An Inducible Mouse Model of Melanoma Expressing a Defined Tumor Antigen

Ivo J. Huijbers,1 Paul Krimpenfort,2 Patrick Chomez,1 Martin A. van der Valk,3 Ji-Ying Song,3 Else-Marit Inderberg-Suso,1 Anne-Marie Schmitt-Verhulst,1 Anton Berns,2 and Benoît J. Van den Eynde1

1Ludwig Institute for Cancer Research and Cellular Genetics Unit, Université catholique de Louvain, Brussels, Belgium; Divisions of 2Molecular Genetics and Centre of Biomedical Genetics and 3Experimental Animal Pathology, The Netherlands Cancer Institute, Amsterdam, the Netherlands; and 4Centre d’Immunologie de Marseille-Luminy, Centre National de la Recherche Scientifique/Institut National de la Sante et de la Recherche Medicale, Université de la Méditerranée, Marseille, France

Abstract
Cancer immunotherapy based on vaccination with defined tumor antigens has not yet shown strong clinical efficacy, despite promising results in preclinical models. This discrepancy might result from the fact that available preclinical models rely on transplantable tumors, which do not recapitulate the long-term host-tumor interplay that occurs in patients during progressive tumor development and results in tumor tolerance. To create a faithful preclinical model for cancer immunotherapy, we generated a transgenic mouse strain developing autologous melanomas expressing a defined tumor antigen recognized by T cells. We chose the antigen encoded by P1A, a well-characterized murine cancer germ line gene. To transform melanocytes, we aimed at simultaneously activating the Ras pathway and inactivating tumor suppressor Ink4a/Arf, thereby reproducing two genetic events frequently observed in human melanoma. The melanomas are induced by s.c. injection of 4-OH-tamoxifen (OHT). By activating a CreER recombinase expressed from a melanocyte-specific promoter, this treatment induces the loss of the conditional Ink4a/Arf gene in melanocytes. Because the CreER gene itself is also flanked by loxp sites, the activation of CreER also induces the deletion of its own coding sequence and thereby allows melanocyte-specific expression of genes H-ras and P1A, which are located downstream on the same transgene. All melanomas induced in those mice with OHT show activation of the Ras pathway and deletion of gene Ink4a/Arf. In addition, these melanomas express P1A and are recognized by P1A-specific T lymphocytes. This model will allow to characterize the interactions between the immune system and naturally occurring tumors and thereby to optimize immunotherapy approaches targeting a defined tumor antigen. (Cancer Res 2006; 66(6): 3278-86)

Introduction
In the early 1990s, the molecular definition of human tumor antigens recognized by T lymphocytes has raised interest to use immunologic approaches to treat cancer, and a number of clinical trials have been initiated aimed at boosting antitumor immune responses by vaccination with defined antigens (1, 2). Most of these trials were done in melanoma, a tumor type for which many antigens were defined, including those encoded by cancer germ line genes (3). These genes are expressed in many tumors and encode tumor-specific peptides that are presented to T lymphocytes by molecules of the MHC (1). These cancer germ line genes are silent in normal tissues, except in male germ line cells, which do not express MHC molecules. Vaccination of metastatic melanoma patients with such tumor antigens has produced significant tumor regressions in 10% to 20% of the patients, indicating the potential of the approach (4). However, it proved difficult to improve the success rate, as various immunization modalities all induce a similar proportion of tumor responses (5). It seems that further progress will depend on a better understanding of the mechanisms governing tumor rejection after vaccination, including not only the efficiency of immunization but also the mechanisms of tumor resistance and the state of functional tolerance that may result from the prolonged presence of tumor cells among host normal tissues. Such mechanisms can be studied in preclinical models, and a number of mouse tumor models have been developed. However, most available models are based on transplantable tumors expressing a defined antigen. Transplantable tumor models have a number of drawbacks, including the fact that the tumors do not develop in their natural tissue microenvironment and do not recapitulate this long-term interaction between tumor cells and host tissues. A number of melanoma models were described recently, where tumors develop within the host (6–8). However, these melanomas do not express a defined antigen and therefore are difficult to use for immunologic studies and for optimization of immunotherapy. For these reasons, we decided to develop an inducible mouse model of melanoma expressing a well-defined antigen.

To optimally mimic the human situation, we wanted to use an antigen that is naturally expressed by mouse tumors rather than a surrogate antigen of artificial nature. We chose the tumor-specific antigen encoded by mouse gene P1A, which represents the best-characterized murine cancer germ line gene. This antigen, which consists of a peptide derived from the P1A protein and presented to cytolytic T lymphocytes (CTL) by H-2 L3 molecules, represents the major rejection target of P1A-expressing tumors (9–12).

The pathogenesis of human melanoma is determined by environmental factors, including skin exposure to UV light, and by a number of genetic alterations. Common genetic lesions in melanomas are activating mutations in B-RAF, seen in 66% of primary melanomas, and loss of INK4a/ARF, which is detected in 50% of human melanomas (13–15). B-RAF encodes a serine/
threonine kinase that is activated upon binding to active RAS proteins and signals via the mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway (16). INK4a/ARF contains two overlapping reading frames, which encode two tumor suppressor proteins, p16INK4a and p14ARF (p19Arf in mouse), which exert their functions via distinct pathways (17). INK4a, the founding member of the INK4 family, acts as an inhibitor of cyclin-dependent kinase 4/6 and prevents phosphorylation of the retinoblastoma protein (Rb), thereby inducing cell cycle arrest by preventing release of E2F transcription factors from Rb (18). The Arf tumor suppressor protein increases p53 levels by blocking Mdm2-mediated p53 ubiquitination and degradation (19). An evaluation of the mutational profiles associated with activating B-RAF mutations in human melanomas showed that 17 of 26 B-RAFV600E melanomas have simultaneous deletions of INK4a/ARF (15). Chin et al. (20) showed a similar synergism between the activation of the Ras/Raf/MEK/ERK pathway and the combined loss of p16INK4a and p19Arf in melanoma development in mice. Their transgenic mice overexpressing activated H-Ras in melanocytes developed melanomas with a 43% incidence over a period of 6.5 months when placed on the Ink4a/Arf-deficient background. Based on these findings, we designed an inducible mouse model of melanoma using a self-deleting Cre recombinase to simultaneously inactivate Ink4a/Arf and activate both Ras and PIA in melanocytes.

Materials and Methods

Production of transgenic mice. Transgene Tyr-IrasPIA consists of a 2.5-kb fragment of the mouse tyrosinase promoter (−2475/+38), preceded by two copies of a 0.9-kb Sty1 fragment containing an enhancer normally located 15 kb upstream of the tyrosinase gene (21). Downstream of the promoter a chimeric intron composed of the donor site of the β-globin intron and the acceptor site of the IgG intron (pcI-neo; Promega, Madison, WI) was introduced followed by the CreER<sup>D</sup> fusion gene (22) with a bovine growth hormone polyadenylation signal (pRES-EGFP; Clontech, Palo Alto, CA). The CreER<sup>D</sup> and the polyadenylation signal were flanked by two loxP sites. A 380-bp mouse cDNA encoding H-Ras<sup>V12</sup> was obtained by PCR on liver cDNA using a mutant primer inv0107 (5'-CCGTCCAGGGCAGCACATTACAGTGTGGTGGTGGGCTGTTAGCGTCTGGGAAAGATGCCC-3') and primer inv0108 (5'-CGCATGCAATCAGGACAGCACACATTGCGC-3'). The H-Ras<sup>V12</sup> cDNA was cloned downstream of the second loxP site and followed by an encapsidation carditis-virus derived internal ribosome entry site (IRES from pRES-EGFP; Clontech) and by the PIA cDNA with its natural polyadenylation signal. The construct was linearized by digestion with Sall, purified, and injected into fertilized FVB/N mouse oocytes.

Screening of transgenic mice. Eight transgenic founder mice were obtained and named TIRP-1, TIRP-5, TIRP-8/9A, TIRP-8/9B, TIRP-10A, TIRP-10B, TIRP-14, and TIRP-16. They were identified by Southern blot analysis of DNA digested with EcoRV and hybridized with a PIA probe. Transgene TIRP-10B was selected as being susceptible to melanoma induction (see below). We infrequently observed some founder instability in 4 of 34 tested TIRP-10B mice (12%), two of which remained melanoma prone. Genotyping of TiRP-10B transgenic line TiRP-10B was done by Southern blot analysis of DNA digested with EcoRV and hybridized with a PIA probe. They were identified by Southern blot analysis of DNA digested with EcoRV and hybridized with a PIA probe. TiRP-10B;R26R mice were of a pure FVB/N background. Based on these findings, we designed an inducible mouse model of melanoma using a self-deleting Cre recombinase to simultaneously inactivate Ink4a/Arf and activate both Ras and PIA in melanocytes.

Preparation and administration of 4-OH-tamoxifen. TIRP transgenic mice were crossed to the Rosa26 Cre reporter strain (R26R), which has an ubiquitously expressed transgene containing a STOP cassette flanked by loxP sites and followed by the LacZ gene (24). The resulting TIRP26R mice received a topical 4-OH-tamoxifen (OHT) treatment: OHT (Sigma, St Louis, MO) was dissolved in DMSO to a concentration of 0.2 mg/mL, and 20 μL were applied to the right ear. The left ear was treated with 20 μL of DMSO alone. The mice were treated four times with 2-day intervals. Systemic treatment of TIRP-10B;R26R mice was done by injecting twice 4 mg OHT s.c. in the neck 3 days apart, or by injecting four times 2 mg OHT i.p. at 2-day intervals. The TIRP26R mice were of a pure FVB/N background. For tumor induction, TIRP-10B mice were crossed with Ink4a/Arf<sup>Flox/Flox</sup> mice. The resulting mice, named TIRP-10B;Ink4a/Arf<sup>Flox/Flox</sup>, received two s.c. injections of 4 mg OHT into the neck area 2 weeks apart. OHT was dissolved as described (25). In brief, 50 mg OHT was dissolved in 250 μL ethanol. A solution of 20 mg/mL OHT was obtained by adding 2.25 mL autoclaved sunflower oil followed by a 30-minute sonication in an ultrasonicator; 200 μL of the fresh solution were used for s.c. injection into 5- to 14-week-old transgenic mice. The OHT-treated TIRP-10B;Ink4a/Arf<sup>Flox/Flox</sup> mice were monitored for 18 months.

Staining for β-galactosidase activity. Ear samples of TIRP26R reporter mice were taken 6 days after the final OHT treatment. Ears were fixed for 1 hour in 0.2% glutaraldehyde (Sigma) at room temperature. The ears were washed three times in a permeabilization buffer (2 mmol/L MgCl<sub>2</sub>, 0.01% Na-deoxycholate, and 0.02% NP40) and stained overnight at 37°C in a X-gal buffer (1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, 2 mmol/L MgCl<sub>2</sub>, 0.01% Na-deoxycholate, 0.02% NP40). Rinsing the ears with PBS stopped the staining. The ears were postfixed overnight in 4% (w/v) paraformaldehyde at 4°C and embedded in paraffin by standard methods. Sections were counterstained with nuclear fast red.

Establishing stable tumor cell lines from primary tumors. Primary tumor tissue was dissected from melanoma-bearing TIRP-10B;Ink4a/Arf<sup>Flox/Flox</sup> mice, cut into small pieces, and plated on a feeder layer of mitomycin C–treated immortal murine X-2 keratinocytes (26). Cells were grown in DMEM/F12 (Invitrogen/Life Technologies, Grand Island, NY) with 10% fetal bovine serum, 1 × insulin/transferrin/selenium (Invitrogen/Life Technologies), 1 μg/mL hydrocortisone (Sigma), 50 μg/mL cholera toxin (Sigma), and 5 ng/mL epidermal growth factor (Invitrogen/Life Technologies). X-2 feeders were omitted after three to five passages. The cultures were considered as cell lines when the melanoma cells grew indefinitely without the X-2 feeders. As indicated in Supplementary Table S1, the following cell lines were obtained: line M-1, from a nonpigmented tumor of mouse 124632; lines M-2a and M-2b, from two distinct pigmented tumors of mouse 117354; and line M-3, from a pigmented tumor of mouse 124634. M-1 and M-3 are two clones that were isolated manually from the primary culture of line M-3.

Immunohistochemistry. Melanoma-bearing TIRP-10B;Ink4a/Arf<sup>Flox/Flox</sup> mice were sacrificed by CO<sub>2</sub> inhalation. Normal and tumor samples were fixed in 4% (w/v) paraformaldehyde and embedded in paraffin. Sections (5 μm) were stained with H&E, and serial sections were used for immunohistochemical analysis. The slides were stained with anti-tyrosinase related protein 1 (TRP-1; goat polyclonal; I:1000; sc-10446; Santa Cruz Biotechnology, Santa Cruz, CA), anti-si100 (rabbit polyclonal; I:18000; Z0311; DakoCytomation, Glostrup, Denmark), and anti-p75<sup>TCR</sup> (rabbit polyclonal, I:1000; AB1554; Chemicon, Temecula, CA). Antibodies were used with microwave heating in citric acid (pH 6) was done before p75<sup>TCR</sup>-staining. Heavily pigmented slides were pretreated with 0.1% K<sub>3</sub>CrO<sub>7</sub> for 20 minutes followed by 0.3% oxalic acid for 12 minutes to remove pigmentation before TRP-1 staining. Biotinylated rabbit-antisig (1:600; E0966; DakoCytomation) and biotinylated goat-antirabbit (1:800; E0542; DakoCytomation) served as secondary antibodies and were visualized with StreptABC complex/horseradish peroxidase and the AEC substrate-chromogen (DakoCytomation).

Analysis of Cre-mediated recombination. The recombination occurring in the Ink4a/Arf locus was monitored by a three-primer PCR: two of which were also used for genotyping (i.e., p16IIIS-5’ sense and p16IIIS-3’...
antisense) and one additional sense primer located upstream of the first loxp site, p16 intron 234 bp < loxp (5'-CTAACCTCTCGCTCTACAC-3'). The recombined Ink4a/Arf allele yielded a 427-bp product, whereas the unrecombined allele yielded a band of 350 bp. Thermocycling conditions consisted of 35 cycles of 40 seconds at 94°C, 30 seconds at 58°C, and 50 seconds at 72°C. For each PCR reaction, 20 to 40 ng of tail or tumor DNA were used.

Analysis of transgene expression. The presence of the H-Ras-RES-P1A transcript in melanoma cultures was monitored by reverse transcription-PCR (RT-PCR). Total RNA was extracted with the TriPure Isolation Reagent (Roche Molecular Biochemicals, Mannheim, Germany), and 2 μg of each sample served as a template for cDNA synthesis with an oligo-dT primer. Primers Ivo041, located 7 bp upstream of the intron (5'-AACCTTCTCAGGAGT-3') and Pcho6, positioned on the P1A stop codon (5'-ACCTCGATGCTAAGTGAGACG-3'), monitored the expression of the H-Ras-RES-P1A transcript. Thermocycling conditions consisted of 30 seconds at 94°C, 30 seconds at 60°C, and 120 seconds at 72°C.

Western blot analysis. Total protein lysates from the cell lines were obtained by lysis of 5 × 10^6 cells in 1 mL lysis buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 0.5% NP40] containing a protease inhibitor mix (Complete Mini; Roche). Protein concentrations were determined by the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL). Cell lysates (10 μg/sample) were subjected to electrophoresis through 12% polyacrylamide gels and blotted on Hybond-C nitrocellulose membranes (Amersham Biosciences, Chalfont St. Giles, United Kingdom). The membranes were blocked in 5% dried skim milk for 1 hour at room temperature and incubated overnight at 4°C in the blocking buffer with a 1:500 dilution of the rabbit polyclonal anti-P1A antisum (27), a 1:200 dilution of the rabbit polyclonal D1 antibody (sc-753; Santa Cruz Biotechnology), or a 1:1,000 dilution of a monoclonal antibody against murine β-actin (A3316; Sigma). The membranes were developed with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibodies (sc-2031 or sc-2302; Santa Cruz Biotechnology) and the SuperSignal West Pico Chemiluminescent Substrate (Pierce) and exposed to a BioMax MR film (Kodak, Rochester, NY). For tumor samples, the Western blot analysis was done similarly, using the maximal available amount of proteins, which varied from 10 to 36 μg per sample.

Ras pull-down assay. The active Ras pull-down assay on cell lines was done according to the EZ-detach Ras Activation kit protocol (Pierce) with 0.42 to 1.39 mg of total protein per lysate. For the tumor samples, the Ras pull-down protocol was modified, and all samples, ranging from 0.336 to 1.183 mg of protein per sample, were incubated with 0.1 mmol/L GTPγS for 15 minutes at 30°C, before the affinity purification of activated Ras. For the Western blots, we used either an anti-Ras antibody (Pierce) or a mouse monoclonal antibody specific for Ras proteins mutated at position 12 (G12V; OP38; Calbiochem, San Diego, CA).

CTL stimulation assay. The 1.1-kb full-length cDNA of the murine MHC class I molecule, H-2 Ld, was cloned into expression vector pEF6-V5-His-TOPO (Invitrogen/Life Technologies). Cells (2.5 × 10^6) of tumor cell lines M-1 and M-3.2 were electroporated with 25 μg of the pEF6-V5-His-TOPO + H-2Ld and selected for 3 weeks in medium containing 5 to 10 μg/mL blasticidin (Invitrogen/Life Technologies). Clones were picked and verified for their H-2 Ld expression by fluorescence-activated cell sorting (FACS) analysis with the 28-14-8S hybridoma supernatant (American Type Culture Collection, Rockville, MD). The M-1 and M-3.2 clones were seeded at 5,000 or 30,000 cells per microwell, respectively, 2 days before the CTL stimulation assay. At day 2, 10,000 CD8+ T cells derived from the TCRP1A transgenic mice (28) were added in 100 μL stimulation medium with 5 units/mL human recombinant interleukin-2. The supernatant was harvested after 20 hours of coculture, and the IFN-γ content was determined with a mouse IFN-γ ELISA (Biosource Europe SA, Nivelles, Belgium). P511 and P1.204 (10) served as controls and were plated at 20,000 cells per microwell on the day of the CTL stimulation assay. As controls, some microcultures of the M-1 and M-3.2 clones were pulsed for 1 hour at 37°C with 10 μg/mL of antigenic peptide P1A 35-43 (PVGWGWLVF; ref. 10), before the coculture with the CD8+ T cells.

MS-1 is a tumor line derived from a TiRP-10B(Ink4a/Arf)−/−;H-2d/d male mouse, obtained after four backcrosses to B10.D2 mice (H-2d). A melanoma appeared on the flank of this mouse 117 days after injection of OHT. It was resected and adapted to in vitro culture. MS-1 cells (10^5 irradiated cells) were used to stimulate 10^5 TCRP1A CD8+ T cells labeled with CFSE (5- and 6-carboxyfluorescein diacetate succinimidyl ester; Molecular Probes, Eugene, OR). The number of T-cell divisions was determined by FACS analysis (29). Expression of T-cell activation markers was assessed by FACS using antibodies APC-anti-CD25, PE-anti-CD44, and PCP-Cys5.5-anti-CD8α (PharMingen, Becton Dickinson, Mountain View, CA).

Figure 1. Transgenic approach for the construction of an inducible mouse model of melanoma. A, map of transgene Tyr-RasP1A. The tyrosinase promoter is preceded by two copies of a tyrosinase enhancer element (LCR, locus control region) and followed by a chimeric intron, a loxp site (triangle), a CreER<sup>12</sup> fusion gene followed by a polyadenylation signal, a second loxp site, the Harvey-Ras cDNA harboring an activating mutation in codon 12 (G12V), an IRES, and the P1A gene with its polyadenylation signal. Dotted lines, Cre-mediated recombination induced by OHT. Gray bar, P1A probe used for Southern blotting on EcoRV-digested DNA. s and as arrows, positions of primers Ivo41 and Pcho6, used for the detection of the H-Ras-RES-P1A transcript by RT-PCR. B, schematic representation of the inducible melanoma model. The transgene above is introduced into mice with a homozygous conditional Ink4a/Arf locus, where exons 2 and 3 of Ink4a/Arf are flanked by loxp sites (triangles). In normal melanocytes (left), CreER<sup>12</sup> is expressed but remains inactive, trapped in the cytosol by hsp90. Upon treatment with OHT, CreER<sup>12</sup> will translocate to the nucleus and recombine genes flanked by loxp sites (triangles). In normal melanocytes (left), CreER<sup>12</sup> is expressed but remains inactive, trapped in the cytosol by hsp90. Upon treatment with OHT, CreER<sup>12</sup> will translocate to the nucleus and recombine genes flanked by loxp sites (triangles). In normal melanocytes (left), CreER<sup>12</sup> is expressed but remains inactive, trapped in the cytosol by hsp90.
Results

Generation and characterization of TiRP transgenic mice. We constructed a transgene containing the murine melanocyte-specific tyrosinase promoter followed by the CreER<sup>DD</sup> fusion gene, which encodes a OHT-responsive Cre recombinase, and a polyadenylation signal (22, 30). The CreER<sup>DD</sup> gene and the polyadenylation signal are flanked by two loxP sites and act as a STOP cassette preventing expression of the Harvey-Ras<sup>G12V</sup> and P1A genes, which are located downstream and separated by an IRES (Fig. 1A). This transgene will be introduced into the previously obtained Ink4a/Arf flox/flox conditional knock-out mice, which have exons 2 and 3 of the Ink4a/Arf gene flanked by loxP sites (23). Activation of CreER<sup>DD</sup> by OHT will result in deletion of both the CreER<sup>DD</sup> and polyadenylation sequences, thereby allowing expression of genes H-Ras<sup>G12V</sup> and P1A. In addition to its self-deletion, activation of CreER<sup>DD</sup> will induce deletion of exons 2 and 3 of the Ink4a/Arf gene, thereby prohibiting expression of tumor suppressors p16<sup>Ink4a</sup> and p19<sup>Arf</sup>. Due to the tyrosinase promoter, these events will occur only in melanocytes and promote their transformation by the combined effects of H-Ras<sup>G12V</sup> activation and Ink4a/Arf deletion (Fig. 1B). The use of a self-deleting Cre as a STOP cassette in transgenic mice has a number of advantages, including the fact that Cre is only transiently active, limiting the risk of undesired further recombination events resulting from sustained Cre activity (31).

Eight transgenic founders were obtained and named TiRP. They seemed normal and transmitted the transgene to their offspring.

Table 1. Tumor incidence and latency in transgenic line TiRP-10B

<table>
<thead>
<tr>
<th>OHT</th>
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<th>Melanoma-bearing mice</th>
<th>Tumor incidence (%)</th>
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<td>Pigmented</td>
<td>Nonpigmented</td>
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<tr>
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<tr>
<td>Yes</td>
<td>33</td>
<td>7</td>
<td>4</td>
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<td>Total melanoma-bearing:</td>
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<td>4</td>
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<td>Latency (days after induction):</td>
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<td>253 (± 57)</td>
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<td>Survival period (days after diagnosis):</td>
<td>161 (± 31)</td>
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Abbreviation: ND, not determined.

*These tumors grew extremely fast, so that in most cases the tumor load already present at diagnosis required immediate euthanasia of the mouse.
mice, either s.c. or i.p., and screened for TiRP-10B;R26R (C). To further evaluate the specificity of the tyrosinase (Fig. 2), bulbs, whereas line TiRP-16, resulted in numerous large macrophages loaded with melanin (melanophages; Fig. 3, A-C). These macrophages were not observed in the surrounding tissue. The epidermis remained intact in all mice upon treatment with OHT. A-C, multiple heavily pigmented melanomas observed after OHT treatment of TiRP-10B;Ink4a/Arf fb/fb mice #117348 (A), #117354 (B), and #1172958 (C). D-E, histologic view of tumors from mice #85294 (D) and #117348 (E), showing invasive cutaneous spindle-cell melanomas with numerous melanophages (H&E counterstaining). F, immunohistochemical staining for TRP-1 after depigmentation of a section from the melanoma of the ear of mouse #117348. G-H, draining lymph node of melanoma-bearing mouse #124634, infiltrated by large rounded melanophages and melanoma cells. I, immunohistochemical staining for TRP-1 on a depigmented serial section of the lymph node portrayed in (H). J-K, nonpigmented late-appearing tumors showing fascicular structures (J; mouse #117365) and signs of Schwann cell differentiation (K; mouse #161749). Inset, immunohistochemical stainings for S-100 (J-K). L, heavily pigmented spindle-cell melanoma overgrown by late-appearing nonpigmented tumor with Schwann cell differentiation (mouse #151126).

They were screened for expression of CreER3D in melanocytes by crossing them to the Rosa26 Cre reporter strain (R26R; ref. 24). This strain has an ubiquitously expressed transgene containing a STOP cassette flanked by loxP sites and followed by the LacZ gene. It therefore expresses β-galactosidase only upon recombination by Cre. Topical treatment of OHT on the ear of TiRP26R mice resulted in β-galactosidase activity in melanocytes in three different transgenic lines, TiRP-10B, TiRP-14, and TiRP-16. These Cre-recombined blue melanocytes were observed at two locations: in the hair bulbs and in the epidermis (Fig. 2A and B). The distribution was variable between the transgenic lines. For instance, TiRP-10B showed more blue melanocytes in the hair bulbs, whereas line TiRP-16 showed more in the epidermis (Fig. 2C). To further evaluate the specificity of the tyrosinase promoter, we did a systemic OHT treatment of TiRP-10BR26R mice, either s.c. or i.p., and screened for β-galactosidase activity in the skin, brain, eye, spleen, thymus, kidney, liver, heart, and lung. Blue staining was observed only in melanocytes of the skin.

The three selected transgenic lines, TiRP-10B, TiRP-14, and TiRP-16, all contained multiple copies of the transgene in a concatemeric array (Fig. 2D). Upon recombination by Cre, all these copies should be reduced to a single copy because the outermost loxP sites should recombine (Fig. 2E). To ensure that the recombined transgene still contained intact H-Ras and PIA genes at the 3′ end, we crossed the three TiRP transgenic lines with the Cre-deleter strain DCre, which imposes ubiquitous deletion of loxP-flanked DNA segments (32). Southern blot analysis using a PIA-specific probe showed that the recombined TiRP-16 line carried a fragmented transgene with a break in the CreER3D gene, resulting in the loss of both H-Ras and PIA (Fig. 2D). The fully recombined TiRP-14 line seemed to have both genes intact based on the Southern blot, but subsequent PCR analysis showed that the polyadenylation signal of PIA was lost (data not shown). The fully recombined TiRP-10B line had genes H-Ras and PIA intact, including the polyadenylation signal.

Tumor susceptibility of TiRP transgenic mice. Lines TiRP-10B and TiRP-14 were then crossed to the conditional Ink4a/Arf fb/fb knock-out background. In theory, the activation of the CreER3D protein by OHT in melanocytes will remove two exons from the Ink4a/Arf locus, resulting in the loss of both p16ymMy and p19arf. We generated one compound line bearing transgene TiRP-10B and two copies of the conditional Ink4a/Arf locus (TiRP-10BInk4a/Arf fb/fb), and another compound line bearing transgene TiRP-14 with one copy of the conditional Ink4a/Arf locus and the other copy fully deleted (TiRP-14Ink4a/Arf fb/del). These mice were injected s.c. twice with 4 mg OHT, with the injections 2 weeks apart. No tumors appeared in OHT-treated TiRP-14Ink4a/Arf fb/del mice, possibly related to the absence of a correct polyadenylation signal. However, OHT-treated TiRP-10B;Ink4a/Arf fb/fb mice developed tumors with a 33% incidence (Table 1). This compares favorably with the 25% tumor incidence reported in the previously described inducible melanoma model (33).

Most of the tumors that developed in the OHT-treated TiRP-10B;Ink4a/Arf fb/fb mice were heavily pigmented skin tumors that appeared with an average latency of 73 days and showed the pathologic features of cutaneous spindle-cell melanoma (Table 1; Supplementary Table S1). These tumors were often multiple and usually appeared on hairy skin but rarely on the tail (Fig. 3A-C). The tumor mass consisted of spindle-shaped melanoma cells and numerous large macrophages loaded with melanin (melanophages; Fig. 3D and E). The melanocytic origin of the tumor cells was confirmed by staining of depigmented lesions with melanocyte marker TRP-1 (Fig. 3F). The melanomas were locally invasive into the surrounding tissue. The epidermis remained intact in all melanomas and showed moderate hyperplasia. The tumors grew slowly, and the affected mice were monitored for an average period of 161 days (i.e., time between observation of the first pigmented lesion and death). Upon necropsy, draining and regional lymph nodes were found enlarged and pigmented in several mice (Fig. 3G and H; Supplementary Table S1). These lymph nodes contained both melanoma cells and melanophages (Fig. 3I). We searched for distant metastases in organs, such as lung, liver, kidney, and spleen, but did not find any.

In some mice, nonpigmented tumors were induced by treatment with OHT with a much longer latency (253 days; Table 1). These tumors grew faster than the pigmented melanomas and showed the pathologic features of malignant peripheral nerve sheath tumors (MPNST), with areas reflecting Schwann cell differentiation, including dense and hypodense lacerations and thickened papillae.
fascicles and spindle cells with irregular nuclei (Fig. 3f and K). These tumors were usually positive for neural crest markers S-100 and p75NGFR and negative for melanocyte marker TRP-I (Supplementary Table S1). Because melanocytes and nerve sheath cells have a common neuroectodermal origin, melanomas and MPNST may represent two different facets of the same oncologic entity induced in these mice by treatment with OHT. In line with this concept, we observed in two mice the later appearance of an MPNST underneath a pigmented melanoma (Fig. 3f). In addition, some of the pigmented melanomas showed signs of Schwann cell differentiation. We considered therefore that these MPNSTs might represent transdifferentiated forms of melanoma as suggested for some spontaneous melanomas observed in another transgenic mouse model and some case reports of human MPNST-like melanoma (34, 35).

CreER<sup>ΔB</sup>-mediated recombination in induced tumors. We established four cell lines from three pigmented and one non-pigmented melanomas (Supplementary Table S1). To confirm the loss of Ink4a/Arf<sub>G12V</sub> in the induced tumors, we designed a three-primer PCR that amplifies either a 350-bp product representing the unrecombined (flox) conditional Ink4a/Arf allele or a 427-bp product representing the recombined (del) Ink4a/Arf allele (Fig. 4A). As expected, when done on DNA from tails of TIRP-10BInk4a/Arf<sup>flox/flox</sup> mice, the three-primer PCR gave only the unrecombined product. Both products were amplified on DNA from tumor samples, which contain both stromal cells and tumor cells. Tumor cell lines showed only the recombined product, indicating that both copies of the Ink4a/Arf gene had recombined in the tumor cells (Fig. 4).

Recombination of the transgene was analyzed by Southern blotting on genomic DNA extracted from TIRP-10BInk4a/Arf<sup>flox/flax</sup> tails and tumor cell lines using a P1A-specific probe as above. The results showed a complete Cre-mediated recombination in cell lines M-2a and M-2b (Fig. 4B), with the expected 3.5-kb band already seen in Fig. 2D. Cell lines M-3.2 and M-3.3 showed partial recombination (i.e., recombination of some of the transgene copies

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**Figure 4.** Genetic characterization of melanomas. A, loss of conditional Ink4a/Arf alleles in OHT-induced melanomas. A PCR with three primers can distinguish between conditional and recombined Ink4a/Arf alleles on genomic DNA extracted from tails, tumor samples, or tumor cell lines M-1, M-2a/b, and M-3.2/3. Two primers flanking the loxP site downstream of exon 3 of Ink4a/Arf amplify a product of 350 bp on the conditional allele. An additional sense primer located upstream of the loxP site in intron 1 of Ink4a/Arf amplifies, together with the antisense primer, a product of 427 bp on the recombined allele. Numbers on top indicate the mouse of origin. Genomic DNA isolated from tails of Ink4a/Arf<sup>flox/flox</sup>, Ink4a/Arf<sup>flox/del</sup>, or Ink4a/Arf<sup>delt/delt</sup> mice serve as controls in the PCR. B, recombination of the transgene in tumor cell lines. Southern blot analysis of genomic DNA isolated from tumor cell lines M-2a, M-2b, M-3.2, and M-3.3, and from corresponding tails, digested with EcoRV and hybridized with a P1A probe. The fully recombined transgene is expected at 3.5 kb, as indicated. The expected sizes of the nonrecombined transgene s are indicated in Fig. 2 legend. C, the presence of Ras-IRE5-P1A transcripts in early primary melanoma cultures and in tumor line M-1 detected by RT-PCR. The location of the primers used is indicated in Fig. 1A. The expected size of the PCR product is 1.93 kb. D, expression of the activated Ras, cyclin D1, and P1A proteins in tumor cell lines. Activated Ras was purified by GST-Raf1 pull down and analyzed by Western blotting. Top blot was incubated with a general anti-Ras antibody, and the second blot was incubated with an antibody specific for the Ras<sup>G12V</sup> mutation. The actin control (bottom) relates to the cyclin D1 and P1A part of the figure, where each lane contained 10 μg of proteins from the cell lysate, except for the P1A-positive control lane, which contained only 1 μg of lysate from mouse mastocytoma cell line P815 (subline P511). The P1A-negative control was P815 subline P1.204, which has lost gene P1A (10). Non-specific signals observed with the anti-P1A serum (*). E, expression of the activated Ras and P1A proteins in primary melanoma samples. Top, activated Ras purified by GST-Raf1 pull down was analyzed by Western blotting using an antibody specific for the Ras<sup>G12V</sup> mutation. Bottom, 10 to 36 μg of proteins from tumor lysates were analyzed using a P1A-specific rabbit serum. Positive and negative controls were lysates of wild type (1 μg) and P1A knockout 78.50 (10 μg) embryonic stem cells, respectively (I.H., unpublished results).
Activation of the H-Ras pathway in melanomas. In theory, recombination of the transgene should induce expression of genes H-RasG12V and P1A. By RT-PCR, we confirmed the presence of a long transcript containing the H-Ras– and P1A-coding sequences in the tumor lines (Fig. 4C). Expression of the active H-RasG12V protein was confirmed in tumor lines and in primary tumor samples by Western blotting using an anti-RasG12V antibody on Ras proteins purified by a Ras pull-down assay (Fig. 4D). In one primary melanoma sample (117348.2), we failed to detect H-RasG12V proteins. This sample also showed low levels of total Ras proteins, which might be related to a higher proportion of stromal cells in that particular sample (data not shown).

A consequence of high levels of activated Ras is the accumulation of cyclin D1 (36). Western blot analysis showed high cyclin D1 levels in the tumor lines compared with B16F1 and NIH-3T3, indicating that the oncogenic Ras expressed in the tumor lines is able to activate one of its downstream targets (Fig. 4D).

Expression of P1A in tumor lines and recognition by P1A-specific CD8+ T cells. Expression of P1A was detected in all tumor lines by Western blot with a rabbit polyclonal anti-P1A serum (Fig. 4D). P1A expression was equal between the tumor lines, as already observed for H-RasG12V. P1A was also detected in most primary melanoma samples (Fig. 4E). The level of P1A expression in the tumor lines was ~10-fold lower compared with control line P815 (Fig. 4D). To determine whether this expression level was sufficient to elicit a T-cell response, we transfected tumor lines with a plasmid construct encoding H-2 Ld, the MHC class I molecule required for presentation of the P1A peptide to CD8+ T lymphocytes. Two transfected clones showing moderate expression of H-2 Ld by FACS analysis were selected: one derived from tumor line M-1 and one from line M-3.2. CTL stimulation assays done with these clones showed that the M-1/2Ld and M-3.2/2Ld clones were recognized by P1A-specific CD8+ T cells, whereas the vector-transfected control clones were not (Fig. 5A). These results indicated that the tumor lines produced sufficient levels of endogenous P1A from the transgene and that the protein was normally processed to give rise to the antigenic peptide. This was further confirmed when we started backcrossing TiRP-10B;Ink4a/Arf flox/flox mice to H-2d mice B10.D2 to bring constitutive expression of the proper MHC molecules. A tumor cell line established from a melanoma induced with OHT in a backcrossed mouse was also recognized by P1A-specific CD8+ T cells, as indicated by the induction of their proliferation and expression of activation markers (Fig. 5B).

Discussion

The described inducible mouse model of melanoma fulfills the key elements for a useful experimental system to test the efficacy of immunotherapy on naturally occurring tumors in immunocompetent mice. The mice develop primary tumors within their natural tissue microenvironment. These tumors progress to lymph node metastases and express a defined tumor-specific antigen. The expression of the antigen-encoding gene P1A was observed in all tested melanomas and was sufficient to trigger activation of P1A-specific T lymphocytes and sustain their proliferation. Because H-RasG12V and P1A are produced from the same mRNA transcript and because oncogenic Ras is required for tumor maintenance (33), it is unlikely that P1A-negative tumors will emerge. The model also has some practical benefits, such as the superficial location of the tumors, which makes it easy to monitor progression, and the slow growth rate, which provides ample time for immunotherapeutic treatment and monitoring. In addition, the inducible nature of the model offers the opportunity to compare the fate of T cells induced by vaccination either before or after tumor induction.

The inducible mouse model of melanoma that we have designed is based on the melanoma models of Chin et al. (20, 33), who showed that loss of Ink4a/Arf in combination with activation of the Ras pathway in melanocytes confers melanoma susceptibility in
mice. Although the similarities between the models are evident, some differences are worth noting. The melanomas that developed in our model were mainly located on the hairy skin, whereas both the constitutive and the inducible melanoma mice of Chin et al. showed a preference for melanoma development on skin areas with sparse or no hairs, such as the pinna of the ear, the tail, or the anus (20, 33). This difference in tumor location might be due to different tyrosinase promoter and enhancer elements used in the transgenic constructs. We used the mouse 0.8-kb enhancer/2.5-kb promoter, and Chin et al. used the mouse 3.6-kb enhancer/5.5-kb promoter. In our melanoma-susceptible mouse line, Cre-mediated recombination was mainly observed in follicular melanocytes, which are located in the hair bulbs. Consequently, appearance of melanomas on hairy skin was to be expected. We never observed Cre-mediated recombination in dermal melanocytes, whereas this was the only type of melanocyte that showed expression of Ras in the models of Chin et al. (20). In adult mice, these dermal melanocytes are primarily present in regions that are characterized by sparse or absent hairs (38). This could explain the preferential development of melanoma at these sites in their models.

Another main difference between the models is the pigmentation of the tumors: most of the melanomas that developed in our model were heavily pigmented, whereas melanomas in the models of Chin et al. were mainly amelanotic (20, 33). Because most human melanomas are also heavily pigmented, our model may serve more adequately to recapitulate human melanoma. The reason for this difference between the models is unclear but might lie in the status of the Ink4a/Arf gene. Chin et al. used mice that were fully deficient for Ink4a/Arf, whereas we combined a conditional Ink4a/Arf<sup>fl/fl</sup> background with a melanocyte-specific Cre recombinase. This approach prevented loss of Ink4a/Arf in nonmelanocytic cells. It is possible that the presence or absence of Ink4a/Arf in stromal cells influences tumor phenotype. Indeed, preliminary results indicate that TIRP transgenic mice on the fully deficient Ink4a/Arf background exclusively develop amelanotic tumors with MPNST-like features, which grow considerably faster than the heavily pigmented melanomas seen on the conditional Ink4a/Arf<sup>fl/fl</sup> background (data not shown). Whether these nonpigmented tumors are similar to the amelanotic melanomas observed in Chin et al.’s models remains to be established. The use of a conditional Ink4a/Arf<sup>fl/fl</sup> background in our transgenic mice also prevents the spontaneous development of tumors, such as fibrosarcomas and lymphomas, which are caused by systemic loss of p16<sup>Ink4a</sup> and p19<sup>Arf</sup> (39). Such additional tumors are unwanted in a melanoma therapy model. Moreover, this approach makes the model more representative of sporadic melanoma as opposed to familial melanoma.

The melanomas we observed were locally invasive and caused lymph node metastases but not distant metastases. This allows for a long observation period of the animals and is therefore convenient for a model aimed at testing therapeutic approaches. It also indicates that the loss of Ink4a/Arf and the activation of the Ras pathway do not give rise to a fully metastatic phenotype. In line with this notion, it was observed recently that patients with melanomas harboring mutations in these two pathways only, had a better prognosis than patients with more complex mutational profiles (15). The metastatic phenotype may, however, depend on the Ras subtype that is mutated and the location of the activating mutation (8). Thus, another use of our model will be to help defining pathways involved in metastasis, by identifying genes whose alteration could convert locally invasive into metastatic tumors. This could be done by crossing the mice described here with mice either deficient or transgenic for additional genes potentially involved in metastasis.

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