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Absence of Distinguishing Senescence Traits in Human Melanocytic Nevi

Sieu L. Tran[1], Sebastian Haferkamp[2], Lyndee L. Scurr[1], Kavitha Gowrishankar[1], Therese M. Becker[1], Chitra Desilva[3], John F. Thompson[3,4], Richard A. Scolyer[3,5,6], Richard F. Kefford[1,3,7] and Helen Rizos[1]

Cellular senescence permanently restricts the replicative capacity of cells in response to various stress signals, including aberrant activation of oncogenes. The presence of predictive senescence markers in human premalignant lesions suggests that senescence may function as a genuine tumor suppressor. These markers are not exclusive to the senescence program, however, and it is possible that their expression *in vivo* does not discriminate irreversible from reversible forms of proliferative arrest. In this study, we aimed to clarify whether human nevus cells can be distinguished from primary and transformed melanocytes by examining the expression of eight senescence markers, including those previously purported to define nevi as senescent tumors. Specifically, we analyzed effectors of senescence, including $p16^{INK4a}$, p53, and DNA damage (γ-H2AX), as well as predictive markers of senescence including Ki67, PML, senescence-associated β-galactosidase, heterochromatic foci (H3K9Me, 4′-6-diamidino-2-phenylindole), and nuclear size. We found that these commonly accepted senescence markers do not in fact distinguish nevi from precursor/normal and transformed/malignant melanocytes. We conclude that on the basis of current evidence it cannot be reasonably inferred that nevi are permanently growth arrested via senescence.

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INTRODUCTION

Cellular senescence permanently restricts the proliferative capacity of cells in response to various stress signals, including aberrant activation of oncogenes. For instance, the sustained activation of oncogenic RAS and its downstream kinase effector, B-RAF, promotes a rapid cell cycle arrest *in vitro*, known as oncogene-induced senescence (Michaloglou *et al.*, 2005; Gray-Schopfer *et al.*, 2006). Importantly, senescent cells are unresponsive to physiological mitogenic stimuli, and senescence is thought to act as a genuine tumor suppressor *in vivo*. Senescent cells display a combination of markers that are not exclusive to the senescence program but in combination represent powerful predictors of this form of arrest. Senescence markers include the upregulation of $p16^{INK4a}$, induction of senescence-associated β-galactosidase (SA-β-gal) activity, the formation of senescence-associated heterochromatin foci, and the accumulation of DNA damage foci (reviewed in Campisi and d'Adda di Fagagna, 2007; Collado and Serrano, 2006). Compelling recent data, based on the expression of a limited number of senescence markers in human premalignant lesions of the skin, colon, prostate, and nervous system, support the concept that senescence acts as a genuine tumor suppression mechanism (Chen *et al.*, 2005; Michaloglou *et al.*, 2005; Bartkova *et al.*, 2006; Courtois-Cox *et al.*, 2006; Kuilman *et al.*, 2008).

Evidence demonstrating that B-RAF promotes senescence *in vivo* comes from studies in animal models and human nevi. Nevi are benign tumors of melanocytes that frequently harbor oncogenic mutations in B-RAF (Pollock *et al.*, 2003). Nevi remain growth arrested for decades and rarely develop into melanomas (Kuwata *et al.*, 1993; Maldonado *et al.*, 2004), presumably because aberrant B-RAF signaling induces a potent senescence response (Michaloglou *et al.*, 2005; Gray-Schopfer *et al.*, 2006; Dankort *et al.*, 2009; Dhomen *et al.*, 2009; Goel *et al.*, 2009). Mutant B-RAF has been shown to promote nevus formation in murine and fish melanoma models, and these nevi are growth arrested and express $p16^{INK4a}$ and SA-β-gal (Dhomen *et al.*, 2009; Goel *et al.*, 2009). Human nevi also display some features of oncogene-induced senescence, including intact telomeres, increased $p16^{INK4a}$ expression, and positive SA-β-gal activity (Miracco *et al.*, 2002; Michaloglou *et al.*, 2005; Mooi and Peeper, 2006), although the expression of this enzyme in

[1]*Westmead Institute for Cancer Research, University of Sydney at Westmead Millennium Institute, Westmead Hospital, Westmead, New South Wales, Australia;* [2]*Department of Dermatology, Venereology and Allergology, University of Würzburg, Würzburg, Germany;* [3]*Melanoma Institute Australia, Sydney, New South Wales, Australia;* [4]*Discipline of Surgery, Sydney Medical School, The University of Sydney, Sydney, New South Wales, Australia;* [5]*Tissue Pathology and Diagnostic Oncology, Royal Prince Alfred Hospital, Camperdown, Sydney, New South Wales, Australia;* [6]*Department of Pathology, Sydney Medical School, The University of Sydney, Sydney, New South Wales, Australia and* [7]*Department of Medicine, Sydney Medical School, The University of Sydney, Sydney, New South Wales, Australia*

Correspondence: Helen Rizos, Westmead Institute for Cancer Research, University of Sydney at Westmead Millennium Institute, Westmead Hospital, Westmead, New South Wales 2145, Australia.
E-mail: helen.rizos@sydney.edu.au

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human nevus cells *in vivo* remains controversial (Cotter *et al.*, 2007, 2008; Michaloglou *et al.*, 2008). The role of oncogenic B-RAF in initiating human melanocytic proliferation and nevus formation is also contentious, as mutant B-RAF is not present in every cell within a nevus (Ichii-Nakato *et al.*, 2006; Lin *et al.*, 2009).

There is clinical and histological evidence that ~25% of melanomas arise from, or are associated with, a pre-existing nevus (Marks *et al.*, 1990; Bevona *et al.*, 2003; Tsao *et al.*, 2003), and this suggests that either senescence does not prevent tumorigenesis or that not all nevus cells have undergone senescence. In fact, the concept that human nevi are irreversibly arrested via senescence has not been rigorously tested, and there is clear evidence for low levels of mitotic activity in congenital, common acquired, and dysplastic nevi (Soyer *et al.*, 1989; Moretti *et al.*, 1990; Rudolph *et al.*, 1997; Florell *et al.*, 2002, 2005; Jensen *et al.*, 2007; Lebe *et al.*, 2007; Nasr and El-Zammar, 2008; Glatz *et al.*, 2010; McCarthy and Scolyer, 2010; Ruhoy *et al.*, 2011).

In this study, we sought to clarify whether human nevi display a consistent and conclusive senescence signature by examining the expression of eight senescence markers, including those previously purported to define nevi as senescent tumors, namely $p16^{INK4a}$, Ki67, and SA-β-gal. We examined a series of fresh-frozen and paraffin-embedded nevi and melanomas and melanocytic cell lines. Importantly, we compared whether these markers differentiate nevus cells from primary and metastatic melanomas, as well as individual normal melanocytes scattered along the dermal–epidermal junction in adjacent normal skin. We confirm that although several senescence markers, including $p16^{INK4a}$ and SA-β-gal, are common in human nevi, they also occur frequently in melanoma samples. Further, both nevus cells and individual skin melanocytes are uniformly negative for the proliferation marker Ki67. Thus, it cannot be reasonably concluded on the basis of current evidence that nevi are permanently growth arrested via senescence.

RESULTS AND DISCUSSION

In this report, we assessed the expression of eight senescence markers previously identified *in vitro* in a series of fresh-frozen and formalin-fixed, paraffin-embedded human benign nevi ($n = 46$) and melanomas ($n = 46$). We analyzed putative effectors of oncogene-induced senescence, including $p16^{INK4a}$, p53, and DNA damage (detected using γ-H2AX), as well as markers of senescence including Ki67, PML, SA-β-gal, senescence-associated heterochromatin foci (detected using 4′-6-diamidino-2-phenylindole (DAPI) stain and histone H3 methylated at lysine 9 (H3K9Me)), and nuclear size (reviewed in Kuilman *et al.*, 2010; Muller, 2009). Where possible, the genotype of *B-RAF* at codon-600 was also examined and the V600E mutation was detected in 11/17 and 8/17 nevi and metastatic melanomas, respectively (Supplementary Table S1 online).

Initially, we analyzed the behavior of our panel of senescence markers in cultured human neonatal melanocytes expressing oncogenic $B\text{-}RAF^{V600E}$. As expected, $p16^{INK4a}$, γ-H2AX, and PML were all substantially increased in $B\text{-}RAF^{V600E}$-transduced melanocytes (Figure 1). Staining of 19 formalin-fixed paraffin-embedded nevi and 18 metastatic cutaneous melanomas (Supplementary Table S1 online) with antibodies against $p16^{INK4a}$, γ-H2AX, and PML revealed negligible staining in individual melanocytes in adjacent normal skin (Figure 2). In contrast, γ-H2AX and PML proteins were equally, highly expressed in both human nevi and metastatic melanomas (Figure 2, Table 1). The expression of $p16^{INK4a}$ clearly distinguished nevi from metastatic melanomas; most nevi displayed heterogeneous $p16^{INK4a}$ staining irrespective of B-RAF status, and the expression of $p16^{INK4a}$ was significantly diminished in the metastatic melanomas (Figure 2). Elevated expression of $p16^{INK4a}$ in nevi has supported the notion that oncogene-induced senescence promotes cell cycle arrest in nevus cells (Michaloglou *et al.*, 2005), although $p16^{INK4a}$ was shown to be uniformly expressed in melanomas *in situ* (Reed *et al.*, 1995). We explored the link between $p16^{INK4a}$ induction and melanoma progression by conducting additional immunohistochemical analyses using paraffin-embedded tumor tissue arrays of an additional 21 primary cutaneous melanomas and 20 nevi (Supplementary Table S2 online). As shown in Figure 3, $p16^{INK4a}$ expression was usually retained in primary melanomas at levels comparable to those seen in nevi (Figure 3, Table 2). Thus, although $p16^{INK4a}$ induction is commonly associated with oncogene-induced senescence, it is not unique to growth-arrested nevi but is also expressed in proliferating primary melanomas.

As with B-RAF-transduced melanocytes (Figure 1), we observed no evidence of proliferation (Ki67 positivity) within nevi or in individual skin melanocytes. In contrast, Ki67 was expressed in a high proportion of metastatic melanomas cells (Figure 2). The appearance of heterochromatin foci in B-RAF-transduced melanocytes coincided with cell cycle arrest, and these foci were enriched for the heterochromatin markers H3K9Me (Figure 1) and HMGA2 (data not shown). DAPI-stained nuclear foci were not detected in nevi or metastatic melanomas (Figure 2), and although the heterochromatin marker H3K9Me was highly expressed it did not differentiate nevi from these metastatic melanomas (Figure 2, Table 1). It has been shown that expression of H3K9Me is significantly increased in human tumors (head and neck squamous carcinomas and lung and colon cancers) compared with normal tissue (Di Micco *et al.*, 2011), and we confirmed that only a small percentage of individual normal melanocytes stained positive for this marker. Nuclear size enlargement, an established marker of *in vitro* senescence, was not observed in cultured melanocytes expressing $B\text{-}RAF^{V600E}$ compared with control and wild-type B-RAF-transduced cells (Figure 4a), and there was no evidence that nevus cell nuclei were enlarged compared with normal epidermal melanocytes or metastatic melanoma cells (Figure 4b).

Senescence can also be initiated by p53 in response to DNA damage checkpoints triggered by activated oncogenes (Bartkova *et al.*, 2006; Di Micco *et al.*, 2006). In human melanocytes, however, p53 does not appear to be a critical effector of oncogene-induced senescence. In particular, p53

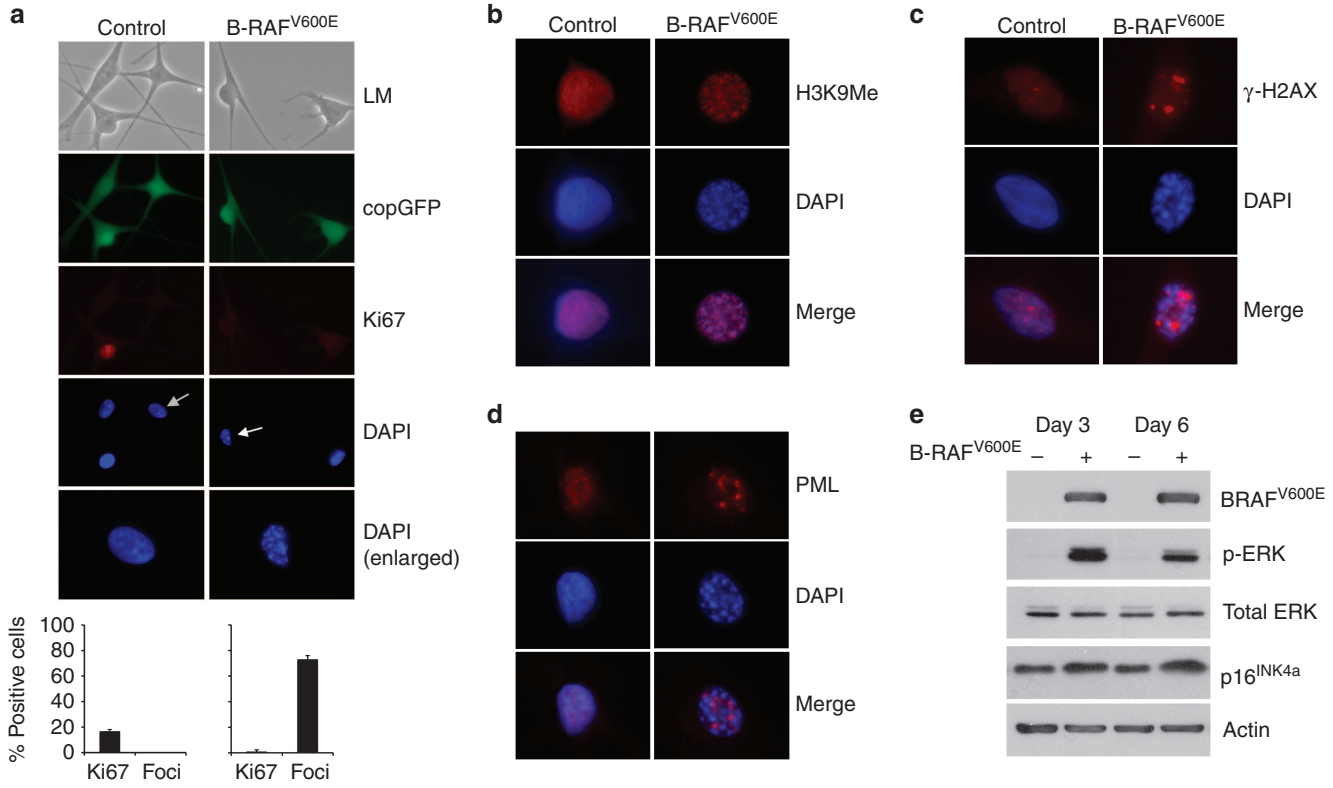

Figure 1. Senescence program induced by oncogenic B-RAF^{V600E} in cultured human melanocytes. (**a**) Human melanocytes were infected with lentiviruses carrying B-RAF^{V600E} or control vector. The efficiency of transduction was controlled with the co-expression of copGFP and was consistently above 90%. Cell proliferation (Ki67) and chromatin condensation (4′-6-diamidino-2-phenylindole (DAPI)) were analyzed and quantified 5 days after infection of melanocytes. Cells enlarged to show DAPI-stained chromatin foci are indicated with arrows. LM, light microscopy. (**b**–**d**) Representative examples of chromatin condensation (DAPI) in human epidermal melanocytes expressing B-RAF^{V600E} (day 5) and staining for (**b**) H3K9Me, (**c**) γ-H2AX, and (**d**) PML. (**e**) Expression of the indicated proteins was determined by western blot analysis after infection of melanocytes with lentiviruses expressing copGFP (−) or B-RAF^{V600E} (+). Transduced B-RAF^{V600E} (MYC tagged) was detected using the MYC antibody.

was not induced by B-RAF in cultured melanocytes (data not shown; Scurr *et al.*, 2010; Zhuang *et al.*, 2008), and oncogene-induced senescence is effectively initiated and maintained in human melanocytes lacking p53 expression (Denoyelle *et al.*, 2006; Zhuang *et al.*, 2008; Haferkamp *et al.*, 2009b). Accordingly, accumulation of p53 was not associated with arrested nevi or skin melanocytes, and showed higher expression in our panel of metastatic melanomas (Figure 2, Table 1). The lack of p53 induction in human nevi has been reported previously (Cristofolini *et al.*, 1993; Michaloglou *et al.*, 2005), and is intriguing considering that p53 prevents the conversion of benign nevi to melanomas in transgenic animals expressing oncogenic RAS or B-RAF. Indeed, the loss of p53 eliminated nevus formation and initiated melanoma formation *de novo* in these animal models (Patton *et al.*, 2005; Ferguson *et al.*, 2010; Terzian *et al.*, 2010).

Finally, we analyzed SA-β-gal activity, which reflects lysosomal expansion rather than senescence (Kurz *et al.*, 2000), in a small set of frozen human nevi and metastatic melanomas (Supplementary Table S3 online). As expected, most nevi (5/7) accumulated detectable levels of this enzyme, but so did a subset of metastatic melanomas (3/7) stained in parallel (Figure 5, Supplementary Table S3 online). We found only two previous reports showing positive SA-β-gal staining in human nevi. In both reports, enzyme activity was detected in congenital nevi but not in normal skin, and melanomas were not included in the analyses (Michaloglou *et al.*, 2005; Gray-Schopfer *et al.*, 2006). We found only one study looking at SA-β-gal activity in human malignant and non-neoplastic lesions, and in this report enzyme activity was limited to regions of prostate hyperplasia and was not detected in prostate cancer (Chen *et al.*, 2005). The value of SA-β-gal as an *in vivo* marker of senescence requires further investigation; our data indicate that it does not consistently discriminate between arrested benign nevi and proliferating melanoma, and its specificity as a marker of aging tissue remains controversial (Dimri *et al.*, 1995; Severino *et al.*, 2000). *In vitro*, the expression of this enzyme correlates strongly with the senescence state, although it can also be induced by stresses such as serum withdrawal and prolonged cell culture (Severino *et al.*, 2000).

It is clear from our data that it is not easy to recognize senescent cells *in vivo*, and this reflects the nature and specificity of the predictive markers currently available. Our findings indicate, however, that none of the commonly

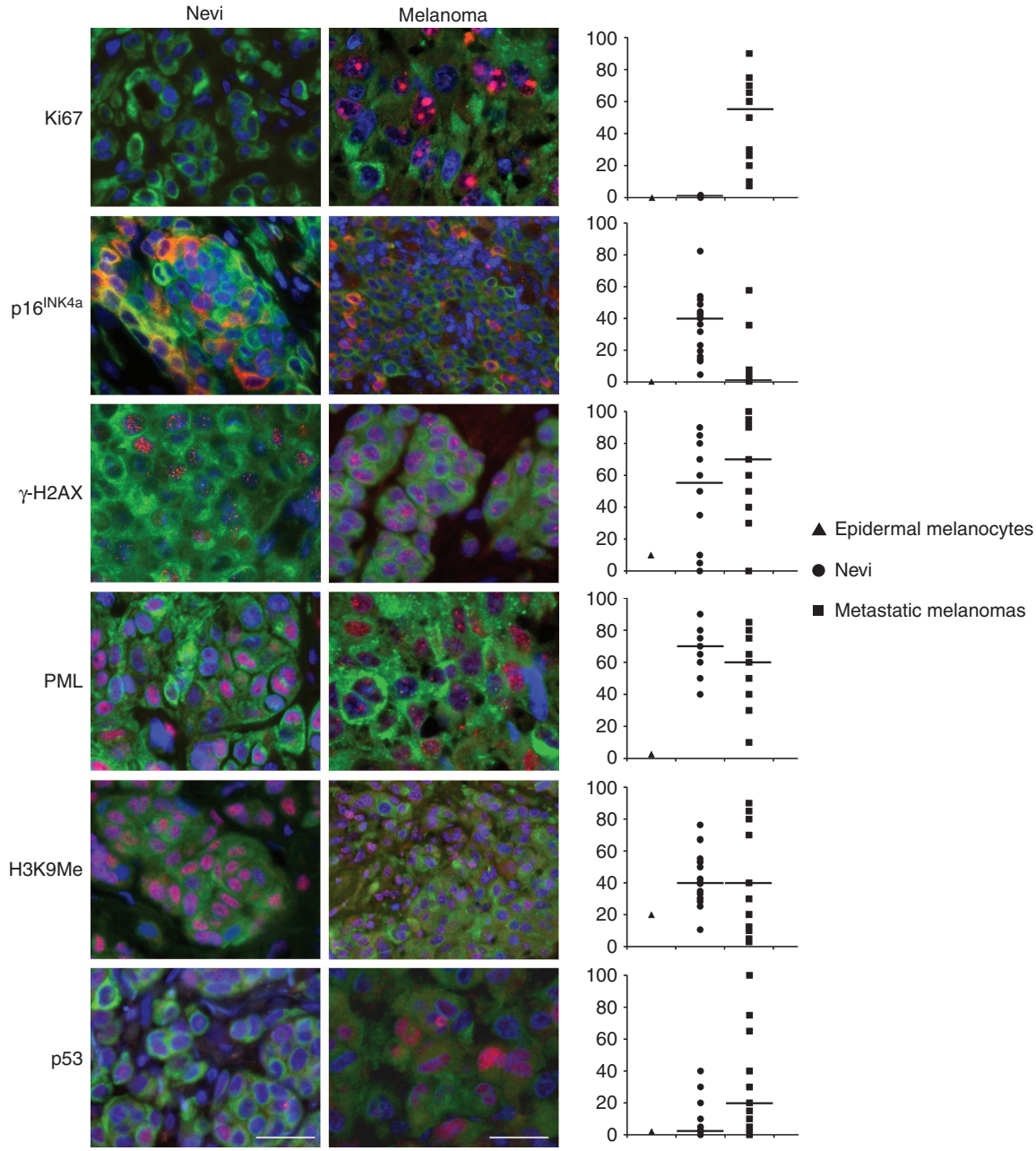

Figure 2. Expression of senescence markers in human nevi and melanoma. (Left) Paraffin-embedded sections of human nevi and metastatic melanomas were subjected to dual-fluorescence immunohistochemistry with the indicated antibodies. The pan-melanoma cocktail (antibodies against HMB45, MART1, and tyrosinase (green)) was used to identify melanocytes. Representative examples of nevi and metastatic melanomas demonstrating Ki67, $p16^{INK4a}$, γ-H2AX, PML, H3K9Me, and p53 staining are shown (red). Nuclei were stained with 4′-6-diamidino-2-phenylindole (blue). Bar = 20 μm. (Right) Scatter plot analysis showing the percentage of cells scoring positive for each marker in nevi, metastatic melanoma tissue, and in individual melanocytes scattered along the epidermal–dermal junction in adjacent normal skin. The horizontal bar indicates the median expression values in nevi and melanoma samples.

accepted senescence-associated markers differentiate benign nevus cells from melanoma cells or skin melanocytes. Moreover, the critical markers used to define nevi as senescent tumors in previous studies, namely Ki67 negativity, and $p16^{INK4a}$ and SA-β-gal positivity, did not distinguish nevi from their precursor and transformed melanocyte counterparts. For instance, both nevi and individual skin melanocytes were Ki67 negative, $p16^{INK4a}$ displayed a mosaic pattern of expression in nevi but also in primary melanomas, and SA-β-gal was expressed in a subset of nevi and melanoma metastases. These data were not age dependent, as there was no significant difference in the expression of any of the eight senescence markers in nevi excised from younger patients (<40; $n=10$) versus older patients (>40; $n=9$; data not shown). There is no doubt that benign nevi are usually growth arrested, but considering that the majority of individual skin

Table 1. Median and interquartile ranges of senescence markers by melanocytic tumor type together with associated *P*-value (Mann–Whitney test)

	Nevus		Metastatic melanoma		
Marker	Median	(LQ, UQ)	Median	(LQ, UQ)	*P*-value
H3K9Me	40	(30, 53)	40	(13, 80)	0.945
Ki67	0	(0, 0)	55	(15, 68)	<0.001
$p16^{INK4a}$	40	(16, 44)	0	(0, 2)	<0.001
p53	4	(0, 15)	20	(5, 65)	0.028
PML	70	(65, 80)	60	(60, 80)	0.165
γ-H2AX	55	(10, 80)	70	(50, 92)	0.570

Abbreviations: LQ, lower quartile; UQ, upper quartile.

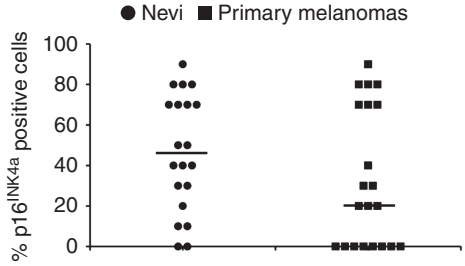

Figure 3. $p16^{INK4a}$ expression in melanocytic tumors. Tissue microarray results of $p16^{INK4a}$ staining in 20 nevi and 21 primary melanomas. Scatter plot analysis showing the percentage of cells positive for $p16^{INK4a}$. The horizontal bar indicates the median expression values.

Table 2. Median and interquartile ranges of $p16^{INK4a}$ protein expression by melanocytic tumor type together with associated *P*-value (Mann–Whitney test)

	Nevus		Primary melanoma		
Marker	Median	(LQ, UQ)	Median	(LQ, UQ)	*P*-value
$p16^{INK4a}$	45	(25, 70)	20	(0, 70)	0.159

Abbreviations: LQ, lower quartile; UQ, upper quartile.

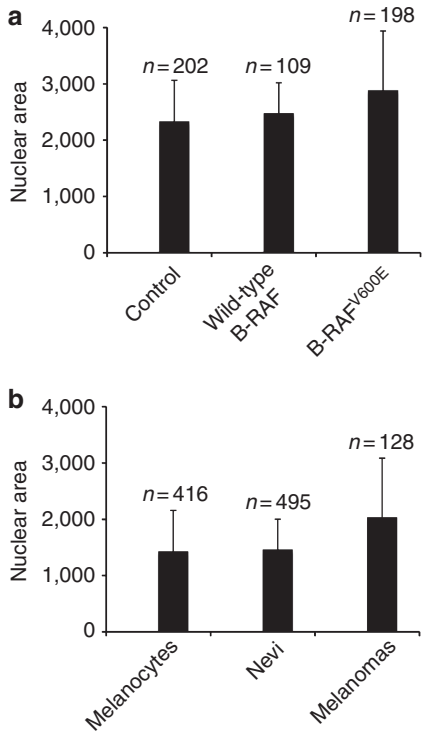

Figure 4. Increased nuclear size is not apparent in senescent cultured melanocytes or nevi. (**a**) Human melanocytes transduced with control vector, wild-type B-RAF, or $B\text{-}RAF^{V600E}$ were stained with 4′-6-diamidino-2-phenylindole (DAPI) 5 days post transduction. Average nuclear area was determined using the Image J software and is shown as histograms, which correspond to the mean and standard deviation of at least two independent transduction experiments. The number of cells analyzed (*n*) are also indicated. (**b**) Nuclear area of DAPI-stained epidermal melanocytes, nevus cells, and metastatic melanoma cells, from paraffin-embedded sections, was determined using the Image J software. The average nuclear area is shown as histograms, which correspond to the mean ± SD derived from 13, 17, and 5 independent sections, respectively. Total number of cells analyzed (*n*) is indicated.

melanocytes are also arrested, that nevi show no distinguishing senescence markers, and that proliferation of nevus cells can occur *in vitro* and *in vivo* (Soyer *et al.*, 1989; Moretti *et al.*, 1990; Rudolph *et al.*, 1997, 1998; Tronnier *et al.*, 1997; Florell *et al.*, 2002, 2005; Lebe *et al.*, 2007; Chan *et al.*, 2010), it is premature to define nevi as senescent lesions. In addition, the mix of B-RAF wild-type and B-RAF mutant cells within clonal nevi (Ichii-Nakato *et al.*, 2006; Lin *et al.*, 2009) suggests that these tumors may not be permanently restricted from transformation via oncogene-driven arrest.

There is also substantial clinical evidence that nevus cells are capable of proliferation. For instance, low-level mitotic activity has been reported in nevi (Soyer *et al.*, 1989; Moretti *et al.*, 1990; Rudolph *et al.*, 1997; Florell *et al.*, 2002, 2005; Jensen *et al.*, 2007; Lebe *et al.*, 2007; Nasr and El-Zammar, 2008; Glatz *et al.*, 2010; McCarthy and Scolyer, 2010; Ruhoy *et al.*, 2011), and nevus cell proliferation can be induced during pregnancy (Gerami *et al.*, 2009; Chan *et al.*, 2010) by UV irradiation (Tronnier *et al.*, 1997; Rudolph *et al.*, 1998) and upon incomplete surgical removal of a benign nevus (Scolyer *et al.*, 2004, 2006; Sommer *et al.*, 2011). Spitz nevi are considerably more mitotically active than other melanocytic nevi (Hofmann-Wellenhof *et al.*, 1993; Crotty *et al.*, 2002; Dahlstrom *et al.*, 2004; Florell *et al.*, 2005), and over 30% of choroidal nevi showed slow growth over several years without evidence of transformation (Mashayekhi *et al.*, 2010). In addition, growing melanocytic nevi are more likely to carry the $B\text{-}RAF^{V600E}$ mutation compared with lesions showing no changes in structure or growth (Loewe *et al.*, 2004; Zalaudek *et al.*, 2011). Further, the histological recognition of nevi in contiguity with 20–40% of melanomas indicates that a substantial proportion of melanomas may have arisen from the proliferation of benign or dysplastic nevus cells (Rhodes *et al.*, 1983; Duray and Ernstoff, 1987;

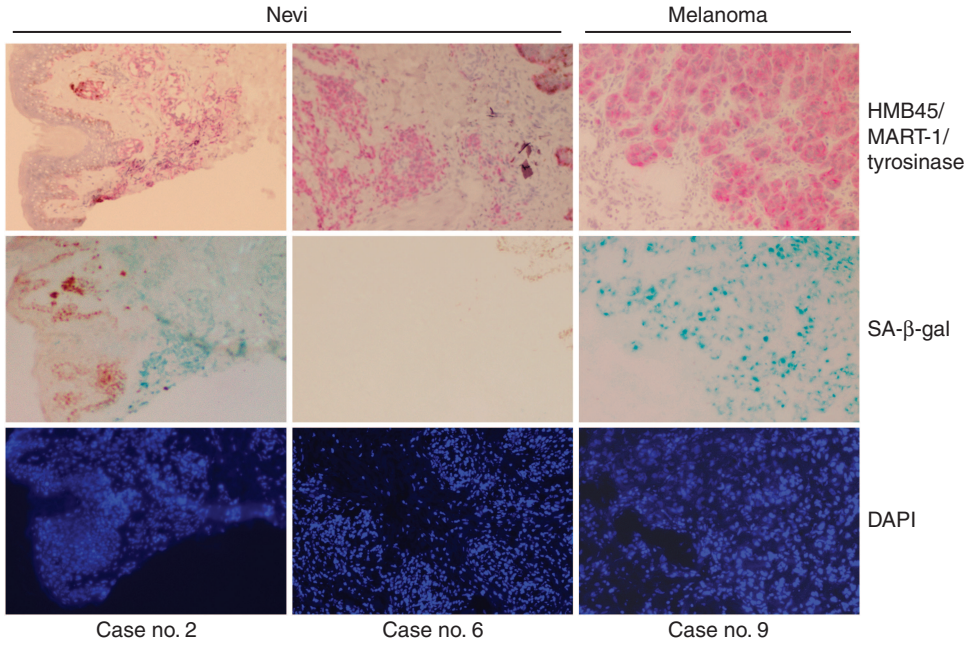

Figure 5. Human nevi and melanomas display increased senescence-associated β-galactosidase (SA-β-gal) activity. Sequential frozen sections of human nevi and metastatic melanomas were subjected to immunohistochemistry with antibodies against HMB45/MART1/tyrosinase (red) to identify melanocytes and SA-β-gal staining. 4′-6-Diamidino-2-phenylindole (DAPI) co-staining was used to identify nuclei. Cases 2 and 9 stained positive for SA-β-gal expression.

Bevona *et al.*, 2003). Finally, whereas melanocytes from adult skin proliferate only for 4 weeks *in vitro*, melanocytes from dysplastic and congenital nevi (which express SA-β-gal; Michaloglou *et al.*, 2005) proliferate in the presence of mitogens for 4–6 weeks in culture before becoming quiescent (Halaban *et al.*, 1986). Importantly, only a small fraction of explanted nevus cells grow *in vitro*, highlighting the heterogeneity in the proliferative potential of these precursor lesions (Soo *et al.*, 2011).

Taken together, these data challenge the prevailing view that benign nevus cells have undergone permanent proliferative arrest via oncogene-induced senescence.

MATERIALS AND METHODS

Specimen collection

The formalin-fixed, paraffin-embedded, and fresh-frozen human nevi, as well as primary and metastatic melanomas (Supplementary Tables S1–3 online), were surgically excised between 1993 and 2010. The use of these specimens for this study was approved by the Sydney South West Area Health Service Institutional Ethics Review Committee (RPAH Zone) under Protocol No. X08-0155/HREC 08/RPAH/262 and Protocol No. X11-0023/HREC 11/RPAH/32. The fresh-frozen nevi and melanomas were obtained under sample procurement Protocol X07-0202/HREC/07/RPAH/30. The histopathologic features of each sample were reviewed by a pathologist (RAS) to confirm diagnosis and tumor content.

DNA extraction and genotyping

DNA of nevi from three consecutive 4-μm sections from the paraffin blocks was isolated using the QIAamp DNA FFPE Tissue kit (Qiagen, Venlo, Netherlands) according to the manufacturer's recommendations. Subsequently, *B-RAF* exon 15 (encompasses Val-600) was PCR amplified and sequenced in both directions using the following primers: B-RAF_exon15_Fwd 5′-TCATAATGCTTGCTCTGATAGGA-3′ and B-RAF_exon15_Rev 5′-GGCCAAAAATTTAATCAGTGGA-3′. B-RAF testing of the archival paraffin-embedded melanoma samples was performed at the Peter MacCallum Cancer Centre, Department of Diagnostic Molecular Pathology (Melbourne, Australia). Samples were microdissected and subjected to high-resolution melting analysis using primers flanking codon 600 in the *B-RAF* gene. These primers identify variations in exon 15 of the *B-RAF* gene between nucleotides c.1788 and c.1823 in reference sequence NM_004333.4, corresponding to codons 597–607. All abnormal high-resolution melting traces were subjected to bidirectional DNA sequencing using the primers described above.

Cell culture

Human neonatal epidermal melanocytes (HEM1455) were obtained from Cell Applications (San Diego, CA). Melanocytes were grown in HAM's F10 media supplemented with ITS premix (Becton Dickinson, Franklin Lakes, NJ), 12-O-tetradecanoylphorbol-13-acetate (Sigma-Aldrich, St Louis, MO), 3-isobutyl-1-methylxanthine (Sigma-Aldrich), cholera toxin (List Biological Laboratories, Campbell, CA), 20% fetal bovine serum, and glutamine (Gibco BRL, Carlsbad, CA; modified from Halaban *et al.*, 1986). Cells were cultured in a 37 °C incubator with 5% CO_2.

Lentiviral transductions

Lentiviruses were produced in HEK293T cells as described previously (Haferkamp *et al.*, 2009a). Cells were infected using a multiplicity of infection between 5 and 10 to provide an efficiency of infection above 90%.

Constructs

The wild-type and mutant *B-RAF* complementary DNAs were kindly provided by Professor R Marais (London, United Kingdom). Cloning of MYC-tagged wild-type and mutant *B-RAF* complementary DNAs into the *pCDH-CMV-MCS-EF1-copGFP* lentiviral vector, which co-expresses copGFP (System Biosciences, Mountain View, CA), has been described previously (Scurr *et al.*, 2010).

Western blotting

Total cellular proteins were extracted at 4 °C using RIPA lysis buffer containing protease inhibitors (Roche, Basel, Switzerland). Proteins (30–50 μg) were resolved on 12% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Western blots were probed with antibodies against $p16^{INK4a}$ (N20; Santa Cruz, Santa Cruz, CA), c-MYC (A14; Santa Cruz), phosphorylated ERK (E4; Santa Cruz), total ERK (137F5; Cell Signaling, Danvers, MA), and β-actin (AC-74; Sigma-Aldrich).

Indirect immunofluorescence

Cells were seeded on coverslips in 12-well plates at 3×10^4 cells per well at each time point and incubated overnight. Cells were washed in phosphate-buffered saline (PBS) and fixed with 4% formaldehyde/PBS for 15 minutes at room temperature. Cells were rinsed three times with PBS, permeabilized with 0.2% Triton-X100/PBS for 10 minutes, and then rinsed and blocked in 10% fetal calf serum/PBS for 1 hour. Cells were incubated with primary antibodies for 50 minutes, and then washed and incubated with Alexa Fluor-594 and -488 secondary antibodies (Invitrogen, Carlsbad, CA) and 1 $\mu g\,ml^{-1}$ of the nuclear DNA stain 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 50 minutes. The following primary antibodies were used: Ki67 (MIB-1; Dako, Glostrup, Denmark), trimethyl-histone H3 (Lys 9) (H3K9Me; 07-442; Millipore), γ-H2AX (20E3; Cell Signaling, Boston, MA), p53 (FL-393; Santa Cruz), and $p16^{INK4a}$ (N20; Santa Cruz) and PML (H-238; Santa Cruz). SA-β-Gal activity was detected as described previously (Dimri *et al.*, 1995). Sections were mounted using ProLong Gold antifade with DAPI (Invitrogen).

Immunohistochemistry

Tissue arrays and paraffin-embedded sections were dewaxed before they were rehydrated by washing twice with absolute ethanol, twice with 70% ethanol, and finally once with bi-distilled water. Samples were antigen retrieved by heating at 90 °C for 20 minutes in antigen-retrieval solution (pH 6; S1699; Dako), cooled for 20 minutes, and then blocked in 50% fetal calf serum/1% BSA/1% Tween 20 in TBS (0.9% NaCl, 20 mM Tris-Cl, pH 7.4) for 1 hour. Tissue arrays were incubated with biotinylated anti-goat antibody (BA-5,000; Vector Laboratories, Burlingame, CA or P0449; Dako) for 25 minutes; slides were washed with PBS, incubated in streptavidin–HRP (K0690; Dako) or streptavidin/biotin-HRP (Invitrogen) for 25 minutes, and placed in Vector NovaRed or 3,30-diaminobenzidine substrate for 10–15 minutes (Vector Laboratories). Sections were counterstained with hematoxylin and mounted using Faramount (Dako). A pathologist performed the evaluation of the staining in a blinded manner.

For dual immunofluorescence staining of paraffin tissue, sections were dewaxed as detailed above and then incubated with primary antibodies for 2 hours, followed by washing three times in TBS containing 0.05% Tween 20. Subsequently, the slides were incubated with Alexa Fluor-594 and -488 secondary antibodies (Invitrogen) for 1 hour, washed with TBS/0.05% Tween 20, and mounted using Prolong antifade with DAPI (Invitrogen). Staining of pan-melanoma-positive cells was evaluated by at least two investigators in a blinded manner. The following primary antibodies were used: pan-melanoma cocktail (HMB45 + M2-7C10 + M2-9E3 + T311; BIOCARE Medical, Concord, CA), Ki67 (MIB-1; Dako), trimethyl-histone H3 (Lys 9) (07-442; Millipore), γ-H2AX (20E3; Cell Signaling), p53 (FL-393; Santa Cruz), $p16^{INK4a}$ (N20 and C20; Santa Cruz), and PML (H-238; Santa Cruz). SA-β-Gal activity was detected as described previously (Dimri *et al.*, 1995). The specificity of all antibodies was validated using paraffin-embedded control samples (Supplementary Figure S1 online).

Nuclear area was calculated using Image J v10.2 (NIH Image, Bethesda, MA) on DAPI-stained images. Outlined nuclei were manually selected to include only complete, well-separated nuclei (DeCoster, 2007).

Statistical analysis

Scatter plots were used to illustrate the distribution of gene expression. Medians and interquartile ranges were applied to summarize the distributions, and the Mann–Whitney test was used to determine the differences between the B-RAF wild-type and B-RAF mutant populations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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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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