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REVIEW ARTICLE

PAX3 across the spectrum: from melanoblast to melanoma

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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 factors respectively.

Keywords: *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 tumorigenesis 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 factors 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 tumorigenesis (for reviews see Chi and Epstein, 2002; Robson *et al.*, 2006; Ziman and White, 2006; Lang *et al.*, 2007; Blake *et al.*, 2008; Frost *et al.*, 2008; Wang *et al.*, 2008). Pax3 protein contains two DNA binding domains, a paired domain and a homeodomain which can be utilized alone or in combination to bind downstream target genes (Epstein *et al.*, 1993; Chalepakis *et al.*, 1994; Chalepakis and Gruss, 1995; Corry and Underhill, 2005). In addition Pax3 contains a C-terminal transcription activation domain and an octapeptide (Jostes *et al.*, 1990; Chalepakis *et al.*, 1994; Vorobyov *et al.*, 1997). 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 1) (Goulding *et al.*, 1991; Tsukamoto *et al.*, 1994; Barber *et al.*, 1999; Parker *et al.*, 2004). 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 (Tsukamoto *et al.*, 1994; Underhill and Gros, 1997; Seo *et al.*, 1998).

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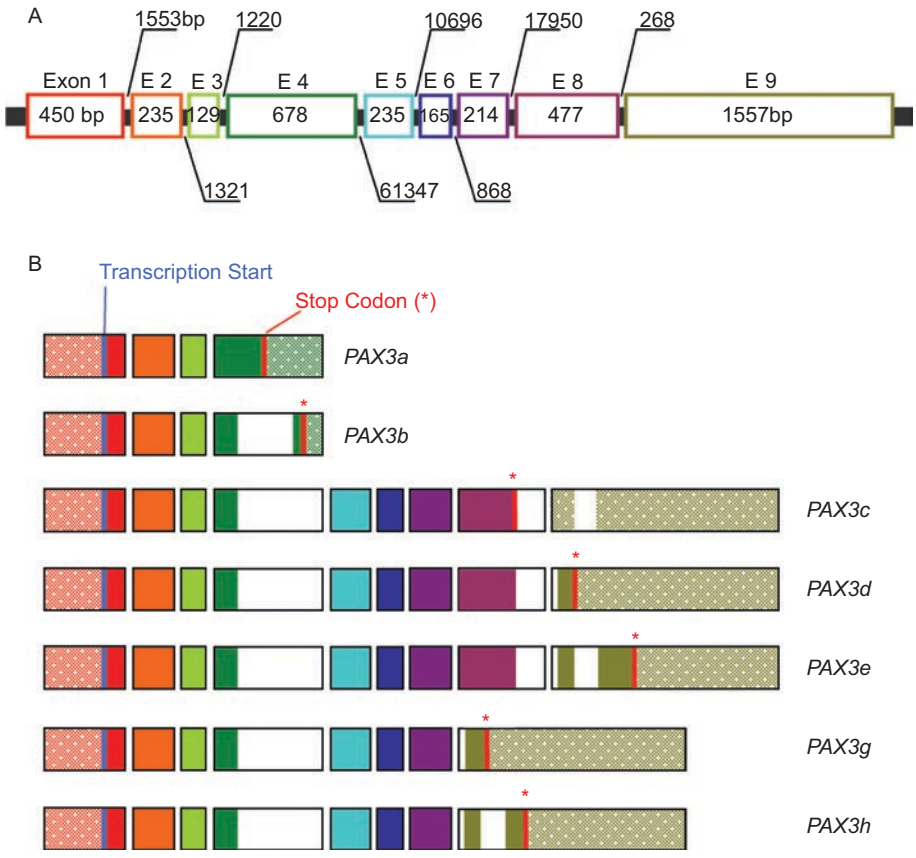

Figure 1. Schematic representation of human *PAX3* mRNA splice variants. (A) shows the exons (E) (E1 to E9) and introns and their respective sizes. (B) shows the structure of alternative transcripts a, b, c, d, e, g, and h. Filled boxes depict sequences retained in mature mRNA, clear boxes represent 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).

Even though Pax3 is recognized 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.

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 (Yanfeng *et al.*, 2003). As the cells divide and migrate, multipotent neural crest cells acquire more lineage-specific phenotypes, including that of melanoblast, which upon reaching its destination in the epidermis terminally differentiates into a melanocyte.

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 2A). *Pax3* is first expressed in neural crest precursors as they differentiate from the neural ectoderm (Bang *et al.*, 1997; Meulemans and Bronner-Fraser, 2004); expression continues as melanoblasts develop and migrate from the neural crest and persists in these cells in developing hair follicles (Blake and Ziman, 2005). Similar temporal expression is observed for *Sox10*, an early neural crest marker essential for the survival of undifferentiated neural crest cells (Mollaaghababa and Pavan, 2003).

Specification along the melanocyte lineage, first observed in neural crest cells overlaying and lateral to

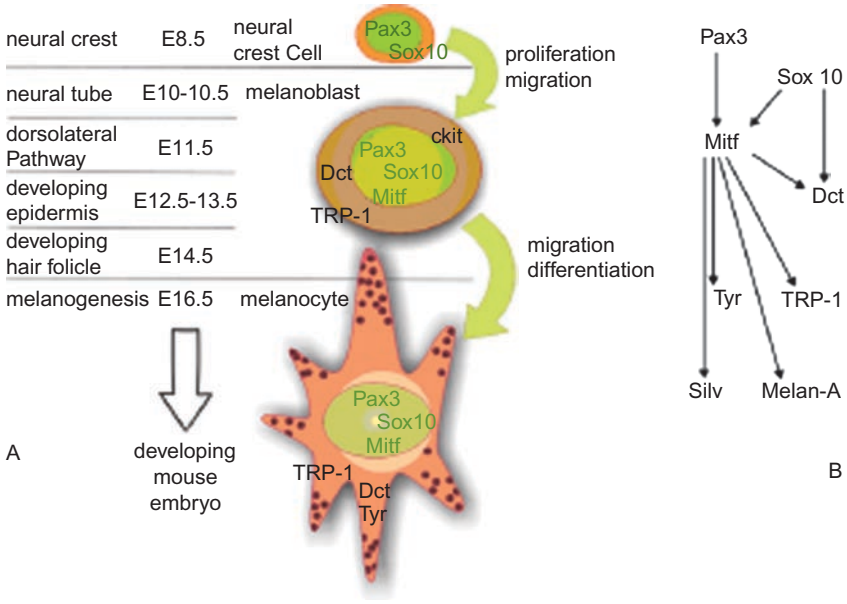

Figure 2. Gene expression patterns in murine melanocytes during development. (A) represents temporal expression of the crucial genes during sequential stages of embryonic melanocyte development. (B) represents the hierarchy of melanocyte-specific gene activation.

the neural tube at E10-10.5, is denoted by the expression of melanoblast markers *Mitf*, *Kit* (*Kit oncogene*) and *Dct* (*Dopachrome Tautomerase*) (Steel *et al.*, 1992; Opdecamp *et al.*, 1997; Nakayama *et al.*, 1998; Hou *et al.*, 2000; Hornyak *et al.*, 2001; Baxter and Pavan, 2002). 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) (Kunisada *et al.*, 1996; Yoshida *et al.*, 1996; Blake and Ziman, 2005). 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) (Steel *et al.*, 1992). Melanogenesis marks the emergence of differentiated melanocytes (E16.5) (Steel *et al.*, 1992).

Melanoblasts migrate to the epidermis and in humans differentiate into melanocytes which lie at the epidermal/dermal border (Drochmans, 1960; Quevedo *et al.*, 1969; Commo *et al.*, 2004; Gershon *et al.*, 2005), whereas in mouse these cells die off (Hirobe and Takeuchi, 1977; Hirobe, 1984; Mak *et al.*, 2006). 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* (Osawa *et al.*, 2005).

Key factors in melanocyte development

Pax3, also known as MSF (melanocyte specific factor) (Galibert *et al.*, 1999), 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 (Gershon *et al.*, 2005). Pax3 is crucial for neural crest specification (Goulding *et al.*, 1991; Bang *et al.*, 1997), and later for expansion of committed melanoblasts formed early in development (Hornyak *et al.*, 2001). 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 (Hornyak *et al.*, 2001), 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 (Mollaaghababa and Pavan, 2003). 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 (Bondurand *et al.*, 2000; Potterf *et al.*, 2000; 2001).

Both Pax3 and Sox10 are required, and precede expression of the transcriptional regulator **Mitf** (Watanabe *et al.*, 1998; Bondurand *et al.*, 2000; Potterf *et al.*, 2000). 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) (Hornyak *et al.*, 2001). 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, characterized by initially normal pigmentation which is lost during the next hair follicle cycle (Lerner *et al.*, 1986; Nishimura *et al.*, 2005).

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 (Yasumoto *et al.*, 2002; Jiao *et al.*, 2004; Ludwig *et al.*, 2004; Lang *et al.*, 2005). The *Dct* promoter region contains an Mitf binding site (M-box) directly adjacent to upstream Lef1 binding sites (Jiao *et al.*, 2004; Lang *et al.*, 2005; Schwahn *et al.*, 2005); Mitf and Lef1 act in synergy to activate the *Dct* promoter (Yasumoto *et al.*, 2002). Pax3 and Mitf share the same binding site within the *Dct* promoter, and compete for occupancy (Lang *et al.*, 2005).

In summary, the hierarchy of melanocyte-specific gene activation (Figure 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 stabilization, such as *SILV* and *Melan-A*, begin in unpigmented undifferentiated melanoblasts, where they show perinuclear localization but become cytoplasmic in fully matured melanocytes (Cook *et al.*, 2003). Unpigmented differentiating melanoblasts possess early immature, stage I and II melanosomes containing melanogenic enzymes (Kawa *et al.*, 2000). Melanogenesis is however, a characteristic of later stage III and IV melanosomes which with maturation take position at the periphery of the cytoplasm (Kushimoto *et al.*, 2003).

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 (Mak *et al.*, 2006; Nishikawa and Osawa, 2007). These quiescent cells are characterized as being Dct- and Pax3-positive (Osawa *et al.*, 2005). 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 dependant upon Wnt signaling (Takeda *et al.*, 2000; Lang *et al.*, 2005). The prerequisite for maintenance of quiescent melanocyte stem cells is downregulation of the Wnt-dependant differentiation programme. 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 (Tumbar *et al.*, 2004; Ohyama *et al.*, 2006). These inhibitors decrease Wnt signaling as well as Mitf levels (Takeda *et al.*, 2000), and as a result induce cell cycle exit (Carreira *et al.*, 2006) 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 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.

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 (Lang *et al.*, 2005). 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 (Nakazaki *et al.*, 2008) and myogenic (reviewed in Wang *et al.*, 2008) 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 (Nakazaki *et al.*, 2008). Hes1 is also implicated in maintenance of both embryonic melanoblasts and melanocyte stem cells in the bulge area of

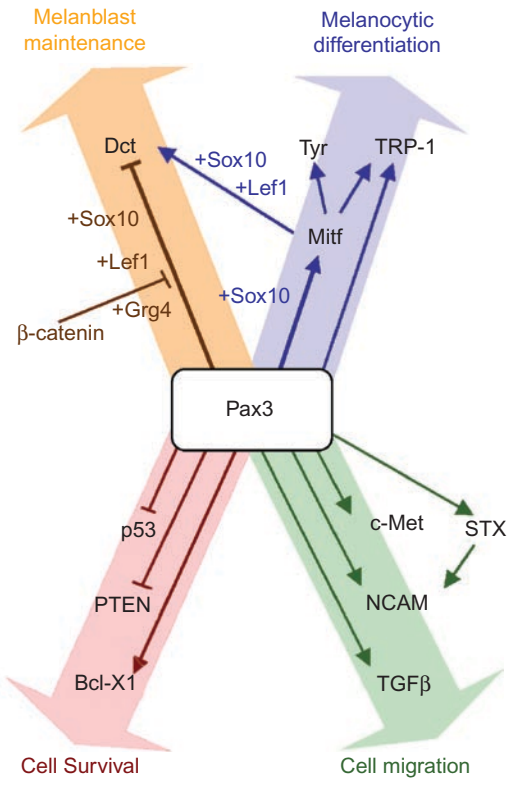

Figure 3. Multiple roles of Pax3 in melanocyte development and maturation. Schematic representation of the cooperation between Pax3 and other factors involved in regulating "stemness", differentiation, survival and migration.

adult mouse hair follicles, possibly via Pax3 (Moriyama *et al.*, 2006).

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 its 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 signaling), 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 (Lang *et al.*, 2005). 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 (Kulhbrodt *et al.*, 1998; Watanabe *et al.*, 1998; Bondurand *et al.*, 2000) 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 (Yavuzer and Goding, 1994; Galibert *et al.*, 1999) thus enhancing the melanogenic cascade. In summary, Pax3 interacts with distinct recognition motifs found in the promoters of *MITF*, *TRP-1* and *Dct* (Budd and Jackson, 1995; Corry and Underhill, 2005). 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 (Corry and Underhill, 2005). Different Pax3 isoforms may mediate these different Pax3 binding activities (Ziman and White, 2006).

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 tumorigenesis and metastasis in melanoma.

Antiapoptotic role for Pax3

Mounting evidence supports an antiapoptotic role for Pax3. Several known antiapoptotic factors, such as tumor suppressors p53, PTEN and Bcl-Xl (see later), are direct downstream targets of Pax3 and thus 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 (Pani *et al.*, 2002). 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 (Pani *et al.*, 2002).

Pax3 has a dual effect on p53: it represses transcription of p53-dependant genes, *BAX* and *HDM2-P2*; and promotes p53 protein degradation (Underwood *et al.*, 2007). p53 exhibits its pro-apoptotic function by promoting transcription of $p21^{Cip/Waf\text{-}1}$, 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 $p21^{Cip/Waf\text{-}1}$ (Underwood *et al.*, 2007). In contrast, the Pax3 target, Mitf regulates $p21^{Cip1}$ expression both directly and indirectly, inducing G1 arrest (Carreira *et al.*, 2005). Mitf cooperates with the hypophosphorylated form of Rb1, to activate $p21^{Cip1}$ expression, which contributes to cell cycle exit and activation of the differentiation programme.

PTEN expression is also directly inhibited by Pax3 (Li *et al.*, 2007), at least in myogenesis. PTEN regulates

progression through the G1 cell cycle check point, by negatively regulating PI3K/AKT signaling, 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 $p27^{Kip1}$ (Li *et al.*, 2007). PTEN also directly regulates p53 activity (Freeman *et al.*, 2003; Zhou *et al.*, 2003).

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 (Lang *et al.*, 2000; Lang and Epstein, 2003), and **c-Met** during limb muscle and melanocyte development (Epstein *et al.*, 1996; Mayanil *et al.*, 2001; Relaix *et al.*, 2004; Tomescu *et al.*, 2004; Gupta *et al.*, 2005; Wang *et al.*, 2007).

Additionally, Pax3 directly represses expression of ***NCAM1*** (Chalepakis *et al.*, 1994; Hsieh *et al.*, 2006), a cell surface molecule involved in cell–cell adhesion. Pax3 also activates expression of ***STX***, which causes post-translational polysialylation of NCAM preventing NCAM–NCAM-mediated homophilic adhesion, leading to decreased cell adhesion and increased migratory properties (Mayanil *et al.*, 2000; 2001).

Other key genes involved in embryonic neural crest migration are *TGFα* and ***TGFβ*** (reviewed in Frost *et al.*, 2008), both of which are directly regulated by Pax3 (Barber *et al.*, 2002; Mayanil *et al.*, 2006). TGFβ signaling regulates genes responsible for remodeling 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 (Mayanil *et al.*, 2006). *TGFβ* knock-out mice show neural tube defects (Sanford *et al.*, 1997), 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 (Mayanil *et al.*, 2006).

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 from 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 the differentiated melanocyte 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 (Blake *et al.*, 2008; Kubic *et al.*, 2008). Since Pax3 may have both activating and repressing roles in transcriptional regulation (Ziman and White, 2006; Kubic *et al.*, 2008), 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 (Blake *et al.*, 2008).

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 (Lang *et al.*, 2005), 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 pathways, i.e. *MITF* and *TRP-1* (Cook *et al.*, 2005) 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 the cell cycle regulator pRB (retinoblastoma protein) (Wiggan *et al.*, 1998). pRB family proteins have a dual role in both cell cycle regulation, and in cell fate determination (Wiggan *et al.*, 1998). 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 (Bandara and La Thangue, 1991; Helin *et al.*, 1992; Buck *et al.*, 1995) 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 (Wiggan *et al.*, 1998). 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 (Miller and Hollenbach, 2007; Miller *et al.*, 2008). Pax3 protein stability is also regulated by ubiquitination and proteasomal degradation during adult muscle stem cell activation (Boutet *et al.*, 2007).

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 concentrations (together with Sox10) may need to reach a certain threshold in order to activate *Mitf* transcription and commit the cells to a melanogenic lineage (Galibert *et al.*, 1999). 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 (Hong and Saint-Jeannet, 2007).

Once Mitf is activated in melanoblasts it is possible that Pax3 functions 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 (Lang *et al.*, 2005) repression of differentiation is no longer possible and the melanogenic cascade is initiated.

Factors that upregulate *Pax3* may also provide a clue to its temporal functions. Transcription factors **N-Myc** and **c-Myc** are both regulators of *Pax3* transcription (Harris *et al.*, 2002). *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 (Harris *et al.*, 2002). 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 corepressor KAP1 (Hsieh *et al.*, 2006). 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 (Pruitt *et al.*, 2004; Zhu and Pruitt, 2005). 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 (Rhee *et al.*, 1998). *In vitro* experiments show BRN2 protein levels and DNA-binding affinity decrease during melanocyte differentiation (Cook *et al.*, 2005). By contrast, OCT1 levels increase during the differentiation process (Cook *et al.*, 2005) indicating that either BRN2 or OCT-1 can regulate and maintain PAX3 levels.

PAX3 expression in melanoma

While PAX3 function in developing melanocytes is reasonably clear, its precise role in tumorigenesis is undefined. Perplexingly, expression is observed in melanocytes of normal skin (Gershon *et al.*, 2005; our own unpublished observations), in benign naevi (Plummer *et al.*, 2008) and in melanomas (Barber *et al.*, 1999; Barr *et al.*, 1999; Galibert *et al.*, 1999; Scholl *et al.*, 2001; Muratovska *et al.*, 2003; Plummer *et al.*, 2008). In fact, PAX3 has been identified as a significant marker for melanoma staging (Takeuchi *et al.*, 2004; Koyanagi *et al.*, 2005) and for detection of circulating melanoma cells (Koyanagi *et al.*, 2005; Ziman *et al.*, 2008). It has also been identified as an immunogenic protein in melanomas (Matsuzaki *et al.*, 2005; Rodeberg *et al.*, 2006; Himoudi *et al.*, 2007), with several epitopes able to induce the host's immune response – stimulation of the immune response against *PAX3*-expressing tumor cells results in tumor growth suppression (Rodeberg *et al.*, 2006; Himoudi *et al.*, 2007). Based on the information gleaned thus far it is clear that the function of PAX3 in melanoma is more than merely a marker of the cell type.

PAX3 role(s) in melanoma

As in development, PAX3 plays an antiapoptotic role in cancers such as melanoma and paediatric rhabdomyosarcoma (Barr *et al.*, 1993; Galili *et al.*, 1993; Shapiro *et al.*, 1993; Bernasconi *et al.*, 1996; Borycki *et al.*, 1999). Transfection with *PAX3*-specific antisense nucleotide (*PAX3*-As) induces increased cell detachment, growth reduction and increased apoptosis in transfected melanoma cell lines (He *et al.*, 2005). *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 (He at al., 2005).

Additionally, inactivation of the tumor suppressor PTEN is often found in PAX3-positive human tumors and tumor cell lines, and its overexpression in tumors results in cell cycle arrest and apoptosis via induction of $p27^{Kip}$ (Di Cristofano and Pandolfi, 2000).

Transcription of *BCL-XL*, a member of the *BCL-2* family of antiapoptotic genes, is also directly regulated by PAX3 in rhabdomyosarcoma (Margue *et al.*, 2000). 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 (Margue *et al.*, 2000). Additionally, the PAX3 target MITF regulates *BCL-2* in melanocytes and melanoma (McGill *et al.*, 2002).

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 immortalized melanocytes resulted in their malignant transformation (Gupta *et al.*, 2005). 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 (McGill *et al.*, 2006). Thus PAX3 regulates c-Met either directly or indirectly via Mitf (McGill *et al.*, 2006; Wang *et al.*, 2007).

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 signaling; the other that is strongly metastatic showing upregulation of genes involved in modifying the extracellular environment through TGFβ signaling (Hoek *et al.*, 2006; Hoek, 2007). Induction of TGFβ-like signaling in melanoma may inhibit Wnt signaling 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 tumor, 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 signaling 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* (Mayanil *et al.*, 2006), whereas MITF is functionally regulated by Wnt3a (Takeda *et al.*, 2000). 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 favor differentiation, and intermediate levels promote proliferation (McGill *et al.*, 2002; Carreira *et al.*, 2006). Indeed, overexpression of *Mitf* in a highly aggressive melanoma cell line resulted in morphological and behavioral 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 (Lekmine *et al.*, 2007). Compared to the original cell line, altered melanoma cells were less tumorigenic as evidenced by late development of tumors and lack of liver metastases in injected mice.

PAX3 and MITF lie on opposing sides of the differentiation regulation pathway, determining less and more differentiated melanocytes respectively; similarly perhaps they may dictate less or more 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 (Cook *et al.*, 2005). This suggests that in melanocytic transformation, PAX3 is involved in regulation of 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 tumor cells is via BRAF mediated pathways. One of the genes most frequently mutated in both naevi and melanomas is the *BRAF* gene (Brose

et al., 2002; Davies *et al.*, 2002; Pollock and Meltzer, 2002; Shinozaki *et al.*, 2004). Activating *BRAF* mutations direct two downstream regulatory pathways: one stimulates the *Brn-2* promoter, increasing its expression which drives *Pax3* expression (Zhu and Pruitt, 2005). 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 (Cook *et al.*, 2005). This may explain the persistent expression of *PAX3* commonly observed in melanomas (Barber *et al.*, 1999; Barr *et al.*, 1999; Galibert *et al.*, 1999; Scholl *et al.*, 2001; Muratovska *et al.*, 2003; Plummer *et al.*, 2008).

The second pathway activated by *BRAF* mutations is the MEK-ERK pathway which leads to decreased MITF levels as a result of degradation of the MITF protein (Garraway *et al.*, 2005; Gray-Schopfer *et al.*, 2007). This is evident since in tumors with *MITF* amplification, often seen together with a *BRAF* mutation, the actual levels of MITF are not elevated accordingly (Gray-Schopfer *et al.*, 2007). 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 (Wellbrook *et al.*, 2008).

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 differential growth characteristics in differentiated melanocytes (Wang *et al.*, 2006); *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 (Wang *et al.*, 2006).

It is interesting to note that *PAX3c* increases the migratory ability of transfected melanocytes (Wang *et al.*, 2006), and microarray analysis of *PAX3c*-transfected cells show upregulation of MCAM (also known as MUC18, and CD146) (Mayanil *et al.*, 2001; Wang *et al.*, 2007). MCAM is frequently upregulated in melanomas (Hoek, 2007) and associated with is invasion and metastasis (Shih *et al.*, 1997).

Notably, expression of PAX3 isoforms varies in different PAX3-associated cancers: c and d isoforms are predominant in melanoma and small-cell lung cancer (Parker *et al.*, 2004; Matsuzaki *et al.*, 2005), and g and h in neuroblastoma (Wang *et al.*, 2006); a, b and e are expressed at low or undetectable levels in all of the above tumors (Wang *et al.*, 2006). This suggests that full length isoforms might promote tumorigenesis, whereas shortened isoforms might repress tumor propagation. Indeed, microarray analysis showed downregulation of *PAX3a* and *PAX3b* transcripts in aggressive melanomas compared to normal melanocytes (Ryu *et al.*, 2007).

One explanation is that shortened PAX3 isoforms may compete with full-length isoforms and alter or inhibit their function (Parker *et al.*, 2004). Since PAX3a and b isoforms lack a homeodomain theoretically they cannot bind *Mitf*, but may bind and induce *TRP-1* (Corry and Underhill, 2005) 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 tumors in general, contain tumor "stem" cells harboring metastatic potential (Reya *et al.*, 2001; Lee and Herlyn, 2007; Grichnik, 2008; Schatton and Frank, 2008; Vermeulen *et al.*, 2008; Visvader and Lindeman, 2008). Given that melanoma stem cells may promote tumor growth and metastasis (Fang *et al.*, 2005; Grichnik *et al.*, 2006; Keshet *et al.*, 2008; Schatton *et al.*, 2008) and since PAX3 is involved in maintenance of progenitor cells (Lang *et al.*, 2005; Graf Finckenstein *et al.*, 2008; Nakazaki *et al.*, 2008), 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 area of the hair follicle – i.e. positive for Pax3 and Wnt inhibitors, but negative for Mitf and its downstream melanogenic targets (Osawa *et al.*, 2005) – it seems likely that the melanoma stem cells would have a similar signature. Indeed, melanoma stem cells are in fact quiescent, slow growing, non-melanized cells with a stem cell marker signature, including ABCB5+ (Grichnik *et al.*, 2006; Schatton *et al.*, 2008).

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.

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