Regulation of Merkel cell development by Pax6

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ABSTRACT  Merkel cells are mechanoreceptors widely distributed in the vertebrate skin. In rodents, Merkel cells within the whisker pads are innervated by free sensory nerve endings derived from the maxillary branch of the trigeminal nerve. This study identified expression of the transcription factor Pax6 in Merkel cells and investigated its role. Immunohistochemistry and Western blot for Pax6 and Merkel cell markers, cytokeratin-8 (K8) and cytokeratin-20 (K20) were performed in wild-type and Pax6−/− fetuses. The subcellular localisation of Pax6 in Merkel cells in vitro was manipulated using hydrogen peroxide. Pax6 was primarily localised within the cytoplasm of the Merkel cells at birth, but postnatally was also detected within the nuclei. In vitro, after 4 days in culture Pax6 protein was completely relocated to the nuclei of fetal-derived Merkel cells, mimicking the in vivo situation, suggesting that Pax6 acts as an active nucleo-cytoplasmic shuttling protein in common with many other homeodomain transcription factors. The subcellular localisation of Pax6 could be modulated in vitro by changing the redox potential of the culture medium for Merkel cells. Differentiation of cultured Pax6−/− Merkel cells was shown to be inhibited. At perinatal stages, it was found that Pax6 is required for maintaining cytokeratin-8 expression, an early Merkel cell marker, whereas cytokeratin-20 was retained by the Pax6−/− mutant cells. Pax6 is expressed in developing Merkel cells as a nucleo-cytoplasmic shuttling protein and its activity is required for normal differentiation, possibly through regulating cell maturation.

KEY WORDS: Pax6, Merkel cell, nucleo-cytoplasmic shuttling, oxidative stress

Introduction

The sensory neurons derived from the maxillary branch of the trigeminal nerve have free endings that innervate skin mechanoreceptors, the Merkel cells (Merkel, 1875). The Merkel cells are mainly located within the basal layer of the epidermis and are found in the hairy skin, including whisker pads (Narisawa et al., 1993, 1994; Halata et al., 2003), and tactile areas within the glabrous skin e.g. foot pads (Moll et al., 1990; Narisawa et al., 1994). Merkel cells contribute to touch perception and are located in the outer root sheath of vibrissal follicles, forming cell-neurite complexes called touch domes (Iggo and Muir, 1969; Halata et al., 2003; Woodbury and Koerber, 2007). A characteristic of the Merkel cells that allows them to be distinguished from keratinocytes is the cytoskeletal structure constituted by the epithelial keratin, cytokeratin-8 (K8 - recognised by the TROMA I monoclonal antibody) and cytokeratin-20 (K20) that have been used as markers for identification of these cell types (Moll et al., 1996; Vielkind et al., 1995).

The mouse atonal homologue 1 (Math1) is the earliest gene identified as being required for the specification and postnatal differentiation of Merkel cells (Ben-Arie et al., 2000; Haeberle et al., 2004; Maricich et al., 2009). Atoh1 null embryos show a decreased number of Merkel cells within the developing skin (Ben-Arie et al., 2000). Atoh1 conditional knock-out (Atoh1 cKO) in the skin resulted in the complete absence of Merkel cells within the touch dome, footpad and whisker pads (Maricich et al., 2009). In Drosophila, atonal controls the development and function of chordotonal stretch receptors (Eberl, 1999; van Staaden and Romer, 1998; Moulins, 1976). The ability of Math1, like ato, to induce ectopic chordotonal organs in Drosophila suggests some level of deep conservation of genetic control of mechanosensory structures (Ben-Arie et al., 2000).

From the earliest stages of embryonic development, the expression of the gene encoding the developmentally regulated transcription factor, Pax6, is detected in the non neural ectoderm identified as being required for the specification and postnatal differentiation of Merkel cells (Ben-Arie et al., 2000; Haeberle et al., 2004; Maricich et al., 2009). Atoh1 null embryos show a decreased number of Merkel cells within the developing skin (Ben-Arie et al., 2000). Atoh1 conditional knock-out (Atoh1 cKO) in the skin resulted in the complete absence of Merkel cells within the touch dome, footpad and whisker pads (Maricich et al., 2009). In Drosophila, atonal controls the development and function of chordotonal stretch receptors (Eberl, 1999; van Staaden and Romer, 1998; Moulins, 1976). The ability of Math1, like ato, to induce ectopic chordotonal organs in Drosophila suggests some level of deep conservation of genetic control of mechanosensory structures (Ben-Arie et al., 2000).

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Abbreviations used in this paper: K8, cytokeratin-8; K20, cytokeratin-20; MCC, Merkel cell carcinoma.
from where the olfactory and lens placodes will develop (Hogan et al., 1986; Grindley et al., 1995; reviewed in Baker and Bronner-Fraser, 2001). As a consequence, homozygous null (Pax6–) mice fail to develop eyes and olfactory epithelia (Hill et al., 1991; Grindley et al., 1995). Pax6 regulates cell-sorting events that ensure the exclusion of Pax6-negative cells from the developing lens and olfactory placode (Collinson et al., 2000). The crucial role of Pax6 in regulating many aspects of eye and nasal development, in embryonic and adult neurogenesis, has been intensively studied (Hill et al., 1991; reviewed in Chalepaakis et al., 1993; Simpson and Price 2002; Osumi et al., 2008).

In mice, Pax6 expression starts at embryonic day 8 (E8) in the neural plate. At E10, after neural tube regionalization, Pax6 is expressed in the forebrain, hindbrain and spinal cord (Walther & Gruss, 1991; Osumi, 2001; Inoue, 2000), where it plays a crucial role in regulating dorsoventral patterning and specification of different neuronal cell types (Ericson et al., 1997; Osumi et al., 1997; Pratt et al., 2000). Pax6 has pivotal roles during central nervous system regionalization, e.g., by regulating boundary formation along the anteroposterior axis (Mastrick et al., 1997; Warren et al., 1997; Matsunaga et al., 2000).

Although some reports suggested a neural crest derivation of the Merkel cells (Grim and Halata, 2000; Szeder et al., 2003), an epidermal lineage origin is nowadays well documented (Moll et al., 1990; Morrison et al., 2009; VanKeymeulen et al., 2009; Woo et al., 2010). Notwithstanding microarray studies on isolated Merkel cells describing expression of other transcription factors involved in neural development, such as Islet1, Bm3b, Sox2 and Mash1 (Haeberle et al., 2004), their specific role in controlling the differentiation and maturation of the Merkel cells is not yet fully understood. The increased interest in studying the genetics and biology of this specialized skin cell type is partly due to the occurrence of a very detrimental and rare tumour, the Merkel cell carcinoma (MCC) (Toker, 1972; Gould et al., 1985).

In this study a previously undescribed domain of Pax6 expression is reported in Merkel cells. Putative roles for Pax6 in regulating Merkel cell development were analysed and characterised. It was observed that Pax6 regulates perinatal development of the Merkel cell, acting as a nucleo-cytoplasmic shuttling protein whose localisation can be modulated by oxidative stress. The requirement for Pax6, a tumour-suppressor, in these cells is relevant to understanding the development and the progression of MCC.

Results

Pax6 is expressed within Merkel cells in wild-type embryos in vivo

Pax6-positive cells were observed within the developing whisker follicles from E16.5 onward (Fig. 1A). Pax6 was absent from whisker follicles at E14.5 (Fig. 1A), but was detected at E16.5 in a subset of cells (Fig. 1B). Coronal sections at E18.5 demonstrated that these Pax6-positive cells were specifically localised around the papillae (Fig. 1C). As expected, high immuno-reactivity was also detected within the nasal epithelium that was used as positive control for Pax6 expression (Fig. 1D). A role of Pax6 in regulating hair growth was hypothesised and whisker follicle morphology at E16.5 and E18.5 was analysed. At both developmental stages, Pax6-null mutant embryos showed normal structure of the hair follicles (Fig. 1E-H), including, starting from the outmost cell layer, the capsule, dermal sheath, matrix, follicular papillae and the growing hair (Fig. 1E). Normal patterns of hair growth were observed in Pax6-null fetuses at E18.5 (Fig. 1I,J). The involvement of Pax6 in

![Pax6 expression in the whisker follicles](image)

**Fig. 1.** Pax6 expression in the whisker follicles. (A) Sagittal section through the wild-type whisker pad at E14.5 showing absence of Pax6-positive cells. (B) Presence of Pax6-positive cells at E16.5, the whisker follicle is outlined by dashes and the cells are indicated by arrowheads. (C) Pax6-positive cells localised around the whisker papillae at E18.5 (arrowheads). (D) Pax6 expression in the E18.5 nasal epithelium, used as positive control. (E-H) Haematoxylin-eosin staining at E16.5 (E, F) and E18.5 (G, H) in wild-type (E, G) and Pax6–/– mutant embryos (F, H) revealed that hair follicle structure was grossly normal. (I, J) Growing hairs within the E18.5 whisker pad in wild-type (I, arrowheads) and Pax6-null mutant embryos (J, arrowheads). Abbreviations: cp, capsule; ds, dermal sheath; m, matrix; fp, follicular papilla; gh, growing hair; Nep, nasal epithelium; WF, whisker follicles. Scale bars: (A, D, E-H), 90 μm; (B) 45 μm; (C) 23 μm.
the regulation of the whisker follicle structure and hair growth was therefore potentially excluded.

Merkel cells fully differentiate postnatally but can be identified from E14.5 through the expression of cytokeratin-8 (K8 - detected using the TROMA-I monoclonal antibody) (Moll et al., 1996; Vielkind et al., 1995). At E18.5, Pax6 was found to be expressed in cells which, on the basis of their localisation in the outer root sheath of the follicles, in a pattern similar to K8-positive cells (Fig. 2 A,B), were thought to be Merkel cells. As expected, at the same developmental stage, Pax6 was undetectable within the whisker follicles of Pax6<sup>−/−</sup> embryos (Fig. 2 C). Co-immunolabeling for Pax6 (Fig. 2 D) and K8 (Fig. 2 E) confirmed the two markers are co-localized within the Merkel cells (Fig. 2 F). The expression of cytokeratin-20 (K20), a late marker of the Merkel cells (Tachibana et al., 2000) was also analysed at E18.5. The Pax6 immunolocalisation (Fig. 2 G) also overlapped K20 expression (Fig. 2 H), confirming the new expression domain within the Merkel cells (Fig. 2 I). In order to clarify whether Pax6 is exclusively present within the fetal Merkel cells, its expression was also analysed in hair follicles from postnatal (P4) body skin (Fig. 2 J-L). Pax6 (Fig. 2 J) was still co-localised with K20 (Fig. 2 K) within the Merkel cells in the outer sheath root of the neonatal hair follicles (Fig. 2 L) suggesting it may have a role also in regulating postnatal aspects of Merkel cell development. Interestingly, while Pax6 was mainly localised in the cytoplasm of these cells at E18.5, at P4 the protein was also detected within the nuclei of the Merkel cells (Fig. 2 J',K',L', inset of the boxed regions in J, K, L respectively). The results suggested that the subcellular localisation of Pax6 changes from cytoplasm to nucleus at around the time of birth in mice.

Furthermore, the localisation of another molecular marker, the vasoactive intestinal peptide (VIP), which is known to be expressed in adult Merkel cells, was investigated. VIP was undetectable in perinatal Merkel cells in accordance with previous studies suggesting it is highly expressed only in adults (Hartschuh et al., 1984; Cheng Chew and Leung, 1994). VIP expression was detected only in cells in the dermal matrix or inner root sheath of hair follicles from the body skin of P4 mice (Fig. S1B), not consistent with a Merkel cell localisation, and not overlapping Pax6 (Fig. S1 A,C) thus indicating VIP is not expressed within Merkel cells at P4 in mice.

**Pax6 is required for the perinatal development of Merkel cells**

In Pax6<sup>−/−</sup> embryos, K8 and K20 staining of mutant Merkel cells was found at E16.5, without any differences between the wild-type (Fig. 3 A-C) and the mutant cells (Fig. 3 D-F), suggesting that Pax6 is likely not required for early specification of this cell type. Interestingly, while K20 expression was retained by both wild-type and Pax6<sup>−/−</sup> Merkel cells, K8 staining, in contrast to wild-type, was not detected after E16.5 in Pax6-null mutants (n = 30 follicles serially sectioned from 3 different embryos) (Fig. 3 G-L). At earlier stages K8 expression is likely to be under the primary control of other transcription factors such as Math1 (Morrison et al., 2009; Maricich et al., 2009). The results suggested that Pax6 may also be required to maintain Merkel cell differentiation by directly and/or
indirectly regulating exclusively at least one early molecular marker, K8, of this cell type.

**Pax6 expression is retained by Merkel cells in vitro and dynamically changes its subcellular localisation**

In order to study in more detail the subcellular distribution of Pax6 protein within Merkel cells, primary cultures of epidermal sheets dissected from the wild-type whisker pads of E18.5 fetuses were set up (modified from Boulais et al., 2009a, 2009b) as described in Materials and Methods. The dissociated cell culture included keratinocytes and in addition, cells were observed that reflected the oval shape and morphology of Merkel cells (Iggo and Muir, 1969; Holbrook and Smith, 1993). It was hypothesised that those showing cytoplasmic protrusions and intimate interactions with the keratinocytes were Merkel
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cells as previously described (Moll et al., 1984; Toyoshima et al., 1993) (Fig. 4D). After two days of cell culture, these were found to be labelled by overlapping cytoplasmic localisation of Pax6 and Merkel cell markers K8 (Fig. 4 A-D) and K20 (Fig. 4 E-G). K8 and K20 were colocalised, confirming the identity of these cells as Merkel cells (Fig. 4 H-J). These in vitro data recapitulated the results in vivo and confirmed Pax6 expression in Merkel cells. After 2 days in culture the Merkel cells represented 33.8% ± 6% (expressed as mean value ± SEM) of the total cells in culture, while the remaining 66.2% ± 6.8% were keratinocytes (n = 250 cells analysed from five replicates) (Fig. 4K).

When the cells were cultured for more than two days a change in the sub-cellular localisation of Pax6 was identified which recapitulated that observed in vivo from perinatal to postnatal stages. From exclusively cytoplasmic localisation at two days in vitro (Fig. 5 A-D), after 3 days some of the cells analysed showed Pax6 in both nuclei and cytoplasm (Fig. 5 E-H). At 4 days Pax6 was completely relocated to the nuclei of the Merkel cells (Fig. 5 I-L) suggesting that dynamic changes in the subcellular localisation of Pax6 occur in vitro as they do in vivo, possibly through an intrinsic mechanism controlling differentiation.

Many lines of evidence suggest that activity of homeodomain transcription factors may be controlled by changes in their subcellular localisation (Prochiantz, 2000; Prochiantz and Joliot, 2003). Nucleocytoplasmic export is a regulated event in eukaryotic cells that occurs through the highly elaborate nuclear pore complexes (NPCs) (Stoffler et al., 1999; Ryan and Wente, 2000).

Fig. 5. Subcellular localization of Pax6 is modulated by oxidative stress. Pax6 and K8 immunocytochemistry on Merkel cells in vitro. (A-D) After 2 days in culture some cells labelled for both Pax6 and K8, with Pax6 localized in the cytoplasm. After 3 days some of the cells have Pax6 both in the cytoplasm and in the nuclei (E-H). After 4 days Pax6 was completely relocated to the nuclei of the Merkel cells (I-L). (M-P) Upon the addition of 0.3 mM of H₂O₂ to 4-day cultures, 76.06% of the cultured cells relocated Pax6 to the cytoplasm. (Q-T) When 1.5 mM of H₂O₂ was added to the primary cell culture nearly all of the cells had Pax6 in the cytoplasm. (U) After addition of H₂O₂ the percentage of Merkel cells in culture did not change significantly (P = 0.67). (V) Quantification of percentage of 4-day cultured Merkel cells with nuclear or cytoplasmic localisation of Pax6 under different concentrations of H₂O₂. Scale bars (A-H) 23 μm; (I-T) 45 μm.
Active transport requires specific nuclear localisation signals (NLS) and nuclear export signals (NES) within the protein (reviewed in Fabbro and Henderson, 2003; Kohler and Hurt, 2010), amino acid sequences previously described also for Pax6 (LeSaffre et al., 2007), (Fig. S2A, modified from LeSaffre et al., 2007). The online availability of NES prediction server (La Cour et al., 2004; http://www.cbs.dtu.dk/index.shtml) and protein conformation prediction server (https://www.predictprotein.org) confirmed the potential presence respectively of one NES and two NLSs within the Pax6 protein (LeSaffre et al., 2007), (Fig. S2 A-C).

The in silico analysis was carried out for the three major isoforms of Pax6 protein (Pax6, Pax6(5a), Pax6ΔPD) and resulted in the prediction of 7 residues participating in the formation of a NES in the Pax6ΔPD isoform, that lacks the paired domain (Fig. S1B). The same amino acid sequence is present in all three Pax6 isoforms, but does not yield an equally strong prediction of NES function suggesting the leucine-rich NES sequence may be dynamically masked or unmasked depending on the paired-domain conformation and as consequence of protein folding. Furthermore, western blot analysis carried out on whisker and foot pads from E18.5 embryos, confirmed that Pax6 (with the paired domain) is the major isoform to be expressed within the Merkel cells, as in most Pax6-expressing tissues, and also indicated expression of Pax6 (presumably in Merkel cells) within the glabrous skin, although at a lower level than within the whisker pad (Fig. S2 D).

Oxidative stress modulates Pax6 subcellular localisation

We previously showed that Pax6 cytoplasmic localisation in the adult corneal epithelium is a consequence of oxidative stress (Ou et al., 2008). In order to analyze if the same mechanism also occurs in Merkel cells, we induced experimental oxidative stress by adding different concentrations of H₂O₂ to the cell cultures after 4 days in vitro when Pax6 was completely relocated into the cell nuclei. Interestingly, upon addition of 0.3 mM or 1.5 mM H₂O₂ to Merkel cells at 4 days of culture, Pax6 was found to be excluded from the nucleus (Fig. 5 M–T). The percentage of Pax6/K8-positive Merkel cells in culture did not change significantly. As summarised in Fig. 5U, the percentage of Merkel cells in control culture was 32.40 ± 5.8% (expressed as mean value ± SEM); after the addition of 0.3 mM H₂O₂ this was 28.4 ± 2.26% and after the addition of 1.5 mM H₂O₂ was 32.45 ± 6.2%, suggesting that the induced oxidative stress does not significantly affect the number of Merkel cells in short-term culture (one-way ANOVA: P = 0.67). The differences between the control cells and those treated with the two different concentration of H₂O₂, 0.3 mM (Fig. 5 M-P) and 1.5 mM (Fig. 5 Q-T), were statistically significant. Whereas 99.6 ± 0.4 % of Pax6/K8-positive Merkel cells had nuclear localisation of Pax6 after 4 days in control culture (n = 250 cells analysed from five replicates), 76.06 ± 1.3% of cells showed Pax6 in the cytoplasm upon addition of 0.3 mM H₂O₂ (n = 250 cells), (Fig. 5V), (1-way ANOVA: P < 0.0001). Moreover, 95.06 ± 1.5% of Merkel cells had Pax6 relocated in the cytoplasm after the treatment with 1.5 mM H₂O₂ (n = 250 cells), (Fig. 5V) (1-way ANOVA: P < 0.0001). The difference between cultures with 0.3 mM and 1.5 mM was statistically significant (t-test: P < 0.001) suggesting the relocation of Pax6 into the cytoplasm may also be dose-dependent (Fig. 5V).

Pax6 modulates differentiation of Merkel cells in vitro

As described above, the Merkel cells cultured from the E18.5 whisker pad were positive for both K8 and K20 (Fig. 6 A-C). In spite of the undetectable expression of K8 within the Pax6–/– Merkel cells found in vivo at E18.5, both K8 and K20 labelling were detected in Pax6–/– cells cultured for 4 days (Fig. 6 D-F). This may suggest that lack of Pax6 delays but does not prevent Merkel cell differentiation, or more likely it may indicate a degree of de-differentiation of fetal Merkel cells in culture. Although K8 and K20 labelling were visible in cultured Pax6–/– Merkel cells, there were proportionately significantly fewer such cells in culture compared to wild-type. While the K8–K20 double positive Merkel cells from wild-types represented 31.3 ± 2.1% (expressed as mean ± SEM) of the total number of cells, those from Pax6–/– mutants were only 21.3 ± 1.9%, (Fig. 6G) (t-test: P < 0.05). This finding suggested that, although the Merkel cells from Pax6–/– mutants are able in vitro to undergo differentiation, this process may not be as efficient as that occurring in wild-types.

Fig. 6. K8/K20 double-positive Merkel cells are reduced in Pax6–/– mutant cultures. (A–F) K8 and K20 expression was retained by both wild-type (A–C) and Pax6-null Merkel cells in vitro (D–F). (G) Quantification of the percentage of double-labelled Merkel cells with K8 and K20 showed a significant reduction of the number of Pax6-null Merkel cells. While the Merkel cells from wild-types represented 31.33 ± 2.08% (expressed as mean percentage ± SEM) of the total number of cells, those from Pax6-null mutants were only 21.30 ± 1.9% (t-test: P < 0.05). Scale bar: 45 μm.
Discussion

Pax6 is required for Merkel cell development

This study showed for the first time that the gene encoding the developmentally regulated transcription factor, Pax6, is expressed in Merkel cells and is required for their normal development. Furthermore the subcellular localisation of Pax6 protein changes from cytoplasmic to nuclear during differentiation of the cells. Mature K20-positive Merkel cells could nevertheless develop in absence of Pax6 protein. Whereas expression of K8, taken as an earlier marker of Merkel cells, was normal at E16.5 in Pax6-/- fetuses, it was absent from E18.5, suggesting that Pax6 perhaps contributes to maintenance of the immature, proliferative differentiation state of Merkel cells. In contrast, K8 expression was maintained within the Pax6+ Merkel cells in vitro that have perhaps undergone a degree of dedifferentiation as a result of culture. It is hypothesised that Pax6 may have a role in controlling rates of cell division in Merkel cells and the proportionate underrepresentation of Merkel cells in Pax6-/- culture may be a consequence of cell cycle retardation.

Development of Merkel cells

Although the epidermal origins of Merkel cells have been extensively reported (Moll et al., 1990; Morrison et al., 2009; Van-Keymeulen et al., 2009; Woo et al., 2010), recent studies described the expression of SOX2, a stem cell neural crest transcription factor, in a subpopulation of Merkel cells and melanocytes within the human skin (Laga et al., 2010). Like Sox2, Pax6 is also a neural transcription factor involved in controlling neural stem cell self-renewal (Sakurai and Osumi, 2007; 2008; Sansom et al., 2009). As stated by Boulais and Misery (2007), the indication of two different developmental origins may not be considered as mutually exclusive. The presence of epidermal neural crest stem cells (eNCSs) has been proposed, which initially migrate as neural crest precursors and later differentiate only in the epidermis (Boulais and Misery, 2007). A dual ontogenesis for Merkel cells is very intriguing possibility, but the eNCS population has not yet been experimentally identified.

Given the intense investigation of the roles of Pax6 in embryonic sensory system development, since its identification as the gene responsible for human anhidria and mouse Small eye (Hill et al., 1991; Ton et al., 1991), the discovery of a new expression domain in Merkel cells of hair follicles was surprising.

Pax6 acts as a nucleo-cytoplasmic shuttling transcription factor

Pax6 is not a nuclear transcription factor, however we have shown that, in common with other transcription factors, it undergoes cytoplasmic shuttling (Camarata et al., 2006; Bimber et al., 2007; Ou et al., 2008). Sox10, which regulates many aspects of embryonic development, and Vax2, which participates with Pax6 in retinal development, both dynamically shuttle between the nucleus and the cytoplasm with consequences for their transcriptional activity (Rehberg et al., 2002; Kim and Lemke, 2006). Pax6 interacts with some secreted cytoplasmic proteins such as SPARC (secreted protein acidic and rich in cysteine), a matricellular protein that regulates transport and processing of Pax6 during neuronal primary culture, in the cerebellum and cortex of the mouse brain (Tripathi and Mishra, 2010). Furthermore, there is evidence that Pax6 may be localised in the cytoplasm during synaptic signal-ling (Cooper and Hanson, 2005). Analysis of Pax6 expression in the chick retina showed that Pax6 is localized in the cytoplasm of some cell types (Shin et al., 2003). Pax6 may be considered as an active nucleocytoplasmic protein.

The complete relocation of Pax6 into the nuclei of the Merkel cells in vitro after 4 days in culture indicates that Pax6 may control some aspects of the postnatal maturation of this cell type. In vivo before birth, only cytoplasmic localisation was observed, but there was significant nuclear relocation of the protein at postnatal stages. In vivo, the predominantly cytoplasmic Pax6 protein may modulate some aspect of Merkel cell differentiation, for example maintenance of K8 expression. Either there is enough nuclear Pax6 protein in vivo to autonomously control K8 gene expression, or Pax6 has non-genomic roles similar to those described above. The evidence from Pax6-/- mutants however was that Pax6 is not required for expression of K20, a marker of mature Merkel cells.

Nucleo-cytoplasmic export is a regulated event characterised by several mechanisms, but the leucine-rich NES has been the most intensively studied (Ossareh-Nazari et al., 2001). It requires the mediation of a specific evolutionarily conserved importin, CRM1 (Haassen et al., 1999), with the participation of a member of the Ras superfamily, Ras-related- nuclear protein (Ran) (Fornerod et al., 1997, Ossareh-Nazari and Dargemont, 1999). CRM1-mediated nuclear export is finely regulated through the masking and unmasking of the NES within the shuttling protein by chemical modifications, such as phosphorylation (Brunet et al., 2002) and formation of disulfide bonds after oxidation (Kuge et al., 2001). Our in silico analysis of the Pax6 protein confirmed the presence of a typical leucine-rich NES sequence that may be dynamically masked or unmasked depending on the paired domain conformation.

Pax6 is required for maintaining cytokeratin-8 expression in vivo

We also demonstrate that Pax6 is controlling some aspects of Merkel cell development by the direct or indirect regulation of K8. The data suggest that Pax6 is involved in their perinatal maturation. We could only analyse prenatal Pax6-/- mice because the Pax6 mutants die at birth, which limited our observations, as was previously the case with investigation on Atoh-1 null embryos (Ben-Arie et al., 2000). Analysis of Pax6-/- cells in a fully developed context by conditional knockout would be a valuable line of future inquiry.

Previous studies in our lab have shown that cytoplasmic Pax6 localisation in the adult corneal epithelium is a consequence of oxidative stress. This was shown to be one of the molecular mechanisms responsible for the corneal abnormalities observed in Pax6 heterozygotes that represent the main model for studying the human congenital eye syndrome, aniridia-related keratopathy (aniridic keratopathy) (Ou et al., 2008). The dose-dependent response of the Merkel cells to addition of H2O2 was perhaps due to the rise of intracellular calcium and elevated mitogen-activated protein kinase (MAPK) signalling that is activated by H2O2, events that are commonly associated with inflammation and disease (Nakamura et al., 1997). The findings in the current study extend the observation of dynamic changes in Pax6 subcellular localisation to a physiological developmental process, in addition to the pathological conditions described for the cornea. On the basis of the well-documented role of Pax6 as a tumour suppressor (Salem et al., 2000; Hellwinkel et al., 2008; Ballestar et al., 2003; Zhou et al., 2005; Myers et al., 2006; Nitta et al., 2004; Shyr et al., 2010), it
is possible to propose a hypothetical model of Pax6 activity within the Merkel cells. Relocation of Pax6 into the Merkel cells nuclei may transcriptionally activate other genes involved in cell cycle arrest.

Merkel cell differentiation is incompletely understood and Merkel cell carcinoma has a generally poor prognosis. The involvement of a tumour suppressor, Pax6, is therefore of wider interest in a clinical setting. Many tumour-suppressors and oncogenes are active nucleo-cytoplasmic proteins with an NES (reviewed in Fabbro and Henderson, 2003; Kau et al., 2004). Because nuclear transport represents a critical mechanism to regulate transcription factor localisation and function, misregulation of subcellular localisation of tumour-suppressors has been linked to cancer development (Fabbro and Henderson, 2003; Kau et al., 2004; Kohler and Hurt, 2010).

Although the expression of Pax6 in Merkel cells is new to this study, it is intriguing in light of the previously elucidated role of Math1 in these cells. Math1 and Pax6 are both developmentally regulated genes showing many similarities in their expression pattern and genetic regulation together with well documented roles as tumour-suppressors. Furthermore many lines of evidence support mechanistic cross-regulation of these two genes. Binding sites of Pax6 have been identified within the regulatory sequences of Math1 (Hufnagel et al., 2007). Conversely, a recent study indicated Pax6 to be a direct target of Math1 (Klisch et al., 2011). In addition, studies carried out on eyeless and atonal (Pax6 and Math1 homologues respectively) in Drosophila suggested that both transcription factors share cis-regulatory target regions and thus cooperatively regulate a number of genes (Aerts et al., 2010).

Loss of Math1 promotes tumour formation in colorectal cancer (CRC) mouse models and the Math1 locus is highly mutated and hypermethylated in human tumour samples (Bossuyt et al., 2009), probably through the regulation of Notch signalling pathways (Van Es et al., 2010). The regulation of Pax6 and Math1 in different cell populations offers a very promising field of investigation that may elucidate the link between developmentally regulated events and cancer formation, using Merkel cells as an experimental model.

Materials and Methods

Mice

The Pax6 allele used in the present study, Pax6<sup>Cre<sub>neo</sub></sup>, is an ethynitrosoare-a-induced mutation on the CBA/Ca genetic background which acts as a genetic null (Hill et al., 1991). Heterozygous Pax6<sup>Cre<sub>neo</sub></sup>-/<sup>+</sup> (Pax6<sup>+</sup>) parents were crossed under Home Office (UK) licence in order to obtain homozygous, heterozygous and wild-type littermates. Genotypes were confirmed by polymerase chain reaction (PCR) (Quinn et al., 1996).

Immunohistochemical staining

Embryos were fixed in 4% paraformaldehyde (PFA), PBS, overnight, and then processed to wax. Seven-μm sections were cut. Embryonic heads were sectioned in sagittal and transverse planes. Coronal sections of whisker follicles at E16.5 and E18.5 were stained with haematoxylin and eosin.

Immunostaining was performed as described previously (Collinson et al., 2003). The following primary antibody were used: anti-PAX6 (Developmental Studies Hybridoma Bank, University of Iowa, mouse IgG, diluted 1:200); anti-cytokeratin 8 endo-A (TROMA-I, Developmental Studies Hybridoma Bank, University of Iowa, rat IgG, diluted 1:20); anti-cytokeratin 20 (Abcam, rabbit IgG, diluted 1:200). The following secondary antibodies were used: biotinylated rabbit anti-mouse IgG (1:300, Sigma), goat anti-rat IgG biotinylated (1:300, Sigma), Alexa488 goat anti-mouse IgG (1:250, Molecular Probes); Alexa 488 or Alexa 594 rabbit anti-rat IgG (1:250, Molecular Probes) and Alexa 594 goat anti-rabbit (1:250, Molecular Probes).

Sections were inspected and representative images of immunoreactivity were acquired on Nikon Eclipse E400 fluorescence or Zeiss 780LSM confocal laser-scanning microscopes. Images were processed using the ZEN2009 software (Zeiss, Jena, Germany). Multi-panel figures were assembled in CorelDraw X3 (Corel Corp., Ottawa, ON, Canada).

Merkel cell primary culture

Whisker pads were dissected out from fetal mice (E18.5). The culture method was modified from Boulaïs et al., (2009a, 2009b). The epidermal sheet was separated from the dermis by enzymatic digestion, incubating the tissue overnight in 5 mg/ml Dispase II (Sigma, Poole, UK) at 4°C. Cells were dissociated by digestion with 1% trypsin-EDTA (Invitrogen, Paisley, UK), assisted by gentle pipetting. Cultured cells (including Merkel cells and keratinocytes) were plated onto plastic tissue-culture dishes (Thermo Fisher Scientific) in Dulbecco’s Modified Eagle Medium (DMEM/F12, Invitrogen, Paisley, UK), supplemented with 10% fetal calf serum (FCS) (Sigma), NGF 20 ng/ml (Sigma), 20 ng/ml NT-3 (Sigma) and 25 μg/ml gentamycin (Invitrogen, Paisley, UK) and maintained at 37°C 5% CO<sub>2</sub> for 2 - 4 days. Experimental oxidative stress was induced by adding 0.3 mM or 1.5 mM H<sub>2</sub>O<sub>2</sub> to the cells for two hours. The cells were fixed with 4% paraformaldehyde, permeabilized with methanol (10 minutes at -20°C) and blocked with 10% serum from the same animal species as the secondary antibody diluted in 0.3% BSA, PBS. Immunocytochemistry was performed using the combination of primary and secondary antibodies as described in the previous section.

Statistical methods

Primary cells stained with anti-K8, anti-K20 and anti-Pax6 antibody from both genotypes were imaged and antigen-positive cells were counted using ImageJ (http://rsbweb.nih.gov/ij/). On each cell dish, at least five fields of view were photographed for a total number of 250 cells from five replicates. The cells stained with DAPI represented the total number of cells. The cells immunoreactive for K6, K20 and Pax6 were then counted and expressed as a percentage of the total number of cells. Percentages of positive cells of different genotypes were compared by t-test. A two-tailed unpaired t-test was used to compare the subcellular localisation of Pax6 within the Merkel cells before and after the addition of H<sub>2</sub>O<sub>2</sub>. Chi-squared distribution tests were used to determine the statistical significance of differences in the percentage of Merkel cells treated and not treated with H<sub>2</sub>O<sub>2</sub> (n = 250 cells analysed from five replicates) All reported values are means ± standard error of the mean (S.E.M.).

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