal melanocytes (NHEM-a (P) and NHEM-a (I)). A Mann-Whitney U test was utilised to determine whether there was a significant difference in *PAX3* mRNA expression between cell lines of the same cell type. The primary cell line with a significantly lower *PAX3* expression than the others was MM622 (p= 0.007). Additionally, the M14 cell line exhibited a significantly higher expression of *PAX3* mRNA than the other metastatic melanoma cell lines (p= 0.007).

To statistically compare the relative expression levels per cell type, a test for normality was performed, but indicated that the data was not normally distributed, thus a non-parametric Kruskal-Wallis test was required for further statistical analysis. Upon comparison of relative *PAX3* expression between primary melanoma, metastatic melanoma and melanocyte groups using the Kruskal-Wallis test, a significant difference in *PAX3* mRNA levels was found between groups (p=0.035). In order to determine which of the groups were significantly different from the others, Mann-Whitney U tests were performed. Whereas the levels of *PAX3* mRNA in the primary melanoma group of cell lines were not significantly different from those in the metastatic group of cell lines (p=0.644), there was a significant difference in mRNA levels between primary melanoma cells and melanocytes (p=0.001). There was a significant difference in *PAX3* mRNA levels between the metastatic group of melanoma cell lines and the melanocytes (p=0.002) (Table 3). Furthermore, it was found that there was a significantly higher expression of *PAX3* in the adult melanocytes, both NHEM-a (P) and NHEM-a (I), compared to the neonatal melanocytes (p=0.006, p=0.004, Table 3).

Table 3: mRNA levels of *PAX3* in melanocytes, primary melanoma cells and metastatic melanoma cells

	Melanocytes			Primary melanoma						Metastatic melanoma				
Cell line/culture	NHEM-n	NHEM-a (P)	NHEM-a (I)	MM200	MM229	MM329	MM540	MM622	WM115	A2058	M14	SKMEL2	SKMEL5	UACC62
Mean fold change ($x10^{-3}$)	52.16	909.28	208.84	13.09	1.99	5.01	8.68	1.10	6.35	4.51	22.95	10.97	3.32	3.59
Std dev ($x10^{-3}$)	±22.33	±56.65	±5.31	±7.33	±0.20	±2.55	±4.14	±0.09	±0.05	±3.09	±16.15	±7.15	±1.22	±2.27
Between group p-values (Kruskal-Wallis test)	Melanocytes v Primary melanoma: p= 0.001* Melanocytes v Metastatic melanoma: p= 0.002*			Primary melanoma v Melanocytes: p= 0.001* Primary melanoma v Metastatic melanoma: p= 0.644						Metastatic melanoma v Melanocytes: p= 0.002* Metastatic melanoma v Primary melanoma: p= 0.644				

***Statistically significant, p ≤ 0.05**

7.1.3 Western blot

Western blot was used to determine the relative levels of PAX3 protein in all cell types. For each sample, a standard amount of protein (20µg total nuclear protein) was loaded onto the gel. The levels of PAX3 protein in each of the six primary melanoma cell lines, five metastatic melanoma cell lines, one primary neonatal melanocyte culture and two adult melanocyte primary cultures were assessed.

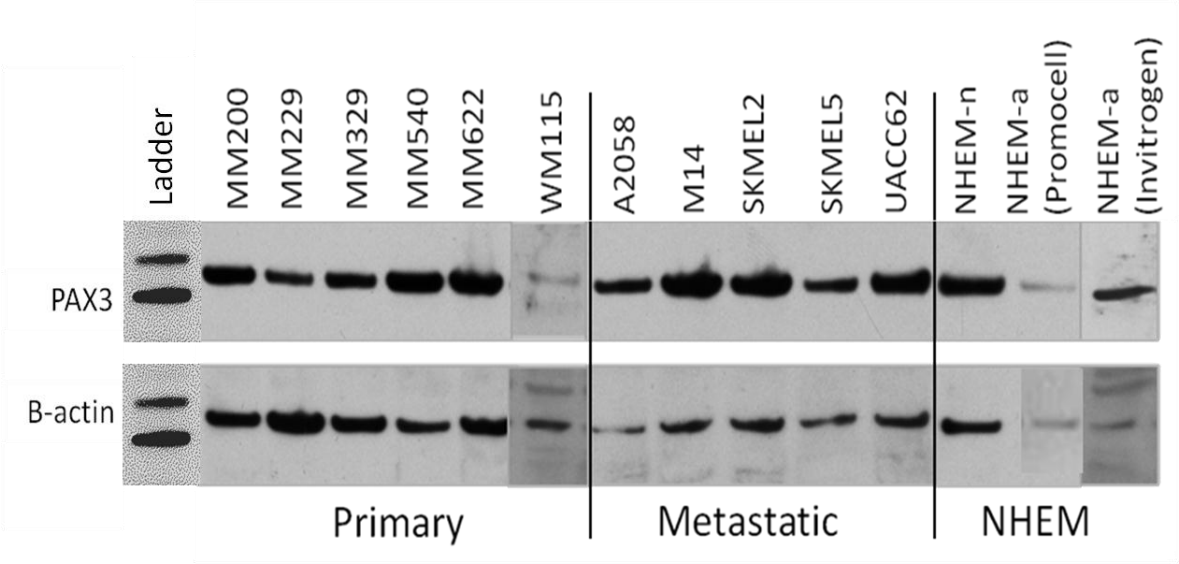

Figure 17: Western blot results. Each sample was run in duplicate on each of two biological replicates on separate gels and blotted against PAX3 (DSHB, 1:1000) and β-actin (Abcam, 1:1000). The representative western blots are shown here.

The western blot (Figure 17) revealed a single band when blotted with anti-PAX3 antibody (mouse monoclonal, DSHB) of around 55kDa. This corresponds to isoforms PAX3C, D and/or E which are 53kDa, 53kDa and 56kDa, respectively. Furthermore, primary melanoma cell line WM115 also showed a band around 44kDa, which could correspond to isoforms PAX3G or PAX3H. PAX3 protein levels were assessed by densitometry to quantitatively assess expression levels relative to that of β-actin and the average fold change above that of β-actin for each sample was graphed (Figure 18).

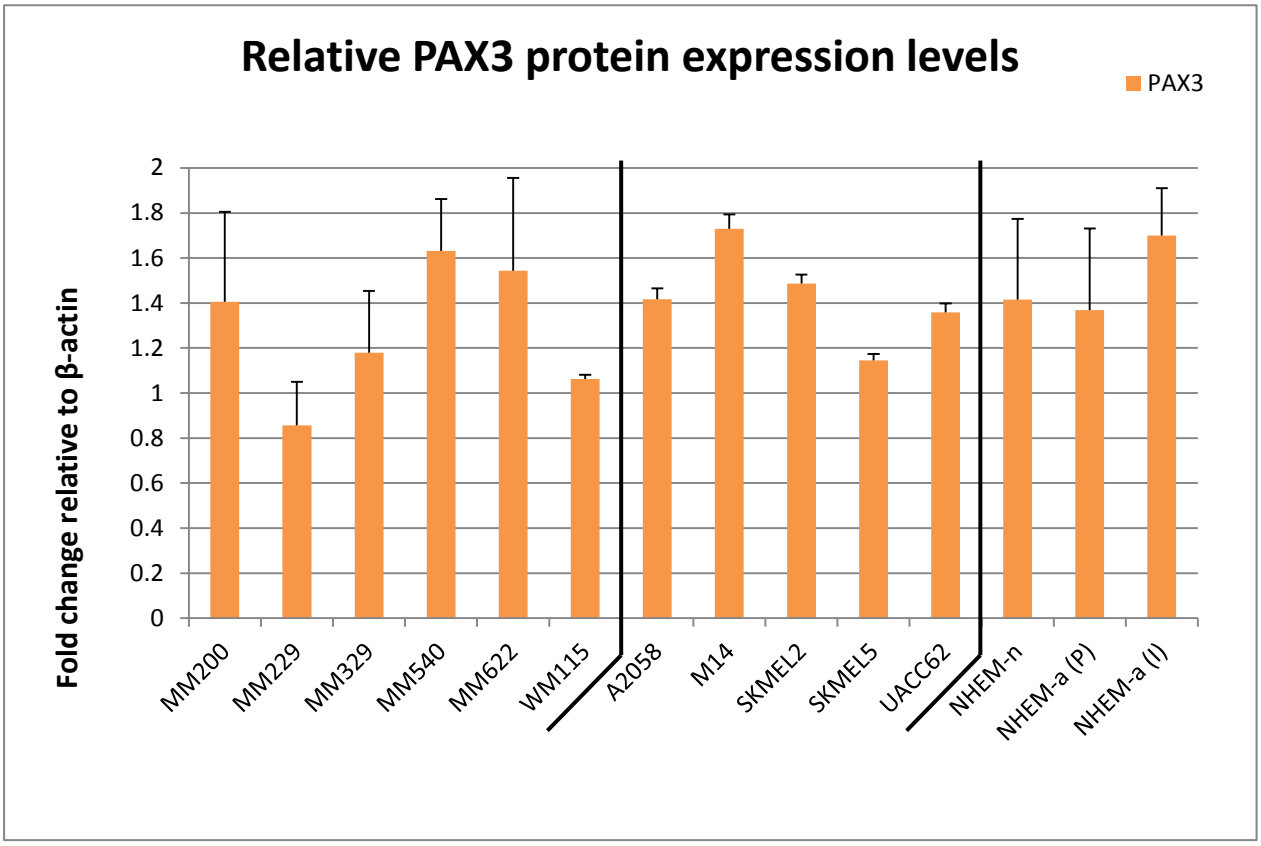

Figure 18: PAX3 protein expression relative to β-actin expression. Each sample was run in duplicate on separate gels and the density of each band was assessed by densitometric scanning using the GS-800 Calibrated Densitometer (Biorad). The density of each PAX3 band was compared to that of the housekeeping gene β-actin for each lane on the same membrane and the fold change calculated and graphed. The error bars represent the standard deviation between duplicate samples.

Densitometry results were normalised to the background and PAX3 protein expression was calculated relative to β-actin. The data were normally distributed and so a one-way ANOVA was used to compare relative PAX3 protein expression levels between groups. There was no significant difference in relative PAX3 protein expression between the primary melanoma cell lines, metastatic melanoma cell lines or melanocytes when the groups were compared using a one-way ANOVA test (p= 0.088, Table 4). Furthermore, a student's T-test was used to determine whether there was significant difference in PAX3 protein expression within each of the groups. There was no significant difference in relative PAX3 protein expression between the primary melanoma cell lines, however, within the metastatic melanoma cell line panel, PAX3 protein was significantly higher in M14 than in A2058, SKMEL5 and UACC62

(p= 0.037, p= 0.035 and p= 0.032, respectively). Comparison of PAX3 protein expression within the melanocyte cells showed no statistically significant difference. These results differ considerably to the results obtained from qPCR analysis of mRNA expression, where the *PAX3* mRNA expression in adult melanocytes was significantly higher than that found in the primary and metastatic melanoma cell lines and neonatal melanocytes.

Primary melanoma cell line MM540 and metastatic melanoma cell line M14 were chosen for further comparative experiments along with neonatal (NHEMn) and adult (NHEMa (I)) primary melanocyte cultures.

Table 4: Protein levels of PAX3 in neonatal and adult melanocytes and primary and metastatic melanoma cells

	Melanocytes			Primary melanoma						Metastatic melanoma				
Cell line/culture	NHEMn	NHEMa (P)	NHEMa (I)	MM200	MM229	MM329	MM540	MM622	WM115	A2058	M14	SKMEL2	SKMEL5	UACC62
Mean and standard deviation	1.415 ±0.254	1.369 ±0.257	1.700 ±0.149	1.405 ±0.283	0.857 ±0.136	1.179 ±0.195	1.632 ±0.162	1.543 ±0.292	1.063 ±0.013	1.417 ±0.034	1.730 ±0.046	1.487 ±0.028	1.145 ±0.020	1.359 ±0.028
Between group p-values (one-way ANOVA)	Melanocytes v Primary melanoma: p= 0.331 Melanocytes v Metastatic melanoma: p= 0.901			Primary melanoma v Melanocytes: p= 0.331 Primary melanoma v Metastatic melanoma: p= 0.482						Metastatic melanoma v Melanocytes: p= 0.901 Metastatic melanoma v Primary melanoma: p= 0.482				

7.2 RNAseq to assess differential expression of *PAX3* transcripts

One representative cell line from each of the melanoma cell groups, as well as one neonatal and adult melanocyte primary culture, were analysed by RNAseq for relative *PAX3* transcript expression profiles. Total messenger RNA from metastatic melanoma cell line M14, primary melanoma cell line MM540, neonatal normal human epidermal melanocytes (NHEMn) and adult normal human epidermal melanocytes (NHEMa, Promocell) was sequenced using paired end sequencing on the Illumina HiSeq2000 platform at the Australian Genome Research Facility.

Primary data analysis was conducted by the Australian Genome Research Facility. Primary sequence data was generated using the Illumina CASAVA 1.8.2 pipeline as a FASTQ format. Cleaned sequence reads were aligned against the human genome using the Tophat aligner and transcripts were assembled with the Cufflinks tool using the reference annotation based assembly (RABT) option. Differential expression of the *PAX3* transcripts was then analysed through the use of Cufflinks applications Cuffmerge and Cuffdiff, merging the two samples for comparison and assessing the differential expression of the transcripts. Each of the transcript expression levels were normalised to the highest expressed transcript level and the fold change for each transcript and significant difference of transcript expression between cell types was calculated.

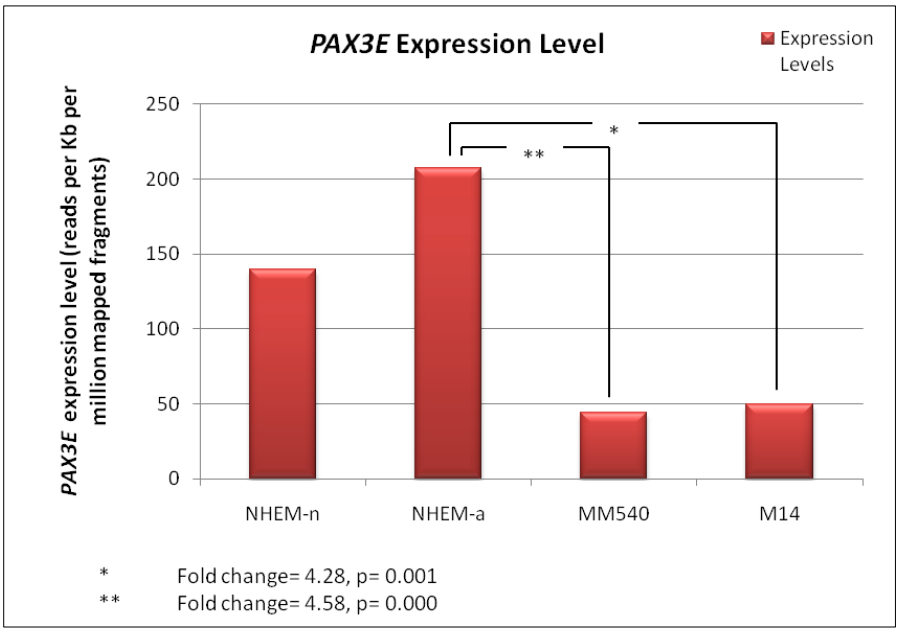

Figure 19: A comparison of *PAX3E* expression levels between neonatal and adult melanocytes and MM540 and M14 melanoma cell lines measured as number of reads per kilobase of exon model per million mapped fragments. Asterisk (*, **) denotes significant difference. No significant difference was found between neonatal melanocytes and the primary or metastatic melanoma cell lines.

Adult melanocytes were compared against neonatal, primary and metastatic melanomas. The only *PAX3* transcript shown to exhibit significant differential expression between cell types was *PAX3E*, which was significantly downregulated in primary and metastatic melanoma cells compared to adult melanocytes (Figure 19), with a significant fold change of 4.28 between metastatic melanoma cells (M14) and adult melanocytes (NHEMa) ($p<0.001$), and a significant fold change of 4.58 between primary melanoma cells (MM540) and adult melanocytes (NHEMa) ($p<0.001$). There was no statistically significant difference between *PAX3* transcript expression profiles between neonatal and adult melanocytes.

7.3 Testing for post-translational modifications

7.3.1 Optimisation of immunoprecipitation procedure

In order to ensure optimal immunoprecipitation procedures, a number of optimisation conditions were trialled in order to find the most effective immunoprecipitation method. Optimisation steps included testing direct versus indirect methods, the use of different anti-PAX3 antibodies, different temperatures for antibody-antigen binding, as well as different incubation times, and different immunoprecipitation beads.

As indicated below, the direct immunoprecipitation procedure failed to capture the PAX3 protein, evident from the bands present in the lane containing the supernatant (lanes 3, 5, 7 and 9), whilst no bands were present in the lanes containing the immunoprecipitated sample (lanes 2, 4, 6 and 8, Figure 20A). Furthermore, it was not possible to determine whether PAX3 was immunoprecipitated by the indirect procedure due to the presence of the antibody heavy chains at 50 kDa, which could mask the presence of PAX3 protein at 56 kDa (lanes 2, 6 and 8, Figure 20B). It was apparent, however, that any of the three bead types (ChIP, protein A or protein G) could be used in further immunoprecipitation experiments, as they produced the same result, or lack of it (lanes 2-5 and 6-9, Figure 20B).

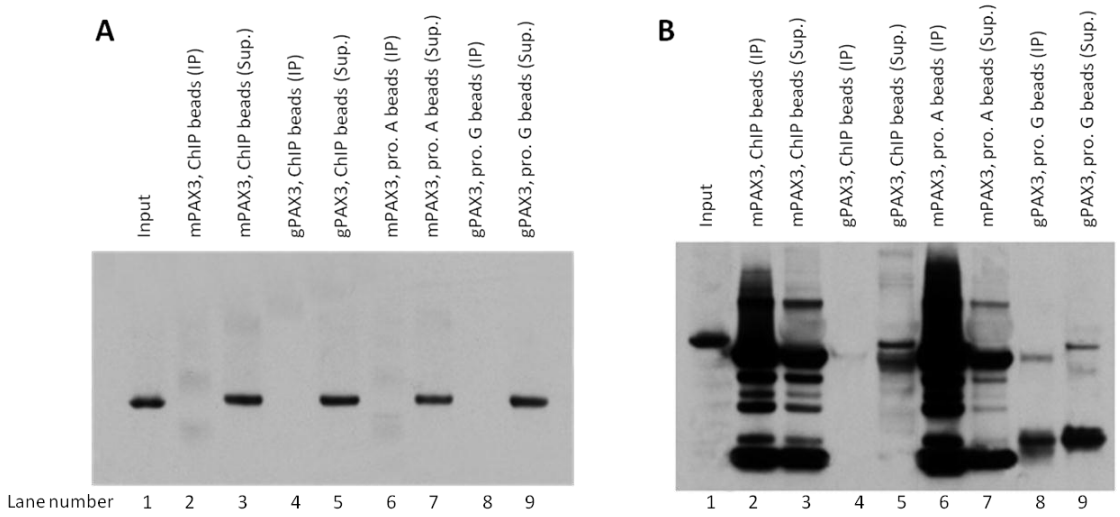

Figure 20: Optimisation of the immunoprecipitation procedure using direct (A) and indirect (B) procedures. Temperature (4˚C) and incubation times (overnight) were kept constant. Anti-PAX3 antibodies raised in mouse and goat were used and ChIP beads and protein A (mouse) and G (goat) beads were also tested. The supernatant (the flow-through that was collected after incubation of the beads, antibody and protein) was run alongside each immunoprecipitation sample to determine whether the PAX3 antibody sufficiently immunoprecipitated the PAX3 protein. mPAX3, rPAX3 and gPAX3 = PAX3 antibody raised in mouse, rabbit and goat respectively. IP = immunoprecipitation, Sup. = supernatant.

Due to the presence of multiple bands in the indirect immunoprecipitation procedure, the direct method was used to determine the optimal concentrations of the immunoprecipitating antibody (using mouse, rabbit and goat anti-PAX3 antibodies). Concentrations of 1µg and 10µg of antibody were used along with protein A (mouse and rabbit) and protein G (goat) coated Dynabeads (Invitrogen) to immunoprecipitate PAX3. Again, the result showed that PAX3 was not being pulled out of the total nuclear proteins by the bead and antibody complex, despite changing the concentration of the antibody (Figure 21).

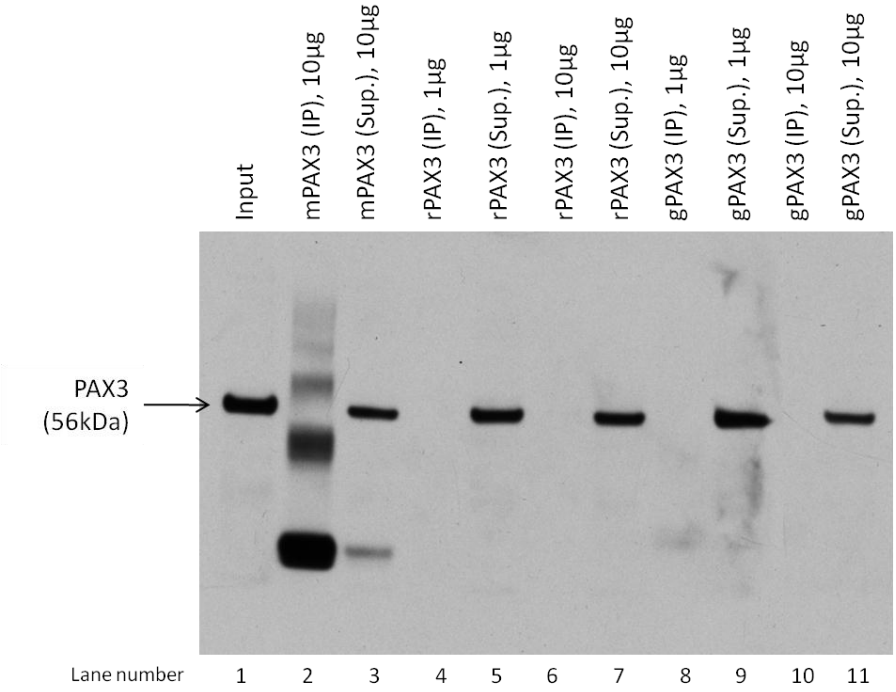

Figure 21: Optimisation of the direct immunoprecipitation procedure using different anti-PAX3 antibody concentrations (1µg or 10µg) to immunoprecipitate the PAX3 protein. Protein A (mouse and rabbit) and protein G (goat) Dynabeads were used for immunoprecipitation. mPAX3, rPAX3 and gPAX3 = PAX3 antibody raised in mouse, rabbit and goat respectively. IP = immunoprecipitation, Sup. = supernatant.

Due to the absence of PAX3 protein in the lanes containing the immunoprecipitated sample when the direct method was used, it was clear that the indirect method should be used for further optimisation. Further experiments were conducted using anti-PAX3 antibodies raised in different species for immunoprecipitation and western blot (Figure 22A). Antibody heavy and light chains appear when only the antibodies were used in the immunoprecipitation procedure, without cellular protein (lanes 2-7, Figure 22B). The flow through from washing after incubation of the antibody and antigen was also included on the gel in order to determine whether the antibody was binding the PAX3 protein but detaching during washing (lanes 5 and 8, Figure 22A).

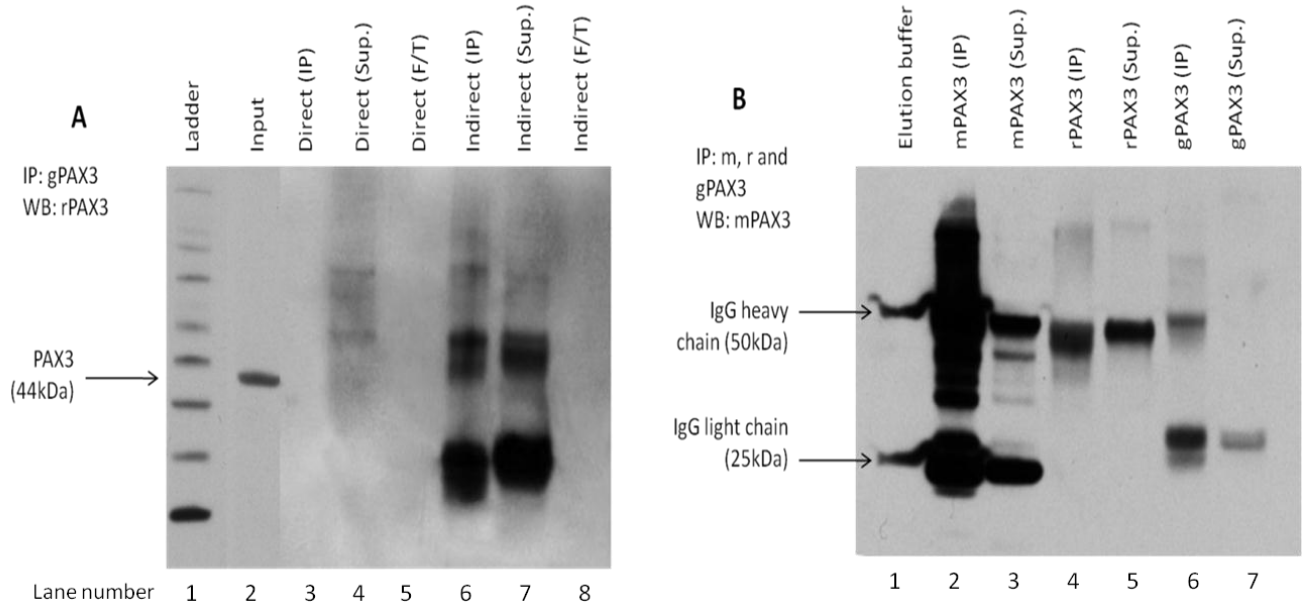

Figure 22: Optimisation of the immunoprecipitation procedure. A) Immunoprecipitation was performed using anti-PAX3 (goat) and immunoblotted with anti-PAX3 (rabbit) to attempt to eliminate antibody heavy chain interference, however, results indicate cross-reactivity between species. Additionally, a band appears at 44kDa in the input when blotted with anti-PAX3 raised in rabbit, which corresponds to isoforms PAX3G and PAX3H (lane 2). B) Anti-PAX3 antibodies (mouse (m) (lanes 2 and 3), rabbit (r) (lanes 4 and 5) and goat (g) (lanes 6 and 7)) were subject to the IP procedure, without cellular protein, and blotted with mouse anti-PAX3 to determine where antibody heavy and light chains appeared on the gel to allow us differentiate between antibody IgG and our PAX3 product. mPAX3, rPAX3 and gPAX3 = PAX3 antibody raised in mouse, rabbit and goat respectively. IP = immunoprecipitation, Sup. = supernatant; F/T = flow through.

For these reasons, after extensive optimisation of the immunoprecipitation procedures, the indirect method was chosen for all further analyses, along with anti-PAX3 raised in rabbit as the immunoprecipitating antibody and anti-PAX3 raised in mouse as the immunoblotting primary antibody, due to its lower background. Anti-PAX3 raised in mouse was unable to be used as the immunoprecipitating antibody as it produced multiple bands when immunoblotted with anti-PAX3 raised in both rabbit and mouse.

7.3.2 Immunoprecipitation and western blot

Total nuclear proteins were extracted from the metastatic cell line, M14, and the primary melanoma cell line, WM115, as well as from neonatal (NHEMn) and adult (NHEMa (I)) melanocytes, and subject to immunoprecipitation using anti-acetyl lysine, anti-phosphoserine and anti-ubiquitin antibodies, respectively. Mouse anti-PAX3 antibody was then used to blot the membranes to determine if PAX3 was included in the proteins that were acetylated, phosphorylated or ubiquitinated.

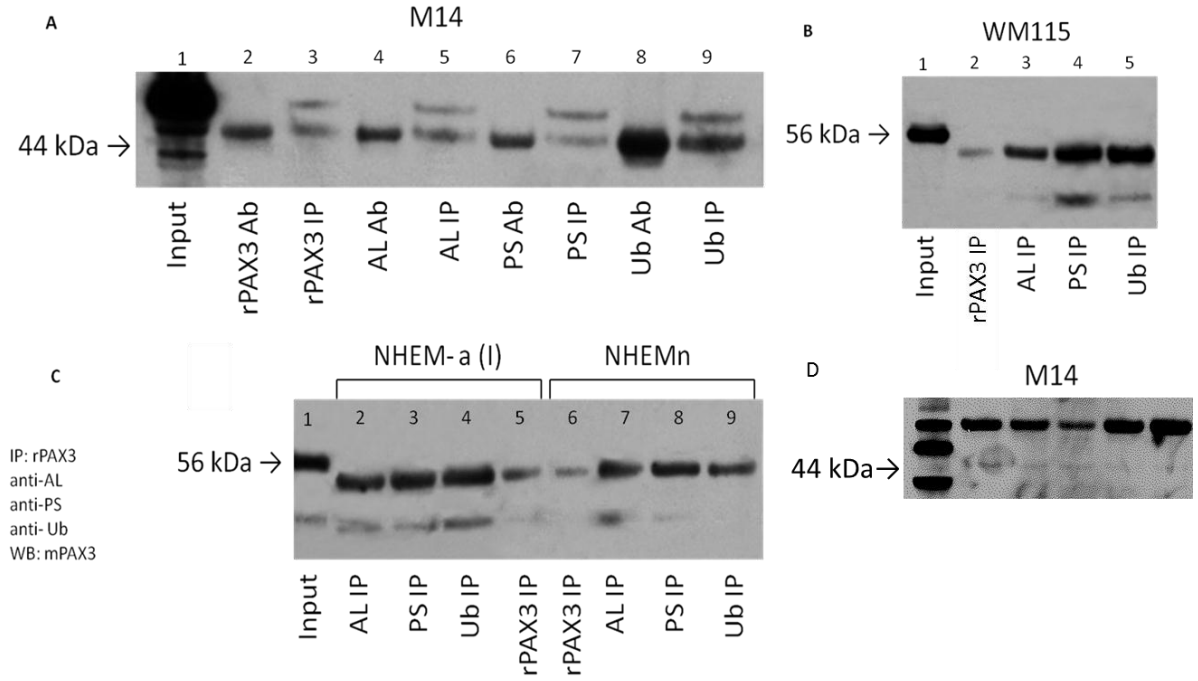

Figure 23: Immunoprecipitation (IP) using anti-acetylysine (AL), anti-phosphoserine (PS) and anti-ubiquitin (Ub) immunoblotted with anti-PAX3 (mouse monoclonal, DSHB) in the metastatic melanoma cell line M14 (A), the primary melanoma cell line WM-115 (B), adult human epidermal melanocytes (NHEM-a (I)) and neonatal human epidermal melanocytes (NHEM-n) (C). Figure 23D shows the presence of a 44kDa product using M14 cell lysate and immunoblotting with mouse anti-PAX3 antibody (DSHB). Al= acetylated, PS = phosphorylated, Ub = ubiquitinated; mPAX3, rPAX3 and gPAX3 = PAX3 antibody raised in mouse, rabbit and goat respectively. IP = immunoprecipitation, Sup. = supernatant.

Post-translational modifications of PAX3 were analysed in the metastatic cell line M14 (Figure 23A). Lane 1 contains M14 total nuclear protein input, blotted using anti-PAX3 (mouse monoclonal, DSHB) for comparison with other sample lanes. Each precipitating antibody (anti-PAX3, anti-acetylysine, anti-phosphoserine and anti-ubiquitin) was run alongside its respective immunoprecipitation (IP) sample to confirm the presence of the desired product relative to the presence of antibody heavy chains (at approximately 50kDa). Lanes 3, 5, 7 and 9 contain the immunoprecipitated PAX3 protein, acetylated proteins, phosphorylated proteins and ubiquitinated proteins, respectively. The membrane was then blotted for PAX3 and, after ruling out the product being the heavy chain of the antibody, it can be seen that a product that measures approximately 56kDa is present in each of the immunoprecipitation lanes, which could correspond to PAX3 isoform E (56 kDa), or potentially PAX3C and/or D (53 kDa). This result suggests that these isoforms are acetylated, phosphorylated and ubiquitinated in the M14 metastatic melanoma cell line.

PAX3 protein from the primary melanoma cell line, WM-115, was also tested for acetylation, phosphorylation and ubiquitination using the respective antibodies, as described above. As shown in Figure 23B, upon immunoprecipitation with the anti-acetyl lysine, anti-phosphoserine and anti-ubiquitin antibodies and imunoblotting with anti-PAX3, a product of 44kDa, corresponding to the expected size of PAX3g and PAX3h isoforms, suggests that these isoforms are possibly acetylated, phosphorylated and ubiquitinated in primary melanoma cell line WM115, whereas a band of 56kDa was not detected.

Furthermore, PAX3 acetylation, phosphorylation and ubiquitination were assessed in both the adult primary melanocytes and the neonatal primary melanocytes (Figure 23C). Again, a product of 44kDa is present in each of the samples, whereas a band of 56kDa was not detected, suggesting that PAX3g or PAX3h are acetylated, phosphorylated and ubiquitinated in normal human epidermal melanocytes. Although a 44kDa band is not detected in the input in Figures 23B and 23C, previous western blot results have detected a product of 44kDa in the input using the mouse anti-PAX3 monoclonal antibody when exposed for a longer period of time (e.g. Figure 23D).

Interestingly, it appears that there is the possibility of different isoforms of the PAX3 protein being differentially post-translationally modified in the metastatic melanoma cell line compared to those of the primary cell line and neonatal and adult melanocytes.

7.4 *PAX3* silencing and downstream targets

PAX3 silencing was used to confirm the differential regulation of previously identified downstream target genes by PAX3 in primary and metastatic melanoma and adult and neonatal melanocytes. The expression levels of each of the downstream target genes were calculated by normalising the Ct value of the gene to that of 18S (ΔCt). The fold change in siPAX3 was then calculated relative to the negative control siRNA sample (ΔΔCt), and converted to a percentage, and this was performed for each of the four cell types over the course of a three day silencing experiment. The knockdown experiment included *GAPDH* silencing as a positive control to ensure that the procedure was effective (Figure 24). Once the efficacy of the procedure was confirmed, *PAX3* silencing was performed on neonatal and adult melanocytes and primary and metastatic melanoma cells and downstream target gene expression was analysed by qPCR.

Figure 24 shows a dramatic knockdown of *GAPDH* in primary melanoma (WM115) (>98%) and metastatic melanoma (M14) (>78%) cells following treatment with *GAPDH* siRNA, indicating that the knockdown procedure was effective in these two cell lines. *PAX3* was also sufficiently silenced in the primary melanoma cells (>75%), however, knockdown of *PAX3* in the metastatic melanoma cells was not efficient following treatment with *PAX3* siRNA (<56%). Primary melanocyte cultures did not show efficient knockdown of either *GAPDH* or *PAX3* after treatment with siRNA, with adult melanocytes showing <50% knockdown of *GAPDH* levels and <52% knockdown of *PAX3* levels, and neonatal melanocytes showed <57% and <40% *GAPDH* and *PAX3* knocked down, respectively. Although the knockdown procedure was not considered efficient for the adult and neonatal melanocyte cultures (with knockdown of *GAPDH* <70%), remaining *PAX3* expression was significantly lower than negative controls in all cell types following treatment with siPAX3 and therefore downstream target gene expression was analysed further.

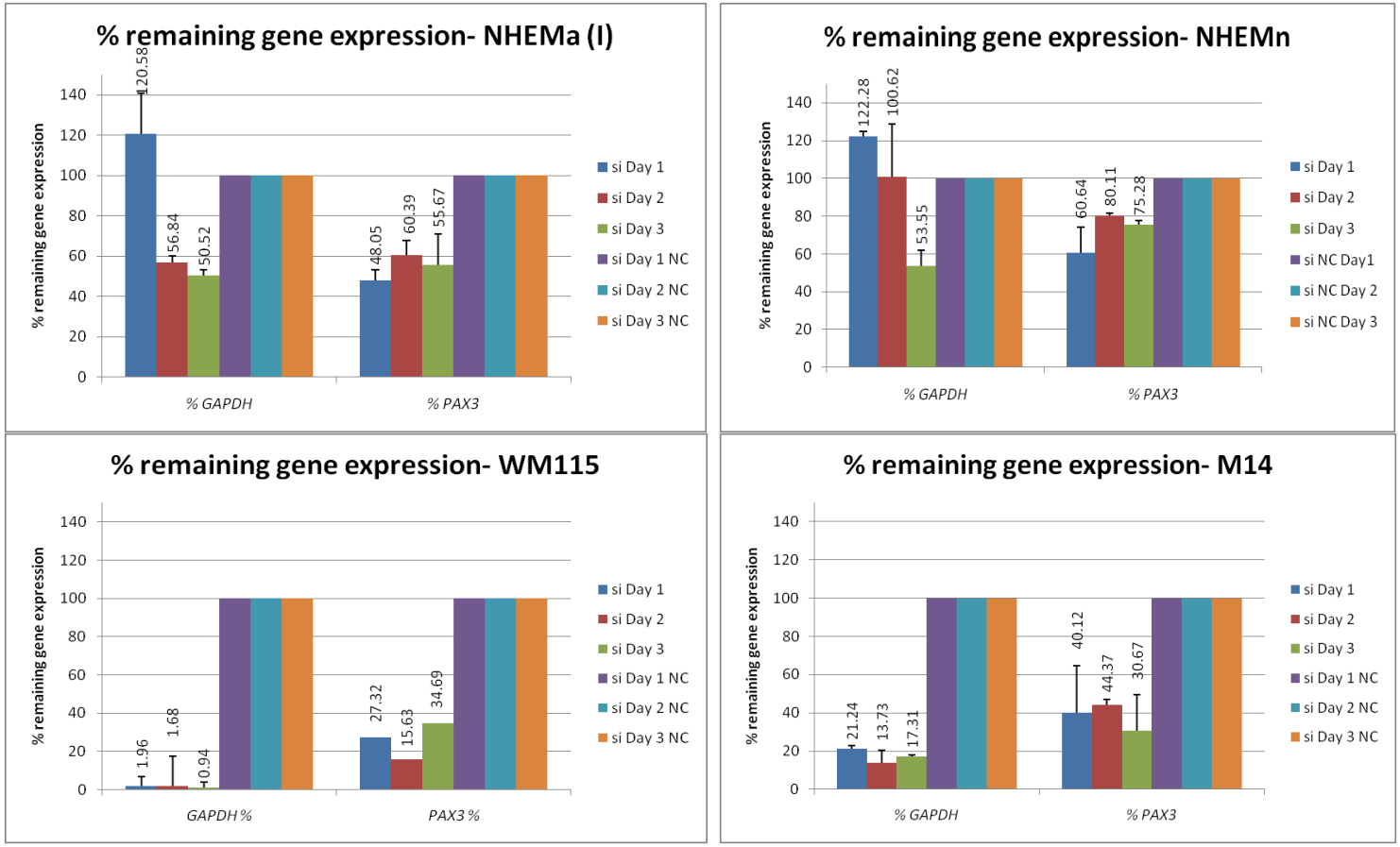

Figure 24: Percentage of remaining mRNA expression of *PAX3* following silencing with siPAX3 and *GAPDH* following silencing with siGAPDH in adult melanocytes, neonatal melanocytes, primary melanoma cells and metastatic melanoma cells. Percentage of remaining mRNA expression was calculated relative to 18S and then as fold change relative to the negative control (NC) siRNA and converted to a percentage. si DAY 1, 2, 3 = Day 1, 2, 3 of *GAPDH* or *PAX3* silencing experiments; si DAY 1, 2, 3 NC = Day 1, 2, 3 of negative control silencing experiments run in parallel to *GAPDH* and *PAX3* silencing. Each silencing experiment was performed in duplicate using a biological replicate.

7.4.1 Expression levels of PAX3 downstream targets involved in cell differentiation following *PAX3* silencing

Figures 25-28 represent the percentages of remaining mRNA expression of *PAX3* and those of its downstream target genes relative to the negative control in each of the four cell types, after knockdown of *PAX3* with siRNA. Percentage of remaining expression was analysed by RT-qPCR at 24, 48 and 72 hours following transfection with siRNA to silence *PAX3*.

Following transfection of cells from all cell types with *PAX3* siRNA, *PAX3* was shown to be significantly (but not efficiently) decreased post-knockdown in neonatal (NHEMn, $p=0.045$) and adult (NHEM (I), $p=0.046$) melanocytes, and in primary (WM115, $p<0.05$) and metastatic (M14, $p=0.020$) melanoma cells (Figure 25).

Furthermore, *MITF* was shown to be downregulated in neonatal melanocytes after *PAX3* siRNA transfection ($p=0.027$) and in the primary and metastatic melanoma cell lines ($p=0.004$; $p=0.033$, respectively). Although *MITF* expression levels in adult melanocytes were not significantly reduced following *PAX3* silencing, there is a trend toward lower MITF expression levels post-silencing. *DCT* also exhibited a significant and striking decrease in the primary metastatic cell line only following silencing of *PAX3*, ($p<0.05$). *NES* is significantly downregulated in primary melanoma cells after *PAX3* silencing ($p=0.029$), and, *HES1* expression is downregulated only in neonatal melanocytes after transfection with *PAX3* siRNA ($p=0.017$) (Figure 25). These findings indicate that differentiation genes are not generally affected by PAX3 in metastatic melanoma cells, even though PAX3 was effectively knocked down in these cells. Although two biological replicates were included, the lack of knockdown of *MITF* and other differentiation genes in adult melanocytes after silencing of *PAX3* was not surprising considering the lack of effective *PAX3* inhibition in these cells and additional experiments are required to confirm this.

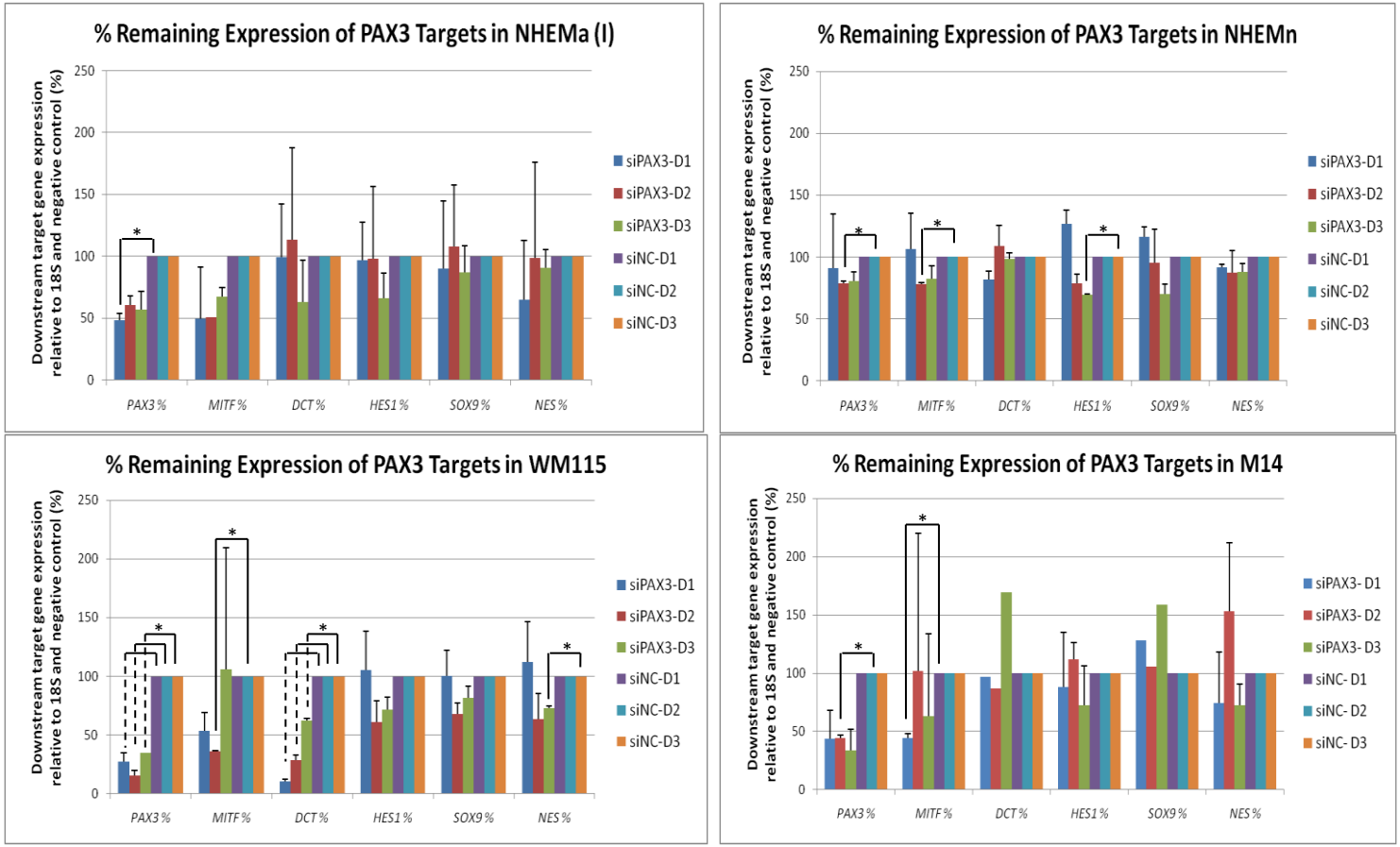

Figure 25: Remaining expression levels (%) of PAX3 downstream targets *MITF*, *DCT*, *HES1*, *SOX9* and *NES* in adult (NHEM (I)) and neonatal (NHEMn) melanocytes and primary melanoma (WM115) and metastatic melanoma (M14) cell lines following *PAX3* knockdown with siRNA (siPAX3). Target gene expression levels were calculated as the fold change over the negative control (siNC) and converted to a percentage. Asterisk (*) indicates significant difference, p≤0.05. siPAX3 –D1, 2, 3 = Day 1, 2, 3 of *PAX3* silencing experiments; si NC D1, 2, 3 = Day 1, 2, 3 of negative control silencing experiment run in parallel with *PAX3* silencing. Each silencing experiment was performed in duplicate using a biological replicate.

7.4.2 Expression levels of PAX3 downstream targets involved in cell proliferation following *PAX3* silencing

The results in Figure 26 show the percentage of remaining expression of *CCNA2* and *TPD52*, genes involved in cell proliferation, after transfection of cells with *PAX3* siRNA. The results indicate that *CCNA2* is significantly downregulated in adult melanocytes following *PAX3* knockdown ($p<0.05$). By contrast, primary and metastatic melanoma cells express significantly less *TPD52* after *PAX3* knockdown ($p=0.021$, $p=0.004$, respectively). These findings suggest that key cell proliferation genes are differently regulated by PAX3 in adult melanocytes and primary and metastatic melanoma cells.

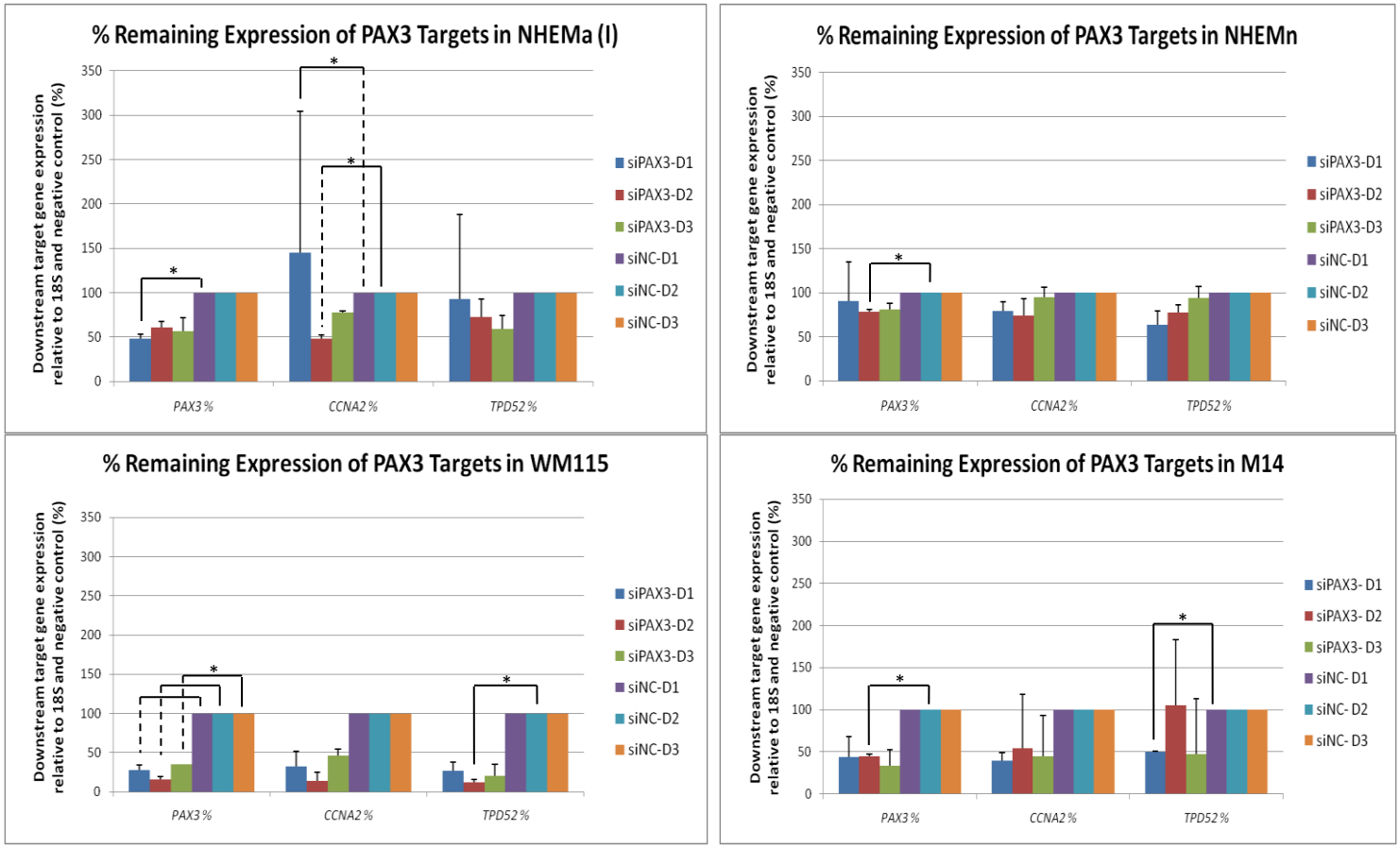

Figure 26: Expression levels (%) of PAX3 downstream targets *CCNA2* and *TPD52* in adult (NHEM (I)) and neonatal (NHEMn) melanocytes and primary melanoma (WM115) and metastatic melanoma (M14) cell lines following *PAX3* knockdown with siRNA (siPAX3). Target gene expression levels were calculated as the fold change over the negative control (siNC) and converted to a percentage. Asterisk (*) indicates significant difference, p≤0.05. siPAX3 –D1, 2, 3 = Day 1, 2, 3 of *PAX3* silencing experiments; si NC D1, 2, 3 = Day 1, 2, 3 of negative control silencing experiment run in parallel with *PAX3* silencing. Each silencing experiment was performed in duplicate using a biological replicate.

7.4.3 Expression levels of PAX3 downstream targets involved in cell survival following *PAX3* silencing

When analysed after transfection of cells with *PAX3* siRNA, the metastatic melanoma cell line showed significant downregulation of *BCL2L1* expression ($p<0.05$) on the second day post-transfection (Figure 27). Interestingly, BCL2L1 expression was significantly higher on days 1 and 3 post-transfection ($p<0.05$). The primary melanoma cell line exhibited a significant decrease in *PTEN* mRNA expression post *PAX3* silencing ($p=0.046$) (Figure 27). Thus, PAX3 potentially differentially regulates genes responsible for cell survival in primary and metastatic melanoma cells compared to melanocytes.

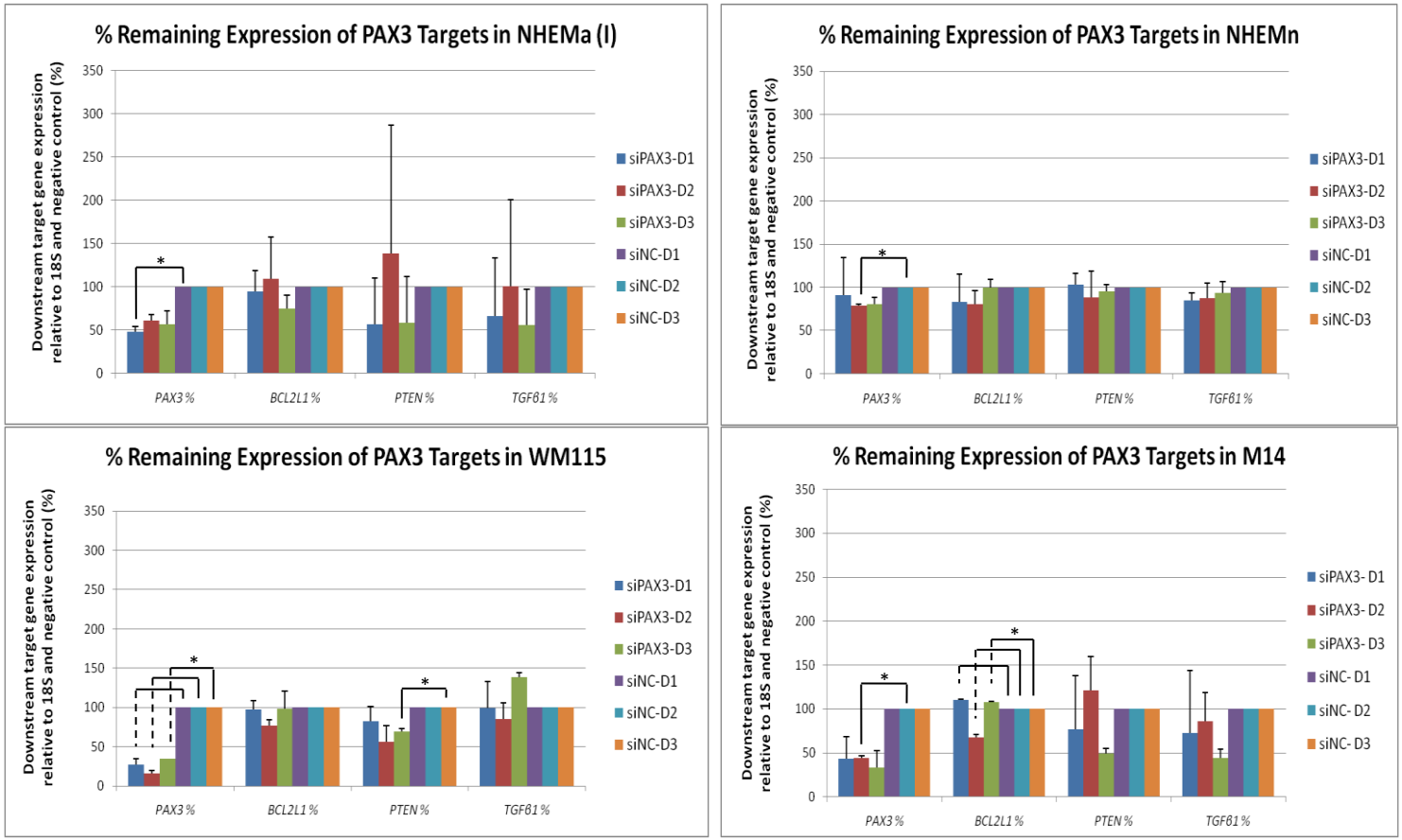

Figure 27: Expression levels (%) of PAX3 downstream targets *BCL2L1*, *PTEN* and *TGFβ1* in adult (NHEM (I)) and neonatal (NHEMn) melanocytes and primary melanoma (WM115) and metastatic melanoma (M14) cell lines following *PAX3* knockdown with siRNA (siPAX3). Target gene expression levels were calculated as the fold change over the negative control (siNC) and converted to a percentage. Asterisk (*) indicates significant difference, $p \leq 0.05$. siPAX3 –D1, 2, 3 = Day 1, 2, 3 of *PAX3* silencing experiments; si NC D1, 2, 3 = Day 1, 2, 3 of negative control silencing experiment run in parallel with *PAX3* silencing. Each silencing experiment was performed in duplicate using a biological replicate.

7.4.4 Expression levels of PAX3 downstream targets involved in cell migration following *PAX3* silencing

The *CXCR4* gene was the only cell migration gene that was shown to be downregulated following *PAX3* silencing, particularly in neonatal melanocytes (p= 0.016) and in the primary melanoma cell line (p= 0.013) (Figure 28).

In summary, *PAX3* knockdown caused a dramatic and significant decrease in genes associated with differentiation in primary melanoma cells, but less so in metastatic melanoma cells. Moreover, different proliferation genes were associated with *PAX3* knockdown in melanocytes relative to melanoma cells, confirming previous work indicating differential regulation of these pathways in melanocytes relative to melanoma cells.

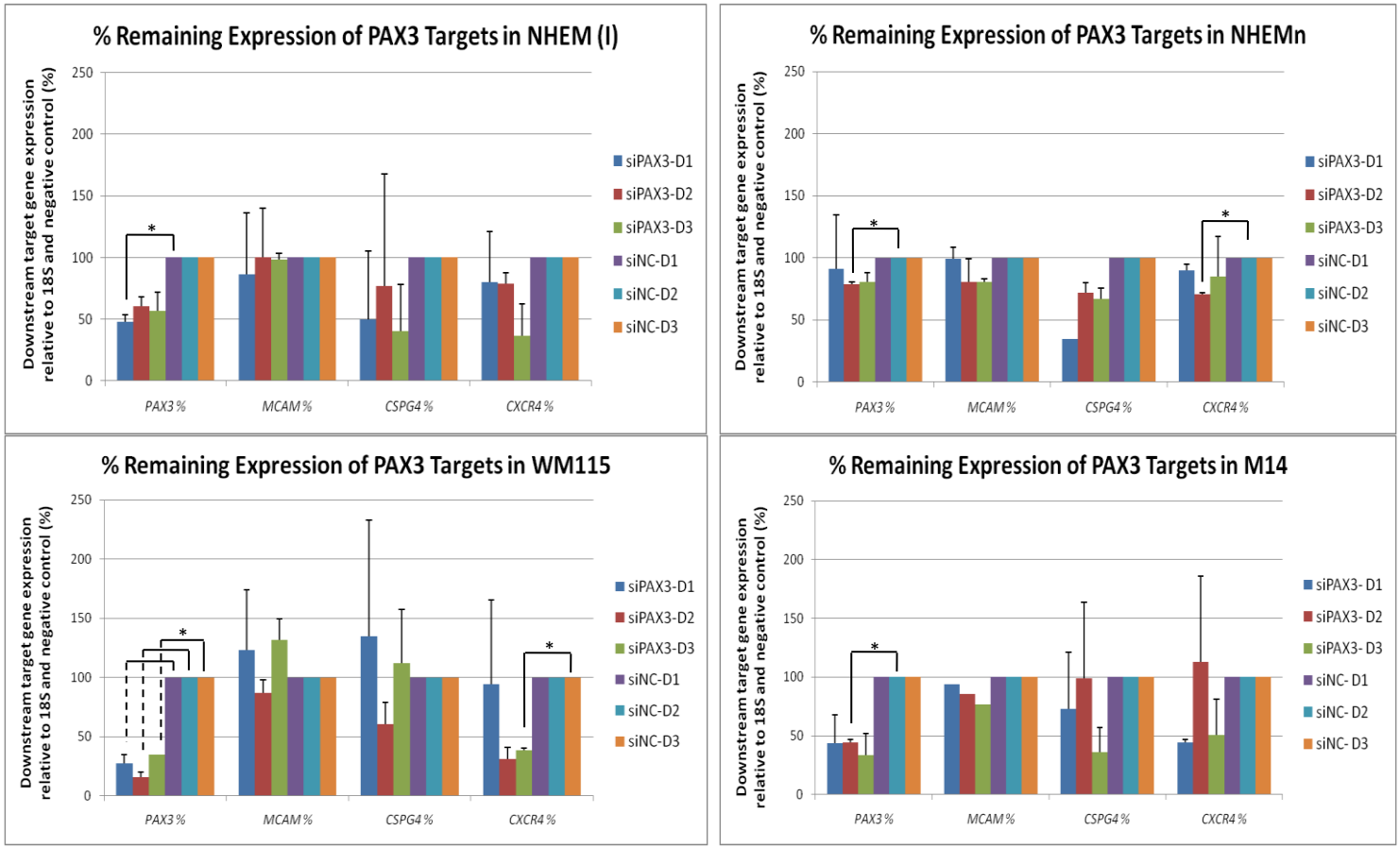

Figure 28: Expression levels (%) of PAX3 downstream targets *MCAM*, *CSPG4* and *CXCR4* in adult (NHEM (I)) and neonatal (NHEMn) melanocytes and primary melanoma (WM115) and metastatic melanoma (M14) cell lines following *PAX3* knockdown with siRNA (siPAX3). Target gene expression levels were calculated as the fold change over the negative control (siNC) and converted to a percentage. Asterisk (*) indicates significant difference, p≤0.05. siPAX3 –D1, 2, 3 = Day 1, 2, 3 of *PAX3* silencing experiments; si NC D1, 2, 3 = Day 1, 2, 3 of negative control silencing experiment run in parallel with *PAX3* silencing. Each silencing experiment was performed in duplicate using a biological replicate.

8. Discussion

The overall aim of this project was to confirm the regulation of selected downstream target genes by PAX3 and to determine the mechanism of their differential regulation in melanoma cells compared to melanocytes. It was hypothesised that differential regulation results from different *PAX3* alternate transcript expression or different post-translational modifications of the protein in these cells.

We showed here that there are indeed differences in *PAX3* expression in melanocytes and melanoma cells. Firstly, we identified a discrepancy between *PAX3* mRNA and protein levels in adult melanocytes, indicating that ubiquitination or miRNA activity play a role in *PAX3* regulation in these cells. Furthermore, we found that *PAX3* transcript expression profiles differ in melanoma cells compared to melanocytes. Our results also suggested that PAX3 protein isoforms undergo different post-translational modifications in metastatic melanoma cells compared to melanocytes. Lastly we demonstrated that selected downstream target genes are indeed regulated by PAX3, with some of these downstream targets being differentially regulated in melanocytes compared to melanoma cells.

Given the significant role of PAX3 in melanocyte development, and the significant expression of *PAX3* in melanoma, the role of PAX3 in melanoma cells needs to be comprehensively clarified. The outcomes of this study further our understanding of the role of PAX3 in melanoma and ultimately, through further research, may lead to *PAX3* becoming a target for therapeutic treatment.

8.1 *PAX3E* expression is downregulated in melanoma cells compared to melanocytes

One way in which downstream target genes of PAX3 could be differentially regulated is through alternate transcript expression. Each of the *PAX3* transcripts has been shown to produce a protein with a different function. Our data from RNA sequencing revealed that the *PAX3E* transcript is expressed at significantly lower levels in metastatic and primary melanoma cells compared to adult melanocytes (Figure 19). The role of PAX3E is thought to

be to reduce proliferation of melanocytes and to increase apoptosis (Wang et al., 2006). If *PAX3E* is downregulated in primary and metastatic melanoma cells, this could result in an increase in the proliferation of melanoma cells and a decrease in activation of apoptosis pathways, resulting in melanoma progression. This needs to be further investigated using additional cell lines and confirmed in melanoma tissue samples. Moreover, confirmation of a decrease in PAX3E protein should be confirmed in melanoma tissue samples with PAX3E specific antibodies.

Further research is also required to identify a mechanism whereby the action of this individual PAX3 isoform may influence melanomagenesis, perhaps giving melanoma cells the ability to proliferate, migrate, survive and maintain a "stem cell-like" state as in melanoblasts.

8.2 Differential PAX3 acetylation, phosphorylation and ubiquitination in metastatic melanoma cells compared to primary melanoma cells and melanocytes

Another way that PAX3 could differentially regulate downstream target genes is through post-translational modifications. Previous research has demonstrated acetylation, phosphorylation and ubiquitination of PAX3 in neuroblasts and myoblasts, causing these precursor cells to differentiate into their pre-determined adult forms (Boutet et al., 2010; Boutet et al., 2007; Dietz et al., 2011; Ichi et al., 2011; Miller et al., 2008). Our results suggest that PAX3 possibly undergoes acetylation, phosphorylation and ubiquitination in adult melanocytes, neonatal melanocytes, primary melanoma cells and metastatic melanoma cells, however, the isoforms that undergo these modifications differ between metastatic melanoma cells relative to the other three cell types. Our results show that the acetylated, phosphorylated and ubiquitinated PAX3 product in the metastatic melanoma cell line could be one, or a combination, of PAX3C, PAX3D and/or PAX3E isoforms. By contrast, the primary melanoma cell line and adult and neonatal melanocytes have isoforms PAX3G and/or PAX3H, with post-translational modifications.

Thus it is possible, from our results, that different PAX3 isoforms are postranslationally modified in metastatic melanoma cells compared to primary melanoma cells and

melanocytes and this could be one important mechanism by which differential downstream target gene regulation occurs in metastatic melanoma cells. Interestingly, neonatal melanocytes seem to lack ubiquitination of all PAX3 isoforms, indicating that all isoforms are possibly required at maximal levels during this time period. The presence of differential protein modifications in the four cell types could explain the differential regulation of downstream target genes in these cells, leading to differences in cellular function and phenotype and may contribute to the differential regulation of pathways associated with melanoma.

Different PAX3 isoforms possesses different amino acid residues and thus different sites at which acetyl, phosphate and ubiquitin moieties can attach (Boutet et al., 2010; Boutet et al., 2007; Dietz et al., 2011; Ichi et al., 2011; Miller et al., 2008). Although the sites for acetylation, phosphorylation and ubiquitination have been identified in PAX3, not all PAX3 isoforms possess all of these sites.

Acetylation of the PAX3 protein has been demonstrated by Ichi, et al. (2011) to occur at lysine residues 437 and 475 of the PAX3 protein. More recently, potential acetylation sites have been discovered at lysine residues 107, 116, 182 and 183 (Figure 29)(Cell Signaling Technology). Isoforms PAX3G and PAX3H contain only the more recently discovered potential sites for acetylation, while isoforms PAX3C, PAX3D and PAX3E contain both sites required for acetylation. Since acetylation of the isoforms will only increase their molecular weight by a maximum of 252Da (if all six sites are acetylated), which is not separable by SDS-PAGE, it is indeed possible that PAX3 isoforms C, D and/or E are differentially acetylated in metastatic melanoma cells, affecting downstream target gene regulation.

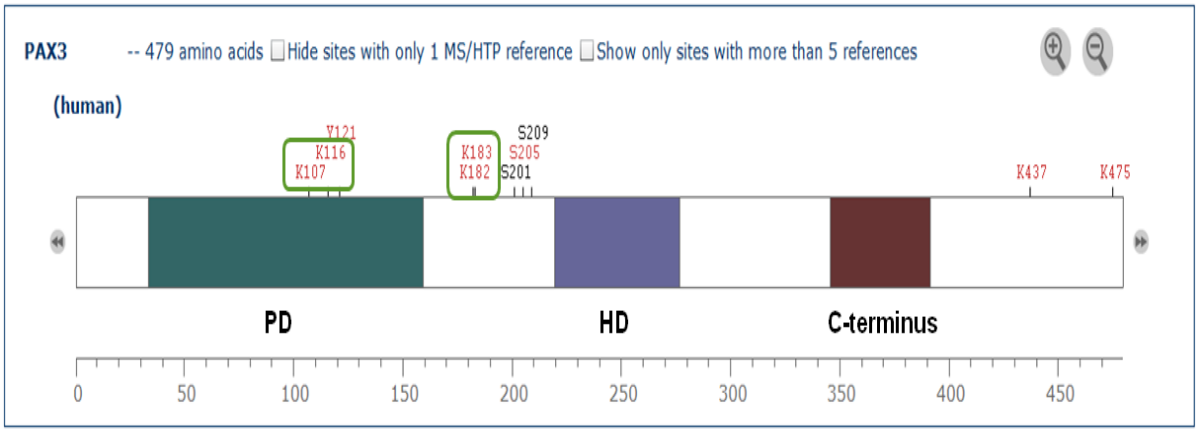

Figure 29: Additional potential sites of acetylation on the PAX3 protein (lysine (K) residues 107, 116, 182 and 183) (Cell Signaling Technology).

Previous research has demonstrated that acetylation of PAX3 causes it to repress, rather than activate, the same promoter on downstream target genes (Ichi et al., 2011). Since alternate PAX3 isoforms differentially regulate cellular function (Wang et al., 2006). , and these isoforms are potentially variably acetylated, it is plausible that differential acetylation of these isoforms differentially regulate cellular function, adding a layer of complexity in the role of PAX3 regulation of downstream target genes.

Serine residues 201, 205 and 209 of the PAX3 protein are possible sites of phosphorylation (Dietz et al., 2011; Miller et al., 2008). Isoform PAX3A does not contain serine residues at positions 201, 205 and 209 and therefore evades phosphorylation at these sites. The remaining PAX3 isoforms (PAX3B-PAX3I) do possess these identified sites for phosphorylation.

Posttranslational phosphorylation of PAX3 is actioned by GSK-3β (glycogen synthase kinase 3 beta) and CK2 (formerly casein kinase II) (Dietz et al., 2011; Kubic et al., 2012). Inhibition of GSK-3β results in a decrease in PAX3 expression in melanoma cells thereby altering proliferation states and regulation of apoptosis and terminal differentiation (Kubic et al., 2012). It is possible that the differential phosphorylation of PAX3 by GSK-3β could alter these cellular processes through either degradation of the protein, similar to that which has been shown with β-catenin (Aberle et al., 1997), or stabilisation and activation of the protein and alteration of its function via activation or repression of downstream targets

(Takeda et al., 2000a; Zeng et al., 2005). Again, the alternate PAX3 isoforms have the potential to differentially regulate cellular functions and this could be achieved through the variable phosphorylation of PAX3 isoforms, as we have demonstrated here, which could lead to differential activation or repression of downstream target genes. Here, we identified that between each of the cell lines, several downstream target genes of PAX3 were differentially regulated. Furthermore, PAX3 was found to be differentially phosphorylated in metastatic melanoma cells compared to primary melanoma cells and adult and neonatal primary melanocyte cells cultures. Thus, it is possible that phosphorylation of the PAX3 protein contributes to differential downstream target gene regulation.

Ubiquitination of the PAX3 protein occurs at the same lysine residues as acetylation (K437 and K475) (Boutet et al., 2010; Boutet et al., 2007), meaning that, like acetylation, ubiquitination of isoforms PAX3C, PAX3D and PAX3E can occur. Ubiquitination of the PAX3 protein enables it to be recognised by the proteosomal degradation pathway and, at the required time during development, has been shown to cause cells to differentiate (Boutet et al., 2010; Boutet et al., 2007). This indicates that PAX3 degradation would remove its activation or repression of downstream target genes and the differential ubiquitination of PAX3 isoforms could be a mechanism underpinning differential downstream target gene regulation.

Ubiquitin moieties would increase the molecular weight of the protein by up to 52.8kDa if all six possible lysine residues are ubiquitinated, a size difference separable by SDS page. Since our suspected product has the same molecular weight as acetylated and phosphorylated PAX3 in primary and metastatic melanoma cells and adult melanocytes, it is possible that fewer sites on the PAX3 protein are ubiquitinated in these cell types.

Although PAX3 isoforms are shown to be ubiquitinated in metastatic melanoma, primary melanoma and adult melanocytes, they appear not to be so in neonatal melanocytes. There is however no significant difference in PAX3 protein levels between these four cell types, so it is possible that the PAX3 isoforms ubiquitinated in these cell types are not the predominant forms or all PAX3 isoforms are not ubiquitinated and this is evident from our results.

Real-time qPCR and western blot analyses on each of the cell lines and primary melanocytes were performed to confirm PAX3 expression in all of these cell types. However, from these results, it can be seen that there is a discrepancy between the levels of mRNA and protein in adult melanocytes (Figures 16 and 18, respectively). RT-qPCR results show that *PAX3* is expressed at significantly higher levels in adult melanocytes, while PAX3 protein levels in adult melanocytes do not differ significantly from those of primary or metastatic melanoma cells. Although this was not an expected finding from our research, it supports our hypothesis that there is differential regulation of PAX3 in melanocytes relative to melanoma cells and this is possibly by ubiquitination. Furthermore, our results indicate that PAX3 ubiquitination is not present in neonatal melanocytes. By contrast it is present in primary and metastatic melanoma and adult melanocytes, however different isoforms are ubiquitinated. Ubiquitination of PAX3 in adult melanocytes may therefore contribute to the lower PAX3 protein levels in these cells.

PAX3 is ubiquitinated differently in melanoma and melanocyte cells, meaning it is possible that the PAX3E isoform is differentially lost in these cells, influencing downstream pathway regulation. However, although the *PAX3E* mRNA transcript is downregulated in melanoma cells, this is not evident in overall PAX3 protein levels, since there is no significant difference in protein levels between cell types. A difference in levels of this protein may not be evident on its own, or the difference in mRNA levels may not translate to differences in protein levels and further research is required to demonstrate differences in PAX3E protein levels in the different cell types, where differences may be due to ubiquitination.

Changes in mRNA and protein expression are generally attributable to ubiquitination or regulation by miRNA. To date, the miRNAs shown to regulate *Pax3* expression in the myogenic lineage are miR-1, miR-27b and miR-206, particularly in embryonal and alveolar rhabdomyosarcoma (Li et al., 2012) and could possibly regulate *PAX3* expression in melanoma. In addition miR-211 has been associated with melanoma, and is shown to repress an upstream regulator of *PAX3,* BRN2 (Boyle et al., 2011). miR-211 is reduced in melanoma cell lines in comparison to melanocytes and overexpression of miR-211 in melanoma cell lines inhibits cell invasion (Boyle et al., 2011). In this way, it is possible that other miRNAs exist that have the potential to regulate *PAX3*.

8.3 Knockdown of *PAX3* differentially downregulates key genes involved in cell differentiation, proliferation, migration and survival in melanoma cells compared to melanocytes

Previous research conducted by our group identified 13 potential downstream target genes of PAX3, several of which were differentially regulated in melanoma cells compared to melanocytes (Medic et al., 2011). In order to further clarify the role of PAX3 in melanoma, regulation of these genes in different cell types of melanocytic lineage were assessed following *PAX3* silencing and then downstream target gene expression was analysed. Ultimately, the aim was to confirm the regulation of these genes by PAX3 in normal melanocytes, through primary melanoma cells, to metastatic melanoma cells and to determine whether PAX3 plays a role in cell migration, proliferation, survival and differentiation in all of these cell types through regulation of specific target genes.

MITF is a key gene involved in melanocyte differentiation, survival and proliferation (Watanabe et al., 1998). Our results indicate that *MITF* expression following *PAX3* silencing is decreased significantly in primary neonatal melanocytes and melanoma cells and, although not significant, trends toward lower expression in adult melanocytes. This suggests that PAX3 regulates *MITF* in melanocytes and melanoma cells. Furthermore, the regulation of *MITF* by PAX3 in neonatal melanocytes and primary and metastatic melanoma cells indicates that PAX3 maintains tight control of differentiation pathways in these cells to regulate proliferation, migration and maintenance of an undifferentiated phenotype, which, in melanoma cells, may contribute to melanoma progression (Cronin et al., 2009). The knockdown of *HES1*, a marker of undifferentiated cells which aids in survival of melanocyte precursors (Moriyama, 2006), observed here in neonatal melanocytes, confirms the role of PAX3 in maintenance of undifferentiated cell phenotype through this gene in melanocytes but not melanoma cells.

By contrast, our results showed that in adult melanocytes, *PAX3* silencing did not cause a significant downregulation of any of the tested genes involved in cell differentiation (*MITF*, *DCT*, *HES1*, *SOX9*, *NES*). This is presumably due to the fact that the silencing was not efficient or possibly that differentiation genes are not as effectively regulated by PAX3 in these already differentiated cells.

Following *PAX3* silencing, only primary melanoma cells exhibited *DCT* downregulation. In normal melanoblasts, the inhibition of *DCT* by PAX3 causes these cells to remain in their undifferentiated state, allowing them to maintain their proliferation, migration and survival properties (Lang et al., 2005), so in primary melanoma cells, this regulation of DCT by PAX3 could similarly maintain a relatively undifferentiated state.

As shown previously by Wang et al. (2007), we observed regulation of *CCNA2* by PAX3 in normal melanocytes. Notably, cell cycle regulators *CCNA2* and *TPD52* were differentially regulated in adult melanocytes and melanoma cells following *PAX3* silencing. We showed here that PAX3 regulates *CCNA2* in adult melanocytes, whereas *TPD52* is regulated by PAX3 in melanoma cells. This confirms earlier findings by Medic et al. (2011), who also showed differential regulation of *TPD52* in melanocytes and melanoma cells. The regulation of *TPD52* by PAX3 in melanoma cells could be one mechanism underpinning an alternative *PAX3*-driven mechanism of cell survival of melanoma cells relative to melanocytes and requires further investigation.

Here we also demonstrated that PAX3 regulates two key cell survival genes in melanoma, while these do not appear to be controlled by PAX3 in melanocytes. *BCL2L1* and *PTEN* are both downregulated in response to PAX3 silencing in melanoma cells. In particular, since *BCL2L1* inhibits apoptosis, its regulation by PAX3 in metastatic melanoma cells may be a cell survival mechanism (Margue et al., 2000). *PTEN* is knocked down in response to *PAX3* silencing in primary melanoma cells, indicating that *PTEN* is regulated by PAX3. However regulation may be in an indirect manner, activating a separate pathway involved in *PTEN* regulation, as previously PAX3 has been shown to inhibit *PTEN* expression in melanoma cells (Li et al., 2007). Furthermore, *PTEN* has previously been shown to be differentially regulated in melanoma cells compared to melanocytes (Medic et al., 2011).

The variable results demonstrated in differential downstream target gene regulation limit the efficacy of this experiment. The presence of very few significantly downregulated downstream target genes in neonatal and adult melanocytes may be attributable to the insufficient knockdown of *PAX3*. Although the silencing procedure was tested for efficiency using siGAPDH as a positive control, and *GAPDH* was found to be sufficiently knocked down in adult and neonatal melanocytes, it is possible that *PAX3* silencing was not as effective in

these cells due to their slow turnover rate. Alternately, other potential downstream target genes are differentially regulated by PAX3 in melanocytes, thus further investigation is needed, in more samples and possibly in tissues, in order to gain an improved representation of the role of PAX3 in regulating these downstream target genes.

In summary, in this project, we have demonstrated PAX3 regulation of downstream target genes that drive key pathways in cell differentiation, proliferation, migration and survival. Furthermore, we have shown that this regulation differs in melanocytes relative to melanoma cells and that this differential regulation could be attributable to alternate transcript expression, shown through the downregulation of *PAX3E* in melanoma, or differential protein modifications of the PAX3 isoforms.

8.4 Limitations of the study and suggested future experiments

The most significant limitation of this study is that all experiments were performed in one cell line per cell phenotype limiting the general applicability of these findings. Additional experiments in additional cell lines for each phenotype *in vitro* and in tissue are required to further identify the role of PAX3 in differential activation of downstream target genes. There is however only one commercially available neonatal cell line and very few adult melanocyte primary cell cultures, limiting what is possible *in vitro*. Alternately, additional experiments could be conducted using tissue explants or fresh tissue samples to gain a more comprehensive understanding of the complex role of PAX3 in skin homeostasis and in melanomagenesis.

Different antibodies against PAX3 preferentially recognise and bind to different PAX3 isoforms. When used for immunoprecipitation and western blotting, this contributed significantly to difficulties with experiment optimisation. New transcript-specific antibodies have recently become available commercially however these have not been confirmed in the literature, as useful for immunoprecipitation. Furthermore, optimisation of these experiments with additional cell lines, and alternative immunoprecipitation kits with a range of PAX3 antibodies, is required to further our understanding of these isoforms.

The lack of change in downstream target gene regulation indicated by our results suggests that either these genes do not depend on PAX3 for their regulation, or more likely, insufficient *PAX3* silencing prevented us from observing any effect on the target. This means that further silencing experiments in these cell types is required to determine whether PAX3 was sufficiently knocked down and whether the results acquired are a true representation of these cell types. The limited number of biological replicates reduces confidence in the *PAX3* silencing data and therefore an increase in the number of biological replicates is required for each cell line. In order to increase the efficiency of *PAX3* knockdown in melanocytes, the transfection procedure should take place over 48 hours, rather than the 24 hour time lapse used here. It is possible that the inefficient knockdown of PAX3 is due to the slow turnover of melanocytes compared to melanoma cells. Improving the efficiency of this experiment will enable us to determine which downstream target genes are differentially regulated in melanocytes compared to melanoma cells and will help to broaden our understanding of the mechanisms underpinning differential downstream target gene regulation in melanoma cells compared to melanocytes.

Additionally, immunoprecipitation of the individual PAX3 isoforms, using specific antibodies, followed by assessment of the isoforms for differential post-translational modifications in melanoma cells compared to melanocytes will further our understanding of the individual roles of the PAX3 isoforms in melanoma compared to normal skin melanocytes.

9. Conclusion

In conclusion, melanoma is an extremely aggressive skin cancer and is highly drug resistant (Chen et al., 2006; Chin et al., 2006), emphasising the need to define mechanisms involved in the development and progression of this cancer so as to make advances in the therapeutic treatment of melanoma. Previous research has demonstrated that potential downstream target genes of PAX3 are differentially regulated in metastatic melanoma compared to normal human epidermal melanocytes (Medic et al., 2011). We hypothesised that this differential regulation of downstream targets by PAX3 could be attributable to alternate *PAX3* transcript expression or differences in post-translational modifications of the PAX3 protein between metastatic melanoma cells and normal adult human melanocytes. Thus, we assessed the *PAX3* transcript expression, PAX3 post-translational modification profiles and the regulation of downstream target genes in normal human adult and neonatal melanocytes relative to primary and metastatic melanoma,

Here we have identified that the *PAX3E* transcript is downregulated in metastatic and primary melanoma cells compared to normal adult human melanocytes. Since the role of PAX3E in normal melanocytes is to reduce proliferation and increase apoptosis (Wang et al., 2006), our results suggest that its downregulation in melanoma could be one of the mechanisms underpinning the ability of melanoma cells to evade apoptosis and increase cell proliferation. Furthermore, we have shown that PAX3 is regulated at a post-translational level in normal adult and neonatal melanocytes and in primary and metastatic melanoma cells, with differential modifications of PAX3 isoforms in metastatic melanoma cells compared to normal melanocytes and primary melanoma cells. Moreover, we showed that PAX3 in normal neonatal human melanocytes is not ubiquitinated, in contrast to the other three cell types, confirming differential regulation of PAX3 at different developmental stages. Finally, we have confirmed differential regulation of downstream target genes involved in differentiation, proliferation, migration and cell survival between normal human adult and neonatal melanocytes, and primary and metastatic melanoma cells.

The major limitation of this study was the apparent preferential binding of the PAX3 antibodies to different isoforms, making it difficult to immunoprecipitate the PAX3 protein and effectively visualise it using western blotting. Thus, future experiments should look into

using isoform specific antibodies that have recently become commercially available. Furthermore, silencing experiments need to be repeated and expanded to include more samples, in order to confirm differential regulation of downstream targets between melanocytes and primary and metastatic melanoma cells. Finally, transcript expression profiling should be performed on an increased number of samples.

By further analysis, the mechanisms by which PAX3 differentially regulates downstream target genes could become clearer, furthering our knowledge of the potential role of PAX3 in melanoma progression. Given the importance of *PAX3* in the development of melanocytes and its expression in the majority of melanoma cells, knowledge of differences in PAX3 regulated mechanisms between normal cells and melanoma cells that lead to melanoma progression could provide tools for therapeutic manipulation of melanoma cells while sparing the normal cells.

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