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Targets for personalized treatment of conjunctival melanoma

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Chapter 4

A murine model for metastatic conjunctival melanoma

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Abstract

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 help to further our understanding of this disease and allows 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 IL2 γ^{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 NOD/SCID IL2 γ^{null} mice led to excellent subconjunctival tumor growth in all inoculated mice ($n=101$) within two 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.

Key words: conjunctiva, melanoma, metastatic, mouse model.

Introduction

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 five 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 which 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 $\text{r}\gamma^{\text{null}}$ 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 Gordan Nareyeck (Essen, Germany) [8], and kindly provided by Michele Madigan (Sydney, Australia). The cell line CM2005.1 was created in our lab 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 (1x) supplemented with 10% fetal bovine serum (FBS) (both from Invitrogen, Grand Island, NY), 1% 200mM L-Glutamine and 1% Penicillin/Streptomycin/Amphotericin-B mixture (both from Lonza, Walkersville, MD) at a 5% CO₂ atmosphere. The CM2005.1 cells were grown in RPMI-1640 (Lonza) supplemented with 10% FBS, 1% 200mM L-Glutamine, with 20mM Hepes and 1g/L NaHCO₃ (Gibco, Grand Island, NY) 1% Penicillin/Streptomycin/Amphotericin-B mixture, in 5% CO₂ atmosphere. In Leiden, the F-12K Nutrient Mixture, Kaighn's Modification (1x) was provided by Gibco (Life Technologies, UK), supplemented with 10% FBS (Greiner Bio-one, 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, UK) based on the procedure recommended by The International Cell Line Authentication Committee (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). After culturing, cells were transferred into 96-well micro-culture plates (Costar 3596, Corning, NY) at four different concentrations and placed in an incubator at 37°C. Growth was measured daily for four days: first 10µl 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

DNA material from the 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 (PCR) analysis was performed to amplify BRAF exon 15, NRAS exon 2 and NRAS exon 3, as previously described [27, 28]. Following the PCR, the reverse primer was added to the purified PCR products. Sequencing was examined (Baseclear), and mutations were identified using Mutation Surveyor software (SoftGenetics, State College, PA, USA).

Table 1. Short Tandem Repeat Profiles of In Vitro Grown CM cells.

Cellline	Locus														
	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338	D19S433	vWA	TPOX	D18S51	AM	D5S818
CRM-1	14	29;32.2	8;11	10;13	15;18	7	12	11	17;20	13;14	15	8;11	12;17	x	12;13
CRM-2	12;14	28;31.2	8;11	12	15	7;9.3	8;11	9;13	17;23	13;14	16	8	14;17	xy	11
CM2005.1	14;15	29;30	12;14	10;12	17	9	11;14	12;13	17;25	16;2	17	8;11	14;16	x	11;12

We used all three cell lines described above. Short tandem analysis was carried out using the AmpFLSTR® Identifier™ PCR Amplification Kit (Life Technologies, UK) based on the procedure recommended by ICLAC in Basedclear [26].

Antibodies

The following primary antibodies were used: mouse anti-HMB45 mAb (1:100 dilution; ab787; 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 E0433; 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 cytopins of the three CM cell lines. Cytopins were made from a cell suspension of 0.3×10^6 cells/ml cell suspension of 2% BSA/ PBS using a Shandon Cytospin 4 cytocentrifuge (Thermo Fisher Scientific, Waltham, USA). Cytopins were made from the three different cell lines at different times. Air-dried cytosin 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 haematoxylin (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. Non-obese 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 (IP) 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 0.4×10^6 cells in a total volume of 5 μ l. 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 haematoxylin 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 xenograft, 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 [8]. CRMM-2 also originated from a recurrent melanoma

located at the bulbar part of the conjunctiva, with a predominantly epithelioid cell type [8]. 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 [25]. 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-1 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 the 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 exhibit the BRAF mutation (Table 2). The cell line CRMM-2 and the corresponding xenografts harbors the NRAS c.182A>T mutation. There was no tissue of in vivo passaged tumors available for this cell line.

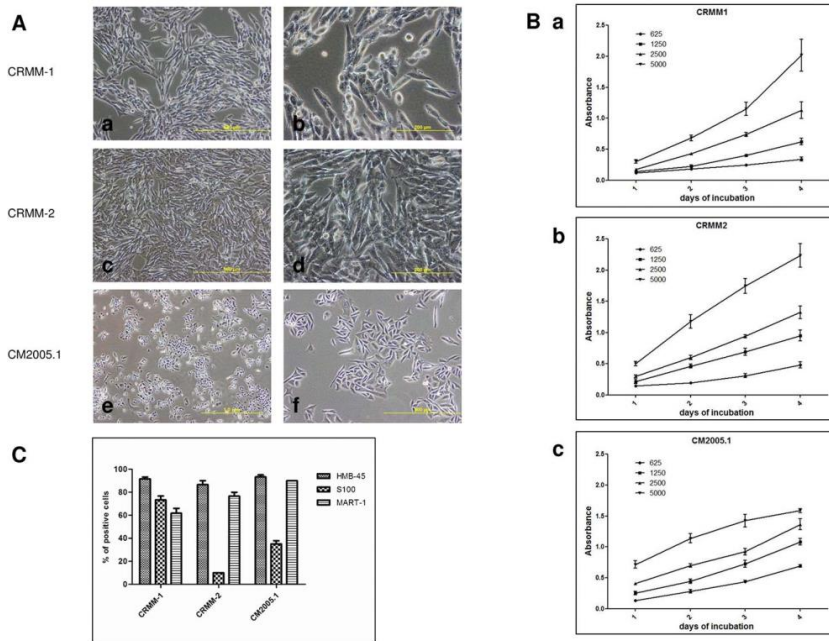


Figure 1. Characteristics of the CM cell lines. (A) Representative light microscopic images of the cultured cell lines CRMM-1, CRMM-2 and CM2005.1. Photographs were taken 72 hours after seeding. Images at original magnification of $\times 4$ (a, c, e) and $\times 10$ (b, d, f) respectively. (B) Growth kinetics of the CM cell lines (a) CRMM-1, (b) CRMM-2, and (c) CM2005.1, determined with a WST cell proliferation assay. Measurements were taken daily for 4 days. (C) Representative bar graph showing immunocytochemistry analysis on cytopins of the expression of HMB-45, S100B and MART-1 in the CM cell lines.

In Vivo Growth of CM Cells

Since 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 IL2 γ^{null} mice. Of the 101 inoculated mice, 100% developed a detectable tumor within two 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

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

Material	Type of mutations	
	BRAF (T1799A)	NRAS (A182T)
CRMM-1		
Cell line	+	-
Primary tumor	+	-
In vivo-passaged tumor	+	-
CRMM-2		
Cell line	-	+
Primary tumor	-	+
CM2005.1		
Cell line	+	-
Primary tumor	+	-
In vivo-passaged tumor	+	-

There was not enough material to study mutations in metastases. +, presence of mutation; -, absence of mutation.

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 non-pigmented, 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 (Figure 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 xeno-

grafts from cell line CM2005.1 some sclerosis was observed (Fig. 2B).

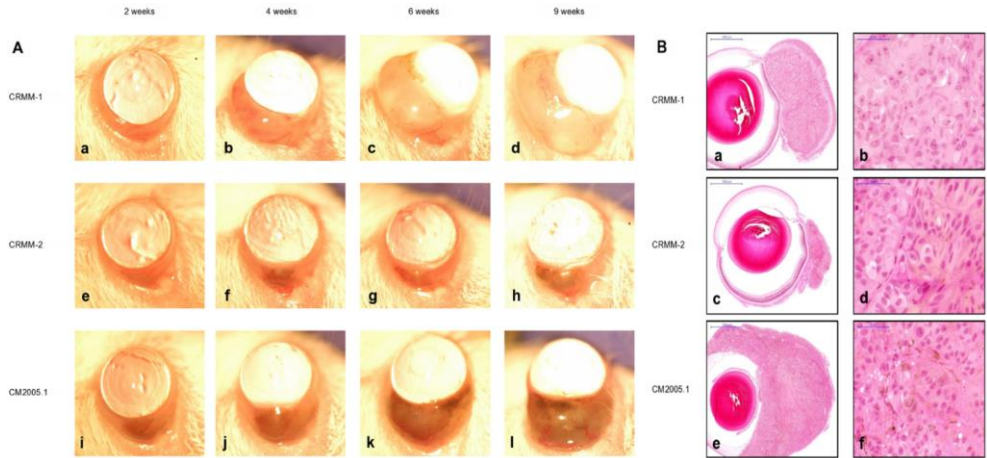


Figure 2. Assessment of tumor development and histological analysis of the xenografts. (A) Tumor development after subconjunctival injection of 0.4×10^6 human CM cell lines into NOD/SCID IL2 $r\gamma^{\text{null}}$ mice. Tumors developed after injection with cell line CRMM-1 (a-d), CRMM-2 (e-h), and CM2005.1 (i-l). Day 0 is the time of injection; photos were taken 2, 4, 6 and 9 weeks post-injection. (B) Histological analysis of whole eye sections derived from mice previously injected with CM cells. Haematoxylin/eosin staining shows a non-invasive growth pattern in the tumor xenografts when harvested nine weeks post injection (a, c, e). Image at original magnification of $\times 10$ (a, c, e) and $\times 400$ (b, d, f).

A total of 44 mice (CRMM-1 $n = 14$, CRMM-2 $n = 15$, CM2005.1 $n = 15$) was examined for systemic spread at six weeks post-injection, and another 22 mice were inspected at nine weeks post-injection (CRMM-1 $n = 6$, CRMM-2 $n = 10$, 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.

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 IL2 $r\gamma^{\text{null}}$ 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 (0.4×10^6 cells in a total volume of 5 μ l). All 21 inoculated mice developed a visible subconjunctival tumor within two 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 nine 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 makers. 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 73% 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 S100B (70%), with a lower expression of MART-1 (30%) (Figs. 4I-L). We also examined a lung metastasis from cell line CM2005.1: it showed a high expression of all three antigens (80% with anti-HMB-45; 90% with anti-S100B; 80% for anti-MART-1) (Figs. 4M-P). 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.

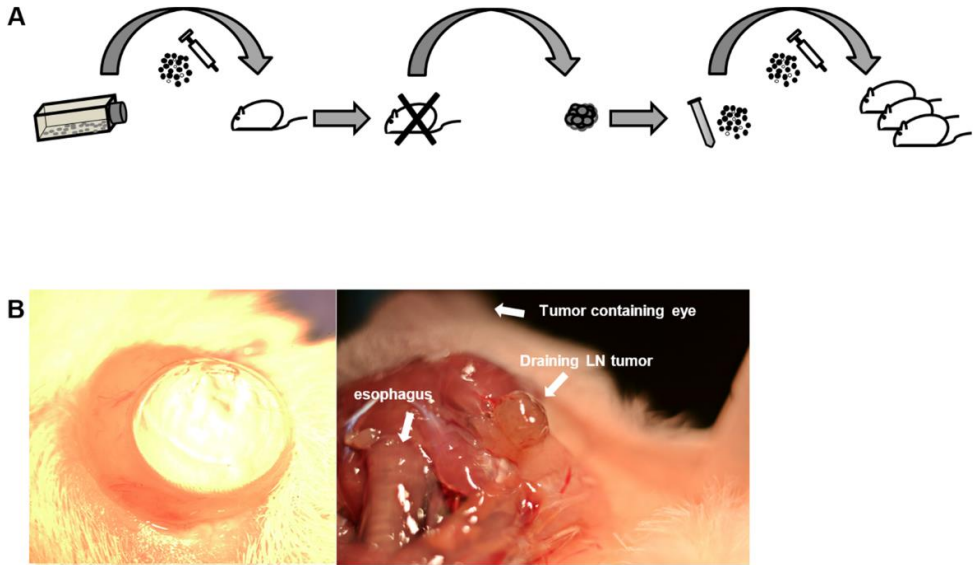


Figure 3. Serial passaging of human CM cells in immunodeficient mice. Tumor development after in vivo passaging. **(A)** Orthotopic xenografts were first established as described previously; tumor growth was monitored up to 9 weeks. Subsequently, mice were euthanized and surgical dissection of the primary 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 IL2 γ^{null} mice. **(B)** Primary tumor 8 weeks after subconjunctival injection of 0.4×10^6 xenograft-derived human CM cells (cell line CRMM-1) into NOD/SCID IL2 γ^{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.

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, the lack of a reliable animal model has hampered thorough investigation of new therapies [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 are

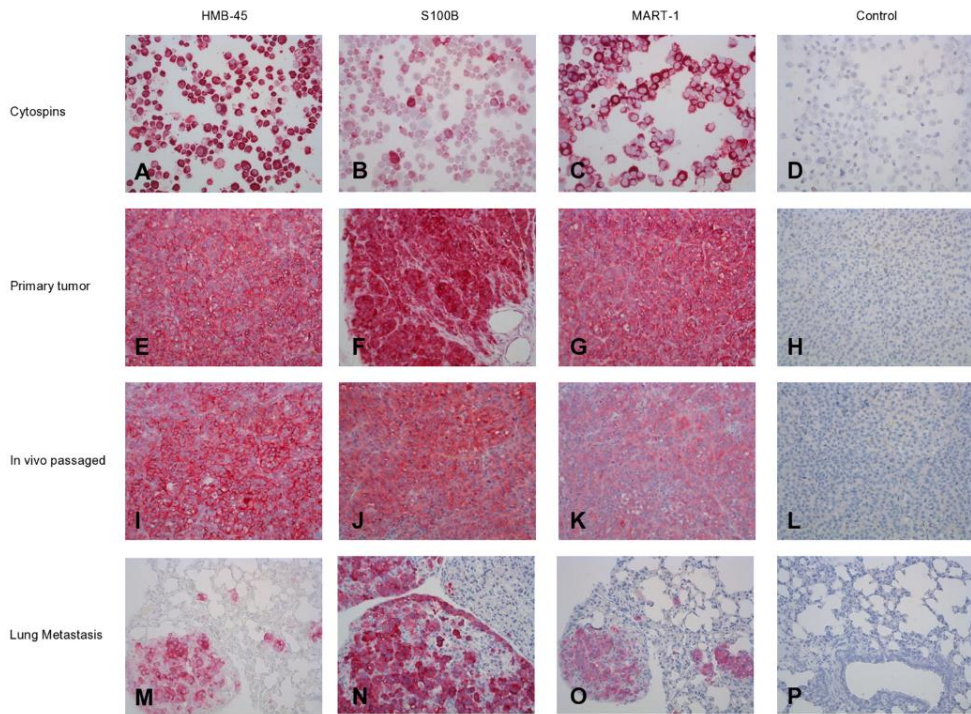


Figure 4. Immunohistochemical analysis to determine the expression of melanocytic markers during tumor development. Immunohistochemical staining of cytopins, tumor xenografts, and metastasis for the melanocyte markers HMB-45, S100B and MART-1; all tumor material was derived from cell line CM2005.1. (A-D) Staining of cytopins (original magnification $\times 200$). (E-H) Primary CM. (I-L) In vivo passaged orthotopic tumor xenograft. (M-P) Lung metastasis (original magnification $\times 200$). Negative controls were stained with secondary antibody alone.

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 intraepithelial 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. [32,33] We injected the tumor cells directly below

the conjunctiva, thereby placing them at a site from where 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. [34]. 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 [35]. 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 [36]. 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 melanocyte 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 IL2 IL2 γ^{null} mice, as recent studies have shown that these mice are highly susceptible to the establishment of metastasis when compared with less immunodeficient mice [13, 37-39]. Nonetheless, formation of metastases was observed only after applying the method of in vivo passaging, indicating that the in vivo environment plays a crucial role in enhancing tumorigenicity when compared with the in vitro environment.

In fact, previous studies have indeed shown that the in vivo passage of tumorigenic cells resulted in more aggressively growing tumor cells, thereby suggesting that the in vivo environment exerts selective pressure and favors malignant transformation. [40, 41] It was noticed that stromal activation and an increase in the expression of hematopoietic growth factors occur due to a switch from paracrine to autocrine growth control. Independence of growth-regulatory mechanisms, together with an improved adaptation of cells for growth in the in vivo environment and an increase in angiogenesis, leads to accelerated enlargement of tumors after in vivo passaging. [40] Together, these data suggest that in vivo passaging will lead to tumor cells with a higher metastatic ability than exhibited by the original cell line, as a result of better adaptation to their microenvironment [40-42]. Moreover, in vivo passaging also helped to overcome one problem frequently associated with the formation of metastasis: the long latency that is often required [29, 43].

At this point we do not know which molecular changes have occurred in the CM cells, causing the in vivo passaged cells to be more malignant. To date, it has not been shown that the subconjunctival space is immunosuppressive as is the

intraocular environment, and we do not know which molecular changes have occurred in the CM cells, causing in vivo-passaged cells to be more malignant. However, we speculate that epigenetic regulation might be one of the options, as has been described in other tumors, in highly specialized environments. An example of epigenetic regulation of metastases comes from an intraocular study: LS174T colon cancer cells were placed in the eye of immunodeficient mice, and subsequently removed and grown in vitro [44]. Analysis showed a downregulation of the expression of CXCR4, a chemokine which plays an important role in tumor cell migration. The cells derived from the anterior chamber showed a higher methyltransferase and deacetylase activity, and the decreased CXCR4 expression was reversed by addition of 5-Aza-2-deoxycytidine, a demethylating agent. We are investigating this option.

Additionally, considering the identification of specific mutations in cutaneous melanoma that has led to the development of new and targeted chemotherapy treatments, we sought to define the mutational status of the cell lines as well as their established xenografts before and after the in vivo passaging. It is of great interest that we detected the BRAF c.1799T>A (V600E) mutation in both the cell lines and tumor xenografts from CRMM-1 and CM2005.1, and the NRAS c.182A>T mutation in cell line CRMM-2 and the corresponding xenografts. This highlights the importance of our murine model, as it facilitates further research to investigate treatments that specifically target these different mutations. Also, it allows the testing of multiple drugs, with or without the use of, for instance, additional MEK inhibitors [23].

Although we show that the use of NOD/SCID IL2 γ^{null} mice offers the opportunity to establish a mouse model for human CM, the mice's lack of a functional immune system limits the possibility for effective studies to investigate therapeutic advances of, for instance, natural immunological treatments. On the other hand, our model provides the ability to test the antitumor effect of specific T cells following direct transfer, from mice with a functional immune system. Furthermore, this model may be used to determine the expression of various immunologically

relevant factors, such as those of major histocompatibility complex and adhesion molecules during tumor progression.

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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References

- [1] Shields, C.L., Markowitz, J.S., Belinsky, I., et al. Conjunctival melanoma: outcomes based on tumor origin in 382 consecutive cases. *Ophthalmology*. 2011;118:389-395. e1-e2.
- [2] Errington, J.A., Conway, R.M., Walsh-Conway, N., et al. Expression of cancer-testis antigens (MAGE-A1, MAGE-A3/6, MAGE-A4, MAGE-C1 and NY-ESO-1) in primary human uveal and conjunctival melanoma. *Br J Ophthalmol*. 2012;96:451-458.
- [3] Shields, J.A., Shields, C.L., and De Potter, P. Surgical management of circumscribed conjunctival melanomas. *Ophthal Plast Reconstr Surg*. 1998;14:208-15.
- [4] Westekemper, H., Freistuehler, M., Anastassiou, G., et al. Chemosensitivity of conjunctival melanoma cell lines to chemotherapeutic agents. *Int J Clin Pharmacol Ther*. 2011;49:78-80.
- [5] Lim, L.A., Madigan, M.C., and Conway, R.M. Conjunctival melanoma: a review of conceptual and treatment advances. *Clin Ophthalmol*. 2013;6:521-531.
- [6] Damato, B. and Coupland, S.E. Management of conjunctival melanoma. *Expert Rev Anticancer Ther*. 2009;9:1227-1239.
- [7] Karim, R. and Conway, R.M. Conservative resection and adjuvant plaque brachytherapy for early-stage conjunctival melanoma. *Clin Experiment Ophthalmol*. 2011;39:293-298.
- [8] Nareyck, G., Wuestemeyer, H., von der Haar, D., Anastassiou, G. Establishment of two cell lines derived from conjunctival melanomas. *Exp Eye Res*. 2005;81:361-362.
- [9] Li, M., Zhou, M., Gong, M., et al. A novel animal model for bone metastasis in human lung cancer. *Oncol Lett*. 2012;3:802-806.
- [10] Greiner, D.L., Hesselton, R.A., and Shultz, L.D. SCID mouse models of human stem cell engraftment. *Stem Cells*. 1998;16:166-177.
- [11] Quintana, E., Shackleton, M., Sabel, M.S., et al. Efficient tumour formation by single human melanoma cells. *Nature*. 2008;456:593-598.
- [12] Schatton, T., Murphy, G.F., Frank, N.Y., et al. Identification of cells initiating human melanomas. *Nature*. 2008;451:345-349.
- [13] Shtivelman, E., Namikawa, R. Species-specific metastasis of human tumor cells in the severe combined immunodeficiency mouse engrafted with human tissue. *Proc Natl Acad Sci U S A*. 1995;92:4661-4665.
- [14] Iorns, E., Drews-Elger, K., Ward, T.M., et al. A new mouse model for the study of human breast cancer metastasis. *PLoS One*. 2012;7:e47995.
- [15] Folberg, R., Baron, J., Reeves, R.D., Stevens, R.H., Bogh, L.D. Animal model of conjunctival primary acquired melanosis. *Ophthalmology*. 1989;96:1006-1013.
- [16] Alexandrescu, D.T., Ichim, T.E., Riordan, N.H., et al. Immunotherapy for melanoma: current status and perspectives. *J Immunother*. 2010;33:570-590.
- [17] Jandus, C., Speiser, D., Romero, P. Recent advances and hurdles in melanoma immunotherapy. *Pigment Cell Melanoma Res*. 2009;22:711-723.
- [18] Gear, H., Williams, H., Kemp, E.G., Roberts, F. BRAF mutations in conjunctival melanoma. *Invest Ophthalmol Vis Sci*. 2004;45:2484-2488.
- [19] Spendlove, H.E., Damato, B.E., Humphreys, J., et al. BRAF mutations are detectable in conjunctival but not uveal melanomas. *Melanoma Res*. 2004;14:449-452.

- [20] Goldenberg-Cohen, N., Cohen, Y., Rosenbaum, E., et al. T1799A BRAF mutations in conjunctival melanocytic lesions. *Invest Ophthalmol Vis Sci.* 2005;46:3027-3030.
- [21] Lake, S.L., Damato, B.E., Dopierala, J., et al. Multiplex ligation-dependent probe amplification analysis of uveal melanoma with extraocular extension demonstrates heterogeneity of gross chromosomal abnormalities. *Invest Ophthalmol Vis Sci.* 2011;52:5559-5564.
- [22] Griewank, K.G., Westekemper, H., Murali, R., et al. Conjunctival melanomas harbor BRAF and NRAS mutations and copy number changes similar to cutaneous and mucosal melanomas. *Clin Cancer Res.* 2013;19:3143-3152.
- [23] Flaherty, K.T., Infante, J.R., Daud, A., et al. Combined BRAF and MEK inhibition in melanoma with BRAF V600 mutations. *N Engl J Med.* 2012;367:1694-1703.
- [24] Flaherty, K.T., Puzanov, I., Kim, K.B., et al. Inhibition of mutated, activated BRAF in metastatic melanoma. *N Engl J Med.* 2010;363:809-819.
- [25] Keijser, S., Maat, W., Missotten, G.S., de Keizer, R.J. A new cell line from a recurrent conjunctival melanoma. *Br J Ophthalmol.* 2007;91:1566-1567.
- [26] Capes-Davis, A., Reid, Y.A., Kline, M.C., et al. Match criteria for human cell line authentication: where do we draw the line? *Int J Cancer.* 2013;132:2510-2519.
- [27] Maat, W., Kilic, E., Luyten, G.P., et al. Pyrophosphorolysis detects B-RAF mutations in primary uveal melanoma. *Invest Ophthalmol Vis Sci.* 2008;49:23-27.
- [28] Wong, C.W., Fan, Y.S., Chan, T.L., et al. BRAF and NRAS mutations are uncommon in melanomas arising in diverse internal organs. *J Clin Pathol.* 2005;58:640-644.
- [29] Francia, G., Cruz-Munoz, W., Man, S., Xu, P., Kerbel, R.S. Mouse models of advanced spontaneous metastasis for experimental therapeutics. *Nat Rev Cancer.* 2011;11:135-141.
- [30] Missotten, G.S., Keijser, S., De Keizer, R.J., De Wolff-Rouendaal, D. Conjunctival melanoma in the Netherlands: a nationwide study. *Invest Ophthalmol Vis Sci.* 2005;46:75-82.
- [31] Paridaens, A.D., Minassian, D.C., McCartney, A.C., Hungerford, J.L. Prognostic factors in primary malignant melanoma of the conjunctiva: a clinicopathological study of 256 cases. *Br J Ophthalmol.* 1994;78:252-259.
- [32] Damato B, Coupland SE. Conjunctival melanoma and melanosis: a reappraisal of terminology, classification and staging. *Clin Experiment Ophthalmol.* 2008;36:786–795.
- [33] Eagle RC. *Eye Pathology: An Atlas and Text.* Philadelphia:Williams & Wilkins; 2011.
- [34] Heegaard, S., Jensen, O.A., Prause, J.U. Immunohistochemical diagnosis of malignant melanoma of the conjunctiva and uvea: comparison of the novel antibody against melan-A with S100 protein and HMB-45. *Melanoma Res.* 2000;10:350-354.
- [35] Sharara, N.A., Alexander, R.A., Luthert, P.J., Hungerford, J.L., Cree, I.A. Differential immunoreactivity of melanocytic lesions of the conjunctiva. *Histopathology.* 2001;39:426-431.
- [36] Keijser, S., Missotten, G.S., Bonfrer, J.M., et al. Immunophenotypic markers to differentiate between benign and malignant melanocytic lesions. *Br J Ophthalmol.* 2006;90:213-217.
- [37] Nemeth, J.A., Harb, J.F., Barroso, U., Jr., et al. Severe combined immunodeficient-hu model of human prostate cancer metastasis to human bone. *Cancer Res.* 1999;59:1987-1993.
- [38] Yonou, H., Yokose, T., Kamijo, T., et al. Establishment of a novel species- and tissue-specific metastasis model of human prostate cancer in humanized non-obese diabetic/severe combined immunodeficient mice engrafted with human adult lung and bone. *Cancer Res.* 2001;61:2177-

- 2182.
- [39] Kuperwasser, C., Dessain, S., Bierbaum, B.E., et al. A mouse model of human breast cancer metastasis to human bone. *Cancer Res.* 2005;65:6130-6138.
 - [40] Mueller, M.M., Fusenig, N.E. Constitutive expression of G-CSF and GM-CSF in human skin carcinoma cells with functional consequence for tumor progression. *Int J Cancer.* 1999;83:780-789.
 - [41] Mueller, M.M., Peter, W., Mappes, M., et al. Tumor progression of skin carcinoma cells in vivo promoted by clonal selection, mutagenesis, and autocrine growth regulation by granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor. *Am J Pathol.* 2001;159:1567-1579.
 - [42] Bruns, C.J., Harbison, M.T., Kuniyasu, H., Eue, I., Fidler, I.J. In vivo selection and characterization of metastatic variants from human pancreatic adenocarcinoma by using orthotopic implantation in nude mice. *Neoplasia.* 1999;1:50-62.
 - [43] Khanna, C., Hunter, K. Modeling metastasis in vivo. *Carcinogenesis.* 2005;26:513-523.
 - [44] Li, H., Niederkorn, J.Y., Sadegh, L., Mellon, J., Chen, P.W. Epigenetic regulation of CXCR4 expression by the ocular microenvironment. *Invest Ophthalmol Vis Sci.* 2013;54:234-243.

