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

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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 resemble 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 stimuli. Melanoma tumors regressed when H-Ras V12G 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 codon 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/).

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and polyadenylation sequences, cloned into the vector tyr(hs3.6/6.1)-lacZ (18). The gel-purified insert was injected into oocytes derived from superovulated B6D2F1 (F1[C57BL/6J × DBA/2]) male and female matings. Tyr::N-RasQ61K transgenic mice (Tg[N;Tyr::N-RasQ61K];Ebec) 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 (>87%) and DBA/2 (<13%). To generate N-ras deficiency phenotype. A line was established with offspring of founder 3 (94%) developed cutaneous melanomas and 11 enlarged lymph nodes were tumor cells and tumorigenicity. Tumors were dissociated from Tyr::N-RasQ61K transgenic mice by a toxigenic approach and analyzed in detail. As expected, transgenic N-ras expression of N-ras in dermis or epidermis but due to alterations in ras 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% of mice), the upper and lower extremities (22%), and head and neck (39%; n = 36 mice). Acral melanomas and melanomas of the genitalia were observed in some 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). 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. The gel-purified insert was injected into oocytes derived from superovulated B6D2F1 (F1[C57BL/6J × DBA/2]) male and female matings. Tyr::N-RasQ61K transgenic mice (Tg[N;Tyr::N-RasQ61K];Ebec) 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 (>87%) and DBA/2 (<13%). To generate N-ras deficient mice (6). The resulting mice were of a mixed genetic background. 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 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 tumors, 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 paraffin sections. αPEP7 (provided by Vinc Hearing, Laboratory of Cell Biology, National Institutes of Health, Bethesda, MD), anti-rabbit S100 (DAKO Co., Carpinteria, CA), and anti-nestin (Developmental Studies Hybridoma bank, University of Iowa) were used as primary antibodies followed by cy3-coupled (Jackson ImmunoResearch, West Grove, PA) and biotinylated (Amersham Biosciences Europe, Freiburg, Germany) donkey anti-rabbit IgG as secondary antibodies. Fast Red substrate (Sigma, St. Louis, MO) was used as the final chromogen and hematoxylin was used as nuclear red.

Primary tumor cells and tumorigenicity. Tumors were analyzed in detail. They were symmetrical with sharp circumscription and invaded the reticular dermis, subcutis and in some cases the underlying muscle (Fig. 2A). The lower incidence and longer latency for mice developing melanoma was f; the melanocyte lineage leads to hyperpigmentation, and persistent expression of N-ras in dermis or epidermis but due to alterations in ras, 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 wild type at INK4a. In these latter two groups, the average latency for mice developing melanoma was ~36 mice). Acral melanomas and melanomas of the genitalia were observed in some 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

Results

Generation and characterization of tyrosinase::N-RasQ61K transgenic mice. We used the 6.1-kb promoter sequence of the mouse tyrosinase gene in combination with the 3.6-kb distal control region (18) to target expression of a mutant human N-ras gene (N-RasQ61K) to the melanocytic lineage (Fig. 1A). Five Tyr::N-RasQ61K transgenic founders were obtained all of which exhibited hyperpigmentation of the skin, the paws, the ears and the tail. Two founders died before adulthood, another founder displayed patchy pigmentation, and two founders showed a similar hyperpigmentation phenotype. A line was established with offspring of founder 3 and analyzed in detail. As expected, transgenic N-ras is expressed in this line and leads to the activation of MAPK (Fig. 1A). Dorsal skin of adult transgenic mice displayed a band-like distribution of pigmented melanocytes in the papillary dermis that was closely associated with the epidermis. In contrast, few melanocytes were histologically identifiable within the interfollicular epidermis and the hair follicles (Fig. 1B). Numerous melanocytes were present in the epithelium of the upper, portion of the hair follicle and seemed to disrupt certain follicles particularly during anagen. Melanin-containing macrophages were also present particularly in the reticular dermis and the s.c. fat. Occasionally, epidermal hyperplasia, hyperkeratosis, and melanin deposits were observed in epidermis, and hair follicles often showed disordered architectural features such as pseudocysts (Fig. 1B). Thickening of the epidermis, in combination with branching and fusion of interfollicular and follicular epithelium is equally observed in human lentigines as for example lentigo simplex (24), which is characterized by increased number of melanocytes and melanization. No apparent morphologic changes were observed in the retinal pigment epithelium, but the choroidal layer was thickened in adult eyes resulting in folding of the neuroretina (Fig. 1C). 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).
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. 3B). 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 located 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 often two to three small nodules were detected at autopsy. Lung and liver metastases uniformly express S100, whereas tyrosinase immunoreactivity was much more heterogeneous (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 and metastases 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−/−/C0 background. Similarly, melanocyte-specific expression of an oncogenic form of H-ras (H-rasV12G) on a INK4a−/−/C0 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 cavedae 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 epidermisd 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 appeared to originate from the dermis. Such a nonpidermal 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.
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 heterogenous. 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 characteristics 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 dermal-epidermal 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.3 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.

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3 Unpublished data.


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