2011

# New perspectives on melanoma: The role of PAX3

Sandra Medic
*Edith Cowan University*

# Edith Cowan University

# Copyright Warning

**Doctor of Philosophy**

**(Human Biology)**

**Thesis**

# New Perspectives on Melanoma: The Role of PAX3

**PhD Candidate:**

**MSc Sandra Medic**

**Supervisor:**

**Dr Melanie Ziman**

**Edith Cowan University**

**Faculty of Computing, Health and Science**

**School of Medical Sciences**

**2011**

# USE OF THESIS

The Use of Thesis statement is not included in this version of the thesis.

## *DECLARATION*

*I certify that this thesis does not, to the best of my knowledge and belief:*

*(i) incorporate without acknowledgement any material previously submitted for a degree or diploma in any institution of higher education;*

*(ii) contain any material previously published or written by another person except where due reference is made in the text; or*

*(iii) contain any defamatory material.*

*I also grant permission for the Library at Edith Cowan University to make duplicate copies of my thesis as required.*

*21st November 2011*

*(Signature of Candidate)* *(Date)*

## ABSTRACT

**Background:** Cutaneous melanoma is an aggressive form of skin cancer, arising in cutaneous melanocytes. The transcription factor PAX3 is critical for the proper development of neural crest lineages including melanocytes. Melanocytic cells show continued PAX3 expression from melanoblast formation in the neural crest to their differentiation into melanocytes. While many studies clarify the importance of PAX3 in embryonic development of melanocytes, less well understood, and more perplexing, is the continued PAX3 expression in adult skin melanocytes. By contrast PAX3 is frequently found in melanomas and naevi, and its expression correlates with melanoma staging.

In this study we explore the multiple roles of PAX3 in melanocyte genesis and melanoma progression. While PAX3 is known to regulate melanocyte differentiation, survival, proliferation and migration during embryonic development, it is not clear if these same functions are maintained in adult melanocytes or melanoma cells. Drawing on evidence from development, we propose here a more encompassing theory that PAX3 is a key regulator of the myriad steps in melanocytic cell determination and function. We discuss the possibility that these roles may be accomplished by differential association with cofactors, via alternate transcripts or posttranslational protein modification(s). Moreover, we consider its possible roles in melanoma and provide a comprehensive consideration of the significance of PAX3 expression in melanoma.

**Methodology and Principal Findings:** In this study we firstly analysed the expression of PAX3 across the spectrum of melanocytic cells, from normal melanocytes to cells of benign and malignant lesions. PAX3 expression was analysed by immunohistochemistry and RT-qPCR. In order to get a better understanding of PAX3 functions in these various tissues, we have characterised PAX3-expressing cells with respect to their proliferative, migratory and antiapoptotic potential, as well as differentiation status. For this, immunofluorescence was used to analyse PAX3 co-expression with markers representative of these processes that are also its downstream targets.

As expected, PAX3 expression was observed in all naevi and melanoma samples, but it was also found in both follicular and epidermal melanocytes of normal skin. Melanocytic origin of PAX3-expressing cells was confirmed by co-expression with known melanocyte markers, MITF and MLANA. Co-expression with its downstream target, antiapoptotic factor BCL2L1 confirmed PAX3 as a cell survival regulator. PAX3 was also co-expressed with melanoma cell migration marker MCAM in dermal naevi, melanoma cell nests and normal melanocytes in growing hair follicles, but this downstream target of PAX3 was not present in normal epidermal melanocytes, suggesting differential roles for PAX3 in normal epidermal melanocytes and melanoma cells. Most interestingly, a proportion of PAX3-positive epidermal melanocytes in normal skin show HES1 and Ki67 co-expression, indicating their less differentiated proliferative phenotype.

To clarify PAX3 roles in normal melanocytes and melanoma cells, we thought to analyse its direct target genes in these cell types. An intensive literature research was employed to identify the potential and likely PAX3 targets in normal melanocytes and melanoma cells. This generated a list of 56 genes, from which we selected 14 genes for further investigation, based on their significance for melanoma development and progression. Direct PAX3 binding to the selected genes in melanocytes and melanoma cells was assessed by ChIP-qPCR. Differential expression of identified direct targets was then analysed in melanoma cells compared to normal melanocytes, by RT-qPCR.

Results distinguish genes that are commonly regulated by PAX3 in melanocytes and melanoma cells, from those that are restricted to melanoma cells. Further, we show that similar to its role in development, PAX3 controlled complex differentiation networks (via NES and SOX9) in both melanoma cells and melanocytes, in order to maintain cells in a less differentiated, stem cell-like state. We also show that mediators of migration (MCAM and CSPG4) are common to both cell types, but are more highly expressed in melanoma cells. By contrast, PAX3-mediated regulation of melanoma cell proliferation (through TPD52) and survival (through BCL2L1 and PTEN) differs from that of melanocytes.

**Conclusions and Significance:** Our results suggest that previously identified roles for PAX3, as a regulator of an undifferentiated plastic state, and of cell proliferation, migration and survival, may operate in melanoma cells as well as in melanocytes of normal skin. By controlling these crucial cellular processes in melanoma cells, PAX3 may contribute to development of malignant melanocytic lesions and disease progression. Moreover its expression and action in normal melanocytes may confer upon them a less differentiated, proliferative and motile phenotype, thus predisposing them to aggressive malignant transformation. Results presented here provide avenues for exploring PAX3 as a potential tool for developing targeted therapy for melanoma treatment.

## ACKNOWLEDGEMENTS

I would first like to thank to my supervisor Mel, for guidance and endless support, optimism and patience. I am most thankfull however to my family, especially my dear Petar and Helena, for the patience and support through this (what seemed sometimes like a never-ending) journey. Finally, I would like to thank to my colleagues and friends for the assistance and encouragement.

## *PREFACE*

***Policy Content:*** *A PhD by publication shall conform to Rule 52 plus the following:*

*(i) completion of the requirements for Confirmation of Candidature (Rule 34);*

*(ii) a full explanatory introduction, general discussion and conclusion shall be included to link the separate papers and to place them in the context of an established body of knowledge;*

*(iii) if an adequate contextual literature review is not otherwise presented, it shall be included as a separate chapter;*

*(iv) if detailed data and descriptions of methods are not otherwise given, they shall be included as appendices;*

*(v) only papers published in refereed scholarly media during the period of enrolment may be included in a thesis submitted in the form of a series of published papers. However, papers which have been accepted for publication but have not yet appeared in refereed scholarly media may also be included as part of the thesis;*

*(vi) the number of papers submitted should be sufficient for the body of work to constitute a substantial and original contribution to knowledge;*

*(vii) any published paper of which the candidate is a joint author may only be included in the thesis provided the work done by the candidate is clearly identified. The candidate must provide to the University Research Students and Scholarships Committee, at the time of submission of the thesis, a written statement from each co-author attesting to the candidate's contribution to a joint publication included as part of the thesis; and*

*(viii) the sources from which the candidate's information is derived, the extent to which the work of others has been used and/or for which the assistance of individuals, associations or institutions has been obtained, shall be acknowledged generally in a preface or introduction, and specifically in notes, references and appendices.*

## LIST OF PUBLICATIONS INCLUDED

The following publications are included as part of this thesis:

- Medic S, Ziman M (2009) PAX3 across the spectrum: from melanoblast to melanoma. Crit Rev Biochem Mol Biol: 1-13.

- Medic S, Ziman M (2010) PAX3 expression in normal skin melanocytes and melanocytic lesions (naevi and melanomas). PLoS One 5: e9977.

- Medic S, Rizos H, Ziman M (2011) Differential PAX3 functions in normal skin melanocytes and melanoma cells. Biochem Biophys Res Commun 411: 832-837.

***STATEMENT OF CONTRIBUTION OF THE CANDIDATE***

*The purpose of this statement is to summarise and identify the nature and extent of the intellectual input by the candidate, and any other co-authors. Signed statement from H. Rizos is included in this thesis as an Appendix.*

*I,* ***Sandra Medic****, contributed to conceiving, designing, and performing the experiments, analysing the data, and writing all manuscripts included in this thesis, as well as writing the thesis itself.*

*(Signature of Candidate)*

*I, as a supervisor and co-author on all publications included here, endorse that this level of contribution by the candidate indicated above is appropriate.*

***Mel Ziman****,*

*(Signature of Co-Author)*

*29th September 2011*

*(Date)*

## LIST OF CONFERENCE PRESENTATIONS

Additional to the publications included as part of the thesis, the following conference presentations have relevance to the thesis:

- Ziman M, Medic S, Pearce R, Millward M, Kumarasinghe P (2011) PAX3 as a marker of melanocytes, melanocytic lesions, circulating melanoma cells, and both primary and metastatic melanoma. IPCC, France.

- Medic S, Ziman M (2010) The role of PAX3 in development of melanoma. International Society of Differentiation, Japan.

- Medic S, Ziman M (2009) PAX3 expression in normal skin melanocytes and melanocytic lesions. WA Cancer Research Symposium, Australia.

- Medic S, Ziman M (2009) Novel Perspectives on Cutaneous Malignant Melanoma: A role for PAX3. ASMR, Australia.

## TABLE OF CONTENTS

## LIST OF FIGURES

## LIST OF TABLES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| αMSH | α melanocytes stimulating hormone, currently designated as propiomelanocortin (POMC) |
| ACTH | adenocorticotropic hormone |
| AKT | serine/threonin protein kinase AKT |
| ATCC | American Type Culture Collection |
| BAX | BCL2-associated X protein |
| BCL2L1 | BCL2 like protein 1, also known as BCL-XL |
| BRAF | v-raf murine sarcoma viral oncogene homologue B1 |
| BRN2 | PUO class 3 homeobox 2 (POU3F2) |
| CCNA2 | cyclin A2 |
| CCND1 | cyclin D1 |
| CDK4 | cyclin-dependant kinase 4 |
| ChIP | chromatin immuno-precipitation |
| c-Met | met proto-oncogene, also known as hepatocyte growth factor receptor |
| CSPG4 | chondroitin sulphate proteoglycan 4, also known as MCSP, and HMW-MAA |
| CXCR4 | CXC chemokine receptor 4 |
| DCT | dopachrome tautomerase, also known as tyrosinase-related protein 2 (TYRP2) |
| DKK1 | dickkopf homologue 1 |
| DMEM | Dulbecco's Modified Eagle Medium |
| DNA | deoxyribonucleic acid |
| DSHB | Developmental Studies Hybridoma Bank |
| EDNRB | endothelin receptor type B |
| ET | endothelin |
| FBS | foetal bovine serum |
| FGF | fibroblasts growth factor |
| FGFR | fibroblasts growth factor receptor |
| GAPDH | glyceraldehydes-3-phosphate dehydrogenase |
| GRG4 | groucho 4, currently designated as transduction-like enhancer of split 4 (E (sp1) homologue, Drosophila) (TLE4) |
| HDAC10 | histone deacetylase 10 |
| HDM2 | currently known as Mdm2 p53 binding protein homologue (MDM2) |
| HES1 | hairy and enhancer of split 1 |
| HGF | hepatocyte growth factor |
| LEF1 | lymphoid enhancer-binding factor 1 |
| IgG | immunoglobulin G |
| IP | immunoprecipitation |

| | |
|---|---|
| KIT | v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homologue, also known as c-Kit |
| MAPK | mitogene-activated protein kinase |
| MC1R | melanocortin 1 receptor |
| MCAM | melanoma cell adhesion molecule, also known as MUC18 |
| MITF | microphthalmia-associated transcription factor |
| MLANA | melan-A, also known as melanoma antigen recognise by T cells (MART1) |
| NES | nestin |
| NF-Kβ2 | nuclear factor kappa β 2 |
| NGS | normal goat serum |
| NRAS | neuroblastoma RAS viral oncogene homologue |
| p16 | cyclin-dependant kinase inhibitor 2A (CDKN2A), also known as p16-INK4A |
| PAX3 | paired box 3 |
| PMEL | premelanosome protein, also known as PMEL17, gp100, or SILV |
| PRAME | preferentially expressed antigen in melanoma |
| PTEN | phosphatase and tensin homolog |
| qPCR | quantitative (real time) polymerase chain reaction |
| RB | retinoblastoma protein |
| RNA | ribonucleic acid |
| RT | reverse transcriptase |
| SCF | stem cell factor, also known as steel factor (SLF), currently designated as KIT ligand (KITLG) |
| SIRT1 | sirtuin 1 |
| SMAD2 | SMAD family member 2 |
| SMAD3 | SMAD family member 3 |
| SOX9 | SRY (sex determining region Y) - box 9 |
| SOX10 | SRY (sex determining region Y) - box 10 |
| TGFα | transforming growth factor α |
| TGFβ | transforming growth factor β |
| TP53 | tumour protein p53 |
| TPD52 | tumour protein 52 |
| TYR | tyrosinase, also known as oculocutaneous albinism IA (OCA1A) |
| TYRP1 | tyrosinase-related protein 1 |
| WNT | wingless-type MMTV integration site family |

**N.B. the following conventional notation is used:**

*Pax3* (italicised) indicates gene
Pax3 (non-italicised) indicates protein
*PAX3* (italicised, capital) indicates human gene
PAX3 (non-italicised, capital) indicates human protein

# *Chapter I – Thesis overview*

## i. GENERAL INTRODUCTION

### Cutaneous melanoma

Cutaneous melanoma (it will be referred to in the remainder of the text as 'melanoma') is the most aggressive form of skin cancer arising from melanocytic cells in the skin. While it represents only around 4% of all skin cancers, melanoma accounts for nearly 80% of skin cancer related deaths [4]. Recent statistics are alarming, showing that the worldwide incidence of melanoma is increasing at a rate of 3-7% per annum [5,6]. Australia has the highest incidence of melanoma in the world, with Western Australia following closely behind Queensland [7]. Official reports from the Western Australia Cancer Registry for the year 2008 (http://www.health.wa.gov.au/wacr) rate melanoma as the third most common cancer overall (with 1082 new cases reported in 2008). It is the most common cancer in young males and the second most common type in young females (age 15-39). The 5-year survival rate for early stage melanoma patients is high (around 95%), however, the fifteen year survival rate for some of those patients drops to around 40%, and for advanced stage patients 5-year survival is less that 20% [8,9]. It is important therefore to understand in detail the mechanisms driving melanoma development and progression.

### PAX3 transcription factor

PAX3 (paired box 3) is a paired box transcription factor, expressed during early embryonic development in cells of neural crest origin, the cells which give rise to skin melanocytes. In fact, PAX3 plays a key role in specification of melanoblasts and in maintenance of their stem-like/undifferentiated state. PAX3 also regulates proliferation and migration to their final location in the skin, where they terminally differentiate into melanocytes [10-15]. PAX3 regulates these processes through direct activation (or repression) of its downstream target genes which control these pathways.

While PAX3 expression and its roles during embryonic development have been well described, its persistent expression in normal adult epidermal melanocytes has been confirmed only recently [16-18], and the precise role of PAX3 in these cells is less well understood. It is possible that PAX3 controls maintenance [19] and survival of melanocyte stem cells, required for continuous repopulation of skin melanocytes, and facilitates their proper migration. It might also regulate survival of differentiated epidermal melanocytes and their expansion/ proliferation in response to sun exposure [20].

Notably, PAX3 is highly expressed in melanoma, and its expression correlates with melanoma staging/progression [21-26]. While PAX3 is known to contribute to melanoma cell survival [27,28], it is not known whether it continues to control pathways of differentiation, migration and proliferation in melanoma cells as it does during embryonic development, and whether this contributes to melanoma progression and metastatic spread. The analogy between developmental processes and tumourigenesis, and the pivotal role that PAX3 has during melanocyte development, has instigated us to further investigate PAX3 involvement in melanoma development/progression. Furthermore, due to a lack of conclusive information on the precise role of PAX3 in normal adult epidermal melanocytes, the significance of its continued expression in normal melanocytes as they progress through to melanoma is unknown. Further studies are required to determine the causal relationship between PAX3 and tumourigenesis and to determine the downstream molecular mechanisms involved in melanoma metastasis.

**Significance of the study**

For melanoma patients complete surgical removal of a primary lesion is the first line of treatment and this remains the most successful therapy [29]. Current therapies for advanced melanomas have limited success, but new targeted therapies are proving more successful [30-34]. Identification of the key regulators of melanoma progression would provide a tool for development of more effective therapies to halt metastatic spread.

PAX3 controls maintenance of the undifferentiated cell state, as well as proliferation, migration and survival, throughout melanocyte development and maturation. PAX3 might act similarly during melanoma-genesis, ie as a key intrinsic factor driving melanoma development and progression. We hypothesise that its activity drives the cells towards an undifferentiated proliferative motile state, which predisposes them to an aggressive phenotype upon malignant transformation.

This study is aimed therefore at clarifying the role/s of PAX3 in normal melanocytes and in melanoma cells. To achieve this aim we performed a detailed analysis of PAX3 expression in normal skin and in various melanocytic lesions, and identified downstream PAX3-regulated targets and associated pathways in melanocytes and in melanoma cells. Key differences, if any, in PAX3 function between these two cell types, are likely to provide strategic clues to the process of melanoma-genesis.

## ii. LITERATURE REVIEW

In order to uncover mechanisms of melanoma development and progression, we first need to understand the regulatory mechanisms acting in normal cells from which melanoma originates. This literature review is focused firstly on melanocytes, describing their development, function, key regulatory mechanisms, and the involvement of PAX3 in these processes. The review then focuses on melanoma and on PAX3-regulated pathways driving its development and progression.

### 1. Melanocytes: Development and function

Melanocytes are specialised pigment producing cells located in the skin, brain, inner ear and choroid layer of the eye (pigmented cells in the retinal pigmented epithelium are not of neural crest origin, rather they develop from locally derived neural epithelium of the optic cup) [35]. Human melanocytes of the skin are located in the hair follicles (termed follicular melanocytes) and at the epidermal-dermal border, above the basal lamina (termed interfollicular or epidermal melanocytes). In contrast, mouse melanocytes are generally located in the hair follicles, except for the hairless parts of the body (tail, nose and ears) where they are located in the epidermis [36].

### 1.1. Melanocyte development: From neural crest through melanoblasts to melanocytes

**Melanocytic origin and embryonic development**

Melanocytes originate from the neural crest in early developing embryos. The neural crest is an ectodermal derivative of vertebrate embryos, which produces a transient population of multipotent progenitor cells (Figure 1.1.1.A). These cells arise at the lateral edge of the neural plate, adjacent to the non-neural ectoderm, and following the closure of the neural tube, they become located on the dorsal side of the neural tube. After delamination and migration from the neuroepithelium, these progenitor cells differentiate, committing to several

lineages, including melanocytes, neurons, bone and endocrine cells, smooth muscles and craniofacial cartilage (Figure 1.1.1.B) [37,38].

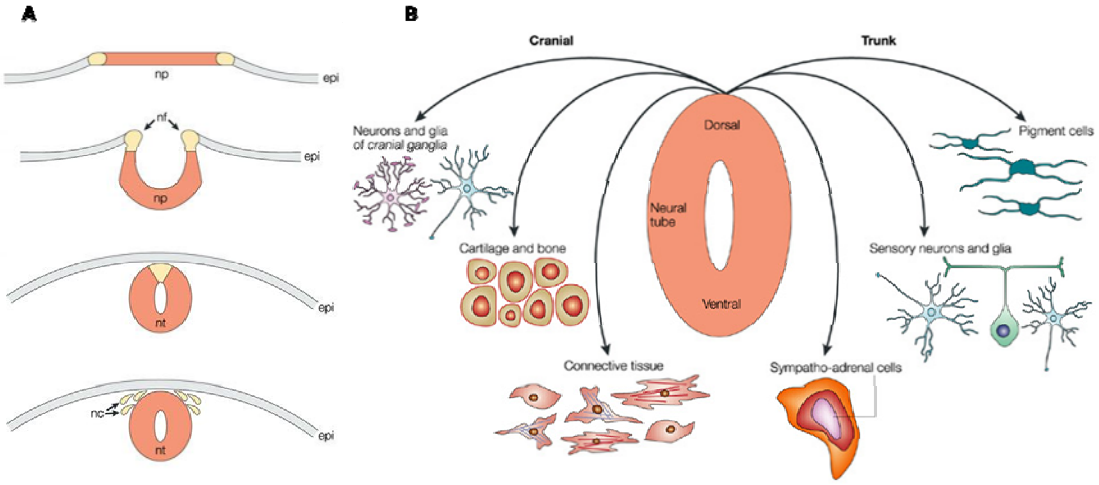


***Figure 1.1.1.*** **Neural crest formation and diversification.** A) Schematic representation of the emergence of neural crest progenitors at the boundary between the neural plate (np) and the non-neural epidermis (epi). Following the closure of neural folds (nf), neural crest cells become located at the top of the closed neural tube (nt), from where migrating neural crest (nc) cells are observed. B) Diagram of various cell types arising from multipotent neural crest progenitors (adapted from [38]).

A subset of neural crest cells (*Pax3*, *Sox10* (*SRY (sex determining region Y)- box 10*)-expressing cells) give rise to melanocyte precursors, termed melanoblasts, which are firstly observed in the cells overlaying and lateral to the neural tube. These cells are further characterised by expression of the melanoblast markers *Kit* (*v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene*), *Mitf* (*microphthalmia-associated transcription factor*) and *Dct* (*dopachrome tautomerase*) [12,39-43]. Melanoblasts expand/proliferate in the migration staging area, before entering the dorsolateral pathway and start migrating towards the developing epidermis [12,44,45]. Upon reaching their final destination in the skin, melanoblasts differentiate into melanocytes, expressing *Tyr* (t*yrosinase*) and *Tyrp1 (tyrosinase-related protein 1)*, marking the start of melanogenesis (melanin synthesis) [43]. In prenatal skin, a proportion of melanoblasts become incorporated into developing hair follicles, while others remain located at the border between the epidermis and dermis; the latter are lost in murine postnatal skin [46-48], whereas in human skin they persists postnatally [17,49-51].

**Regulation of melanocyte development: Role of Pax3 during embryogenesis**

Pax3 is one of nine members of the paired box (Pax) family of transcription factors, which have all been implicated in regulation of organogenesis and described as key factors in stem cell maintenance during development and maturation (reviewed in [52,53]). Pax3 is involved in development of the central and peripheral nervous system, skeletal muscle and cardiac tissue, gastrointestinal enteric ganglia, and skin melanocytes.

Pax3 is the first regulator of neural crest specification [10,11,54]. Its expression is observed in precursor cells prior to neurulation [17]. During neurulation, Pax3 plays a crucial **antiapoptotic** role, regulating proper development and closure of the neural tube, via inhibition of p53 (tumour protein p53)-mediated apoptosis, and activation of its targets, *BAX* (*BCL2-associated X protein*) and *HDM2* (*Mdm2 p53 binding protein homologue*) [55,56]. Here Pax3 is also crucial for proper **migration** of cells, via direct regulation of transcription of both *TGFα* (*transforming growth factor α*) and *TGFβ* (*transforming growth factor β*) [57,58]. TGFβ signalling pathways are involved in remodelling of the extracellular matrix, and cell cytoskeleton required for cellular migration [58-61]. Mutations or knock out models of either *Pax3*, *p53* or *TGFβ*, result in neural tube defects, confirming that Pax3-mediated regulation of cell survival and migration are crucial at this stage of development [55,62].

Following neural tube closure, expression of Pax3 continues in a number of progenitor cells, including those in the dorsal root ganglion, in the cells entering the migratory pathway in the dermomyotome, myoblasts, and in developing melanoblasts throughout their development (Figure 1.1.2.) [17,63]. Pax3 is crucial for **melanoblast specification**, being at the very pinnacle of the hierarchy of melanocyte-specific gene regulators, directly activating other key melanocytic regulators, including *Mitf* [64,65]. Pax3 is also required for **proliferation** of committed melanoblasts prior to their entry into the early migration staging area [12]. By contrast, Mitf promotes melanoblast survival during and immediately following migration from the dorsal neural tube to the migration staging area [12].

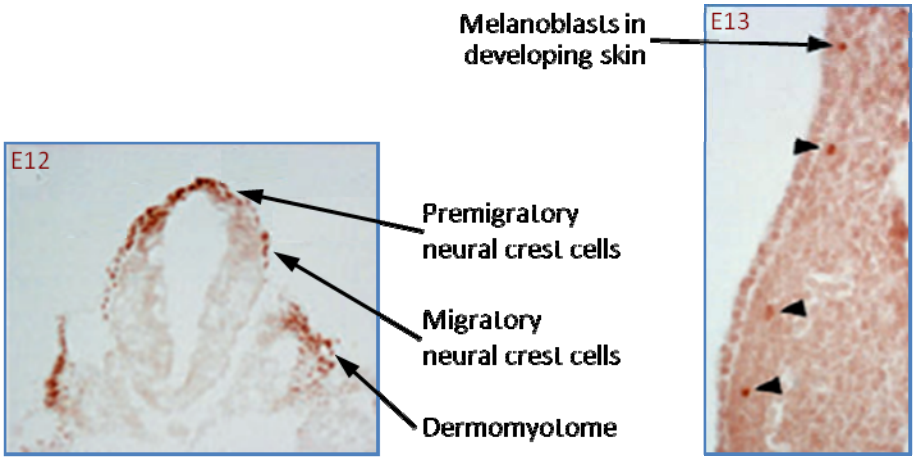


***Figure 1.1.2. Pax3* expression during embryonic development.** *Pax3* expression is observed in premigratory and migratory neural crest cells, and developing dermomyotome at E12, and in melanoblasts in developing skin at E13 of murine development (adapted from [66]).

It is clear therefore, that during embryonic development of melanocytes, Pax3 functions to specify, maintain and expand progenitor cells during completion of the morphogenic program, thus facilitating their proper migration at key points.

**The central role of Pax3 in melanocyte differentiation**

The regulation of differentiation is probably, thus far, the best described role of Pax3 in melanocytes. Pax3 directly binds to the *Mitf* promoter and, in synergy with Sox10, activates *Mitf* transcription [64,65,67]. On the other hand, Mitf is the key melanogenic regulator, activating the complete melanogenic cascade, from the melanogenic enzyme-coding genes (such as *Tyr*, *Tyrp1* and *Tyrp2*/*Dct*), to the genes encoding the structural components of melanosomes (such as *Pmel* (*premelanosome protein*, also known as *PMEL17*, *gp100*, or *SILV*) and *Mlana* (*melan-A*, also known as *MART1*)) (Figure 1.1.3.A) [68-71]. Thus by activating *Mitf*, Pax3 drives the cell along a differentiation pathway. At the same time, however, it competes with Mitf for occupancy of the *Dct* promoter, repressing its transcription and preventing terminal differentiation (Figure 1.1.3.B). Pax3 binding to the *Dct* promoter is mediated by the Groucho co-repressor (Grg4), which physically interacts with both Pax3 and Lef (lymphoid enhancer-binding factor 1); mutations in the Lef binding site in the *Dct* promoter abolish Pax3-mediated repression of *Dct* [19]. Lef1 also acts synergistically with Mitf to activate the *Dct* promoter [72]. In

the presence of nuclear β-catenin, however, Grg4, and hence Pax3, are displaced from the *Dct* promoter, allowing Mitf to bind to the response element within the *Dct* promoter (common to both Pax3 and Mitf), initiating its transcription (Figure 1.1.3.C) [19].

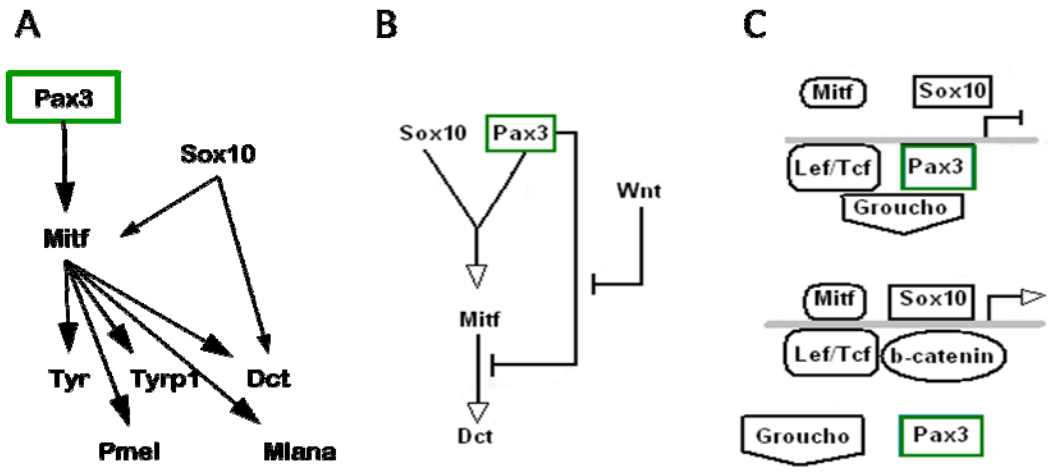


***Figure 1.1.3.*** **Schematic representation of regulation of melanogenic gene expression.** A) Pax3 activates a cascade of melanocytic gene expression. B) Pax3 activates *Mitf* (in synergy with Sox10) and represses *Dct* transcription at the same time. Wnt signalling abolishes Pax3 mediated repression of *Dct*. C) Pax3 binding to the *Dct* promoter is mediated by formation of a Groucho/ Lef/Tcf complex while β-catenin, activated by Wnt signalling, can displace Pax3 from the complex (Adapted from [19])

Pax3 also directly binds to the promoter region of the melanogenic enzyme, *Tyrp1* [73,74]. Pax3 can therefore regulate melanocyte differentiation both indirectly, through *Mitf*, or directly by regulating *Tyrp1* and *Dct* [75,76]. It is not certain however whether these mechanisms act at all stages of melanocytic development, or whether the latter mechanism is a common regulatory mechanism at all. Nevertheless, it is clear that Pax3 determines melanocytic cell fate while keeping the cell in an undifferentiated state, poised to differentiate in response to external stimuli [52]. It is likely that Pax3 prevents premature differentiation of melanoblasts during their migration, while contributing to their proliferation and expansion. Less clear is the function of Pax3 in melanocytes once they reach the skin.

## 1.2. Melanocytes of the adult skin

Human skin melanocytes are located both in the hair follicles and at the epidermal-dermal boundary (Figure 1.1.4.). Interfollicular/ **epidermal melanocytes** are lodged between basal cells, and form close relationships with epidermal keratinocytes, to which they distribute melanin, giving skin its pigmentation. **Follicular melanocytes** are located in the bulb of the hair follicle where they contribute to pigmentation of the growing hair. These two melanocyte populations are predominantly mature differentiated pigment-producing cells.

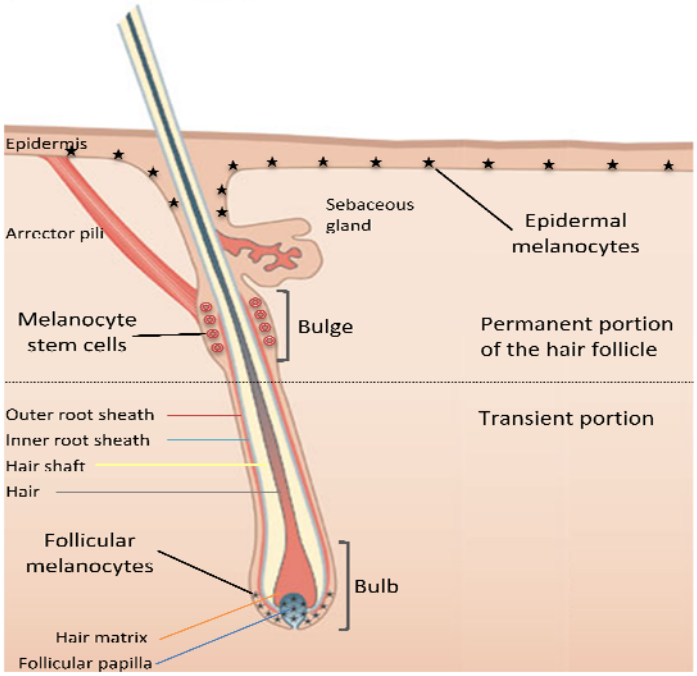


***Figure 1.1.4.*** **Distribution of three distinct populations of melanocytic cells in human skin.** This diagram represents a simplified structure of human skin and associated hair follicle. Two populations of mature differentiated melanocytes are located in the epidermis and in the bulb of the hair follicle. Melanocyte stem cells are located in the bulge region of the hair follicle (adapted from [77]).

Adult skin does however contain another population of melanocytic cells, melanocyte stem cells, located in the lower permanent portion of the hair follicle, just below the musculus arrector pili. This stem cell niche (called a 'bulge'), provides a reservoir of adult stem cells that give rise to new hair [78,79]. Bulge melanocyte stem cells represent a pool of progenitor cells serving to repopulate

lost melanocytes in the hair and presumably in the epidermis [77,80-82]. Melanocyte stem cells are immature, slow cycling and self-maintaining cells that remain quiescent in the niche until appropriate external stimuli initiate their proliferation, migration and differentiation [78].

Since the hair and hair follicle go through continuous cycles of loss and regeneration (Figure 1.1.5.), so therefore do follicular melanocytes. During the hair cycle, the upper portion of the hair follicle remains permanent (Figure 1.1.4.), in contrast to the lower transient portion, which undergoes cyclical stages of hair growth and loss. In the anagen phase, the newly formed hair shaft grows and hair protrudes through the skin surface. Following this, during the destructive catagen phase, the lower part of the follicle undergoes apoptosis. The dermal papilla is then brought to rest below the bulge, and after the resting phase (telogen), a critical threshold of activating factors is reached and the bulge stem cells become activated to regrow the hair. During the subsequent early anagen phase, hair follicle regeneration starts from the bulge area, followed by the downward growth of the basal portion of the follicle to form the hair matrix. This is accompanied by simultaneous formation of the new hair that grows upwards, finally protruding through the epidermis.

Bulge melanocyte stem cells are activated at early anagen to divide and generate a population of cells that remain in the bulge as stem cells as well as amplifying progeny that extend towards follicular papilla [78]. These two populations become segregated in the growing follicle and amplifying progeny localise to the hair matrix, proliferate and mature into fully differentiated melanocytes which produce melanin, transfer it to the hair keratinocytes and then die by apoptosis during catagen [83].

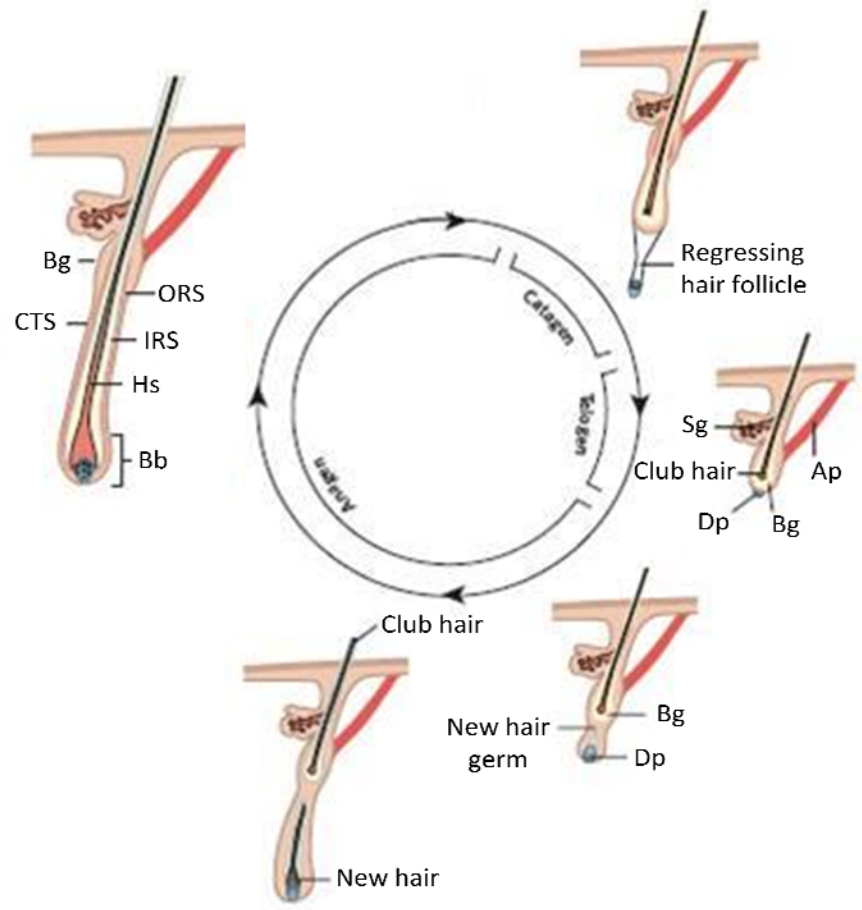


***Figure 1.1.5.*** **Schematic representation of the hair cycle.** The figure shows successive stages of the hair cycle: anagen, catagen and telogen. **Sg** is sebaceous glad, **Ap** is arrector pilli muscle, **Bg** is bulge, **Dp** is dermal papilla, **Hs** is hair shaft, **IRS** is inner root sheath, **ORS** is outer root sheath, **CTS** is connective tissue sheath (adapted from [84]).

## 1.3. Melanocyte function: Regulation of melanogenesis

The main function of melanocytes is pigment production (or melanogenesis), providing photoprotection and thermoregulation, thus contributing to the skin's protective function as the body's main barrier to the external environment. During melanogenesis, the precursor tyrosine is initially converted into dopaquinone, from which biosynthesis proceeds, via independent mechanisms, to produce two forms of melanin pigment, pheomelanin and eumelanin (Figure 1.1.6.). Production of pheomelanin is spontaneously initiated in the presence of cysteine, whereas eumelanin synthesis (in the absence of cysteine) involves action of three main

melanocytic enzymes: tyrosinase (TYR), tyrosinase-related protein-1 (TYRP1) and dopachrome tautomerase (DCT/TYRP2) [85,86].

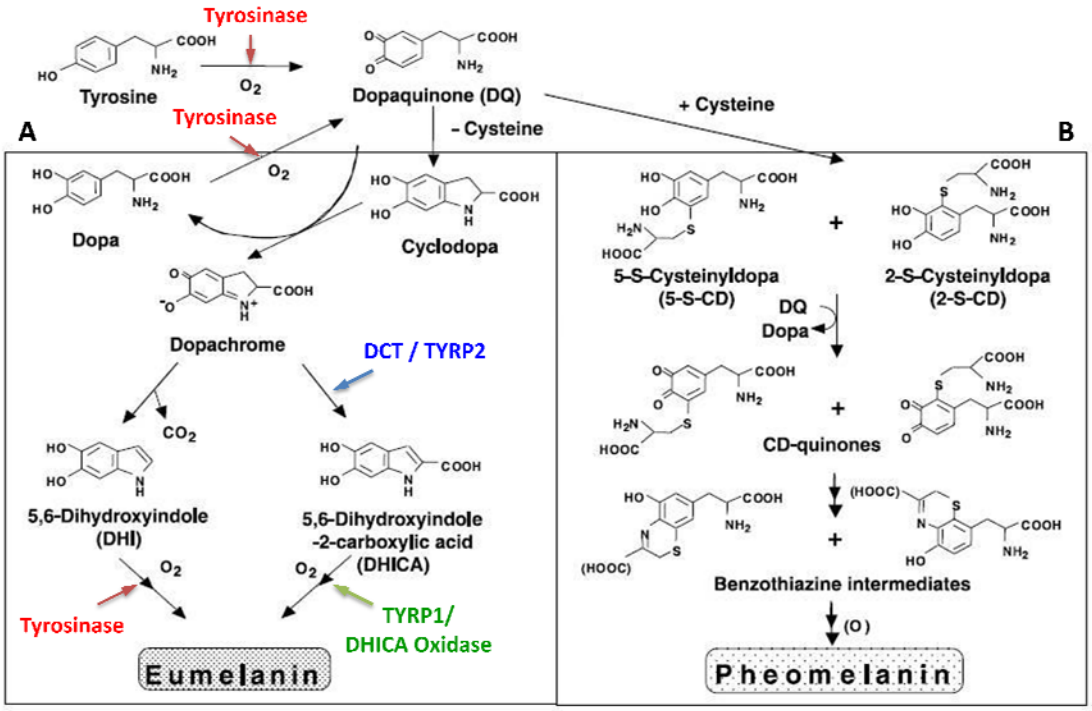


***Figure 1.1.6.*** **Biochemistry of melanogenesis.** When tyrosine is oxidized by tyrosinase, dopaquinone is produced as the immediate product. From dopaquinone two different reactions are possible, formation of eu- or pheomelanin. A) In the absence of cysteine, dopaquinone undergoes the addition of the amino group giving cyclodopa (leucodopachrome). The redox exchange between leucodopachrome and dopaquinone then gives rise to dopachrome (and dopa). Dopachrome gradually decomposes to give mostly DHI, and to a lesser extent DHICA. This latter process is catalysed by DCT. Finally, these dihydroxyindoles are oxidized to eumelanin: DHI oxidizes spontaneously (or faster with tyrosinase action); DHICA transformation is slower but needs TYRP1 catalysis. B) In the presence of cysteine, dopaquinone rapidly reacts with cysteine to give 5-S-cysteinyldopa and to a lesser extent 2-S-cysteinyldopa. Cysteinyldopas are then oxidized to give benzothiazine intermediates and finally to produce pheomelanin (adapted from [85]).

Melanogenesis takes place in the specialised melanocyte organelles known as melanosomes [87], where the pigment is synthesised and "packaged". The melanosomes are then transported to the adjacent keratinocytes via dendritic cytoplasmic extensions. Four morphologically distinctive stages are observed that correlate with successive stages of melanosome 'maturation' and biogenesis:

stages I and II are described as non-pigmented immature pre-melanosomes; whereas stages III and IV melanosomes are capable of melanogenesis (Figure 1.1.7.) [88-91].

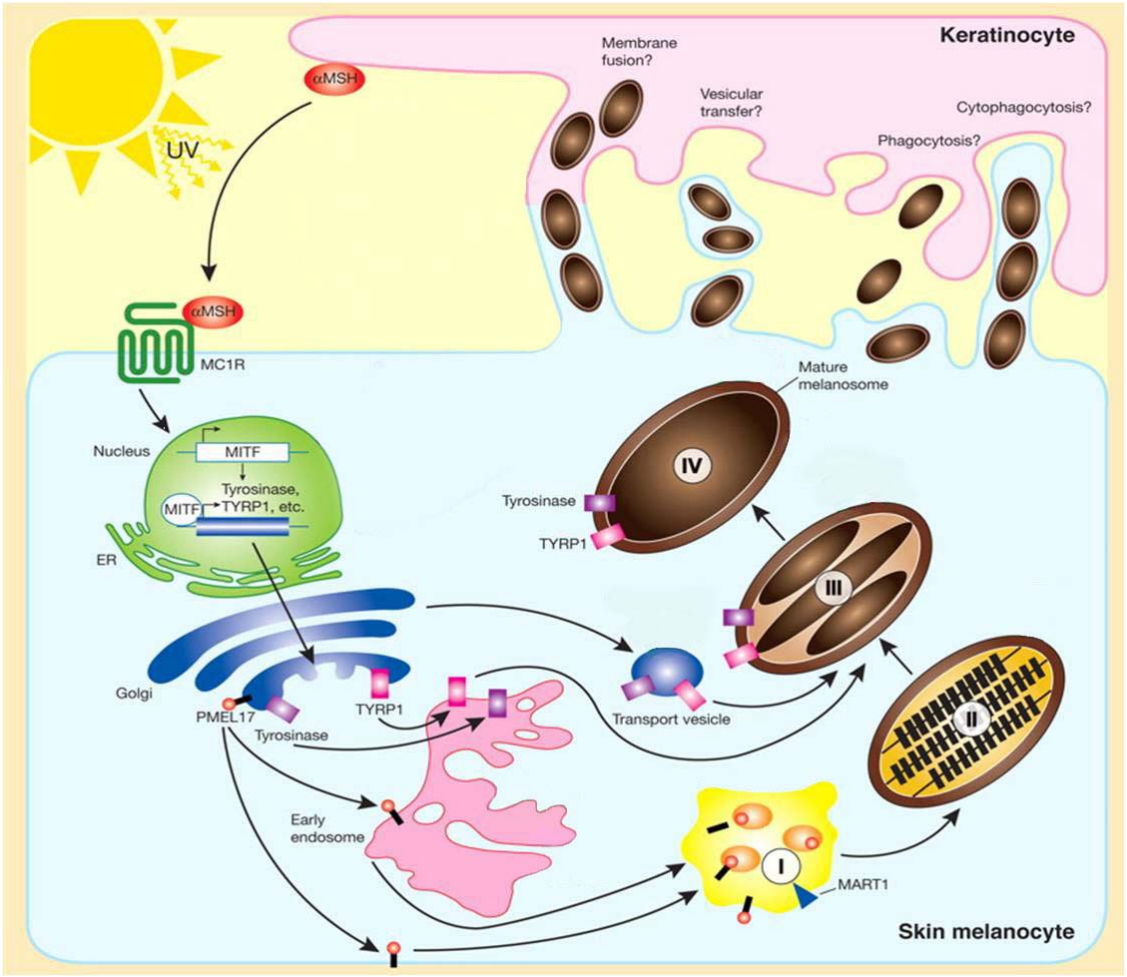


***Figure 1.1.7.*** **Melanosome biogenesis, maturation and secretion.** Melanosome stages I through IV are indicated. Non-pigmented stage I melanosomes contain the melanosomal protein Pmel17 sorted into intralumenal vesicles, and MART1. Stage II melanosomes are characterised by Pmel17 undergoing proteolytic cleavage, forming intralumenal fibrillar striations. The melanogenic enzymes TYR and TYRP1 are delivered to preformed stage II melanosomes from early endosomes or transport vesicles from Golgi complex which marks transition into stage III (adapted from [91]).

Stage I melanosomes are vacuolar early endosomes. Premelanosome protein PMEL plays an essential role in the structural organization of premelanosomes: in stage I melanosomes it is sorted into intralumenal vesicles, and later it undergoes proteolytic cleavage to generate intralumenal fibrils of stage II melanosomes [89,92-94]. Stage I melanosomes also contain MLANA, required for proper function

of PMEL and melanosome maturation [95,96]. The melanogenic enzymes TYR and TYRP1 are delivered to stage II melanosomes via secretory pathway from the Golgi complex and early endosomes (Figure 1.1.7.) [88,89]. Here they initiate melanogenesis and further maturation into stage III melanosomes, characterised by melanin deposition onto internal striations. Finally, stage IV melanosomes are fully mature melanised organelles.

Epidermal melanocytes and surrounding keratinocytes form a symbiotic functional relationship, termed the epidermal-melanin unit that consists of around 36 keratinocytes to each melanocyte [97,98]. The precise process of melanosomal transfer from melanocyte dendrites into keratinocytes is not clear, but possible mechanisms include: cytophagocytosis of dendrite tips by keratinocytes; exocytosis of melanin from melanocytes followed by their uptake by keratinocytes via phagocytosis; transfer via membrane-bound vesicles; or direct fusion of plasma membranes of the melanocyte and associated keratinocytes (Figure 1.1.7.) [99]. Once transferred from the melanocyte, melanin accumulates above the nuclei of keratinocytes, where it serves to protect mitotic keratinocytes from the ionising effects of UV irradiation [100]. This is achieved by scattering UV radiation or absorbing it and converting it into heat, or by absorbing DNA damage-causing free radicals generated in the cytoplasm (reviewed in [101]. In the event of extreme or repeated exposure to UV irradiation, increased synthesis of melanin is evident by "tanning". Complex signalling networks between keratinocytes, melanocytes and skin fibroblasts maintain skin homeostasis, and in the case of increased UV exposure result in an increase in melanogenesis as well as in the number and dendricity of melanocytes [20,102,103].

**Melanocyte signalling pathways**

A very complex and meticulously coordinated network of signalling pathways is involved in the regulation of the fate and function of adult epidermal melanocytes, with some pathway components contributing to more than one regulation pathway (reviewed in [36]). Keratinocytes have a significant effect on melanocyte proliferation as well as on melanogenesis, being the cells that are in a direct contact with the external environment, and thus mediating external environmental

signals to melanocytes. Keratinocytes stimulate melanocytes via secretion of **mitogens** and **melanogens**, such as α melanocyte-stimulating hormone (αMSH), adenocorticotropic hormone (ACTH), basic fibroblast growth factor (FGF), stem cell factor (SCF), and endothelins (ETs) [36].

Melanogenic signals and their receptors (like MSH, ACTH/MC1R (melanocortin 1 receptor), ET/EDNRB (endothelin receptor type B), Wnt (wingless-type MMTV integration site family)/frizzled family receptor) converge mainly on *MITF*, increasing its transcription and upregulation of pigmentation-specific genes, leading to increased melanogenesis. On the other hand mitogenic signals mediated by their membrane-bound receptors (like SCF/cKit, bFGF/FGFR, HGF (hepatocyte growth factor)/c-Met (met proto-oncogene)) activate receptor tyrosine kinases, and initiate a cascade of phosphorylation events involving cytoplasmic mitogen-activated protein kinases (MAPK) (reviewed in [36]).

Disruption in crucial signalling pathways regulating normal melanocyte function, usually as a result of mutations in key genes involved in these pathways, is strongly linked to melanoma-genesis (reviewed in [104]). Some of these pathways, relevant to this study, are described below.

Activation of **MAPK** (RAS-RAF-MER-ERK) pathway in melanocytes results in increased proliferation, survival, but also in posttranslational MITF phosphorylation [77]. MITF phosphorylation stabilises the MITF-p300 transactivation complex, enhancing its transcriptional activity on target genes, further contributing to melanogenesis [105]. Disruption and constitutive activation of this pathway, through activating *BRAF* (*v-raf murine sarcoma viral oncogene homologue B1*) or *NRAS* (*neuroblastoma RAS viral oncogene homologue*), or *cKit* mutations is recognised in many melanomas [104,106].

Another signalling pathway important for melanocytes is the canonical **Wnt signalling** pathway, acting through β-catenin and LEF-1/TCF. Wnt proteins bind to receptor complexes, including Frizzled-3 and co-receptor LDL Related Proteins (LRP5/6). The most proximal intracellular component in a cascade of activation is

molecular adaptor dishevelled (dsh) which gets recruited to the macromolecular complex containing adenomatous polyposis coli (APC) gene product, axin and glycogene synthase kinase-3β (GSK-3β). The formation of the complex blocks the phosphorylation of β-catenin by GSK-3β, which would target β-catenin for proteosome-mediated degradation [107]. This results in translocation of hypophosphorylated β-catenin into the nucleus where it cooperates with LEF-1 on target gene promoters, such as *Mitf or Dct*, enhancing their transcription, thus driving melanocyte differentiation and melanogenesis [19].

Two of the Wnt proteins that are also implicated in melanoma, Wnt1 and Wnt3a, are found crucial for development of the melanoblast [108]. Wnt1 promotes melanoblast precursor expansion or specification, and Wnt3a promotes differentiation along the melanocytic lineage at the expense of other cell lineages [109]. Both of these Wnt proteins are associated with key melanocytic regulators, Pax3 and Mitf; *Wnt1* expression is activated by Pax3 [110], whereas Wnt3a activates *Mitf* [111]. In adult epidermal melanocytes, inhibition of Wnt signalling by dickkopf homologue 1 (DKK1) secreted from fibroblasts in the dermis, suppresses melanocyte density and differentiation [112].

The opposing signalling pathway to Wnt, in terms of its effect on melanocytes, is **TGFβ signalling**. The TGFβ family of signalling molecules plays a role in regulation of a multitude of cellular processes in both normal and cancer cells, including wound healing, epithelial-to-mesenchymal transition (EMT), differentiation, proliferation, and response to the immune system [113-115]. Binding of one of the three main isoforms of TGFβ (TGFβ1, 2 and 3) to a type I and II TGFβ receptor complex, activates phosphorylation of the intracellular mediators SMAD family members 2 and 3 (SMAD2/3), resulting in their translocation into the nucleus, complexing with Smad4, and transcriptional activation/repression of target genes [116].

TGFβ (both 1 and 2) signalling has been shown to exhibit a repressive effect on both melanocyte differentiation and melanogenesis (via downregulation of *MITF* and *PAX3*) and to play a major role in driving immaturity and quiescence of

melanocyte stem cells in the bulge [20,117,118]. But TGFβ also inhibits melanocyte proliferation and induces apoptosis [116,117]. In skin, under normal conditions, secretion of TGFβ by keratinocytes inhibits proliferation of epidermal melanocytes, via *PAX3* suppression [20]. UV exposure of skin, however, induces downregulation of TGFβ, followed by *PAX3* upregulation, and increased melanocyte proliferation and melanogenesis [20].

Secretion of TGFβ1 by proliferating normal melanocytes is found to be induced by exogenous factors [119,120]. It is interesting that both *TGFβ1* and *TGFβ2* are upregulated by PAX3 [58,121], and *PAX3* itself is repressed by TGFβ1 [20], suggesting a negative feedback mechanism, involving PAX3 and TGFβ, which keeps melanocyte proliferation upon mitogenic stimulation under control [119]. A close functional relationship between TGFβ2 and PAX3 is even more evident since they share some common targets (such are *MITF*, *hairy and enhancer of split 1* (*HES1*) and *SRY (sex determining region Y)- box 9* (*SOX9*)), while having opposing effects on their regulation [122,123].

In contrast to normal melanocytes, loss of responsiveness to TGFβ–induced growth inhibition by melanoma cells is strongly linked to metastatic melanoma progression [119,124,125]. It is interesting that TGFβ1 treatment in growth inhibition-resistant melanoma cells does not downregulate *PAX3* expression, and overexpression of *PAX3* in TGFβ-sensitive melanoma cells results in their desensitisation [20], suggesting that resistance to TGFβ observed in some melanoma cells might be PAX3-mediated.

## 2. Melanoma

Melanoma is a highly metastatic tumour arising from cutaneous melanocytic cells. Some melanomas retain the physical characteristics of melanocytes, namely pigmentation, giving melanomas a highly pigmented phenotype; on the other hand some melanomas lose this ability and are amelanotic. Melanomas are, in general, significantly heterogeneous, in terms of cell morphology, level of pigmentation and marker expression [29,126]. Melanoma cells are also highly resistant to drug treatment by employing various mechanisms that confer multidrug resistance, including controlled drug uptake and export via membrane transporter systems, as well as drug trapping and export via melanosomes (Figure 1.2.1.) [127,128]. In addition, melanomas show a higher propensity to metastasise, relative to other skin cancers [129].

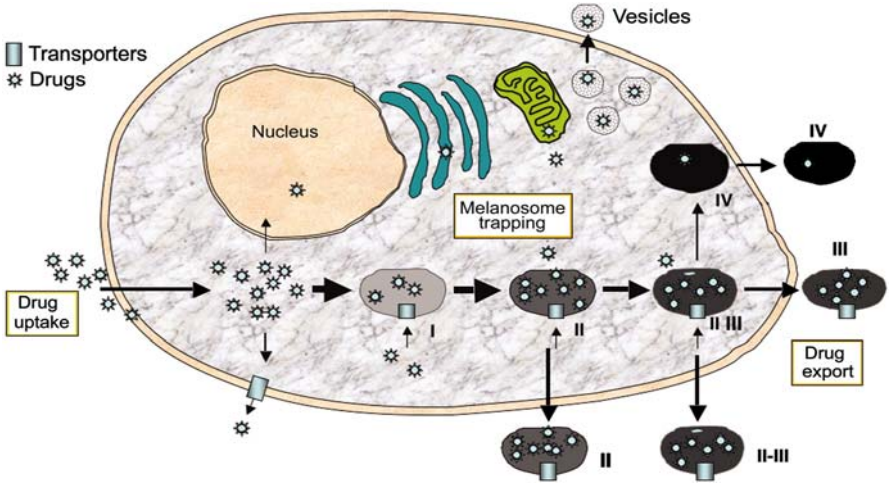


***Figure 1.2.1.*** **Drug resistance mechanisms in melanomas.** Melanoma cells become resistant to anticancer drugs by several sequentially occurring mechanisms. Initially drug resistance occurs as a result of reduced drug influx (e.g., reduced endocytosis, reduced activity of an importer, or increased energy-dependent efflux). Once drugs enter cells, they can be trapped in subcellular organelles, such as melanosomes or other vesicles, and exported from cells by enhanced melanosome transfer mechanisms. I–IV refers to stage I–IV melanosomes (adapted from [127]).

Morphologically, melanomas consist of atypical, rounded (epithelioid) or spindle (lentiginous) shaped cells, which initially aggregate in the epidermis and form 'nests' or 'branches' above the basal membrane. Histologically, several stages of melanoma development are evident, associated with worsening prognosis and

decreasing patient survival [8]. At early stages, melanoma is constrained within the epidermis. At this stage it is thought to have a low metastatic potential; with complete surgical removal, 5-year survival rates are high. As the tumour continues to develop, cells penetrate the basal membrane, and continue to expand in the dermis. The risk of metastasis gradually increases as the tumour invades deeper into the dermis. Once the melanoma reaches the subcutaneous fat tissue, the risk of metastasis is high and systemic metastases are likely to occur. Cells that are shed from the primary lesion infiltrate the circulatory and lymphatic system, and migrate to new sites where they adhere to the walls of the capillary and invade a new organ. It has been reported that 3-4 x $10^6$ cells are shed daily, it is not known however how many of these cells have metastatic potential [130]. At the secondary site, micrometastases can survive for several years before they become proliferative, stimulate angiogenesis, and begin to form a metastatic tumour [131]. Advanced stage melanomas are described by the presence of regional or distant metastases.

**Melanoma origin and progression**

The precise origin of melanoma is still uncertain and the exact mechanism of its development and progression remain to be elucidated. Several possibilities exist which explain melanoma origin, from epidermal melanocytes, or alternatively from melanocytic stem cells, or even from dermis-derived stem cells, suggested to give rise to epidermal melanocytes and potentially also to melanoma [132]. The likelihood of melanomas arising from bulge melanocyte stem cells or even follicular matrix melanocytes is low since most of the primary melanomas are observed at the epidermal-dermal junction. A more likely origin would be from epidermal melanocytic cells, since the existence of interfollicular melanocyte stem cells have not yet been confirmed [133].

Large numbers of melanomas, around 40%, are observed to arise from pre-existing precursor lesions [134]. A linear step-wise model has been suggested to explain melanoma progression from a melanocytic naevus through to a dysplastic naevus, followed by radial and vertical growth phases, via clonal evolution and acquisition of additional mutations (Figure 1.2.2) [104,106,135]. The majority of

melanomas however arise *de novo*, and do not necessarily follow this model of progression.

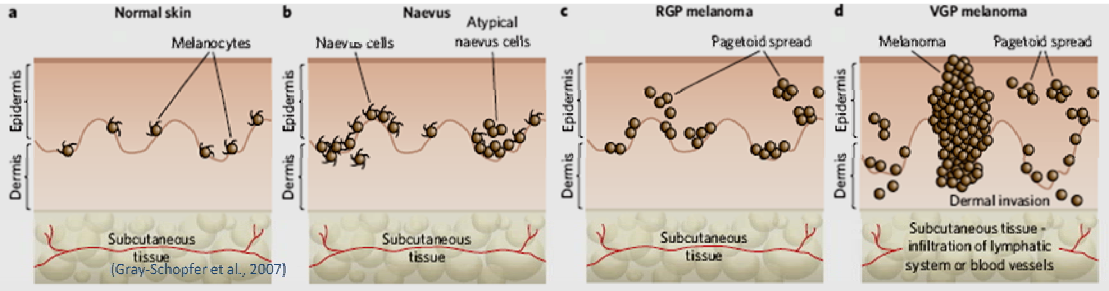


***Figure 1.2.2.*** **Schematic representation of the linear model of melanoma progression.** This diagram shows consecutive steps in melanoma progression, from (a) normal epidermal melanocytes, (b) through the formation of naevi, (c) pagetoid spread of atypical cells in radial growth phase, (d) to the vertical growth of the lesion and dermal invasion (Adapted from [136]).

According to the linear model, three major steps/events are required for melanoma progression, including clonal expansion of cells harbouring initial mutation/s, acquisition of additional mutations allowing cells to overcome senescence, and finally acquisition of mutations which result in suppression of apoptosis [104,106,137].

Initial mutations in melanocytes, such as activating mutations in *BRAF*, *NRAS,* or *KIT*, or amplification of *cyclin D1* (*CCND1*) or *cyclin-dependant kinase 4* (*CDK4*), result in their increased proliferation. Over-activation of proliferative signals (oncogenic stress) is known to trigger cellular senescence, and benign pigmented moles/naevi can be described as a senescent, clonal proliferation of melanocytes [104,106,137]. Additional mutations, such as inactivation of cyclin-dependant kinase inhibitor p16/ retinoblastoma protein (RB) pathways or activation of telomerase, are required to enable cells to overcome senescence and to become proliferative and immortalised [104,106].

Lesions with changes described above, are early malignant melanocytic lesions, known as superficial spreading melanomas. Melanoma cells at this stage are still dependant on keratinocytes and their signalling network for maintaining survival and can only proliferate close to the epidermis. This is described as the radial growth phase (RGP) of melanoma. Additional changes are required to allow

melanoma cells to escape keratinocyte dependence and invade deeper layers of the skin, a stage that characterises the vertical growth phase (VGP) of melanoma progression. This stage requires mutations that actively suppress apoptosis. Genetic alterations consistent with advanced melanomas include loss of phosphatase and tensin homolog (PTEN) (which inhibits apoptosis by serine/threonin protein kinase AKT activation), *RAS* and *RAF* activating mutations and β-catenin activation [106,138].

Changes also occur in a network of cell adhesion molecules that enable melanoma cell detachment. These include loss of E-Cadherin, essential for melanocyte homeostasis [139], and upregulation of N-Cadherin which mediates gap junctions with N-cadherin-expressing dermal fibroblasts [140]. Further changes that affect basal membrane integrity include upregulation of matrix metalloproteinase-2 that enables invasion of the deeper layers [141]. Additionally, dermal invasion correlates with the production of immune modifying factors such as interleukins, chemokines and TGFβ [125]. Finally, vascularisation and melanoma growth, seen at later metastatic stages, are regulated by several factors including connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) [142].

An alternative to the linear progression model, is the melanoma 'stem cell' model, in which progression towards a metastatic fate is driven by melanoma stem-like cells, as observed in other cancers [143-147]. According to this model tumours contain two distinctive cell subpopulations: one slow cycling population, that resembles normal stem cells in terms of ability to self-renew and give rise to a differentiating progeny, carry tumourigenic potential; and another that derives from these tumourigenic cells, has higher proliferative potential (maybe limited), but will not seed new tumours. Further research suggests that the two subpopulations of melanoma cells, with stem-like and more differentiated proliferative phenotype, are only temporarily distinct and that these phenotypes are actually interchangeable [124,148].

Interestingly, microarray analysis has identified two clearly distinguishable gene-expression profiles corresponding to the phenotype of two melanoma cell subpopulations: one population of proliferative, more differentiated, weakly metastatic cells which show a Wnt-signalling gene expression signature, reflected through upregulation of *MITF* and its downstream melanogenic targets; and a less differentiated/stem-like, highly motile aggressive population of cells, which show upregulation of TGFβ-signalling genes, involved in modifying the extracellular environment [124,125]. Induction of TGFβ-like signalling in melanoma, inhibits Wnt signalling by driving the expression of Wnt-inhibitor factors, thus leading to less proliferative but more metastatic melanoma cells. This scenario is reminiscent of normal melanocyte stem cells, residing in the bulge that is a Wnt–protected area, lacking MITF and other melanogenic markers, in contrast to their differentiating progeny in the Wnt-responsive hair follicle [118,149,150].

## 3. PAX3 expression in melanoma and other neural crest derived malignancies

The involvement of PAX proteins in cancer is well known [14,15,53]. A multitude of studies consistently show *PAX3* expression in melanoma [21-26,73,151]. Research from our own lab, shows *PAX3* in peripheral blood of melanoma patients at various stages of melanoma, including many years after removal of the primary lesion [152].

In addition to melanoma, PAX3 has also been found in tumours arising from other neural crest-derived tissues, such as classic medulloblastoma, benign peripheral glial tumour neurofibroma (precursor of malignant nerve sheath tumour), Erwin's sarcoma, and supratentorial primitive neuroectodermal tumour [17]. PAX3 is also know to be associated with paediatric alveolar rhabdomyosarcoma (ARMS), which is caused by the gene fusion resulting from the t(2;13)(q35;q14) translocation which juxtaposed the *PAX3* (or its analogous counterpart *PAX7*, t(1;13)(p36;q14)) N-terminus to the C-terminus of the Forkhead (*FKHD*) transcription factor [53,153-156].

Several studies have identified PAX3 as an immunogenic protein in melanoma, with several epitopes known to induce the host immune response [157-159]. Experimental stimulation of the immune response against *PAX3*-expressing tumour cells resulted in melanoma growth suppression and increased apoptosis [157,159]. In addition, inhibition of *PAX3* expression by siRNA causes apoptosis in both paediatric rhabdomyosarcoma and melanoma cells [25,28,155,156,160-162]. All of this evidence highlights the importance of PAX3 in tumourigenesis, clearly supporting its role in anti-apoptosis. It is not clear however if this is the main or only role for PAX3 in melanoma. Given the importance of PAX3 during embryonic development and suggested role in tumour progression, the possibility that PAX3 plays additional roles in tumourigenesis required further exploration.

## iii. SUMMARY

Many expression studies provide strong support for PAX3 involvement in melanoma-genesis, from the primary lesion, through circulating melanoma cells, to metastatic melanoma [21-26,73,151]. The suggestion that melanoma progression might be driven by melanoma cells showing less differentiated stem-like phenotype [124,144,147], and the crucial roles that PAX3 plays in regulation of melanocyte stem cells during development, implies that it might replicate these roles in melanoma. The analogy between developmental processes and tumour progression, such as delamination from the site of origin, migration through tissues and adhesion at secondary sites, provide further support for this hypothesis. Moreover, PAX3 is shown to be a nodal point in melanocyte differentiation, since it simultaneously functions to initiate the melanogenic cascade while preventing terminal differentiation, thus keeping the cell in a lineage restricted stem cell-like state [19].

Its expression in adult epidermal melanocytes has been uncertain, with only a few studies reporting PAX3 expression in human melanocytes [16,17]. It has been suggested that TGFβ-mediated *PAX3* upregulation under increased UV exposure induces melanocyte proliferation [20]. Similarly, ectopic expression of *PAX3* promotes growth, proliferation and migration of transfected melanocytes [163]. Taken together, the results highlight the importance of PAX3 in melanocytes, and justify the need to identify the precise mechanism of its regulation, not just in melanocytes but also in melanoma.

Normal skin melanocytes reside in a very dynamic microenvironment; on one side they are exposed to the outer macroenvironment, and on the inside to a continuum of cyclical changes. They have a close functional relationship with surrounding cells, regulated by cell-cell cross talk, to maintain homeostasis.

It is clear then that melanocytes are required to consistently maintain responsiveness to environmental cues. This includes cyclic melanocyte stem cell activation to proliferate giving rise to transient amplifying progeny cells that

migrate in the growing hair towards the matrix of the bulb, where the mature melanocytes will commence melanogenesis. Different signalling networks exist in the niche (Wnt protected area) to that in the growing hair follicle [118,149,150]. Once the progenitor cells exit the niche, further signals determine their migration and maturation. So here, melanocyte stem cell progeny respond to endogenous/intrinsic/ innate cycles dictated by the environment.

By contrast, epidermal melanocyte response is driven by exogenous/external signals, predominantly UV exposure (reviewed in [164]. As a result, melanocytes respond, by increasing in number and with increased melanogenesis [20]. It is not clear however, whether increases in epidermal melanocyte numbers result from their proliferation or from the activation and migration of bulge melanocyte stem cells. A different mechanism to that of UV induced melanocyto-genesis seems to be employed in wound healing and in epidermal repigmentation in the case of vitiligo, where newly formed melanocytes originate from the hair follicle [80,82,165-167]. Those processes are clearly driven, in both of these situations, by the loss of local epidermal melanocytes, and only melanocytic bulge cells are available for repopulation of the epidermis.

In summary, normal melanocytic cells have an in-built capacity to proliferate and migrate when required. Since PAX3 is one of the key regulators of melanocyte proliferation and migration during embryonic development, it is likely that its role is maintained in melanocytes of the adult skin.

Moreover, it is not known whether the same developmental processes are likely to be employed in melanoma development and progression. Initially malignant cells proliferate intensively, resulting in growth of the primary lesion. Some of these cells will be undifferentiated stem-like cells, being able to self-renew as well as produce progeny able to differentiate. Eventually, some of the melanoma cells will pass the basal membrane and invade the deeper layers of the dermis. This step requires cells to be able to actively migrate, modify their immediate surrounding, intercellular matrix, and survive independently of keratinocytes. Again, highly

motile melanoma cells will migrate into the circulation and spread to distal locations where they can generate metastases.

Given that PAX3 is a key regulator of these crucial cellular processes of differentiation, proliferation, survival and migration during melanocyte development, it may regulate similar processes in normal melanocytes, predisposing them to malignant transformation. Similarly, it might regulate these same processes in melanoma, given its high expression here, driving its progression.

## iv. RESEARCH DESIGN, HYPOTHESIS AND AIMS

Evidence supports a key role for PAX3 in melanocyte development during embryogenesis, regulating cellular processes such as differentiation, survival, proliferation and migration [10-12,14,15,19]. It is also known that PAX3 is highly expressed in melanoma [23-25,168]. The continued *PAX3* expression in postnatal/adult melanocytes, however, requires confirmation [16,17].

If PAX3 is indeed continuously expressed throughout melanocyte cell maturation the following question then remains: what is the role of this developmental regulator in adult differentiated cells? The logical assumption is that the roles would be similar to those of development. In addition, can it be assumed that the roles of PAX3 in melanoma are similar to those in melanoblasts and melanocytes, and if this is correct, does the mere presence of PAX3 in normal melanocytes predispose them to a 'plastic' state that increases their tumourigenic potential and then to a more aggressive phenotype upon malignant transformation?

Evidence suggests that at least some aspects of developmental PAX3 regulation are active in adult melanocytes and melanoma cells. When *PAX3* is transfected into a nontumourigenic mouse melanocyte cell line, it increases proliferation and migration [163], and inhibition of PAX3 results in melanoma growth suppression and increased apoptosis [28]. These results support PAX3 involvement in the regulation of melanocyte proliferation and migration, and similarly in regulation of melanoma cell proliferation and survival. A key role of PAX3 in development is in maintenance of an undifferentiated state, and it may be that PAX3 continues to perform this role in adult melanocytes, so they remain 'plastic' and responsive to environmental cues. Moreover, PAX3 may regulate survival and maintenance of melanoma stem-like cells, allowing their proliferation and migration. In this case PAX3 might play an active role in melanoma-genesis and metastasis.

**Hypothesis:** Given the persistent expression of PAX3 in cells of the melanocytic lineage, from the very precursor melanoblast cells of the developing embryo, to postnatal melanocytes, and finally throughout the progression of melanocytic lesions, we hypothesise that PAX3 is the key regulator of similar cellular processes in both normal melanocytes and melanoma cell as observed in developing melanoblasts. We also hypothesise that by regulating the 'plasticity' of melanocytic cells, PAX3 enhances their tumourigenic potential and increases the aggressive phenotype of melanoma cells upon malignant transformation, thus contributing to melanoma progression.

**The primary aim** of this project is to identify the role of the PAX3 in melanocytes and melanoma cells.

**Specific aims:**

1. To compare the functions of PAX3 in melanoblasts, melanocytes and melanoma cells.
2. To assess PAX3 expression in normal skin and in a variety of melanocytic lesions (including benign naevi and malignant melanomas), and to characterise PAX3-expressing cells with respect to markers of differentiation, proliferation, survival and migration.
3. To identify the roles of PAX3 by analysis of its target genes in melanocytes and in melanoma cells.

***Aim 1: To compare the functions of PAX3 in melanoblasts, melanocytes and melanoma cells.***

To address this aim, an intensive literature search was performed to identify the known roles for PAX3 during melanocyte development and melanoma-genesis. The outcome of this study was an all encompassing view of the role of PAX3 in melanoma progression, published in Medic and Ziman, 2009, the first publication towards this thesis (Chapter II).

***Aim 2:*** ***To assess PAX3 expression in normal skin and in a variety of melanocytic lesions, and characterise PAX3-expressing cells with respect to markers of differentiation, proliferation, survival and migration.***

To achieve this aim, PAX3 expression was assessed in various tissue samples, including normal skin, naevi and melanoma (in situ, invasive, systemic and lymph node metastases). PAX3 expression across the variety of samples was analysed by immunohistochemistry, and by quantitative RT-PCR.

To characterise PAX3-expressing cells, we initially performed co-expression analysis of PAX3 with a known melanocytic marker MITF to confirm that PAX3-positive cells were indeed melanocytic cells. Immunofluorescent double-staining was then used to further characterise the differentiation status of these cells. Based on the known PAX3-regulated pathways involved in normal melanocyte development and differentiation, the following markers were used here to more precisely determine the differentiation status of the cells: HES1 (marker of less differentiated melanocytic cells), and MLANA (marker of differentiated melanocytes).

Previous studies have implicated PAX3 in regulation of melanoma proliferation, survival and migration, providing us with an array of markers to use for further characterisation of PAX3-expressing cells in melanoma tissue. Markers used were for: proliferation (Ki67), survival (BCL2L1), and migration (MCAM). Again co-expression of PAX3 with these markers was analysed by immunofluorescence. The frequency of PAX3 co-expression with each marker was quantified in normal skin, in naevi, and in primary and metastatic melanoma samples.

The results of these experiments were published in Medic and Ziman, 2010, the second publication towards this thesis (Chapter III).

***Aim 3: To identify the roles of PAX3 by analysis of its target genes in melanocytes and in melanoma cells.***

To clarify the roles of PAX3 in melanocytes and melanoma cells we sought to identify its direct targets in both cells types. By investigating published literature we made a selection of confirmed and potential PAX3 target genes. These genes are representative of the specific PAX3-dependant regulatory mechanisms involved in differentiation, proliferation, cell survival and migration. ChIP-qPCR assay was used to analyse PAX3-binding to the promoter of the selected genes *in vitro*, in a metastatic melanoma cell line, and in primary neonatal melanocytes. By investigating the difference/similarity in PAX3 binding to its targets between melanocytes and melanoma cells, we were able to identify mechanisms exclusive to melanoma cells, but also confirmed mechanisms of PAX3 regulation common to both melanocytes and melanoma cells. Levels of expression of target genes were quantified to substantiate these findings.

The results of these experiments were published in Medic *et al.*, 2011, the third publication towards this thesis (Chapter IV).

# *Chapter II*

REVIEW ARTICLE

## PAX3 across the spectrum: from melanoblast to melanoma

**Authors and Affiliations**

Sandra Medic[1] and Melanie Ziman[1,2*]

[1] School of Exercise, Biomedical and Health Sciences, Edith Cowan University, Perth, Western Australia

[2] School of Pathology and Laboratory Medicine, University of Western Australia, Perth, Western Australia

[*] **Corresponding author**

Melanie Ziman

School of Exercise, Biomedical and Health Sciences,

Edith Cowan University,

100 Joondalup Drive, Joondalup WA 6027, Australia

Phone: (61 8) 6304 5171

Fax: (61 8) 6304 5851

E-mail: m.ziman@ecu.edu.au

**Running Title:** *PAX3* expression in melanocyte development and melanoma

**Citation:** Medic S, Ziman M (2009) PAX3 across the spectrum: from melanoblast to melanoma. Crit Rev Biochem Mol Biol: 1-13.

**Impact factor** (2010): 10.125

**ERA rank** (2010): A

**Abstract**

The PAX3 transcription factor is critical for the proper development of neural crest lineages including melanocytes. These cells show continued PAX3 expression from formation to differentiation. While many expression, misexpression and mutation studies clarify the importance of PAX3 in melanocyte development, less well understood, and more perplexing, is the continued PAX3 expression in the adult skin. In this article we explore the multiple roles of PAX3 in melanocyte genesis, and draw on evidence from expression in developing melanoblasts, adult melanocytes and melanocyte stem cells. From this, we present a more encompassing theory that PAX3 is a key regulator of the myriad steps in melanocytic cell determination. These roles may be accomplished by differential association with cofactors, via alternate transcripts or posttranslational protein modification(s). In light of the plethora of information gleaned from development we then consider its roles in melanoma and provide here a comprehensive consideration of the significance of PAX3 expression in melanoma. PAX3 and Pax3 indicate human and mouse transcription factor respectively.

**Key words:** *Pax genes; melanocytes; melanoblasts; melanocyte stem cells; MITF; melanoma*

## Introduction

Cutaneous Malignant Melanoma is the most aggressive form of skin cancer, thought to be derived from cutaneous melanocytes. Its aggressiveness is attributed to frequent metastasis and high drug resistance. Intensive research of the mechanisms regulating melanoma tumourigenesis has included investigation of the factors and pathways of normal melanocyte development and function. One key factor is the developmental transcriptional regulator PAX3.

PAX3/Pax3 (PAX3 and Pax3 indicate human and mouse transcription factor respectively) is a member of the Pax family of transcription factors which are highly conserved throughout phylogeny. All play a crucial role in embryogenesis but are also implicated in tumourigenesis (for reviews see [13,14,52,53,169-171]). Pax3 protein contains two DNA binding domains, a paired domain and a homeodomain which can be utilised alone or in combination to bind downstream target genes [76,172-174]. In addition Pax3 contains a C-terminal transcription activation domain and an octapeptide [173,175,176]. The ability of Pax3 to employ one or both DNA binding domains accounts for its ability to regulate a variety of downstream targets. Moreover, a single *Pax3* gene encodes multiple transcripts produced by alternate splicing (Figure 2.1) [11,151,168,177]. The resultant protein isoforms provide functional diversity for Pax3, as they differ in structure and ultimately in activity of their paired, homeodomain and alternate transactivation domains [177-179].

Even though Pax3 is recognised as a key embryonic regulator of melanocyte specification and development, its expression and function in differentiated epidermal melanocytes of adult human skin is uncertain and its role in melanoma remains unclear. By clarifying its functions during embryonic and adult melanocyte development we provide insights into its roles in melanoma.

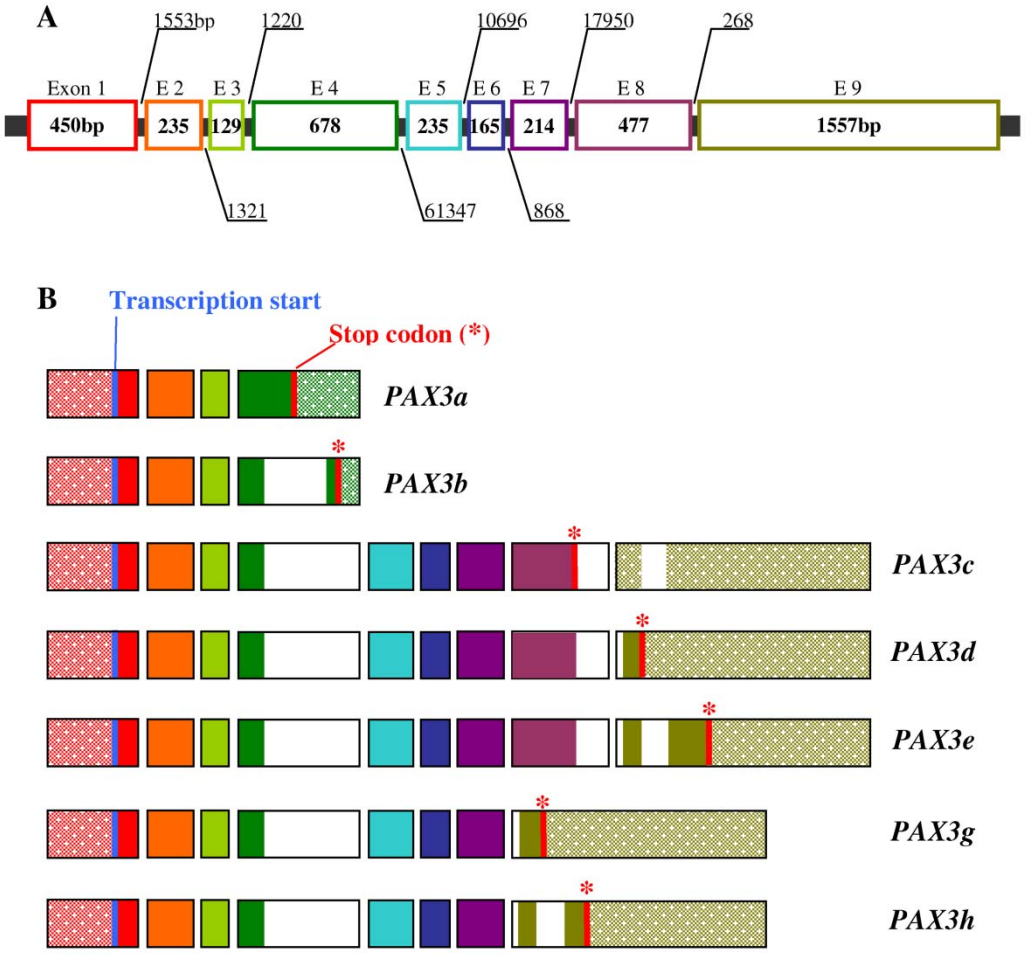


***Figure 2.1.*** **Schematic representation of human *PAX3* mRNA splice variants.** (A) shows the exons (E) (E1 to E9) and introns and their respective sizes. (B) shows the structure of alternative transcripts a, b, c, d, e, g, and h. Filled boxes depict sequences retained in mature mRNA, clear boxes represent sequences spliced out and patterned boxes are non-transcribed sequences; the vertical blue lines represent the transcription start sites and the vertical red lines and asterisks (*) indicate the positions of the alternate stop codons. This representation is based upon current information for human PAX3 mRNA available on NCBI (Evidence Viewer Tool).

## Melanocyte development during embryogenesis

Mammalian skin melanocytes originate from neural crest cells formed early in developing embryos. Neural crest cells are ectodermal derivatives characteristic of vertebrate embryos and represent a transient population of multipotent progenitor cells arising at the lateral edge of the neural plate adjacent to the non-neural ectoderm. After delamination and migration from the neuroepithelium,

these cells differentiate and contribute to various tissues, such as pigment cells, neurons, bone and endocrine cells, smooth muscles and craniofacial cartilage [37]. As the cells divide and migrate, multipotent neural crest cells acquire more lineage-specific phenotypes, including that of melanoblasts, which upon reaching its destination in the epidermis terminally differentiate into melanocytes.

Pathways crucial for the regulation of melanocyte development have been detailed in mouse studies. Key genes regulating these pathways are those encoding transcription factors Pax3 (Paired box 3), Sox10 (Sry-like HMG box 10) and Mitf (Microphthalmia transcription factor) (Figure 2.2A). *Pax3* is first expressed in neural crest precursors as they differentiate from the neural ectoderm [10,54]; expression continues as melanoblasts develop and migrate from the neural crest and persists in these cells in developing hair follicles [63]. Similar temporal expression is observed for *Sox10*, an early neural crest marker essential for the survival of undifferentiated neural crest cells [180].

Specification along the melanocyte lineage, first observed in neural crest cells overlaying and lateral to the neural tube at E10-10.5, is denoted by the expression of melanoblast markers *Mitf*, *Kit* (*Kit oncogene*) and *Dct* (*Dopachrome Tautomerase*) [12,39-43]. The melanoblasts expand a few hours later in the migration staging area from where they enter the dorsolateral pathway and migrate to the epidermis (E12.5-E13.5) [44,45,63]. Once in the epidermis, melanoblasts are incorporated into developing hair follicles and begin to express *Tyr* (*Tyrosinase*) and *TRP-1* (*Tyrosine related protein-1*) (E14.5) [43]. Melanogenesis marks the emergence of differentiated melanocytes (E16.5) [43].

Melanoblasts migrate to the epidermis and in humans differentiate into melanocytes which lie at the epidermal/dermal border [17,49-51], whereas in mouse these die off [46-48]. Melanocytes also populate the hair follicle matrix in both mouse and humans. These melanocytes show a molecular expression profile characteristic of maturing melanocytes, expressing *Pax3*, *Sox10*, *Mitf*, *Kit*, *Dct*, *Tyr*, *TRP-1* and *SILV* [150].

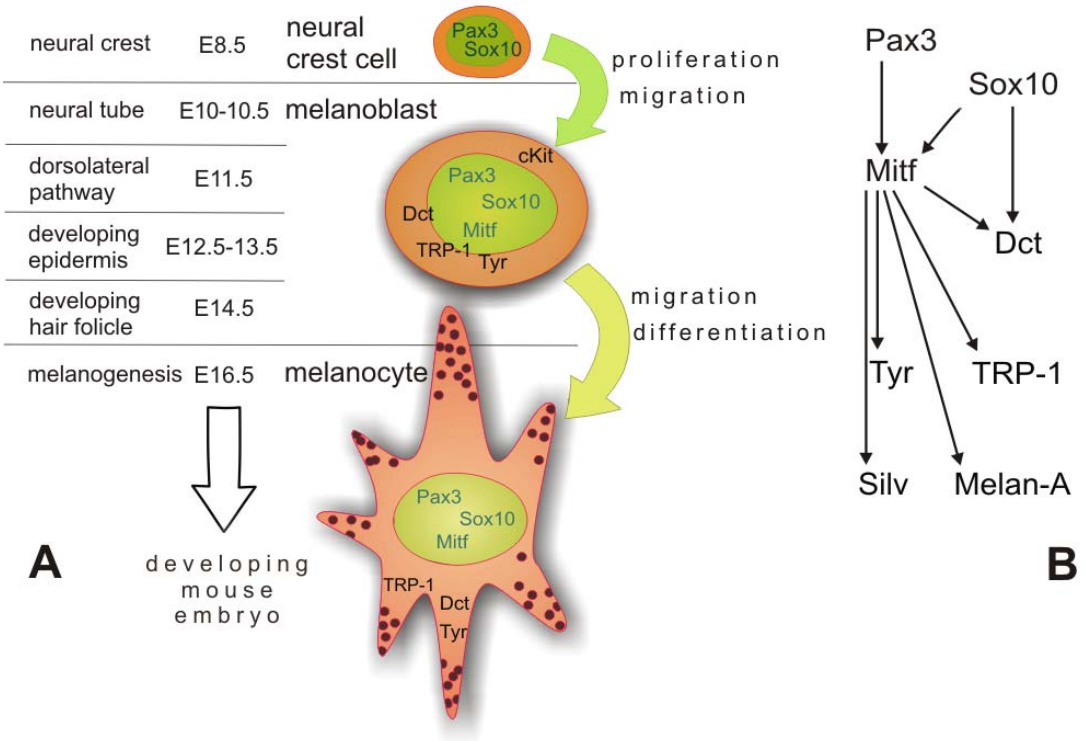


***Figure 2.2*. Gene expression patterns in murine melanocytes during development.** (A) represents temporal expression of the crucial genes during sequential stages of embryonic melanocyte development. (B) represents the hierarchy of melanocyte-specific gene activation.

### Key factors in melanocyte development

**Pax3,** also known as MSF (melanocyte specific factor) [73], is one of the earliest neural crest markers. It is expressed in neural crest precursors during neurulation, and later in the dorsal neural tube, dorsal root ganglion (DRG) and in cells entering the migration pathway in the dermomyotome [17]. Pax3 is crucial for neural crest specification [10,11], and later for expansion of committed melanoblasts formed early in development [12]. Mice that are homozygous for a mutation in *Pax3* show greatly reduced numbers of melanoblasts but the cells are able to migrate to characteristic locations along the migratory pathway [12], suggesting a role in specification and proliferation of melanoblast precursors but not in the migration process, at least at this stage of melanoblast/melanocyte development.

**Sox10** is a transcription factor critical for the survival of neural crest cell progenitors and proper differentiation of melanocytes [180]. Mice that are homozygous for a mutation in *Sox10,* lack *Mitf*- and *Dct*-expressing cells and have reduced numbers of *Kit*-expressing cells, due to the essential role that Sox10 plays in activating the promoters of these genes [64,181,182].

Both Pax3 and Sox10 are required, and precede expression of the transcriptional regulator **Mitf** [64,65,181]. Mitf is crucial for melanoblast survival during and immediately following migration from the dorsal neural tube to the migration staging area; mice that are heterozygous for a mutation in the *Mitf* gene show diminished numbers of melanoblasts but only in early stages of development, whereas during the migratory phase this number increases rapidly. $Mitf^{mi}/Mitf^{mi}$ mutant melanoblasts do not undergo dorsolateral migration (either they are not capable of migration or they don't survive) [12]. Mitf also has a role in melanocyte stem cell survival in adult tissue, since mice homozygous for $Mitf^{mi\text{-}vit}$ (vitiligo spontaneous mutation) exhibit vitiligo, characterised by initially normal pigmentation which is lost during the next hair follicle cycle [183,184].

***Dct*** encodes a melanogenic enzyme which marks the emergence of early melanoblasts. Several transcription factors are involved in the regulation of *Dct* expression including Pax3, Sox10, Mitf and Lef1 which all bind directly to the *Dct* promoter and act together to activate transcription [19,72,185,186]. The *Dct* promoter region contains an Mitf binding site (M-box) directly adjacent to upstream Lef1 binding sites [19,185,187]; Mitf and Lef1 act in synergy to activate the *Dct* promoter [72]. Pax3 and Mitf share the same binding site within the *Dct* promoter, and compete for occupancy [19].

In summary, the hierarchy of melanocyte-specific gene activation (Figure 2.2B) proposed by Opdecamp and colleagues (1997) suggests that in committed melanoblasts, Pax3 and Sox10 synergistically induce *Mitf* expression. Mitf and Sox10 then cooperate to immediately activate expression of *Dct.* Induction of *Tyr* and *TRP-1* by Mitf follows a few days later. Expression of most of the melanogenic enzyme genes, *Tyr*, *TRP-1* and *Dct*, as well as genes for melanosome biogenesis and

melanin stabilisation, such as *SILV* and *Melan-A*, begin in unpigmented undifferentiated melanoblasts, where they show perinuclear localisation but become cytoplasmic in fully matured melanocytes [188]. Unpigmented differentiating melanoblasts possess early immature, stage I and II melanosomes containing melanogenic enzymes [189]. Melanogenesis is however, a characteristic of later stage III and IV melanosomes which with maturation take position at the periphery of the cytoplasm [190].

**Melanocyte stem cells in the adult skin**

During embryonic development some melanoblasts will undergo transformation towards quiescent cells and form a population of melanocyte stem cells remaining in the bulge area of the hair follicles of adult mice and humans [48,191]. These quiescent cells are characterised as being Dct- and Pax3-positive [150]. Interestingly, other melanoblast markers expressed during embryogenesis, such as Sox10 and Kit, are not detected in bulge melanocyte stem cells, suggesting different mechanisms act to regulate production and maintenance of embryonal melanoblasts and adult melanocyte stem cells.

Pax3, Sox10 and Mitf determine the balance between melanocyte differentiation and maintenance of melanoblast and melanocyte stem cells in a process which is dependent upon Wnt signalling [19,111]. The prerequisite for maintenance of quiescent melanocyte stem cells is downregulation of the Wnt-dependant differentiation program. Indeed, the bulge area is described as a Wnt “protected” area with increased expression of Wnt inhibitors such as Sfrp1, Dab2 and Dkk3 in bulge cells [118,192]. These inhibitors decrease Wnt signalling as well as Mitf levels [111], and as a result induce cell cycle exit [193] and melanocyte stem cell quiescence.

**Multifunctional role of Pax3 during melanocyte development and maturation**

Studies of temporal gene patterning of melanocytes in the developing embryo indicate that although Pax3 is one of the first genes in the melanocyte specification hierarchy, it clearly plays a much broader, multifunctional role during normal melanocyte development (Figure 2.3). Here, we review the involvement of Pax3 in differentiation, survival and migration of melanocytes. By analysis of Pax3 function in normal melanocyte development we seek to gain not only a better understanding of its involvement in adult differentiated melanocytes, but also a greater appreciation of its role in melanoma, where it is commonly expressed.

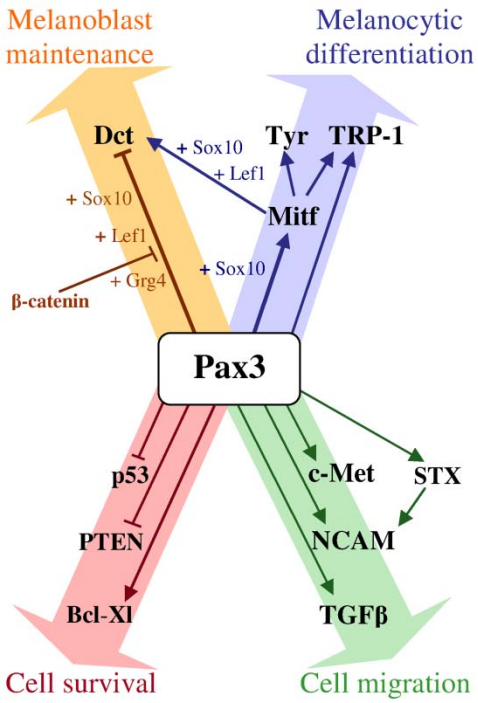


***Figure 2.3.*** **Multiple roles of Pax3 in melanocyte development and maturation.** Schematic representation of the cooperation between Pax3 and other factors involved in regulating "stemness", differentiation, survival and migration.

**Pax3 and maintenance of the undifferentiated state**

One of the best described roles for Pax3 is regulation of melanocyte differentiation; as recently suggested it acts as a "switch" or a "nodal point" in differentiation of these cells [19]. Pax3 is thought to activate melanocyte lineage specification but at the same time it acts to block terminal differentiation, thus acting to maintain a pool of undifferentiated melanoblast cells. A role in maintaining committed progenitor cells is similarly observed in other Pax3-dependant lineages, namely in neuronal [122] and myogenic (reviewed in [15]) lineages. In regulating neuronal precursors, Pax3 plays a dual role: at early stages of development it acts to maintain "stemness" of migratory neural cells, via the repressor Hes1. Later it initiates neuronal lineage specification via the proneural activator Ngn2 [122]. Hes1, is also implicated in maintenance of both embryonic melanoblasts and melanocyte stem cells in the bulge area of adult mouse hair follicles, possibly via Pax3 [194].

The pathways by which Pax3 maintains "stemness" have been detailed for melanocyte stem cells of the bulge area of the adult mouse hair follicle. Pax3 inhibits differentiation by binding to the *Dct* promoter acting to repress *Dct.* Grg4 (Groucho co-repressor) is also required for this interaction; it physically binds both Pax3 and Lef1 to forms a complex on the *Dct* promoter. Lef1 is also a cofactor for β-catenin (activated by Wnt signalling), which displaces Grg4 together with Pax3, allowing Mitf to bind to the response element within the *Dct* promoter. Pax3 has a higher affinity for the *Dct* promoter, thus replacing Mitf when present at equal or higher concentrations [19]. Mitf binding to *Dct* and other genes encoding enzymes for melanin synthesis *Tyr*, *TRP-1* initiates the melanogenic cascade. Thus Pax3 acts as a molecular switch to direct this process by binding either to the *Dct* promoter to inhibit differentiation or to the *Mitf* promoter in synergy with Sox10 to activate *Mitf* transcription [64,65,67] and the differentiation pathway.

In fact, Pax3 plays an even more complex role in regulation of melanocyte differentiation; Pax3 also has the ability to directly bind to and positively regulate the *TRP-1* promoter [73,74] thus enhancing the melanogenic cascade. In summary, Pax3 interacts with distinct recognition motifs found in the promoters of *MITF*,

*TRP-1* and *Dct* [75,76]. Notably, binding to the *MITF* promoter requires both the paired and homeodomain of the Pax3 protein in contrast to the *TRP-1* and *Dct* promoters where only the paired domain is required [76]. Different Pax3 isoforms may mediate these different Pax3 binding activities [53].

As noted above, the role of Pax3 in melanocyte development is far broader than just that of regulation of differentiation. Here we also describe the roles of Pax3 in melanocyte survival, maintenance and migration - roles that could implicate Pax3 in promotion of tumourigenesis and metastasis in melanoma.

**Antiapoptotic role for Pax3**

Mounting evidence supports an antiapoptotic role for Pax3. Several known antiapoptotic factors, such as tumour suppressors p53, PTEN and Bcl-Xl (see later), are direct downstream targets of Pax3 or mediators of Pax3-induced survival.

During embryogenesis, Pax3 regulates neural tube development via inhibition of **p53**-mediated apoptosis, keeping cells alive until the morphogenetic program is completed [55]. A neural tube defect observed in *Pax3*-deficient *Splotch* mice is mediated in part by p53-dependant apoptosis. Pax3 regulation of p53 may be via alteration of protein levels, rather than transcriptional repression, since there are no identifiable Pax3 binding sites in the promoter of the *p53* gene [55].

Pax3 has a dual effect on p53: it represses transcription of p53-dependant genes, *BAX* and *HDM2-P2*; and promotes p53 protein degradation [56,195]. p53 exhibits its pro-apoptotic function by promoting transcription of $p21^{Cip/Waf-1}$, cyclin-dependant kinase inhibitor, and members of the *BH3* family of pro-apoptotic genes (*BAX*, *PUMA* and *NOXA*). In overexpression experiments, Pax3 suppresses p53-dependant activation of both *BAX* and *HDM2* promoters, but not that of $p21^{Cip/Waf-1}$ [56]. In contrast, the Pax3 target, Mitf regulates $p21^{Cip1}$ expression both directly and indirectly, inducing G1 arrest [196]. Mitf cooperates with the hypophosphorylated form of Rb1, to activate $p21^{Cip1}$ expression, which contributes to cell cycle exit and activation of the differentiation program.

***PTEN*** expression is also directly inhibited by Pax3 [197], at least in myogenesis. PTEN regulates progression through the G1 cell cycle check point, by negatively regulating PI3K/AKT signalling, through cell cycle inhibitor (CDK inhibitor) $p27^{Kip1}$. Increased expression of Pax3 causes *PTEN* downregulation and a decrease in apoptosis through the PTEN/AKT pathway, accompanied by downregulation of $p27^{Kip1}$ [197]. PTEN also directly regulates p53 activity [198,199].

Thus, the apparent antiapoptotic function described for Pax3, a function presumably designed to facilitate migration of undifferentiated cells from the neural crest to the epidermis, may in fact enhance the survival of melanoma cells.

**Pax3 role in migration**

Embryonic melanoblast migration is important for movement of cells from the neural crest position to the epidermis. During embryogenesis Pax3 regulates several genes that promote cell migration, including receptor tyrosine kinases; c-Ret during enteric ganglia formation [200,201], and **c-Met** during limb muscle and melanocyte development [121,202-206].

Additionally, Pax3 directly represses expression of ***NCAM1*** [173,207], a cell surface molecule involved in cell-cell adhesion. Pax3 also activates expression of ***STX***, which causes posttranslational polysialylation of NCAM preventing NCAM-NCAM-mediated homophilic adhesion, leading to decreased cell adhesion and increased migratory properties [204,208].

Other key genes involved in embryonic neural crest migration are *TGFα* and ***TGFβ*** (reviewed in [209]), both of which are directly regulated by Pax3 [57,58]. TGFβ signalling regulates genes responsible for remodelling the cell-extracellular matrix and adhesion molecule-receptors and the cytoskeleton, thus playing a critical role in the regulation of cell-cell adhesion, growth, differentiation and migration [58]. *TGFβ* knock-out mice show neural tube defects [62], and similarly Pax3-deficient *Splotch* mice show diminished levels of TGFβ and neural tube defects. Pax3 binds to a *cis*-regulatory element within the *TGFβ* promoter region, directly regulating its transcription [58].

Migration of melanoblasts is an important step in melanocyte development and Pax3 appears to facilitate this process. Presumably, Pax3 is also important for the movement of developing melanocytes form the bulge area to the matrix along the hair shaft and into the epidermis. Moreover, migration and dissemination of melanoma cells, a key factor in metastasis may indeed be PAX3 dependent.

**Pax3 function in differentiated melanocytes**

The wide spectrum of Pax3 functions performed at given points along the developmental pathway may be operational in adult melanocytes and may in fact continue in melanoma. Alternatively, Pax3 functions associated with embryonic melanocyte genesis may differ from those of adult cells and melanoma cells, or perhaps only a select few functions are activated in each of these cell types. In melanocytes, Pax3 probably functions together with Sox10 in maintenance of upregulation of Mitf and its downstream melanogenic genes to continually produce melanin. Its role in maintenance of differentiated melanocytes remains to be determined.

A key question that remains then is how are Pax3 functions regulated temporally? Pax3 accomplishes specific temporal functions via interactions with several specific cofactors present in a particular cell at any given stage of melanocyte development [52,210]. Since Pax3 may have both activating and repressing roles in transcriptional regulation [53,211], it might be that at one point in development it is responsible for repressing and at another moment activating differentiation processes, mediating and coordinating the cell fate in response to environmental cues [52].

Changes in binding affinity and efficiency to downstream targets are important determinants of Pax3 functional activity. In fact, in melanoblasts it preferentially binds to the *Dct* promoter and blocks its activation by Mitf [19], but still retains moderate activation of *Mitf* to maintain intermediate protein levels required for melanoblast proliferation; it could also be involved in migration and survival of undifferentiated committed melanoblasts. In melanocytes however, it shows

preferential binding to both components of the differentiation pathway, i.e. *MITF* and *TRP-1* [16], where it maintains melanocyte cell function. These results support the idea that PAX3 has different role/s at different stages of melanocyte development.

**Regulation of Pax3 function**

Another mechanism by which functional switching of Pax3 occurs is via modulation of protein activity by cell cycle regulator pRB (retinoblastoma protein) [212]. pRB family proteins have a dual role in both cell cycle regulation, and in cell fate determination [212]. As a check point in the cell cycle, pRB acts as a negative regulator by complexing with and inactivating E2F family members, repressing their transcriptional function [213-215] thus preventing cell cycle progression. Secondly, as a determinant of cell fate, active unphosphorylated pRB forms a stable complex with Pax3, repressing its transcriptional activity [212]. The implications of this are that pRB repression of E2F transcriptional activity facilitates cell cycle exit, and pRB repression of Pax3 transcriptional activity enables terminal differentiation (by reducing levels of active Pax3 protein, thus allowing accumulated Mitf to activate *Dct* expression and induce rapid terminal differentiation) or apoptosis (due to an antiapoptotic role of Pax3). In other words during cell proliferation active Pax3 protein levels are maintained. Upon exiting the cell cycle however, Pax3 activity as an inhibitor of terminal differentiation is suppressed, allowing the cell cycle to proceed.

Pax3 protein activity may also be regulated by phosphorylation and ubiquitination. A Ser205 phosphorylation site has recently been identified; but to date phosphorylated Pax3 has only been seen in proliferating mouse primary myoblasts [216,217]. Pax3 protein stability is regulated by ubiquitination and proteasomal degradation during adult muscle stem cell activation [218].

**Function determined by Pax3 levels**

It is not certain how Pax3 protein levels determine its function(s). In neural crest cells that are Pax3- and Sox10-positive but Mitf-negative, Pax3 expands the pool of undifferentiated cells. Pax3 protein concentration (together with Sox10) may need to reach a certain threshold in order to activate *Mitf* transcription and commit cells to melanogenic lineage [73]. Indeed the amount of Pax3 protein appears to be a key factor in determining its role in neural crest determination in developing Xenopus embryos where different levels of Pax3 are required for activation of different downstream targets. Intermediate doses induce Snail2 expression and neural crest formation, and in high doses Pax3 strongly induces Xhe, thus changing the cell fate towards that of a hatching gland cell [219].

Once Mitf is activated in melanoblasts it is possible that Pax3 function may be driven, to some extent, by relative Mitf levels; Mitf needs to exceed a certain threshold, much higher that the amount of Pax3, in order to drive the differentiation pathway. *In vitro* experiments suggest that once the level of Mitf reaches an amount significantly greater than that of Pax3 [19] repression of differentiation is no longer possible and the melanogenic cascade is initiated.

Factors that upregulate *Pax3* may also provide a clue as to its temporal functions. Transcription factors **N-Myc** and **c-Myc** are both regulators of *Pax3* transcription [220]. *Myc* is actively transcribed in proliferating cells but very little is found in senescent or differentiated cells. The cell cycle oscillation of *Myc* and *Pax3* mRNA levels was studied in cells *in vitro*; both are undetectable during starvation-induced growth arrest, but increase after addition of medium, yet decrease again when cells enter S-phase [220]. Peak *Pax3* expression lags behind *Myc* by a couple of hours, as expected for Myc-regulated transcription of *Pax3*.

In turn, Pax3 itself represses the activity of cell cycle regulatory genes *Rb*, *Myc*, and *p21* by interacting with co-repressor KAP1 [207]. Thus it appears that the levels of Pax3 may be regulated via a negative feedback loop, since Myc upregulates Pax3 which subsequently downregulates *Myc*.

Additionally, two POU transcription factors, **Brn-2** and **Oct-1**, are positive *Pax3* transcriptional regulators [221,222]. Bound as a monomer Brn-2 has a positive role in *Pax3* expression in B16 (mouse melanoma cell line) cells, however when bound as a homodimer it decreases *Pax3* expression [223]. *In vitro* experiments show BRN2 protein levels and DNA-binding affinity decrease during melanocyte differentiation [16]. By contrast, OCT1 levels increase during the differentiation process [16] indicating that either BRN2 or OCT-1 can regulate and maintain stable PAX3 levels.

**PAX3 expression in melanoma**

While PAX3 function in developing melanocytes is reasonably clear, its precise role in melanoma is undefined. Perplexingly, expression is observed in melanocytes of normal skin [17]; our own unpublished observations), in benign naevi [24] and in melanomas [21,23-25,73,151]. In fact, PAX3 has been identified as a significant marker for melanoma staging [22,26] and for detection of circulating melanoma cells [22,224]. It has also been identified as an immunogenic protein in melanomas [157-159], with several epitopes able to induce the host's immune response - stimulation of the immune response against PAX3-expressing tumour cells results in tumour growth suppression [157,159]. Based on the information gleaned thus far it is clear that the function of PAX3 in melanoma is more than merely a marker of cell type.

**PAX3 role(s) in melanoma**

As in development, PAX3 plays an antiapoptotic role in cancers such as melanoma and paediatric rhabdomyosarcoma [155,156,160-162]. Transfection with *PAX3*-specific antisense nucleotide (*PAX3*-As) induces increased cell detachment, growth reduction and increased apoptosis in transfected melanoma cell lines [28]. *PAX3*-As-transfected cells show increased numbers of p53-positive cells, but no change in *TP53* mRNA levels, confirming that PAX3 regulation of p53 is posttranscriptional [28].

Additionally, inactivation of the tumour suppressor PTEN is often found in PAX3-positive human tumours and tumour cell lines, and its overexpression in tumours results in cell cycle arrest and apoptosis via induction of $p27^{Kip}$ [225].

Transcription of *BCL-XL*, a member of the *BCL-2* family of antiapoptotic genes, is also directly regulated by PAX3 in rhabdomyosarcoma [226]. Treatment with *PAX3* or *BCL-XL* antisense oligonucleotides individually or in combination decreases cell viability to a similar extent, suggesting that they lie in the same antiapoptotic pathway [226]. Additionally, the PAX3 target MITF regulates *BCL-2* in melanocytes and melanoma [227].

In a manner similar to its regulation of neural crest-derived cell migration and possibly melanocyte stem cells from the bulge area of the hair follicle, PAX3 may facilitate dissemination of melanoma cells and metastatic progression. The mechanism by which PAX3 may mediate melanoma metastasis is via regulation of c-Met, the HGF (hepatocyte growth factor) receptor involved in the regulation of migration and cell motility in development. c-Met transfection of immortalised melanocytes resulted in their malignant transformation [203]. On the other hand, overstimulation of HGF induces activation of the MAPK pathway and Mitf phosphorylation which in turn induces recruitment of the transcriptional co-activator p300. This results in an increase in *c-Met* mRNA and protein since c-Met is a direct transcriptional target of Mitf [228]. Thus PAX3 regulates c-Met either directly or indirectly via Mitf [121,228].

**Opposing role(s) of PAX3 and MITF in melanoma**

Recent microarray analysis of melanoma tissue was able to distinguish two melanoma subgroups; one that is proliferative and weakly metastatic with a neural crest-like transcriptional signature maintained through Wnt signalling; the other that is strongly metastatic showing upregulation of genes involved in modifying the extracellular environment through TGFβ signalling [125,229]. Induction of TGFβ-like signalling in melanoma may inhibit Wnt signalling by activating the

expression of Wnt-inhibitors, leading to less proliferative but more metastatic melanoma cells.

Given that microarray data reflect the profile of the majority of cells within the tumour, it is possible that individual melanoma cells possess different metastatic potential determined by their individual gene expression profile; more differentiated melanoma cells expressing differentiation genes under Wnt signalling would have low metastatic potential whereas less differentiated and more stem cell-like melanoma cells would have higher metastatic potential regulated by TGFβ.

PAX3 is known to directly regulate *TGFβ* [58], whereas MITF is functionally regulated by Wnt3a [111]. Interestingly, levels of MITF are an important determinant of melanoma cell fate: depletion or complete loss of MITF results in cell cycle arrest and/or apoptosis; increased expression levels favour differentiation; and intermediate levels promote proliferation [193,227]. Indeed, overexpression of *Mitf* in a highly aggressive melanoma cell line resulted in morphological and behavioural changes towards a more differentiated and less aggressive phenotype, evident by an increase in *Tyr* and *TRP-1* expression, as well as an increase in p21 and p27 and arrest in G2/G1 cell cycle stage, together with a decrease in Ki57 and an increase in Bcl-2 [230]. Compared to the original cell line, altered melanoma cells were less tumourigenic as evidenced by later development of tumours and lack of liver metastases in injected mice.

PAX3 and MITF lie on the opposing sides of the differentiation regulation pathway, determining less and more differentiated melanocytes respectively; similarly perhaps they may dictate more or less differentiated melanoma cells that are more or less metastatic. Intriguingly, MITF does not appear to be regulated by PAX3 in melanoma, since PAX3 DNA-binding to *MITF* promoter sequences is relatively less efficient in melanoma cells than in melanocytes [16]. This suggests that in melanocytic transformation, PAX3 is involved in some other aspects of melanoma progression not *MITF* regulation.

**PAX3 and BRAF-regulated pathways in melanoma**

An additional mechanism by which PAX3 and MITF levels are regulated within the tumour cells as via BRAF mediated pathways. One of the genes most frequently mutated in both naevi and melanomas is the *BRAF* gene [231-234]. Activating *BRAF* mutations direct two downstream regulatory pathways: one stimulates the *Brn-2* promoter, increasing its expression which drives *Pax3* expression [222]. Interestingly, in a *BRN2*-negative melanoma cell line, OCT-1 levels are high, whereas levels of OCT-1 are low in a *BRN2*-positive melanoma cell line [16]. This may explain the persistent expression of *PAX3* commonly observed in melanomas [21,23-25,73,151].

The second pathways activated by *BRAF* mutation is MEK-ERK pathway which leads to decrease MITF levels as a result of degradation of the MITF protein [136,235]. This is evident since in tumours with *MITF* amplification, often seen together with a *BRAF* mutation, the actual levels of MITF are not elevated accordingly [136]. Interestingly, oncogenic BRAF exerts control over MITF on two levels. It downregulates the protein by stimulating its degradation, but then counteracts this by increasing transcription through BRN2. Thus oncogenic BRAF plays a critical role in regulating MITF expression resulting in protein levels compatible with proliferation and survival of melanoma cells [236].

Clearly, PAX3 and MITF levels are regulated independently and even synonymously by numerous disregulated pathways in melanoma and together these genes may contribute significantly to melanoma progression.

**Isoform mediated roles**

The myriad roles detailed for Pax3 may also be mediated by different isoforms during both development and differentiation. A recent *in vitro* study detailed transcript-mediated growth characteristics in differentiated melanocytes [163]: *PAX3a*, *b* or *e* transcripts showed decreased proliferation and migration; by contrast *PAX3c*, *d* and *h* transfected melanocytes showed increased proliferation,

migration and survival; *PAX3g* had no affect on melanocyte proliferation or apoptosis, but reduced migration; and *PAX3c*, *d*, *g* and *h* isoforms were shown to be associated with anchorage-independent growth, conferring the ability of otherwise anchorage-dependant melanocytes to grow in soft-agar [163].

It is interesting to note that *PAX3c* increases the migratory ability of transfected melanocytes [163], and microarray analysis of *PAX3c*-transfected cells show upregulation of MCAM (also known as MUC18, and CD146) [121,204]. MCAM is frequently upregulated in melanomas [229] and is associated with invasion and metastasis [237].

Notably, expression of PAX3 isoforms varies in different PAX3-associated cancers: c and d isoforms are predominant in melanoma and small-cell lung cancer [158,168], and g and h in neuroblastoma [163]; a, b and e are expressed at low or undetectable levels in all of the above tumours [163]. This suggests that full length isoforms might promote tumourigenesis, whereas shortened isoforms might repress tumour propagation. Indeed, microarray analysis showed downregulation of *PAX3a* and *PAX3b* transcripts in aggressive melanomas compared to normal melanocytes [238].

One explanation is that shortened PAX3 isoforms may compete with full-length isoforms and alter or inhibit their function [168]. Since PAX3a and b isoforms lack a homeodomain theoretically they cannot bind *Mitf*, but may bind and induce *TRP-1* [76] having an "immediate" effect on melanocyte differentiation. Thus the PAX3-induced migration, proliferation and survival of melanocytes may be mediated either by MITF, or requires a fully functional homeodomain or a specific subset of isoforms for activation of other target genes required for these processes.

**Conclusion**

Recently it has been proposed that melanoma, and tumours in general, contain tumour "stem" cells harbouring metastatic potential [145,146,239-242]. Given that melanoma stem cells may promote tumour growth and metastasis

[143,144,147,243] and since PAX3 is involved in maintenance of progenitor cells [19,122,244], its role in melanoma may be similar, i.e. to maintain the melanoma stem cell population. Further experiments to confirm this are underway.

Based upon the molecular signature of melanocyte stem cells in the bulge are of the hair follicle - i.e. positive for Pax3 and Wnt inhibitors, but negative for Mitf and its downstream melanogenic targets [150] - it seems likely that the melanoma stem cells would have a similar signature. Indeed, melanoma stem cells are in fact quiescent, slow growing, non-melanised cells with a stem cell marker signature, including ABCB5 [144,240].

Based on the information provided in this review, it is clear that the expression of PAX3 in melanoma is much more than merely a marker of the cell lineage. It may in fact be a key factor in determining melanoma cell fate as well as its migratory properties, thus influencing its metastatic potential and ultimately the course of the disease. Further research to clarify its specific role(s) in melanoma is required.

**Acknowledgement**

***Declaration of Interest:*** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

## *Chapter III*

EXPERIMENTAL ARTICLE

# PAX3 Expression in Normal Skin Melanocytes and Melanocytic Lesions (Naevi and Melanomas)

**Authors and Affiliations**

Sandra Medic[1] and Mel Ziman[1,2*]

[1] School of Exercise, Biomedical and Health Sciences, Edith Cowan University, Perth, Western Australia

[2] School of Pathology and Laboratory Medicine, University of Western Australia, Perth, Western Australia

*** Corresponding author**

A/Prof. Mel Ziman

School of Exercise, Biomedical and Health Sciences

Edith Cowan University

Joondalup Campus

PH: +61-8-63045171

Fax: +61-8-63045717

Email: m.ziman@ecu.edu.au

**Running Title:** PAX3 in Melanocytes

**Citation:** Medic S, Ziman M (2010) PAX3 expression in normal skin melanocytes and melanocytic lesions (naevi and melanomas). PLoS One 5: e9977.

**Impact factor** (2010): 4.411

**ERA rank** (2010): A

**Abstract**

***Background:*** Cutaneous Malignant Melanoma is an aggressive form of skin cancer, arising in cutaneous melanocytes. The transcription factor PAX3 regulates melanocyte specification from neural crest cells during development but expression in differentiated melanocytes is uncertain. By contrast it is frequently found in melanomas and naevi and is a marker for melanoma staging and detection. In this study we analysed the expression of PAX3 across the spectrum of melanocytic cells, from normal melanocytes to cells of benign and malignant lesions to better assess its function in these various tissues. *Pax3* and *PAX3* (italicized) refer to the mouse and human gene, respectively; whereas Pax3 and PAX3 (non-italicized) refer to the corresponding mouse and human protein.

***Methodology and Principal Findings:*** PAX3 expression was analysed by immunohistochemistry and qRT-PCR. Immunofluorescence was used for co-expression with differentiation, migration and survival markers. As expected PAX3 expression was observed in naevi and melanoma cells. It was also found in melanocytes of normal skin where it coexpressed with melanocyte markers, MITF and MLANA. Co-expression with its downstream target, antiapoptotic factor BCL2L1 confirms PAX3 as a cell survival regulator. PAX3 was also co-expressed with melanoma cell migration marker MCAM in dermal naevi and melanoma cell nests, but this downstream target of PAX3 was not present in normal epidermal melanocytes, suggesting differential roles for PAX3 in normal epidermal melanocytes and melanoma cells. Most interestingly, a proportion of PAX3-positive epidermal melanocytes in normal skin show HES1 and Ki67 co-expression, indicating their less differentiated proliferative phenotype.

***Conclusions and Significance:*** Our results suggest that a previously identified role for PAX3, that of regulator of an undifferentiated plastic state, may operate in melanocytes of normal skin. This role, possibly required for cellular response to environmental stimuli, may contribute to formation and development of melanocytic lesions in which PAX3 expression is prominent.

## Introduction

Cutaneous Malignant Melanoma is a highly aggressive form of skin cancer, thought to be derived from cutaneous melanocytes or melanoblasts. Its aggressiveness is attributed to frequent metastasis and high drug resistance. Intensive research into the mechanisms regulating melanoma tumourigenesis has included investigation of the factors and pathways of normal melanocyte development and function. One key factor that regulates melanocyte development from neural crest derived progenitor cells is the transcriptional factor PAX3.

PAX3 is very frequently expressed in melanomas and naevi [21,23-25,73,151]. In fact, PAX3 has been identified as a significant marker for melanoma staging [22,26] and for detection of circulating melanoma cells [22]. It has also been identified as an immunogenic protein in melanomas [157-159], with several epitopes able to induce the host's immune response - stimulation of the immune response against *PAX3*-expressing tumour cells results in tumour growth suppression [157,159].

PAX3 is a member of the PAX family of transcription factors which are highly conserved throughout phylogeny. All play a crucial role in embryogenesis but are also implicated in tumourigenesis [13-15,52,53,169,209]. Although PAX3 is recognised as a key embryonic regulator of melanocyte specification and development, its expression and function in differentiated epidermal melanocytes of adult human skin is largely unexplored and its role in melanoma remains unclear [1].

Pax3 starts its expression in early embryos, in neural crest precursor cells that later give rise to melanocyte precursors, melanoblasts [1,10,11,17,54,63]. Here the main function of Pax3 besides neural crest specification is regulation of melanoblast survival and proliferation [11,12]. Together with SRY (sex determining region Y)-box 10 (Sox10), Pax3 regulates expression of key melanocyte specification factor *Microphthalmia-associated transcription factor* (*Mitf*). It has been suggested that Pax3 controls a "nodal point" in melanocyte differentiation; it simultaneously activates *Mitf* and represses *Dopachrome-*

*tautomerase (Dct)* transcription thus blocking terminal differentiation [19]. Once Mitf levels reach a certain threshold this repression is removed, allowing Mitf, in the presence of β-catenin, to activate *Dct* expression and melanocyte maturation. This model of regulation of melanocyte differentiation suggests that *Pax3* expression is either negligible or significantly reduced in adult differentiated melanocytes.

Several studies report *PAX3* expression in naevi and melanomas [21,23], and show no expression in melanocytes of normal skin [24,25], thus concluding that *PAX3* re-expression might be involved in melanocyte transformation. In contrast to this widely accepted assumption, other studies note *PAX3* expression in melanocytes of normal skin [17,18], and more specifically its upregulation as a result of the UV-induced loss of TGFβ signalling from keratinocytes [20]. Additionally, *PAX3* expression has been described in cultured melanocytes [16,73] and melanoblasts with no significant change in levels of either mRNA or protein between these two cell types [16].

Here we confirm that PAX3 is present in the majority of naevus and melanoma cells. We also show conclusively that PAX3 is expressed in melanocytes of normal skin where it is co-expressed with known melanocyte markers. Since the precise role played by PAX3 in melanomas is not clear, we have looked into potential PAX3 regulated pathways within normal melanocytes. We have chosen pathway-representative markers that are also downstream targets of PAX3 and analysed their co-expression with PAX3 in normal skin melanocytes, naevi and melanomas. Interestingly, while the majority of PAX3-positive melanocytes in the epidermis of normal skin show a mature phenotype, a portion (around 20%) show a less differentiated and proliferative phenotype. Moreover, PAX3-positive melanocytes frequently show expression of BCL2-like 1 (BCL2L1, also known as Bcl-X, bcl-xL or BCL-XL/S), an antiapoptotic marker. Taken together these results indicate that melanocytes of the normal skin display a phenotype that is predisposed to malignant transformation (antiapoptotic, undifferentiated and proliferative). We also show that PAX3-positive epidermal melanocytes of normal skin are molecularly distinct from those of the hair follicle, which like melanoma cells

express Melanoma cell adhesion molecule (MCAM, also known as MUC18/CD146), associated with cell migration and melanoma progression and metastasis.

## Results

### PAX3 expression in normal skin, naevi and melanoma

PAX3 expression was analysed by immunohistochemistry in tissue sections from paraffin embedded samples of normal skin, naevus, primary melanoma and melanoma metastases. Positive staining for PAX3 was obtained after performing heat-induced antigen retrieval in an EDTA/Tris buffer, pH 8.0 and incubating the primary antibody (anti-PAX3, DSHB) at room temperature (Figure 3.1A, top panel). Antigen retrieval using the most common 10mM Citrate buffer, pH 6.0 with the same staining protocol, failed to produce a positive signal in several of these samples, and in those that were positive after citrate antigen retrieval, the intensity of staining was dramatically reduced. Negative controls included omitting the primary antibody from the procedure resulting in no immunohistochemical signal (not shown). Mouse monoclonal anti-PAX3 antibody (DSHB) has been previously confirmed by immunohistochemistry to be specific to PAX3 protein [245]. PAX3-specific staining was also confirmed using a different commercially available anti-PAX3 antibody (Invitrogen) (Figure 3.2, A-C and E). However, we have observed less intensive staining overall with this polyclonal antibody (Invitrogen) compared to that observed with the monoclonal (DSHB).

All of the samples analysed showed positive PAX3 staining in more than 10% of cells (Figure 3.1A, top panel). In normal skin PAX3-positive cells showed a pattern of distribution that is characteristic of melanocytes; i.e. they were found at the epidermal-dermal boundary and along the hair follicle (Figure 3.1A-D). When adjacent sections were stained with MITF, a known marker of melanocytes, a comparable pattern of distribution was observed (Figure 3.1A, bottom panel).

The intensity of PAX3 staining varied between cells within the same lesion and between different samples. There was no obvious correlation between either intensity or number of PAX3-positive cells and lesion type. However, PAX3-

positive cells of normal skin samples generally showed weaker staining and far fewer cells were positive, compared to those in naevi and primary melanoma samples. In contrast, melanoma metastases have an overall low level of PAX3 expression and these cells were mainly located at the periphery of the lesion.

Additional analysis by quantitative RT-PCR was performed on a separate set of samples, to confirm *PAX3* expression in normal skin, naevi, primary melanomas, and melanoma metastases. *PAX3* expression was detected in all naevi (5/5), primary melanomas (5/5) and melanoma metastases analysed (5/5), and in one out of two (1/2) normal skin samples. The highest levels of *PAX3* expression were found in melanoma metastases (mean fold change of 2583±1198), followed by naevi and primary melanomas with 101±39 and 73±55- fold change, respectively (Figure 3.1E). Even though these results suggest that *PAX3* might be upregulated in advanced melanomas (melanoma metastases vs. primary melanomas) comparison with immunohistochemistry results show that expression levels are not due to increased amounts of *PAX3* per individual cell, but rather they reflect the number of cells expressing *PAX3* (Figure 3.1A). Nevertheless RT-PCR results confirm *PAX3* expression in melanocytes of normal skin.

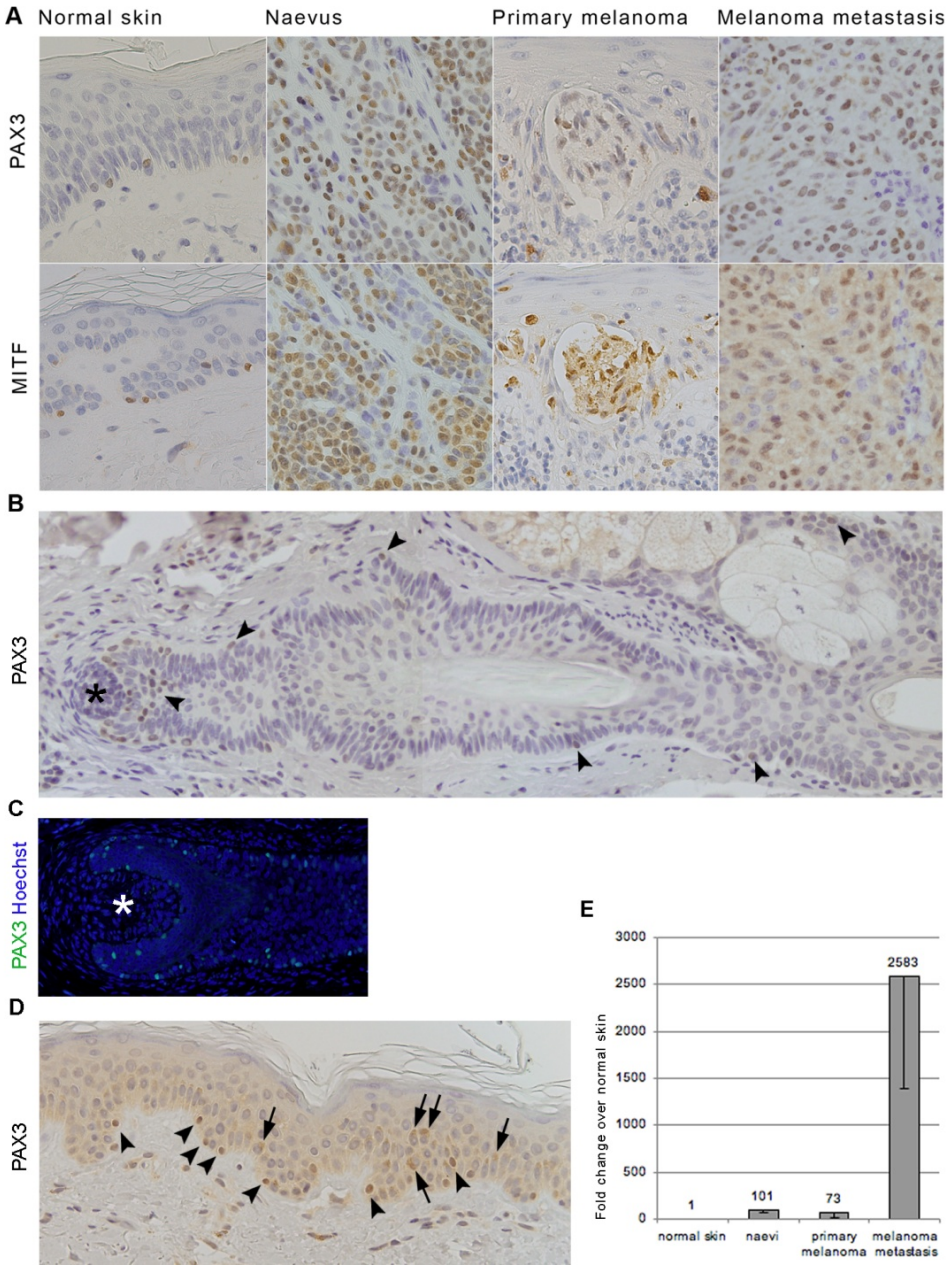


***Figure 3.1*. PAX3 expression in melanocytic and melanoma cells.** A) Immunohistochemistry shows PAX3 expression (top panel) in representative samples of normal skin, naevus, primary melanoma and melanoma metastasis, compared to MITF expression in adjacent sections (bottom panel). B-D) Show the distribution of PAX3-positive melanocytes (arrowheads) in normal skin: along the hair follicle (early (B) and late (C) anagen); and in the epidermis (D). Arrows in D point to the cytoplasmic melanin deposit distinguishable from the nuclear PAX3 staining (arrowheads). Asterisk in (B) and (C) marks a dermal papilla. PAX3 was labelled with mouse monoclonal antibody (DSHB). E) *PAX3* expression was analysed by RT-PCR and the graph shows the mean fold increase of *PAX3* expression in naevi, primary melanomas and lymph node metastases normalised to the expression in normal skin.

**Characterisation of PAX3-positive cells: Co-expression of PAX3 and MITF**

In order to validate PAX3 expression in naevi and melanomas as well as to confirm that PAX3-positive cells in normal skin are in fact melanocytes, co-staining of PAX3 and MITF was assessed by immunofluorescence. Indeed, in all of the samples analysed, including normal skin, PAX3-positive cells co-express MITF (Figure 3.2, A-C and E). However, in normal skin (as in other samples) a small number of cells showed only MITF and not PAX3 expression, highlighting the variation in normal epidermal melanocyte cell phenotype.

In normal skin samples 81.6% of all MITF-positive melanocytes were also PAX3-positive (Figure 3.2D). Both naevi and primary melanoma samples show similar numbers of MITF-positive cells that also express PAX3 (97.2% and 94.5%, respectively) (Figure 3.2D). Interestingly, melanoma metastases show significantly less PAX3-positive than MITF-positive cells (Figure 3.2D), and generally these were weakly stained for PAX3. However, this was observed primarily when using rabbit polyclonal antibody to PAX3 (Invitrogen) for immunofluorescent staining. When the mouse monoclonal anti-PAX3 antibody (DSHB) was used for immunohistochemistry staining, many more PAX3-positive cells were observed on the same sample. The discrepancy between these two results is probably due to the sensitivity of the two antibodies. Nevertheless, the intensity of PAX3 staining varied when either antibody was used, whereas MITF levels were uniform across samples (Figure 3.2, A-C). This is shown clearly in the A2058 metastatic cell line (Figure 3.2C) and may also reflect different cell status/phenotype.

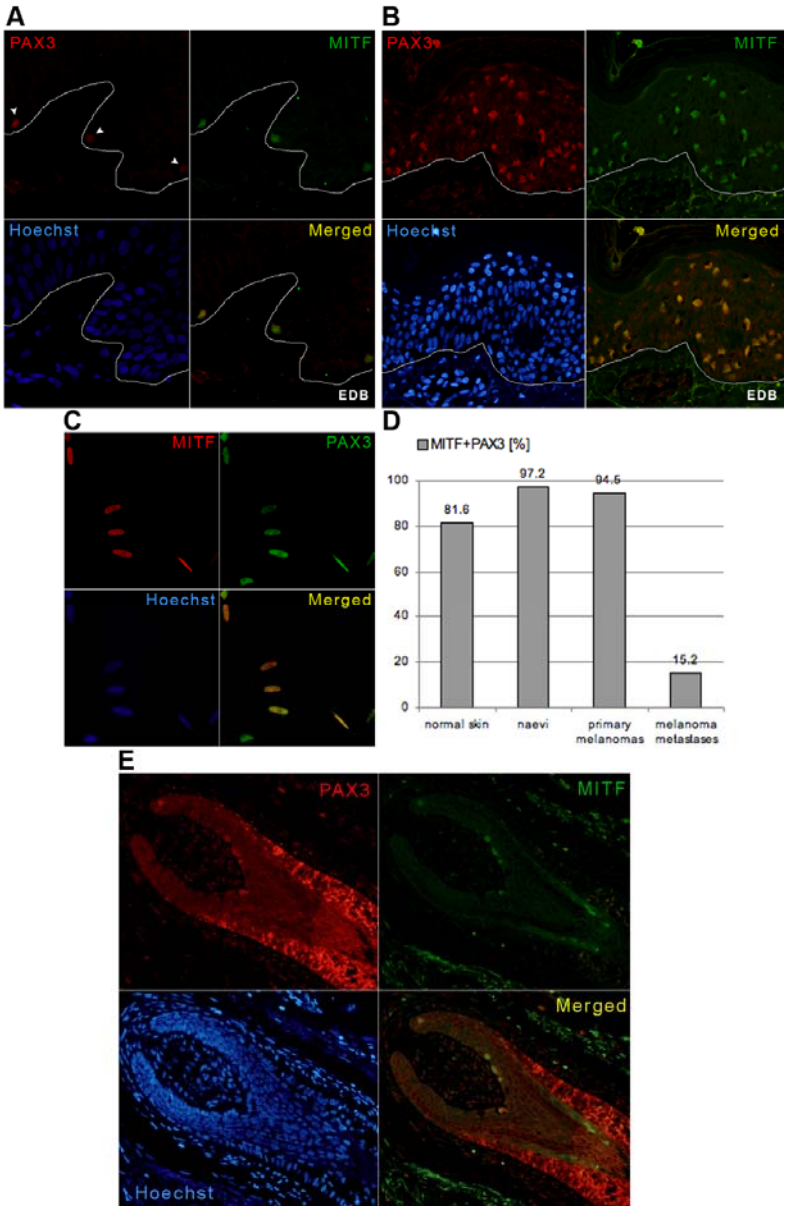


***Figure 3.2.*** **Co-expression of PAX3 and MITF in melanocytic and melanoma cells.** A-C) Double immunofluorescent staining showing co-expression of PAX3 and MITF in: (A) epidermal melanocytes of normal skin; (B) primary melanoma; and (C) in an A2058 metastatic melanoma cell line. Arrowheads in (A) show PAX3-positive normal epidermal melanocytes. Lines in (A) and (B) demarcate epidermal-dermal border (EDB). The variable PAX3 expression was clearly visible in A2058 cell line (C). For all these experiments depicted in the figure, PAX3 was labelled with rabbit polyclonal antibody (Invitrogen). D) Graph showing overall number of MITF and PAX3 double labelled cells in normal skins, naevi, primary melanomas and melanoma metastases. Each column represents a percentage of MITF-positive cells that are also PAX3-positive averaged across all samples. E) Double immunofluorescent staining shows PAX3 and MITF co-expressing melanocytes (yellow-orange) in the bulb of the hair follicle of normal skin. Note the single MITF-labelled melanocytes (green) at the base of the hair.

**PAX3 in melanocyte differentiation: Co-expression of PAX3 with MITF, HES1 and MLANA**

Even though the overall percentage of MITF-positive melanocytes that were also PAX3-positive in normal skin was not significantly different to either benign or malignant melanocytic lesions (Figure 3.2D), the distribution of cells that co-expressed PAX3 and MITF differed between the epidermis and hair follicles of normal skin. Around 90% of MITF-positive melanocytes in the epidermis and the outer root sheath (ORS) of the hair follicle were PAX3-positive, compared to only 65% of those in the bulbar area (Figure 3.3D). Interestingly there was a distinct location in the hair bulb, at the very base of the hair, populated exclusively by MITF-positive melanocytes (Figure 3.2E).

Given this difference between epidermal and follicular melanocytes, we thought it necessary to further characterise PAX3-positive melanocytes in the normal skin. We therefore co-stained PAX3 cells with Hairy and enhancer of split 1 (HES1) (Figure 3.3A and 3.4A-C) or Melan-A (MLANA, also known as MART-1) (Figure 3.3B and 3.4D, E), markers of less or more differentiated cell phenotype respectively. Upon analysis of the distribution of double-labelled cells we found that HES1 was expressed in a proportion of PAX3-positive melanocytes, while cells that were only HES1-positive were identified as keratinocytes [246,247] (Figure 3.3A). The number of PAX3-positive melanocytes co-expressing HES1 was lowest in the matrix of the hair bulb (10.5%), compared to the ORS (23.7%) and the epidermis (27.9%) (Figure 3.3D). By contrast, MLANA was expressed in more that 60% of both epidermal and ORS PAX3-positive melanocytes, compared to 36% of those in the matrix of the hair bulb (Figure 3.3D). We have also observed a significant number of single MLANA-labelled melanocytes (Figure 3.3B) (30.5% in the epidermis, 3.9% in ORS, and 40% in the hair bulb).

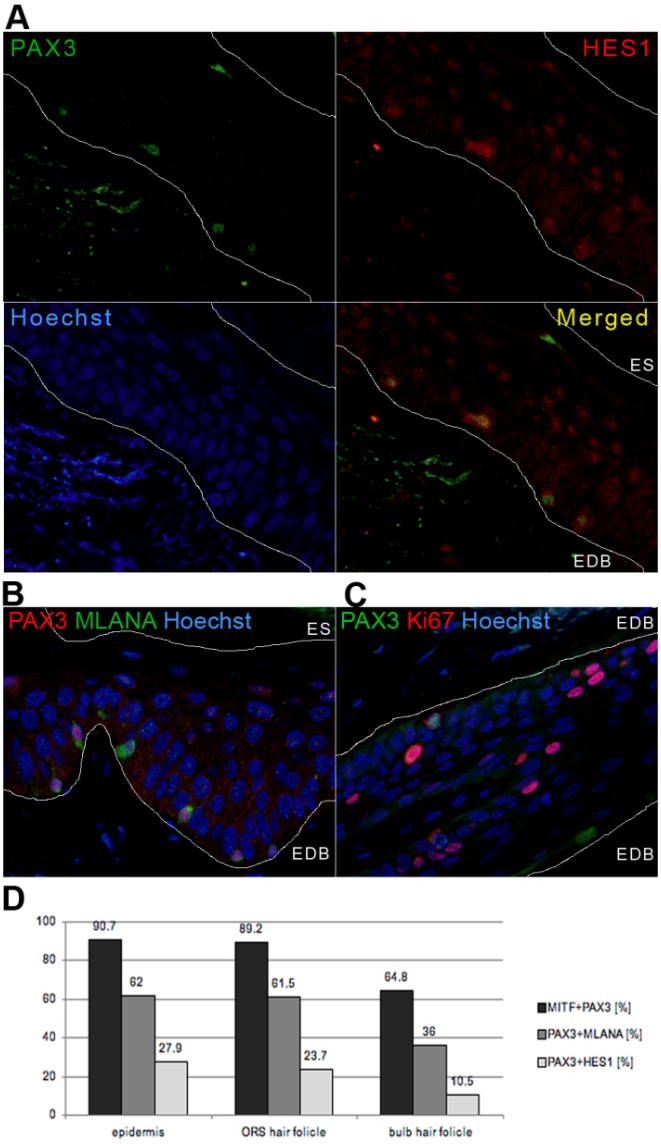


***Figure 3.3.*** **Co-expression analysis of PAX3-expressing epidermal melanocytes of normal skin.** Epidermal melanocytes of normal skin show variable differentiation status: less differentiated melanocytes co-expressing PAX3 (mouse monoclonal antibody, DSHB) and HES1 (A); more differentiated co-expressing PAX3 (rabbit polyclonal, Invitrogen) and MLANA (B); and mature melanocytes expressing only MLANA (B). Single HES1-labbeled cells in the epidermis are keratinocytes (A). C) PAX3 (mouse monoclonal antibody, DSHB) and Ki67 co-expressing melanocytes are also observed in the epidermis of the sun-exposed skin. Lines in (A), (B) and (C) demarcate epidermal-dermal border (EDB) or epidermal surface (ES). D) Graph shows the distribution of differentiation marker expression in normal skin melanocytes with respect to melanocyte location (in epidermis, outer root sheath (ORS), or hair follicle bulb).

This indicates that melanocytes, both follicular and epidermal, have a variable differentiation status: from less differentiated PAX3, MITF and HES1-positive to more differentiated PAX3, MITF and MLANA-positive. The observed PAX3-negative, MLANA-positive epidermal melanocytes may represent mature terminally differentiated melanocytes.

In addition, Yang and colleagues [20] have recently proposed that in UV-treated skin TGF-β signalling from keratinocytes is downregulated, which increases PAX3 expression in epidermal melanocytes and stimulates their proliferation. In order to check if PAX3-positive melanocytes observed here are proliferating we co-stained PAX3 with a marker of proliferation Ki67, and found 18.1% of epidermal melanocytes in samples of sun-exposed skin (scalp) to be double-labelled (Figure 3.3C). In contrast to this, in samples that had relatively little sun exposure (breast) one single double labelled cell was observed in the epidermis (accounting for 1.4% of PAX3-positive cells).

**PAX3 in cell survival: Co-expression with BCL2L1**

To further characterise the phenotype of PAX3-positive melanocytes relative to melanoma cells, expression of an antiapoptotic factor BCL2L1 was assessed in PAX3-positive cells (Figure 3.5A). We have observed that a similar proportion of PAX3-positive cells were also BCL2L1-positive in melanocytes of normal skin, in naevi and in melanoma cells in all of the samples analysed (Figure 3.5D). The exception is melanoma metastases, in which BCL2L1 was detected in only one sample (out of four) and both PAX3 and BCL2L1 were weakly stained and very rarely co-stain. BCL2L1 expression in epidermal keratinocytes, as reported previously [248], served as an internal positive control for the staining.

BCL2L1 expression in normal PAX3-positive melanocytes indicates that they might utilise the same cell survival regulatory mechanism as melanoma cells, in order to sustain continuous renewal in the ever-changing environment of the epidermis. It is possible that one of the roles PAX3 plays in adult melanocytes (similar to that in development) is to ensure their maintenance and survival, while regulating their specification and proliferation.

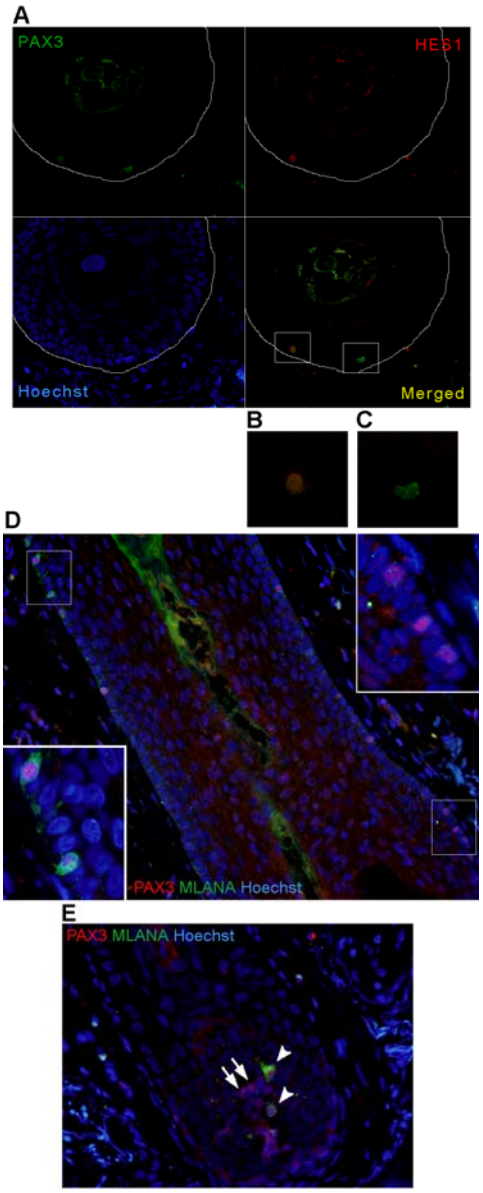


***Figure 3.4.*** **Co-expression analysis of PAX3-positive follicular melanocytes.** A) The transverse section of the hair follicle shows both PAX3 and HES1 co-expressing (enlarged in B), and single PAX3-expressing (enlarged in C), melanocytes in the outer root sheath (ORS). The line circumcises the hair follicle. PAX3 was labelled with mouse monoclonal antibody (DSHB). D) The longitudinal section of the hair follicle shows PAX3 and MLANA co-expressing (enlarged in the insert on the left) and single PAX3-expressing (enlarged in the insert on the right) melanocytes in the outer root sheath (ORS). E) Single PAX3-expresing (arrows) and PAX3 and MLANA co-expressing (arrowheads) melanocytes in the matrix of the hair bulb. PAX3 was labelled with rabbit polyclonal antibody (Invitrogen).

**PAX3 in cell migration: Co-expression with MCAM**

Expression of the melanoma progression marker MCAM was also analysed relative to PAX3. MCAM expression was observed in most naevi and melanoma samples analysed; 80% (4/5) naevi samples, 66.7% (4/6) primary melanomas and 100% (5/5) of melanoma metastases. There was no MCAM expression in epidermal melanocytes of normal skin (Figure 3.5B). Notably we frequently observed MCAM expression in hair follicle cells of normal skin [192,249,250], mainly in the ORS and not exclusively in melanocytic cells (Figure 3.5C). MCAM expression in the endothelial cells of the blood vessels served as an internal positive control [250,251].

In naevi samples MCAM expression was observed in specific areas, mainly in nests of cells in the dermal component of the naevus and only a small proportion (14.7%) of a PAX3-positive cells were MCAM-positive (Figure 3.5B, E). Compared to naevi, primary melanomas showed MCAM expression in a larger proportion of PAX3-positive cells, 56.4% (Figure 3.5B, E), which are again primarily located in the dermal component of the lesion. Samples of melanoma metastases showed MCAM expression in the majority (93.2%) of PAX3-positive cells (Figure 3.5B, E).

In general, MCAM co-expressed with PAX3 in all MCAM-positive samples. In accordance with previous reports [249], number of MCAM expressing PAX3-positive cells increased with malignant and particularly metastatic transformation. The expression of MCAM in follicular melanocytes, in contrast to the absence of expression in epidermal melanocytes, highlights again the difference between these two melanocyte populations.

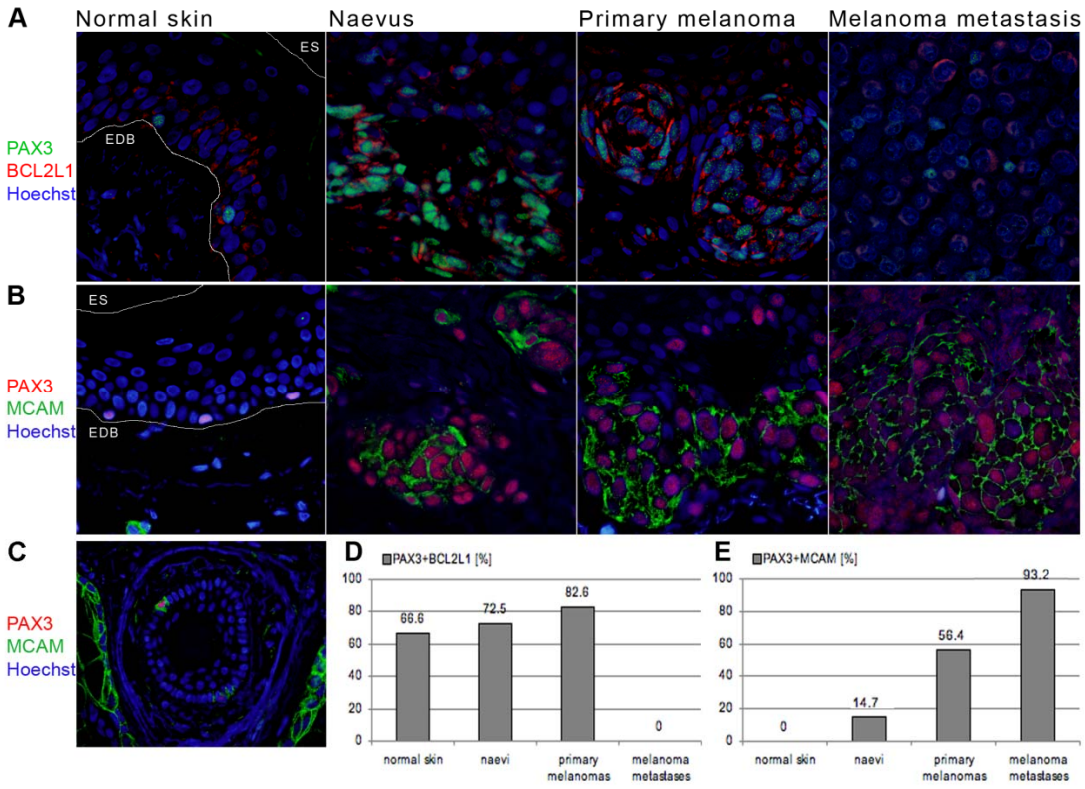


***Figure 3.5.*** **Co-expression of PAX3 with markers of cell survival and migration in melanocytic and melanoma cells.** A) Double immunofluorescent staining showing PAX3 (mouse monoclonal antibody, DSHB) and BCL2L1 co-expression in representative samples of normal skin, naevus, primary melanoma and melanoma metastasis. B) PAX3 (mouse monoclonal antibody, DSHB) and MCAM co-expression in normal skin (epidermal melanocytes), naevus, primary melanoma and melanoma metastasis. Lines in (A) and (B) demarcate epidermal-dermal border (EDB) or epidermal surface (ES). C) In contrast to the epidermal melanocytes, some PAX3-positive melanocytes in the outer root sheath (ORS) co-express MCAM. D) Graph showing overall number of PAX3, BCL2L1 double-labelled cells in normal skins, naevi, primary melanomas and melanoma metastases. Each column represents a percentage of PAX3-positive cells that are also BCL2L1-positive, averaged across all samples. E) Graph showing overall number of PAX3, MCAM double-labelled cells in normal skins, naevi, primary melanomas and melanoma metastases. Each column represents a percentage of PAX3-positive cells that are also MCAM-positive, averaged across all samples.

## Discussion

### PAX3 expression in normal skin, naevi and melanoma

In agreement with previous reports, we confirm here that PAX3 is expressed in all melanoma and naevi samples analysed. There is however a paucity of studies analysing PAX3 expression in differentiated melanocytes in human skin. Traditionally considered a critical embryonic regulator, PAX3 has been suggested to maintain melanocyte stem cells in the bulge area of the hair follicles in mice [19], but was thought to be downregulated upon terminal differentiation of melanocytes. This belief has been supported by reports detailing absence of PAX3 expression in melanocytes of normal human skin in contrast to naevi and melanomas [24,25]. Our results show conclusively that PAX3 is expressed in both epidermal and follicular melanocytes of human skin. Furthermore, co-expression with MITF confirms the melanocytic origin of these PAX3-positive cells. We also show here, for the first time, the variable phenotype of epidermal melanocytes of normal skin.

The observed frequency of PAX3 expression in naevi, melanomas and specifically in melanocytes in normal skin is much higher that reported elsewhere [24,25]. One possible explanation could be the methodology we used, mainly EDTA/Tris antigen retrieval buffer, rather than the citrate buffer commonly used by other researchers, which was, in our experience, less efficient. It might also reflect the influence of environmental factors, namely sun exposure and solar radiation. It has recently been suggested that in UV-treated skin TGF-β signalling from keratinocytes is downregulated, increasing PAX3 expression in epidermal melanocytes thus stimulating their proliferation [20]. All the samples used in this study are collected from people living in Australia, where the solar radiation levels are in the high to extreme range for much of the year. A recent report from New Zealand [18] shows PAX3 expression in all naevi, melanomas and normal skin samples, confirming our findings. Further studies are required to investigate differences in PAX3 expression in skin samples derived from the Southern vs. the Northern hemisphere.

Normal skin samples used in this study, mainly originating from anatomic locations frequently exposed to the sun (such as limbs and face), were free of any melanocytic lesion having been used for other diagnostic purposes. We have included here two normal skin samples from breast reductions which would have lower sun exposure levels and these samples also show PAX3-positive melanocytes in the epidermis. We have looked to see if PAX3-positive melanocytes in our samples were proliferative, by co-staining with Ki67. In our samples of sun-exposed skin, 18.1% of epidermal PAX3-positive melanocytes were proliferative, but the majority were not. This was in contrast to samples subjected to low levels of sun exposure, where we observed one single PAX3, Ki67-positive cell. However, to accurately address the question whether the high frequency of PAX3 expression in normal melanocytes observed in this study, relative to previously published papers, is caused in any way by the extent of sun exposure, a more thorough analysis of skin subjected to chronic and/or acute UV exposure relative to sun-protected skin, would need to be performed.

**PAX3 expression in melanocytes of normal adult skin: A role in regulating differentiation?**

PAX3 and MITF are crucial regulators of melanocyte development and not surprisingly have overlapping expression. To some extent they lie on opposing sides of the differentiation pathway, PAX3 being upstream of MITF and MITF initiating the melanogenic cascade and differentiation. We were interested to see if there is an association between expression of PAX3 and MITF (indicated by their co-expression) and the stage of melanocyte differentiation (based on the cell location, mainly in the hair follicle), but found no correlation. Studies on mice and human skin both show the existence of skin stem cells in the lower permanent portion of the hair follicle, or bulge [78,192,252]. It is reported that mouse bulge melanocyte stem cells are PAX3-positive and MITF-negative [150]; however no reports confirm this to be the case in human skin. In this study we were not able to find such cells. Instead the majority of melanocytes we observed, both in the epidermis and the outer root sheath of the hair follicle, were PAX3 and MITF-positive. In contrast to this, the matrix of the hair bulb has far fewer PAX3 and MITF-positive melanocytes and the base of the hair comprises solely MITF-positive

(PAX3-negative) melanocytes. Similarly, the number of PAX3, MLANA-double-labelled melanocytes in the epidermis and ORS were around 60%, but were much lower (around 36%) in the hair matrix. This would indicate that some epidermal melanocytes resemble more the undifferentiated transient amplifying cells of the growing hair rather than differentiated cells of the hair matrix.

On the other side of differentiation is the transcription factor HES1, suggested to prevent premature differentiation of melanocytes. Hes1 is reported to be a mediator of Notch signalling in skin development and in hair follicle maintenance where it regulates commitment of epidermal keratinocytes to terminal differentiation [247,253]. Hes1 is expressed in spinous layers and mediates spinous cell gene induction and thus terminal differentiation of epidermal keratinocytes [247]. In a mature hair follicle it is most prominent in the inner root sheath, in matrix cells committed to terminally differentiate to form the hair shaft [246,247]. On the other hand, in the melanocytic lineage, Notch signalling, acting through Hes1, plays a crucial role in survival of immature melanoblasts and melanocyte stem cells, by preventing initiation of apoptosis [194]. Moreover, it prevents differentiation of melanoblasts and melanocyte stem cells before they reach the hair bulb and regulates their proper location in the ORS and in the hair matrix [254]. Therefore it is not surprising that we see a decrease in the number of PAX3 and HES1-positive cells in the hair bulb compared to the ORS (10.5% and 23.7%, respectively). What is surprising is that around 28% of PAX3-positive epidermal melanocytes are also HES1-positive, indicating they are not terminally differentiated.

In summary, it seems that epidermal melanocytes are not all terminally differentiated and similar to those in the hair follicle they exhibit variable differentiation status, with a persistent PAX3 expression. Melanocytes in the ORS of the hair follicle are mostly PAX3 and MITF-positive and not all are fully differentiated (23.7% HES1-positive, 61.5% MLANA-positive). In the matrix of the hair bulb the number of PAX3-positive melanocytes is lower (around 65%), with only a small proportion still showing a less differentiated phenotype (10.5% HES1-positive and 36% MLANA-positive). Epidermal PAX3-positive melanocytes show a

similar profile to those in the ORS (27.9% HES1-positive, 62% MLANA-positive). Interestingly, there are a similar number of MLANA-positive (but PAX3-negative) melanocytes in the epidermis (30.5%) and the hair matrix (40%), in contrast to the ORS where such cells are rare (3.9%). PAX3 expression seems to correlate with undifferentiated (co-expressing HES1 and MITF) and differentiating (co-expressing MLANA and MITF) cells, whereas it might be diminished in terminally differentiated cells (PAX3-negative but MLANA or MITF-positive) that are further along the continuum of differentiation. It is possible that PAX3 is involved in maintenance of melanocyte "stemness", at least while they are migrating towards their destination, either in the hair follicle or the epidermis.

**PAX3 in apoptosis**

It is also very likely that PAX3 is involved in keeping melanocytes alive, since three quarters of epidermal and more than half ORS and matrix PAX3-positive melanocytes show expression of the PAX3 target, antiapoptotic BCL2L1. PAX3 is previously reported as an antiapoptotic regulator in tumours, including melanoma [28,155,156,160-162]. Several known antiapoptotic factors, such as tumour suppressors p53, PTEN and BCL2L1, are involved in Pax3-induced cell survival [28,225,226]. *BCL2L1* is directly transcriptionally regulated by PAX3, and in rhabdomyosarcoma treatment with *PAX3* or *BCL2L1* antisense oligonucleotides, individually or in combination, decreases cell viability to a similar extent, suggesting that they lie in the same antiapoptotic pathway [226]. Similarly, BCL2L1 is associated with melanoma cell survival, since its expression correlates with melanoma progression [255,256] and treatment with antisense oligonucleotides resulted in reduction of cell viability [257,258]. However, its expression is observed in naevi and normal skin [255,256] and in melanocytes it also affects cell viability [258].

As expected we have observed positive BCL2L2 staining in all normal skin, naevi and primary melanoma samples analysed. The majority of PAX3-positive cells in melanomas and naevi samples and melanocytes in normal skin co-express BCL2L2, at a similar frequency. These results support the potential role of PAX3 as one of many cell survival regulators. The results also indicate that normal melanocytes

might utilise the same cell survival regulatory pathway as melanoma cells, both in the epidermis and in hair follicles.

**PAX3 in migration**

The cell adhesion molecule MCAM has been associated with melanoma progression and metastatic potential [259-262]. Even though MCAM is frequently expressed in naevi [250] its increased expression in melanomas shows significant correlation with poor disease free survival and mortality [250,259-261,263,264]. Upregulation of MCAM, together with loss of keratinocyte-dependence, is one of the crucial events that allows melanoma cells to invade the dermis and progress to vertical growth phase (for review see [265]). MCAM can mediate both homotypic adhesion between melanoma cells, promoting local tumour growth, and heterotypic adhesion between melanoma cells and endothelial cells of blood vessels, facilitating metastatic spread [237,262,266].

Pax3-transfected melanocyte cells show upregulation of MCAM, both at an mRNA and protein level, suggesting that MCAM is a downstream target of Pax3 [121,204]. Our results confirm co-expression of PAX3 and MCAM in melanomas and naevi, mainly in the cells that form nests located in the dermal component of the lesion, with melanoma samples showing larger number of PAX3, MCAM-positive cells compared to naevi. This indicates that some PAX3-positive cells in naevi and melanomas have the capability to undergo vertical spread/migration. It suggests that regulation of migration might be one of the roles of PAX3 in melanoma, as it is in development, where Pax3 regulates cMet [202].

The frequent observation of MCAM expression in cells of the hair follicle might be associated with downwards growth into the dermis and migration of follicular cells. The observation that some PAX3-positive melanocytes in the ORS show MCAM expression, in contrast to those in the epidermis, suggests that MCAM is associated with migration of normal melanocytes from the bulge area to the developing hair follicle and to the epidermis. Upon reaching the epidermis, MCAM expression is downregulated. Alternatively, epidermal melanocytes do not arise and migrate from the bulge, but rather arise from interfollicular melanocyte stem

cells, similar to those believed to maintain interfollicular epidermal homeostasis [165,267,268]. Even though some epidermal melanocytes resemble the transient amplifying melanocytic cells of the hair follicle with respect to their differentiation status, they are not thought to have the same migratory propensity.

In summary, in addition to detection in the majority of naevus and melanoma cells, we conclusively show PAX3 expression in melanocytes of normal skin. Epidermal PAX3-positive melanocytes, like follicular cells, exhibit a variable differentiation status; the majority show a more mature phenotype, but a proportion (around 20%) show a less differentiated and proliferative phenotype. Moreover, these melanocytes frequently show expression of a cell survival marker. Our results suggest that a previously identified role for PAX3, as a regulator of an undifferentiated plastic state, may operate in melanocytes of normal skin. This role, possibly required for cellular response to environmental stimuli, may predispose a proportion of these cells to a successful malignant transformation as they are antiapoptotic, undifferentiated and proliferative.

In addition, our findings that PAX3 expression is specific for both melanocytic lineage and melanocytic lesions, could have potential applications in the clinical setting, as an immunohistochemical assay for the differential diagnosis of melanoma. The effectiveness of the commercially available anti-PAX3 antibodies and optimisation of staining protocols shown in this paper might also be proved useful in a clinical setting.

## Materials and methods

### Sample collection

De-identified archival tissue samples were obtained from several pathology laboratories in accordance with the Human Research Ethics Committee of Edith Cowan University (ethics approval code 07-189 MEDIC). Written consent was obtained for the use of patient archival tissue samples. Samples were previously diagnosed by certified pathologists. 4μm thick formalin fixed paraffin embedded tissue sections were used for immunohistochemistry and immunofluorescence.

The following samples were analysed by immunohistochemistry and immunofluorescence: 10 normal skins (including 3 hairy, 2 limb and 2 breast skin samples, and 3 samples had no anatomic location specified), 14 naevi (including 5 intradermal, 6 compound and 3 junctional samples), 15 primary (including 8 superficial spreading melanoma samples, 2 of radial growth phase, and 1 sample each of acral lentiginous, nodular, neurotropic, recurrent and lentigo maligna melanoma) and 5 systemic melanoma metastases (2 small bowel and 3 lymph node). Tissue samples characterized here as normal skins were free of any melanocytic lesion, having been used for other diagnostic purposes (solar keratosis, seborrhoeic keratosis and granulomatous folliculitis) or tissue obtained after breast reduction. Normal skin samples originated mainly from limbs or face (referred to as sun-exposed skin) or breast (referred to as reduced sun-exposed skin). Additionally, a separate set of 12 archival samples, including 2 normal skin, 5 naevi and 5 primary melanomas as well as 5 cryopreserved lymph node melanoma metastases were analysed by qRT-PCR.

**RNA extraction and qRT-PCT**

Total RNA was extracted from the paraffin embedded and cryopreserved tissue samples using Aurum Total RNA Mini Kit (Bio-Rad) according to the manufacturer's recommendations. Slight modifications were made to the protocol used for paraffin embedded samples. Mainly, sections were dewaxed in Xylene and absolute Ethanol (each twice for 10 minutes), before tissue was scratched from the slides and lysed in a mixture of Tissue Lysis Buffer (High Pure RNA Paraffin Kit, Roche), Proteinase K and 10% SDS by incubation at $55^0$C overnight.

The quality and integrity of extracted RNA was assessed by gel electrophoresis and subsequent amplification of the housekeeping gene *GAPDH*. 250ng of the total RNA was reverse transcribed with Omniscript RT kit (Qiagen), according to the manufacturers' instructions and PCR was performed using Taq DNA Polymerase (Qiagen). Primers used were, for *GAPDH:* 5'-GGG TGT GAA CCA TGA GAA GT-3' (forward) and 5'-GAC TGT GGT CAT GAG TCC T-3' (reverse) [269]; and for *PAX3*: 5'-AGA CTG ATT ACG CGC TCT CC-3' (forward) and 5'-GGC TGC GAA GAC CAG AAA C-3' (reverse). Real time PCR was performed using iQ SYBR Green Supermix (Bio-

Rad). Each sample was run in duplicate and assays included negative controls (reagents without RNA or cDNA) and positive controls (plasmid DNA containing a *PAX3* insert). The calibration curve was generated using the threshold cycle (Ct) of 8 serial dilutions of plasmid DNA template with known copy number. The Ct value of the sample was interpolated from the standard curve and mRNA copy number, mean value and standard deviation were calculated using iQ5 RealTime Detection System Software (Bio-Rad Laboratories). The increase in the mean values of mRNA copy number for each sample relative to that in the normal skin was expressed as a fold-change increase in mRNA.

**Antibodies**

The following primary antibodies were used: mouse monoclonal to PAX3 (DSHB, 1/10), rabbit polyclonal to PAX3 (Invitrogen, 1/500), mouse monoclonal to MITF (Merck, 1/20), mouse monoclonal to MLANA (Merck, 1/50), rabbit polyclonal to HES1 (Abcam, 1/100), rabbit monoclonal to BCL2L1 (Abcam, 1/50), rabbit monoclonal to MCAM (Abcam, 1/500) and rabbit polyclonal to Ki67 (Abcam, 1/25). For immunofluorescent staining the following secondary and tertiary antibodies were used: anti-mouse conjugated AlexaFluor-488 (1/500), anti-rabbit conjugated AlexaFluor-488 (1/500), anti-mouse conjugated AlexaFluor-546 (1/500), or biotinylated anti-rabbit IgG (1/500) linked to streptavidin-conjugated AlexaFluor-546 (1/500). All antibodies were diluted in PBST (0.2% TritonX-100 in PBS) together with 1%NGS for immunofluorescent staining.

**Immunohistochemistry**

Paraffin embedded sections were dewaxed in xylene (3x 5 minutes) and dehydrated in ethanol series (3x 5 minutes in 100% ethanol, 1 minute in each of 95% and 70% ethanol, and 3x 10 dips in dd$H_20$). Antigen retrieval was routinely performed in EDTA/Tris, pH 8.0, by heating for 3x 5 minutes in a microwave oven, at approximately 750 Watts. Slides were left to cool to room temperature for at least 20 minutes. Sections were washed in PBS and endogenous peroxidases were blocked with 3% $H_20_2$ for 10 minutes. After rinsing in PBS, sections were blocked with 10% FCS for one hour, followed by incubation with primary antibodies for one hour at room temperature. After washing in PBS, the secondary antibody

linked to biotin (Dako, LSAB kit) was applied and sections were incubated for 20 minutes at room temperature. After washing in PBS-Tween20 (0.05%), sections were incubated with streptavidin conjugated horseradish peroxidase (Dako, LSAB kit) for 20 minutes at room temperature, washed in PBS-Tween20, then incubated with DAB (Dako) for several minutes. The reaction was stopped by washing in dd$H_20$, and the signal was enhanced by applying a solution of $CuSO_4$ and NaCl, for 3 minutes. Hematoxylin staining was performed before the slides were mounted with DPX neutral mounting medium. Results were analysed on an Olympus BX51 microscope and images were captured using an Olympus DP71 camera. Controls with primary antibodies withheld were immunonegative. The specific staining of nuclear markers PAX3 and MITF was generally distinguishable from the cytoplasmic melanin deposit in the tissue by their sub-cellular localisation. Intensity of PAX3 staining was compared across all samples analysed and described as either weak or strong.

**Immunofluorescence**

The same dewaxing, rehydration and antigen retrieval procedures described above were followed for immunofluorescence. Sections were blocked with 10%NGS for one hour at room temperature, followed by incubation with primary antibodies at $4^0$C overnight (for rabbit PAX3 and MITF co-staining) or at room temperature for one hour (for all other antibodies). After washing in PBS, sections were incubated with the appropriate secondary antibody (followed by tertiary only for rabbit PAX3 and HES1) for one hour and washed in PBS. For counterstaining Hoechst 33342 was used. Sections were mounted with FluorSave Reagent (Calbiochem) and analysed on an epifluorescent Olympus BX51 microscope equipped with an Olympus DP71 camera. Controls with primary antibodies withheld were immunonegative. For each sample, quantification of cells positive for a given marker was performed by analysis of 5-10 representative regions of the section. The number of double-labelled cells was calculated relative to the number of cells positive for a comparative marker and expressed as a percentage.

**Acknowledgments**

The authors would like to thank Dr. Mark Brown for thoughtful discussions, Dr. Jennifer Thompson for technical assistance with microscopy and Dr. Robert White for constructive comments. The Pax3 monoclonal antibody, developed by C.P. Ordahl, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

**Author Contributions**

Conceived and designed the experiments: SM MZ.

Performed the experiments: SM.

Analysed the data: SM MZ.

Contributed reagents/materials/analysis tools: SM MZ.

Wrote the paper: SM.

Edited the paper: MZ.

## *Chapter IV*

EXPERIMENTAL ARTICLE

# Differential PAX3 functions in normal skin melanocytes and melanoma cells

**Authors and Affiliations**

Sandra Medic[1], Helen Rizos[2] and Mel Ziman[1,3*]

[1]School of Exercise, Biomedical and Health Sciences, Edith Cowan University, Perth, WA, Australia,

[2]Westmead Institute for Cancer Research and Melanoma Institute of Australia, University of Sydney at Westmead Millennium Institute, Westmead, NSW, Australia,

[3]School of Pathology and Laboratory Medicine, University of Western Australia, Perth, WA, Australia

*** Corresponding author**

A/Prof. Mel Ziman

School of Exercise, Biomedical and Health Sciences

Edith Cowan University

Joondalup Campus

PH: +61-8-63045171

Fax: +61-8-63045717

E-mail: m.ziman@ecu.edu.au

**Citation:** Medic S, Rizos H, Ziman M (2011) Differential PAX3 functions in normal skin melanocytes and melanoma cells. Biochem Biophys Res Commun 411: 832-837.

**Impact factor** (2010): 2.59

**ERA rank** (2010): B

**Abstract**

The PAX3 transcription factor is the key regulator of melanocyte development during embryogenesis and is also frequently found in melanoma cells. While PAX3 is known to regulate melanocyte differentiation, survival, proliferation and migration during development, it is not clear if its function is maintained in adult melanocytes and melanoma cells. To clarify this we have assessed which genes are targeted by PAX3 in these cells. We show here that similar to its roles in development, PAX3 regulates complex differentiation networks in both melanoma cells and melanocytes, in order to maintain cells as "stem" cell-like (via NES and SOX9). We show also that mediators of migration (MCAM and CSPG4) are common to both cell types but more so in melanoma cells. By contrast, PAX3-mediated regulation of melanoma cell proliferation (through TPD52) and survival (via BCL2L1 and PTEN) differs from that in melanocytes. These results suggest that by controlling cell proliferation, survival and migration as well as maintaining a less differentiated "stem" cell like phenotype, PAX3 may contribute to melanoma development and progression.

**Keywords:**
PAX3, melanoma, melanocyte

## Introduction

Melanoma is an aggressive skin cancer and mortality rates remain high for advanced stage patients where the five year survival rate is less that 20% [8]. Current therapies have limited success, and while targeted therapies are proving more successful [30-32], knowledge of the mechanisms that drive disease progression is urgently required. Of particular interest here are the key regulators of cellular process in both normal skin melanocytes and melanoma cells, as differences between these two are likely to provide strategic clues to the process of melanoma-genesis.

The transcription factor, Paired box 3 (PAX3), is at the top of the hierarchy of genes that regulate melanocyte specification, maintenance of the undifferentiated state, proliferation and migration during embryonic development (reviewed in [1]). PAX3 is also highly expressed in melanoma [24,25], where it is shown to contribute to melanoma cell survival [27,28]; however, it is not known whether it continues to control pathways of differentiation, migration and proliferation and whether this contributes to melanoma progression.

In contrast to its well known role in embryonic development and in maintenance of the undifferentiated postnatal melanocyte stem cell [19], not much is known about the role of PAX3 in adult epidermal melanocytes, since its expression there has only recently been confirmed [2,18]. Our own studies indicate that PAX3-positive epidermal melanocytes exhibit a variable phenotype, from a more mature to a less differentiated and proliferative form, frequently showing expression of the antiapoptotic factor BCL2 like protein 1 (BCL2L1) [2]. Taken together with other studies [20,163], the results suggest that even in normal adult skin melanocytes PAX3 regulates differentiation and proliferation, as it does during development. It seems likely then, that PAX3 would continue to control maintenance of the undifferentiated state, proliferation and migration, throughout melanocyte development and maturation, and melanoma-genesis, possibly playing a significant role in driving melanoma development and progression.

To clarify its role and identify any potential differences in the functional repertoire of PAX3 between melanocytes and melanoma, we analysed its direct target genes, and assessed their differential activation in each cell type. We show here that PAX3 is similarly involved in promoting a "stem" cell phenotype in both melanoma cells and melanocytes (via Nestin (NES) and SRY (sex determining region Y)-box 9 (SOX9)). There is however, a hitherto unreported difference in PAX3 regulation of melanoma cell proliferation relative to melanocytes (through Tumour protein D52 (TPD52)). We also confirm that its role in cell survival (via BCL2L1 and Phosphatase and tensin homologue deleted from chromosome 10 (PTEN)) is distinct for melanoma cells. Moreover, while PAX3 commonly regulates cell migration through Melanoma cell adhesion molecule (MCAM) and Chondroitin sulphate proteoglycan 4 (CSPG4) in both melanocytes and melanoma cells, binding and expression of these genes are significantly enhanced in melanoma cells, presumably affecting the functional outcome. Therefore, PAX3 may indeed 'tick all the boxes' as an intrinsic factor in melanoma development and progression.

## Materials and Methods

### Cell culture

Human primary neonatal foreskin melanocytes HEM1455 (Cell Applications) were cultured as previously described [270]. A2058 metastatic melanoma cells (ATCC) were cultured in DMEM medium (Invitrogen) supplemented with 10% FBS (Interpath). Cell cultures were incubated at 37°C in a humidified incubator supplemented with 5% $CO_2$.

### Immunocytochemistry

Immunofluorescent analysis was done as previously described [2]. Briefly, $5x10^4$ cells/ cover slip were grown for 24 hours, as described above. Following fixation in 4% paraformaldehyde, and blocking with 10% NGS, cells were double-stained with anti-PAX3 (Invitrogen, 1/500) and anti-MITF (Merck, 1/200) antibody overnight. Controls with primary antibodies withheld were immunonegative.

**Western blotting**

Total cellular proteins were extracted using RIPA lysis buffer containing protease inhibitors (Roche). Proteins (30–50 mg) were resolved on 12% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). Western blots were probed with antibodies against PAX3 (DSHB) and β-actin (Abcam), and proteins detected with ECL Western Blot Detection Kit (Amersham).

**ChIP**

Chromatin immunoprecipitation was performed using the EZ-Magna ChIP A kit (Millipore) according to the manufacturer's recommendations. Briefly, melanocyte and melanoma cells were fixed in 1% formaldehyde, harvested, lysed, and DNA was sheared using a Branson450 Sonifier. Immunoprecipitation (IP) was performed with 5μg of either anti-PAX3 (from either DSHB or Invitrogen) or anti-IgG antibody (Millipore) as negative control. A 2% aliquot of sheared DNA prior to immunoprecipitation was used as input. All IPs were done in duplicate. Positive control promoters of Microphthalmia associated transcription factor (*MITF*) (-332/+5) and Dopachrome tautomerase (*DCT*) (-186/-3), encompassing the confirmed PAX3 binding sites [64,65,187], were amplified with 5'-TCCTCCAAAGGGGCATTCTGCT and 5'-TCCCGAGACACCACCGGAAA (*MITF*); and 5'-TGCCCTCCTGAAATAAAGCC and 5'-AAGCCAAACACCGTGCTG (*DCT*). Negative control primers (Millipore) amplified a non-related *GAPDH* promoter sequence. All PCRs were amplified using a Taq DNA Polymerase kit (Qiagen) for 40 cycles.

**qPCR analysis of the gene promoter**

Profiling of ChIP samples was done with custom designed ChampionChIP PCR array (Qiagen) containing 12 genomic sites of interest (Supplementary Table S1). qPCR analysis was performed according to the manufacturer's recommendations. Briefly, PAX3 or IgG immunoprecipitated DNA samples (IPs) and input DNA samples were loaded with RT$^2$qPCR SYBR Green/Fluorescein Master Mix (Qiagen) into arrays and amplified for 40 cycles on iQ5 cycler (BioRad). Additionally, *MITF*, *DCT* and *GAPDH* genomic sites were amplified with KAPA SYBR FAST qPCR Master Mix (KapaBiosystems) for 40 cycles. Each PCR reaction was performed in duplicate and mean Ct values were used for quantification. Each IP Ct value was normalised

against the Input Ct value for the same PCR assay (ΔCt), before the fold enrichment in PAX3-IP over negative control IgG-IP (ΔΔCt) was calculated for each genomic site. Enrichment for the non-specific site (*GAPDH*) was determined to be the background and only enrichment above this value was considered true enrichment for each specific genomic site. Specific genomic enrichment was then calculated for each individual ChIP experiment, by subtracting the background from it, and the mean value for duplicate ChIP experiments was calculated.

**RNA extraction and RT-qPCR**

Total RNA was extracted from cultured HEM1455 and A2058 cells using either Isolate RNA Mini Kit (Bioline), or Tryzol reagent (Invitrogen) with a column clean up (Qiagen). Quantity and quality of RNA were measured with a nanodrop spectrophotometer and assessed by agarose gel electrophoresis. 250ng of total RNA was reverse transcribed using Omniscript RT kit (Qiagen) and PCR products were amplified with either KAPA SYBR FAST qPCR Master Mix (KapaBiosystems) or SYBR GreenER qPCR SuperMix (Invitrogen), for 40 cycles. Each PCR reaction was performed in triplicate and the mean Ct value was used to calculate fold change over *GAPDH* (ΔΔCt). Primers used for RT-qPCR are listed in the supplementary information (Supplementary Table S2).

**Statistical analyses**

Statistical analyses of specific site enrichment and gene expression were performed using a Student's t-test.

## Results

### PAX3 expression in HEM1455 primary melanocytes and A2058 melanoma cells

We have previously reported persistent PAX3 expression in melanocytic lesions and normal skin melanocytes, both follicular and epidermal [2]. Here we confirm *PAX3* expression in both HEM1455 and A2058 cell lines (Fig. 4.1), showing slightly lower (however not statistically significant) *PAX3* levels in A2058 compared to HEM1455 cells. This is most likely due to the variable expression of PAX3 in A2058 cells, as evidenced by differences in PAX3-staining intensity across the cultured cells, including some PAX3-negative cells. Others have also reported high variation in PAX3 levels across a variety of melanoma cell lines, but more uniform staining and higher expression in melanocytes [27].

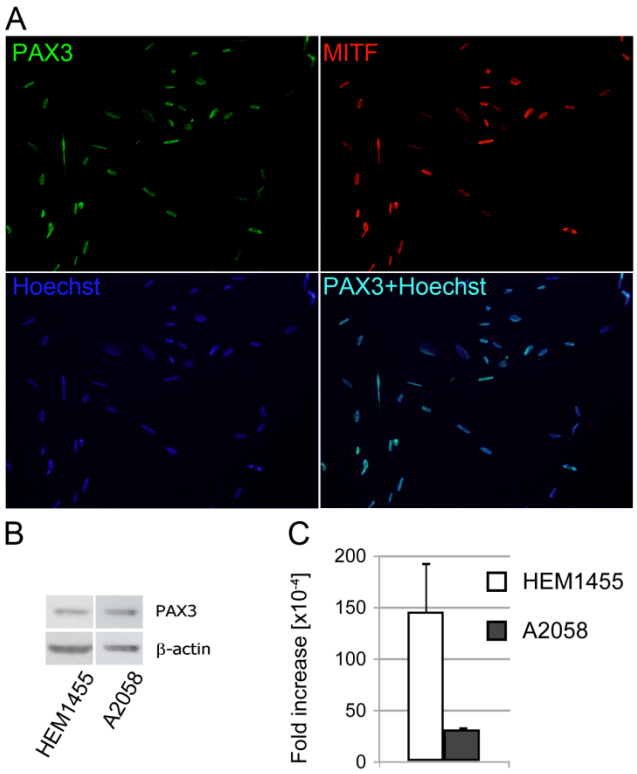


***Figure 4.1.*** **PAX3 expression in melanocytes and melanoma cells.** (A) PAX3 expression in A2058 cells confirmed by immunocytochemistry, showing co-expression of PAX3 with MITF, a known marker of the melanocytic lineage. (B) PAX3 expression in HEM1455 and A2058 cells confirmed by western blotting. (C) Graph shows *PAX3* mRNA levels in HEM1455 and A2058 (relative to *GAPDH*) assessed by RT-qPCR.

**PAX3 target genes in HEM1455 and A2058 cell lines**

In order to assess PAX3 function/s and identify differential regulation mediators between melanocytes and melanoma cells, we have analysed 12 out of 56 potential direct PAX3 target genes (Supplementary Table S3). These genes were selected as indicators of key PAX3-regulated processes, ie differentiation (*SOX9*, *NES*, and Hairy and enhancer of split 1 (*HES1*)), proliferation (*TPD52*, Cyclin A2 (*CCNA2*), and Nuclear factor kappa β 2 (*NF-Kβ2*)), cell survival (*BCL2L1*, *PTEN*, and Transforming growth factor-beta 1 (*TGFβ1*) and migration (*MCAM*, *CSPG4*, and CXC chemokine receptor 4 (*CXCR4*)).

In addition *MITF* and *DCT*, known PAX3 targets in the melanocytic cell lineage, were chosen as positive controls to assess ChIP assay efficiency. ChIP with anti-PAX3 antibody (DSHB) showed enrichment for both *MITF* and *DCT* promoters over the IgG control in A2058 cells, and this was confirmed using a different anti-PAX3 antibody (Invitrogen), albeit with less efficiency (Fig. 4.2A).

After validating the PAX3 ChIP assay on A2058 cells, we assessed binding of our selected genes (Table S1) in HEM1455 and A2058 cells. Enrichment in PAX3-IP relative to control IgG-IP was observed for most of the genomic sites analysed (Fig. 4.2B), which is consistent with these genes being direct downstream targets of PAX3 in these cell types. However, there was no enrichment in *NFKβ2* genomic site in either HEM1455 or A2058, indicating it is not a target of PAX3 in either cell.

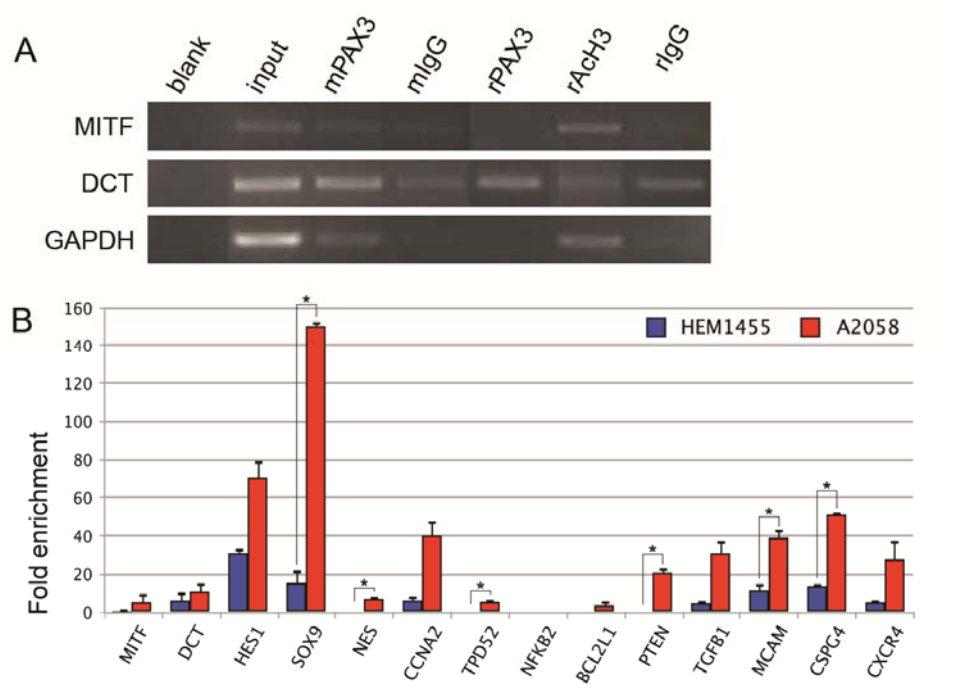


***Figure 4.2.*** **PAX3 binding to target genes in melanocytes and melanoma cells.** (A) PAX3 binding to *MITF* and *DCT* promoters in A2058 cells was assessed by end-point PCR. ChIP assay was performed with two different PAX3 antibodies (from DSHB, lane 3; or Invitrogen, lane 5) and matching control IgG antibodies (lanes 4 and 7). Non-specific *GAPDH* promoter was included as a negative control for PAX3-IP; and IP with AcH3 (acetylated Histone H3, lane 6) serves as positive IP control. Blank (lane 1) is no-template control for PCR reaction. (B) Graph shows PAX3 binding to potential target genes in HEM1455 and A2058 cells, quantified by qPCR. For each target site, enrichment in PAX3-IP was normalised to the input DNA and calculated as a fold increase over normalised IgG-IP. Only enrichment above the enrichment value for the non-specific site (*GAPDH*) was considered true enrichment. Therefore, for each IP, specific promoter enrichment was subtracted from non-specific promoter enrichment (which was 5.58-fold for both HEM1455 IP1 and 2 and 2.6 and 5.135-fold for A2058 IP1 and 2, respectively). Asterisk (*) indicates statistically significant fold difference, $p<0.05$.

Interestingly the overall fold enrichment at all sites is higher in A2058 compared to HEM1455, indicating a stronger binding of PAX3 to its targets in melanoma cells. In melanocytes four genomic sites, *NES*, *TPD52*, *BCL2L1* and *PTEN*, do not show enrichment in PAX3-IP, suggesting that PAX3 regulates different pathways in melanocytes and melanoma cells. The fact that these genes are involved in differentiation (*NES*), proliferation (*TPD52*), and cell survival (*BCL2L1* and *PTEN*) signifies PAX3 involvement in regulation of these processes in melanoma by instigating melanoma-exclusive mechanisms.

In addition, genomic sites for *SOX9*, *MCAM* and *CSPG4*, even though common for both cell types, show significantly higher fold enrichment in melanoma cells compared to melanocytes. This suggests that PAX3 utilises the same mechanisms (via *MCAM* and *CSPG4*) to regulate migration of melanocytes and melanoma cells.

In summary, the fourteen potential PAX3 target genes analysed here can be categorised as follows: i) PAX3 targets specific for melanoma (*NES, TPD52*, *PTEN* and *BCL2L1*); ii) PAX3 targets common to both melanocytes and melanoma (*MITF*, *DCT*, *HES1, SOX9*, *CCNA2*, *TGFβ1*, *MCAM*, *CSPG4*, and *CXCR4*); and iii) not a PAX3 target, such is *NFKβ2*).

**Expression of PAX3 targets in HEM1455 and A2058**

We next analysed the expression of the thirteen identified target genes in the two cell lines to confirm that the mRNA profile corresponds to PAX3 binding (Fig. 4.3). Results here show downregulation of *MITF*, *BCL2L1*, and *PTEN* in A2058 compared to HEM1455, by 4.96*, 2.47, and 1.81*-fold, respectively (asterisk indicates statistical significance, $p<0.05$). *HES1* shows no change in expression levels between the two cell lines (0.86-fold). By contrast, the remaining nine genes show upregulation in A2058 cells compared to HEM1455: *DCT* (2.74), *SOX9* (6.13*), *NES* (10.66*), *CCNA2* (2.17*), *TPD52* (6.09*), *TGFβ1* (3.24*), *MCAM* (38.04*), *CSPG4* (2.37*), and *CXCR4* (11.35*) (asterisk indicates statistical significance, $p<0.05$).

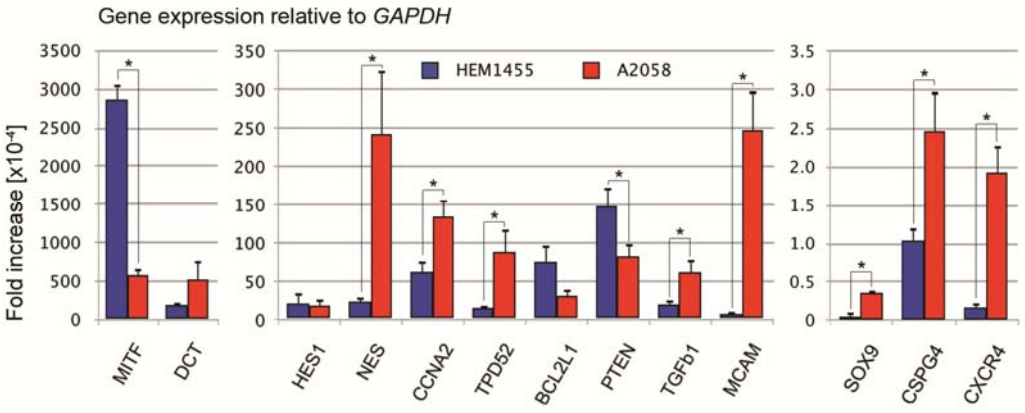


***Figure 4.3.*** **PAX3 "target" gene expression levels in melanocyte and melanoma cells.** Relative expression of PAX3 targets was analysed by RT-qPCR. Asterisk (*) indicates statistically significant fold difference, $p<0.05$

We observe here a correlation between PAX3 binding to the target gene and its expression level; ie genes showing statistically significant enrichment in PAX3 binding in the melanoma cells vs. melanocytes, also show a significant difference in expression levels. Melanoma specific PAX3 targets, *NES* and *TPD52* show significant upregulation in A2058, whereas *BCL2L1* is downregulated although this is not statistically significant. Also, *PTEN* is significantly downregulated in melanoma. Furthermore, targets that are common in melanoma and melanocytes, but show significantly higher PAX3 binding in melanoma (such as *SOX9*, *MCAM* and *CSPG4*) also show significant upregulation in A2058 suggesting a role for PAX3 in their increased activation.

**Discussion**

To elucidate the role of PAX3 in melanoma relative to its role in melanocytes, we focused on processes known to be regulated by PAX3 during melanocyte development that might also be critical for melanoma progression. Fourteen chosen genes associated with these processes were analysed here as potential PAX3 targets, and proved to be informative in clarifying PAX3 involvement not just in melanoma, but also in melanocyte cellular processes.

Traditional developmental roles of PAX3 in regulating differentiation, proliferation, cell survival and migration, are retained in melanocytes and melanoma cells, utilising common pathways/ mediators (such are *MITF*, *DCT*, *HES1, SOX9*, *CCNA2*, *TGFβ1*, *MCAM*, *CSPG4*, and *CXCR4*). However, additional regulatory mechanisms are employed specifically in melanoma (exemplified by *NES, TPD52*, *PTEN*, and *BCL2L1*). Moreover, several of the common targets, namely *SOX9*, *MCAM* and *CSPG4*, show significant fold enrichment in melanoma cells compared to melanocytes, suggesting that PAX3 mediated mechanisms are amplified in melanoma. Interestingly, those targets that are more strongly activated in melanoma are those that determine a less differentiated (*SOX9* and *NES*), more motile (*MCAM* and *CSPG4*) cell phenotype, characteristic of melanomas with a higher metastatic potential [125]. In addition PAX3 targets that appear

restricted to melanoma cells, promote cell survival (via *PTEN*) and proliferation (via *TPD52*).

For the first time, we identify *TPD52* as a novel PAX3 target, and this PAX3-mediated activation is restricted to melanoma cells rather than melanocytes. Overexpression of *TPD52* associated with cellular proliferation is frequently observed in cancer, and in melanoma it is the fifth most upregulated gene [229,271,272]. Thus we show here that PAX3 regulates melanocyte and melanoma proliferation [20,163], and it does so in melanoma via TPD52.

We also identify that the clearest difference in PAX3 function between melanoma and melanocytes is in regulating cell survival. PAX3 binds to antiapoptotic factor *BCL2L1* and tumour suppressor gene *PTEN* in melanoma cells. PTEN overexpression results in cell cycle arrest and apoptosis, and mutations and inactivation of this gene are common in tumours [197,225]. Moreover PAX3 was previously reported to repress *PTEN* transcription in rhabdomyosarcoma and myoblasts [197]. Indeed, we show here strong PAX3 binding and downregulation of *PTEN* in melanoma. The strong decrease in expression may be exacerbated by the presence of three heterozygous *PTEN* mutations in A2058 cells including a 35bp deletion and two missense point-mutations [273].

The mechanism of melanocytes and melanoma cell migration appears to be similar, since it binds to *MCAM, CSPG4* and *CXCR4* in both cell types, suggesting that the mere presence of PAX3 predisposes both cells to a highly motile phenotype. However, this is the first time that *MCAM* and *CSPG4*, known to promote cell motility [274,275], are shown to be direct targets of PAX3. We also confirm PAX3 binding to *CXCR4*, associated with metastatic spread of melanoma [276]. CXCR4, and its ligand CXCL12, regulate chemotactic migration and "homing" of tumour cells to a secondary organ/site, and facilitate tumour cell extravasation [277,278]. While PAX3 might regulate melanocyte and melanoma cell migration via the same mechanisms, it does so with increased strength in melanoma. By contrast, the low *MCAM* expression and PAX3 binding in melanocytes are consistent with low levels of PAX3/MCAM co-expression restricted to melanocytes of the growing hair

follicles of normal skin, whereas epidermal melanocytes show no *MCAM* expression [2]. These observations highlight the likelihood of PAX3 involvement in melanoma progression to metastasis.

The overall higher fold enrichment at all gene sites, observed in melanoma compared to melanocytes, suggests a higher affinity of PAX3 for its targets in melanoma, since there is a similar level of PAX3 expression in both cell types. The increased binding might be due to the upregulation of "appropriate" cofactors in melanoma cells, providing a more favourable environment for PAX3 binding to target genes; or perhaps chromatin changes allow increased binding to targets. PAX3 binding efficiency to genes can also be altered in the presence of competitors (such as MITF) and transcriptional activation affected by cofactors (like SOX10, GRG4, DAXX) or antagonists (TGFβ2) [19,64,123]. For example, we did see here a very high increase of *TGFβ2* expression in melanoma cells (180.84-fold, data not shown).

Finally, our results show that PAX3 is a key element in the complex regulating networks defining differentiation in melanocytes and melanoma cells, balancing the cells between a less or more differentiated state, through MITF, DCT and TRP1 [19,64,76], together with transcription factors SOX9 and HES1, as well as the TGFβ signalling pathway. We confirm here that *MITF*, *DCT*, *SOX9* and *HES1* are all direct targets of PAX3. It is evident from our results that regulation of the differentiation status of melanocytes and melanoma cells operates via PAX3-regulated mechanisms. We see exceptionally strong PAX3 binding to *SOX9* and *HES1* in melanoma, presumably to maintain the less differentiated phenotype. SOX9 promotes melanocyte differentiation, whereas HES1 maintains a less differentiated phenotype [254,279]. PAX3 represses *SOX9*, and activates *HES1*, whereas TGFβ2 has the opposite effect [123]. However, we do not see the expected changes in *SOX9* and *HES1* expression levels in these cells. One likely explanation is the significant upregulation of *TGFβ2* in melanoma, known to antagonise PAX3, which could override PAX3-regulated *SOX9* repression and *HES1* activation.

Moreover, we find that *NES* is regulated by PAX3 exclusively in melanoma cells. NES is thought to mark a subpopulation of quiescent melanoma 'stem' cells with higher metastatic potential [144], which contribute to melanoma progression.

It is interesting to note that even though PAX3 is known to activate *MITF*, and *MITF* expression in melanocytes is very high, its promoter shows minimal occupancy by PAX3. This suggests limited involvement of PAX3 in transcriptional regulation of *MITF* in melanocytes. Indeed, melanocyte transfection with Pax3 did not result in an increase in *Mitf* [121], confirming that Pax3 is not required to activate Mitf in these cells. It is possible that, in contrast to embryonic development, once the required level of MITF has been established in postnatal melanocytes, PAX3 is no longer necessary for its maintenance. In melanomas, however, PAX3 might once again regulate *MITF*, maintaining low to intermediate levels required to promote proliferation rather than differentiation [193].

In conclusion, the key transcription factor PAX3 is expressed both in normal melanocytes and melanoma cells and regulates a number of processes similarly in both cells. In melanoma cells however, PAX3 appears to have a higher affinity /binding capacity for target genes, which regulate cell proliferation and cell survival. Since PAX3 acts as an internal rheostat to regulate cell differentiation, and maintenance of the stem cell phenotype, its expression in melanocytes and then in melanoma cells may increase the aggressive phenotype associated with metastatic melanoma, by promoting proliferation and preventing apoptosis, a lethal mixture. Moreover, by conferring migratory properties to melanocytes PAX3 predisposes them to a more motile phenotype upon malignant transformation thus contributing to metastatic spread of melanoma cells. Future studies to evaluate PAX3 as a potential target for melanoma treatment are suggested.

**Acknowledgments**

The authors would like to thank Dr. Mark Brown and Dr. Rob White for thoughtful discussions. The Pax3 monoclonal antibody, developed by C.P. Ordahl, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

## Supplementary data

***Table S1.*** Selected PAX3 target genes included in the custom promoter binding qPCR array.

| *Gene* | *ChIP-qPCR Assay cat #* | *Binding site position* | *Biding sequence* |
|---|---|---|---|
| ***HES1*** (Hairy and enhancer of split 1) | GPH1009779(+)04A | 193857155 | TTGGTCATGCCCCA |
| ***SOX9*** (SRY (sex determining region Y)-box 9) | GPH1005991(-)01A | 70117017 | CTCGTCACCCAGCC |
| ***NES*** (Nestin) | GPH1015041(+)01A | 156646502 | CGCGCCACGCCTCG |
| ***CCNA2*** (Cyclin A2) | GPH1023871(+)02A | 1227743650 | ATCTTCACGCTCTA |
| ***TPD52*** (Tumour protein D52) | GPH1026252(+)01A | 81083671 | CTCGCCGCGGTCCA |
| ***NF-Kβ2*** (Nuclear factor kappa β 2 (p100)) | GPH1001851(-)01A | 104154208 | GCGAGGCGTGACGC |
| ***BCL2L1*** **(**BCL2 like protein 1) | GPH1022053(-)01A | 30311475 | CGGAAGCGGGACGG |
| ***PTEN*** (Phosphatase and tensin homologue deleted from chromosome 10) | GPH1001746(-)02A | 89622131 | CTCGCCCCGCCCAC |
| ***TGFβ1*** (Transforming growth factor-beta isoform 1) | GPH1020707(-)05A | 41863841 | ATGGGGTGGGACCA |
| ***MCAM*** (Melanoma cell adhesion molecule) | GPH1016778(+)05A | 119183639 | ACAGTCTGGGACGA |
| **CSPG4** (Chondroitin sulphate proteoglycan 4) | GPH1018524(-)05A | 76009320 | AGTGGCTGTGACCA |
| | GPH1021572(+)01A | 136872816 | TTGGAGTGTGACAG |
| ***CXCR4*** (CXC chemokine receptor 4) | GPH1021572(+)01A | 136873229 | GGGAAGCGTGATGA |

***Table S2.*** Primers used in RT-qPCR analysis.

| *Gene* | *Forward primers* | *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' |
| ***TGFβ2*** | 5'-CTGTAGCCCCATAACTTGG-3' | 5'-GAAAGCACGTGGCTCTTGT-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' |
| ***NFKβ2*** | 5'-GCCCAGAGACATGGAGAGTTGCT-3' | 5'-TGGCTCCTTGGGTTCCACAATGG-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' |

**Selection of potential PAX3 target genes**

Various genes have been reported to be directly regulated by PAX3 in different cell lineages and at different time points of development. Our interest here, however, was to identify those that are differentially regulated in melanocytes and melanoma cells. We initially searched the literature for those genes that show direct transcriptional regulation by PAX3, as well as those that show up/downregulation following PAX3 transfection [121,204,280]. Table below lists 56 genes commonly reported to be affected by PAX3 in either melanocytic cells or tumours. We chose those genes that are indicative of key functional pathways of proliferation, differentiation, cell survival and migration. Additionally, we considered those genes that show promoter occupancy by PAX3 as assessed by ChIP-sequencing [281] and/or are significant to melanoma development [229] (Table S1). Our final list included 12 genes potentially regulated by PAX3 in melanocytes and/or melanoma cells (Table S1), in addition to the *MITF* and *DCT*.

***Table S3*.** Potential PAX3 target genes.

| ***Gene*** | ***Gene description and function*** | ***Gene expression*** | ***PAX3 binding*** | ***Melanoma top genes*** |
|---|---|---|---|---|
| ***Genes involved in melanocytic differentiation:*** | | | | |
| MITF | Microphthalmia associated transcription factor (D) | Up [64,65] | | |
| TYR | Tyrosinase (D) | | χ [282] | |
| TRP1 | Tyrosinase related protein 1 (D) | Down [204] | χ [282] | |
| DCT / TRP2 | Dopachrome tautomerase (D) | Down [19] | | |
| HES1 | Hairy and enhancer of split 1 (D) | Up [122] | χ [282] | |
| SOX9 | SRY (sex determining region Y)-box 9 (D) | Down [122] | χ [280] | |
| NES | Nestin (D) | | χ [280] | |
| CITED1/ MSG1 | Cbp/p300-interacting transactivator 1 (D, P) | Up [121,280] | | |
| ***Genes involved in proliferation and cell cycle regulation:*** | | | | |
| CCNA1 | Cyclin A1 (P, Ap) | Up [121] | | |
| CCNA2 | Cyclin A2 (P) | Up [121] | χ [280] | **(59$^{th}$) [229] |
| CCNE1 | Cyclin E1 (P) | Up [121,204] | | |
| TPD52 | Tumour protein D52 (P, anti-Ap) | Up [121] | χ [280] | **(5$^{th}$) [228] |
| c-KIT | C-kit receptor (P, D, M) | Up [121] | | |
| KITL | KIT ligand (P, Ap, M) | Up [121,280] | | |
| APOD | Apolipoprotein D (P, D) | Up [121] | | *(45$^{th}$) [228] |

| | | | | |
|---|---|---|---|---|
| ***Growth factors involved in proliferation and angiogenesis:*** | | | | |
| TGFβ1 | Transforming growth factor-beta isoform 1 (P, Ap, M) | Up [121] | χ [280] | |
| TGFβ2 | Transforming growth factor-beta isoform 2 (P, Ap, An, EMT) | Up [204] | X [281] | |
| TGFα | Transforming growth factor-alpha (P) | Up [57] | | **(62nd) [228] |
| FGF1 | Fibroblast growth factor 1 (P, Ad) | Up/down [121] | | |
| FGF2 | Fibroblast growth factor 2 (P, Ap, An, M) | Up [121] | | |
| FGF8 | Fibroblast growth factor 8 (P, An) | | X [281] χ [280] | |
| FGF10 | Fibroblast growth factor 10 (P, An, M) | | X [279] χ [280] | |
| FGF17 | Fibroblast growth factor 17 (P, D, M) | Up [121] | χ [280] | |
| FGFR1 | Fibroblast growth factor receptor 1 (P, D) | Up/down [121] | | |
| IGF1R | Insulin-like growth factor 1 receptor (P, M, anti-Ap) | | X [279] | |
| VEGFC | Vascular endothelial growth factor C (An, M, P) | | X [279] χ [280] | |
| ***Genes involved in apoptosis:*** | | | | |
| NF-Kβ2 | Nuclear factor kappa B 2 (p100) (anti-Ap) | Up [204] | χ [280] | |
| BCL2L1 | BCL2 like protein 1 (anti-Ap) | Up [226] | χ [280] | |
| PTEN | Phosphatase and tensin homologue deleted from chromosome 10 (Ap) | Down [197] | χ [280] | |
| MDM1 | Mdm1 nuclear protein homologue (mouse) (Ap) | | X [279] | **(120th) [228] |
| TIMP2 | Tissue inhibitor of metalloproteinase 2 (An, D, P) | Up [121] | | |
| TIMP3 | Tissue inhibitor of metalloproteinase 3 (Ap, An) | Up [121,204]/ down [280] | χ [280] | |
| ***Genes involved in migration and adhesion:*** | | | | |
| CSPG4/ MCSP | Chondroitin sulphate proteoglycan 4 / HMW-MAA (M, An) | | χ [280] | |
| MCAM/ MUC18 | Melanoma cell adhesion molecule (M, Ad) | Up [121,204] | χ [280] | **(21st) [228] |
| CXCR4 | CXC chemokine receptor 4 (M) | | X [279] χ [280] | |
| EPHA2 | Ephrin type-A receptor 2 (M) | Down [280] | | |
| EPHA4 | Ephrin type-A receptor 4 (M) | Up [280] | X [279] | |
| EPHB2 | Ephrin type-B receptor 2 (M) | Down [280] | X [279] | |
| VCAM1 | Vascular cell adhesion molecule (Ad) | | X [279] | |
| S100A6 | Calcium binding protein A6 (M, P) | Up [121] | χ [280] | |
| VCAN | Versican (V2 splice variant) (Ad) | Up [204] | | |

| | | | | |
|---|---|---|---|---|
| CNR1 | Cannabinoid receptor type 1 (Ap, Im, ) | Up [280] | χ [280] | |
| ***EMT (epithelial to mesenchimal transition):*** | | | | |
| CDH3 | Cadherin 3 type 1/ P-cadherin (Ad) | | X [279] | *(3rd) [228] |
| CDH5 | Cadherin 5 type 2/ VE-cadherin (Ad) | | X [279] | |
| CDH12 | Cadherin 12 type 2/ N-cadherin 2 (Ad) | | X [279] | |
| ***Cancer specific genes:*** | | | | |
| PRAME | Melanoma antigen preferentially expressed in tumours (P, anti-Ap) | Up [204] | | **(4th) [228] |
| MAGEC3 | Melanoma antigen family C, 3 (ND) | | X [279] | |
| MAGEA5 | Melanoma antigen family A, 5 (ND) | | X [279] | **(28th) [228] |
| MAGEB6 | Melanoma antigen family B, 6 (ND) | | X [279] | |
| MAGEB18 | Melanoma antigen family B, 18 (ND) | | X [279] | |
| ***Other genes:*** | | | | |
| MGP | Matrix gla protein (D, M) | Down [204] | | **(20th) [228] |
| DMRT2 | Doublesex and mab-3 related transcription factor 2 (D) | | X [279] χ [280] | |
| ST8SIA1 | ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1 (P) | | X [279] | |
| NAA10 | N (alfa)-acetyletransferase 10 (P, anti-Ap) | Up [121] | X [279] | |
| CD55 | Complement decay-accelerating factor (anti-Ap, Im) | | X [279] | **(98th) [228] |
| WNT5B | Wingless-type MMTV integration site family, member 5B (D, M, An) | | X [279] | |

Table includes list of potential PAX3 target genes, their function, transcriptional regulation by PAX3 (up or downregulation), and presence of a PAX3 binding site; as well as literature references. Function is described as: differentiation (D), proliferation (P), migration (M), adhesion (Ad), apoptosis (Ap), angiogenesis (An), immune response (Im), and not defined (ND). X [4] indicates direct binding assessed by ChIP [281]; and χ [8] indicates presence of PAX3 binding site according to SABioscience database [282]. Single (*) or double asterisk (**), followed by the order number, indicate top downregulated or upregulated gene in melanoma vs. melanocytes, respectively [229].

# *Chapter V – General discussion*

Cutaneous melanoma is a neoplasm that arises from melanocytic cells in the skin. The transcription factor PAX3 is at the top of the hierarchy of factors that regulate melanocyte specification and differentiation, proliferation, survival and migration during embryonic development [10-15]. PAX3 is also highly expressed in melanoma [2,21-26], where it is shown to contribute to melanoma cell survival [27,28]. It is however not known whether PAX3 continues to control pathways of differentiation, proliferation and migration in melanoma cells and whether this contributes to melanoma progression.

Taking into account the possibility that metastatic melanoma progression is driven by melanoma stem-like cells, and taken together with the analogy between developmental processes and tumourigenesis, the pivotal role that PAX3 has during melanocyte development, has instigated us to further investigate PAX3 involvement in melanoma development and progression.

**PAX3 expression in melanocytic lesions and normal skin**

We initially started with a comparative analysis of PAX3 expression in normal skin, relative to its expression in a variety of melanocytic lesions, including benign pigmented naevi, and primary and metastatic melanomas, to see if changes in PAX3 expression were associated with melanoma progression [2]. Our results showed persistent PAX3 expression across all melanocytic lesions. When assessed by RT-qPCR, *PAX3* expression was similar in naevi and primary melanoma samples, and significantly upregulated in melanoma metastases. However, these results reflect the overall increase in the number of PAX3-expressing melanoma cells in samples of melanoma metastasis, rather than levels of expression per cell. Immunohistochemical analysis showed no significant correlation between level of PAX3 protein per cell and melanoma progression. Both intensity of PAX3 staining and frequency of PAX3-expresing cells, relative to a known melanocytic marker MITF, showed similar results for primary melanomas as for naevi and melanocytes. By contrast, metastatic samples showed slightly lower PAX3 staining intensity per cell but the overall number of PAX3-positive cells increased.

Interestingly, we also observed consistent PAX3 expression in normal melanocytes, including epidermal melanocytes presumed to be terminally differentiated mature cells [2]. This was in contrast to the widely accepted belief that this developmental regulator is 'switched off' in differentiated adult melanocytes, and re-expressed or aberrantly expressed in melanoma [19,20,24,25]. Several conflicting reports created uncertainty as to whether PAX3 expression is retained in adult melanocytes, with only a handful of reports detailing PAX3 expression in human primary melanocytes [16,17]. By contrast, murine primary melanocytes were shown to lack endogenous Pax3 [163]. A detailed study done by He and colleagues [18] confirmed our findings that PAX3 expression is retained in human epidermal melanocytes.

This essentially posed a new question- what is the role of PAX3 in differentiated adult melanocytes? Moreover, does PAX3 persistent expression in melanocytes contribute to melanoma-genesis and progression? The likelihood exists that the same developmental roles for PAX3 are operational in adult melanocytes and might continue in melanoma. Logically the next focus of our study was to investigate PAX3 involvement in several developmental regulatory processes, namely, differentiation, proliferation, survival and migration, in both melanocytes and melanoma cells.

**PAX3 in normal skin melanocyte**

The role of PAX3 in melanocyte differentiation is probably the best described role for PAX3 thus far, balancing melanocyte differentiation with maintenance of the undifferentiated state [13,19]. We decided to better characterise PAX3-expressing melanocytes in terms if their differentiation status, utilising previously identified markers of a more or less differentiated state, namely MLANA and HES1, respectively. MLANA is a type III membrane protein, indispensable for correct functioning of the structural melanosomal protein PMEL, and for pigmentation [96]. Like other melanogenic genes (*TYR*, *TYRP1*, *DCT*, and *PMEL*), *MLANA* expression is transcriptionaly regulated by MITF, a direct PAX3 dowstream target [68-71]. MLANA therefore marks melanocytes that are further down the

differentiation path. By contrast, the transcriptional repressor HES1, another direct target of PAX3 [122,123], is found in undifferentiated melanocyte stem cells of the hair follicle [194].

During the hair cycle, melanocytes are known to progress through stages of differentiation and maturation, closely associated with their location in the growing follicle, namely bulge melanocyte stem cells, their differentiating progeny along the outer root sheath (ORS) and finally mature matrix melanocytes [254].

Utilising co-expression analysis of PAX3 with MLANA or HES1, we identified the differentiation status of follicular and epidermal melanocytes; from less differentiated PAX3, MITF and HES1 positive cells, to more differentiated PAX3, MITF and MLANA positive cells, and also identified PAX3-negative, MITF and MLANA positive cells, which are presumably fully mature melanocytes. We found that epidermal melanocytes resemble transit amplifying cells of the outer root sheath, showing similar numbers of undifferentiated and differentiating PAX3-expressing cells, and a small number of PAX3-negative melanocytes. By contrast, the hair matrix contains much larger numbers of PAX3-negative mature melanocytes [2].

Thus melanocytes express PAX3 along the full differentiation continuum, with its expression diminishing at the very end, in fully mature melanocytes. These cells at the terminal end of differentiation might be cells close to the end of their life span, and perhaps there is no need for PAX3 at this point since the melanogenic cascade is already operational; or perhaps the loss of PAX3 causes their termination, since loss of PAX3 allow cells to undergo apoptosis [27,28]. Nevertheless, it is obvious that normal epidermal melanocytes show variable differentiation levels, from less differentiated to fully mature cells.

**PAX3-regulated melanocyte differentiation network**

Networks regulating melanocyte differentiation have been established previously and include the synergistic activity of PAX3 and SOX10 to induce MITF, which then activates the whole cascade of melanogenic targets (refer to the Figure 1.1.3. in literature review). However, another member of the SOX family, SOX9 has also been implicated in melanocyte regulation. SOX9 is commonly expressed in cells of melanocytic lineage, including normal melanocytes, naevi and melanomas [16,279,283,284]. In contrast to SOX10, increased levels of SOX9 are found in differentiating melanocytes, particularly after UVB exposure, playing a role in melanogenesis by upregulating *MITF*, *DCT* and *TYR* [16,279]. By contrast, the transcriptional repressor HES1 promotes stem cell maintenance and prevents premature differentiation [122,123,254]. PAX3 is found to directly regulate both *HES1* and *SOX9* during neural crest development [122,123], to differentially regulate stem cell maintenance and differentiation [285].

We have looked to see if the same PAX3 - HES1, SOX9 regulation axis is operational in melanocytes. Indeed ChIP experiments show *HES1* and *SOX9* promoter occupancy by PAX3, supporting our hypothesis [3]. Moreover, we observed PAX3 binding to the *DCT* promoter, an event shown to direct melanocyte stem cell maintenance [19]. It does seem then that PAX3 is promoting or maintaining a less differentiated melanocyte phenotype via HES1, SOX9 and DCT, shown for the first time here to act together in regulating adult epidermal melanocytes. Interestingly, the well known *MITF* activation by PAX3, known to drive differentiation seems to be lost in adult epidermal melanocytes; we did not observe PAX3 binding to the *MITF* promoter in melanocytes in any of our experiments. Similarly *Pax3* transfection in murine melanocytes did not result in upregulation of *Mitf* or upregulation of other melanogenic factors [121].

Therefore, regulation of melanocyte differentiation, more complex than initially proposed, is driven by a network of factors, including transcription factors PAX3, SOX10, SOX9, MITF, and HES1, and downstream effectors TYR, TYRP1, DCT, MLANA, and PMEL. Moreover, several of the components of this network have

been shown to be affected by two major signalling pathways, Wnt and TGFβ. Canonical Wnt signalling pathway is shown to be essential both during hair cycle and associated melanocyte renewal and melanogenesis, mainly affecting *MITF* activation thus promoting melanocyte differentiation [19,118,149,150]. On the other hand, TGFβ signalling is required for maintenance of bulge melanocyte stem cells [117]. Moreover, it antagonises MITF function reducing melanosome maturation and pigmentation, but also antagonises Wnt signalling, by upregulating Wnt inhibitors [20,124,125,286,287].

Furthermore, TGFβ also suppresses *PAX3* and has the opposite effect on *HES1* and *SOX9* activation to that of PAX3 [20,122,123]. So, perhaps a feedback loop functions between these two factors to maintain melanocyte plasticity, and confers on cells the ability to switch between less and more differentiated phenotypes, given the bidirectional regulation of TGFβ and PAX3 [20,58,121].

Even though PAX3 maintains cells in a less differentiated state, it remains expressed even in cells that are undergoing differentiation, hence we see its co-expression with markers of differentiated melanocytes, like MLANA. It is likely to regulate other cellular processes at this point, like proliferation, survival or migration. It is possible therefore that a loss of PAX3 in terminally differentiated mature melanocytes might signal their terminal end point, and apoptosis.

**Significance of PAX3 expression in normal melanocytes**

Several studies, undoubtedly show an antiapoptotic role for PAX3 [27,28], suggesting regulation of *BCL2L1*, *p53* and *PTEN* as mediators of this action [28,55,197,226].

Here we looked at BCL2L1 and PTEN as potential targets of PAX3 in melanocytes and melanoma cells. We observe co-expression of PAX3 and BCL2L1 in a similar proportion of cells in normal melanocytes, naevi and primary melanomas. No co-expression was observed in metastatic melanoma samples, where in fact only one sample was positive for BCL2L1 [2]. These results do suggest PAX3-mediated survival via BCL2L1. However, ChIP experiments show no PAX3 binding to *BCL2L1*

at all in melanocytes, and low binding in a metastatic melanoma cell line [3]. Taken together, these results could mean that PAX3 acts as a transcriptional repressor of *BCL2L1*, and because it does not bind in melanocytes, both PAX3 and BCL2L1 do co-express in the same cell, whereas in metastatic melanoma cells PAX3 binds to *BCL2L1*, preventing transcription, therefore co-expression is not observed; lower levels of *BCL2L1* mRNA observed in melanoma cells, by RT-qPCR, support this view.

Alternatively, PAX3 does activate *BCL2L1* (as others have suggested), and we see co-expression in most of our samples, but the promoter region we have chosen for ChIP experiments might not be correct. Another explanation might lie in alternative BCL2L1 isoforms shown to have different biological functions; the longer isoform 1 (also known as Bcl-xl) is antiapoptotic, whereas the shorter isoform 2 (also known as Bcl-s) is proapoptotic [288]. These two transcripts do however share the same promoter, in which case we cannot be sure which of them is regulated by PAX3 in our experiments. Both of the transcripts are shown to be expressed in normal skin and melanoma samples, but in different proportions [255]. Presumably the most likely explanation is that the antiapoptotic role of PAX3 in melanocytes is via other factors and not BCL2L1.

An interesting observation is that a proportion of epidermal melanocytes in sun exposed normal skin are proliferative [2]. A causal relationship has been established between sun exposure, PAX3 upregulation and increased melanocyte proliferation [20]. Cyclin A2 (*CCNA2*) is upregulated in melanocytes following *PAX3* transfection [121], and in our ChIP experiments we observed low enrichment of the *CCNA2* promoter in melanocytes, suggesting that PAX3 regulation of melanocytic proliferation might be via this general cell cycle regulator. However, PAX3 binding to *CCNA2* is much more prominent in the melanoma cell line. PAX3-knockdown in melanoma cells has been shown to inhibit cell proliferation; and induce cell cycle arrest in S and G2/M phases, both of which require CCNA for progression through S phase and entry to M phase [27]. These results further support regulation of proliferation, as a generic role for PAX3, in both melanocytes and melanoma cells.

Besides its expected involvement in melanocyte differentiation and survival, it is interesting to observe PAX3 binding to promoters of genes associated with migration, namely *MCAM*, *CSPG4* and *CXCR4*, suggesting that regulation of migration is a common feature of PAX3 in melanocytes, as it is during development and tumourigenesis. It also suggests that melanocytes have an intrinsic propensity to migrate. This feature is required for normal hair follicle regeneration [289], and sub-bulge region of hair follicle shows overexpression of *MCAM* in outer root sheath, where melanocytes reside [192]. Consistent with these results, we have observed PAX3 and MCAM co-expressing melanocytes in the growing hair follicle, but not in epidermal melanocytes [2]. *MCAM* upregulation in melanoma is associated with metastatic progression [229], and *PAX3*-transfection into melanocytes or medulloblastoma cells has been previously shown to result in *MCAM* upregulation [121,204].

CSPG4 is a chondroitin sulphate proteoglycan that promotes cell motility and invasion by recruiting specific MT-MMPs (membrane type matrix metalloproteinases) and MMPs to the cell membrane [290,291]. Interestingly, CSPG4 was found expressed in many cancers including melanoma [292]. Treatments targeting CSPG4 specifically inhibit melanoma growth *in vivo* and *in vitro* [293,294]. CSPG4 has also been associated with epidermal and follicular stem cells, where it is believed to contribute to their patterning and distribution [295,296].

CXCR4 is a chemokine receptor associated with the metastatic spread and progression of many tumours, including melanoma [276,297-300]. CXCR4**,** and its ligand CXCL12, regulate chemotactic migration and "homing" of tumour cells to a secondary organ/site, and facilitate tumour cell extravasation [278,297,301]. CXCL12 and CXCR4 are shown to similarly regulate melanocyte stem cell migration and their proper positioning during hair cycle [302]. It was shown that *CXCR4* is upregulated by PAX3-FKHR in rhabdomyosarcomas [206,280,281,303].

From these results it is clear that melanocytic cells possess the potential and in fact the need to migrate. This intrinsic ability of melanocytes to migrate could possibly contribute to the aggressive and motile phenotype of transformed melanoma cells. The fact that all of the genes associated with metastatic progression of melanoma tested here, such as *MCAM*, *CSPG4* and *CXCR4*, are shown here to be direct targets of PAX3 in both melanocytes and melanoma cells, strongly supports the role of PAX3 in regulating cell motility, possibly contributing to melanoma dissemination.

**Differential mechanisms of PAX3 regulation in melanoma**

In addition to PAX3-regulated processes that are common in melanocytes and melanoma cells, such as stem-like cell maintenance, proliferation and migration, we have identified here some melanoma exclusive PAX3 targets, such as TPD52 and PTEN, which may differentially mediate melanoma cell proliferation, and prevent apoptosis.

TPD52 has a role in membrane trafficking and its overexpression has been reported in numerous cancers [304-308]; in melanoma it is the fifth most highly upregulated gene [229].

*PTEN* mutations and inactivation are often found in tumours, and overexpression of functional *PTEN* results in cell cycle arrest and apoptosis [197,225,309]. PAX3 was previously reported to repress *PTEN* transcription in rhabdomyosarcoma and myoblasts [197], and here we show that it might do the same in melanoma. Normally, PTEN regulates progression through the G1 cell cycle check point, by negatively regulating PI3K/AKT signalling, through the cell cycle inhibitor (CDK inhibitor) p27Kip1 [197]. PTEN also directly regulates p53 activity [198,199]. Loss of functional PTEN (due to mutations or transcriptional suppression) results in the loss of apoptosis and uncontrolled proliferation.

Another differential PAX3 target in melanoma cells is intermediate filament *Nestin* (*NES*), a neuroectodermal stem and progenitor cell marker that is also present in hair follicle stem cells [310,311]. Its expression has been reported in melanocytes, naevi and melanomas [312-315]. In melanomas stronger staining and increased

numbers of NES positive cells correlate with more advanced stages [312]. NES-positive stage I and II melanomas correlate with worse prognosis [315], and increased numbers of NES-positive circulating melanoma cells correlate with tumour burden, number of metastatic sites and to shorter overall survival [316]. NES marks a subpopulation of melanoma cells with stem cell like properties, as does ABCB5 [144,147]. It is therefore suggested to be an indicator of less differentiated and more aggressive melanomas [312] and is implicated in cell migration and metastasis [317]. Stem-like cell maintenance therefore seems to be the generic role of PAX3, not just in melanocytic but also in other PAX3-associated lineages (such are muscle and neurons).

What is interesting is that PAX3 binding to a common melanocyte and melanoma target *SOX9* is significantly higher in melanoma cells. Besides promoting pigmentation, SOX9 might also regulate proliferation, since its overexpression induces a significant decrease in cell proliferation and G1 cell cycle arrest via p21 upregulation (direct binding of SOX9 to p21 promoter) [283]. Additionally, increased levels of SOX9 in melanoma reduce PRAME (preferentially expressed antigen in melanoma) proteins and restore sensitivity to retinoic acid treatment [283]. Therefore, by repressing SOX9 in melanomas, PAX3 might not only drive a less differentiated phenotype, but might also promote their proliferation and drug resistance.

PAX3 regulation of melanoma cell proliferation and survival, however, seems to be driven by mechanisms distinct from those in melanocytes; this is in contrast to cell differentiation and migration mediators, commonly regulated by PAX3 in both melanocytes and melanoma cells. These results provide strong support for our hypothesis that PAX3 contributes to melanoma progression by conferring to cells less differentiated, proliferative, migratory, and antiapoptotic properties, leading these cells to a proliferative motile aggressive phenotype in melanoma.

**Proliferative vs. aggressive motile melanoma cell phenotype and how does PAX3 fit into this**

High levels of cellular heterogeneity are observed in many melanomas with identification of a subpopulation of cells with invasive properties believed to be responsible for tumour dissemination and metastatic seeding. It is not still clear how some melanoma cells gain this ability; whether through acquisition of additional mutations [104,106], and/or having arisen from a defined subpopulation of slow-cycling self-renewing melanoma stem-like cells [239,240,318-320]. The latter assumes that tumours are hierarchically organised, with a small subpopulation of tumourigenic cells with self-renewing capacity that generate phenotypically diverse nontumourigenic progeny, in a similar manner to normal stem cell differentiation [126]. However, even after intensive effort to fully characterise melanoma stem-like cells, research has yielded conflicting results, and has failed to clearly identify uniform sets of markers for these cells (for review see [29,126]). Some groups suggest that most melanoma cells (not just rare stem cells) actually have tumourigenic capacity [148,321-323].

Another, more recent, concept suggests that most melanoma cells have the ability to switch between less and more active malignant states [124,287,322]. According to this model melanoma metastasis and phenotypic heterogeneity are driven by specific gene expression programs that are imposed by the cellular microenvironment, rather than by accumulation of genetic events. Melanoma cells are able to respond to microenvironmental changes by switching between a highly proliferative (low metastatic potential, leading to tumour growth), and highly invasive phenotype (motile and stem cell-like, resulting in tumour dissemination).

A highly proliferative cell phenotype shows Wnt and MITF-driven gene expression profiles with susceptibility to TGFβ-mediated inhibition of proliferation. By contrast, a highly invasive cell phenotype shows a TGFβ-driven gene expression profile, with downregulation of Wnt signalling, MITF and downstream differentiation markers, and low pigmentation [124,125,324].

In support of this concept, an elegant study by Pinner and colleagues [287] has shown that low or non pigmented melanoma cells contained within the primary lesion are motile and these cells enter the vasculature; however this same low pigmented phenotype is not maintained at the secondary site, suggesting phenotype switching. They have identified these low pigmented motile melanoma cells as having high BRN2 (PUO class 3 homeobox 2 (POU3F2)) and high TGFβ expression. The phenotype switch is bidirectional, but the preference is from Brn2 high to low, or from low pigmented (less differentiated) motile to more differentiated, pigment-producing cells.

Transcriptional regulator BRN2 (also known as POU3F2, or N-Oct-3 and N-Oct-5 when it forms a complex with DNA) is also involved in melanocyte differentiation and melanogenesis; high expression is observed in unpigmented melanoma and melanoblast cultures, whereas pigmented melanocytes and melanoma cell lines show low *BRN2* expression [16,188,325,326]. BRN2 plays a role in maintaining an undifferentiated melanocytic phenotype through *TYR* and *MITF* repression [16,188,325,327], and reduced levels of BRN2 upon differentiation are accompanied by melanosome maturation and increased pigmentation [16,326,328]. Additionally, Brn2 is associated with melanoma cell proliferation, and its regulation has been linked to two major pathways associated with melanoma tumourigenesis. Activation of *Brn2*, as a result of either oncogenic BRAF activity [329], or Wnt/β-catenin signaling [330], enhances melanoma cell proliferation. Similarly, BRN2 ablation in melanoma results in decreased proliferation and tumourigenicity [327,331].

Melanoma cells therefore exhibit phenotypic plasticity and toggle between more or less differentiated cells, through a bidirectional reversible process of phenotype switching in response to the environment. This fits into our concept of a role for PAX proteins in adult tissues that is to regulate cell plasticity, driving their response to environmental cues [52]. Factors characterising both proliferative more differentiated (Wnt signalling, and MITF) and less differentiated motile (BRN2, and TGFβ signalling) phenotype are strongly associated with PAX3. In fact BRN2 has been shown to form protein-protein interaction with key melanocytic

transcription factors PAX3 and SOX10 [332], but also to directly bind to the *Pax3* promoter to stimulate its expression [221,222]. Even though functional interactions between PAX3 and BRN2 on one side, and TGFβ on the other, further support a link between PAX3 and the ability of melanoma cells to switch phenotype in response to environmental changes, further studies are required to clarify the precise nature of the interaction between these factors.

PAX3 could therefore be an intrinsic factor in melanocyte and melanoma cell plasticity, controlling their 'appropriate' response. As our results suggest it can promote a less differentiated stem-like cell phenotype (via HES1, SOX9, NES, DCT), but might also switch cells to an MITF-driven pigmented, differentiated phenotype; in corroboration we do see PAX3 binding to the *MITF* promoter in melanoma cells, but not in melanocytes.

In melanoma cells PAX3 bound more frequently to genes involved in determining a less differentiated, motile phenotype, compared to those involved in proliferation and survival [3]. This might reflect the characteristics of the metastatic melanoma cell line used here, A2058, shown to have low pigmentation, with high BRN2 levels [16,188,326]. Nevertheless, significantly higher enrichment of *TPD52*, and *PTEN*, relative to melanocytes, signifies additional differential regulatory mechanisms are operating in melanoma to regulate their proliferation and survival.

By controlling the crucial cellular processes (such as proliferation, cell survival and migration), as well as promoting a less differentiated stem-like cell phenotypes, PAX3 indeed 'ticks all the boxes' as an intrinsic factor driving melanoma development and progression. Moreover, PAX3 seems capable of translating environmental signals into cellular response.

# *Chapter VI – Conclusion*

**Limitations of the study**

This study has conclusively shown continuous expression of the melanocytic developmental regulator, PAX3, in adult normal epidermal melanocytes [2], and this was also confirmed later by He and colleagues [18]. A study from the USA however, reports no PAX3 expression in normal skin and naevi, and expression is only observed in a small proportion of melanomas [24]. This discrepancy might be attributed to the methodology, but might also be a reflection of the geographic origin of the samples used for these studies. It has been shown that UV-induced TGFβ reduction results in *PAX3* upregulation and melanocyte proliferation, linking *PAX3* expression to the extent of sun exposure [20]. There might therefore be a difference between melanoma and skin samples originating from the Northern vs the Southern hemisphere, related to the amount of sun exposure and UV radiation.

The limitation of our study is that a relatively small number of normal skin samples were tested which originated from different anatomical locations. Additional normal skin samples, from both exposed and non sun-exposed areas are required to address this anomaly.

Another limitation of this study relates to the cell lines used for PAX3 target analysis. One metastatic melanoma cell line and one neonatal human epidermal melanocyte primary culture were used for the study. Neonatal melanocytes are known to exhibit different culturing capacity compared to adult epidermal melanocytes. On the other hand the A2058 melanoma cell line is a highly metastatic low pigmented cell line. Further experiments using additional cell lines, in particular adult epidermal melanocytes and a less aggressive melanoma cell line, might provide additional information.

Results from this study provide, for the first time, several novel findings, such as a continuous expression of PAX3 across the whole range of melanocytic cells, from melanoblasts, throughout adult melanocytes, through to melanocytic lesions. We have also identified novel PAX3 targets and mechanisms differentially regulated in

melanoma cells, compared to melanocytes. These results add to our knowledge of melanocyte biology, opening a new perspective on PAX3 and its role in melanoma progression.

**Future directions**

This study has identified differential and common PAX3 targets in melanocytes and melanoma cells, implicating it in regulation of proliferation, survival and migration, as well as in maintenance of a less differentiated cell state, in both cell types. Further investigation is however required to clarify the precise mechanisms of PAX3 regulation of these processes. We have observed that PAX3 binding to its targets, and presumably its activity, is more prominent in melanoma cells compared to melanocytes; however the exact reasons for this are not known. Neither is it known why some PAX3 targets are different in melanoma cells to those in melanocytes.

The main query arising from this study is how does PAX3 employ different mechanisms of action in different cell types? What are the factors that modulate/enhance PAX3 activity in melanoma, and how does that translate into the functional outcome? Further investigation including functional and mechanistic analysis of PAX3 regulation of downstream pathways in melanoma is required to answer these questions.

One possible explanation lies in different PAX3 isoforms. By alternative splicing, a single *PAX3* gene is able to encode multiple isoforms, that differ in the structure of their paired, homeodomain and/or transactivation domains, and ultimately in binding activity and function [177-179]. For example, the inclusion of Glutamine (Q) within the linker region between the paired HTH motifs (PAX3Q+ isoform), alters the binding affinity of the paired domain compared to the Q- isoform [333,334]. In addition to the Q+ and Q- isoforms at least eight more PAX3 isoforms have been reported to date (named as PAX3A-I) [11,151,168,177]. Two shorter isoforms, PAX3A and B, both lack the homeodomain and the C-terminus; while the other longer isoforms differ in the structure of the transactivation domain.

Different *PAX3* transcripts are shown to differentially impact on cellular processes, when transfected into a nontumourigenic murine melanocyte cell line. *PAX3C*, *D* and *H* transcripts for example increase cell transformation, proliferation, migration and survival; whereas *PAX3A* and *B* exhibit the opposite effect, and *PAX3E* and *G* reduce melanocyte growth and increase apoptosis [163]. Interestingly, *PAX3C* and *PAX3D* transcripts are more commonly expressed in melanomas [168], suggesting these isoforms might be driving melanoma progression. It is possible that differences observed between PAX3-regulated pathways in melanoma and melanocytes could be attributed to different predominant isoforms in each cell type. Therefore, the expression pattern of *PAX3* transcripts in melanoma cells lines vs. melanocytes needs to be thoroughly investigated.

Another possible determinant of differential PAX3 activity in melanoma is posttranslational modification of the PAX3 protein. Several recent studies have reported that PAX3 protein modifications, such is ubiquitination or acetylation, can switch PAX3 activity from promoting myogenic and neuronal stem cell maintenance to initiating a differentiation program [218,285]. Similarly, changing patterns of Pax3 phosphorylation (at Ser205, 201, and 209) have been seen in proliferating compared to differentiating mouse primary myoblasts [216,335,336].

According to the model proposed by Dietz and colleagues [336], the initial phosphorylation of Ser205 by CK2 in proliferating myoblasts promotes the GSKβ-dependant phosphorylation of Ser201, followed by the subsequent dephosphorylation of Ser205. Upon induction of differentiation, phosphorylation at Ser205 is completely abolished, whereas phosphorylation at Ser201 persists, and is accompanied by a rapid increase in phosphorylation at Ser209. Loss of phosphorylation at Ser209, consistent with a decrease in Pax3 expression, is observed after 4 hours of differentiation. The authors further suggest that the presence of a single phosphorylation at Ser201 after 8 hours of differentiation is sufficient to target Pax3 for degradation; which is an essential step for the progression of the differentiation program in activated satellite cells [218]. Transient Pax3 expression is observed in proliferative intermediate progenitor cell populations during satellite cell activation [337], but its sustained expression was

found to inhibit myogenic differentiation and maintain myogenic progenitors in an undifferentiated state [218]. Pax3 protein stability is regulated by the ubiquitin/proteasome system. Monobiquitination (at the two C-terminal lysine residues, K475 and K437) by Taf1, targets Pax3 for proteasomal degradation, via Rad23B-shuttling to the proteasome [218,338].

Interestingly the same two lysine residues, K475 and K437, have been recently shown to be critical for acetylation as well, associated with Pax3-regulated neuronal differentiation [285]. Acetylated Pax3 may downregulate *Hes1* and upregulate *Neurog2*, resulting in switching from stem cell proliferation to neuronal differentiation. Acetylated Pax3 is a substrate for NAD-dependant deacetylase sirtuin 1 (SIRT1), associated with stem cell maintenance; and in the presence of SIRT1 Pax3 is deacetylated and thus able to activate *Hes1* and repress *Neurog2* transcription, maintaining undifferentiated cell state [285].

Pax3 deacetylation is also found to correlate with melanogenesis and possibly with melanocytic differentiation [339]. Histone deacetylase HDAC10 is found to physically interact with Pax3 via its paired domain, forming a ternary complex that maintains Pax3 in a deacetylated state; however, it is not clear if it actively deacetylates Pax3. HDAC10 decreases repressional activity of Pax3 and co-repressor KAP1 on Pax3-target promoters, namely *MITF*, *TRP-1* and *DCT*, thus activating melanogenic program in a murine melanoma cell line B16F10 [339].

It is possible that similar mechanisms are involved in determining differential roles for PAX3 in melanocytes vs melanoma. However, the above mentioned modifications still have to be assessed in melanocytes and melanoma cells.

Finally, PAX3 regulation of cell proliferation, migration and apoptosis in melanocytes and melanoma cells and the mechanism of PAX3 regulation of its downstream targets need to be confirmed *in vitro*. Several studies have reported effects of either exogenous *PAX3* expression or *PAX3* silencing on melanocyte and melanoma cell behaviour [27,28,163], but the effect of *PAX3* silencing or

overexpression on the expression of PAX3 target genes identified in this study also need to be confirmed. Finally, these processes need to be confirmed *in vivo*.

Overall, our findings support an importance of PAX3 for melanocytic lineage and give insight into its role in adult melanocytes. Moreover, they open a new perspective on melanoma progression and role of PAX3 in melanoma-genesis. Here we have paved the way for future studies on the differential role of PAX3 in melanocytes and melanoma, and provided the basis for several prospective projects.

1. Medic S, Ziman M (2009) PAX3 across the spectrum: from melanoblast to melanoma. Crit Rev Biochem Mol Biol: 1-13.
2. Medic S, Ziman M (2010) PAX3 expression in normal skin melanocytes and melanocytic lesions (naevi and melanomas). PLoS One 5: e9977.
3. Medic S, Rizos H, Ziman M (2011) Differential PAX3 functions in normal skin melanocytes and melanoma cells. Biochem Biophys Res Commun 411: 832-837.
4. Lewis TB, Robison JE, Bastien R, Milash B, Boucher K, et al. (2005) Molecular classification of melanoma using real-time quantitative reverse transcriptase-polymerase chain reaction. Cancer 104: 1678-1686.
5. Diepgen TL, Mahler V (2002) The epidemiology of skin cancer. Br J Dermatol 146 Suppl 61: 1-6.
6. Rigel DS (2010) Trends in dermatology: melanoma incidence. Arch Dermatol 146: 318.
7. Lens MB, Dawes M (2004) Global perspectives of contemporary epidemiological trends of cutaneous malignant melanoma. Br J Dermatol 150: 179-185.
8. Balch CM, Buzaid AC, Soong SJ, Atkins MB, Cascinelli N, et al. (2001) Final version of the American Joint Committee on Cancer staging system for cutaneous melanoma. J Clin Oncol 19: 3635-3648.
9. Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB, et al. (2009) Final version of 2009 AJCC melanoma staging and classification. J Clin Oncol 27: 6199-6206.
10. Bang AG, Papalopulu N, Kintner C, Goulding MD (1997) Expression of Pax-3 is initiated in the early neural plate by posteriorizing signals produced by the organizer and by posterior non-axial mesoderm. Development 124: 2075-2085.
11. Goulding MD, Chalepakis G, Deutsch U, Erselius JR, Gruss P (1991) Pax-3, a novel murine DNA binding protein expressed during early neurogenesis. Embo J 10: 1135-1147.
12. Hornyak TJ, Hayes DJ, Chiu LY, Ziff EB (2001) Transcription factors in melanocyte development: distinct roles for Pax-3 and Mitf. Mech Dev 101: 47-59.
13. Lang D, Powell SK, Plummer RS, Young KP, Ruggeri BA (2007) PAX genes: roles in development, pathophysiology, and cancer. Biochem Pharmacol 73: 1-14.
14. Robson EJ, He SJ, Eccles MR (2006) A PANorama of PAX genes in cancer and development. Nat Rev Cancer 6: 52-62.
15. Wang Q, Fang WH, Krupinski J, Kumar S, Slevin M, et al. (2008) Pax genes in embryogenesis and oncogenesis. J Cell Mol Med 12: 2281-2294.
16. Cook AL, Smith AG, Smit DJ, Leonard JH, Sturm RA (2005) Co-expression of SOX9 and SOX10 during melanocytic differentiation in vitro. Exp Cell Res 308: 222-235.
17. Gershon TR, Oppenheimer O, Chin SS, Gerald WL (2005) Temporally regulated neural crest transcription factors distinguish neuroectodermal tumors of varying malignancy and differentiation. Neoplasia 7: 575-584.
18. He S, Yoon HS, Suh BJ, Eccles MR (2010) PAX3 Is Extensively Expressed in Benign and Malignant Tissues of the Melanocytic Lineage in Humans. J Invest Dermatol.
19. Lang D, Lu MM, Huang L, Engleka KA, Zhang M, et al. (2005) Pax3 functions at a nodal point in melanocyte stem cell differentiation. Nature 433: 884-887.

20. Yang G, Li Y, Nishimura EK, Xin H, Zhou A, et al. (2008) Inhibition of PAX3 by TGF-beta modulates melanocyte viability. Mol Cell 32: 554-563.
21. Barr FG, Fitzgerald JC, Ginsberg JP, Vanella ML, Davis RJ, et al. (1999) Predominant expression of alternative PAX3 and PAX7 forms in myogenic and neural tumor cell lines. Cancer Res 59: 5443-5448.
22. Koyanagi K, Kuo C, Nakagawa T, Mori T, Ueno H, et al. (2005) Multimarker quantitative real-time PCR detection of circulating melanoma cells in peripheral blood: relation to disease stage in melanoma patients. Clin Chem 51: 981-988.
23. Muratovska A, Zhou C, He S, Goodyer P, Eccles MR (2003) Paired-Box genes are frequently expressed in cancer and often required for cancer cell survival. Oncogene 22: 7989-7997.
24. Plummer RS, Shea CR, Nelson M, Powell SK, Freeman DM, et al. (2008) PAX3 expression in primary melanomas and nevi. Mod Pathol 21: 525-530.
25. Scholl FA, Kamarashev J, Murmann OV, Geertsen R, Dummer R, et al. (2001) PAX3 is expressed in human melanomas and contributes to tumor cell survival. Cancer Res 61: 823-826.
26. Takeuchi H, Morton DL, Kuo C, Turner RR, Elashoff D, et al. (2004) Prognostic significance of molecular upstaging of paraffin-embedded sentinel lymph nodes in melanoma patients. J Clin Oncol 22: 2671-2680.
27. He S, Li CG, Slobbe L, Glover A, Marshall E, et al. (2010) PAX3 knockdown in metastatic melanoma cell lines does not reduce MITF expression. Melanoma Res.
28. He SJ, Stevens G, Braithwaite AW, Eccles MR (2005) Transfection of melanoma cells with antisense PAX3 oligonucleotides additively complements cisplatin-induced cytotoxicity. Mol Cancer Ther 4: 996-1003.
29. Hoek KS, Goding CR (2010) Cancer stem cells versus phenotype-switching in melanoma. Pigment Cell Melanoma Res 23: 746-759.
30. Bhatia S, Tykodi SS, Thompson JA (2009) Treatment of metastatic melanoma: an overview. Oncology (Williston Park) 23: 488-496.
31. Flaherty KT, Puzanov I, Kim KB, Ribas A, McArthur GA, et al. (2010) Inhibition of mutated, activated BRAF in metastatic melanoma. N Engl J Med 363: 809-819.
32. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, et al. (2010) Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 363: 711-723.
33. Smalley KS, Xiao M, Villanueva J, Nguyen TK, Flaherty KT, et al. (2009) CRAF inhibition induces apoptosis in melanoma cells with non-V600E BRAF mutations. Oncogene 28: 85-94.
34. Wolchok JD, Neyns B, Linette G, Negrier S, Lutzky J, et al. (2010) Ipilimumab monotherapy in patients with pretreated advanced melanoma: a randomised, double-blind, multicentre, phase 2, dose-ranging study. Lancet Oncol 11: 155-164.
35. Dupin E, Le Douarin NM (2003) Development of melanocyte precursors from the vertebrate neural crest. Oncogene 22: 3016-3023.
36. Hirobe T (2005) Role of keratinocyte-derived factors involved in regulating the proliferation and differentiation of mammalian epidermal melanocytes. Pigment Cell Res 18: 2-12.

37. Yanfeng W, Saint-Jeannet JP, Klein PS (2003) Wnt-frizzled signaling in the induction and differentiation of the neural crest. Bioessays 25: 317-325.
38. Knecht AK, Bronner-Fraser M (2002) Induction of the neural crest: a multigene process. Nat Rev Genet 3: 453-461.
39. Baxter LL, Pavan WJ (2002) The oculocutaneous albinism type IV gene Matp is a new marker of pigment cell precursors during mouse embryonic development. Mech Dev 116: 209-212.
40. Hou L, Panthier JJ, Arnheiter H (2000) Signaling and transcriptional regulation in the neural crest-derived melanocyte lineage: interactions between KIT and MITF. Development 127: 5379-5389.
41. Nakayama A, Nguyen MT, Chen CC, Opdecamp K, Hodgkinson CA, et al. (1998) Mutations in microphthalmia, the mouse homolog of the human deafness gene MITF, affect neuroepithelial and neural crest-derived melanocytes differently. Mech Dev 70: 155-166.
42. Opdecamp K, Nakayama A, Nguyen MT, Hodgkinson CA, Pavan WJ, et al. (1997) Melanocyte development in vivo and in neural crest cell cultures: crucial dependence on the Mitf basic-helix-loop-helix-zipper transcription factor. Development 124: 2377-2386.
43. Steel KP, Davidson DR, Jackson IJ (1992) TRP-2/DT, a new early melanoblast marker, shows that steel growth factor (c-kit ligand) is a survival factor. Development 115: 1111-1119.
44. Kunisada T, Yoshida H, Ogawa M, Shultz LD, Nishikawa S (1996) Characterisation and isolation of melanocyte progenitors from mouse embryos. Development, Growth and Differentiation 38: 87-97.
45. Yoshida H, Kunisada T, Kusakabe M, Nishikawa S, Nishikawa SI (1996) Distinct stages of melanocyte differentiation revealed by anlaysis of nonuniform pigmentation patterns. Development 122: 1207-1214.
46. Hirobe T (1984) Histochemical survey of the distribution of the epidermal melanoblasts and melanocytes in the mouse during fetal and postnatal periods. Anat Rec 208: 589-594.
47. Hirobe T, Takeuchi T (1977) Induction of melanogenesis in the epidermal melanoblasts of newborn mouse skin by MSH. J Embryol Exp Morphol 37: 79-90.
48. Mak SS, Moriyama M, Nishioka E, Osawa M, Nishikawa S (2006) Indispensable role of Bcl2 in the development of the melanocyte stem cell. Dev Biol 291: 144-153.
49. Commo S, Gaillard O, Thibaut S, Bernard BA (2004) Absence of TRP-2 in melanogenic melanocytes of human hair. Pigment Cell Res 17: 488-497.
50. Drochmans P (1960) Electron microscope studies of epidermal melanocytes, and the fine structure of melanin granules. J Biophys Biochem Cytol 8: 165-180.
51. Quevedo WC, Szabo G, Virks J (1969) Influence of age and UV on the populations of dopa-positive melanocytes in human skin. J Invest Dermatol 52: 287-290.
52. Blake JA, Thomas M, Thompson JA, White R, Ziman M (2008) Perplexing Pax: from puzzle to paradigm. Dev Dyn 237: 2791-2803.
53. Ziman M, White R (2006) *PAX* genes in cell differentiation, lineage development and pathogenesis. In: Sherbet GV, editor. The Molecular and Cellular

Pathology of Cancer Progression and Prognosis. Kerala: Research Signpost. pp. 235-259.
54. Meulemans D, Bronner-Fraser M (2004) Gene-regulatory interactions in neural crest evolution and development. Dev Cell 7: 291-299.
55. Pani L, Horal M, Loeken MR (2002) Rescue of neural tube defects in Pax-3-deficient embryos by p53 loss of function: implications for Pax-3-dependent development and tumorigenesis. Genes Dev 16: 676-680.
56. Underwood TJ, Amin J, Lillycrop KA, Blaydes JP (2007) Dissection of the functional interaction between p53 and the embryonic proto-oncoprotein PAX3. FEBS Lett 581: 5831-5835.
57. Barber TD, Barber MC, Tomescu O, Barr FG, Ruben S, et al. (2002) Identification of target genes regulated by PAX3 and PAX3-FKHR in embryogenesis and alveolar rhabdomyosarcoma. Genomics 79: 278-284.
58. Mayanil CS, Pool A, Nakazaki H, Reddy AC, Mania-Farnell B, et al. (2006) Regulation of murine TGFbeta2 by Pax3 during early embryonic development. J Biol Chem 281: 24544-24552.
59. Berking C, Takemoto R, Schaider H, Showe L, Satyamoorthy K, et al. (2001) Transforming growth factor-beta1 increases survival of human melanoma through stroma remodeling. Cancer Res 61: 8306-8316.
60. Wight TN (2002) Versican: a versatile extracellular matrix proteoglycan in cell biology. Curr Opin Cell Biol 14: 617-623.
61. Zavadil J, Bitzer M, Liang D, Yang YC, Massimi A, et al. (2001) Genetic programs of epithelial cell plasticity directed by transforming growth factor-beta. Proc Natl Acad Sci U S A 98: 6686-6691.
62. Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, et al. (1997) TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. Development 124: 2659-2670.
63. Blake JA, Ziman MR (2005) Pax3 transcripts in melanoblast development. Dev Growth Differ 47: 627-635.
64. Bondurand N, Pingault V, Goerich DE, Lemort N, Sock E, et al. (2000) Interaction among SOX10, PAX3 and MITF, three genes altered in Waardenburg syndrome. Hum Mol Genet 9: 1907-1917.
65. Watanabe A, Takeda K, Ploplis B, Tachibana M (1998) Epistatic relationship between Waardenburg syndrome genes MITF and PAX3. Nat Genet 18: 283-286.
66. Lacosta AM, Muniesa P, Ruberte J, Sarasa M, Dominguez L (2005) Novel expression patterns of Pax3/Pax7 in early trunk neural crest and its melanocyte and non-melanocyte lineages in amniote embryos. Pigment Cell Res 18: 243-251.
67. Kulhbrodt K, Herbarth B, Sock E, Hermans-Borgmeyer I, Wegner M (1998) SOX10, a novel transcriptional modulator in glia cells. J Neurosci 18: 237-250.
68. Bertolotto C, Busca R, Abbe P, Bille K, Aberdam E, et al. (1998) Different cis-acting elements are involved in the regulation of TRP1 and TRP2 promoter activities by cyclic AMP: pivotal role of M boxes (GTCATGTGCT) and of microphthalmia. Mol Cell Biol 18: 694-702.

69. Du J, Miller AJ, Widlund HR, Horstmann MA, Ramaswamy S, et al. (2003) MLANA/MART1 and SILV/PMEL17/GP100 are transcriptionally regulated by MITF in melanocytes and melanoma. Am J Pathol 163: 333-343.
70. Yasumoto K, Yokoyama K, Shibata K, Tomita Y, Shibahara S (1995) Microphthalmia-associated transcription factor as a regulator for melanocyte-specific transcription of the human tyrosinase gene. Mol Cell Biol 15: 1833.
71. Yasumoto K, Yokoyama K, Takahashi K, Tomita Y, Shibahara S (1997) Functional analysis of microphthalmia-associated transcription factor in pigment cell-specific transcription of the human tyrosinase family genes. J Biol Chem 272: 503-509.
72. Yasumoto K, Takeda K, Saito H, Watanabe K, Takahashi K, et al. (2002) Microphthalmia-associated transcription factor interacts with LEF-1, a mediator of Wnt signaling. Embo J 21: 2703-2714.
73. Galibert MD, Yavuzer U, Dexter TJ, Goding CR (1999) Pax3 and regulation of the melanocyte-specific tyrosinase-related protein-1 promoter. J Biol Chem 274: 26894-26900.
74. Yavuzer U, Goding CR (1994) Melanocyte-specific gene expression: role of repression and identification of a melanocyte-specific factor, MSF. Mol Cell Biol 14: 3494-3503.
75. Budd PS, Jackson IJ (1995) Structure of the mouse tyrosinase-related protein-2/dopachrome tautomerase (Tyrp2/Dct) gene and sequence of two novel slaty alleles. Genomics 29: 35-43.
76. Corry GN, Underhill DA (2005) Pax3 target gene recognition occurs through distinct modes that are differentially affected by disease-associated mutations. Pigment Cell Res 18: 427-438.
77. Lin JY, Fisher DE (2007) Melanocyte biology and skin pigmentation. Nature 445: 843-850.
78. Nishimura EK, Jordan SA, Oshima H, Yoshida H, Osawa M, et al. (2002) Dominant role of the niche in melanocyte stem-cell fate determination. Nature 416: 854-860.
79. Yu H, Fang D, Kumar SM, Li L, Nguyen TK, et al. (2006) Isolation of a novel population of multipotent adult stem cells from human hair follicles. Am J Pathol 168: 1879-1888.
80. Cui J, Shen LY, Wang GC (1991) Role of hair follicles in the repigmentation of vitiligo. J Invest Dermatol 97: 410-416.
81. Kanwar AJ, Dogra S, Parsad D (2004) Topical tacrolimus for treatment of childhood vitiligo in Asians. Clin Exp Dermatol 29: 589-592.
82. Falabella R, Barona MI (2009) Update on skin repigmentation therapies in vitiligo. Pigment Cell Melanoma Res 22: 42-65.
83. Chase HB, Rauch R, Smith VW (1951) Critical stages of hair development and pigmentation in the mouse. Physiol Zool 24: 1-8.
84. Fuchs E (2007) Scratching the surface of skin development. Nature 445: 834-842.
85. Ito S (2003) The IFPCS presidential lecture: a chemist's view of melanogenesis. Pigment Cell Res 16: 230-236.
86. Simon JD, Peles D, Wakamatsu K, Ito S (2009) Current challenges in understanding melanogenesis: bridging chemistry, biological control, morphology, and function. Pigment Cell Melanoma Res 22: 563-579.

87. Seiji M, Shimao K, Birbeck MS, Fitzpatrick TB (1963) Subcellular localization of melanin biosynthesis. Ann N Y Acad Sci 100: 497-533.
88. Hearing VJ (2005) Biogenesis of pigment granules: a sensitive way to regulate melanocyte function. J Dermatol Sci 37: 3-14.
89. Raposo G, Marks MS (2007) Melanosomes--dark organelles enlighten endosomal membrane transport. Nat Rev Mol Cell Biol 8: 786-797.
90. Setaluri V (2003) The melanosome: dark pigment granule shines bright light on vesicle biogenesis and more. J Invest Dermatol 121: 650-660.
91. Wasmeier C, Hume AN, Bolasco G, Seabra MC (2008) Melanosomes at a glance. J Cell Sci 121: 3995-3999.
92. Harper DC, Theos AC, Herman KE, Tenza D, Raposo G, et al. (2008) Premelanosome amyloid-like fibrils are composed of only golgi-processed forms of Pmel17 that have been proteolytically processed in endosomes. J Biol Chem 283: 2307-2322.
93. Theos AC, Truschel ST, Tenza D, Hurbain I, Harper DC, et al. (2006) A lumenal domain-dependent pathway for sorting to intralumenal vesicles of multivesicular endosomes involved in organelle morphogenesis. Dev Cell 10: 343-354.
94. Valencia JC, Rouzaud F, Julien S, Chen KG, Passeron T, et al. (2007) Sialylated core 1 O-glycans influence the sorting of Pmel17/gp100 and determine its capacity to form fibrils. J Biol Chem 282: 11266-11280.
95. De Maziere AM, Muehlethaler K, van Donselaar E, Salvi S, Davoust J, et al. (2002) The melanocytic protein Melan-A/MART-1 has a subcellular localization distinct from typical melanosomal proteins. Traffic 3: 678-693.
96. Hoashi T, Watabe H, Muller J, Yamaguchi Y, Vieira WD, et al. (2005) MART-1 is required for the function of the melanosomal matrix protein PMEL17/GP100 and the maturation of melanosomes. J Biol Chem 280: 14006-14016.
97. Fitzpatrick TB, Breathnach AS (1963) [the Epidermal Melanin Unit System.]. Dermatol Wochenschr 147: 481-489.
98. Jimbow K, Quevedo WC, Jr., Fitzpatrick TB, Szabo G (1976) Some aspects of melanin biology: 1950-1975. J Invest Dermatol 67: 72-89.
99. Van Den Bossche K, Naeyaert JM, Lambert J (2006) The quest for the mechanism of melanin transfer. Traffic 7: 769-778.
100. Kamaraju AK, Bertolotto C, Chebath J, Revel M (2002) Pax3 down-regulation and shut-off of melanogenesis in melanoma B16/F10.9 by interleukin-6 receptor signaling. J Biol Chem 277: 15132-15141.
101. Park HY, Kosmadaki M, Yaar M, Gilchrest BA (2009) Cellular mechanisms regulating human melanogenesis. Cell Mol Life Sci 66: 1493-1506.
102. Grichnik JM, Burch JA, Burchette J, Shea CR (1998) The SCF/KIT pathway plays a critical role in the control of normal human melanocyte homeostasis. J Invest Dermatol 111: 233-238.
103. Halaban R, Langdon R, Birchall N, Cuono C, Baird A, et al. (1988) Basic fibroblast growth factor from human keratinocytes is a natural mitogen for melanocytes. J Cell Biol 107: 1611-1619.
104. Bennett DC (2008) How to make a melanoma: what do we know of the primary clonal events? Pigment Cell Melanoma Res 21: 27-38.

105. Hemesath TJ, Price ER, Takemoto C, Badalian T, Fisher DE (1998) MAP kinase links the transcription factor Microphthalmia to c-Kit signalling in melanocytes. Nature 391: 298-301.
106. Bennett DC (2003) Human melanocyte senescence and melanoma susceptibility genes. Oncogene 22: 3063-3069.
107. Reya T, Clevers H (2005) Wnt signalling in stem cells and cancer. Nature 434: 843-850.
108. Ikeya M, Lee SM, Johnson JE, McMahon AP, Takada S (1997) Wnt signalling required for expansion of neural crest and CNS progenitors. Nature 389: 966-970.
109. Dunn KJ, Brady M, Ochsenbauer-Jambor C, Snyder S, Incao A, et al. (2005) WNT1 and WNT3a promote expansion of melanocytes through distinct modes of action. Pigment Cell Res 18: 167-180.
110. Fenby BT, Fotaki V, Mason JO (2008) Pax3 regulates Wnt1 expression via a conserved binding site in the 5' proximal promoter. Biochim Biophys Acta 1779: 115-121.
111. Takeda K, Yasumoto K, Takada R, Takada S, Watanabe K, et al. (2000) Induction of melanocyte-specific microphthalmia-associated transcription factor by Wnt-3a. J Biol Chem 275: 14013-14016.
112. Yamaguchi Y, Passeron T, Watabe H, Yasumoto K, Rouzaud F, et al. (2007) The effects of dickkopf 1 on gene expression and Wnt signaling by melanocytes: mechanisms underlying its suppression of melanocyte function and proliferation. J Invest Dermatol 127: 1217-1225.
113. Massague J (2008) TGFbeta in Cancer. Cell 134: 215-230.
114. Chin D, Boyle GM, Parsons PG, Coman WB (2004) What is transforming growth factor-beta (TGF-beta)? Br J Plast Surg 57: 215-221.
115. Javelaud D, Alexaki VI, Mauviel A (2008) Transforming growth factor-beta in cutaneous melanoma. Pigment Cell Melanoma Res 21: 123-132.
116. Hibino T, Nishiyama T (2004) Role of TGF-beta2 in the human hair cycle. J Dermatol Sci 35: 9-18.
117. Nishimura EK, Suzuki M, Igras V, Du J, Lonning S, et al. (2010) Key roles for transforming growth factor beta in melanocyte stem cell maintenance. Cell Stem Cell 6: 130-140.
118. Tumbar T, Guasch G, Greco V, Blanpain C, Lowry WE, et al. (2004) Defining the epithelial stem cell niche in skin. Science 303: 359-363.
119. Rodeck U, Bossler A, Graeven U, Fox FE, Nowell PC, et al. (1994) Transforming growth factor beta production and responsiveness in normal human melanocytes and melanoma cells. Cancer Res 54: 575-581.
120. Van Belle P, Rodeck U, Nuamah I, Halpern AC, Elder DE (1996) Melanoma-associated expression of transforming growth factor-beta isoforms. Am J Pathol 148: 1887-1894.
121. Wang Q, Kumar S, Mitsios N, Slevin M, Kumar P (2007) Investigation of downstream target genes of PAX3c, PAX3e and PAX3g isoforms in melanocytes by microarray analysis. Int J Cancer 120: 1223-1231.
122. Nakazaki H, Reddy AC, Mania-Farnell BL, Shen YW, Ichi S, et al. (2008) Key basic helix-loop-helix transcription factor genes Hes1 and Ngn2 are regulated by Pax3 during mouse embryonic development. Dev Biol 316: 510-523.

123. Nakazaki H, Shen YW, Yun B, Reddy A, Khanna V, et al. (2009) Transcriptional regulation by Pax3 and TGFbeta2 signaling: a potential gene regulatory network in neural crest development. Int J Dev Biol 53: 69-79.
124. Hoek KS, Eichhoff OM, Schlegel NC, Dobbeling U, Kobert N, et al. (2008) In vivo switching of human melanoma cells between proliferative and invasive states. Cancer Res 68: 650-656.
125. Hoek KS, Schlegel NC, Brafford P, Sucker A, Ugurel S, et al. (2006) Metastatic potential of melanomas defined by specific gene expression profiles with no BRAF signature. Pigment Cell Res 19: 290-302.
126. Shackleton M (2010) Normal stem cells and cancer stem cells: similar and different. Semin Cancer Biol 20: 85-92.
127. Chen KG, Valencia JC, Lai B, Zhang G, Paterson JK, et al. (2006) Melanosomal sequestration of cytotoxic drugs contributes to the intractability of malignant melanomas. Proc Natl Acad Sci U S A 103: 9903-9907.
128. Haimeur A, Conseil G, Deeley RG, Cole SP (2004) The MRP-related and BCRP/ABCG2 multidrug resistance proteins: biology, substrate specificity and regulation. Curr Drug Metab 5: 21-53.
129. Nakayama K (2010) Growth and progression of melanoma and non-melanoma skin cancers regulated by ubiquitination. Pigment Cell Melanoma Res 23: 338-351.
130. Ireland A, Millward M, Pearce R, Lee M, Ziman M (2011) Genetic factors in metastatic progression of cutaneous melanoma: the future role of circulating melanoma cells in prognosis and management. Clin Exp Metastasis 28: 327-336.
131. Pantel K (1999) Minimal residual disease. Introductory overview. Cancer Metastasis Rev 18: 1-2.
132. Toma JG, McKenzie IA, Bagli D, Miller FD (2005) Isolation and characterization of multipotent skin-derived precursors from human skin. Stem Cells 23: 727-737.
133. Zabierowski SE, Fukunaga-Kalabis M, Li L, Herlyn M (2011) Dermis-derived stem cells: a source of epidermal melanocytes and melanoma? Pigment Cell Melanoma Res 24: 422-429.
134. Weatherhead SC, Haniffa M, Lawrence CM (2007) Melanomas arising from naevi and de novo melanomas--does origin matter? Br J Dermatol 156: 72-76.
135. Clark WH, Jr., Elder DE, Guerry Dt, Braitman LE, Trock BJ, et al. (1989) Model predicting survival in stage I melanoma based on tumor progression. J Natl Cancer Inst 81: 1893-1904.
136. Gray-Schopfer V, Wellbrock C, Marais R (2007) Melanoma biology and new targeted therapy. Nature 445: 851-857.
137. Bennett DC, Medrano EE (2002) Molecular regulation of melanocyte senescence. Pigment Cell Res 15: 242-250.
138. Govindarajan B, Sligh JE, Vincent BJ, Li M, Canter JA, et al. (2007) Overexpression of Akt converts radial growth melanoma to vertical growth melanoma. J Clin Invest 117: 719-729.
139. Haass NK, Smalley KS, Li L, Herlyn M (2005) Adhesion, migration and communication in melanocytes and melanoma. Pigment Cell Res 18: 150-159.

140. Hsu M, Andl T, Li G, Meinkoth JL, Herlyn M (2000) Cadherin repertoire determines partner-specific gap junctional communication during melanoma progression. J Cell Sci 113 ( Pt 9): 1535-1542.
141. Vaisanen A, Tuominen H, Kallioinen M, Turpeenniemi-Hujanen T (1996) Matrix metalloproteinase-2 (72 kD type IV collagenase) expression occurs in the early stage of human melanocytic tumour progression and may have prognostic value. J Pathol 180: 283-289.
142. Hsu MY, Meier F, Herlyn M (2002) Melanoma development and progression: a conspiracy between tumor and host. Differentiation 70: 522-536.
143. Fang D, Nguyen TK, Leishear K, Finko R, Kulp AN, et al. (2005) A tumorigenic subpopulation with stem cell properties in melanomas. Cancer Res 65: 9328-9337.
144. Grichnik JM, Burch JA, Schulteis RD, Shan S, Liu J, et al. (2006) Melanoma, a tumor based on a mutant stem cell? J Invest Dermatol 126: 142-153.
145. Lee JT, Herlyn M (2007) Old disease, new culprit: tumor stem cells in cancer. J Cell Physiol 213: 603-609.
146. Reya T, Morrison SJ, Clarke MF, Weissman IL (2001) Stem cells, cancer, and cancer stem cells. Nature 414: 105-111.
147. Schatton T, Murphy GF, Frank NY, Yamaura K, Waaga-Gasser AM, et al. (2008) Identification of cells initiating human melanomas. Nature 451: 345-349.
148. Quintana E, Shackleton M, Sabel MS, Fullen DR, Johnson TM, et al. (2008) Efficient tumour formation by single human melanoma cells. Nature 456: 593-598.
149. Morris RJ, Liu Y, Marles L, Yang Z, Trempus C, et al. (2004) Capturing and profiling adult hair follicle stem cells. Nat Biotechnol 22: 411-417.
150. Osawa M, Egawa G, Mak SS, Moriyama M, Freter R, et al. (2005) Molecular characterization of melanocyte stem cells in their niche. Development 132: 5589-5599.
151. Barber TD, Barber MC, Cloutier TE, Friedman TB (1999) PAX3 gene structure, alternative splicing and evolution. Gene 237: 311-319.
152. Ziman M, Medic S, Slattery R, Pearce R (2008) Blood test for cutaneous malignant melanoma. Anticancer Research Proceedings of the 8th Anticancer Research Conference.
153. Barr FG (2001) Gene fusions involving PAX and FOX family members in alveolar rhabdomyosarcoma. Oncogene 20: 5736-5746.
154. Blake J, Ziman MR (2003) Aberrant PAX3 and PAX7 expression. A link to the metastatic potential of embryonal rhabdomyosarcoma and cutaneous malignant melanoma? Histol Histopathol 18: 529-539.
155. Galili N, Davis RJ, Fredericks WJ, Mukhopadhyay S, Rauscher FJ, 3rd, et al. (1993) Fusion of a fork head domain gene to PAX3 in the solid tumour alveolar rhabdomyosarcoma. Nat Genet 5: 230-235.
156. Shapiro DN, Sublett JE, Li B, Downing JR, Naeve CW (1993) Fusion of PAX3 to a member of the forkhead family of transcription factors in human alveolar rhabdomyosarcoma. Cancer Res 53: 5108-5112.
157. Himoudi N, Nabarro S, Yan M, Gilmour K, Thrasher AJ, et al. (2007) Development of anti-PAX3 immune responses; a target for cancer immunotherapy. Cancer Immunol Immunother 56: 1381-1395.

158. Matsuzaki Y, Hashimoto S, Fujita T, Suzuki T, Sakurai T, et al. (2005) Systematic identification of human melanoma antigens using serial analysis of gene expression (SAGE). J Immunother 28: 10-19.
159. Rodeberg DA, Nuss RA, Elsawa SF, Erskine CL, Celis E (2006) Generation of tumoricidal PAX3 peptide antigen specific cytotoxic T lymphocytes. Int J Cancer 119: 126-132.
160. Barr FG, Galili N, Holick J, Biegel JA, Rovera G, et al. (1993) Rearrangement of the PAX3 paired box gene in the paediatric solid tumour alveolar rhabdomyosarcoma. Nat Genet 3: 113-117.
161. Bernasconi M, Remppis A, Fredericks WJ, Rauscher FJ, 3rd, Schafer BW (1996) Induction of apoptosis in rhabdomyosarcoma cells through down-regulation of PAX proteins. Proc Natl Acad Sci U S A 93: 13164-13169.
162. Borycki AG, Li J, Jin F, Emerson CP, Epstein JA (1999) Pax3 functions in cell survival and in pax7 regulation. Development 126: 1665-1674.
163. Wang Q, Kumar S, Slevin M, Kumar P (2006) Functional analysis of alternative isoforms of the transcription factor PAX3 in melanocytes in vitro. Cancer Res 66: 8574-8580.
164. Miyamura Y, Coelho SG, Wolber R, Miller SA, Wakamatsu K, et al. (2007) Regulation of human skin pigmentation and responses to ultraviolet radiation. Pigment Cell Res 20: 2-13.
165. Ito M, Liu Y, Yang Z, Nguyen J, Liang F, et al. (2005) Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. Nat Med 11: 1351-1354.
166. Ito M, Yang Z, Andl T, Cui C, Kim N, et al. (2007) Wnt-dependent de novo hair follicle regeneration in adult mouse skin after wounding. Nature 447: 316-320.
167. Rusfianti M, Wirohadidjodjo YW (2006) Dermatosurgical techniques for repigmentation of vitiligo. Int J Dermatol 45: 411-417.
168. Parker CJ, Shawcross SG, Li H, Wang QY, Herrington CS, et al. (2004) Expression of PAX 3 alternatively spliced transcripts and identification of two new isoforms in human tumors of neural crest origin. Int J Cancer 108: 314-320.
169. Chi N, Epstein JA (2002) Getting your Pax straight: Pax proteins in development and disease. Trends Genet 18: 41-47.
170. Frost V, Grocott T, Eccles MR, Chantry A (2008) Self-Regulated Pax Gene Expression and Modulation by the TGFbeta Superfamily. Crit Rev Biochem Mol Biol: 1.
171. Wang Q, Fang WH, Krupinski J, Kumar S, Slevin M, et al. (2008) Pax Genes in Embryogenesis and Oncogenesis. J Cell Mol Med.
172. Chalepakis G, Gruss P (1995) Identification of DNA recognition sequences for the Pax3 paired domain. Gene 162: 267-270.
173. Chalepakis G, Jones FS, Edelman GM, Gruss P (1994) Pax-3 contains domains for transcription activation and transcription inhibition. Proc Natl Acad Sci U S A 91: 12745-12749.
174. Epstein DJ, Vogan KJ, Trasler DG, Gros P (1993) A mutation within intron 3 of the Pax-3 gene produces aberrantly spliced mRNA transcripts in the splotch (Sp) mouse mutant. Proc Natl Acad Sci U S A 90: 532-536.

175. Jostes B, Walther C, Gruss P (1990) The murine paired box gene, Pax7, is expressed specifically during the development of the nervous and muscular system. Mech Dev 33: 27-37.
176. Vorobyov E, Mertsalov I, Dockhorn-Dworniczak B, Dworniczak B, Horst J (1997) The genomic organization and the full coding region of the human PAX7 gene. Genomics 45: 168-174.
177. Tsukamoto K, Nakamura Y, Niikawa N (1994) Isolation of two isoforms of the PAX3 gene transcripts and their tissue-specific alternative expression in human adult tissues. Hum Genet 93: 270-274.
178. Seo HC, Saetre BO, Havik B, Ellingsen S, Fjose A (1998) The zebrafish Pax3 and Pax7 homologues are highly conserved, encode multiple isoforms and show dynamic segment-like expression in the developing brain. Mech Dev 70: 49-63.
179. Underhill DA, Gros P (1997) The paired-domain regulates DNA binding by the homeodomain within the intact Pax-3 protein. J Biol Chem 272: 14175-14182.
180. Mollaaghababa R, Pavan WJ (2003) The importance of having your SOX on: role of SOX10 in the development of neural crest-derived melanocytes and glia. Oncogene 22: 3024-3034.
181. Potterf SB, Furumura M, Dunn KJ, Arnheiter H, Pavan WJ (2000) Transcription factor hierarchy in Waardenburg syndrome: regulation of MITF expression by SOX10 and PAX3. Hum Genet 107: 1-6.
182. Potterf SB, Mollaaghababa R, Hou L, Southard-Smith EM, Hornyak TJ, et al. (2001) Analysis of SOX10 function in neural crest-derived melanocyte development: SOX10-dependent transcriptional control of dopachrome tautomerase. Dev Biol 237: 245-257.
183. Lerner AB, Shiohara T, Boissy RE, Jacobson KA, Lamoreux ML, et al. (1986) A mouse model for vitiligo. J Invest Dermatol 87: 299-304.
184. Nishimura EK, Granter SR, Fisher DE (2005) Mechanisms of hair graying: incomplete melanocyte stem cell maintenance in the niche. Science 307: 720-724.
185. Jiao Z, Mollaaghababa R, Pavan WJ, Antonellis A, Green ED, et al. (2004) Direct interaction of Sox10 with the promoter of murine Dopachrome Tautomerase (Dct) and synergistic activation of Dct expression with Mitf. Pigment Cell Res 17: 352-362.
186. Ludwig A, Rehberg S, Wegner M (2004) Melanocyte-specific expression of dopachrome tautomerase is dependent on synergistic gene activation by the Sox10 and Mitf transcription factors. FEBS Lett 556: 236-244.
187. Schwahn DJ, Timchenko NA, Shibahara S, Medrano EE (2005) Dynamic regulation of the human dopachrome tautomerase promoter by MITF, ER-alpha and chromatin remodelers during proliferation and senescence of human melanocytes. Pigment Cell Res 18: 203-213.
188. Cook AL, Donatien PD, Smith AG, Murphy M, Jones MK, et al. (2003) Human melanoblasts in culture: expression of BRN2 and synergistic regulation by fibroblast growth factor-2, stem cell factor, and endothelin-3. J Invest Dermatol 121: 1150-1159.
189. Kawa Y, Ito M, Ono H, Asano M, Takano N, et al. (2000) Stem cell factor and/or endothelin-3 dependent immortal melanoblast and melanocyte populations derived from mouse neural crest cells. Pigment Cell Res 13 Suppl 8: 73-80.

190. Kushimoto T, Valencia JC, Costin GE, Toyofuku K, Watabe H, et al. (2003) The Seiji memorial lecture: the melanosome: an ideal model to study cellular differentiation. Pigment Cell Res 16: 237-244.
191. Nishikawa S, Osawa M (2007) Generating quiescent stem cells. Pigment Cell Res 20: 263-270.
192. Ohyama M, Terunuma A, Tock CL, Radonovich MF, Pise-Masison CA, et al. (2006) Characterization and isolation of stem cell-enriched human hair follicle bulge cells. J Clin Invest 116: 249-260.
193. Carreira S, Goodall J, Denat L, Rodriguez M, Nuciforo P, et al. (2006) Mitf regulation of Dia1 controls melanoma proliferation and invasiveness. Genes Dev 20: 3426-3439.
194. Moriyama M, Osawa M, Mak SS, Ohtsuka T, Yamamoto N, et al. (2006) Notch signaling via Hes1 transcription factor maintains survival of melanoblasts and melanocyte stem cells. J Cell Biol 173: 333-339.
195. Epstein DJ, Vekemans M, Gros P (1991) Splotch (Sp2H), a mutation affecting development of the mouse neural tube, shows a deletion within the paired homeodomain of Pax-3. Cell 67: 767-774.
196. Carreira S, Goodall J, Aksan I, La Rocca SA, Galibert MD, et al. (2005) Mitf cooperates with Rb1 and activates p21Cip1 expression to regulate cell cycle progression. Nature 433: 764-769.
197. Li HG, Wang Q, Li HM, Kumar S, Parker C, et al. (2007) PAX3 and PAX3-FKHR promote rhabdomyosarcoma cell survival through downregulation of PTEN. Cancer Lett 253: 215-223.
198. Freeman DJ, Li AG, Wei G, Li HH, Kertesz N, et al. (2003) PTEN tumor suppressor regulates p53 protein levels and activity through phosphatase-dependent and -independent mechanisms. Cancer Cell 3: 117-130.
199. Zhou M, Gu L, Findley HW, Jiang R, Woods WG (2003) PTEN reverses MDM2-mediated chemotherapy resistance by interacting with p53 in acute lymphoblastic leukemia cells. Cancer Res 63: 6357-6362.
200. Lang D, Epstein JA (2003) Sox10 and Pax3 physically interact to mediate activation of a conserved c-RET enhancer. Hum Mol Genet 12: 937-945.
201. Lang D, Chen F, Milewski R, Li J, Lu MM, et al. (2000) Pax3 is required for enteric ganglia formation and functions with Sox10 to modulate expression of c-ret. J Clin Invest 106: 963-971.
202. Epstein JA, Shapiro DN, Cheng J, Lam PY, Maas RL (1996) Pax3 modulates expression of the c-Met receptor during limb muscle development. Proc Natl Acad Sci U S A 93: 4213-4218.
203. Gupta PB, Kuperwasser C, Brunet JP, Ramaswamy S, Kuo WL, et al. (2005) The melanocyte differentiation program predisposes to metastasis after neoplastic transformation. Nat Genet 37: 1047-1054.
204. Mayanil CS, George D, Freilich L, Miljan EJ, Mania-Farnell B, et al. (2001) Microarray analysis detects novel Pax3 downstream target genes. J Biol Chem 276: 49299-49309.
205. Relaix F, Rocancourt D, Mansouri A, Buckingham M (2004) Divergent functions of murine Pax3 and Pax7 in limb muscle development. Genes Dev 18: 1088-1105.
206. Tomescu O, Xia SJ, Strezlecki D, Bennicelli JL, Ginsberg J, et al. (2004) Inducible short-term and stable long-term cell culture systems reveal that

the PAX3-FKHR fusion oncoprotein regulates CXCR4, PAX3, and PAX7 expression. Lab Invest 84: 1060-1070.
207. Hsieh MJ, Yao YL, Lai IL, Yang WM (2006) Transcriptional repression activity of PAX3 is modulated by competition between corepressor KAP1 and heterochromatin protein 1. Biochem Biophys Res Commun 349: 573-581.
208. Mayanil CS, George D, Mania-Farnell B, Bremer CL, McLone DG, et al. (2000) Overexpression of murine Pax3 increases NCAM polysialylation in a human medulloblastoma cell line. J Biol Chem 275: 23259-23266.
209. Frost V, Grocott T, Eccles MR, Chantry A (2008) Self-regulated Pax gene expression and modulation by the TGFbeta superfamily. Crit Rev Biochem Mol Biol 43: 371-391.
210. Kubic JD, Young KP, Plummer RS, Ludvik AE, Lang D (2008) Pigmentation PAX-ways: The role of Pax3 in melanogenesis, melanocyte stem cell maintenance, and disease. Pigment Cell Melanoma Res.
211. Kubic JD, Young KP, Plummer RS, Ludvik AE, Lang D (2008) Pigmentation PAX-ways: the role of Pax3 in melanogenesis, melanocyte stem cell maintenance, and disease. Pigment Cell Melanoma Res 21: 627-645.
212. Wiggan O, Taniguchi-Sidle A, Hamel PA (1998) Interaction of the pRB-family proteins with factors containing paired-like homeodomains. Oncogene 16: 227-236.
213. Bandara LR, La Thangue NB (1991) Adenovirus E1a prevents the retinoblastoma gene product from complexing with a cellular transcription factor. Nature 351: 494-497.
214. Buck V, Allen KE, Sorensen T, Bybee A, Hijmans EM, et al. (1995) Molecular and functional characterisation of E2F-5, a new member of the E2F family. Oncogene 11: 31-38.
215. Helin K, Lees JA, Vidal M, Dyson N, Harlow E, et al. (1992) A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. Cell 70: 337-350.
216. Miller PJ, Dietz KN, Hollenbach AD (2008) Identification of serine 205 as a site of phosphorylation on Pax3 in proliferating but not differentiating primary myoblasts. Protein Sci 17: 1979-1986.
217. Miller PJ, Hollenbach AD (2007) The oncogenic fusion protein Pax3-FKHR has a greater post-translational stability relative to Pax3 during early myogenesis. Biochim Biophys Acta 1770: 1450-1458.
218. Boutet SC, Disatnik MH, Chan LS, Iori K, Rando TA (2007) Regulation of Pax3 by proteasomal degradation of monoubiquitinated protein in skeletal muscle progenitors. Cell 130: 349-362.
219. Hong CS, Saint-Jeannet JP (2007) The activity of Pax3 and Zic1 regulates three distinct cell fates at the neural plate border. Mol Biol Cell 18: 2192-2202.
220. Harris RG, White E, Phillips ES, Lillycrop KA (2002) The expression of the developmentally regulated proto-oncogene Pax-3 is modulated by N-Myc. J Biol Chem 277: 34815-34825.
221. Pruitt SC, Bussman A, Maslov AY, Natoli TA, Heinaman R (2004) Hox/Pbx and Brn binding sites mediate Pax3 expression in vitro and in vivo. Gene Expr Patterns 4: 671-685.
222. Zhu BK, Pruitt SC (2005) Determination of transcription factors and their possible roles in the regulation of Pax3 gene expression in the mouse B16 F1 melanoma cell line. Melanoma Res 15: 363-373.

223. Rhee JM, Gruber CA, Brodie TB, Trieu M, Turner EE (1998) Highly cooperative homodimerization is a conserved property of neural POU proteins. J Biol Chem 273: 34196-34205.
224. Ziman M, Medic S, Slattery R, Pearce R. Blood Test for Cutaneous Malignant Melanoma. In: Delinassios JG, editor; 2008 October 17-22 2008; Kos, Greece. International Institute of Anticancer Research. pp. 3552-3553.
225. Di Cristofano A, Pandolfi PP (2000) The multiple roles of PTEN in tumor suppression. Cell 100: 387-390.
226. Margue CM, Bernasconi M, Barr FG, Schafer BW (2000) Transcriptional modulation of the anti-apoptotic protein BCL-XL by the paired box transcription factors PAX3 and PAX3/FKHR. Oncogene 19: 2921-2929.
227. McGill GG, Horstmann M, Widlund HR, Du J, Motyckova G, et al. (2002) Bcl2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability. Cell 109: 707-718.
228. McGill GG, Haq R, Nishimura EK, Fisher DE (2006) c-Met expression is regulated by Mitf in the melanocyte lineage. J Biol Chem 281: 10365-10373.
229. Hoek KS (2007) DNA microarray analyses of melanoma gene expression: a decade in the mines. Pigment Cell Res 20: 466-484.
230. Lekmine F, Chang CK, Sethakorn N, Das Gupta TK, Salti GI (2007) Role of microphthalmia transcription factor (Mitf) in melanoma differentiation. Biochem Biophys Res Commun 354: 830-835.
231. Brose MS, Volpe P, Feldman M, Kumar M, Rishi I, et al. (2002) BRAF and RAS mutations in human lung cancer and melanoma. Cancer Res 62: 6997-7000.
232. Davies H, Bignell GR, Cox C, Stephens P, Edkins S, et al. (2002) Mutations of the BRAF gene in human cancer. Nature 417: 949-954.
233. Pollock PM, Meltzer PS (2002) A genome-based strategy uncovers frequent BRAF mutations in melanoma. Cancer Cell 2: 5-7.
234. Shinozaki M, Fujimoto A, Morton DL, Hoon DS (2004) Incidence of BRAF oncogene mutation and clinical relevance for primary cutaneous melanomas. Clin Cancer Res 10: 1753-1757.
235. Garraway LA, Widlund HR, Rubin MA, Getz G, Berger AJ, et al. (2005) Integrative genomic analyses identify MITF as a lineage survival oncogene amplified in malignant melanoma. Nature 436: 117-122.
236. Wellbrock C, Rana S, Paterson H, Pickersgill H, Brummelkamp T, et al. (2008) Oncogenic BRAF regulates melanoma proliferation through the lineage specific factor MITF. PLoS One 3: e2734.
237. Shih IM, Speicher D, Hsu MY, Levine E, Herlyn M (1997) Melanoma cell-cell interactions are mediated through heterophilic Mel-CAM/ligand adhesion. Cancer Res 57: 3835-3840.
238. Ryu B, Kim DS, Deluca AM, Alani RM (2007) Comprehensive expression profiling of tumor cell lines identifies molecular signatures of melanoma progression. PLoS ONE 2: e594.
239. Grichnik JM (2008) Melanoma, nevogenesis, and stem cell biology. J Invest Dermatol 128: 2365-2380.
240. Schatton T, Frank MH (2008) Cancer stem cells and human malignant melanoma. Pigment Cell Melanoma Res 21: 39-55.
241. Vermeulen L, Sprick MR, Kemper K, Stassi G, Medema JP (2008) Cancer stem cells--old concepts, new insights. Cell Death Differ 15: 947-958.

242. Visvader JE, Lindeman GJ (2008) Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer 8: 755-768.
243. Keshet GI, Goldstein I, Itzhaki O, Cesarkas K, Shenhav L, et al. (2008) MDR1 expression identifies human melanoma stem cells. Biochem Biophys Res Commun 368: 930-936.
244. Graf Finckenstein F, Shahbazian V, Davicioni E, Ren YX, Anderson MJ (2008) PAX-FKHR function as pangenes by simultaneously inducing and inhibiting myogenesis. Oncogene 27: 2004-2014.
245. Venters SJ, Argent RE, Deegan FM, Perez-Baron G, Wong TS, et al. (2004) Precocious terminal differentiation of premigratory limb muscle precursor cells requires positive signalling. Dev Dyn 229: 591-599.
246. Ambler CA, Watt FM (2007) Expression of Notch pathway genes in mammalian epidermis and modulation by beta-catenin. Dev Dyn 236: 1595-1601.
247. Blanpain C, Lowry WE, Pasolli HA, Fuchs E (2006) Canonical notch signaling functions as a commitment switch in the epidermal lineage. Genes Dev 20: 3022-3035.
248. Krajewski S, Krajewska M, Shabaik A, Wang HG, Irie S, et al. (1994) Immunohistochemical analysis of in vivo patterns of Bcl-X expression. Cancer Res 54: 5501-5507.
249. Lehmann JM, Holzmann B, Breitbart EW, Schmiegelow P, Riethmuller G, et al. (1987) Discrimination between benign and malignant cells of melanocytic lineage by two novel antigens, a glycoprotein with a molecular weight of 113,000 and a protein with a molecular weight of 76,000. Cancer Res 47: 841-845.
250. Shih IM, Elder DE, Speicher D, Johnson JP, Herlyn M (1994) Isolation and functional characterization of the A32 melanoma-associated antigen. Cancer Res 54: 2514-2520.
251. Bardin N, Anfosso F, Masse JM, Cramer E, Sabatier F, et al. (2001) Identification of CD146 as a component of the endothelial junction involved in the control of cell-cell cohesion. Blood 98: 3677-3684.
252. Lyle S, Christofidou-Solomidou M, Liu Y, Elder DE, Albelda S, et al. (1998) The C8/144B monoclonal antibody recognizes cytokeratin 15 and defines the location of human hair follicle stem cells. J Cell Sci 111 ( Pt 21): 3179-3188.
253. Yamamoto N, Tanigaki K, Han H, Hiai H, Honjo T (2003) Notch/RBP-J signaling regulates epidermis/hair fate determination of hair follicular stem cells. Curr Biol 13: 333-338.
254. Aubin-Houzelstein G, Djian-Zaouche J, Bernex F, Gadin S, Delmas V, et al. (2008) Melanoblasts' proper location and timed differentiation depend on Notch/RBP-J signaling in postnatal hair follicles. J Invest Dermatol 128: 2686-2695.
255. Leiter U, Schmid RM, Kaskel P, Peter RU, Krahn G (2000) Antiapoptotic bcl-2 and bcl-xL in advanced malignant melanoma. Arch Dermatol Res 292: 225-232.
256. Zhuang L, Lee CS, Scolyer RA, McCarthy SW, Zhang XD, et al. (2007) Mcl-1, Bcl-XL and Stat3 expression are associated with progression of melanoma whereas Bcl-2, AP-2 and MITF levels decrease during progression of melanoma. Mod Pathol 20: 416-426.

257. Heere-Ress E, Thallinger C, Lucas T, Schlagbauer-Wadl H, Wacheck V, et al. (2002) Bcl-X(L) is a chemoresistance factor in human melanoma cells that can be inhibited by antisense therapy. Int J Cancer 99: 29-34.
258. Olie RA, Hafner C, Kuttel R, Sigrist B, Willers J, et al. (2002) Bcl-2 and bcl-xL antisense oligonucleotides induce apoptosis in melanoma cells of different clinical stages. J Invest Dermatol 118: 505-512.
259. Luca M, Hunt B, Bucana CD, Johnson JP, Fidler IJ, et al. (1993) Direct correlation between MUC18 expression and metastatic potential of human melanoma cells. Melanoma Res 3: 35-41.
260. Pacifico MD, Grover R, Richman PI, Daley FM, Buffa F, et al. (2005) Development of a tissue array for primary melanoma with long-term follow-up: discovering melanoma cell adhesion molecule as an important prognostic marker. Plast Reconstr Surg 115: 367-375.
261. Pearl RA, Pacifico MD, Richman PI, Wilson GD, Grover R (2008) Stratification of patients by melanoma cell adhesion molecule (MCAM) expression on the basis of risk: implications for sentinel lymph node biopsy. J Plast Reconstr Aesthet Surg 61: 265-271.
262. Xie S, Luca M, Huang S, Gutman M, Reich R, et al. (1997) Expression of MCAM/MUC18 by human melanoma cells leads to increased tumor growth and metastasis. Cancer Res 57: 2295-2303.
263. Denton KJ, Stretch JR, Gatter KC, Harris AL (1992) A study of adhesion molecules as markers of progression in malignant melanoma. J Pathol 167: 187-191.
264. Kraus A, Masat L, Johnson JP (1997) Analysis of the expression of intercellular adhesion molecule-1 and MUC18 on benign and malignant melanocytic lesions using monoclonal antibodies directed against distinct epitopes and recognizing denatured, non-glycosylated antigen. Melanoma Res 7 Suppl 2: S75-81.
265. Melnikova VO, Bar-Eli M (2006) Bioimmunotherapy for melanoma using fully human antibodies targeting MCAM/MUC18 and IL-8. Pigment Cell Res 19: 395-405.
266. Johnson JP, Bar-Eli M, Jansen B, Markhof E (1997) Melanoma progression-associated glycoprotein MUC18/MCAM mediates homotypic cell adhesion through interaction with a heterophilic ligand. Int J Cancer 73: 769-774.
267. Ghazizadeh S, Taichman LB (2005) Organization of stem cells and their progeny in human epidermis. J Invest Dermatol 124: 367-372.
268. Levy V, Lindon C, Harfe BD, Morgan BA (2005) Distinct stem cell populations regenerate the follicle and interfollicular epidermis. Dev Cell 9: 855-861.
269. Takeuchi H, Kuo C, Morton DL, Wang HJ, Hoon DS (2003) Expression of differentiation melanoma-associated antigen genes is associated with favorable disease outcome in advanced-stage melanomas. Cancer Res 63: 441-448.
270. Scurr LL, Pupo GM, Becker TM, Lai K, Schrama D, et al. (2010) IGFBP7 is not required for B-RAF-induced melanocyte senescence. Cell 141: 717-727.
271. Byrne JA, Nourse CR, Basset P, Gunning P (1998) Identification of homo- and heteromeric interactions between members of the breast carcinoma-associated D52 protein family using the yeast two-hybrid system. Oncogene 16: 873-881.

272. Thomas DD, Frey CL, Messenger SW, August BK, Groblewski GE (2010) A role for tumor protein TPD52 phosphorylation in endo-membrane trafficking during cytokinesis. Biochem Biophys Res Commun 402: 583-587.
273. Pollock PM, Walker GJ, Glendening JM, Que Noy T, Bloch NC, et al. (2002) PTEN inactivation is rare in melanoma tumours but occurs frequently in melanoma cell lines. Melanoma Res 12: 565-575.
274. Campoli MR, Chang CC, Kageshita T, Wang X, McCarthy JB, et al. (2004) Human high molecular weight-melanoma-associated antigen (HMW-MAA): a melanoma cell surface chondroitin sulfate proteoglycan (MSCP) with biological and clinical significance. Crit Rev Immunol 24: 267-296.
275. Yang J, Price MA, Li GY, Bar-Eli M, Salgia R, et al. (2009) Melanoma proteoglycan modifies gene expression to stimulate tumor cell motility, growth, and epithelial-to-mesenchymal transition. Cancer Res 69: 7538-7547.
276. Scala S, Giuliano P, Ascierto PA, Ierano C, Franco R, et al. (2006) Human melanoma metastases express functional CXCR4. Clin Cancer Res 12: 2427-2433.
277. Burger JA, Kipps TJ (2006) CXCR4: a key receptor in the crosstalk between tumor cells and their microenvironment. Blood 107: 1761-1767.
278. Gassmann P, Haier J, Schluter K, Domikowsky B, Wendel C, et al. (2009) CXCR4 regulates the early extravasation of metastatic tumor cells in vivo. Neoplasia 11: 651-661.
279. Passeron T, Valencia JC, Bertolotto C, Hoashi T, Le Pape E, et al. (2007) SOX9 is a key player in ultraviolet B-induced melanocyte differentiation and pigmentation. Proc Natl Acad Sci U S A 104: 13984-13989.
280. Begum S, Emami N, Cheung A, Wilkins O, Der S, et al. (2005) Cell-type-specific regulation of distinct sets of gene targets by Pax3 and Pax3/FKHR. Oncogene 24: 1860-1872.
281. Cao L, Yu Y, Bilke S, Walker RL, Mayeenuddin LH, et al. (2010) Genome-wide identification of PAX3-FKHR binding sites in rhabdomyosarcoma reveals candidate target genes important for development and cancer. Cancer Res 70: 6497-6508.
282. SABiosciences EpiTech ChIP Search Portal.
283. Passeron T, Valencia JC, Namiki T, Vieira WD, Passeron H, et al. (2009) Upregulation of SOX9 inhibits the growth of human and mouse melanomas and restores their sensitivity to retinoic acid. J Clin Invest 119: 954-963.
284. Rao P, Fuller GN, Prieto VG (2010) Expression of Sox-9 in metastatic melanoma--a potential diagnostic pitfall. Am J Dermatopathol 32: 262-266.
285. Ichi S, Boshnjaku V, Shen YW, Mania-Farnell B, Ahlgren S, et al. (2011) Role of Pax3 acetylation in the regulation of Hes1 and Neurog2. Mol Biol Cell 22: 503-512.
286. Martinez-Esparza M, Jimenez-Cervantes C, Beermann F, Aparicio P, Lozano JA, et al. (1997) Transforming growth factor-beta1 inhibits basal melanogenesis in B16/F10 mouse melanoma cells by increasing the rate of degradation of tyrosinase and tyrosinase-related protein-1. J Biol Chem 272: 3967-3972.
287. Pinner S, Jordan P, Sharrock K, Bazley L, Collinson L, et al. (2009) Intravital imaging reveals transient changes in pigment production and Brn2

expression during metastatic melanoma dissemination. Cancer Res 69: 7969-7977.
288. Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, et al. (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 74: 597-608.
289. Oshima H, Rochat A, Kedzia C, Kobayashi K, Barrandon Y (2001) Morphogenesis and renewal of hair follicles from adult multipotent stem cells. Cell 104: 233-245.
290. Iida J, Pei D, Kang T, Simpson MA, Herlyn M, et al. (2001) Melanoma chondroitin sulfate proteoglycan regulates matrix metalloproteinase-dependent human melanoma invasion into type I collagen. J Biol Chem 276: 18786-18794.
291. Iida J, Wilhelmson KL, Ng J, Lee P, Morrison C, et al. (2007) Cell surface chondroitin sulfate glycosaminoglycan in melanoma: role in the activation of pro-MMP-2 (pro-gelatinase A). Biochem J 403: 553-563.
292. Wang X, Wang Y, Yu L, Sakakura K, Visus C, et al. (2010) CSPG4 in cancer: multiple roles. Curr Mol Med 10: 419-429.
293. Hafner C, Breiteneder H, Ferrone S, Thallinger C, Wagner S, et al. (2005) Suppression of human melanoma tumor growth in SCID mice by a human high molecular weight-melanoma associated antigen (HMW-MAA) specific monoclonal antibody. Int J Cancer 114: 426-432.
294. Schwenkert M, Birkholz K, Schwemmlein M, Kellner C, Kugler M, et al. (2008) A single chain immunotoxin, targeting the melanoma-associated chondroitin sulfate proteoglycan, is a potent inducer of apoptosis in cultured human melanoma cells. Melanoma Res 18: 73-84.
295. Legg J, Jensen UB, Broad S, Leigh I, Watt FM (2003) Role of melanoma chondroitin sulphate proteoglycan in patterning stem cells in human interfollicular epidermis. Development 130: 6049-6063.
296. Ghali L, Wong ST, Tidman N, Quinn A, Philpott MP, et al. (2004) Epidermal and hair follicle progenitor cells express melanoma-associated chondroitin sulfate proteoglycan core protein. J Invest Dermatol 122: 433-442.
297. Kim M, Koh YJ, Kim KE, Koh BI, Nam DH, et al. (2010) CXCR4 signaling regulates metastasis of chemoresistant melanoma cells by a lymphatic metastatic niche. Cancer Res 70: 10411-10421.
298. Robledo MM, Bartolome RA, Longo N, Rodriguez-Frade JM, Mellado M, et al. (2001) Expression of functional chemokine receptors CXCR3 and CXCR4 on human melanoma cells. J Biol Chem 276: 45098-45105.
299. Scala S, Ottaiano A, Ascierto PA, Cavalli M, Simeone E, et al. (2005) Expression of CXCR4 predicts poor prognosis in patients with malignant melanoma. Clin Cancer Res 11: 1835-1841.
300. Tucci MG, Lucarini G, Brancorsini D, Zizzi A, Pugnaloni A, et al. (2007) Involvement of E-cadherin, beta-catenin, Cdc42 and CXCR4 in the progression and prognosis of cutaneous melanoma. Br J Dermatol 157: 1212-1216.
301. Bartolome RA, Ferreiro S, Miquilena-Colina ME, Martinez-Prats L, Soto-Montenegro ML, et al. (2009) The chemokine receptor CXCR4 and the metalloproteinase MT1-MMP are mutually required during melanoma metastasis to lungs. Am J Pathol 174: 602-612.

302. Belmadani A, Jung H, Ren D, Miller RJ (2009) The chemokine SDF-1/CXCL12 regulates the migration of melanocyte progenitors in mouse hair follicles. Differentiation 77: 395-411.
303. Libura J, Drukala J, Majka M, Tomescu O, Navenot JM, et al. (2002) CXCR4-SDF-1 signaling is active in rhabdomyosarcoma cells and regulates locomotion, chemotaxis, and adhesion. Blood 100: 2597-2606.
304. Byrne JA, Balleine RL, Schoenberg Fejzo M, Mercieca J, Chiew YE, et al. (2005) Tumor protein D52 (TPD52) is overexpressed and a gene amplification target in ovarian cancer. Int J Cancer 117: 1049-1054.
305. Chen SL, Maroulakou IG, Green JE, Romano-Spica V, Modi W, et al. (1996) Isolation and characterization of a novel gene expressed in multiple cancers. Oncogene 12: 741-751.
306. Dhanasekaran SM, Barrette TR, Ghosh D, Shah R, Varambally S, et al. (2001) Delineation of prognostic biomarkers in prostate cancer. Nature 412: 822-826.
307. Malek RL, Irby RB, Guo QM, Lee K, Wong S, et al. (2002) Identification of Src transformation fingerprint in human colon cancer. Oncogene 21: 7256-7265.
308. Roesch A, Becker B, Bentink S, Spang R, Vogl A, et al. (2007) Ataxia telangiectasia-mutated gene is a possible biomarker for discrimination of infiltrative deep penetrating nevi and metastatic vertical growth phase melanoma. Cancer Epidemiol Biomarkers Prev 16: 2486-2490.
309. Sun H, Lesche R, Li DM, Liliental J, Zhang H, et al. (1999) PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. Proc Natl Acad Sci U S A 96: 6199-6204.
310. Amoh Y, Li L, Katsuoka K, Penman S, Hoffman RM (2005) Multipotent nestin-positive, keratin-negative hair-follicle bulge stem cells can form neurons. Proc Natl Acad Sci U S A 102: 5530-5534.
311. Li L, Mignone J, Yang M, Matic M, Penman S, et al. (2003) Nestin expression in hair follicle sheath progenitor cells. Proc Natl Acad Sci U S A 100: 9958-9961.
312. Brychtova S, Fiuraskova M, Hlobilkova A, Brychta T, Hirnak J (2007) Nestin expression in cutaneous melanomas and melanocytic nevi. J Cutan Pathol 34: 370-375.
313. Flammiger A, Besch R, Cook AL, Maier T, Sturm RA, et al. (2009) SOX9 and SOX10 but not BRN2 are required for nestin expression in human melanoma cells. J Invest Dermatol 129: 945-953.
314. Klein WM, Wu BP, Zhao S, Wu H, Klein-Szanto AJ, et al. (2007) Increased expression of stem cell markers in malignant melanoma. Mod Pathol 20: 102-107.
315. Piras F, Perra MT, Murtas D, Minerba L, Floris C, et al. (2010) The stem cell marker nestin predicts poor prognosis in human melanoma. Oncol Rep 23: 17-24.
316. Fusi A, Ochsenreither S, Busse A, Rietz A, Keilholz U (2010) Expression of the stem cell marker nestin in peripheral blood of patients with melanoma. Br J Dermatol 163: 107-114.

317. Kleeberger W, Bova GS, Nielsen ME, Herawi M, Chuang AY, et al. (2007) Roles for the stem cell associated intermediate filament Nestin in prostate cancer migration and metastasis. Cancer Res 67: 9199-9206.
318. Zabierowski SE, Herlyn M (2008) Melanoma stem cells: the dark seed of melanoma. J Clin Oncol 26: 2890-2894.
319. Frank NY, Schatton T, Frank MH (2010) The therapeutic promise of the cancer stem cell concept. J Clin Invest 120: 41-50.
320. Schatton T, Frank MH (2010) The in vitro spheroid melanoma cell culture assay: cues on tumor initiation? J Invest Dermatol 130: 1769-1771.
321. Quintana E, Shackleton M, Foster HR, Fullen DR, Sabel MS, et al. (2010) Phenotypic heterogeneity among tumorigenic melanoma cells from patients that is reversible and not hierarchically organized. Cancer Cell 18: 510-523.
322. Roesch A, Fukunaga-Kalabis M, Schmidt EC, Zabierowski SE, Brafford PA, et al. (2010) A temporarily distinct subpopulation of slow-cycling melanoma cells is required for continuous tumor growth. Cell 141: 583-594.
323. Shackleton M, Quintana E, Fearon ER, Morrison SJ (2009) Heterogeneity in cancer: cancer stem cells versus clonal evolution. Cell 138: 822-829.
324. Jeffs AR, Glover AC, Slobbe LJ, Wang L, He S, et al. (2009) A gene expression signature of invasive potential in metastatic melanoma cells. PLoS One 4: e8461.
325. Eisen T, Easty DJ, Bennett DC, Goding CR (1995) The POU domain transcription factor Brn-2: elevated expression in malignant melanoma and regulation of melanocyte-specific gene expression. Oncogene 11: 2157-2164.
326. Sturm RA, O'Sullivan BJ, Thomson JA, Jamshidi N, Pedley J, et al. (1994) Expression studies of pigmentation and POU-domain genes in human melanoma cells. Pigment Cell Res 7: 235-240.
327. Goodall J, Carreira S, Denat L, Kobi D, Davidson I, et al. (2008) Brn-2 represses microphthalmia-associated transcription factor expression and marks a distinct subpopulation of microphthalmia-associated transcription factor-negative melanoma cells. Cancer Res 68: 7788-7794.
328. Sturm RA, Bisshop F, Takahashi H, Parsons PG (1991) A melanoma octamer binding protein is responsive to differentiating agents. Cell Growth Differ 2: 519-524.
329. Goodall J, Wellbrock C, Dexter TJ, Roberts K, Marais R, et al. (2004) The Brn-2 transcription factor links activated BRAF to melanoma proliferation. Mol Cell Biol 24: 2923-2931.
330. Goodall J, Martinozzi S, Dexter TJ, Champeval D, Carreira S, et al. (2004) Brn-2 expression controls melanoma proliferation and is directly regulated by beta-catenin. Mol Cell Biol 24: 2915-2922.
331. Thomson JA, Murphy K, Baker E, Sutherland GR, Parsons PG, et al. (1995) The brn-2 gene regulates the melanocytic phenotype and tumorigenic potential of human melanoma cells. Oncogene 11: 691-700.
332. Smit DJ, Smith AG, Parsons PG, Muscat GE, Sturm RA (2000) Domains of Brn-2 that mediate homodimerization and interaction with general and melanocytic transcription factors. Eur J Biochem 267: 6413-6422.
333. Vogan KJ, Gros P (1997) The C-terminal subdomain makes an important contribution to the DNA binding activity of the Pax-3 paired domain. J Biol Chem 272: 28289-28295.

334. Vogan KJ, Underhill DA, Gros P (1996) An alternative splicing event in the Pax-3 paired domain identifies the linker region as a key determinant of paired domain DNA-binding activity. Mol Cell Biol 16: 6677-6686.
335. Dietz KN, Miller PJ, Hollenbach AD (2009) Phosphorylation of serine 205 by the protein kinase CK2 persists on Pax3-FOXO1, but not Pax3, throughout early myogenic differentiation. Biochemistry 48: 11786-11795.
336. Dietz KN, Miller PJ, Iyengar AS, Loupe JM, Hollenbach AD (2011) Identification of serines 201 and 209 as sites of Pax3 phosphorylation and the altered phosphorylation status of Pax3-FOXO1 during early myogenic differentiation. Int J Biochem Cell Biol 43: 936-945.
337. Conboy IM, Rando TA (2002) The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. Dev Cell 3: 397-409.
338. Boutet SC, Biressi S, Iori K, Natu V, Rando TA (2010) Taf1 regulates Pax3 protein by monoubiquitination in skeletal muscle progenitors. Mol Cell 40: 749-761.
339. Lai IL, Lin TP, Yao YL, Lin CY, Hsieh MJ, et al. (2010) Histone deacetylase 10 relieves repression on the melanogenic program by maintaining the deacetylation status of repressors. J Biol Chem 285: 7187-7196.

***STATEMENT OF CONTRIBUTION OF THE CANDIDATE***

*To Whom It May Concern,*

*I, **Sandra Medic**, contributed to conceiving and designing the experiments. I performed the majority of experiments, analysed the data, and wrote the manuscript entitled **Differential PAX3 functions in normal skin melanocytes and melanoma cells**. Biochem Biophys Res Commun (2011) 411: 832-837.*

*Exceptions to the experiments performed by S. Medic were: culturing the HEM1455 melanocyte cell line, RNA extraction and Western blotting from HEM1455 cells, as well as preparation of the cell pellet for ChIP experiments, which were done by H. Rizos.*

_____

*(Signature of Candidate)*

*I, as a Co-Author, endorse that the level of contribution by the candidate indicated above is appropriate.*

***Helen Rizos**,*

_____

*(Signature of Co-Author)*

*Westmead Institute for Cancer Research and Melanoma Institute of Australia, University of Sydney at Westmead Millennium Institute,*
*Westmead, NSW, Australia*

*27 September 2011*
*(Date)*