A Murine Model for Metastatic Conjunctival Melanoma

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PURPOSE. Conjunctival melanoma (CM) is an ocular malignancy with a high rate of local recurrences after treatment, and can give rise to deadly metastases. The establishment of a murine model will further our understanding of this disease and allow in vivo testing of new therapies. We therefore analyzed the ability of three CM cell lines to grow orthotopically and spread to distant sites. Furthermore, we determined the characteristics of the xenografts and their metastases.

METHODS. Orthotopic xenografts of human CM were established by subconjunctival injection of three different CM cell lines into NOD/SCID IL2γc null mice. Single-cell suspensions were generated from the primary tumors and placed subconjunctivally in another set of mice, which were then screened for metastases. The presence of melanoma markers was determined on the cell lines and during tumor development.

RESULTS. Subconjunctival injection of cultured CM cells into immunodeficient mice led to excellent subconjunctival tumor growth in all inoculated mice (n = 101) within 2 weeks; however, no metastases were found at the time of autopsy. Serial in vivo passage of primary tumor cells resulted in metastatic tumors in the draining lymph nodes (n = 21). The CM cell lines, as well as the tumor xenografts and their metastases, were positive for the melanoma markers HMB-45, S100B, and MART-1. Two cell lines and their corresponding xenografts carried a BRAF mutation, the third showed an NRAS mutation.

CONCLUSIONS. We established a murine model for CM that shows excellent formation of metastases in a pattern that accurately resembles metastatic human CM following in vivo passaging.

Keywords: conjunctiva, melanoma, metastatic, mouse model

Conjunctival melanoma (CM) is an ocular malignancy that originates from conjunctival melanocytes. Although it is a rare form of cancer, CM has a high rate of local recurrence after treatment, and recurs in more than one in three cases.1 Tumor-related mortality following the diagnosis of CM is 15% after 5 years and 30% after 10 years, with metastatic spread as the main cause of death.2

Current therapies focus on radical excision of the tumor, and are frequently supplemented with cryotherapy or local therapy.3,4 The type of additional local treatment is a matter of discussion, although some studies support the use of adjuvant therapy regardless of clinical staging due to the high risk of local recurrence.5–7 However, the rarity of this disease limits the ability to develop randomized clinical trials to determine which adjuvant chemotherapy or new treatment will improve prognosis.8 To overcome these limitations, the use of a murine model is a good alternative; several studies have recently shown evidence that immunodeficient mice can be used successfully for hosting xenogeneic tumor cells.9–14 Hence, the development of a mouse model that follows the natural progression from primary to metastatic disease will provide a unique opportunity to examine in vivo tumor growth and progression. Although several animal models have been developed to study uveal melanoma, very few models are available for CM. There is one report that describes the induction of conjunctival primary acquired melanosis by using topical 7,12-dimethylbenz[a]anthracene in rabbit eyes. The pigmented conjunctival lesions are intraepithelial precursors of melanoma and recapitulate the human local conjunctival disease, but metastases were not detected in this model.15

Conjunctival melanoma exhibits a high rate of local recurrence and metastases; therefore, more effective treatments directed against the local and distant recurrences are needed. Recent findings have demonstrated that immunotherapy targeting specific tumor-associated antigens may reduce recurrences in cutaneous melanoma.16,17 The presence of melanoma markers during different stages of CM tumor growth may provide an opportunity for the development of adequate immunotherapy. Conjunctival melanomas exhibit BRAF and NRAS mutations similar to cutaneous and mucosal melanomas.18–21 A large cohort study demonstrated that 29% of CMs harbor a BRAF mutation, and 18% an NRAS mutation, in a mutually exclusive pattern.22 The presence of these mutations may allow specific targeted therapy, as is already used for the treatment of metastases from cutaneous melanoma carrying these mutations.23,24
The establishment of a murine model for human CM would help to further our understanding of the disease and provide us with a strategy to test new treatments, thereby increasing the efficiency of therapeutic modalities and reduce recurrences. So we examined the possibility of CM growing orthotopically in nonobese diabetic (NOD)/severe combined immunodeficiency (SCID) IL-2 rγc null mice, and to create a model in which metastatic spread resembles human disease.

MATERIALS AND METHODS

Cell Culture

Three CM cell lines, all derived from locally recurrent tumors, were used. The cell lines CRMM-1 and CRMM-2 were created by Gordon Nareyeck (Essen, Germany),8 and kindly provided by Michele Madigan (Sydney, Australia). The cell line CM2005.1 was created in our laboratory by Sander Keijser (Leiden University Medical Center, Leiden, The Netherlands).25 In Boston, CRMM-1 and CRMM-2 cells were grown in F-12K Nutrient Mixture, Kaighn’s Modification (1:3) supplemented with 10% fetal bovine serum (FBS) (both from Invitrogen, Grand Island, NY, USA), 1% 200 mM L-glutamine and 1% penicillin/streptomycin/amphotericin-B mixture (both from Lonza, Walkersville, MD, USA) at a 5% CO2 atmosphere. The CM2005.1 cells were grown in RPMI-1640 (Lonza) supplemented with 10% FBS, 1% 200 mM L-glutamine, with 20 mM Hepes and 1 g/L NaHCO3 (Gibco, Grand Island, NY, USA), 1% penicillin/streptomycin/amphotericin-B mixture, in 5% CO2 atmosphere. In Leiden, the F-12K Nutrient Mixture, Kaighn’s Modification (1:3) was provided by Gibco (Life Technologies, Paisly, UK), supplemented with 10% FBS (Greiner Bio-one, Alphen aan den Rijn, The Netherlands), and amphotericin-B was not used. Photographs of the cultured cells were taken with a microscope (Zeiss AX10; Carl Zeiss Meditec, Jena, Germany) equipped with a camera (Axiocam MRm).

Short Tandem Repeat (STR) Profiles

Short tandem analysis was carried out using the AmpFLSTR Identifiler PCR Amplification Kit (Life Technologies) based on the procedure recommended by The International Cell Line Authentication Committee (ICLAC) in Baseclear (Baseclear, Leiden, The Netherlands)26 (see Table 1). We used all three cell lines described above. Short tandem analysis was carried out using the AmpFLSTR Identifiler PCR Amplification Kit (Life Technologies) based on the procedure recommended by ICLAC in Baseclear (Baseclear, Leiden, The Netherlands)26 (see Table 1).

Cell Line Growth Kinetics

Proliferation of the CM cell lines was measured by mitochondrial function using the water-soluble tetrazolium salt (WST-1) assay (Roche Diagnostics, Indianapolis, IN, USA). After culturing, cells were transferred into 96-well micro-culture plates (Costar 3596; Corning Enterprises, Corning, NY, USA) at four different concentrations and placed in an incubator at 37°C. Growth was measured daily for 4 days: first 10 μL of WST-1 (Roche) was added to each well, followed by a 2-hour incubation at 37°C; analysis was performed using a microplate spectrophotometer (μQuant, MQX200; BioTek, Winooski, VT, USA).

Isolation of DNA and Sanger Sequencing

The DNA material from three CM cell lines as well as paraffin-embedded tumors of CM xenografts were extracted with, respectively, the QIAamp DNA mini kit (Qiagen, Venlo, The Netherlands) and the ReliaPrep FFPE gDNA miniprep system (Promega, Fitchburg, WI, USA). Polymerase chain reaction analysis was performed to amplify BRAF exon 15, NRAS exon 2, and NRAS exon 3, as previously described.27,28 Following
Antibodies

The following primary antibodies were used: mouse anti-HMB45 mAb (1:100 dilution, ab7877; Abcam, Cambridge, UK), rabbit anti-S100B pAb (1:3200 dilution, Code Z0311; Dako, Glostrup, Denmark) and mouse anti-MART-1 mAb (1:80 dilution, Clone M2-7C10; Covance, Denver, PA, USA). As secondary antibodies, we used a goat anti-mouse IgG pAb (1:200 dilution, Code E0435; Dako) and a biotinylated goat anti-rabbit antibody (1:300 dilution, BA-1000; Vector, Burlingame, CA, USA).

Immunocytochemistry

Expression of HMB-45, S100B, and MART-1 was determined on cytospins of the three CM cell lines. Cytospins were made from a cell suspension of 0.3 × 10⁶ cells/mL cell suspension of 2% BSA/PBS using a Shandon Cytospin 4 cytocentrifuge (Thermo Fisher Scientific, Waltham, MA, USA).

Cytospins were made from the three different cell lines at different times. Air-dried cytospin preparations were fixed in acetone for 10 minutes. The slides were washed in PBS and blocked with normal goat serum in 1% BSA/PBS (1:50 dilution, Code X0907; Dako) at room temperature for 30 minutes. Excess serum was removed before overnight incubation at 4°C with the primary antibodies at the required concentrations, diluted in 1% BSA/PBS. Thereafter, cells were washed three times in PBS followed by incubation with either one or two secondary antibodies for 60 minutes, and washed again.

Staining was visualized with nuclear Fast Red (Vectastain ABC-AP kit; Vector). Sections were then counterstained with Mayer’s hematoxylin (Klinipath, Duiven, The Netherlands) and embedded in Kaiser’s glycerin. Evaluation of the staining was done by light microscopy; two independent observers determined scores and the average result was reported. In case of a difference, consensus was reached during a simultaneous grading session.

Eyes were fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin-embedded whole eye sections were used for immunohistochemistry to investigate the expression of HMB-45, S100B, and MART-1. Tissue sections were baked at 60°C overnight. After sections were deparaffinized and rehydrated in a graded series of alcohol, they underwent antigen retrieval with citrate buffer for 20 minutes and were rinsed in PBS. Slides were blocked with normal goat serum in 1% BSA/PBS at room temperature for 30 minutes. The rest of the staining method was performed as described above. Control sections were incubated with secondary antibody alone, and did not show staining.

In Vivo

Animals. Nonobese diabetic/SCID IL-2 receptor gamma chain null mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The animals were maintained under defined conditions in accordance with institutional guidelines of The Schepens Eye Research Institute and experiments were performed according to approved experimental protocols, following the guidelines for the use of animals in research of the Association for Research in Vision and Ophthalmology. Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (9 mg/kg). Mice were euthanized using CO₂ asphyxia, followed by cervical dislocation. All internal organs were inspected macroscopically at the time of autopsy with special attention given to the regional lymph nodes, heart, lung, and liver.

Conjunctival Melanoma Xenografts and Follow-up. Orthotopic xenografts of human CM were established by subconjunctival injection of the cell lines CRMM-1, CRMM-2, and CM2005.1. The cells were harvested from an in vitro cell culture, and, after counting, resuspended in media. Mice were anesthetized as described and injections were delivered under a microscope using a 33-gauge needle, inserted into the nasal subconjunctival space. Each injection contained 0.4 × 10⁶ cells in a total volume of 5 µL. Tumor formation and growth were monitored weekly, at least up to the endpoint of 10 weeks, unless excessive tumor growth required euthanasia earlier. For preparation of whole eye sections, the eyes were enucleated and tissue was fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. Staining with hematoxylin and eosin was used for histopathologic analysis. At the time mice were euthanized, all mice were autopsied and screened for visible metastases. When metastases were found, some of them were removed and fixed in 4% paraformaldehyde for quantification and histopathological analysis.

In Vivo Passaging. In vivo passaging of the primary tumor xenografts was carried out by surgical dissection of the xenografts, and its processing into a single cell suspension. Cells were counted, washed, and resuspended in media. Orthotopic xenografts of the in vitro cultured melanoma cells were then established as described above.

Results

Morphology and Growth of Human CM Cells

Three cell lines derived from three different CMs were cultured in vitro and their growth characteristics were determined. The CRMM-1 had been obtained from a nodular tumor located in the bulbar conjunctiva, which was the third local recurrence of a mainly epithelioid CM. The CRMM-2 also originated from a recurrent melanoma located at the bulbar part of the conjunctiva, with a predominantly epithelioid cell type. We observed that, in culture, both cell lines displayed a spindle-cell type appearance with large nuclei, mostly mononucleated, containing prominent nucleoli (Fig. 1A, a–d). The third cell line, CM2005.1, had also been established from a recurrent CM, located at the palpebral part of the conjunctiva with extension into the nasal cavity. In vitro, this cell line had a spindle-cell appearance with dendritic extensions, abundant cytoplasm, and large round nuclei with prominent nucleoli (Fig. 1A, e–f). All three cell lines showed a slow but steady proliferation rate at four different seeding densities in the WST proliferation assay (Fig. 1B). Immunocytochemistry was performed on cytospin preparations at three different moments, and the percentage of positive cells and the intensity of the staining were determined. All three cell lines showed a high expression of HMB-45. Staining for S100B revealed a weak expression pattern on most of the cells from cell line CRMM-1 (80%), and strong expression on a lower percentage of cells from cell lines CRMM-2 and CM2005.1 (10% of CRMM-2; 35% of CM2005.1); MART-1 was moderately expressed on cell lines CRMM-1 and CRMM-2 (on 62% of CRMM-1 and 77% of CRMM-2), with a strong expression on cell line CM2005.1 on almost all cells (90%) (Fig. 1C).

BRAF and NRAS Mutations

We determined the presence of specific mutations in CM by using all three cell lines, and material obtained from the
primary and in vivo passaged tumor xenografts of CRMM-1 and CM2005.1. We detected the BRAF c.1799T > A (V600E) mutation in both cell lines CRMM-1 and CM2005.1, as well as their established xenografts and in vivo passaged tumors. Also, the metastasis derived from cell line CRMM-1 exhibited the BRAF mutation (Table 2). The cell line CRMM-2 and the corresponding xenografts harbored the NRAS c.182A > T mutation. There was no tissue of in vivo passaged tumors available for this cell line.

**In Vivo Growth of CM Cells**

Because the availability of an animal model will create the opportunity to test new therapies, we set out to establish orthotopic xenografts of human CM by injecting the cell lines CRMM-1, CRMM-2, and CM2005.1 subconjunctivally in NOD/SCID IL-2 rγnull mice. Of the 101 inoculated mice, 100% developed a detectable tumor within 2 weeks of the subconjunctival injection (CRMM-1 n = 33, CRMM-2 n = 34, CM2005.1 n = 35). Tumor formation was closely monitored and a difference was observed in the in vivo growth rate of the cell lines. However, obtaining accurate in vivo measurements of tumor volume was difficult, and therefore typical examples of clinical tumor behavior are shown in Figure 2A. The degree of in vivo pigmentation varied, with CRMM-1-derived tumors being nonpigmented, tumors from CRMM-2 being slightly pigmented, and those from cell line CM2005.1 being heavily pigmented (Fig. 2A). Histological analysis of more than 50 tumor xenografts showed that all the tumors generated from the three cell lines had comparable morphological characteristics: the subconjunctival grafts grew on the ocular surface and into the sclera, with an epithelioid cell morphology with large nuclei and prominent nucleoli. The tumor cells had large cytoplasmic compartments with vacuoles (Fig. 2B). In all specimens, mitoses were observed. Histologically, tumor xenografts derived from the cell lines CRMM-1 and CRMM-2 did not contain pigment, in contrast to xenografts from cell line CM2005.1, which were pigmented. Only CRMM-2-derived tumors showed some necrotic areas, whereas in the xenografts from cell line CM2005.1 some sclerosis was observed (Fig. 2B).

A total of 44 mice (CRMM-1 n = 14, CRMM-2 n = 15, CM2005.1 n = 15) were examined for systemic spread at 6 weeks after injection, and another 22 mice were inspected at 9 weeks after injection (CRMM-1 n = 6, CRMM-2 n = 10,
Although subconjunctival injection of cultured CM cells into immunodeficient mice led to excellent local growth, at the time of autopsy, no metastatic sites were found in any of the mice, showing that this model does not mimic human disease.

To select for more aggressive cancer cells, we decided to try in vivo passaging. A single cell suspension was generated from an established conjunctival tumor xenograft, and subsequently transplanted into the subconjunctival space of another set of immunodeficient NOD/SCID IL-2 null mice (Fig. 3A). We choose to use two cell lines (CRMM-1 and CM2005.1), as they displayed a high and similar in vivo growth rate (Fig. 2A). Exactly the same number of cells was injected each time (0.4 \( \times \) \( 10^6 \) cells in a total volume of 5 \( \mu L \)). All 21 inoculated mice developed a visible subconjunctival tumor within 2 weeks (CRMM-1 \( n = 9 \), CM2005.1 \( n = 12 \)). Tumor formation was monitored closely, and growth pattern and degree of pigmentation of these in vivo–passaged tumors was similar to their original tumor xenografts. During tumor growth, there was no indication of necrosis. We consistently observed that the mice who had received an in vivo transfer of tumor cells lost weight and developed symptoms of pain. Subsequently, the mice were killed 9 weeks after injection, after which an autopsy was performed (Fig. 3B). Metastases were present in all mice that had received a subconjunctival transfer and were mainly found in the cervical lymph node region, the heart, and lungs.

To verify the melanocytic origin of the tumor material, we examined the tumor xenografts and metastases immunohistochemically for the presence of pigment cell markers. Most cells in the primary xenografts derived from cell line CRMM-1 were strongly positive for the melanoma marker HMB-45 (80% of the cells), whereas S100B and MART-1 were moderately expressed on a low number of cells (33% and 20%, respectively). Most cells in the primary xenografts from cell line CRMM-2 stained strongly with all three antibodies (83% with anti-HMB-45, \( n = 3 \); 92% with anti-S100B, \( n = 3 \); and 75% with anti-MART-1, \( n = 3 \)). The primary tumors derived from the third cell line, CM2005.1, showed similarly high expression levels (83% with anti-HMB-45, 90% with anti-S100B, and 78% with anti-MART-1) (Figs. 4E–H). Examination of xenografts derived from an in vivo–passaged tumor from cell line CM2005.1 showed a high expression of HMB-45 (70% and 70%), with a lower expression of MART-1 (30%) (Figs. 4I–L). The CRMM-1 metastases seen in lung tissue were strongly positive for all three markers. Altogether, these findings show that we established a murine CM model that leads to metastatic disease.

**DISCUSSION**

Although it has long been known that CM exhibits a high rate of local recurrence and can lead to death due to metastatic spread, it is important to note that the establishment of a murine model that recapitulates human metastatic disease is a significant step forward in understanding the mechanisms of CM metastasis. This model can be used to identify novel therapeutic targets and to test the efficacy of existing treatments in a preclinical setting. The use of immunodeficient mice allows for a controlled environment where the immune system does not interfere with tumor growth and metastasis, making it possible to study the tumor dynamics in isolation.

**TABLE 2.** BRAF and NRAS Mutations in Three CM Cell Lines and Their Corresponding Tumor Primary Subconjunctival Xenografts, and Passaged Tumors

<table>
<thead>
<tr>
<th>Material</th>
<th>Type of Mutations</th>
<th>BRAF (T1799A)</th>
<th>NRAS (A182T)</th>
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<tbody>
<tr>
<td>CRMM-1</td>
<td>Cell line</td>
<td>+</td>
<td>–</td>
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<tr>
<td></td>
<td>Primary tumor</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>In vivo–passaged tumor</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>CRMM-2</td>
<td>Cell line</td>
<td>–</td>
<td>+</td>
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<tr>
<td></td>
<td>Primary tumor</td>
<td>–</td>
<td>+</td>
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<tr>
<td>CM2005.1</td>
<td>Cell line</td>
<td>+</td>
<td>–</td>
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<td></td>
<td>Primary tumor</td>
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<tr>
<td></td>
<td>In vivo–passaged tumor</td>
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There was not enough material to study mutations in metastases. +, presence of mutation; –, absence of mutation.

CM2005.1 \( n = 6 \). Although subconjunctival injection of cultured CM cells into immunodeficient mice led to excellent local growth, at the time of autopsy, no metastatic sites were found in any of the mice, showing that this model does not mimic human disease.

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spread, the lack of a reliable animal model has hampered thorough investigation of new therapies.\textsuperscript{14,29–31} In this study, we report the establishment of a murine CM model in which metastatic spread follows human disease.

Recurrences of human CMs stem from failure to eradicate a part of the tumor, either the “in situ” (i.e., the intraepithelial) or the invasive component. A difference between our model and human CM is that, in our murine model, the tumor cells

![Figure 3](image1.png)

**Figure 3.** Serial passaging of human CM cells in immunodeficient mice. Tumor development after in vivo passaging. (A) Orthotopic xenografts were first established as described previously; tumor growth was monitored up to 9 weeks. Subsequently, mice were euthanized and surgical dissection of the primary tumor was performed to generate a single cell suspension. Cells were washed, counted, and resuspended before reinjection into the subconjunctival space of a new set of NOD/SCID II-2 \( \gamma^\text{null} \) mice. (B) Primary tumor 8 weeks after subconjunctival injection of \( 0.4 \times 10^6 \) xenograft-derived human CM cells (cell line CRMM-1) into NOD/SCID II-2 \( \gamma^\text{null} \) mice (left) and metastasis found at time of autopsy (right). The cells have been passaged in vivo once. Day 0 is the time of injection.

![Figure 4](image2.png)

**Figure 4.** Immunohistochemical analysis to determine the expression of melanocytic markers during tumor development. Immunohistochemical staining of cytopsins, tumor xenografts, and metastasis for the melanocyte markers HMB-45, S100B, and MART-1; all tumor material was derived from cell line CM2005.1. (A–D) Staining of cytopsins (original magnification \( \times200 \)). (E–F) Primary CM. (I–L) In vivo passaged orthotopic tumor xenograft. (M–P) Lung metastasis (original magnification \( \times200 \)). Negative controls were stained with secondary antibody alone.
are injected into the subconjunctival space. However, in the human eye, melanomas develop in the epithelium and enter the vertical growth phase at a later stage. Transition to the vertical growth phase is the critical step in conversion to malignant CM. In conjunctival melanocytic neoplasia with atypia, the atypical melanocytes are confined within the conjunctival epithelium by the conjunctival basement membrane; in contrast, in CM the atypical melanocytes have broken through the epithelial basement membrane and invaded the underlying stroma, which contains lymph and blood vessels. We injected the tumor cells directly below the conjunctiva, thereby placing them at a site from which the malignant cells could spread to regional lymph nodes.

To assess the potential of using these tumors for immunotherapy and to confirm the melanocytic nature of the tumor cells, we determined the presence of melanoma markers. Conjunctival melanoma can be quite heterogeneous in its clinical and histological features and can lack pigmentation; therefore, monoclonal antibodies are being used to investigate the melanocytic nature of conjunctival lesions, for instance to examine the expression of the markers HMB-45, S100B, and MART-1. A previous study on CM compared the expression of MART-1/Melan-A with the expression of HMB-45, which identifies gp100, and of S100B. Expression of the melanoma markers HMB-45 and S100B were usually strong on CM, whereas MART-1 staining intensity was mostly weak or moderate. In this study, tissues were fixed in 4% buffered formaldehyde and embedded in paraffin. A different antibody was used for MART-1/Melan-A: they used NCL-Melan-A (Clone A103, code no. 4450, NovoCastra, Newcastle, UK), whereas we used a mouse anti-MART-1 mAb (Clone 2-7C10, Covance). In another study, the expression of HMB-45 and S100B in different types of conjunctival melanocytic lesions was compared, including conjunctival naevi and melanomas arising in the context of primary acquired melanosis with and without atypia. An association was observed between increased malignancy and a higher expression of HMB-45, whereas S100B was expressed to the same extent in all different groups. In this study, formalin-fixed tissues were used. A similar study was performed using monoclonal antibodies against three different S100 antigens: S100A1, S100A6, and S100B, and against MART-1. On average, conjunctival naevi often had a high expression of S100B and MART-1, whereas CMs were strongly positive for S100A and S100B, but not for MART-1. We found a more comparable staining intensity for all three antigens HMB-45, S100B, and MART-1. Tumor xenografts derived from cell line CRMM-1 were strongly positive for the melanoma marker HMB-45, whereas S100B and MART-1 were only moderately expressed. Tumors derived from cell lines CRMM-2 and CM2005.1 showed strong staining with all three different antibodies. The high expression levels of HMB-45 agree with the fact that all tumors were derived from a malignant melanoma. We did not find a clear association between increased malignancy and the expression levels of HMB-45, as staining of in vivo–passaged tumors and metastases did not show a further increase in expression of HMB-45. Nonetheless, the presence of these three melanocytic markers on the cells indicates that we are indeed dealing with pigment cells.

To grow the human CM cell lines in a murine model, we used the severely immunodeficient NOD/SCID IL-2 r−/− mice, as recent studies have shown that these mice are highly susceptible to the establishment of metastasis when compared with less immunodeficient mice. Nonetheless, formation of metastases was observed only after applying the method of in vivo passegging, indicating that the in vivo environment plays a crucial role in enhancing tumorigenicity when compared with the in vitro environment.

In conclusion, we established a mouse model for human CM in which metastatic spread follows and recapitulates the clinical disease. This model will provide opportunities for finding new and more effective treatments to prevent the
development of metastasis, as well as the ability to analyze the changes in tumor cell biology that coincide with disease progression and metastatic dissemination.

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