We examined the potential interactions of anatomic site (head and arms versus trunk and legs) and CSD (CSD versus non-CSD melanoma) as proxy measures of sun exposure on the relationship between *MC1R* status (any variant) and *BRAF*-mutant melanoma. No statistically significant interactions were found (data not shown).

In a study by Landi *et al.* (2006), D84E, R142H, and c.86_87insA were defined as ''r'' rather than ''R''. Reclassification of these variants as ''r'' did not significantly change the results (data not shown). For purposes of comparison with cases included by Landi *et al.* and Fargnoli *et al.*, we repeated all analyses excluding two cases with melanomas on acral skin and four cases with positive or indeterminate germline *CDKN2A* mutations (Orlow *et al.*, 2007). These reanalyses did not reveal any significant associations (data not shown).

Our data do not support a strong association of *MC1R* variants with *BRAF* mutations in our North Carolina population. Differences in the findings between studies may be due, in part, to differing frequencies of distinct *MC1R* variants between study populations, which may not all be similarly associated with *BRAF* mutation. Another possibility is that the risk-modifying effect of *MC1R* variants may vary between populations based on unidentified genetic factors. Climate differences, such as ambient sun exposure, could also influence the relationship between *MC1R* status and *BRAF*-mutant melanoma. However, despite our study being the largest to date, the sample sizes are relatively modest in all the studies, and further investigation is necessary to clarify the relationship of germline *MC1R* variants and *BRAF*-mutant melanomas among different populations.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by a Lineberger Comprehensive Cancer Center grant and the National Cancer Institute grants CA112243, CA112243-05S109, and CA112524.

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PAX3 Is Extensively Expressed in Benign and Malignant Tissues of the Melanocytic Lineage in Humans

Journal of Investigative Dermatology (2010) **130,** 1465–1468; doi:10.1038/jid.2009.434; published online 28 January 2010

TO THE EDITOR

The paired box transcription factor, paired box 3 (PAX3), has an essential role in embryonic melanocyte development (Epstein, 2000). In mice, Pax3 is expressed in the bulge region of adult hair follicles, where melanocyte stem cells are found (Nishimura *et al.*, 2002; Lang *et al.*, 2005). Together with microphthalmia transcription factor (MITF), PAX3 regulates the balance between

Abbreviation: PAX3, paired box 3

committed melanoblast and terminal differentiation (Lang *et al.*, 2005). It is generally thought that upon terminal differentiation, the expression of PAX3 is reduced such that PAX3 is not expressed in normal skin melanocytes (Scholl *et al.*, 2001; Lang *et al.*, 2005), and only sporadically in benign melanocytic lesions (Plummer *et al.*, 2008). PAX3 expression in melanoma has been interpreted as ''re-expression'', which is suggested to contribute to uncontrolled cell growth and loss of terminal differentiation of melanocytes during tumorigenesis (Scholl *et al.*, 2001; Blake and Ziman, 2005; Plummer *et al.*, 2008).

Our results using PAX3 immunohistochemistry differed from that expected. In comparing the expression of PAX3 in adult human skin melanocytes, nevi, and melanomas with the expression of other melanogenesis factors, including microphthalmia transcription factor, Melan-A, and tyrosinase, medium to strong nuclear PAX3 staining was observed in the melanocytes of the epidermal basal layer and in hair follicles from adult skin tissues (12/12 cases), which was similar to the expression of microphthalmia transcription factor (Figure 1a). PAX3 was also strongly expressed in the outer root sheath external layer melanocytes of hair follicles (Figure 1a), which may represent both hair follicle melanocyte stem cells and amplifying progenitor cells.

Dual immunofluorescence staining of PAX3 and tyrosinase, or PAX3 and Melan-A revealed that PAX3-positive cells in the epidermis were also positive for tyrosinase (Figure 1d) and Melan-A (Figure 1e). Hair follicles contained both mature melanocytes that expressed PAX3 with tyrosinase and Melan-A (see Supplementary Figure S1 online), and melanoblasts that expressed PAX3 without tyrosinase or Melan-A, such as in the outer root sheath basal layer and dermal papilla (Figure 1d and e, Supplementary Figure S1 online). These results suggested that PAX3 is expressed in both melanoblasts and differentiated melanocytes. Strong PAX3 expression was also observed in

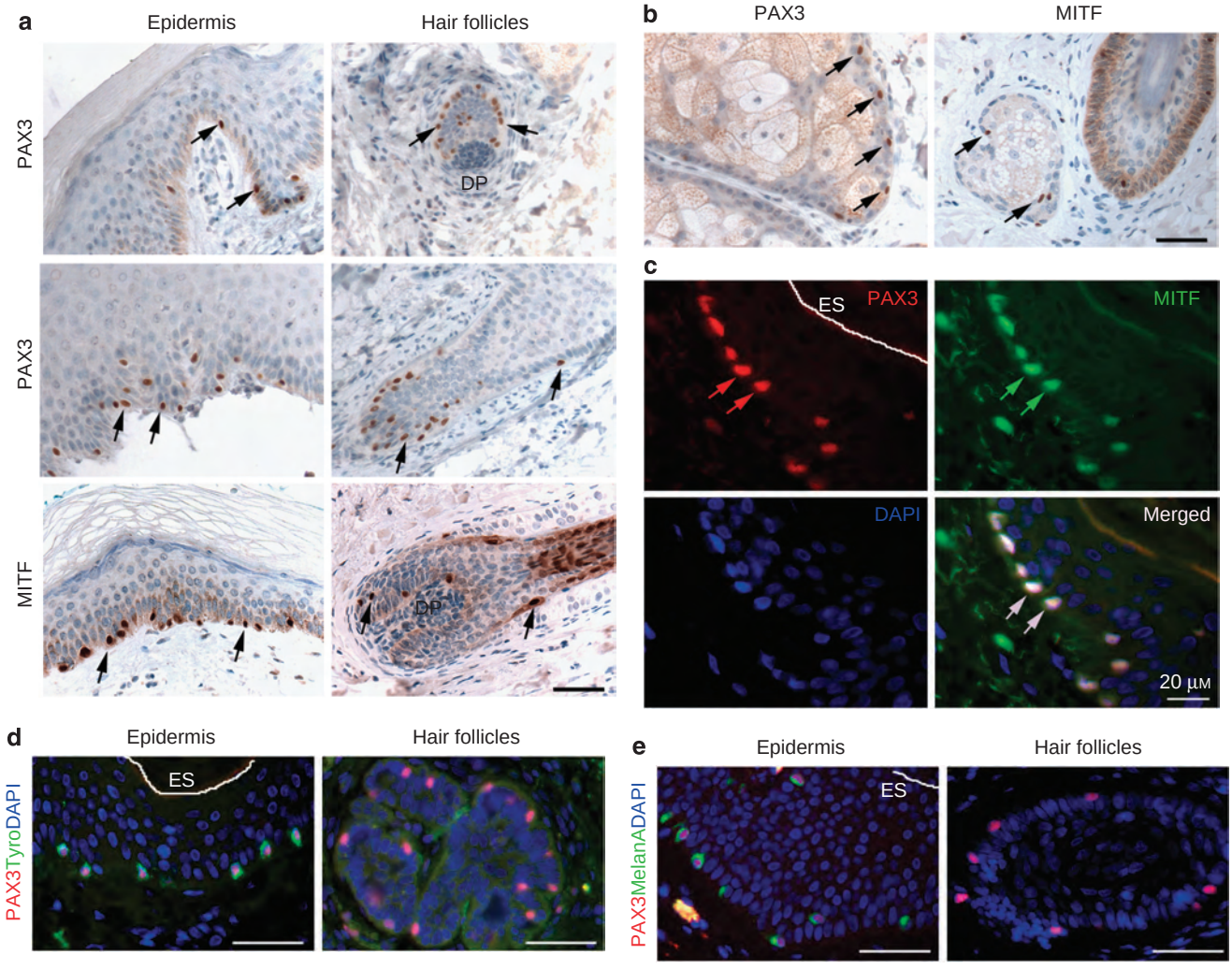

Figure 1. Paired box 3 (PAX3) and microphthalmia-associated transcription factor (MITF) are expressed in normal adult human skin melanocytes and hair follicles. (**a**, **b**) Immunohistochemistry shows PAX3- or MITF-positive melanocytes (arrows) within the epidermal basal layer and external root sheath, and/or the cortex of hair follicle bulbs in the anagen or telogen phase (**a**) and sebaceous glands (**b**). (**c**–**e**) Dual immunofluorescent staining shows the co-expression of (**c**) PAX3 (red) and MITF (green), (**d**) PAX3 (red) and tyrosinase (green), and (**e**) PAX3 (red) and Melan-A (green) in normal skin epidermis (**c**–**e**) and hair follicles (**d**, **e**). DAPI, 4′,6-diamidino-2-phenylindole; DP, dermal papilla; ES, epidermal surface; Tyro, tyrosinase. Bar = 50 μM.

nevus melanocytes, either as single melanocytes or as clusters of melanocytes appearing as nests of cells, which could represent either acquired or congenital nevi (Figure 2 and Supplementary Table S1 online). All 11 nevi examined showed strong PAX3 expression.

PAX3 was expressed in primary melanomas (31/33) as well as metastatic melanomas (10/11) at various levels (Figure 2, Supplementary Table S1 online and summarized in Supplementary Table S2 online). A comparison of immunohistochemical and clinical data failed to demonstrate a relationship between the presence or absence of PAX3, and melanoma subtype, the site of the primary, patient age, patient sex, metastasis, growth phase, Clark level, or Breslow thickness (Supplementary Table S1 online).

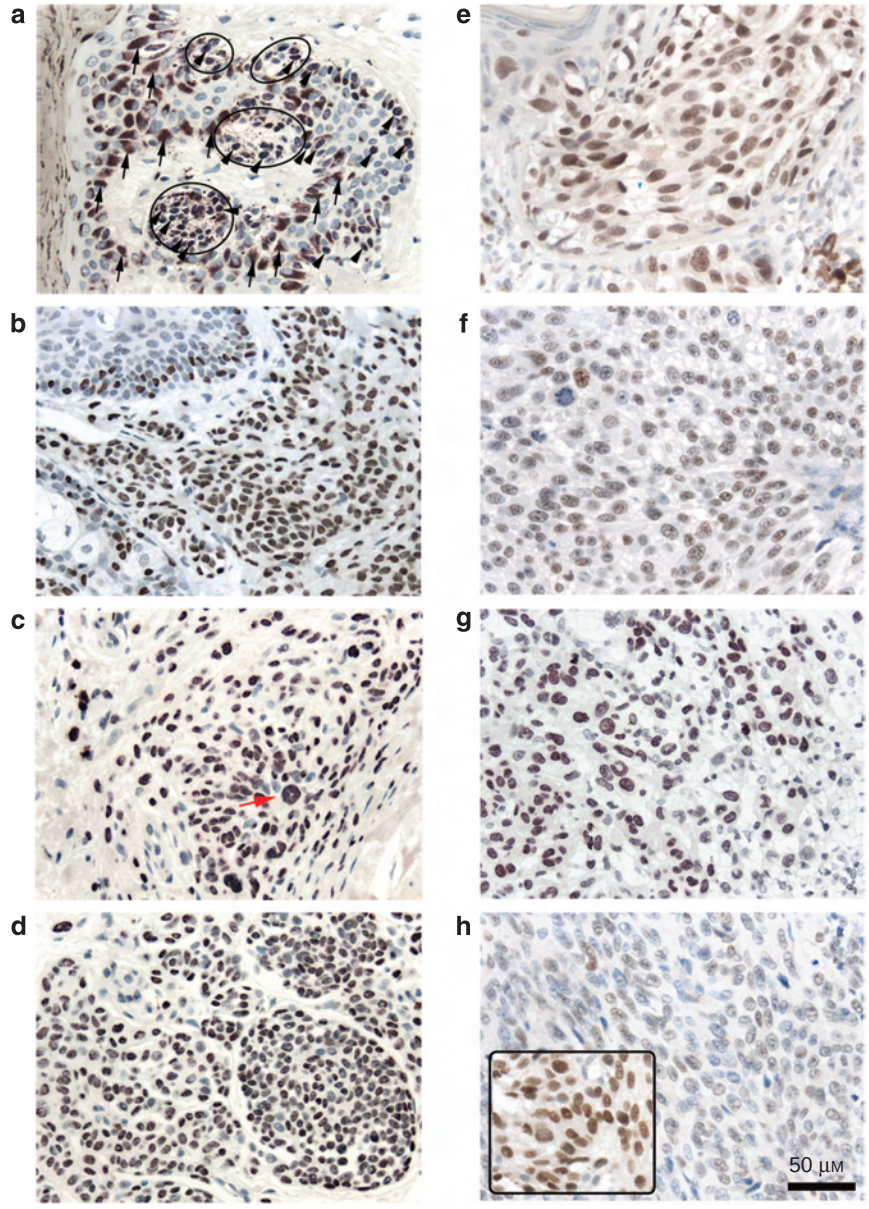

Figure 2. Expression of paired box 3 (PAX3) in nevi and melanomas. Immunohistochemical staining of PAX3 in the (**a**) junctional nevus, (**b**) congenital nevus, (**c**) intradermal nevus, and (**d**) compound nevus. Note, profound melanin was observed in the cytoplasm of epidermal keratinocytes of a junctional nevus (**a**, arrows). Arrowheads indicate PAX3-positive melanocytes. Circles indicate nests of melanocytes. Strong PAX3 staining in a multinucleated giant melanocyte of an intradermal nevus (**c**, arrow). (**e**) Immunohistochemical staining of PAX3 in superficial spreading melanoma, (**f**) acral lentigo malignant melanoma, (**g**) nodular melanoma, and (**h**) metastatic melanoma. Stronger staining of PAX3 was observed in parts of the same tissue section as shown in the inset of (**h**). The use of 3,3′diaminobenzidine enhancing solution rendered PAX3-positive staining different from the melanin color, which was brown, whereas the former was dark gray. Aside from the color difference, nuclear staining for PAX3 was always discernible from melanin in nevi melanocytes, or in melanoma cells because melanin granules were located exclusively in the cytoplasm. Bar = 50 μm.

Although previous reports have suggested a lack of, or variable detection of PAX3 in melanocytes of normal human skin, nevi, and melanomas, or that the expression of PAX3 tended to occur more often in melanomas from younger individuals (Scholl *et al.*, 2001; Plummer *et al.*, 2008), we found no evidence to support such variable detection of PAX3 expression in skin melanocytes, nevi, or in melanomas with respect to patient age, melanoma stage, sun damage, or solar elastosis. For instance, we observed the expression of PAX3 in relatively normal skin areas and in scattered melanocytes within the tumor in three cases of basal cell carcinoma, all with signs of severe solar elastosis (these three cases were not counted as normal skin). We surmise that the differences between our study and previous studies could be technical; we have used the same anti-Pax3 antibody as used previously (Plummer *et al.*, 2008). In our study, PAX3-positive staining was always discernable from melanin because of a clear color difference and cytoplasmic localization of melanin (see Figure 2 legend; see Supplementary Figure S2 and Supplementary Methods online).

In our study, most of the PAX3-positive cells in normal skin represented differentiated melanocytes co-expressing tyrosinase and Melan-A. A role for PAX3 in maintaining the undifferentiated phenotype of Schwann cells, neural crest precursors, and lineage-committed melanocytes has been suggested (Kioussi *et al.*, 1995; Lang *et al.*, 2005; Wu *et al.*, 2008). PAX3 expression was thought to switch off in fully differentiated cells, and re-expression of PAX3 was suggested to promote de-differentiation and contribute to melanoma cell survival, thereby promoting melanoma pathogenesis (Scholl *et al.*, 2001). However, our observation of PAX3 expression in non-malignant differentiated melanocytes contradicts the notion that persistent expression of PAX3 is pathogenic, and

suggests that further clarification of the role of PAX3 expression in non-malignant differentiated melanocytes is now required. Extensive PAX3 expression in benign and malignant tissues of the melanocyte lineage, and absence of expression in other types of skin cancer, nevertheless suggests that PAX3 could be used as an immunohistochemical marker to differentially diagnose melanoma.

Ethics approval for this study was obtained from the New Zealand Multi-Regional Ethics Committee.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

The anti-Pax3 antibody was developed by Dr C. Ordahl and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, USA. This research was supported by grants from the Health Research Council of New Zealand and Dunedin School of Medicine.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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Cathepsin S Elicits Itch and Signals via Protease-Activated Receptors

Journal of Investigative Dermatology (2010) **130,** 1468–1470; doi:10.1038/jid.2009.430; published online 14 January 2010

TO THE EDITOR

Cathepsin S is a cysteine protease linked to inflammatory processes, including atherosclerosis and asthma. The possibility that this or other cysteine proteases might evoke itch or be part of a classical ligand-receptor signaling cascade has not been considered previously. We show here that human cathepsin S evokes itch and activates human protease-activated receptors (PARs) 2 and 4.

The sensation of itch is mediated by two distinct non-overlapping populations of cutaneous nerve fibers that evoke comparable degrees of itch (Johanek *et al.*, 2008; Namer *et al.*, 2008). One set of fibers, the mechano-insensitive population, is more responsive to histamine than to cowhage. The other set is mechanosensitive and is more responsive to cowhage than to histamine (Johanek *et al.*, 2008; Namer *et al.*, 2008). Histamine is a classical mediator of itch and is associated with a wheal and flare. As most clinical itches do not have a wheal or flare and do not respond to antihistamines, histamine is not thought to contribute to most itches (Ikoma *et al.*, 2006). Cowhage refers to a tropical legume or, in this case, the loose hairs that cover the pods of *Mucuna pruriens* and evoke itch. The active component of cowhage is mucunain, a cysteine protease that serves as a ligand for PARs 2 and 4 (Reddy *et al.*, 2008). The identification of an endogenous mediator with properties similar to cowhage could lead to insights into non-histamine-mediated itch. We focused on human cathepsin S because it shares active site sequence homology with mucunain and is selectively up-regulated in human keratinocytes upon stimulation with interferon-gamma, consistent with a possible pruritic role in inflammatory skin disease (Schwarz *et al.*, 2002).

Abbreviation: PAR, protease-activated receptor

There are 15 human cathepsins, including 11 cysteine, 2 aspartic, and 2 serine proteases. Cathepsins were traditionally considered lysosomal proteases. It is now recognized that the broad expression and range of pH dependence of some cathepsins reveal that they have many functional roles, including tissue remodeling, metastasis and inflammation. Examples of cysteine cathepsin activities include cleavage of collagen by cathepsin L to generate endostatin (Felbor *et al.* 2000), an endogenous inhibitor of angiogenesis, and cleavage of the invariant chain in antigen-presenting cells by cathepsin S (Nakagawa *et al.*, 1999) as part of the inflammatory cascade.

There are four PARs and they are members of the G-protein-coupled receptor family. Their identified endogenous activators are all serine proteases. These proteases trigger the activation of PARs by unmasking 'tethered ligand' sequences near the N-termini of the receptors. Certain kallikrein-related peptidases and mast