Metastasizing Melanoma Formation Caused by Expression of Activated N-Ras<sup>Q61K</sup> on an INK4a-Deficient Background

Julien Ackermann, Manon Frutschi, Kostaš Kaloulis, Thomas McKee, Andreas Trumpf, and Friedrich Beermann

1ISREC, Swiss Institute for Experimental Cancer Research, National Center of Competence in Research Molecular Oncology, Epalinges, Switzerland and 2Institute of Pathology, University of Lausanne, Lausanne, Switzerland

Abstract

In human cutaneous malignant melanoma, a predominance of activated mutations in the N-ras gene has been documented. To obtain a mouse model most closely mimicking the human disease, a transgenic mouse line was generated by targeting expression of dominant-active human N-ras (N-Ras<sup>Q61K</sup>) to the melanocyte lineage by tyrosinase regulatory sequences (Tyr::N-Ras<sup>Q61K</sup>). Transgenic mice show hyperpigmented skin and develop cutaneous metastasizing melanoma. Consistent with the tumor suppressor function of the INK4a locus that encodes p16<sup>INK4a</sup> and p19<sup>ARF</sup>, >90% of Tyr::N-Ras<sup>Q61K</sup> INK4a<sup>-/-</sup> transgenic mice develop melanoma at 6 months. Primary melanoma tumors are melanotic, multifocal, microinvasive the epidermis or epithelium of hair follicles, and disseminate as metastases to lymph nodes, lung, and liver. Primary melanoma can be transplanted s.c. in nude mice, and if injected i.v. into NOD/SCID mice colonize the lung. In addition, primary melanomas and metastases contain cells expressing the stem cell marker nestin suggesting a hierarchical structure of the tumors comprised of primitive nestin-expressing precursors and differentiated cells. In conclusion, a novel mouse model with melanotic and metastasizing melanoma was obtained by recapitulating genetic lesions frequently found in human melanoma. (Cancer Res 2005; 65(10): 4005-11)

Introduction

In the past decades, cutaneous melanoma incidence and mortality rates have been steadily increasing. The availability of mouse models retracing the malignant nature of melanoma has helped to unveil the biology of melanoma and might contribute to ameliorate future diagnosis and therapy in the clinic (1). However, the utility of mouse melanoma models is hampered by the fact that they only rarely closely mimic the human disease and often show quite different pathology. Most melanoma models described to date are of dermal origin but lack the usual epidermal or junctional activity that characterizes the radial growth phase of human melanoma. An exception is the recently described HGF/SF transgenic mouse model for which intraepidermal lesions resembling the pagetoid spread of human melanoma was shown (2, 3).

To obtain a mouse melanoma model closely resembling the human disease, efforts are being made to introduce the same genetic lesions found in humans into the mouse genome. One of the most common factors predisposing to melanoma formation in humans is the INK4a/ARF locus (4), which is inactivated with high frequency in human melanoma. This locus encodes two distinct proteins by alternative exon usage, p16<sup>INK4a</sup> and p19<sup>ARF</sup> (p19<sup>ARF</sup> in mice), which function as tumor suppressor genes in the pRB and p53 pathways, respectively (4, 5). Gene-targeted mice, where p16<sup>INK4a</sup> and p19<sup>ARF</sup> are deleted, develop a large variety of tumors but fail to develop melanoma (6). Mice deficient in p16<sup>INK4a</sup> but which retain one copy of p19<sup>ARF</sup> show carcinogen-induced susceptibility to metastatic melanoma (7, 8). Transgenic mice that express a mutant form of H-ras specifically in melanocytes showed melanocytic hyperplasia with intense skin pigmentation (9), which after treatment with carcinogens progressed into skin melanoma with metastasis formation in lymph nodes and lung (10). Breeding of Tyr::H-Ras<sup>V12G</sup> transgenic mice on an INK4a/ARF- or p53-deficient background resulted in the development of highly vascularized but amelanotic melanomas resembling nodular melanoma (11, 12). Unlike human nodular melanoma, no metastasis was observed in these mice suggesting lack of additional genetic stimuli. Melanoma tumors regressed when H-Ras<sup>V12G</sup> expression was removed in an inducible melanoma model (13). This suggests that ras signaling is essential for initiation and maintenance of melanoma (13).

Activating mutations affecting N-ras are frequent events in nevi and early-stage melanomas. For example, 56% of congenital nevi harbored activating point mutations in codons 12 or 61 of the N-ras gene (14), and 33% of primary and 26% of metastatic clinical melanoma samples carried mutations in codons 12, 18, or 61 of N-ras (15). Moreover, in hereditary melanoma with germ line p16<sup>INK4a</sup> mutations, 95% of patients have N-ras mutated at codon 61 (16). Mutations affecting the serine/threonine kinase B-Raf are equally often found in melanoma, melanocytic nevi, and metastases (17). Importantly, B-Raf and N-ras mutations were mutually exclusive, strongly suggesting that both oncogenic activities are in the same linear pathway presumably deregulating the mitogen-activated protein kinase (MAPK) pathway. Taken together, these data indicate that activation of N-ras or B-Raf in combination with inactivation of the INK4a locus are key components in initiating and maintaining melanoma formation in human.

Here, we report the generation of a transgenic mouse line which expresses the oncogenic form of human N-ras (N-Ras<sup>Q61K</sup>) in melanocytes on an INK4a-deficient background. These mice develop melanotic melanomas with high penetrance and acquire a metastatic phenotype thus mimicking the human condition.

Materials and Methods

Generation of transgenic mice. The Tyr::N-Ras<sup>Q61K</sup> construct was generated using a mutant human N-Ras<sup>Q61K</sup> (provided by Corlien Aarnoudse and Peter Schrier, Leiden, the Netherlands) and SV40 splice

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/)

Requests for reprints: Friedrich Beermann, Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland. Phone: 41-21-692-5914; Fax: 41-21-652-6933; E-mail: Friedrich.Beermann@isrec.ch.

©2005 American Association for Cancer Research.
and polyadenylation sequences, cloned into the vector tyr(hs3.6/6.1)-lacZ (18). The gel-purified insert was inserted into oocytes derived from superovulated B6D2F1 (F1[C57BL/6J × DBA/2] male and female matings. Tyr::N-RasQ61K transgenic mice (Tg[Tyr::N-RasQ61K]) were backcrossed to C57BL/6J mice for two generations and then mated to INK4a-deficient mice (6). The resulting mice were of a mixed genetic background that consisted primarily of C57BL/6J (63% and DBA/2 (13%). To generate Tyr::N-RasQ61K transgenic mice on an albino background, transgenics were bred to BALB/c mice for at least three generations. Toxigenic ablation of melanocytes was obtained by breeding the Tyr::N-RasQ61K transgenic line to Tyr::DT-A (tyr(hs6.8/6.1)-DT-A) transgenic mice that express the attenuated diphtheria toxin-A gene specifically in melanocytes (18). To visualize melanocytes by β-galactosidase staining, Tyr::N-RasQ61K transgenics were mated to Dct-lacZ (19) transgenic mice. For genotyping, genomic DNA was prepared from tail biopsies and analyzed by PCR with N-ras-specific primers or INK4a-specific primers (6). Reverse transcription-PCR (on tumor sample) and PCR using primers on both sides of the SV40 small T intron (in the SV40 poly[A]) was done as described (20).

**Histologic analysis and immunohistochemistry.** Mice were analyzed macroscopically for the occurrence of dyspigmentation, enlarged lymph nodes, and metastasis. A total of 16 melanomas and 11 enlarged lymph nodes were analyzed in detail. These samples were chosen to be representative and subsequent histologic analyses confirmed the macroscopic observations. Histology and immunohistochemistry were done using cryostat sections or subsequent histologic analyses confirmed the macroscopic observations. To show that the observed phenotype is not solely due to melanin deposits, the transgene was crossed onto an albino genetic background. Albino Tyr::N-RasQ61K transgenic mice (Fig. 1D) show disruption of hair follicle architecture, inflammation, and epidermal hyperplasia as observed in the pigmented mice (data not shown). Because this phenotype had not been described in other mouse models with hyperpigmentation, it was necessary to show that this phenotype originated from N-ras-mediated alterations in the melanocyte lineage itself. Thus, skin melanocytes were removed from Tyr::N-RasQ61K transgenic mice by a toxigenic approach expressing attenuated diphtheria toxin-A (Tyr::DT-A; ref. 18). Double transgenic mice were similar to Tyr::DT-A transgenic mice (Fig. 1D), and skin seemed normal (data not shown), which suggests, that the skin phenotype is not caused by promiscuous expression of N-ras in dermis or epidermis but due to alterations in the melanocyte lineage. In conclusion, expression of N-RasQ61K in the melanocyte lineage leads to hyperpigmentation, and persistence of melanocytes in dermis and epidermis.

**Melanoma susceptibility of Tyr::N-RasQ61K mice.** In human melanoma, the INK4a locus is frequently inactivated. Thus, Tyr::N-RasQ61K transgenic mice were crossed with INK4a knockout mice and >50 mice (INK4a+/−, INK4a−/−, wild type) were monitored for >1 year for development of melanoma and signs of morbidity and metastases (Fig. 2; Supplementary Table S1). Seventeen of 18 Tyr::N-RasQ61K INK4a−/− mice (94%) developed cutaneous melanomas within about 6 months of birth (6.8 ± 1.3 months). The incidence rate was slightly lower in Tyr::N-RasQ61K INK4a+/− mice (83%, 15 of 18 mice) but dropped to 29% (4 of 14) in Tyr::N-RasQ61K mice wild type at INK4a. In these latter two groups, the average latency for mice developing melanoma was ∼1 year (INK4a+/−, 11.5 ± 1.8 months; wt, 12.1 ± 4.8 months; Fig. 2). The lower incidence and longer latency in mice heterozygous at the INK4a locus would suggest that melanoma formation is most likely due to the loss of heterozygosity of the INK4a tumor suppressor gene. Macroscopically, melanoma tumors presented as black, frequently, ulcerated nodules. They occurred often simultaneously in multiple anatomic sites, involving trunk (81%) and head and neck (39%; n = 36 mice). Acral melanomas and melanomas of the genitalia were observed in a few cases. Histologically tumors were predominantly melanotic and epitheloid, contained numerous macrophages and were well vascularized. They were symmetrical with sharp circumscription and invaded the reticular dermis, subcutis and in some cases the underlying muscle (Fig. 2A). Tumors stained positive for tyrosinase (Fig. 2A) confirming their melanocytic origin. In some of the tumors, atypical melanocytes were identified in the surface or
adnexal epithelium. In certain cases, they formed nests and/or were present within a rather hyperplastic epidermis or at the epidermal/dermal junction of hair follicles (Fig. 2A), indicating that Tyr::N-RasQ61K melanocytes have the capacity to microinvade the epidermis. Other tumors filled the lower two thirds of the dermis and did not affect the papillary dermis or the epidermis (data not shown). In all animals, the melanocytic expansion affected the follicular epithelium and surrounding dermis to a much greater extent than the interfollicular epithelium. In conclusion, Tyr::N-RasQ61K INK4a/C0/C0 mice develop melanoma in the dermis and/or epidermis with a high incidence (>90%) and short latency (6 months).

Melanomas in lymph nodes of Tyr::N-RasQ61K transgenic mice. In a number of human melanoma patients, a population of melanocytes is detected in sentinel lymph nodes (25). Interestingly, in lymph nodes of tumor-bearing Tyr::N-RasQ61K transgenic mice this population was also seen (Fig. 3C). These melanocytes showed the same morphology as those present in the dermis and were present in the subcapsular and medullary sinuses and associated with melanin-laden macrophages. Melanomas were equally identified in lymph nodes (Fig. 3B), and these differed from the hyperplastic melanocytes in forming compact hypercellular nodules that excluded lymphocytes and contained only rare macrophages. Like the melanomas situated in the skin they often had an epithelioid morphology. Overall, 64% of tumor-bearing mice were diagnosed with enlarged lymph nodes, notably observed in the regional lymph nodes draining the site of melanoma formation.

Melanomas appearing in Tyr::N-RasQ61K transgenic mice give rise to liver and lung metastasis. Thirty-six percent of mice with melanoma showed lung or liver metastases, frequently associated with enlargement and pigmentation of lymph nodes. Pulmonary metastases presented as solitary pigmented nodules (Fig. 3C and D). In conclusion, Tyr::N-RasQ61K transgenic mice develop melanomas, which metastasize to lung and liver.

Tumor formation and experimental metastasis by tumor cells ex vivo. Cells isolated from primary melanomas were able to form colonies in soft agar (data not shown) suggesting that they are capable of forming tumors when injected into immunocompromised mice. When 5 x 10^5 cells (in three...
independent experiments) were injected s.c. into athymic nude mice, primary melanoma cells gave rise to melanocytic tumors in 12 of 13 injections. Interestingly, efficient tumor formation was obtained following inoculation with relatively low numbers of cells (50,000 cells, 10 of 13; 2,500 cells, 5 of 13; Supplementary Table S2). To further characterize their metastatic capacity, melanoma cells of two independent tumors were injected i.v. into the tail vein of NOD/SCID mice. All of the injected mice (n = 15) showed melanotic nodules in the lung, and one mouse also developed liver and kidney metastases (Fig. 4A).

It has recently been suggested that not only hematopoietic but also solid tumors may be hierarchically structured with only a minority of cells inside the tumor maintaining tumor-initiating activity ("cancer stem cells"), whereas the majority of tumor cells are more differentiated and have only little self-renewing activity (26–28). Melanomas are thought to originate from early progenitors of the neural crest lineage. One of the best known markers for stem/progenitor cells of the neuroectodermal lineage is the intermediate filament protein nestin. To test whether the Tyr::N-RasQ61K-driven melanomas contain tumor cells with a neural crest progenitor phenotype tumors were analyzed for the presence of nestin expressing cells. Such cells were detected in primary melanomas andmetastases as well as in experimental lung metastases of NOD/SCID mice (Fig. 4B). Because nestin is also expressed in a number of nontumor host tissues, such as for example in endothelial cells, double staining was done with S100 to show nestin expression specifically in tumor cells (Fig. 4C and D). However, only a fraction (10-50%) of tumor cells expressed nestin suggesting that the melanomas are comprised of at least two subpopulations of tumor cells.

Discussion

In this study, we have established a novel mouse model for melanoma, and we show for the first time that overexpression of a mutant form of human N-ras (N-RasQ61K) in melanocytes results in a hyperpigmented skin phenotype and increases the formation of melanoma lesions and favors the acquisition of a metastatic behavior on an INK4a+/− background. Similarly, melanocyte-specific expression of an oncogenic form of H-ras (H-RasV12G) on a INK4a−/− background induced melanomas with comparable latency and penetrance; however, in contrast to the model presented here, these remain amelanotic and nonmetastatic (11). Although N-RasQ61K and H-RasV12G transgenic constructs contain all the known tyrosinase regulatory elements including the distal control region (18), the melanoma phenotype might still be influenced by transgene expression or the integration site. However, the hyperpigmentation phenotype in the Tyr::N-RasQ61K line was intermediate compared with the other four founder mice generated but similar to the hyperpigmentation reported in the Tyr::H-rasV12G mice, suggesting a similar ras activity in both models. On the other hand, the phenotype might be explained by differences between N-ras and H-ras. The ras proteins differ in their intracellular trafficking (29) and are compartmentalized in distinct plasma membrane domains, with N-ras for example present in lipid rafts, whereas caveolae rather contain H-ras, and both H-ras and N-ras are colocalized at the membrane (30). Activated K-ras and H-ras have been shown to modulate radiation sensitivity of cells very differently, most probably mediated by differential activation of phosphatidylinositol-3 kinase/Akt and MAPK pathways (31). Thus, either the exact location of the activating mutation on the Ras protein (H-rasV12G versus N-rasQ61K) could be important, or, more likely, N-ras and H-ras activation in melanocytic cells has slightly distinct consequences either on MAPK activation or on a thus far unidentified pathway causing the different tumorigenic and metastatic potential. Phenotypically, changes in the epidermis, including reddening of the skin and inflammation, have been observed in the transgenic Tyr::N-rasQ61K mice of the experimental line (Fig. 1D) and in one of the other, now extinct lines, when bred to albino. This inflammation might be generated by melanocyte expression of cylooxygenases and prostaglandins (32) and might contribute to tumor progression and metastatic behavior (33, 34).

The Tyr::N-ras INK4a−/− mouse is a unique genetic model in which expression of the human oncogene in melanocytes reproduces the process of melanoma malignancy and metastatic capacity. With respect to histopathology and molecular pathogenesis, the model resembles human cutaneous malignant melanoma thereby making it an attractive melanoma and tumor model. First, the abnormal distribution and proliferation of melanocytes observed in newborn transgenic mice is reminiscent of congenital melanocytic nevi. In human, such congenital nevi include a band-like distribution of melanocytes in the upper dermis, with the presence of melanocytes around adnexal structures and between collagen bundles in the reticular dermis and with some cells above the basal cell layer of the epidermis (35, 36). Assuming that congenital nevi are regarded as a potential melanoma premalignant stage (particularly if of large size; ref. 37), and that N-ras mutations have been frequently found in congenital nevomelanocytic nevi (14), we suggest that the model presented here recapitulates this lesion which frequently progresses into malignant melanoma.

In addition, Tyr::N-RasQ61K transgenic mice develop multiple melanotic and highly invasive primary melanoma tumors. The development of multiple primary tumors is also characteristic of familial cutaneous melanoma with germ line INK4a mutations (38). In addition, some tumors depict nests of melanocytes along and above the dermal-epidermal junction. This junctional activity was most prominent in the follicular epidermis and sometimes extended to the overlying interfollicular epidermis, although given the presence of hyperplastic melanocytes in the dermis it remains possible that these are the real precursors of invasive tumors. In certain cases, the epidermis was not affected and the tumor seemed to originate from the dermis. Such a nonepidermal origin

Figure 2. Kaplan-Meier graph of melanoma incidence in Tyr::N-RasQ61K transgenic mice which are wild type, heterozygous mutant or deficient for INK4a.

The age of mice was defined by the appearance of a cutaneous melanoma and additional signs of morbidity.

Cancer Res 2005; 65: (10). May 15, 2005 4008 www.aacrjournals.org
of melanoma has been described in human for very large congenital nevomelanocytic nevi.

Furthermore, we have shown that 64% of Tyr::N-RasQ61K mice with primary melanomas also had melanocytic/melanoma deposits in lymph nodes. Given the presence of hyperplastic melanocytes in the lymph nodes, it remains unclear whether these tumors were of primary or metastatic origin. Hematogenous metastases of melanomas were clearly present with 36% of animals bearing cutaneous melanoma developing tumor deposits (often multiple) in lung and liver. In the experimental metastasis assay, tumor cells efficiently colonize the lungs of NOD/SCID mice and are capable of reaching other organs.

Finally, we have shown that the melanomas and metastases are heterogeneous. Only a fraction of the tumors expressed tyrosinase, a marker for differentiated melanin producing cell types such as melanocytes. The tumors also contained cell types expressing the neuronal stem cell marker nestin suggesting that they are comprised of melanin expressing cells as well as undifferentiated neural crest stem/progenitor cell types. This indicates that in this mouse model the melanomas are hierarchically structured, in agreement with recent findings suggesting that tumors may contain a minority of so-called “cancer stem cells,” which are essential for its initiation, expansion and maintenance (26, 28). The mouse model can now be used to elucidate whether nestin expression correlates with malignancy and whether melanoma stem cells exist. Interestingly, human melanomas have also been reported to contain nestin expressing cells (39) suggesting that it might be a general characteristic of melanomas, further confirming the similarity between experimental mouse tumors and those known from the clinic. Further support for the presence of melanoma stem cells was obtained by fluorescence-activated cell sorting Hoechst-negative cells (40). Initial results suggest that only 100 of these cells, that show

**Figure 3.** Primary and metastatic melanomas arising in Tyr::N-RasQ61K mice. A, primary melanoma in the dorsal skin of a Tyr::N-RasQ61K/Ink4a−/− transgenic mouse. Left, H&E-stained section of the tumor. Inset, black melanoma nodule (~0.8 cm). Middle, tyrosinase immunoreactivity (red) of melanin-bleached section of the tumor. Magnification (rectangle) reveals epidermal-dermal localization (right, arrowheads) of tyrosinase-positive melanoma cells in the upper part of a hair follicle. Bar, 100 μm. B, lymph nodes. Top, subcapsular proliferation of melanocytes (H&E staining) in inguinal lymph node (inset), which showed S100 immunoreactivity. Note that dendritic cells might also be stained with S100 but would not contain pigmented granules. Right, pigmented subcapsular melanocytes associated with melanin-laden macrophages. Bottom, H&E-stained section and S100 immunoreactivity of inguinal lymph node (inset) with nodular proliferation (metastasis) of amelanotic melanocytes. Right, nodular amelanotic melanocytes with prominent nucleoli. Bar, 25 or 5 μm (right). C, lung metastasis showing a solitary nodule (inset) and positive immunoreactivity for S100 marker. Only the periphery of the nodule was tyrosinase positive. Bar, 25 μm. D, liver metastasis showing an elevated melanotic nodule (inset) that stained positive for S100 and tyrosinase. Bar, 25 μm.
“side population” characteristics, were sufficient to generate melanomas if grafted s.c. into athymic nude mice. In conclusion, by introducing the genetic lesions frequently found in human melanoma patients, we have been able to generate a mouse model developing metastasizing melanoma at high frequency closely resembling the human disease. These animals will not only be useful for preclinical testing of novel therapeutic approaches but should also allow the study of the molecular and cellular basis of tumor progression from the premalignant-stage to full-blown melanoma including metastasis formation.

**Acknowledgments**

Received 8/17/2004; revised 1/17/2005; accepted 3/2/2005.

Grant support: Swiss Cancer League (F. Beermann and A. Trumpp); Fondation Emma Muschamps (F. Beermann); Swiss National Science Foundation (F. Beermann and A. Trumpp); National Center of Competence in Research Molecular Oncology, a research instrument of the Swiss National Science Foundation (F. Beermann); and UBS Optimus Foundation (A. Trumpp).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank the Developmental Studies Hybridoma Bank developed under the auspices of the NIHCD and maintained by the Department of Biological Sciences, University of Iowa for the nestin antibody developed by Susan Hockfield; Corlien Aarnoudse and Peter Schrier for the human CMV-NRasQ61K plasmid; Manuel Serrano for p16/INK4a knockout mice; Ian Jackson for Dct::lacZ transgenic mice; Vince Hearing, Margot Thome, and Donata Rimoldi for antibodies; Anne Wilson for comments on the article; and the MIM facility for technical assistance.

3 Unpublished data.

**References**


Sharpless NE, Bardeesy N, Lee KH, et al. Loss of p16\textsuperscript{INK4a} and ras in melanoma susceptibility.


Metastasizing Melanoma Formation Caused by Expression of Activated N-Ras Q61K on an INK4a-Deficient Background

Julien Ackermann, Manon Frutschi, Kostas Kaloulis, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/10/4005

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2005/05/09/65.10.4005.DC1

Cited articles
This article cites 38 articles, 6 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/10/4005.full#ref-list-1

Citing articles
This article has been cited by 30 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/65/10/4005.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/65/10/4005.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.