Constitutive Rac Activation Is Not Sufficient to Initiate Melanocyte Neoplasia but Accelerates Malignant Progression

Lucy E. Dalton[1,4,5], Jivko Kamarashev[2,5], Irene Barinaga-Rementeria Ramirez[3], Gavin White[1], Angeliki Malliri[1,6] and Adam Hurlstone[3,6]

Deregulated Ras signaling initiates and maintains melanocyte neoplasia. The Rho-like GTPase Rac has been implicated in Ras-induced neoplastic transformation. Moreover, a recurrent UV-induced mutation activating RAC1 has recently been detected in human melanoma. Here, a role for Rac in melanoma initiation and progression was investigated in human melanomas and zebrafish models of melanocyte neoplasia. Immunohistochemical analysis revealed RAC expression and activity restricted to melanocytes at the junction of the epidermis and dermis in benign neoplasms. Malignant melanocytes displayed elevated RAC activity that extended into the suprabasal epidermis, deeper into the dermis, and was maintained in metastases. Previously, we have used zebrafish transgenic models to demonstrate that deregulated Ras/Raf/mitogen-activated protein kinase signaling can initiate melanocyte neoplasia. Expression of a constitutively active RAC1 mutant (V12RAC1) was not sufficient to initiate melanocyte neoplasia in this organism. Furthermore, we did not detect an additive effect when combined with V600EBRAF, nor could V12RAC1 substitute for suppressed Pi3k signaling to restore melanoma progression. However, coexpression of V12RAC1 and oncogenic RAS accelerated tumor nodule formation. Immunohistochemical analysis revealed that the Rac activator Tiam1 (T-cell lymphoma invasion and metastasis 1) is overexpressed in melanoma tumor nodules in both zebrafish and humans. Thus, our data suggest that Rac contributes to the progression of melanoma and that Tiam1 may activate Rac in nodular presentations.

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INTRODUCTION

The Rho-family small guanine nucleotide triphosphate hydrolase (GTPase) Rac cycles between an active GTP-bound form and an inactive guanosine diphosphate–bound form. Guanine nucleotide exchange factors (GEFs) activate GTPases by catalyzing the replacement of guanosine diphosphate with GTP. The Dbl-family Rho GEF, Tiam1, selectively activates Rac (Michiels *et al.*, 1995). Rac stimulates cellular processes deregulated in neoplasia such as proliferation, survival, motility, and invasiveness (Mack *et al.*, 2010), thereby functioning as an oncogene in cellular transformation assays in cooperation with the Ras effector c-Raf and being required for Ras-induced transformation (Khosravi-Far *et al.*, 1995; Qiu *et al.*, 1995). RAC1 expression and activity is increased in human malignancies (Jordan *et al.*, 1999; Schnelzer *et al.*, 2000; Kamai *et al.*, 2004; Pan *et al.*, 2004). Furthermore, *rac1* conditional knockout mice are resistant to the formation of lung adenocarcinoma driven by oncogenic Kras (Kissil *et al.*, 2007) and the formation of skin tumors driven by oncogenic Hras (Wang *et al.*, 2010), similar to Tiam1 (T-cell lymphoma invasion and metastasis 1)-deficient mice (Malliri *et al.*, 2002b). More recently, selective deletion of the *rac1* gene was shown to reduce the anchorage-independent growth, proliferation, migration, invasion, and ability to form metastatic tumor xenografts of immortalized mouse melanocytes expressing oncogenic Nras (Li *et al.*, 2012). Significantly, a recurrent, presumably UVB-induced, C>T transition at coding nucleotide 85 of the human *RAC1* gene has been identified in ~4% of all melanomas (Hodis *et al.*, 2012), and 9% of sun-exposed melanomas specifically, with the mutation not being detected in melanomas from sites shielded from the sun (Krauthammer *et al.*, 2012). This

[1]*Cancer Research UK Paterson Institute for Cancer Research, University of Manchester, Manchester, UK;* [2]*Department of Dermatology, University Hospital Zürich, Zürich, Switzerland and* [3]*Faculty of Life Sciences, University of Manchester, Manchester, UK*

[4]*Current address: Cambridge Institute for Medical Research, Wellcome Trust/MRC Building, Addenbrooke's Hospital, Cambridge, UK*

[5]*The first two authors contributed equally to this work.*

[6]*The last two authors contributed equally to this work.*

Correspondence: Adam Hurlstone, Faculty of Life Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, UK. E-mail: adam.hurlstone@manchester.ac.uk; Angeliki Malliri, Cancer Research UK Paterson Institute for Cancer Research, University of Manchester, Wilmslow Road, Manchester M20 4BX, UK. E-mail: amalliri@picr.man.ac.uk

Abbreviations: cDNA, complementary DNA; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; GTPase, guanine nucleotide triphosphate hydrolase; PI3K, phosphoinositide 3-kinase; RGP, radial growth phase; Tiam1, T-cell lymphoma invasion and metastasis 1; VGP, vertical growth phase

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mutation results in the substitution of proline at position 29 for serine, conferring a conformational change on the switch I loop, resulting in a more avid binding to effectors such as p21-activated kinase 1 (PAK1; Krauthammer *et al.*, 2012). After valine at position 600 of BRAF and glutamine at position 61 of NRAS, proline 29 of RAC1 is the most frequently mutated amino acid in melanoma. The frequency of P29S mutation was similar in primary and metastatic tumors, suggesting that the mutation arises early in the formation of melanoma (Krauthammer *et al.*, 2012). The mutation occurred in conjunction with NRAS and BRAF mutations, as well as independently of these mutations (Hodis *et al.*, 2012; Krauthammer *et al.*, 2012), suggesting that it confers growth advantages distinct from activation of the RAS/RAF/mitogen-activated protein kinase signaling pathway. In this study, we address how widespread are changes in RAC activity during neoplastic transformation of human melanocytes and, using transgenic zebrafish models, whether constitutive RAC activation can initiate neoplasia in melanocytes or contribute to melanoma progression.

RESULTS AND DISCUSSION

Rac activity increases with melanoma progression *in vivo*

RAC expression and activity were analyzed in human melanocytic neoplasms (29 benign and 31 malignant lesions) by performing immunohistochemical analysis with two independent antibodies: one recognizing total RAC (Woodcock *et al.*, 2010) and a conformational antibody recognizing specifically GTP-bound RAC (that is, active RAC). We confirmed the selectivity of the RAC–GTP antibody for active RAC (Supplementary Figure S1a and b online; see also (Samuel *et al.*, 2011)). In all cases, staining for total RAC and RAC–GTP was analogous, although staining with the RAC–GTP antibody was consistently more intense (for a representative example see Supplementary Figure S1c online). Thus, in tissues, RAC activity correlated with RAC expression level. Furthermore, RAC was overexpressed and hyperstimulated in melanoma relative to benign lesions (Figure 1a). Overexpression was observed even in superficial spreading melanoma with Breslow depth of <0.75 mm that could be considered radial growth phase (RGP) melanoma (as evident for the example in Supplementary Figure S1c online). Moreover, as summarized in Supplementary Table S1 online, distinct patterns of staining in nevi and in malignant melanoma were observed (depicted only for RAC–GTP in representative images in Figure 1c–g). In normal skin, only the basal cell layer of the epidermis stained strongly for total RAC and RAC–GTP (Figure 1b; see also (Benitah *et al.*, 2005)). In nevi, additional staining was observed in nests of nevocytes but only at the junction between the epidermis and dermis (Figure 1c, arrowheads). Dysplastic nevi showed a somewhat stronger staining than in nevi without atypia, but the pattern was otherwise similarly restricted to the junctional zone (Figure 1d). Furthermore, neither total RAC nor RAC–GTP expression was detected in nevocytes of purely dermal nevi (not shown). The junctional zone between the epidermis and dermis is considered a region of active proliferation in nevi, and early melanoma always develops at this junctional zone. In melanomas, total RAC and RAC–GTP expression is observed both suprabasally in the epidermis (where malignant melanocytes are also typically found, so-called Pagetoid spread; arrows in Figure 1e and f) and in the dermal invasive component, although heterogeneously (arrowheads in Figure 1e and f). Metastases also frequently displayed heterogeneous staining (an example is shown in Figure 1g) but were occasionally completely negative. Rac1 is the major ubiquitous isoform of Rac expressed in mammalian tissues and is the only Rac isoform expressed in mouse melanocytes (Li *et al.*, 2011). Rac2 is considered to be specifically expressed in the blood and Rac3 largely in the nervous system (Corbetta *et al.*, 2005). We assume therefore that human melanocytes and, by extension, melanoma cells express predominantly RAC1.

RAC activity levels were next measured by pull down of the GTP-bound form of RAC (see Materials and Methods) in a panel of human cell lines comprising primary human neonatal melanocytes moderately pigmented (HEMn-MP), immortalized but nontumorigenic melanocytes (Hermes 2B and Hermes 4A), a cell line derived from RGP melanoma (WM35), cell lines derived from vertical growth phase (VGP) melanoma (WM98-1, WM1158, and Mel Juso), and from melanoma metastases (WM266-4, SKMEL28, A375P, MM485, and WM1361). This analysis revealed elevated RAC activity in all melanoma cell lines relative to primary melanocytes and immortalized melanocytes (Figure 2a), again consistent with increased RAC activity being implicated in malignant progression. As the melanoma cell line panel comprises both NRAS (Mel Juso, MM485, and WM1361) and BRAF mutant lines (the rest), the elevated RAC activity correlated with malignancy and not mutation status. Similarly, the tumors stained above, which were selected at random, will comprise examples of both oncogenic BRAF- and NRAS-transformed cells, implying that RAC overexpression and hyperstimulation in melanoma reflect malignant status and not simply activation by oncogenic NRAS.

Melanocyte neoplasia can be induced in zebrafish. Zebrafish expressing oncogenic V600EBRAF in their melanocytes display hyperplasia only; progression to melanoma can be elicited through inactivation of the tumor-suppressor p53 (Patton *et al.*, 2005). We have demonstrated that animals expressing G12VHRAS develop melanocyte hyperplasia accompanied by an increased progenitor compartment, as well as infrequent invasion of the epidermis and loose connective tissue of the dermis and subcutaneous tissues by melanocytes. We classified this presentation as RGP melanoma in zebrafish. Furthermore, this condition can spontaneously progress to VGP melanoma (Michailidou *et al.*, 2009). In both the V600EBRAF;$p53^{-/-}$ and G12VHRAS models, the VGP manifests largely as malignant tumor nodules (Patton *et al.*, 2005; Michailidou *et al.*, 2009), whereas in humans VGP arises largely in the context of superficial spreading melanoma but also manifests as malignant tumor nodules (Warycha *et al.*, 2008). Protein was extracted from the skins of wild-type, V600EBRAF;$p53^{-/-}$, and V12HRAS animals and tumor nodules from V600EBRAF;$p53^{-/-}$ and V12HRAS animals and subjected to a G-LISA assay for measurement of active Rac (see Materials and Methods). Rac activity levels were increased by 20% in the