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In-vivo xenograft murine human uveal melanoma model develops hepatic micrometastases

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Abstract

The purpose of the study is to develop a mouse ocular melanoma model with human uveal melanoma cells that forms hepatic micrometastases. Human uveal melanoma Mel290 cells were transfected with a lentiviral-enhanced green fluorescent protein (EGFP) expression vector. Proliferation assays were performed by comparing Mel290-EGFP and Mel290 cells. After stable expression of EGFP and proliferation was ascertained, 1×10^6 Mel290-EGFP cells were introduced into NU/NU mice by posterior compartment (PC) inoculation or tail vein injection. Control groups were inoculated or injected with Mel290 cells. Ocular and hepatic frozen sections were examined by fluorescence microscopy, and the number of hepatic micrometastases was determined. EGFP expression was observed at 24 h after transfection. At 72 h after transfection, more than 70% of Mel290 cells expressed EGFP. At 45 days (six passages), 90% of Mel290 cells stably expressed EGFP. Histologic examination showed that Mel290-EGFP cells formed hepatic micrometastases after either PC inoculation or tail vein injection. A significant difference in the number of hepatic micrometastases between PC inoculation and tail vein injection ($P < 0.01$) was observed. Mel290-EGFP cells stably expressed green fluorescent protein *in vitro* at 45 days (six passages). These cells formed hepatic micrometastases in NU/NU mice after PC inoculation or tail vein injection, with significantly more micrometastases developing in the PC inoculation model than after tail vein injection.

Keywords

animal model; hepatic metastasis; micrometastasis; uveal melanoma

Introduction

Melanoma of the uveal tract is the most common primary intraocular malignancy in adults. Local tumor control has improved in the last several decades. Five-year mortality owing to metastases from uveal melanoma to the liver, however, remains high. The liver is the sole site or initial site of metastasis in more than 50% of patients [1–3]. Ocular melanoma spreads to other sites, and generally occurs in association with hepatic metastases. Lorigan *et al* [3] found that hepatic metastases were developed in 92% of patients and the liver was the only organ involved at initial presentation of metastatic disease in 55% of patients with metastatic uveal

melanoma. The mortality rate of patients who develop metastatic uveal melanoma to the liver is essentially 100% [3]. The autopsy frequency of hepatic metastases is 99% in patients who succumb to metastatic uveal melanoma [4]. Treatment of hepatic metastases is paramount for improving survival of patients with uveal melanoma.

Animal models of metastatic human uveal melanoma are essential for investigating the pathogenesis of these metastases and for developing new therapeutic strategies. Most investigators establish animal models of hepatic metastasis by vein injection [5,6]. The metastatic mechanisms after direct vein injection differ from metastatic mechanisms after tissue implantation of the tumor [7–9]. Metastatic tumor cells originating from cancers of a variety of tissues, such as breast, skin and prostate, may remain dormant for extended periods of time. In uveal melanoma, complete removal of the primary tumor by enucleation may be followed by the growth of metastatic tumor in distant organs, months, years, or even decades after enucleation. The current thinking is that tumor cells have already spread to secondary sites at the time of treatment of the primary tumor and remain as dormant micrometastases [10].

We have developed a murine model of uveal melanoma that forms hepatic micrometastases from mouse cutaneous melanoma cells [11]. To further study the mechanisms of metastasis of human uveal melanoma cells, we transduced a human uveal melanoma cell line with enhanced green fluorescent protein (EGFP) induced by lentivirus and tracked the formation of hepatic micrometastases from the uveal melanoma cells.

Methods

Mice

Six-week-old female NU/NU mice were obtained from Charles River Laboratories (Wilmington, Massachusetts, USA). This immunodeficient nude mouse originated from the National Institutes of Health and was originally considered to be a BALB/c congenic. It was later determined that it was not inbred. This mouse colony is therefore maintained as an outbred colony and is not associated with any stock or strain. These mice are athymic and T-cell deficient. All experiments were conducted according to the Declaration of Helsinki and Guiding Principles in the Care and Use of Animals and conform to the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research.

Cell cultures

Human uveal melanoma Mel290 cells (courtesy of B. Ksander from Schepens Eye Institute, Boston, Massachusetts, USA) were originally established from a biopsy of human uveal melanoma, and exhibit aggressive tumor behavior [12]. These cells were cultured in a 75 cm² flask and maintained at 37°C in a 5% CO₂ incubator. Complete culture medium included Roswell Park Memorial Institute-1640 (Mediatech Inc., Herndon, Virginia, USA) with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, L-glutamine, 10% fetal bovine serum, 1% nonessential amino acids, 1% sodium pyruvate solution, 1% vitamin, and 1% antibiotic–antimycotic solution. After reaching confluence, cells were washed with Hank's solution, and trypsinized with 1 × trypsin–ethylene diamine tetraacetic acid solution. Fresh complete medium was changed twice per week. The cells were expanded to confluence for use in all experiments.

Vector construction

The lentiviral backbone used in these experiments is based on Flip, ubiquitin promoter, GFP, and WRE vector [13,14]. Vector particles were produced in 293FT cells by transient

cotransfection of the transfer vector, the HIV-1 packaging vector Δ R8.9, and the vesicular stomatitis virus glycoprotein envelope glycoprotein into 293T cells. Approximately 72 h after transfection, the virus-containing supernatant was removed, filtered through a 0.45 μ M filter unit, concentrated by centrifugation, aliquoted, and frozen at -80°C . For virus titration, a serial dilution method was used.

Mel290-enhanced green fluorescent protein cells

Human uveal melanoma Mel290 cells were seeded in each well of a six-well plate and incubated. When cells reached 30–50% confluence, 1×10^6 infectious units of lentiviral EGFP expression vector were added. NonEGFP-expressing lentivirus was added to the control wells. At 24 h after transfection, the medium was changed with fresh complete medium. The transfection efficiency was observed daily with a Nikon Eclipse E300 inverted fluorescence microscope (Nikon, Tokyo, Japan) and determined by counting the total number of cells without a fluorescent filter and the number of green fluorescent protein (GFP) cells with a green fluorescent filter (560 nm) in the same field under inverted fluorescent microscopy. Mel290 cells which were transfected with lentiviral EGFP expression vector were designated as Mel290-EGFP cells.

Proliferation assay

The CyQUANT Cell Proliferation Assay Kit (Molecular Probes, Eugene, Oregon, USA) was used for this experiment. Concentrated Mel290 and Mel290-EGFP cells were suspended in growth medium. One thousand cells were seeded in 96-well plates. The cells were incubated for 48 h at 37°C and 5% CO_2 , the medium was removed from the wells, and the cells were frozen in the microplate and stored at -70°C . To create a reference cell number standard curve, 10^6 cells/ml suspensions were prepared in complete medium. The suspensions were centrifuged, the supernatant was removed, and the cell pellet was frozen at -70°C . The cell pellet was thawed to room temperature, and 1.0 ml of cell lysis buffer was added to resuspend the cell pellet. 200 μ l dilutions containing 50–50 000 cells or no cells were used to generate a standard curve for correlating cell number with absorbance. Dilutions corresponding to cell numbers ranged from 50 to 50 000 in 200 μ l. A 200 μ l sample with no cells served as a control. At the time of preparation of the reference cells, the plate with samples was thawed to room temperature, and 200 μ l cell lysis buffer was added to each sample well. The plate was incubated for 5 min. The fluorescence absorbance in each sample and the standard wells was measured directly with a microplate fluorescence reader (Bio-Tec Instruments, Inc., Winooski, Vermont, USA) with approximately 480 nm excitation and approximately 520 nm emission filters. The fluorescence absorbance was automatically adjusted to the number of cells in each well in the microplate reader. Each sample was cultured in triplicate.

Xenograft posterior compartment inoculation model

Details of technique have been described elsewhere [15]. All mice were anesthetized with ketamine and xylazine (45 and 4.5 $\mu\text{g/g}$, respectively). Aliquots of 1×10^6 cells per 2.5 μ l were inoculated into the choroid of the right eyes using a transcleral technique that allows the inoculated cells to remain in the eye [6]. Under an operating microscope, a tunnel was prepared from the limbus within the sclera to the choroid with a 30.5 gauge needle. The tip of a 10 μ l glass syringe with a blunt metal needle (Hamilton, Reno, Nevada, USA) was introduced into the choroid of the right eye through the needle track, and no cells were inoculated until the needle tip was inside the eye. A 2.5 μ l suspension of Mel290-EGFP or Mel290 cells was inoculated. No tumor cell reflux occurred, and the subconjunctival space remained free of tumor cells. The right eyes were enucleated 7 days after inoculation. Digital photographs of the eyes were obtained before enucleation using the Leica MZ16FA microsystem (Leica Microsystems GmbH, Wetzlar, Germany) with GFP2 filter. The mice were killed at 3 weeks

after enucleation and necropsies were performed. Hepatic frozen sections were observed under fluorescence microscopy at 4 weeks after enucleation. Two groups ($n = 10$) were present for posterior compartment (PC) inoculation: group 1 – the choroid of the right eyes in mice was inoculated with Mel290–EGFP cells; and group 2 – the choroid of the right eyes in mice was inoculated with Mel290. This experiment was performed in duplicate and the average values of both experiments were determined.

Xenograft model of tail vein injection

Cells (1×10^6) of Mel290–EGFP or Mel290 were introduced into NU/NU mice by tail vein injection. Hepatic frozen sections were observed under fluorescence microscopy at 4 weeks after tail vein injection. Two groups ($n = 10$) were present for tail vein injection; group 3 – mice were injected with Mel290–EGFP cells via the tail vein; and group 4 – mice were injected with Mel290 cells via the tail vein. This experiment was performed in duplicate and the average values of both experiments were determined.

Tissue preparation

For observation of EGFP expression, four enucleated eyes in group 1 or group 2 that were inoculated with Mel290–EGFP or Mel290 were placed in optimum cutting temperature (Sakura Finetek, Inc., Torrance, California, USA) compound and snap-frozen in liquid nitrogen. Frozen sections ($5 \mu\text{m}$) were fixed in ice-cold acetone, and stained with propidium iodide fluorescent nuclear stain. The slides were examined under fluorescence microscopy. The remaining six eyes in groups 1 or 2 were submitted in 4% neutral buffered formalin and routinely processed for light microscopic examination. Serially adjacent $5\text{-}\mu\text{m}$ thick sections were stained with hematoxylin and eosin and evaluated for the presence and location of melanoma.

For observation of EGFP expression, the livers from four mice in each group (groups 1–4) were collected at 4 weeks after inoculation in optimum cutting temperature compound and snap-frozen in liquid nitrogen. The frozen hepatic tissues were sectioned, fixed in ice-cold acetone, and stained with propidium iodide fluorescent nuclear stain. The slides were examined and photographed with the Nikon Eclipse E800 fluorescence microscope (Nikon). For assessment of hepatic micrometastases, the remaining six livers in each group (groups 1–4) were obtained at necropsy performed 4 weeks after inoculation, submitted in 4% neutral buffered formalin, and routinely processed for light microscopic examination.

Quantification of micrometastasis

Six sections through the center of the liver were microscopically examined (Olympus BX41, Tokyo, Japan) for the presence of micrometastases ($< 100 \mu\text{m}$ diameter) and the average number of micrometastases per section was determined. This has been shown to be a reliable and reproducible method for detecting hepatic micrometastasis in our murine model [15,16].

Statistical analysis

Statistical analyses were performed by the independent t -test. Values reported in the tables are mean $\pm$ SEM.

Results

Mel290-enhanced green fluorescent protein cell line

At 24 h after lentiviral EGFP vector transduction, EGFP expression was observed in Mel290–EGFP cells via inverted fluorescence microscopy. At 45 days, six passages, 90% of Mel290–EGFP cells stably exhibited EGFP (Fig. 1a). Control Mel290 cells did not exhibit green fluorescence (Fig. 1b).

Mel290 and Mel290–enhanced green fluorescent protein proliferation assay

The proliferation rate of Mel290–EGFP cells was similar to that of Mel290 cells, respectively (Fig. 2). No difference in the cell proliferation rate between Mel290 and Mel290–EGFP cells (*t*-test, $P > 0.05$) was observed. This indicated that the proliferation rate of Mel290 cell *in vitro* was not affected by transfection of lentiviral EGFP vector.

Posterior compartment inoculation

Human uveal melanoma Mel290–EGFP or Mel290 cells were successfully inoculated into the choroid of the NU/NU mouse via the translimbal approach. At 1 week after inoculation, a fluorescent filter was used to observe in-vivo fluorescence of the intraocular tumor through a dilated pupil. The fundus of the eye inoculated with Mel290–EGFP cells expressed green fluorescence (group 1, Fig. 3a), which was not observed in mice inoculated with Mel290 cells (group 2, Fig. 3b). Histologic examination of hematoxylin and eosin (H&E)-stained slides of eyes enucleated at 7 days after inoculation demonstrated Mel290–EGFP and Mel290 cells forming tumors in the PC (choroid and vitreous) of the eye (Fig. 4d and h), periodic acid Schiff-positive components of loops and networks (Fig. 5a), and red blood cells in vessels (Fig. 5b). These findings indicated that tumor growth in the mouse eye was similar to uveal melanoma growth in humans. After propidium iodide fluorescent nuclear staining, green fluorescence was found in the tumor located in the choroid in group 1 mice (Fig. 4a–c), but not in the tumor in group 2 (control) mice (Fig. 4e–g). The mice survived tumor growth in the eye and development of micrometastasis. Frozen hepatic tissue obtained during necropsy of group 1 mice at 28 days after inoculation and stained with H&E showed islands of cells (micrometastases) that corresponded with the EGFP expressing micrometastases (Fig. 6a–d). The hepatic micrometastases in group 2 did not exhibit green fluorescence (Fig. 6e–h). The incidences of liver micrometastases in both of groups are 100%.

Xenograft tail vein injection

The mice survived tumor growth in the eye and development of micrometastasis. Histologic examination of H&E-stained slides showed that Mel290–EGFP and Mel290 cells formed hepatic micrometastases (Fig. 7d and h) in NU/NU mice after tail vein injection. EGFP expression was found by fluorescence microscopy in hepatic micrometastases of mice injected with Mel290–EGFP (group 3) compared with no EGFP expression in hepatic micrometastases in the control Mel290 (group 4) specimens (Fig. 7a–g). The micrometastases were confirmed by H&E staining.

Comparison of hepatic micrometastasis in posterior compartment and tail vein models

Hepatic tissue from all of four groups were collected and examined at 4 weeks after inoculation of human uveal melanoma cells. No difference was observed between the numbers of micrometastases in the first and second experiments ($P > 0.05$). The average number of hepatic micrometastasis in duplicated experiments was 59.07 ± 16.09 in group 1 (Mel290–EGFP cells inoculated into the posterior chamber), and 58.64 ± 17.97 in group 2 (Mel290 cells inoculated to posterior chamber). No significant difference in the number of hepatic micro-metastasis between group 1 and group 2 (*t*-test, $P > 0.05$) was observed. The number of hepatic micrometastasis was 23.50 ± 9.88 in group 3 (Mel290–EGFP cells injected into the tail vein) and 24.21 ± 7.65 in group 4 (Mel290 cells were injected into the tail vein). No significant difference in the number of hepatic micrometastasis between group 3 and group 4 (*t*-test, $P > 0.05$) was seen. A significant difference between groups 1 and 3 ($P < 0.01$), and between group 2 and 4 ($P < 0.01$, Fig. 8) was observed.

Discussion

Primary uveal melanoma metastasizes before the clinical diagnosis of the intraocular melanoma [17]. This occurs as micrometastases, which have the potential to grow and become vascularized [18], form in the liver, remain dormant, and are clinically undetectable before the primary uveal melanoma growing large enough for ophthalmologic detection. Single primary uveal melanoma cells initiate the formation of a micrometastasis by dividing and forming small colonies of cells that aggregate as a morula within a hepatic sinusoid. These morulas are limited by diffusion to 1–2 mm diameter. In some cases, the morulas quickly progress to a large size and overcome the function of the target organ. In other cases, the morula does not enlarge for months or years. The morula, or micrometastasis, after remaining dormant for years, may grow and result in the death of the host. New therapies are being designed to ensure that the micrometastases remain small and dormant, thus treating micrometastatic melanoma as a chronic disease [19,20].

Human primary uveal melanoma has been transplanted subcutaneously as a xenograft in nude mice [21]. The current concept is that many tumor cells metastasize within emboli consisting of tumor cells that break free from a primary tumor and become associated with platelets and other host cells before arresting at distant sites. After tail vein injection, cells circulate, often in high numbers, as single cells or in small clusters associated with platelets. These single cells may not arrest or interact with target tissues at distant sites in the same way that spontaneous tumor cells metastasize as emboli. This is because the process of spontaneous metastasis from a primary site may be associated with selection events that yield a distinct profile of successful metastases [22]. The development of metastasis is a complex multistep process. A cell must be capable of detaching from the primary tumor, invading through the extracellular matrix, basement membrane, and endothelial cell wall, surviving in systemic circulation, implanting, and subsequently proliferating at the site of metastasis [23]. Primary tumor cells which input into systemic circulation by vein injection are deficient of the process of detachment and invasion. Owing to the complexity of this process, it is difficult to isolate individual events in patients that may be important in the overall progression of the disease from primary tumor to metastasis. The transcriptional profiles are different in human uveal melanoma cells from a primary intraocular tumor, circulating malignant cells, and metastatic cells [24]. Single disseminated tumor cells in the blood often show different properties compared with cells from the primary tumor, including selection of aggressive clones of cells, and are targets for adjuvant therapy [23]. Therefore, orthotopic (ORT) xenografts of human tumors, or tumor cell lines, in nude mice reproduce the histology and metastatic pattern of most human tumors at an advanced stage. For primary tumors, ORT xenograft models are more promising than the most commonly used subcutaneous xenografts in preclinical drug development. For metastatic tumors, ORT xenograft models also are more promising than the xenografts created by vein injection in preclinical drug development [25]. Humanizing mouse models of cancer will most likely require the combined use of currently available methodologies, not just simply inputting primary tumor cells to produce the secondary tumor. In our experiments, tail vein injection of human uveal melanoma cells results in fewer hepatic micrometastases than posterior chamber injection. It suggests that metastasis from the primary ocular tumor from intravenous injection is unique.

Use of ORT transplantation improves human tumor xenograft models and therefore promotion of metastatic spread of the resultant ‘primary’ tumors. It is argued that this disparity can be significantly minimized by use of ORT transplant/metastatic tumor models in which treatment is initiated after the primary tumor has been removed and the distant metastases are well established and macroscopic – that is, the bar is raised and treatment is undertaken on advanced, high volume, and metastatic disease. In such circumstances, survival should be used as an endpoint; changes in tumor burden using surrogate markers or microimaging techniques can

be used as well to monitor effects of therapies on tumor response. Adoption of such procedures would more accurately recapitulate the phase I/II/III clinical trial situation in which treatment is initiated on patients with advanced, high-volume metastatic disease [8].

ORT models are necessary to predict therapy of transplantable tumors in mice [26]. ORT xenograft tumor mouse models like the nude mouse eye inoculated with human uveal melanoma cell Mel290 introduced in this article has significant advantages in relevant sites for host interactions, required for emergence of metastasis, site-specific dependence of therapy, organ-specific expression of genes, quantitated data, duplicated clinical situations with respect to removal of primary tumor, and adjuvant therapy for occult metastasis. Special technique and intensive labor are often needed when establishing ORT xenograft tumor model. The advantages of the ectopic model (e.g. subcutaneous or vein route) are rapid, minimal labor, relatively inexpensive, and quantitated data. The disadvantages of these models are irrelevant host infiltrate and tumor microenvironment and abundance of false-positive responses with drugs [9]. Another mouse uveal melanoma model recently reported is transgenic Tyr-RAS + Ink4a/Arf^{-/-} mice with spontaneous uveal amelanotic melanoma [27]. In transgenic mice, early tumor formation events can be followed. This allows for prevention strategies and early intervention therapies that can be tested. The disadvantages of transgenic mice are difficulties with timing of potential therapies, expense, and potential lack of sufficient numbers needed for therapy groups. Additionally, transgene products may not be relevant and they may be coalterations of cellular pathways coupled with transgene expression [9].

Our laboratory has established a murine uveal melanoma model by PC inoculation of mouse cutaneous melanoma cell lines (B16LS9, B16F10, Queens) into C57Bl/6 mice. All of the cell lines successfully resulted in hepatic micrometastasis at 4 weeks after inoculation (3 weeks after enucleation) [11]. That heterotopic syngeneic model recapitulated the human situation by forming hepatic micrometastases that had the capability to grow [11] or involute, depending on the host immune status [19] and/or intrinsic properties of the melanoma [17,20,28,29]. A deficiency of that model was that it used heterotopic transplantation of cutaneous, not uveal, melanoma. Therefore, we pursued developing an ORT model using human uveal melanoma cells. In our current experiments, we transplanted cultured human uveal Mel290 melanoma cells into the uvea of nude mice eyes. The Mel290 cells grew within the nude mice eyes during 7 days after inoculation, and resulted in hepatic micrometastases during 4 weeks after inoculation (3 weeks after enucleation). This model reflects the clinical situation with regard to location of the primary uveal melanoma, treatment of the primary uveal melanoma (enucleation), and development of hepatic micrometastases after primary uveal melanoma treatment.

Early stages of tumor progression and micrometastasis formation have been difficult to visualize in current models owing to the inability to identify small numbers of tumor cells in host tissues. The sizes of clusters of dormant uveal melanoma cells in the liver are often so small and inconspicuous to identify with routine light microscopy. In this study, we established a human uveal melanoma cell line with stable GFP expression. We have shown no difference in the proliferation rates of the newly created Mel290-EGFP cells and control Mel290 cells. We also developed a new murine model of human uveal melanoma by ORT transplantation of this cell line into murine eyes, which enabled visualization of fluorescent micrometastases composed of single cells or clusters of cells. This model is useful for investigations of the pathobiology of metastatic human uveal melanoma to the liver, including determining the clonal properties of the micrometastases, and screening the new therapy of antimetastatic agents.

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Fig. 1. Mel290-enhanced green fluorescent protein (EGFP) cell line. EGFP expression was observed in Mel290 cells at 24 h after transduction (a) and not observed in control Mel290 cells (b) under inverted fluorescence microscopy. Mel290 cells stably expressed EGFP after 6 weeks and six passages.

Fig. 2.

Mel290 and Mel290-enhanced green fluorescent protein (EGFP) cell proliferation rates. Quantification of Mel290 and Mel290-EGFP was performed with the CyQUANT Cell Proliferation Assay Kit. One thousand cells were seeded in 96-well plates. The plates were incubated at 37°C for another 48 h. Cell number was determined by incubation with CyQUANT dye, and the fluorescence was measured using a fluorescence microplate reader with filters for 480 nm excitation and 520 nm emission. The values of fluorescence measurements were changed to cell numbers by a standard curve. No difference in the cell proliferation was observed between Mel290 and Mel290-EGFP (cell number, $8.09 \times 10^4 \pm 7.05 \times 10^3$ and $8.14 \times 10^4 \pm 0.23 \times 10^3$ respectively; $P > 0.05$). The results are presented as the mean $\pm$ SD. This indicates that the proliferation rate of Mel290 was not affected after transfection by lentiviral EGFP vector.

Fig. 3.

Gross appearance of xenograft models of human uveal melanoma. External examination with the Leicamz16FA microsystem with green fluorescent protein filter, emission 520 nm, excitation 495 nm, shows green fluorescence from Mel290-enhanced green fluorescent protein in the fundus of mice inoculated in the posterior compartment with Mel290-enhanced green fluorescent protein (a), and not in the fundus of mice inoculated in the posterior compartment with Mel290 (b).

Fig 4.

Microscopic findings in enucleated Mel290-enhanced green fluorescent protein (EGFP) and Mel290 eyes. Fluorescent microscopic examination of enucleated eye at 7 days after Mel290-EGFP (a-c) and Mel290 (e-g) inoculation into the posterior compartment. Green fluorescence was found in the melanoma in the Mel290-EGFP group (a-c) and not in the Mel290 group (e-g). Light microscopic examination shows melanoma in the choroid (*) and vitreous (**) of both the Mel290-EGFP (d) and Mel290 (h) eyes. Propidium iodide fluorescent nuclear stain, 100 × (a-g), hematoxylin and eosin, 40 × (d, h).

Fig. 5. Histology of human uveal melanoma microcirculation in a nude mouse. Tissue sections of mouse eye with human uveal melanoma stained by periodic acid Schiff (PAS), without hematoxylin counterstain, viewed by light microscope under a green filter, demonstrates patterns of vascular loops (arrow) forming networks (a). Hematoxylin–eosin-stained section of mouse eye with human uveal melanoma, the structures corresponding to the PAS-positive components of loops, round channels or tubes contain red blood cells (arrow) (b). PAS without hematoxylin counterstaining, photographed with a green filter, 400 × (a); hematoxylin and eosin, 1000 × (b).

Fig. 6. Microscopic findings in livers of posterior compartment model mice obtained at necropsy. Fluorescent microscopic examination of livers obtained at necropsy at 28 days post-inoculation of Mel290-enhanced green fluorescent protein (EGFP) (a–c) and Mel290 (e–g) into the choroid of NU/NU mice. Fluorescent micrometastases are seen in the Mel290–EGFP group (a–c) and not the Mel290 group (e–g). Light microscopic examination shows micrometastases both the Mel290–EGFP group (d) and the Mel290 group (h). Propidium iodide fluorescent nuclear stain, 100 × (a–g); hematoxylin and eosin, 200 × (d, h).

Fig. 7. Microscopic findings in livers of tail vein injection model mice obtained at necropsy. Fluorescent microscopic examination of livers obtained at necropsy at 28 days postinjection of Mel290-enhanced green fluorescent protein (EGFP) (a–c) and Mel290 (e–g) into the tail vein of NU/NU mice. Fluorescent micrometastases (arrows) are seen in the Mel290–EGFP group (a–c) and not the Mel290 group (e–g). Light microscopic examination shows micrometastases (arrows) in both the Mel290–EGFP group (d) and the Mel290 group (h). Propidium iodide fluorescent nuclear stain, 100 × (a–g); hematoxylin and eosin, 200 ×, (d, h).

Fig. 8.

Number of hepatic micrometastasis in posterior compartment (PC) model and tail vein injection model. Statistical analysis (independent *t*-test) demonstrates that there is a significant difference in the number of micrometastases between the Mel290–EGFP PC inoculation model (59.07 ± 16.09) and Mel290–EGFP PC tail vein injection model (23.50 ± 9.88 , $P = 1.75 \times 10^{-7}$). A significant difference between the Mel290 PC inoculation model (58.64 ± 17.97) and the Mel290 tail vein injection model (24.21 ± 7.65 , $P = 5.43 \times 10^{-6}$) was observed. No significant difference between the Mel290–EGFP PC inoculation model (59.07 ± 16.09) and the Mel290 PC inoculation model (58.64 ± 17.97 , $P = 0.94$) was seen. No significant difference between the Mel290–EGFP tail vein injection model (23.50 ± 9.88) and the Mel290 tail vein injection model (24.21 ± 7.65 , $P = 0.83$) was observed. The data are shown as means $\pm$ SD.