Evaluation of (fli:GFP) Casper Zebrafish Embryos as a Model for Human Conjunctival Melanoma

Kelly Cristine de Sousa Pontes,1 Arwin Groenewoud,2 Jinfeng Cao,1,3 Livia Maria Silva Ataide,4 Ewa Snaar-Jagalska,2 and Martine J. Jager1

1Department of Ophthahmolgy, Leiden University Medical Center, Leiden, The Netherlands
2Institute of Biology, Leiden University, Leiden, The Netherlands
3Department of Ophthalmology, The Second Hospital of Jilin University, Changchun, China
4Department of Population Biology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, The Netherlands

Correspondence: Martine J. Jager, Department of Ophthalmology, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands; m.j.jager@lumc.nl.

Submitted: April 9, 2017
Accepted: October 26, 2017

Purpose. Conjunctival melanoma (CM) is a rare malignant disease that can lead to recurrences and metastases. There is a lack of effective treatments for the metastases, and we set out to develop a new animal model to test potential therapies. Zebrafish are being used as a model for many diseases, and our goal was to test whether this animal could be used to study CM.

Methods. Three human CM cell lines (CRMM-1 and CM2005.1, which both harbor a B-RAF mutation, and CRMM-2, which has an N-RAS mutation) were injected into the yolk sac, around the eye, and into the duct of Cuvier of transgenic (fli:GFP) Casper zebrafish embryos. Fluorescent and confocal images were taken to assess the phenotype and the behavior of engrafted cells and to test the effect of Vemurafenib as a treatment against CM.

Results. While the cells that had been injected inside the yolk sac died and those injected around the eye sporadically went into the circulation, the cells that had been injected into the duct of Cuvier colonized the zebrafish: cells from all three cell lines proliferated and disseminated to the eyes, where they formed clusters, and to the tail, where we noticed extravasation and micrometastases. Vemurafenib, a potent agent for treatment of B-RAF V600E-positive melanoma, inhibited outgrowth of CRMM-1 and CM2005.1 cells in a mutation-dependent way.

Conclusions. The (fli:GFP) Casper zebrafish embryo can be used as an efficient animal model to study metastatic behavior of human CM cells and warrants further testing of drug efficacy to aid care of CM patients.

Keywords: conjunctival melanoma, zebrafish, image analysis, vemurafenib, cancer

conjunctival melanoma (CM) is a rare malignant ocular disease, accounting for 5% to 10% of all human ocular melanomas.1 Over the past decades, its incidence has increased worldwide.2–4 The current treatment of choice for primary CM is wide surgical excision, combined with brachytherapy, cryotherapy, and topical chemotherapy (e.g., mitomycin C). However, effective targeted therapies have not yet been developed to treat this malignancy5; CM’s high recurrence rate is associated with metastasis and poor prognosis.6–8 Furthermore, the mortality rate is high, ranging from 13% to 38% after 10 years.9–11

In this malignancy, essential mutations occur in the B-RAF and N-RAS genes.5,12–14 B-RAF mutations constitutively activate the mitogen-activated protein kinase (MAPK) pathway and its downstream kinases MEK1/2-ERK1/2, promoting tumor proliferation.15,16

Mice have previously been used as a model to study human CM,17,18 but there are some limitations. The major disadvantages are a slow growth and spread of the tumor, which can take weeks to months, and the high cost for reproduction and housing. The cost increases further when immunosuppressive drugs are needed to prevent tumor rejection.19 Therefore, there is a need to find a new animal model.

The zebrafish model has been used widely in research because of its advantages, such as (1) the fish’s high fecundity and short time between generations, (2) the high interspecies conservation of molecular pathways between zebrafish and mammals, (3) their transparency, allowing direct imaging of development, organogenesis, and cancer progression, which enables tracking of transplanted cells, (4) the possibility of xenotransplantation, and (5) their permeability to small molecular weight compounds from water, enabling easy delivery and efficient screening of large numbers of anticancer compounds.19 Furthermore, the fact that their adaptive immune system does not reach maturity until 4-weeks postfertilization allows them to be used without the need for immunosuppression in the embryonic stages.20

There are no studies showing whether zebrafish embryos can be used as an animal model for human CM, and our goal was to determine if this animal can be used as a screening platform based on the xenotransplantation of three human CM cell lines. Our group has shown that two of three available CM cell lines, CRMM125 and CM2005.1,26 harbor a B-RAF V600E mutation, while the third, CRMM2, contains an N-RAS Q61L mutation.17,27 We injected stable red fluorescently labeled (lentiviral tdTomato-blas) CM cells via different routes into the
embryonic zebrafish. Thus, we determined the most effective engraftment strategy for the establishment of CM xenograft tumors in zebrafish and we observed distinct phenotypes after implantation of the three CM cell lines. We subsequently validated the model through the use of the well-known B-RAF inhibitor, vemurafenib.

**MATERIAL AND METHODS**

**Cell Culture**

We used three CM cell lines, CRMM-1, CRMM-2, and CM2005.1, all generated from recurrent primary CM. The CRMM-1 and CRMM-2 cell lines, isolated by Nareyeck et al., were cultured in F-12K nutrient mixture, Kaighn’s modification (Gibco, Life Technologies, Bleiswijk, The Netherlands), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Greiner Bio-one, Alphen aan den Rijn, The Netherlands) and 1% Penicillin/Streptomycin (Gibco). CM2005.1, established in 2007 by Keijser et al., was cultured in RPMI 1640 Dutch modified medium (Gibco), supplemented with 10% FBS (Greiner Bio-one), 1% GlutaMAX, and 1% Penicillin/Streptomycin (Gibco). To generate CM cells with red fluorescence, cells were stably transduced with lentivirus expressing both tandem dimer (td)Tomato and Blasticidin-S, as previously described.

**Virus-containing medium** was replaced with fresh medium containing Blasticidin-S (2 µg/mL) to select transduced cells. Transduction of the cells with the tdTomato-expressing virus did not alter the growth pattern of parental cells. After transduction, cells were incubated with multiplicity of infection (MOI) of 2.0 in medium with 8.0-µg/mL polybrene for 16 hours. For cultivation of stable transgenic tdTomato-expressing cells, BLASticidin-S (2 µg/mL) was added to the complete medium.

**Growth Kinetics of Tomato-Red Cells In Vitro**

Transgenic tdTomato-expressing cell lines were seeded in triplicate in 96-well plates at a density of 600, 1200, and 2400 for CRMM-1 and CRMM-2 cell lines in a total volume of 100 µL of medium. Because the CM2005.1 cell line is smaller than the others, it was seeded in triplicate in 96-well plates at a density of 1000, 2000, and 4000 cells per well, in a total volume of 100 µL of medium. For testing vemurafenib, cells were seeded at a density of 2000 (CRMM-1 and CRMM-2) or 3500 (CM2005.1) cells per well, in a total volume of 100-µL medium. Cell proliferation was analyzed at 1, 3, and 5 days of incubation by an In-Cell Western assay (Odyssey Infrared Imaging System, LI-COR, Leusden, The Netherlands); after removing the medium, cells were fixed for 1 hour in 4% formaldehyde and incubated with 100 µL of primary antibody (1:1000, Abcam, Cambridge, UK) at RT for 2 hours followed by washing three times using PBS-TX for 10 minutes and put in blocking buffer at room temperature (RT) for 1 hour. After washing, 1% Tween-PBS buffer, plates were scanned with an Odyssey Infrared Imaging System (LI-COR). Odyssey 3.0 software was used to quantify signal intensity.

**Animals and Injection Sites**

The *HtGFp Casper* transgenic zebrafish were maintained according to standard protocols (http://ZFIN.org, in the public domain) and in compliance with Dutch animal welfare regulations and European Union Animal Protection Directive 2010/63/EU. Our research followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

When the cells reached 75% to 90% confluency, they were trypsinized (0.05% trypsin/EDTA; Gibco), centrifuged for 4 minutes at 200g, washed with Dulbecco’s phosphate-buffered saline (DPBS; Invitrogen), and diluted to 250 cells/mL in 2% polyvinylpyrrolidone-40 (PVP-40; Calbiochem, San Diego, CA, USA).

At 2-days postfertilization (dpf), dechorionated zebrafish embryos were injected with this CM cell suspension using glass capillary needles with an opening of approximately 20 to 30 µm. Embryos were anesthetized with 2% tricaine (Sigma-Aldrich Corp., Zwijndrecht, The Netherlands) and positioned in a petridish covered with 1% agarose. Using a pneumatic picopump and a manipulator (World Precision Instruments, Sarasota, FL, USA), 200 to 400 cells were injected inside the yolk sac in one group of embryos, or inside the duct of Cuvier in a second group, and 50 to 100 cells were injected around the right eye in a third group of zebrafish. The embryos were each placed individually in a well of a 48-well plate, with 1 mL of egg water (60-µg/mL OceanSalt in demi water) in each well and maintained at 34°C, which was the optimal temperatures for cell growth and zebrafish embryo development. The egg water was refreshed daily and the injected embryos were evaluated at 2, 4, and 6-days postinjection (dpi), using a fluorescence stereo microscope (Leica M205FA; Leica Microsystems, Inc., Buffalo Grove, IL, USA).

**Kaplan-Meier Survival Analysis of Injected Embryos**

After establishing the optimal injection site for CM cells in zebrafish, we determined how injection of cancer cells influenced embryo survival, shown in a Kaplan-Meier survival analysis (cumulative survival curve). Tumor cells were injected into the Duct of Cuvier at 2 dpf. One group (n = 90) was injected with CRMM-1, a second group (n = 201) with CRMM-2, and the third group (n = 221) received an injection with CM2005.1. A fourth group (n = 121) received an injection with PVP-40 and the last group (n = 96) was not injected. The number of injected cells was between 200 and 400 per embryo. After infection, the embryos were maintained at 34°C, and scored daily for survival, without changing the egg water, until 6 dpi.

**Phenotype of CM Cell Lines in Zebrafish and Cell Migration**

The embryos were injected with CRMM-1, CRMM-2, or CM2005.1 cells and screened at 1 dpi under the same conditions as described above. Embryos were anesthetized with 2% tricaine at 1, 4, and 6 dpi to perform image analysis using a fluorescence stereo microscope and a confocal microscope (Leica TCS SPE; Leica Microsystems, Inc.). For cell growth quantification, the pixel numbers that represent the amount of cells were counted at 1 and 6 dpi, using ImageJ software. Statistical analysis was performed using R version 2.15.1.

**Immunohistochemistry (IHC)**

After 6 dpi, injected whole embryos were fixed in 4% paraformaldehyde and stored in 100% methanol at −20°C. To perform IHC, embryos were rehydrated, washed with PBS-TX, and permeabilized with 10 µg/mL of protease K in PBS-TX at 37°C (in a water bath) for 10 minutes. Then, they were washed three times using PBS-TX for 10 minutes and put in blocking buffer at room temperature (RT) for 1 hour. Following this, whole embryos were incubated with Ki67 rabbit antibody at a dilution of 1:200 (Abcam, Cambridge, UK), at RT for 2 hours.
1% low melting agar to take pictures with a confocal microscope. Using ImageJ software, pixel numbers were determined. Photographs taken of the same embryo that had been injected with CM cells around the eye at 1 (A) and 6 dpi (B), showing cells inside the head (white arrows) and in the tail (yellow arrow). Following injection in the yolk sac, an embryo shows the cells in the yolk sac at 1 (C), but not 6 dpi (D). After injection of cells into the duct of Cuvier, cells are seen inside the circulation at 1 dpi (E), mainly in the tail and inside the eye. The same embryo shows a cluster in the tail and cells inside the eye at 6 dpi (F). The stereo fluorescent images (original magnification: >20) are representative of >10 independent experiments.

To establish the model, we tested three different injection sites: engraftment around the eye, in the yolk sack, or in the duct of Cuvier. The duct of Cuvier is the common cardinal vein formed by the left and right posterior cardinal veins joining up with the anterior cardinal vein. The duct of Cuvier functions as an embryonic vein structure collecting all venous blood and leads directly to the heart’s sinus venosus; it carries the blood ventrally across the yolk sac. Using this site of injection ensures a rapid and a near complete dissemination of injected cancer cells throughout the blood circulation.

Injecting tumor cells around the eye was technically challenging because of the small size of the eye, limiting throughvput and increasing lethality. After injecting tumor cells around the eye, the cells disseminated to the head, inside the eye, and inside the circulation (Figs. 1A, 1B). Injecting cells inside the yolk sac was easy to perform, but after 6 dpi, many cells had died (Figs. 1C, 1D). Injections into the duct of Cuvier were relatively easy to perform and cells survived and proliferated (Figs. 1E, 1F).

The injection into the duct of Cuvier ensures that the cells have access to the endothelium and their intrinsic adhesion molecules and nutrients and helps to disseminate the cells throughout the body. As the duct of Cuvier is the most reliable and biologically relevant injection site, we used this site in all subsequent experiments. The cumulative survival curves of all groups were above 80% (Fig. 2).

### PhenoType of CM Cells in Zebrafish

After cells had been injected inside the duct of Cuvier, migration was assessed. With all three cell lines, 10% to 50% of the embryos had cells inside the eye and between 58% and 64% of embryos showed cells in the tail (Fig. 5). At 1 dpi, cells from all three cell lines had disseminated to the eye and to the tail, forming clusters at 4 and 6 dpi, with more prominent clusters occurring when we used cell line CM2005.1 (Fig. 4). Cells from all three cell lines grew inside, outside, and around vessels during the 6 days of observation (Fig. 4). More tumor...
cells were observed at 6 dpi than at 1 dpi (P < 0.001 for CRMM-1, P = 0.04 for CRMM-2, and P = 0.001 for CM2005.1) (Fig. 5).

**Immunohistochemistry (IHC)**

As the image analysis suggested that the cells had divided inside the zebrafish, we tested this using IHC with the Ki67 antibody at 6 dpi. Some cells from all three CM cell lines stained positive for Ki67 at 6 dpi and in some cases, mitotic figures in tumor cells were observed (Fig. 6). These findings show that the CM cells proliferated 6 days after injection inside the duct of Cuvier.

**Toxicity Test and Treatment With Vemurafenib In Vivo**

Vemurafenib inhibits the proliferation of the CM cell lines in vitro in a mutation-dependent way (Supplementary Fig. S2) and was, therefore, used to test the in vivo model. The toxicity test resulted in 94% survival at 7 and 8 dpf at a concentration of 0.25 μM, and 94% at 8 dpf when the 0.5-μM concentration was used (Supplementary Fig. S3). For all the other tested concentrations, survival of the embryos was 100%. As the drug concentration was considered nontoxic when survival was equal or higher than 80%, we concluded that vemurafenib was nontoxic to the zebrafish at the evaluated concentrations.
Considering that the highest concentrations of vemurafenib that had been evaluated in vitro were 3.2 µM (CRMM-1 and CRMM-2) and 0.32 µM (CM2005.1), and that this compound was nontoxic to the embryos up to a concentration of 4.0 µM, we chose a final concentration 4.0 µM/mL to treat engrafted embryos up to 5 dpi because the compound was added in egg water.

At 5-days post treatment with 4 µM of vemurafenib, we noticed inhibition of CRMM-1 (Fig. 7A) and CM2005.1 (Fig. 7C) cell growth when compared with control groups; proliferation of CRMM-2 was not affected by vemurafenib (Fig. 7B; vemurafenib treatment versus control P = 0.013 for CRMM-1, P = 0.007 for CM2005.1, and P = 0.33 for CRMM-2).

**DISCUSSION**

Our results showed that when CM cells were injected around the eye (at 2 dpf), they accidentally passed into the circulation and frequently ended up in the tail, head, and ocular vessels. This is because there is a complex system of retinal blood vessels in the zebrafish's eye and intraocular vessels are already detected at 60-hours postfertilization. The vasculature develops quickly and, due to a slower blood flow, the CHT harbors numerous stem cell components driving metastasis formation and proliferation. The zebrafish embryo can be used to study the interaction between the innate immune system (neutrophils and macrophages) and tumor cell behavior: this is one of the reasons why we set out to develop this CM model.

The mutations involved in CM are more similar to cutaneous melanoma than uveal melanoma. Cutaneous melanoma and CM harbor a B-RAF mutation, while in most uveal melanoma, GNAQ/GNA11 mutations occur. While all three cell lines were derived from primary tumors and not from metastases, all of them migrated into the eyes in a considerably proportion of engrafted zebrafish embryos (50% of CRMM-1, 28% of CRMM-2, 10% of CM2005.1). However, metastatic cutaneous human melanoma did not migrate to the eyes when injected into zebrafish embryos. In a recent study, primary and metastatic uveal melanoma cells were seen to migrate to the eye in 10% of the embryos. This suggests that the migration of eye cancer cells to the eye is not mutation dependent, but controlled by others factors, which should be evaluated in the future.

We determined whether the CM zebrafish model can be used to test drugs: vemurafenib inhibited the growth of cell lines CRMM-1 and CM2005.1 in vivo and in vitro, and not of cell line CRMM-2. The results in vitro were expected as CRMM-1 and CM2005.1 harbor a B-RAF V600E mutation, while the CRMM-2 cell line contains an N-RAS Q61L mutation. Vemurafenib was approved in 2011 by the Food and Drug Administration for treatment of unresectable melanoma harboring B-RAF V600E mutations and is a potent agent for
The zebrafish model that we describe here allows migration and proliferation of three human CM cell lines. These cells induced a phenotype that was highly reproducible when injected via the duct of Cuvier. The engrafted embryos tolerated the treatment with vemurafenib well, while this inhibitor affected the cell proliferation in vivo in a mutation-dependent manner. Thus, we conclude that the \(fli:GFP\) Casper zebrafish embryos can be used as an efficient animal model to study metastatic behavior of CM cells and for preclinical testing of new treatments against human CM.

**Acknowledgments**

The authors thank Aart G. Jochemsen for transducing the cells with tomato-red.

Supported by grants from the National Council of Technological and Scientific Development CNPq (2007/46/2015-4; Brasilia, DF, Brazil), and the China Scholarship Council (Beijing, China).

Disclosure: K.C.S. Pontes, None; A. Groenewoud, None; J. Cao, None; L.M.S. Ataide, None; E. Snaar-Jagalska, None; M.J. Jager, None

**References**


