Functional Analysis of Alternative Isoforms of the Transcription Factor PAX3 in Melanocytes In vitro

Qiuyu Wang,1 Shant Kumar,2 Mark Slevin,1 and Patricia Kumar1

1School of Biology, Chemistry and Health Science, Manchester Metropolitan University and 2Department of Pathology, Manchester University and Christie Hospital, Manchester, United Kingdom

Abstract

Transcription factor PAX3 has seven isoforms of which PAX3c has been studied extensively whereas the functions of the other isoforms are less well known. Here, we found that PAX3 isoforms in a stable transfection system have different biological functions in mouse melanocytes in vitro. PAX3a and PAX3b had negative effects on melanocyte proliferation but had no discernible effect on melanocyte growth in soft agar. PAX3a did not affect cell migration and apoptosis but PAX3b reduced migration and accelerated apoptosis. PAX3c and PAX3d promoted cell proliferation, migration, transformation, and survival. PAX3e reduced melanocyte growth; transformation and migration were unchanged and apoptosis was increased in vitro. PAX3g did not influence cell proliferation or apoptosis. Cells expressing PAX3g were able to grow in soft agar but migration was reduced. PAX3h increased cell proliferation, migration, survival, and transformation. These functional studies have advanced our understanding of the effects of PAX3 isoforms in melanocytes and their potential contribution in tumorigenesis. (Cancer Res 2006; 66(17): 8574-80)

Introduction

PAX3 and Zinc1 coactivation is essential for delamination of the neural crest during embryogenesis (1). PAX3 regulates myogenesis, melanogenesis, and neurogenesis; all require migration of cells from the dorsal dermomyotome or neural crest. Pax3 is first expressed (E8.5) during neural tube closure, after which its expression is maintained for a few days in the dorsal neural tube and diminishes in neural crest cells during cell migration (2, 3).

Human PAX3 contains 10 exons (Fig. 1A); exons 2, 3, and 4 encode a paired domain, exons 5 and 6 encode an octapeptide and a homeodomain, whereas exons 7 and 8 encode a proline, serine, and threonine-rich transactivation domain (4). Seven alternatively spliced isoforms occur: PAX3a, PAX3b, PAX3c, PAX3d, PAX3e, PAX3g, and PAX3h (5–7). PAX3a and PAX3b are composed of exons 1 to 4, truncated prematurely in intron 4, and lack the homeodomain and the carboxyl-terminal transactivation domain. PAX3b is highly expressed in most tissues but PAX3a only in cerebellum, esophagus, and skeletal muscle (5). PAX3c retains intron 8 and translation continues from exon 8 for five codons into intron 8 before termination. PAX3d lacks intron 8 and translation proceeds from exon 8 to 9. In vitro DNA binding and transactivation studies suggest that PAX3d is functionally similar to PAX3c (6, 8). PAX3e contains exons 8, 9, and 10 but lacks introns 8 and 9. PAX3g and PAX3h are truncated isoforms of PAX3d and PAX3e, respectively; both lack part of the transactivation domain encoded by exon 8 (7).

Melanoblasts arise in the mouse neural crest on E10.5 and, following migration and proliferation, reach the limb buds by E12 and the lateral trunk by E13/E14 (9). The expression of Pax3 is higher in melanoblast than their precursors, although the role of Pax3 in melanoblasts determination is still unclear (10). Murine Pax3 binds to the melanocyte-specific element of the Tyrp1 promoter to activate transcription in melanomas (11). Pax3 regulates microphthalmia-associated transcription factor following binding of its E-box motif in the promoter. This regulation fails after Pax3 mutation (12). Pax3 mutations causing human Waardenburg’s syndrome and the mouse Splotch phenotype manifest with congenital sensorineural deafness and pigmentary disturbance of eyes, hair, and skin (6, 13).

Deregulated expression of Pax3 occurs in tumors, such as cutaneous and uveal melanoma derived from neural crest–derived melanocytes, medulloblastoma, rhabdomyosarcoma, and Ewing’s sarcoma (8). Pax3 expression in human melanomas is thought to contribute to tumor cell survival (14). The expression of Pax3 isoform transcripts varies in different cell lines and tumor types. Pax3c and Pax3d are predominantly expressed in melanoma and small-cell lung cancer (7). Matsuzaki et al. (15) also reported that Pax3d is the main isoform expressed in melanoma, as shown by its presence as an antigen in the blood circulation. Pax3g and Pax3h predominate in neuroblastomas (16). Pax3a, Pax3b, and Pax3e are consistently expressed at low or undetectable levels in all the above tumors. It is tempting to speculate that alternative Pax3 transcripts have distinct roles in the regulation of cell differentiation within these lineages.

Specific functions of individual isoforms during development and tumorigenesis have not been determined. In the present study, stable mouse melanocyte cell lines were constructed expressing individual Pax3 isoforms, which have been characterized on cell growth, migration, transformation, and survival in vitro.

Materials and Methods

Cell lines and plasmids. Melan-a, a nontumorigenic cell line of pigmented melanocytes from normal inbred C57BL mouse embryos (17), was a gift from Prof. Ian Hart, Department of Cancer Research, St. Thomas’ Hospital, London, United Kingdom. Pax3 isoform (a-h) cDNAs from melanoma cell lines were amplified with a forward primer to a noncoding region in the Pax3 promoter, 45 bp from the transcription start site, and reverse primers E5R, I8R, I9R, and I10R, respectively (Fig. 1A). The PCR products were ligated into pCR 2.1 TOPO cloning vector (Invitrogen, Paisley, United Kingdom), and the cDNA inserts sequenced as reported (7). The Pax3 isoform cDNAs were subcloned into the BamHI and NotI sites of pcDNA4/HisMax C vector (Invitrogen).

Stable transfection. The Pax3 isoform pcDNA4 constructs, or pcDNA4 alone as the empty vector control, were stably transfected into Melan-a

Requests for reprints: Patricia Kumar, School of Biology, Chemistry and Health Science, Manchester Metropolitan University, Chester Street, Manchester M1 5GD, United Kingdom. Phone: 44-161-247-1218; Fax: 44-161-247-6325; E-mail: P.Kumar@mmu.ac.uk.

©2006 American Association for Cancer Research.
doi:10.1158/0008-5472.CAN-06-0947
cells using Transfectam (Promega, Southampton, United Kingdom) according to the protocol of the manufacturer. Briefly, Melan-a cells were plated at $5 \times 10^5$ per 60-mm culture dish and reached ~60% to 70% confluence on the following day. The medium was replaced with 1.5 mL of PD, paired domain, PD, and plated at 5 cells using Transfectam (Promega, Southampton, United Kingdom) according to the instructions of the manufacturer. RNA (1 μg) from each sample was reverse transcribed into cDNA with a single-strand cDNA synthesis kit (Promega) according to the instructions of the manufacturer. cDNA from individual transfectants (1 μL) was amplified with different primers: primers E3F/ESR amplified 684- and 277-bp amplicons for PAX3a and PAX3b, respectively; primers E7F/ISR amplified a 532-bp (PAX3c) fragment; primers E7F/IR amplified 532- and 286-bp amplicons for PAX3d and PAX3g, respectively; and primers E7F/I0R amplified 584- and 338-bp amplicons for PAX3e and PAX3h, respectively. The nucleotide sequences of primers and the PCR procedure have been described (7). The PCR products were separated on a 1% agarose Tris-borate EDTA (TBE) gel and gelled with ethidium bromide. To ensure the semiquantitative nature of the results, all experiments were carried out at 20, 25, 30, 35, 40, and 45 cycles (data not shown). Under optimal cycles (35 cycles), the data of PAX3 isoform transfectants were normalized to that of vector control and semiquantitatively analyzed with Scion Image Software version 4.02.2 The housekeeping gene $\beta$16 was used as loading control and its primer sequence was previously described (18). Mean and SD were obtained from triplicate experiments.

**Cell proliferation assay.** A CellTiter 96 nonradioactive cell proliferation assay kit (Promega) was used according to the instructions of the manufacturer. Briefly, empty vector- and PAX3 isoform–transfected cells in complete DMEM containing 400 μg/mL zeocin were seeded at $1 \times 10^5$ per well in 96-well-plates. The plates were incubated at 37°C in 5% CO$_2$ in air for 72 hours. Fifteen microliters of dye solution were added per well and incubated for 4 hours. Solubilization solution was added (100 μL/well), incubated at room temperature overnight, and the absorbance at 570 nm recorded with a plate reader.

**Cell cycle analysis.** PAX3 isoform transfectants and vector control cells seeded at $5 \times 10^5$/mL in six-well-plates were grown for 24 hours, then trypsinized and washed twice with PBS. They were centrifuged at 100 × g for 5 minutes, resuspended with 200 μL PBS, and cell pellets fixed in 2 mL of 70% ethanol at 4°C for at least 1 hour. They were centrifuged at 100 × g for 5 minutes, the pellets resuspended in 437 μL PBS, 13 μL of 0.8 units/mL DNase-free RNAse A, and 40 μg/mL propidium iodide, and incubated at 37°C for at least 30 minutes. Fluorescence data were collected with a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and data were analyzed with CytChred software (version WinMDI 2.7).

**Cell transformation assay.** Cells transfected with empty vector or PAX3 isoforms, and A375 and XP44 melanoma cell lines as positive controls, were suspended as single-cell suspensions in 0.33% cell culture agar (Sigma, Dorset, United Kingdom) in complete DMEM and seeded (3 × 10$^4$/well) in six-well plates onto a 0.6% cell culture agar layer on the bottom of each well. The cell layers were allowed to solidify before incubation at 37°C in 5% CO$_2$ in air. Three weeks later, colonies were counted and photographed under a microscope.

**Scratch wound healing assay.** PAX3 isoform and empty vector transfectants were plated ($5 \times 10^5$/well) onto 13-mm round Thermanox coverslips (Nunc) in 24-well plates and grown in complete DMEM for 24 hours until 90% confluence. A linear wound was generated on the coverslips (Nunc) in 24-well plates and grown in complete DMEM for 24 hours until 90% confluence. Lineweaver used a 0.8 units/mL cell culture agar layer on the bottom of each well. The cell layers were allowed to solidify before incubation at 37°C in 5% CO$_2$ in air. Three weeks later, colonies were counted and photographed under a microscope.

**Anoikis assay.** PAX3 isoform and empty vector transfectants were seeded ($2 \times 10^5$/well) in 24-well plates treated with Cellform (ICN

---

Results

Expression of PAX3 isoform transcripts in melanocytes in vitro. PAX3a-h transcripts were undetectable in Melan-a cells before transfection. Similarly, after transfection with pcDNA4 empty vector, they were not detectable (data not shown). The PAX3 isoform/pcDNA4 constructs were stably transfected into Melan-a cells. Clones expressing similar levels of different PAX3 transcripts were identified with a semiquantitative analysis carried out under optimal cycles. Figure 1B shows PCR products of PAX3a (684 bp), PAX3b (277 bp), PAX3c (532 bp), PAX3d (532 bp), PAX3e (584 bp), PAX3g (286 bp), and PAX3h (338 bp; lanes 1–7, respectively). The Melan a-PAX3a cell line also expressed PAX3b. PAX3a seems to break down into PAX3b or stimulates endogenous PAX3b transcription. The relative intensities of PCR products of transfected cells were normalized with that of empty vector and were found to have increased from 2.2- to 2.7-fold (Fig. 1C). No significant difference was seen among different transfectants (P > 0.05, Student’s t-test), which indicated that PAX3 isoform transcripts were expressed at comparable levels in the clones selected. Their protein expression was confirmed indirectly by Xpress epitope expression using Western blotting. The densitometry measurements compared with vector control for PAX3a, b, c, d, e, g, and h transfectants were 2.84, 1.84, 3.46, 2.15, 3.03, 2.49, and 3.80, respectively.

Effect of Pax3 isoforms on melanocyte growth in vitro. PAX3 isoforms have different effects on melanocyte proliferation in vitro (Fig. 2). PAX3c, PAX3d, and PAX3h isoform transfectants grew statistically significantly faster than the empty vector transfectants over 72 hours. PAX3h transfectants grew fastest (P < 0.001), followed by PAX3d (P < 0.001) and PAX3c (P < 0.01) transfectants. In contrast, PAX3a, PAX3b, and PAX3e transfectants proliferated significantly more slowly than control cells (P < 0.001 in all three cases). PAX3g did not affect cell proliferation (P > 0.05).

Effects of PAX3 isoforms on melanocyte cell cycle status. Table 1 shows the increased proportion of G0 and G1 phase cells in PAX3a, PAX3b, PAX3c, and PAX3g transfectants compared with the control. Higher proportions of PAX3c-, PAX3d-, and PAX3h-expressing cells were in S phase than vector control cells, but the situation was reversed for PAX3a, PAX3b, and PAX3e transfectants. Marked differences were observed between PAX3d and PAX3g transfectants (P < 0.05) and between PAX3e and PAX3h transfectants (P < 0.01). These results indicate that PAX3a, PAX3b, and PAX3e transfectants had slower proliferation rates whereas PAX3c, PAX3d, and PAX3h transfectants had higher rates than controls. The proportion of cells in G2-M phase was highest in PAX3c transfectants.

Effect of PAX3 isoforms on melanocyte transformation. The growth of PAX3 isoform transfectants and vector control cells was examined in soft agar. A fast-growing melanoma cell line, A375, and

Table 1. Cell cycle distribution of Melan a-PAX3 isoform transfectants following 24 hours of subculture

<table>
<thead>
<tr>
<th>Subculture Time</th>
<th>Vector</th>
<th>PAX3a</th>
<th>PAX3b</th>
<th>PAX3c</th>
<th>PAX3d</th>
<th>PAX3e</th>
<th>PAX3g</th>
<th>PAX3h</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0–G1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>46.78 ± 4.79</td>
<td>58.46 ± 1.13</td>
<td>73.98 ± 2.60</td>
<td>27.49 ± 6.51</td>
<td>33.27 ± 10.94</td>
<td>53.89 ± 3.44</td>
<td>57.25 ± 3.80</td>
<td>33.26 ± 0.85</td>
</tr>
<tr>
<td>S</td>
<td>34.09 ± 7.54</td>
<td>25.48 ± 1.77</td>
<td>15.62 ± 2.36</td>
<td>43.15 ± 2.61</td>
<td>48.01 ± 1.27</td>
<td>21.43 ± 1.13</td>
<td>27.42 ± 5.16</td>
<td>54.38 ± 0.25</td>
</tr>
<tr>
<td>G2–M</td>
<td>19.12 ± 3.01</td>
<td>16.07 ± 1.14</td>
<td>10.39 ± 0.92</td>
<td>29.36 ± 7.27</td>
<td>18.73 ± 4.87</td>
<td>24.68 ± 2.67</td>
<td>15.33 ± 2.02</td>
<td>12.17 ± 0.59</td>
</tr>
</tbody>
</table>

NOTE: DNA content was analyzed by propidium iodide incorporation. Each value is the percentage of cells at that stage. Data are the mean and SD of three experiments.

*P < 0.05 versus vector control group in G0–G1.
†P < 0.05 versus vector control group in S.
‡P < 0.05 versus PAX3g transfectants.
§P < 0.01 versus PAX3h transfectants (by Student’s t-test).
a slow-growing one, XP44, were used for comparison. Colonies were counted after 3 weeks and photographed (Fig. 3). Except for the A375 cell line, Melan-a cells expressing PAX3h formed most colonies, followed by those expressing PAX3c, PAX3d, and PAX3g; cells expressing PAX3a, PAX3b, and PAX3e formed few or no colonies; the vector control cells failed to form colonies. There was a significant difference (P < 0.01) in colony numbers between PAX3e and PAX3h transfectants but not between PAX3d and PAX3g transfectants (P > 0.05). These results indicate that the expression of PAX3c, PAX3d, PAX3g, and PAX3h is associated with anchorage-independent growth in soft agar, a variable of tumorigenicity, whereas that of PAX3a, PAX3b, and PAX3e isoforms has no effect. The data also suggest a relationship between cell proliferation and the transformation potential of PAX3 isoforms.

**Effects of PAX3 isoforms on melanocyte survival.** The murine Melan-a cell line is anchorage dependent. Melan-a transfectants were cultured on poly-HEMA–coated plates to maintain their suspension for 24 hours and induce apoptosis. Apoptosis was confirmed by fluorescence microscopy (Fig. 5A). Apoptotic cells were characterized by condensation of cytoplasm (Fig. 5A, b-d). The proportions of apoptotic cells were significantly lower in PAX3c (P < 0.05), PAX3d (P < 0.01), and PAX3h (P < 0.05) transfectants than in vector controls (Fig. 5C, solid columns); the percentages of apoptotic cells in PAX3b and PAX3e transfectants were significantly higher than in controls (P < 0.05 in both cases). No significant difference was seen between PAX3a and PAX3g transfectants and vector controls (P > 0.05). As Annexin V staining was used to detect early apoptosis, the terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) method was employed to determine later apoptosis (Fig. 5B). The apoptotic cells were characterized by compaction of nuclear chromatin, with dark brown nuclei. Etoposide induced significantly less apoptosis in PAX3c, PAX3d, PAX3g, and PAX3h transfectants (P < 0.05 in all), but increased it in PAX3b and PAX3e transfectants (P < 0.05 in both), compared with controls. No significant change was observed in PAX3a transfectants (Fig. 5C, open columns). These data indicate that PAX3c, PAX3d, and PAX3h protect melanocytes from apoptosis whereas PAX3b and PAX3e accelerate apoptosis in melanocytes. PAX3a seems to have no effect. PAX3g has different effects on melanocytes when a different apoptotic inducer is used.

---

**Figure 3.** Effects of PAX3 isoforms on melanocyte transformation in vitro. **A,** Melan-a cells (3 × 10⁴) that contained either empty vector or a PAX3 isoform sequence were assayed for their ability to grow in soft agar. Microphotographs of colonies were taken 3 weeks after seeding. Representative results from one of three independent experiments. **B,** columns, mean number of colonies per well in soft agar from three independent experiments; bars, SD. **,** P < 0.01, PAX3 isoform transfectants versus vector control cells; P < 0.05, A375 versus XP44 (Student’s t test).
Discussion

An association between PAX3 and cell proliferation has been observed in neuronal cells (19) and myoblasts (20) in vitro. In the 72-hour proliferation assay, the growth rates of PAX3c, PAX3d, and PAX3h transfectants were higher than vector control cells. The proliferation rates of PAX3g transfectants and control cells were similar. However, PAX3a, PAX3b, and PAX3e transfectants grew significantly slower than control cells. PAX3 antisense oligonucleotides inhibited the growth of the alveolar rhabdomyosarcoma cell line Rh30 and melanoma cell lines (21, 22). PAX3c and PAX3d are the predominant isoforms in Rh30 and melanoma cell lines (7). It seems that limitation of cellular proliferation by PAX3a, PAX3b, or Pax3e may be overridden by the predominant expression of PAX3c and PAX3d. PAX3h has been identified only in melanoma cell lines in vitro and not in the original tumors, but may also enhance the melanoma cell growth.

Pax3 mRNA expression is cell cycle regulated and the 5’ promoter region of Pax3 (bp –1,578 to +56) can direct cell cycle–dependent gene expression (3). Mutagenesis of the E-box site within the Pax3 promoter significantly alters Pax3 expression throughout the cell cycle. Bernasconi et al. (21) showed a decrease in the G1 fraction of Rh1 rhabdomyosarcoma cells following antisense Pax3 treatment. In this study, the fraction of cells in S phase for PAX3c, PAX3d, and PAX3h has been identified only in melanoma cell lines in vitro and not in the original tumors, but may also enhance the melanoma cell growth.

Transformation of melanocytes leads to melanoma, which is characterized by reduction in anchorage dependence and contact inhibition. Melanocytes are unable to grow independently of anchorage; primary melanoma cells form colonies with 5% efficiency (24). Muratovska et al. (22) showed that Pax3 is consistently expressed in melanoma cell lines. Furthermore, Pax3 activity is associated with malignant transformation in muscle cells (25) and skin melanocytes (26). Here, an anchorage-independent growth assay showed that PAX3c, PAX3d, PAX3g, or PAX3h expression in Melan-a cells conferred an ability to grow in soft agar, whereas PAX3a-, PAX3b-, and PAX3e-expressing melanocytes and vector control cells virtually failed to form colonies. It is possible that PAX3c, PAX3d, PAX3g, and PAX3h isoforms induce the expression of essential autocrine or paracrine survival factors during melanocyte transformation. Cell transformation by Pax3-Forkhead fusion protein in vitro requires only the Pax3

---

4 Our unpublished data.

5 Our unpublished pilot data.

---

Figure 4. Effects of PAX3 isoforms on melanocyte migration in vitro. A, PAX3 isoform transfectants in a scratch wound assay. Cell movement into the wound is shown 24 hours after scratching. Typical results from individual cell lines. B, columns, mean distance migrated from three independent experiments; bars, SD. *, P < 0.05; **, P < 0.01, versus vector control cells (Student’s t test).
homeodomain; an intact paired domain apparently is dispensable (27). Likewise, Xia and Barr (28) showed that the transforming and growth-suppressive activities of PAX3-FKHR in NIH 3T3 cells are mediated by distinct functional domains. The PAX3 homeodomain is necessary for transformation but not for growth suppression, whereas the paired domain is not required for transformation but is necessary for growth suppression. This may explain why PAX3a and PAX3b transfectants failed to form colonies and did not enhance cell proliferation.

Pax3 is essential for migration of limb muscle precursors and melanocyte precursors (20, 29, 30). Wound healing assay showed that PAX3c, PAX3d, and PAX3h increased melanocyte migration. Both PAX3b and PAX3g reduced migration compared with controls. The effects of these isoforms on migration are not entirely in agreement with their effects on proliferation, supporting the hypothesis of Clark et al. (31) that specific gene products can regulate migration without altering growth properties. This may explain why PAX3a and PAX3e decrease melanocyte proliferation without affecting migration. Loss or loosening of cellular adhesion is often correlated with early stages of tumor progression as cells break away from the primary tumor. Overexpression of mouse Pax3 in a human medulloblastoma cell line had a direct effect on cell adhesion (32). Whether PAX3 isoforms influence melanocyte migration by altering adhesion needs clarification.

Resistance to apoptosis is important for transition from melanocytes to melanoma (33). Apoptosis was prevalent in somites of Splotch embryos, and inhibition of Pax3 expression with antisense oligonucleotides or expression of an engineered Pax3 fused to a transcriptional repressor domain caused apoptosis in cultured presomitic mesoderm, rhabdomyosarcoma, and melanoma (34). Inactivation of anoikis (apoptosis initiated by the disruption of cell-matrix interactions) is a critical step in cancer invasion and metastasis. Etoposide induces apoptosis in cell culture and various tumors (35). In this study, the transfectants were cultured under suspension conditions or treated with etoposide to induce apoptosis. Both assays showed less apoptosis in PAX3c, PAX3d, and PAX3h transfectants than in controls. Etoposide-induced apoptosis was lower in PAX3g transfectants than in controls, whereas apoptosis was higher in PAX3b and PAX3e transfectants. No difference was observed in PAX3a transfectants. PAX3c and PAX3d are the predominant isoforms in melanoma (7) whereas PAX3g and PAX3h are predominant in neuroblastoma (16). This raises the possibility that PAX3c and PAX3d are important for mediating antiapoptosis in melanoma, and PAX3g and PAX3h in neuroblastoma. Etoposide treatment of metastatic melanomas induced <13% apoptosis (35). In the present study, response rates exceeded 13% in all Melan-a transfectants. Thus, there may be other antiapoptotic molecules at work in melanomas that are not present in the melanocyte transfectants.

Margue et al. (36) suggested that at least part of the antiapoptotic effect of PAX3 and PAX3/FKHR is mediated through Bcl-xl. The ectopic expression of wild-type PTEN can lead to anoikis of cancer cells (37). Given the potent growth-inhibitory capacity of PTEN and the ability of PAX3 to bind to the PTEN promotor, it is suggested that decreased apoptosis shown in suspension cultures of PAX3c, PAX3d, and PAX3b transfectants may be through down-regulation of PTEN expression. Hepatocyte growth factor also protects against apoptosis, up-regulates integrin expression, and activates protein kinase B/Akt. Both are potential antiamoikis pathways (38). The hepatocyte growth factor receptor c-Met is another target gene of PAX3. In a preliminary unpublished study, we found that the c-Met mRNA and protein levels were up-regulated in PAX3c and PAX3d transfectants but not in other isoform transfectants. Therefore, c-Met signaling may also be involved in antiapoptosis induced by PAX3c and PAX3d.

In conclusion, the present study has enhanced our knowledge about the effects of PAX3 isoforms on melanocytes in vitro, but further work is required to more fully elucidate their role in tumorigenesis.

**Acknowledgments**

Received 3/20/2006; revised 5/30/2006; accepted 6/23/2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

---

**Figure 5.** Apoptosis in PAX3 isoform transfectants and empty vector control cells in suspension culture or after etoposide treatment. A, cells were cultured in normal anchorage culture (a) or in suspension culture (b-d) for 24 hours and stained with Annexin V and 6-CFDA: a and b, cells viewed through a blue excitation filter and emission filter; c and d, cells viewed through a green excitation filter in (d) but not in (c). Arrows, apoptotic cells. B, cells stained with TUNEL after treatment with 10 μg/mL etoposide for 24 hours. Apoptotic cells have dark brown nuclei. C, effects of PAX3 isoforms on melanocyte apoptosis. Solid columns, cells grown in suspension culture for 24 hours and then stained with Annexin V (mean of four experiments); bars, SD. Open columns, cells treated with 10 μg/mL etoposide for 24 hours and then stained with the TUNEL system (mean of three experiments); bars, SD. *, P < 0.05; **, P < 0.01, versus vector control cells (Student’s t test).
Sato T, Sasai N, Sasai Y. Neural crest determination by

References

Goulding MD, Chalepakis G, Deutsch U, Er塞尔ius JR,

Harris RG, White E, Phillips ES, Lillycrop KA. The


Barr FG, Fitzgerald JC, Ginsberg JP, Vanella ML, Davis

Baynash AG, Hosoda K, Giaid A, et al. Interaction of

Xenopus

Gruss P. Pax-3, a novel murine DNA binding protein

oncogene Pax-3 is modulated by N-Myc. J Biol Chem

expression of the developmentally regulated proto-

Watanabe A, Takeda K, Ploplis B, Tachibana M.

Galibert MD, Yavuzer U, Dexter TJ, Goding CR. Pax3

related protein-1 promoter. J Biol Chem 1999;274:

expressed during early neurogenesis. EMBO J 1991;10:

Harris RG, White E, Phillips ES, Lillycrop KA. The


Bennett DC, Cooper PJ, Hart IR. A line of non-

tumorigenic mouse melanocytes, syngenic with the

B16 melanoma and requiring a tumour promoter for

Lamery TM, Koenders A, Ziman M, Pax genes in


Reeves FC, Burd Ge G, Fredericks WJ, Rauscher FJ,

Wiggan O, Taniguchi-Sidle A, Hamel PA. Interaction

Mendonsa M, Rempis A, Fredericks WJ, Rauscher

FJ III, Schafer BW. Induction of apoptosis in rhabdo-


Bernasconi M, Remppis A, Fredericks WJ, Rauscher

FJ III, Schafer BW. Apoptosis and regulation of the

fibroblast growth factor induces a transformed pheno-

Muratovska A, Zhou C, He S, Goodyer P, Eccles MR.

Pani L, Horal M, Loeken MR. Rescue of neural tube

defects in Pax-3-deficient embryos by p53 loss of

function: implications for Pax-3-dependent development


Helmhach B, Korn MA, Roensen E, et al. Drug

resistance towards etoposide and cisplatin in human

melanoma cells is associated with drug-dependent


Bennett DC, Cooper PJ, Hart IR. A line of non-
tumorigenic mouse melanocytes, syngenic with the

B16 melanoma and requiring a tumour promoter for

Kumar S. Insulin-like growth factor II and PAX3-FKHR

growth suppressive activities of the PAX3-FKHR onco-


Vance KW, Goding CR. The transcription network


Daston G, Lamar E, Olivier M, Goulding M. Pax-3 is

necessary for migration but not differentiation of limb

muscle precursors in the mouse. Development 1996;122:

1017–27.

Clark EA, Golub TR, Lander ES, Hynes RO. Genomic

analysis of metastasis reveals an essential role for RhoC.


Mayanil CS, O’Shea D, Mancia-Farnell B, Bremer CL,

McLone DG, Bremer E. Overexpression of murine Pax3

increases NCAM polysialylation in a human medullo-


Pani L, Horal M, Loeken MR. Rescue of neural tube
defects in Pax-3-deficient embryos by p53 loss of

function: implications for Pax-3-dependent development


Helmhach B, Korn MA, Roensen E, et al. Drug

resistance towards etoposide and cisplatin in human

melanoma cells is associated with drug-dependent


Marque CM, Bernasconi M, Barr FG, Schafer BW.

Transcriptional modulation of the anti-apoptotic protein

BCL-XL, by the paired box transcription factors


Tamura M, Qui G, Vagenas H, Takiko T, Miyamoto S,

Yamada KM. PTEN interactions with focal adhesion

kinase and suppression of the extracellular matrix-
dependent phosphatidylinositol 3-kinase/Akt cell sur-


Smet F, Chen Y, Wang IJ, Soriano HE. Loss of cell

anchorage triggers apoptosis (anoxia) in primary mouse


Bennett DC, Cooper PJ, Hart IR. A line of non-
tumorigenic mouse melanocytes, syngenic with the

B16 melanoma and requiring a tumour promoter for

Lamery TM, Koenders A, Ziman M, Pax genes in


Bernasconi M, Remppis A, Fredericks WJ, Rauscher

FJ III, Schafer BW. Induction of apoptosis in rhabdo-


Muratovska A, Zhou C, He S, Goodyer P, Eccles MR.

Paired-Box genes are frequently expressed in cancer and

often required for cancer cell survival. Oncogene 2003;


Wiggan O, Taniguchi-Sidle A, Hamel PA. Interaction

of the pRB-family proteins with factors containing


Nesbit M, Nesbit HK, Bennett J, et al. Basic

fibroblast growth factor induces a transformed pheno-
type in normal human melanocytes. Oncogene 1999;18:

26894–900.

Mayanil CS, O’Shea D, Mancia-Farnell B, Bremer CL,

McLone DG, Bremer E. Overexpression of murine Pax3

increases NCAM polysialylation in a human medullo-


Pani L, Horal M, Loeken MR. Rescue of neural tube
defects in Pax-3-deficient embryos by p53 loss of

function: implications for Pax-3-dependent development


Helmhach B, Korn MA, Roensen E, et al. Drug

resistance towards etoposide and cisplatin in human

melanoma cells is associated with drug-dependent


Marque CM, Bernasconi M, Barr FG, Schafer BW.

Transcriptional modulation of the anti-apoptotic protein

BCL-XL, by the paired box transcription factors


Tamura M, Gui G, Danen EH, Takiko T, Miyamoto S,

Yamada KM. PTEN interactions with focal adhesion

kinase and suppression of the extracellular matrix-
dependent phosphatidylinositol 3-kinase/Akt cell sur-


Smet F, Chen Y, Wang IJ, Soriano HE. Loss of cell

anchorage triggers apoptosis (anoxia) in primary mouse

Functional Analysis of Alternative Isoforms of the Transcription Factor PAX3 in Melanocytes *In vitro*

Qiuyu Wang, Shant Kumar, Mark Slevin, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/66/17/8574

Cited articles
This article cites 37 articles, 15 of which you can access for free at:
http://cancerres.aacrjournals.org/content/66/17/8574.full.html#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
/content/66/17/8574.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.