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TIMING THE ESTABLISHMENT OF METASTATIC CELL INTRAVASATION IN A MOUSE MODEL OF MELANOMA

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Raúl Torres

2018

To: Dr. Steven Oberbauer, Chairperson
Department of Biological Sciences

This Undergraduate Honors Thesis in Biological Sciences, written by Raúl Torres entitled "Timing the Establishment of Metastatic Cell Intravasation in a Mouse Model of Melanoma", is submitted to you in partial fulfillment of the requirements for Undergraduate Honors in Biological Sciences. The Biological Sciences Undergraduate Honors Committee and the candidate's research supervisor have read this thesis. We recommend that it be approved.

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This thesis by Raúl Torres is approved.

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THE ROBERT JAMES SMIDDY
EXCELLENCE IN RESEARCH AWARD

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Raul Torres

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Robert James Smiddy
October 2, 1983-September 18, 2004

Robert James Smiddy was born on October 2, 1983 in Baltimore, Maryland, the second of nine children born to William and Julie Smiddy. Robert was always known as the oldest child since Joseph Smiddy died at the age of two years when Robert was two months old. In 1989, Robert moved with his family to Miami, where he lived for the remainder of his life.

Robert started piano lessons at the age of 6. His dedication to practice and a gift for performing won critical acclaim on the local, state, and regional level. He won many piano concerto competitions and swept top honors at the Florida State Federation competitions. He studied with many teachers, including the internationally acclaimed Kemal Gekic at Florida International University. Robert was also an accomplished trumpet player, performed in the Greater Miami Youth Symphony, and won their instrumental and piano concerto competitions.

Robert's competitive swimming career began in 1992 with the Stingray Swim Team at Gulliver Academy. His hard work ethic in training resulted in rapid improvements. He was competitive at the state club level, was named to the All-Dade county swim team four years, was a state championship finalist, and was a National Top 16 relay member. He continued an active physical exercise regimen during college.

Robert was home schooled until college and from an early age demonstrated exceptional self-discipline and a voracious appetite for knowledge. He loved to read and excelled in his studies. Home schooling permitted opportunities for Robert to accompany his father on medical conferences to many domestic and international destinations including France, England, Japan, Switzerland, Germany, and Israel; these opportunities offered him insight into many cultures.

He was introduced to FIU through the dual enrollment program during his last two years of high school. He studied music and sciences during that time, gaining over one year's of future college credit, which positioned him well as a full-time student there. His undergraduate major was Biology. He was the recipient of the NIH MBRS-RISE scholarship and was in the Biology Honors program. He was in the latter stages of his Biology Honors Thesis, studying alginate gene expression in *Pseudomonas aeruginosa*. His diligence and eagerness in the laboratory were exemplary for an undergraduate researcher. He was making plans to graduate and enter a MD-PhD program in 2005.

Robert was well liked among his peers, and his advice was highly valued. Those who could count Robert as their friend had a loyal friend indeed.

Robert's untimely death occurred due to a rare bleeding complication of a common viral disease. Though he was removed from life as his adult life was taking shape, his memory will live on in the hearts of his family, friends, and mentors. Robert was a sociable, friendly, diligent man of great character and intellect. Truly he personified a paragon of an Honors Biology student.

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ABSTRACT

Melanoma is the deadliest type of skin cancer that arises from transformed melanocytes. Due to its aggressiveness, melanoma has a high propensity to metastasize and invade other essential organs to seed distant tumors. The main goal of this study was to define when metastatic melanoma cells leave the primary tumors and reach the circulating system to potentially initiate metastases. To achieve this, a spontaneous melanoma metastatic mouse model (*Dct-Grm1/K5-Edn3*) has been created where metastasis to the lungs is 80% penetrant. The *Dct-Grm1/K5-Edn3* mice were crossed to *TYR-CreER*^{T2}*/mT/mG* mice to indelibly label tyrosinase-expressing cells within the primary tumors by topical application of 4-Hydroxytamoxifen (4HT), which induces the expression of green fluorescence protein (GFP). *In vivo* lineage tracing of GFP-labeled cells showed that tyrosinase-expressing cells derived from the primary tumor, or their progeny, can seed successful metastases in the lung thereby demonstrating their tumor initiating capacity. To establish the timing of metastatic cell intravasation, tyrosinase-expressing cells were labeled in the mice at three different stages during tumor progression: pre-nevus stage, nevus stage and mature tumor stage. As expected, GFP-labeled cells were found entering blood vessels at the time tumors had formed. They were also observed entering blood vessels at the nevus stage. Interestingly, GFP-labeled cells were also found in close association with blood vessels prior to the appearance of detectable nevi at sites where tumors generally develop. These results indicate that tyrosinase-positive cells have the ability to disseminate very early and continue to do so during the process of melanomagenesis. Further characterization of the early aggressive cells in melanoma will allow for the development of new prognostic tests and novel therapeutic strategies to eliminate metastasis.

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INTRODUCTION

Melanoma is the most serious type of skin cancer diagnosed in the United States (National Cancer Institute, 2017). Cancerous growth leads to the formation of malignant tumors that arise as a result of rapid multiplication of mutated skin pigment cells (Skin Cancer Foundation, 2018). The human skin consists of three strata: the epidermis, the dermis, and the hypodermis. Each layer contains a set of specialized cells that contributes to the general functioning of this organ. Melanocytes are pigment producing cells located in the epidermis. They are highly controlled by keratinocytes, another epidermal cell that regulates melanocyte functions such as proliferation, differentiation and melanogenesis (Hirobe, 2014). Melanin photo-pigments are synthesized in the melanosome, a cytoplasmic organelle in melanocytes (Simon et al. 2009). Their development requires tyrosinase (TYR) and tyrosinase-related proteins (TRP1, TRP2), which are copper-containing enzymes that catalyze the production of melanin (Campagne et al. 2018). Ultraviolet (UV) radiation stimulates the production of pigment in the melanosome by releasing autocrine and paracrine factors such as α-melanocyte-stimulating hormone secreted by keratinocytes. This hormone can then activate melanocortin 1 receptor that triggers the expression of microphthalmia-associated transcription factor, a key regulator of melanocyte function and melanogenesis (Cai et al. 2018). It is believed that genetic defect accumulation within melanocytes aids in the development of an atypical phenotype that induces excessive production of pigment and the formation of cancerous tumors (Jamal and Schneider, 2002; Su et al. 2017; Kuphal et al. 2017).

The exact cause of all melanomas is not completely clear. It is likely a combination of environmental and genetic factors, including exposure to UV radiation and spontaneous genetic

mutations in cell cycle regulators (Gaggioli and Sahai, 2007; Kim et al. 2017; Rani et al. 2018). CDKN2A, for example, is a regulator of cell division. Mutations in this gene are the most common cause for inherited melanoma. Patients that carry a mutation in the CDKN2A gene have the risk of developing melanoma as age increases (Begg et al. 2005). Other key cancer-relevant genes such as p53 tumor suppressor, are involved in apoptosis and DNA repair pathways. A defect in p53 will lead to the accumulation of mutated DNA. This genetic evidence has suggested a relationship between p53 and UV-mediated mutagenesis of melanoma. Specifically, UV-light induces DNA damage in human epidermal keratinocytes triggering p53 deactivation, and subsequent apoptosis or tumor formation (Hodis et al. 2012; Desgarnier and Rochette 2018). It is estimated that UV radiation is causative of about 90% of skin cancers and 65% of melanomas (Pleasance et al. 2010). This risk increases in patients who exhibit a fair skin complexion and light-toned features that facilitate sunburns, a weakened immune system, and a family history of melanoma (Tatalovich et al. 2006).

Melanoma is considered a highly invasive disease due to its capacity to metastasize (Tian and Lee, 2010). Metastasis is a process in which tumor cells undergo a series of interconnected, sequential or parallel steps to establish new lesions at secondary sites (Krishna Priya et al. 2016). As metastatic cancer cells leave the primary tumor, they infiltrate the circulatory system, travel to distant organs, and seed secondary tumors. The lymphatic vasculature also plays an important role in metastasis since it provides a route for cancer cells to travel from primary to secondary sites (Moy, 2017). The classic paradigm describing the progression of normal melanocytes to metastatic melanoma is linear (Gaggioli and Sahai, 2007). The first stage in malignant melanoma, called the radial growth phase (RGP), suggests that transformed melanocytes begin to spread laterally, but continue to be highly regulated by keratinocytes via growth factors and

remain in close association with the epidermis. The second stage in malignant melanoma, called the vertical growth phase (VGP), suggests that transformed melanocytes begin to invade the dermis, escape growth factor signals, and interact with surrounding stroma cells. At VGP, malignant melanocytes have the potential to disseminate and seed metastasis in other organs (Chin, 2003; Colebatch and Scoyler, 2018). Because of its aggressive characteristic, melanomas have the potential of being destructive (Krishna Priya et al. 2016). Thus, it is of extreme importance, recognizing the signs of skin cancer at an early stage, to detect and treat cancerous changes before metastasis occurs. This can be achieved by surgical resection of the primary site of melanoma (Flaherty, 2012). There is a 90% survival rate if the cancer is treated in the early stages (Mansh et al. 2011).

Malignant melanoma has been reported in cases of human transplantation. Organ recipients can present recurrent metastasis due to organs from patient donors (Puza et al. 2018). Late recurrence has also been reported after the resection of the initial primary tumor (Long et al. 2017). Due to this malignancy, therapies have been redesigned to prevent the development of local recurrences, but have not yet been successful. It is believed that tumor cells disseminate before the removal of the primary tumor (Harper et al. 2016). These "residual" cells seem to have a highly divergent biology from those of the primary tumor, suggesting that this might be a reason for failure of current therapies (Klein, 2013; Werner-Klein et al. 2018). Clinical observations point to the concept of melanoma-metastatic dormancy, which could explain the incomplete cure of melanoma (Eide et al. 2017).

As an important component of preclinical research, murine models have been developed to aid in understanding the mechanisms underlying melanoma progression, and to develop promising therapies for patients (Combest et al. 2012). Studies have detected early dissemination

of abnormal cells in both murine models and human samples, in contrast with the traditional belief that metastasis is a late event in tumor progression and occurs only after all the required steps are completed (Husemann et al. 2008; Weng et al. 2012; Shenoy et al. 2015).

In order to further study the role of melanoma progression, a novel mouse model was created in the laboratory of Dr. Lidia Kos. This model is based on the aberrant expression of Glutamate metabotropic receptor 1 (*Grm1*), in melanocytes under the melanocyte-specific promoter Dopachrome tautomerase (*Dct*), and environmental Endothelin 3 (*Edn3*) under the regulation of the Keratin 5 (*K5*) promoter. These mice are not only hyperpigmented but also spontaneously develop tumors and show metastasis in the lung (Pollock et al. 2003; Garcia et al. 2008; Chin, 2015). The heterogeneity of the melanoma tumors in our spontaneous mouse model of melanoma metastasis can provide an ideal system to identify the tumor-initiating capacity of differentiated TYR-expressing cells, which account for the majority of the tumor composition (Hearing, 2005; Li, 2017). For that reason, lineage tracing was performed using the Cre/loxP system under a *Tyrosinase* promoter. Prior to Cre recombination, all cells in the *TYR-CreER^{T2}/mT/mG* mice express membrane-localized tdTomato (mT) red fluorescence. After 4-Hydroxytamoxifen (4HT) induction, a selective estrogen receptor modulator, TYR+ Cre recombinase-expressing cells stop tdTomato (mT) fluorescence and begin to display membrane-localized Green Fluorescent Protein (mG) fluorescence instead. Thus, the expression of GFP or tdTomato is mutually exclusive (Muzumdar et al. 2007). These mice do not develop tumors, nor do they show metastasis. However, the mice obtained from the mating of *Dct-Grm1/K5-Edn3* with *TYR-CreER^{T2}/mT/mG*, not only spontaneously develop primary tumors and metastatic melanomas, but also have Cre-inducible TYR+ cells that can be labeled with GFP to distinguish them from the rest of the cells in the body. What is interesting about this labeling system is that

once a TYR+ cell is induced with 4HT, its progeny will also express GFP.

The aim of this study was to establish when metastatic melanoma cells (TYR+) begin to migrate into circulation at different stages of the disease. To achieve this, cryosections of Cre-induced tail tissues were obtained to perform immunostaining using GFP and Cluster of Differentiation 31 (CD31), which labels the endothelial cells of the blood vessels. This allowed the identification of TYR+ GFP-labeled cells that are closely associated with the vasculature and are intravasating into the circulatory system to potentially establish metastasis.

MATERIALS AND METHODS

Generation of Transgenic Mouse Model

The experimental mice used in this study were product of the crossing of *Dct-Grm1/K5-Edn3* (Pollock et al. 2003; Garcia et al. 2008) with *TYR-CreER*^{T2}*/mT/mG* (Muzumdar et al. 2007) to yield the experimental group *Dct-Grm1/K5-Edn3/TYR-CreER*^{T2}*/mT/mG.* These mice spontaneously develop primary tumors and metastatic melanomas, and have Cre-inducible TYR+ cells that can be exclusively labeled with GFP and can be distinguished from the other red-labeled cells in the body. Mice were housed in the University Animal Care Facility at Florida International University. The animal protocol was approved by the Committee on Animal Care and Use, and Office of Sponsored Research at Florida International University. All Institutional Animal Care and Use Committee regulations were followed.

DNA Extraction and Genotyping

DNA was first isolated from tail tip biopsies of 0.5-1.0 cm in length. Samples were immersed in a 500 μL solution made of tail lysis buffer (2M Tris, 0.5M EDTA, 5M NaCl, 20% SDS) and a 1:70 dilution of proteinase K (10mg/mL; Fisher Scientific Inc.). Tails were digested over night in a 55°C water bath. Once the digestion was completed, the solution was centrifuged for 10 minutes at 12,000 x g to separate biopsy detritus from supernatant containing the DNA. An aliquot of 450 μL of the supernatant was mixed vigorously with 450 μL of isopropanol. This solution was then centrifuged for 5 minutes to create a DNA pellet. The supernatant was discarded and residues of isopropanol were left to evaporate. A volume of 100 μL of Tris-EDTA (TE) buffer was added to the dry DNA pellet and the mixture was vortexed to help dissolve DNA in buffer. Each sample was stored at 4°C. DNA was genotyped by Polymerase Chain

Reaction (PCR). Transgenes were detected using standard reagents and amplification cycles, and were amplified by their appropriate primers (Table 1). The PCR products were analyzed by gel electrophoresis using a 1.5% agarose gel along with ethidium bromide. Genotypes were observed through a camera-mounted UV ethidium bromide filter.

Table 1. Primers for mouse genotyping

Gene	**Sequence**	**Primer Type**
Dct-Grm1	5'-CCGGGTCCGCATTAATCTTATCTA-3'	Transgene
	5'-GGTAGCATACGGTTCCACGCA-3'	Transgene
K5-Edn3	5'-CCAGGTGGAGTCACAGGATT-3'	K5 regulatory region FW
	5'-ACAGAGACTGTGGACCACCC-3'	K5 regulatory region RV
	5'-GGCCTGTGCACACTTCTGT-3'	Edn3 cDNA FW
	5'-TCCTTGTGAAACTGGAGCCT-3'	Edn3 cDNA RV
TYR-CreER^{T2}	5'-CTCTGCTGCCTCCTGGCTTCT-3'	Wildtype FW
	5'-CGAGGCGGATCACAAGCAATA-3'	Wildtype RV
	5'-TCAATGGGCGGGGGTCGTT-3'	Mutant RV
mT/mG	5'-GCGGTCTGGCAGTAAAAACTATC-3'	Transgene
	5'-GTGAAACAGCATTGCTGTCACTT-3'	Transgene
	5'-CTAGGCCACAGAATTGAAAGATCT-3'	Internal+Ctrl FW
	5'-GTAGGTGGAAATTCTAGCATCATCC-3'	Internal+Ctrl RV

In Vivo Lineage Tracing

The *Dct-Grm1/K5-Edn3/TYR-CreER^{T2}/mT/mG* mice were grouped into three stages – tumor stage, nevus stage (i.e. benign pre-tumor lesion less than 1mm in height) and pre-nevus stage (i.e. before the appearance of a lesion). *In vivo* lineage tracing in the mice was achieved by topical applications of 4HT, 4-Hydroxytamoxifen (Sigma, H6278, St. Louis, MO) on tail primary tumor sites using a small paintbrush. For the pre-nevus stage group, topical applications of 4HT were made 3.0-4.0 cm from the distal part of the tail. To maximize the labeling of TYR+ cells on the primary tumors, 4HT was administered topically for three consecutive days, twice a day. Mice were euthanized 2 weeks after treatment (Figure 1). *TYR-CreER^{T2}/mT/mG* mice were used as a control group and were subjected to the same conditions as the experimental group.

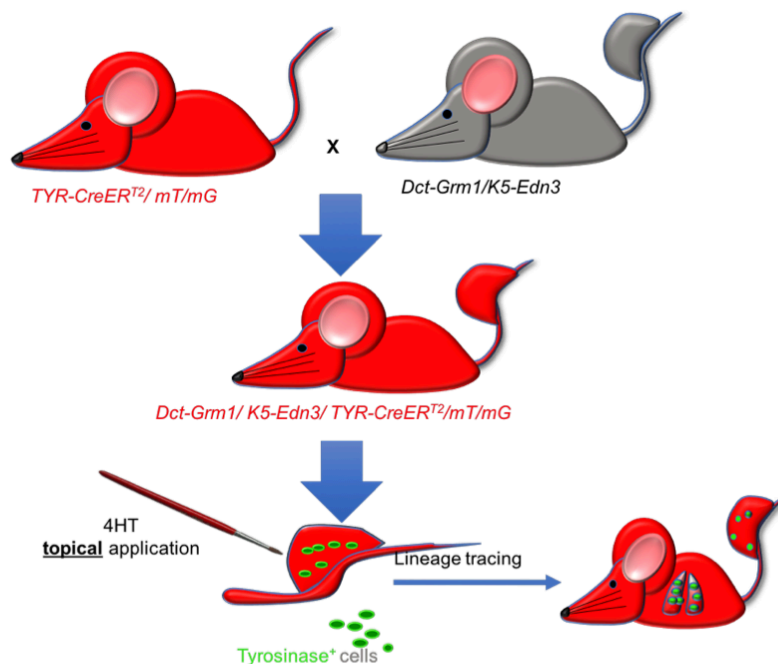

Figure 1. Lineage Tracing. An in vivo lineage tracing system can be established in the *Dct-Grm1/K5-Edn3/TYR-CreER^{T2}/mT/mG* mice. 4-Hydroxytamoxifen (4HT) was topically applied to primary tumor sites.

Tissue Fixation, Embedding, and Sectioning

Upon euthanasia, the portions of the tails with the 4HT painting areas were collected and fixed in 4% paraformaldehyde (PFA) overnight in the dark at 4°C. They were then thoroughly washed in 1X phosphate-buffered saline (PBS). Tails were cryo-protected by sequential immersion in 15% and 30% sucrose, and then embedded in disposable embedding molds using optimum cutting temperature (OCT) compound (Fisher Scientific, Hampton, NH). Samples were stored at -80°C and sectioned at a later time using a cryostat. Ten-micron sections obtained from the tail tissues were placed on glass slides and stored at -80°C for later immunostaining.

Immunostaining

Microscope glass slides with tissue sections were allowed to defrost. Tissue GFP and tdTomato fluorescence was photobleached by treatment with 3% H_2O_2 in methanol in a fluorescent light box for 4 hours at 4°C. Sections were washed with 1XPBS and incubated in blocking buffer (10% normal serum, 0.3% Triton X-100, 1% BSA powder) for 1 hour at room temperature. They were then incubated in primary antibody diluted in dilution buffer (1% normal serum, 0.3% Triton X-100, 1% BSA) for 1 hour at room temperature or overnight at 4°C. Slides were washed exhaustively with 1XPBS and incubated in a secondary antibody dilution at room temperature for 1 hour. Tissues were mounted with fluoroshield mounting medium with DAPI, 4',6-diamidino-2-phenylindole (Abcam ab104139, Cambridge, UK) and visualized on a Leica Leitz DMRB fluorescence microscope.

Since slides were photobleached, GFP antibody was used to amplify the GFP signal from the TYR+ cells, and Cluster of Differentiation 31 (CD31) was used to label the endothelial cells of the blood vessels in the tail tissues (Figure 2). This allowed for the identification of TYR+

cells that are in close association with the circulatory system and with the process of intravasation.

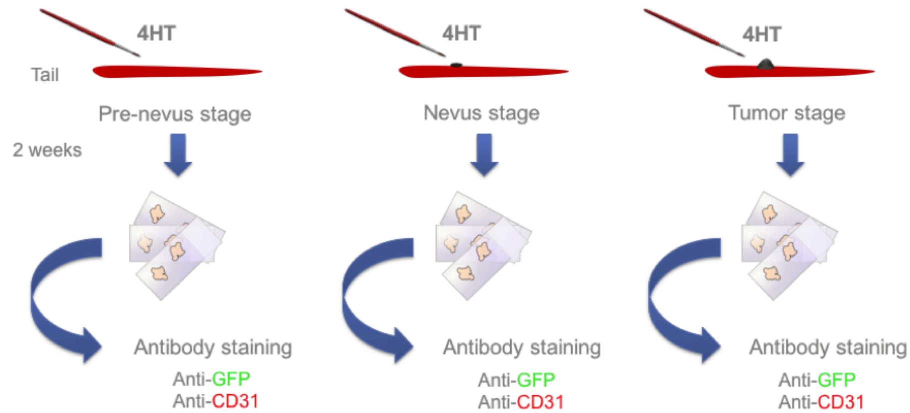

Figure 2. Experimental design. Tails were painted with 4HT for three consecutive days, twice a day. After 2 weeks mice were sacrificed, tail sections were obtained and immunostaining was performed using GFP and CD31.

Table 2. Primary and Secondary Antibodies

	Antibody	**Host and Type**	**Dilution**	**Source**	**Code**
Primary	GFP	Chicken polyclonal	1:500	Aves Labs, Tigard, OR	GFP-1020
	CD31	Rabbit polyclonal	1:200	Abcam, Cambridge, UK	Ab28364
Secondary	Alexa Fluor 488 Anti-Chicken IgY	Goat polyclonal	1:500	Abcam, Cambridge, UK	Ab150169
	Alexa Fluor 594 Anti-Rabbit IgG	Goat polyclonal	1:400	Invitrogen, Carlsbad, CA	A11012

RESULTS

Validation of Lineage Tracing in Mouse Model

Cryosections analyzed under a fluorescent microscope developed a mutually exclusive pattern of Green Fluorescent Protein (GFP) and tdTomato colors inside the primary tumor sites. As expected, only the TYR+ cells that were induced with 4HT within the tumor were labeled with GFP, while those that did not express TYR remained red.

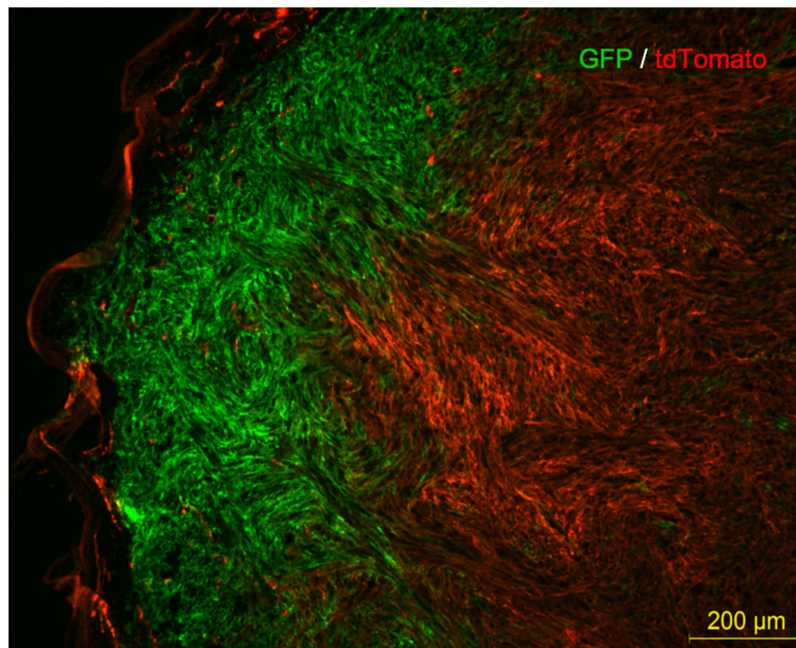

Figure 3. Cryosection of a 4HT induced tail tumor (n=3), visualized under a fluorescent microscope shows the TYR+ cells labeled in green, and the mutually exclusive pattern of GFP and tdTomato colors.

Primary Tumor Derived TYR+ Cells or Their Progeny Can Seed Metastasis in the Lung

Previous studies in our laboratory showed that approximately 80% of the animals present lung metastasis by 6-8 months of age (Chin, 2015). To determine the metastatic potential of TYR+ cells in *Dct-Grm1/K5-Edn3/TYR-CreER*^{T2}*/mT/mG* mice, lungs were sectioned and tissues were examined for the presence of primary tumor derived GFP-labeled cells using a fluorescence microscope. The appearance of metastasis was established to be 2 weeks to 7 months (Figure 4). Immunostaining showed that these GFP-labeled cells in the lung were positive for melanocyte-specific markers TYR and TRP1, demonstrating the tumor initiating capacity of TYR-expressing cells or their progeny (Figure 5). It is of importance to note that not all lung metastatic lesions contained GFP-labeled cells, indicating that either the labeling efficiency of 4HT induction was < 100% or that the metastases originated from non-TYR+ cells.

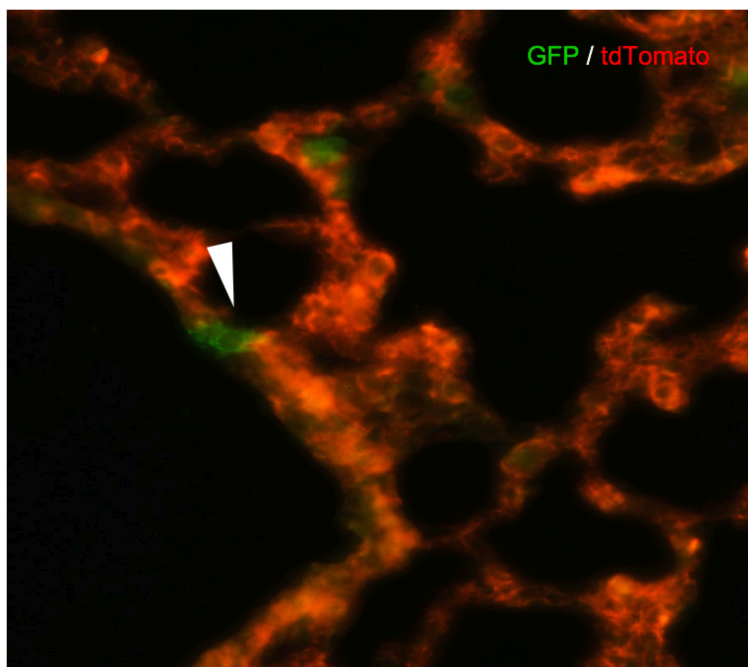

Figure 4. Metastatic capacity of GFP-labeled cells from primary tumor. Mice primary tumor was induced with 4HT (n=3). Cryosections were obtained and analyzed using a fluorescence microscope. GFP-labeled primary tumor cells in the lung tissue (white arrow) demonstrate their metastatic capacity.

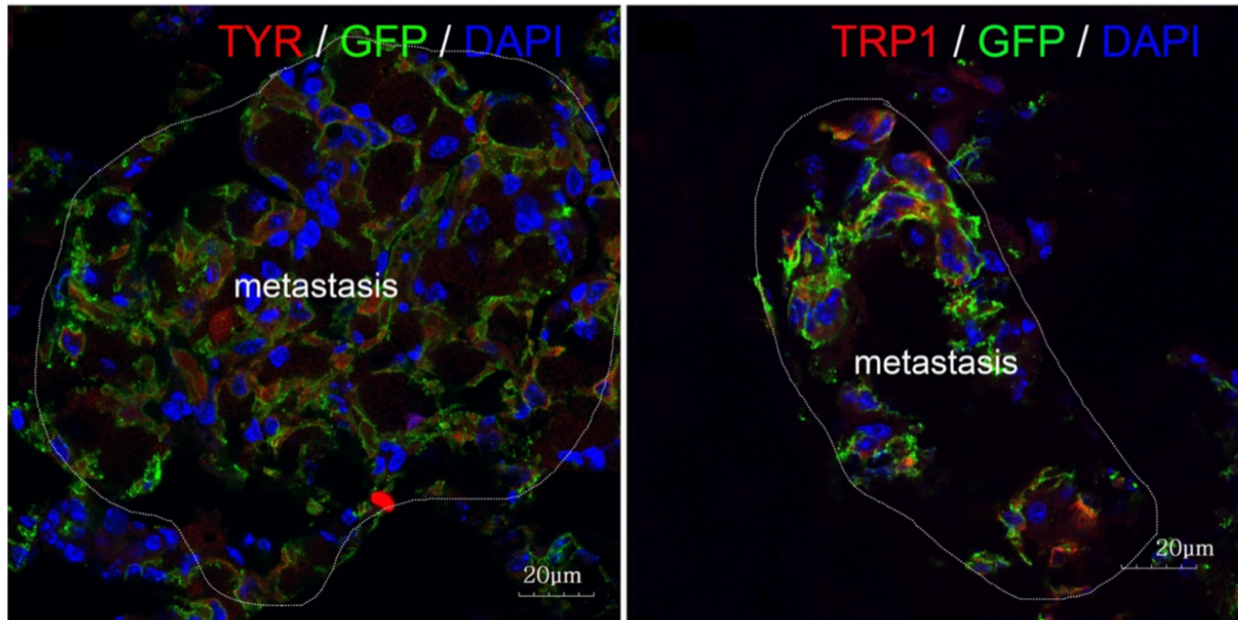

Figure 5. Lineage tracing of tumorigenic cells to the lung. GFP-labeled lung cells were positive for melanoma markers TYR (A) and TRP1 (B), showing the successful seeding of melanoma metastasis (n=3).

Metastatic Melanoma Cells Disseminate Early during Melanomagenesis

Late recurrence has been shown clinically in melanoma patients after the resection of the primary tumor (Weng et al. 2012). This suggests that melanoma cells might disseminate at an early stage into circulation. To determine at which point of tumor progression this happens, the *Dct-Grm1/K5-Edn3/TYR-CreER*^{T2}*/mT/mG* mice were classified into three groups: pre-nevus, nevus, and mature tumor stages. Tails were painted with 4HT and tissue was sectioned for further processing through immunostaining. A total of 15 cryosections per mouse (n=3) were thoroughly analyzed, for each of the three groups, along with a control group. Immunostaining revealed GFP-labeled TYR+ cells entering blood vessels within the tumor and nevi stages. Interestingly, GFP-labeled cells were also found in close association with the vasculature prior to the appearance of detectable nevi (Figure 6). At least 10 cryosections per animal, for each of the

three stages, displayed the presence of GFP-labeled cells associated to the vasculature. Control *TYR-CreER*^{T2}*/mT/mG* mice were subjected to the same conditions, sectioning and analyses as the experimental group. To our surprise, few GFP-labeled cells were found in close association with blood vessels (Figure 7). About 2 cryosections per animal displayed the presence of this result.

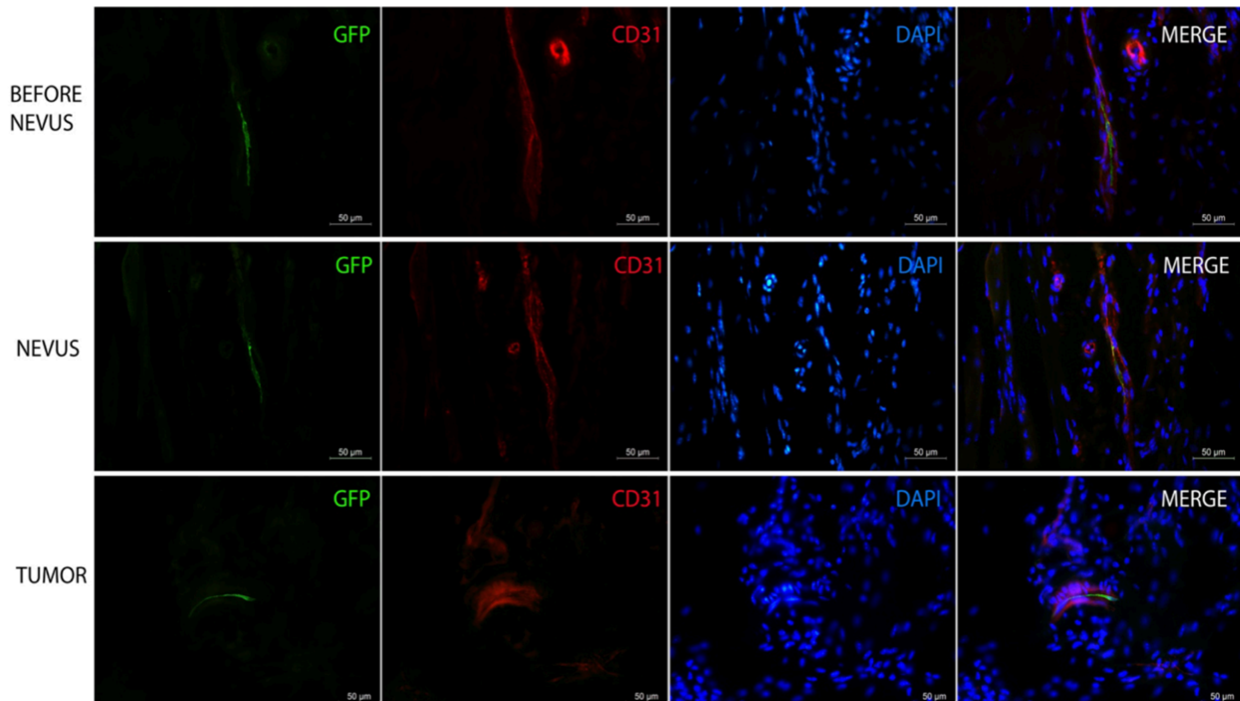

Figure 6. Timing of metastatic GFP-labeled TYR+ cell dissemination. Tails were induced with 4HT (n=3 for each time point). Cryosections were then immunostained using GFP and CD31, which labels the endothelial cells of the blood vessels. GFP-labeled cells were found in close association to blood vessels at all three stages. Only the vasculature underneath the skin induced area is shown, not the actual nevus/tumor/skin.

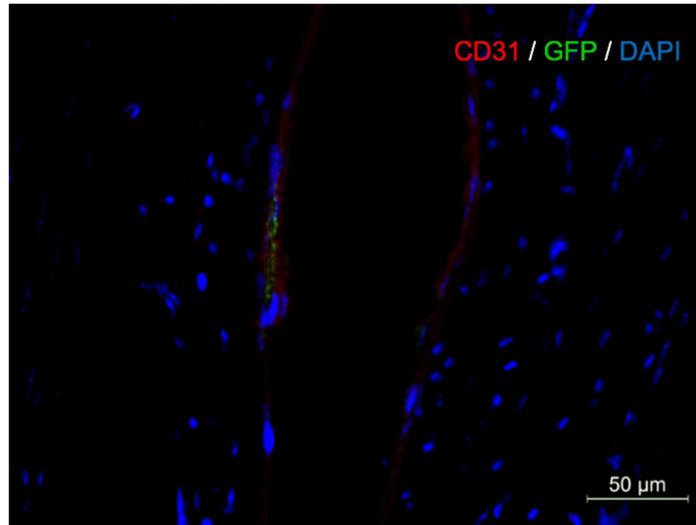

Figure 7. Control *TYR-CreER*^{T2}*/mT/mG* mice. Control group was subjected to the same conditions as the experimental group. Tails were induced with 4HT (n=3), sectioned and immunostained. GFP-labeled cells were found in contact with blood vessels.

DISCUSSION

Melanoma is the deadliest type of skin cancer due to its high capacity to metastasize. Despite the existence of therapies, the number of deaths attributed to this disease continues to rise each year (National Cancer Institute, 2017). Recently, cases of late recurrence of melanoma have been reported in patients after the resection of the initial primary tumor (Puza et al. 2018). Malignant cells that disseminate at an early stage in tumor progression may account for melanoma's aggressiveness.

In vivo lineage tracing, using a murine mouse model of melanoma metastasis, provides a mechanism for labeling cells from the primary tumor and tracing them as they migrate to distant parts of the body. Examination of primary tumors in the *Dct-Grm1/K5-Edn3/TYR-CreER*^{T2}*/mT/mG* mice, validated the mutually exclusive pattern of GFP and tdTomato fluorescence in the mouse model. Lung tissue analysis demonstrated the metastatic capacity of tumor cells derived from the mouse model. Immunostaining revealed the expression of melanocytic markers, such as TYR and TRP1, in lung metastatic cells. Most studies of melanoma metastasis are focused on either the first stage (primary tumors) or last stage (secondary tumors) of the disease (Scott et al. 2011; Chudasama et al. 2017). The pivotal work conducted by Shenoy et al. (2015) used a mouse model to demonstrate the initiation of the metastatic process by tumor cells at an early onset of melanoma. Similarly, the study directed by Weng et al. (2012), suggests that metastatic mammary epithelial cells in mice disseminate and metastasize before full malignant transformation. They found that some of the disseminated cells and lung metastases exhibited stem cell markers. Another corroborating study performed by Rhim et al. (2012) showed the unexpectedly early migration of pancreatic epithelial cells in mice

before the detection of malignancy could be diagnosed using rigorous histologic analysis. In this investigation, the metastatic potential of melanoma cells was studied even before the appearance of a tumorigenic lesion; this provides a more specific time point in which TYR+ begin migrating into circulation. The results obtained in my study support and augment the findings of Shenoy et al. (2015), Weng et al. (2012) and Rhim et al. (2012) by not only showing that metastasis is an early event but also by determining the stage in tumor progression in which intravasation occurs to potentially initiate the metastatic process.

In the *Dct-Grm1/K5-Edn3/TYR-CreER*^{T2}*/mT/mG* experimental group, GFP-labeled cells were found associated with blood vessels at a very early stage. Lineage tracing unveiled TYR+ cells in lung metastasis. However, it is uncertain if the labeled cells associated with the tail vasculature have the capability of giving rise to metastases in distant organs. In the *TYR-CreER*^{T2}*/mT/mG* control group, few GFP-labeled cells were also found associated with blood vessels. Nevertheless, metastasis did not occur in the control group due to the lack of overexpression of Glutamate metabotropic receptor 1 (*Grm1*) and environmental Endothelin 3 (*Edn3*). This comes as no surprise since previous studies conducted in the Kos laboratory suggest that *Grm1* and *Edn3* play a predominate role in tumor progression and metastasis (Pollock et al. 2003; Garcia et al. 2008; Chin 2015). Despite showing a similar result between the two groups, the control mice had a much lower frequency of the presence of GFP-labeled cells intravasating the vasculature compared with the experimental group, suggesting that the frequency of intravasation is correlated to metastatic potential. The association of GFP-labeled cells with the vasculature in the control group might be due to the migratory nature of melanocyte precursors, arguing that some melanocytes maintain this property and migrate towards the blood vessels (Wang et al. 2016).

The results presented here suggest that the onset of metastasis, in *Dct-Grm1/K5-Edn3/TYR-CreER^{T2}/mT/mG* mice, may occur early in tumor progression and can arise in the absence of a tumorigenic lesion. Similar observations are not limited to murine models, but have been shown in humans as well. According to Damsky et al. (2014), 12% of melanoma cases metastasized before there was an identifiable primary tumor. This argues that methods for preventive screening for patients that have a risk of developing melanoma should be done at an earlier stage than is currently established to avoid the likelihood of metastasis.

Previous studies conducted in the laboratory of Dr. Lidia Kos have shown that Endothelin 3, a paracrine factor involved in melanocyte proliferation, migration and survival, has a role in initiating and potentiating metastasis by binding onto EDNRB, Endothelin receptor type B (Chin, 2015). In the future, it would be ideal to use an EDNRB antagonist on the animals, to possibly prevent metastasis from occurring at an early stage. Further characterization of the early aggressive cells in melanoma will allow for the development of new prognostic tests and novel therapeutic strategies to eliminate metastasis.

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