Lack of Genetic and Epigenetic Changes in *CDKN2A* in Melanocytic Nevi

To the Editor:

The strongest known epidemiologic risk factor for melanoma is a large number of melanocytic nevi (Swerdlow and Green, 1987), whereas the most important genetic risk factor is germline mutation of the *CDKN2A* gene, which encodes the cell cycle inhibitor p16 (Kamb *et al*, 1994; Nobori *et al*, 1994). *CDKN2A* mutations exist in some melanoma-prone families (reviewed in Hayward, 1996; Foulkes *et al*, 1997; Ruas and Peters, 1998), but account for only a small fraction of all familial melanoma (Platz *et al*, 1997; Aitken *et al*, 1999).

Some melanoma kindreds include individuals with a high prevalence of "dysplastic" (Greene *et al*, 1985) or "atypical" (Gruis *et al*, 1995) nevi, but this phenotype does not segregate with *CDKN2A* (reviewed in Hayward, 1996); however, evidence for linkage of "common" nevus count with *CDKN2A* has been found in three melanoma pedigrees, only one of which carried an exonic mutation, suggesting that other variants in the region, outside the sequence encoding p16, affect nevus density (Cannon-Albright *et al*, 1994). We have shown by sib-pair linkage analysis that ~30% of variance in moliness is due to genetic variation at or close to the marker D9S942 (Zhu *et al*, 1999), 15 kilobases upstream of *CDKN2A*, and we surmise that D9S942 is in disequilibrium with a functional polymorphism nearby. We hypothesize that as germline mutations in the exons of *CDKN2A* are rare, it is likely that variants in the noncoding regions of this gene are responsible for this major determinant of nevi, and by inference, of melanoma. Somatic changes and loss of heterozygosity (LOH) of *CDKN2A* support a role for this gene in nevus etiology (Healy *et al*, 1996a; Lee *et al*, 1997). Given the above links between *CDKN2A*, melanoma, and nevi, we looked for chromosomal loss, structural and epigenetic changes in the *CDKN2A* gene in benign melanocytic lesions.

A 4 mm punch was used to separate neval from stromal tissue from 10 g paraffin-embedded sections of 25 intradermal nevi and 25 compound nevi. Sections of nevi and stroma were incubated in 80 μl of lysis buffer (10 mM Tris-HG, pH 8.3; 50 mM KCl; 2.5 mM $MgCl_2$) plus 4.4 μl of 10 mg per ml proteinase K and 4 μl of 10% Tween 20. Samples were incubated at 55°C for 18–48 h then boiled for 15 min, adjusted to 1 mM EDTA, and centrifuged for 1 min at 2300 × *g*. LOH at *CDKN2A* was assessed by comparing genotypes at D9S942 as previously described (Pollock *et al*, 1998).

CDKN2A was screened for mutations in nevus samples using SSCP analysis as described (Aitken *et al*, 1999). Samples that showed aberrant band mobility were re-amplified and run on a 2% agarose gel. Appropriate fragments were excised and purified by passing them through a QiaQuick (Qiagen) gel extraction column, then 50–250 ng were sequenced as described (Aitken *et al*, 1999). Matching stromal DNA was analyzed when variants were detected.

The CpG island domain of *CDKN2A*, encompassing the promotor and exon 1 (nucleotides 28602–28856 in GenBank entry AC000048), was screened for methylation using the bisulfite sequencing method under conditions described in Clark *et al* (1994). The control primers used for the standard bisulfite p16 amplification were: outer, p16–4 (28844–28819) and p16–6 (28602–28579), and inner, p16–4 and p16–5 (28516–28541) (Huschtscha *et al*, 1998). The MSP-PCR p16 primers were: outer, p16CG-4 (28962–28939) and p16CG-6 (28641–28616), and inner, p16CG-4 and p16CG-5 (28880–28856); sequence coordinates are from GenBank entry AC000048. Reaction conditions for the standard bisulfite polymerase chain reaction (PCR) and methylation-specific primers PCR (MSP-PCR) were as described (Huschtscha *et al*, 1998).

DNA was successfully extracted from 45 pairs of specimens, of which 41 were constitutionally heterozygous for D9S942. In all nevus samples bands corresponding to each allele were seen at equal intensity to matching somatic DNA, indicating no LOH. No nevus had acquired a somatic change in the protein-coding region of *CDKN2A*, although four individuals carried germline *CDKN2A* variants. Individual 22 was homozygous for the nucleotide 442 G to A variant (ala148thr), and individuals 29 and 43 were heterozygous for this polymorphism. One case possessed a heterozygous nucleotide 412 A to G mutation (arg138gly) (**Fig 1**). To our knowledge this is the first report of an individual with a germline *CDKN2A* mutation who does not have melanoma or a family history of melanoma.

No hypermethylation of the *CDKN2A* promoter, which might lead to loss of p16 expression in the nevus samples, could be

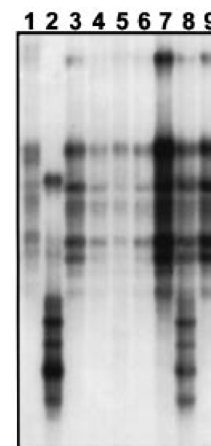

Figure 1. Autoradiograph of a representative SSCP gel showing aberrant band mobility for exon 2 of *CDKN2A*. *Lane 1*, sample 21 (heterozygous for arg138gly mutation); *lane 2*, sample 22 (homozygous for ala148thr polymorphism); *lanes 3–7* and *9*, control samples (homozygous for wildtype alleles); *lane 8*, sample 29 (heterozygous for ala148thr polymorphism).

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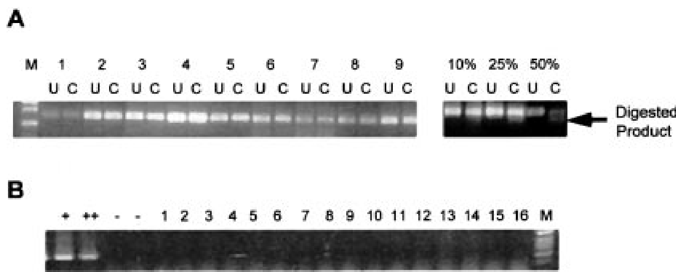

Figure 2. Methylation analysis of the p16 promoter region. (*A*) DNA from nevus samples (1–9) was bisulfite-treated and amplified with primers that amplify methylated and unmethylated DNA in proportion. The PCR was either undigested (U) or digested with *Taq*I (C). Methylated p16 DNA was amplified as a control from bisulfite-treated human DNA that had been spiked at 10%, 25%, and 50% levels (Warnecke *et al*, 1997). *Lane M*; DNA size markers. (*B*) DNA from nevus samples (1–16) was bisulfite-treated and amplified using MSP-PCR primers. Methylated p16 DNA was amplified as a control from bisulfite-treated human DNA that had been spiked at 0.1% (+) and 1% (++) and not spiked as a negative control (–).

detected in any of the 40 nevi tested, either by *Taq*I digestion (**Fig 2*a***) or by MSP-PCR (**Fig 2*b***).

Our results suggest little or no involvement of genetic or epigenetic alterations of *CDKN2A* in nevus etiology, and thus support the data of others who have failed to detect *CDKN2A* mutation (Healy *et al*, 1996b) or methylation (Gonzalgo *et al*, 1997) in various types of nevi; however, our findings do not rule out mutations in noncoding regions nor factors other than methylation affecting expression of this gene. Alternatively, variations in noncoding regions of *CDKN2A* or in a gene adjacent to *CDKN2A* may account for the variance in nevus count we have shown to be linked to this region (Zhu *et al*, 1999).

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Real-Time Evidence of *In Vivo* Leukocyte Trafficking in Human Skin by Reflectance Confocal Microscopy

To the Editor:

The dynamic sequence of events mediating leukocyte emigration from vascular to tissue compartments, including lymphocyte homing and localization of monocytes and neutrophils in inflamed tissue, is directed by adhesive molecules and molecular signals that result in the attachment/rolling of leukocytes followed by their strong adhesion/arrest and diapedesis (Sackstein *et al*, 1988; Shimizu *et al*, 1992; Springer, 1994; Clark and Brugge, 1995; Sackstein, 1995). To date, this multistep process has been investigated utilizing *in vitro* and *in vivo* experimental models requiring invasive procedures such as multiple biopsies to allow *ex vivo* histologic analysis of emigrated leukocytes and, more recently, by intravital

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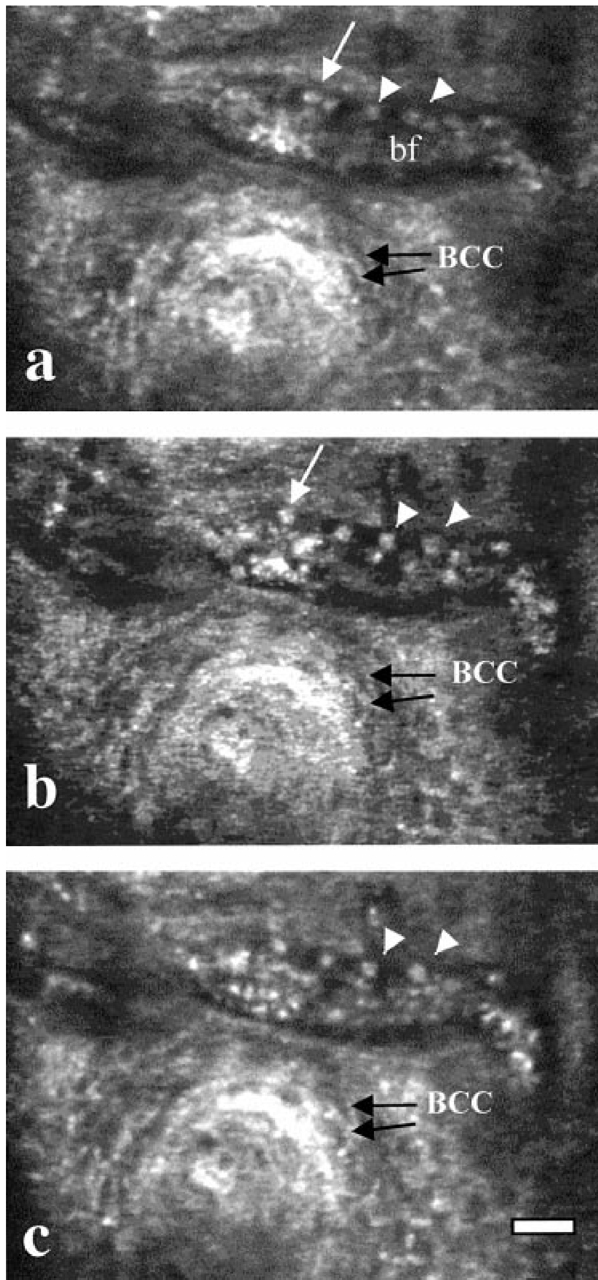

Figure 1. Real time imaging of leukocyte trafficking in human papillary dermis *in vivo*. Sequential en-face optical sections of the same microscopic plane of a superficial basal cell carcinoma (BCC, see elongated atypical nuclei, black arrows) demonstrate the rolling of leukocytes (white arrow). Imaging depth corresponds to 90 μm below the stratum corneum. Within papillary dermis, blood flow (bf) through a capillary loop (35 μm in diameter) is seen. The time period between virtual sections a and c was 3 s. Note that blood flow is more apparent on active real-time video than on still, grabbed images. Individual leukocytes (*arrowheads*) firmly adherent to the endothelial wall are also visualized in real-time (1064 nm Nd:YAG laser; 60X, 0.85 NA. water immersion objective lens; *scale bar*: 25 μm).

microscopy after intravenous administration of fluorescently labeled peripheral blood cells in animals (Lawrence and Springer, 1991; Bargatze *et al*, 1995; von Andrian, 1996; Robert *et al*, 1999). Excisional biopsy permanently changes the native tissue and eventuates in scarring; moreover, most of the fluorescent dyes for studying blood cell flow within vasculature at high resolution are toxic and thus not approved for human use by the FDA.

Recent advances in reflectance confocal microscopy (CM) have resulted in the development of real-time, near-infrared confocal scanning laser microscopes that yield real-time high resolution optical sections of human or animal skin in its native state *in vivo* (Rajadhyaksha *et al*, 1995, > 1999). Similarly, tandem scanning confocal microscopy has been applied for imaging human skin *in vivo* (New *et al*, 1991; Corcuff *et al*, 1993). Optical reflectance imaging relies on the natural variations in refractive indices of tissue microstructures for contrast. Nuclear- and cellular-level detail can be imaged with a resolution comparable with routine histology to a depth that includes papillary and upper reticular dermis. Imaging is obtained at video-rate (30 frames per s), allowing high temporal resolution (33 milliseconds per frame) for visualizing dynamic processes such as the time sequence of histologic events during eczematous reaction (e.g., spongiosis with microvesicle formation and dermal vasodilation) (González *et al*, 1999a), and those occurring after laser-treatment of vascular lesions (González *et al*, 1999b; Agashi *et al*, 2000).

Real-time observation of peripheral blood cells flowing through capillary loops of healthy human skin and oral mucosa *in vivo* has been previously reported (Rajadhyaksha *et al*, 1995, 1999; González *et al*, 1999a; White *et al*, 1999). Based on size, shape, and number density, peripheral blood cells on live images and grabbed images could be identified; however, analysis of leukocyte emigration from vascular to tissue compartments was not performed. In this letter, we report the capability to observe leukocyte–endothelial interactions in native human skin *in vivo* at video-rate with a confocal scanning laser microscope. This methodology makes possible the real-time analysis of the trafficking of human leukocytes *in vivo*.

Near-infrared CM can image live hemodynamic cellular events in inflammatory conditions (e.g., sunburn, psoriasis, skin cancer), including tethering and rolling of leukocytes on the endothelial wall, with adhesion and arrest to the endothelium. In our clinical studies, we consistently observe this in basal cell carcinomas *in vivo* (**Fig 1**). Because the tissue is not harmed, high-resolution CM imaging can be performed frequently and repeatedly, and therefore evaluation of various stages of adhesive interactions between leukocytes and skin endothelium can be investigated *in vivo*.

This novel imaging tool opens new avenues of investigating dynamic events in real time and noninvasively. The potential of confocal imaging to noninvasively view blood flow, and elucidate adhesive interactions and histology, may lead to new criteria for diagnosis of cutaneous diseases and may facilitate treatment and management of significant medical problems where conventional histopathology does not provide a definitive diagnosis. A good example is acute cutaneous graft-*versus*-host disease (GvHD), a major life-threatening complication of allogeneic bone marrow transplantation faces this challenge. This entity does not have pathognomonic histologic features. Dermal lymphocytic infiltrates, mediated by discrete leukocyte–endothelial interactions occurring in the target tissue, are a hallmark of cutaneous GvHD and are the earliest feature of this condition (Sackstein, 1995). The capability to observe emigration of lymphocytes *in vivo* in the early post-transplant period could lead to more accurate recognition and more specific and timely therapy of cutaneous GvHD, and could allow measuring in real-time the response to therapy.

At present, we can observe leukocyte adhesion and rolling on the endothelium with high contrast; however, when the leukocytes migrate out of the blood vessel, they are not always easily seen because the cells lack contrast relative to the surrounding connective tissue. Methods to enhance the contrast of the leukocytes must be developed, using either endogenous or exogenous contrast agents.

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Melanosomal pH, Pink Locus Protein and their Roles in Melanogenesis

To the Editor:

We appreciate the opportunity to respond to the comments on our recent article (Puri *et al*, 2000) by Ancans *et al* (2001).

Our hypothesis that the p protein functions to regulate melanosome pH is based on the following observations:

1 Melanosomes are normally acidic. Indeed, more highly pigmented melanosomes are more acidic than less-pigmented melanosomes (Moellmann *et al*, 1988; Bhatnagar *et al*, 1993; Ramaiah, 1996).

2 Melanosomes, as defined by organelles containing the melanosomal marker protein, Tyrp1, are acidic in wild-type melanocytes, but nonacidic in two independent melanocyte lines lacking expression of the p protein (Puri *et al*, 2000).

3 The p protein is an integral melanosomal membrane protein (Rosemblat *et al*, 1994).

4 The p protein is a 12 membrane spanning domain protein that shares significant homology with anion transporters (Rosemblat *et al*, 1994; Lee *et al*, 1995; Brilliant, 2001), e.g., the oxyanion translocating subunit of the ArsAB ATPase of bacteria, and several dicarboxylate and sulfate transporters, revealed by BLAST search algorithms.

5 Anion transporters are essential partners of vacuolar proton pumps. Together they regulate the luminal pH of a variety of intracellular organelles (Van Dyke, 1996; Grabe and Oster, 2001).

Whether the acidic conditions favor the enzymatic action of tyrosinase or are required for other critical aspects of melanin biosynthesis (e.g., the correct targeting of melanogenic proteins such as Tyrp1 or biogenesis of melanosomes) is indeed an open question as we indicated (Puri *et al*, 2000; Brilliant, 2001). As pointed out by Ancans *et al* (2001), tyrosinase activity *in vitro* is optimal at near neutral pH; however, it may very well be that the *in vivo* activity of tyrosinase (where it is a melanosomal membrane spanning protein) is optimal at a different pH (Devi *et al*, 1987). Like Ancans *et al* (2001), we have also suggested that mistargeting of melanosomal proteins may result from disregulation of melanosomal pH (Puri *et al*, 2000; Brilliant, 2001), consistent with other studies (Potterf *et al*, 1998; Orlow and Brilliant, 1999). Acidification of various intracellular compartments is important for a number of processes, including receptor mediated endocytosis, receptor recycling, and membrane trafficking within the cell (Van Dyke, 1996; Grabe and Oster, 2001).

We agree with Ancans *et al* (2001) that "it is difficult to explain how the p protein, which does not utilize energy from ATP, could function against a proton gradient…". In fact, the p protein does not function against a proton gradient. It works in concert with the melanosome ATP-driven proton pump (Bhatnagar *et al*, 1993) that is very likely the same pump present in endosomes, Golgi-derived vesicles and lysosomes (Al-Awqati, 1995; Orlow, 1995). Based on protein homology, the p protein is most likely an anion transporter. Anion transporters are essential for the acidification of these organelle compartments by the ATP-driven proton pump (Van Dyke, 1996; Grabe and Oster, 2001). Anion (Cl^-, $SO_4^=$, or $HCO3^-$) conductance provides the compensating charge balance to electrogenic proton transport. We do not believe that the p protein directly transports protons or other cations and we are unaware of any data or protein homology algorithm demonstrating that the p protein is homologous to the *E. coli* Na^+/H^+ antiporter as Ancans *et al* (2001) state. The only statistically significant homologies with other proteins are to known anion transporters.

We accept the data by Ancans *et al* (2001) and by others (Ancans and Thody, 2000; Fuller *et al*, 2001) that demonstrate increased melanin synthesis in melanocytes that lack the p protein following bafilomycinA1, monensin, and NH_4Cl treatment; however, in these assays it would be important to know where this melanin synthesis occurs. For example, increased tyrosine in the media promotes increased melanin biosynthesis by p melanocytes (Sidman and Pearlstein, 1965; Rosemblat *et al*, 1998), but this synthesis is not primarily in melanosomes and the p protein is not a tyrosine transporter (Gahl *et al*, 1995; Potterf *et al*, 1998).

In sum, we agree with Ancans *et al* (2001) that the relationship between melanosomal pH and melanin biosynthesis is currently unknown. Clearly further studies are warranted to understand the

role of the p protein in regulating melanosomal pH and melanin biosyntheis

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