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Identification and Phenotypic Plasticity of Metastatic Cells in a Mouse Model of Melanoma

Xiaoshuang Li
liali0721@gmail.com

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

IDENTIFICATION AND PHENOTYPIC PLASTICITY OF METASTATIC CELLS IN A MOUSE MODEL OF MELANOMA

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Xiaoshuang Li

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To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This dissertation, written by Xiaoshuang Li, and entitled Identification and Phenotypic Plasticity of Metastatic Cells in a Mouse Model of Melanoma, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

_____
Manuel A Barbieri

_____
Xiaotang Wang

_____
Zhao-Jun Liu

_____
Fernando G Noriega

_____
Lidia Kos, Major Professor

Date of Defense: June 16, 2017

The dissertation of Xiaoshuang Li is approved.

_____
Dean Michael R. Heithaus
College of Arts, Sciences and Education

_____
Andrés G. Gil
Vice President for Research and Economic Development
and Dean of the University Graduate School

Florida International University, 2017

DEDICATION

I dedicate this dissertation to my family for their support and to my advisor Dr. Lidia Kos who helped me in all things great and small.

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ABSTRACT OF THE DISSERTATION

IDENTIFICATION AND PHENOTYPIC PLASTICITY OF METASTATIC CELLS IN A MOUSE MODEL OF MELANOMA

by

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Florida International University, 2017

Miami, Florida

Professor Lidia Kos, Major Professor

Melanoma is the deadliest form of skin cancer due to its high propensity to metastasize and resistance to current therapies. We have created a spontaneous mouse model of metastatic melanoma (*Dct-Grm1/K5-Edn3*) where metastasis to the lungs is 80% penetrant. The primary tumors of these mice present cellular heterogeneity with cells at varying levels of differentiation. The main goal of this study was to determine the metastatic potential of the primary tumor resident Tyrosinase positive cells and evaluate the dynamic phenotypic changes as those cells move from the primary tumors to the sites of metastasis. To accomplish this aim I crossed the *Dct-Grm1/K5-Edn3* mice to *CreER^{T2}/mT/mG* mice to indelibly label Tyrosinase cell populations within the primary tumor with Green Fluorescent Protein (GFP) by topical application of 4-hydroxytamoxifen (4HT) at the tumor site. *In vivo* lineage tracing and characterization of GFP+ cells were performed in the metastatic lesions.

In the 4HT treated *Dct-Grm1/ K5-Edn3/Tyr-CreER^{T2}/mT/mG* mice, primary tumor derived Tyrosinase positive cells or their progeny (GFP+)

established successful metastases in the distant organs indicating the tumorigenic capacity of the differentiated cell populations. Numerous metastatic melanoma cells were identified in the vasculature of the metastatic organs and established close association with the vascular endothelium. The intravascular cells lost pigmentation and did not express melanocytic markers; however, they mimicked endothelial cell properties and gained the expression of CD31 (also known as platelet endothelial cell adhesion molecule PECAM-1) and vascular endothelial (VE)-Cadherin. In the lung metastatic foci, GFP+ cells resumed pigmentation production and lost the expression of endothelial cell markers. Evidence from other metastatic organs in the mice further supported the phenotypic plasticity of metastatic melanoma cells.

The *in vivo* lineage tracing system established in the melanoma mouse model revealed tumor phenotypic plasticity and will be a powerful model to evaluate and help us understand the etiology and pathogenesis of melanoma metastasis. Further characterization of those more aggressive cells in melanoma will allow for the development of new prognostic tests and novel therapeutic strategies to eliminate metastasis.

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ABBREVIATIONS AND ACCRONYMS

3D..... Three-dimensional

4HT 4-hydroxytamoxifen

a-SMA .....a-smooth muscle actin

AAD.....α1 and α2 domains of HLA-A2 linked to the α3 domain of H2-Dd

ABCB5 ..... ATP binding cassette subfamily B member 5

bHLH..... Basic helix-loop-helix

Bmi1 .....B lymphoma Mo-MLV insertion region 1 homolog

BMP ..... Bone morphogenetic protein

BSA..... Bovine serum albumin

CD31 or PECAM-1 ..... Platelet endothelial cell adhesion molecule

CSCs..... Cancer stem cells

CTCs ..... Circulating tumor cells

Cx43..... Connexin43

Dct..... Dopachrome tautomerase

DMSO .....Dimethyl sulfoxide

E..... Glutamic acid

ECM ..... Extracellular matrix

Edn3..... Endothelin 3

Ednrb .....Endothelin Receptor B

EET ..... Epithelial-to-endothelial transition

EMT.....Epithelial-mesenchymal transition

EndMT ..... Endothelial-mesenchymal transition

EPHA2 .....Erythropoietin-producing hepatocellular carcinoma-A2

EVMM ..... Extravascular migratory metastasis

FDA .....Food and Drug Administration

FOXO3A .....Forkhead Box O3

FSP1 .....Fibroblast-specific protein 1

FZD7 .....Frizzled class receptor 7

G .....Gauge

GBM ..... Glioblastoma

GFP.....Green fluorescent protein

GLIZ .....Glucocorticoid-induced Leucine Zipper

Grm1 ..... Glutamate Metabotropic Receptor 1

H&E..... Hematoxylin and eosin stain

H_2O_2 ..... Hydrogen peroxide

HER-2 .....Human epidermal growth factor receptor 2

HGF.....Hepatocyte growth factor

IHC .....Immunohistochemistry

K5.....Keratin 5

LAMC2 .....Laminin 5 γ2-chain

Lgr5.....Leucine-rich repeat-containing G-protein coupled receptor 5

LLCcm.....Lewis lung carcinoma cells

LYVE-1.....Lymphatic vessel endothelial hyaluronan receptor 1

MCCs ..... Mesenchymal cancer stem cells

mG ..... Green fluorescent protein

mg ..... Microgram

MICs ..... Melanoma-initiating cells

MITF ..... Microphthalmia-associated transcription factor

miPS ..... mouse induced pluripotent stem cells

ml ..... Microliter

mm ..... milimeter

mm ..... Micrometer

MRF ..... Melanoma Research Foundation

mT ..... tdTomato

n ..... Number

NOD-SCID ..... Nonobese diabetic/severe combined immunodeficiency

OCT ..... Optimal cutting temperature

OCT4 ..... Octamer-binding transcription factor 4

PAX3 ..... Paired box 3

PBS ..... Phosphate buffer solution

PCR ..... Polymerase chain reaction

PFA ..... Paraformaldehyde

PyMT ..... Polyoma middle T

RET ..... Rearranged during transfection' proto oncogene

RGP ..... Radial growth phase

RT-PCR ..... Real time-polymerase chain reaction

SF ..... scatter factor

Shh.....Sonic hedhehog

Snail1.....Snail family zinc finger 1

Snail2.....Snail family zinc finger 2

SNP..... Single-nucleotide polymorphism

Sox10 .....Sex determining region Y (SRY)-box 10

SOX2 ..... Sex determining region Y box 2

Tg ..... Thansgene

TGF-b..... Transforming growth factor-beta

TGF-b..... Transforming growth factor-beta

TRP1.....Tyrosinase-related protein 1

TRP2.....Tyrosinase-related protein 2

tTA ..... Tetracycline-sensitive transcriptional activator

Twist..... Twist Family BHLH Transcription Factor

Twist1..... Twist Family BHLH Transcription Factor 1

Tyr or TYR .....Tyrosinase

TRP1..... tyrosinase related protein 1

UV ..... Ultraviolet

V..... Valine

VE ..... Vascular endothelial

VEGF ..... Vascular endothelial growth factor

VGP..... Vertical growth phase

VM..... Vasculogenic mimicry

vs..... Versus

vWF..... Von Willebrand factor

WNT.....Wingless-type MMTV integration site family

ZEB1 ..... Zinc Finger E-box-binding homeobox 1

ZEB2 ..... Zinc Finger E-box-binding homeobox 2

Zn .....Zinc

CHAPTER

1. INTRODUCTION

1.1 Introduction

Most cancer patients die of metastasis [1]. Cancer metastasis is the spread of a cancer from one organ or part of the body to another organ or part of the body that is not directly connected. The process of metastasis consists of a series of interconnected, sequential or parallel steps that must be completed by tumor cells if a metastasis is to develop, although the exact steps for individual tumors are widely context-dependent. The vasculature plays essential roles during cancer metastasis. In addition to the authentic endothelial cell-lined vasculature, new models of vasculature, such as vasculogenic mimicry, vessel co-option and cancer cell-derived endothelial cells, have been reported [2]. Mechanisms underlying tumor metastasis are still under investigation, among which epithelial-mesenchymal transition (EMT) is receiving increasing attention [3, 4].

Melanoma is the type of skin cancer that causes the most deaths because of its high metastatic potential. Progression of normal melanocytes to metastatic melanoma was thought to be linear, but recent evidence has suggested it is more complex and less linear in nature [5]. Clinical cases of late recurrence after the initial primary melanoma resection [6] not only implicated the early dissemination of tumor cells, but also suggested melanoma dormancy. Although a number of studies have proposed an important role for cancer stem cells (CSCs) in melanoma, their existence and relevance remain a controversial issue [7]. The role of circulating tumor cells (CTCs) in metastatic colonization is also extensively discussed [8, 9]. In this chapter, steps of cancer metastasis are summarized and

the putative roles of EMT in metastasis are reviewed. In addition, descriptions of melanoma progression and dormancy are introduced as well as the current available evidence for metastasis-initiating cells focusing on CTCs.

1.2 Cancer Metastasis

1.2.1 Steps in Metastasis

Cancer metastasis involves several steps; metastatic cancer cells leave the primary tumor, travel to distant organs through various mechanisms and eventually seed metastases in the secondary organs. Blood/lymphatic vasculature plays essential roles in cancer metastasis by providing a route for the travel of cancer cells from primary sites to secondary sites. As malignant cancer cells start to proliferate out of control, a solid mass will appear as the primary tumor. Further growth of the primary tumor was originally believed to be angiogenesis-dependent and neovascularization was needed for tumor invasion and metastasis [10]. The promise of this hypothesis has been subjected to clinical evaluations and anti-angiogenesis agents have been used as standard-of-care therapies for various types of cancers [11]. Later, this classic concept was challenged by studies showing that micro-tumors may initiate tumor growth by hijacking the pre-existing host vessels, a process known as vessel co-option (also known as vascular co-option), without inducing angiogenesis in a vascularized tissue [12-15]. Vessel co-option is also responsible for tumor progression after anti-angiogenesis therapies [16-19]. Other than the endothelial cell-lined vasculature in tumors, aggressive tumor cells can form blood-perfused tubular channels, termed as vasculogenic mimicry (VM), linking directly to normal

vessels for the blood supply of the tumor growth [20]. Although aggressive uveal melanoma cells engaged in the formation of VM in 3D Matrigel downregulated invasiveness related genes [21], VM is strongly associated with clinical deaths of metastasis in uveal and cutaneous melanoma patients [22, 23]. The plasticity of tumor cells to gain an endothelial-like phenotype is depicted in another form of vasculature in cancer, the mosaic vessels. Tumor cells can mimic endothelial cells by participating in the formation of blood vessels. Mosaic vessels are marked by discontinued endothelial cell marker CD31 expression and the replacement of some endothelial cells with tumor cells in the vessels [24]. It is reasonable to propose that the presence of cancer cells within the mosaic vessels in contact with the blood lumen indicate tumor dissemination and metastasis [25]. Recent observations in glioma adds another layer to the role played by the tumor vasculature in that glioma cells can transdifferentiate into endothelial cells and express endothelial cell markers [26-31].

Several interactions or phenomena between cancer cells and the vasculature have been suggested or observed. The well-studied interaction between cancer cells and blood vessels is transendothelial migration. Tumor cells can migrate through the vascular endothelium to reach the blood (intravasation) and after transport in the blood stream, some cells can get out of the vasculature (extravasation) to seed metastases. Tumor cells within the blood stream are called circulating tumor cells (CTCs). Metastatic tumor cells can enter circulation as single cells or as cell clusters [32]. Circulating tumor cells have been successfully isolated from human blood in several cancers [33-36]. During

hematogenous dissemination, intravascular rolling behavior of metastatic cancer cells has been reported [37-41]. In contrast to intravascular dissemination or migration, Lugassy and Barnhill suggested “angiotropism” in melanoma as a new mechanism of tumor spread. They termed this phenomenon as 'extravascular migratory metastasis' (EVMM), emphasizing the pericytic mimicry of tumor cells. Extravascular migratory metastasis describes the continuous migration of tumor cells along the abluminal vascular surfaces to nearby or more distant sites [42-44]. The similarity of vessel co-option and angiotropism and the significance of these mechanisms during the metastatic process have been further discussed in a brain melanoma mouse model [15].

At the metastatic sites, tumor cells reach the metastatic organs by whichever migration mechanism (extravasation, vessel co-option, EMVV) and start to proliferate and seed successful metastases. Intravascular proliferation of tumor cells has been suggested as an alternative origin of early metastasis in the lung [40, 45]. Instead of angiogenesis switch for further growth of metastatic tumors, vessel co-option is common in vasculature-rich tissues [18]. The overall process of cancer metastasis is summarized in Figure. 1.1.

1.2.2 Mechanisms underlying Metastasis

1.2.2.1 Epithelial-mesenchymal transition

Epithelial-mesenchymal transition was originally defined in developmental biology as a conversion from embryonic epithelia to individual mesenchymal cells that can invade and migrate through the extracellular matrix (ECM) [46]. During normal development, epithelial cells provide cell-cell adhesion essential to

establish a barrier to the external environment for the development of embryos. In contrast, mesenchymal cells are free to rearrange or move around in three dimensions thus adding more plasticity for body patterning and morphogenesis [47]. Epithelial-mesenchymal transition involves not only morphological changes but also gene expression switch, in particular, the downregulation of E-cadherin and upregulation of N-cadherin. It took a long time for EMT to be recognized as a potential mechanism underlying carcinoma progression. Since local invasion is the first step for cancer metastasis, it is essential for tumor cells to lose cell-cell adhesion and acquire invasive or migratory behaviors. The similarities between the tumor malignancy switch and the developmental signature have solidified the notion that EMT is a major contributor to the escape of tumor cells from the primary tumors [48].

Although clinically carcinomas (tumors of epithelial origin) and sarcomas (tumors of mesenchymal origin) are generally not thought to interconvert, various carcinoma cell lines undergo EMT during specific culturing conditions in vitro [49]. The first evidence of EMT in cancer was noted by studies demonstrating that invasiveness and adoption of a fibroblast morphology can be achieved by down regulation of E-cadherin expression in various epithelial cancer cell lines [50, 51]. In addition, re-expression of E-cadherin can reverse the invasive property of cancer cells [50, 52, 53]. Transforming growth factor-beta (TGF-β) was shown to be a main inducer in the cancer EMT process. Transformed mammary epithelial cells exhibited fibroblastoid properties in the presence of TGF-β, the converted cells themselves produced high levels of TGF-β1 and maintained a highly

invasive phenotype both in vitro and in vivo. Neutralization of TGF-β reverted the epithelial phenotype of the fibroblastoid cells [54]. Blockage of TGF-β receptor abolished metastasis formation in highly metastatic mesenchymal mouse colon carcinoma cells [55]. However, because of the numerous biological interactions TGF-β is involved in, the interpretation that EMT occurs in response to TGF-β is complex [56]. The understanding of EMT in cancer advanced when specific transcriptional modulators of E-cadherin gene promoter were identified. In 2000, two groups showed strong evidence that Snail1 binds directly to the promoter of E-cadherin and represses the transcription of this gene in invasive mouse and human carcinoma cell lines [57, 58]. Other Zn (zinc)-finger transcription factors, including Snail2 [59], ZEB1 [60], ZEB2 [61], were also shown to directly bind to the E-boxes of the E-cadherin promoter to repress its transcription. In addition to these Zn-finger transcription factors, ectopic expression of bHLH family transcription factor Twist1 resulted in the loss of E-cadherin mediated cell-cell adhesion, expression of mesenchymal markers and induction of cell motility, hallmarks of EMT in normal mammalian cells. Suppression of Twist in highly metastatic mammary carcinoma cells inhibited their metastatic capacity from the mammary gland to the lung in a murine breast tumor model [62]. Correlations between Twist1 and Snail2 during EMT in cancer metastasis were also discussed [63]. Other signaling pathways underlying EMT include bone morphogenetic protein (BMP), Wnt–β-catenin, Notch signaling, Hedgehog signaling, and receptor tyrosine kinases. In addition, environmental factors such as hypoxia and ECM also contribute to EMT process [64, 65].

Nonetheless, the abundant stromal fibroblasts and the dynamics of EMT challenge our interpretation of EMT in human tumors [4]. Although numerous clinical studies correlate EMT with poor clinical outcomes, direct evidence is better demonstrated from the studies of CTCs. By lineage tracing, epithelial-origin CTCs acquiring EMT phenotype were identified in a mouse model of pancreatic cancer [66]. Another study, using a spontaneous squamous cell carcinoma mouse model, showed that activation of EMT by Twist1 induction increased CTCs over 2-fold compared with the control group, most important, these CTCs exhibited mesenchymal marker expression [67]. In a human breast cancer xenograft mouse model, Bonnomet et al. showed that EMT in the primary tumor was associated with an enhanced number of CTCs that expressed mesenchymal markers Snail, Slug and Vimentin [68]. Circulating tumor cells with an EMT feature have also been reported in human patients, including breast cancer, lung, colorectal, prostate, bladder, ovarian, and endometrial cancers [69-71].

1.2.2.2 EMT and Stemness

Cancer stem cells (CSCs), also known as cancer-initiating cells, are those cells that have the ability to perpetuate themselves through self-renewal and to generate mature cells of a particular tissue through differentiation [72]. Cancer stem cells share similar stemness as regular stem cells in developmental processes. Recent studies have correlated EMT with stemness or CSCs. Mani and colleagues showed immortalized human mammary epithelial cells acquired the expression of stem-cell markers after EMT induction, and those transformed

cells exhibited stem-cell properties by a higher efficiency in mammosphere and tumor formation [73]. In the context of cancer metastasis, the relationship between EMT and tumor-initiating capacity is better identified by studies on CTCs. Circulating tumor cells isolated from breast cancer patients showed EMT and stem cell characteristics, and inclusion of EMT markers to optimize CTCs detection was suggested [74-76]. In another study, a cell subpopulation with both tumor-initiating feature ($CD44^{+}/CD24^{-/low}$) and in vitro mammosphere-forming potential was identified in conventional therapy treated breast cancer samples. The increased expression of mesenchymal markers in this cell subpopulation has related EMT with tumor-initiating cells [77]. In a study of epithelial lineage tracing in a mouse model of pancreatic cancer, the authors showed that epithelial-derived CTCs expressed mesenchymal markers, and further characterized the tumor initiating properties of those cells by transplantation [66]. However, not all cells that have undergone EMT possess stem cell-like properties, as is shown by the study in a spontaneous model of EMT using a primary prostate cancer derived cell line [78]. The cross talk between EMT and stemness is far more complex than expected. Different research groups have demonstrated that EMT can suppress the tumor-initiating capacities of human epithelial cells [79]. Epithelial-mesenchymal transition and stemness may be coupled, antagonistic or independent processes in cancer metastasis [80].

1.3 Melanoma Skin Cancer

1.3.1 Melanoma Progression

Cutaneous melanoma has been one of the fastest rising cancers over the past several decades. According to the American Cancer Society, about 87,110 new melanomas will be diagnosed and 9,730 people are expected to die of melanoma in 2017. Melanoma is notorious for its tendency to metastasize and for its poor response to current therapeutic regimens.

Melanoma results from the malignant proliferation of melanocytes, the pigment producing cells in the skin. The classic paradigm describing the progression of normal melanocytes to metastatic melanoma is linear [81, 82]. Melanoma starts from the melanocytic nevus, a benign precursor lesion, followed by the dysplastic nevus. The first malignant stage of melanoma is called the radial growth phase (RGP). At RGP, transformed melanocytes start to spread laterally, but remain close to the epidermis and still under the control of the growth factors from the keratinocytes. The continuing invasion of melanocytes from basement membrane to the upper epidermis and lower dermis leads to the next stage, vertical growth phase (VGP). At VGP, melanocytes completely escape from the keratinocyte signals and new interactions between surrounding stroma cells are established. Melanocytes at VGP have the potential to disseminate and seed distant metastases in other organs [81-83].

However, the progression of melanoma may not be sequential or linear; emerging evidence both in mouse models and clinical observations suggest a much more elaborate view of melanoma metastasis [5]. In contrast to the

traditional notion that metastasis is a late event in tumor progression and it only occurs after the completion of all the required stages, early dissemination of abnormal cells was detected in both murine models and human samples. Husemann performed a comprehensive study about the early dissemination of breast cancer cells in both HER-2 and PyMT transgenic mice. In these breast cancer mouse models, transformed epithelial cells disseminated early from the atypical hyperplastic areas and those early disseminating cells can grow into metastases. Another finding of this study is that the dissemination of tumor cells was not associated with tumor size both in the mouse model and ductal carcinomas in situ in women [84]. A similar study using PyMT mice identified the stem cell property of some early disseminating cells. The disseminating and tumorigenic potential of the disseminating cells was further assessed by transplantation assay using GFP PyMT mice [85]. In a study by Podsypanina et al., untransformed mouse mammary cells in circulation were able to acquire malignant growth upon oncogene activation. They demonstrated this by introducing the premalignant mammary cells into circulation directly without going through the transformation within the primary tumors. Although they could not show the timing of the dissemination, they argued the possibility that untransformed cells that disseminated early could account for the later metastases [86]. Early dissemination before tumor formation was also reported in a pancreatic tumor mouse model, in which cells disseminated at premalignant stage reached the liver as single cells albeit no micro- or macro-metastases were observed [66]. In the spontaneous melanoma mouse model RET.AAD, early

dissemination of melanoma cells before primary tumor formation was identified through a series of assays, including SNP genetic analysis, IHC, RT-PCR and H&E staining. The authors also noted the dormancy of disseminating tumor cells in the lung [87]. Clinically, numerous cases of patients with metastatic melanoma free of detectable primary tumors have been reported [88-93].

1.3.2 Melanoma Dormancy

Malignant melanoma is one of the two malignancies with the highest donor tumor transmission rates (74% transmission rate with 58% mortality) in human transplantations [94]. Organs from donors with melanoma history but free of disease for more than 10 years after initial surgery can present with recurrent metastasis in organ recipients. Late recurrence of melanoma after the initial primary tumor resection is reported [6]. Although various adjuvant therapies are designed to prevent the development of local recurrences or metastasis by targeting “residual” disease, the results have not necessarily been successful. Why is it that the “residual” tumor cells are so persistent? The answer may lie in the fact that the biology of some “residual” cells, that may disseminate into other organs before the removal of primary tumors, seem to be highly divergent from that of the primary tumor and/or overt metastasis [95]. All clinical observations listed previously point to an increasing concern surrounding melanoma-metastatic dormancy, which may be the underlying cause for the incomplete cure of melanoma.

Although a handful of reviews on melanoma dormancy are available [96-98], the number of studies specifically focusing on this phenomenon in

melanoma is very limited compared with that for breast and prostate cancers. Despite the well documented clinical observations, evidence of melanoma dormancy in mouse models, which could serve as a useful tool to reveal the mechanisms underlying human disease, is scarce. An early study by Cameron et al. showed dormancy of solitary melanoma cells in lung using B16F10 cell injection. They concluded that solitary cell dormancy is a post-extravasation event contributing to metastasis inefficiency [99]. A similar study, where human melanoma cells were injected into the tail vein of immunodeficient mice, demonstrated that single or clusters of <10 cells in the lung failed to proliferate in vivo up to 60 days but retained tumor initiating capacity after in vitro culture [100]. Another study employing in vivo imaging showed that uveal melanoma cells can persist in the liver for 6 weeks in a dormant state [101]. Nonetheless, these studies did not provide or propose the underlying mechanisms for how these cells kept dormant and were able to escape immune attack in the metastatic organs. Without a mechanistic understanding, the evidence of melanoma dormancy in humans or mice is of little use in reducing recurrence or increasing survival of patients. A very recent study by Touil et al. may offer more clues on melanoma dormancy. In their study, they obtained a mouse model of melanoma dormancy through melanoma-based immunotherapy and ascertained the in vivo existence of immune-resistant dormant melanoma cells in different organs by qRT-PCR one year after the immunotherapy challenge. Unfortunately, no spatial information of those dormant cells was reported and the following characterization of the dormant cells was done through in vitro study of the

dormant cell-derived cell lines. Although they provided evidence that the dormant cells presented stem-like properties and that the GILZ/FOXO3A pathway was involved in the quiescence of those cells, these in vitro results are not as convincing as in vivo characterization [102].

1.3.3 Melanoma and Metastasis-Initiating Cells

Melanoma is less common, but more serious than other types of skin cancers because of its high metastatic potential. The cellular composition of tumors is heterogeneous; there are many subpopulations of cells within the same tumor. Some cells are more differentiated, some are less differentiated showing a stem cell-like phenotype. Recent evidence suggested that the less differentiated cell population (i.e.,CSCs) may be responsible for tumor initiation and resistance to therapies [72]. Although several melanoma stem cell markers have been described [103-107], the robustness of these markers is questionable considering the limitations of the xenotransplantation assay that is widely used in cancer stem cell studies. In addition, the tumor initiating capacity of CSCs in melanoma was mainly discussed in the context of primary tumor formation and little is known about metastasis-initiating capacities. The differentiation levels of melanocytes with respect to their ability to metastasize was discussed by Damsky et al. Microphthalmia-associated transcription factor (MITF) and WNT/ β-catenin signaling are the known players controlling melanocyte differentiation, but their effects on melanoma metastasis seem to be context-dependent. Two hypotheses have been proposed to explain the controversial effects of the two

players on metastasis: the proper balance in the degree of differentiation, and the phenotypic plasticity of melanoma cells [5].

If during metastasis, the dissemination of tumor-initiating cells is required, presence of circulating tumor-initiating cells should be associated with worse patient outcome. A study demonstrated that CD133 and Nestin were expressed on CTCs, and those CTCs showed a similar expression profile with the matched metastatic lesions [108]. Another corroborating study demonstrated that Nestin was expressed in a proportion of melanoma cells enriched from peripheral blood of melanoma patients, and the expression level of Nestin was higher in stage IV patients compared with stage III／IV patients [109]. Clonal dominance of CD133 in primary tumors of patients with recurrence or metastatic lesions was found, suggesting that CD133 subpopulation may be a determinant of tumor metastasis/tumor relapse [105]. Another study using experimental metastasis models, showed that the WNT receptor, FZD7, was required for the initiation and proliferation of metastatic melanoma growth [110]. In addition, Frank's group has identified a subpopulation of tumor-initiating cells in melanoma by ABCB5 expression [111]. Later they showed that human melanoma CTCs contain tumorigenic cell subpopulations capable of causing metastases, most interestingly, ABCB5-positive tumor cells were highly enriched among melanoma CTCs compared to either primary tumors or pulmonary metastases [112].

1.4 Research Questions

Although efforts to identify melanoma-initiating cells (MICs) or CSCs are increasing and strong evidence is emerging, connections between MICs and

metastasis still remain an open question. This is partly because of the complexity of tumor progression in that both external and internal factors may account for tumor metastasis. Another reason is the lack of good melanoma metastasis models. For obvious reasons, it is impossible to follow a specific cell population in human melanoma patients from the primary tumor to the metastatic sites to check their metastasis-initiating capacity. Proper mouse melanoma metastasis models are very limited and in vivo tracing of cells requires numerous optimized conditions, such as timing of metastasis formation and visualization of interested melanoma cells. The effort to fully characterize melanoma metastasis-initiating cells is worthwhile because it will not only enhance our understanding of melanoma metastasis, but also provide insights for the development of metastasis-targeted therapies.

Phenotypic plasticity of cancer cells is a well-recognized phenomenon; we know that tumor cells can switch gene expression profiles as necessary to best adapt to the environment. However, to what extent and how these changes are related to the spatial or temporal dimensions of tumor progression have not been sufficiently investigated. Histological and morphological dynamics may be observable through real-time in vivo imaging on zebrafish models or mouse models for a short period, while genetic changes are hard to be characterized in those setups. A detailed lineage tracing study in melanoma has not been pursued. As such, the establishment of an in vivo lineage tracing system of the tumorigenic cell subpopulations in a spontaneous mouse model of melanoma metastasis is essential. This system will not only delineate the tumor-initiating

capacity of specific cell subpopulations in the context of metastasis but also indicate the phenotypic plasticity of the metastatic cells (Figure 1.2). My overall hypothesis is that **the primary tumor derived cell subpopulation with a more differentiated state possesses metastasis-initiating capacities and exhibit phenotypic plasticity during the metastatic process**. To test this hypothesis, I addressed the following questions:

Question 1: Can primary tumor derived cell subpopulations with a more differentiated state reach the secondary organs and seed successful metastases?

I created a spontaneous melanoma metastasis mouse Tg (Grm1) Epv / K5-tTA-Edn3 (referred to as Dct-Grm1/ K5-Edn3) where metastasis to the lungs is 80% penetrant. Using the Cre/loxp system, I genetically labeled Tyr expressing cell subpopulations within the primary tumors with GFP in these mice and performed lineage tracing of the labeled cells. I hypothesized that the primary tumor derived Tyr expressing cells or their progeny can reach the lung and successfully seed metastases indicating the tumor-initiating capacities of the differentiated melanoma cell subpopulation.

Question 2: When do the metastatic melanoma cells start to disseminate into the circulation system?

My aim was to identify the metastasis-initiating capacities of the primary tumor derived Tyr expressing cells. Experimental mice were monitored for several months after primary tumor formation. Given the variability in the timing of primary tumor and lung metastasis formation, I could not establish the exact

time of arrival of metastatic cells in the lung. Instead, I focused on the timing of metastatic cell intravasation. I labeled Tyr expressing cells in the mice at three different stages during tumor progression: pre-nevus stage, nevus stage and mature tumor stage. I hypothesized that Tyr expressing cells can invade blood vessels even before the appearance of any noticeable nevus.

Question 3: What are the phenotypic changes that cells undergo during the process of metastasis?

The GFP+ metastatic cells are derived from Tyr expressing cells inside the primary tumors. As the cells leave the primary tumors and enter the circulatory system, I hypothesized that the gene expression profile and pigmentation levels of those cells may undergo a reversible switch to facilitate metastasis. I used immunofluorescence staining with various markers to characterize the phenotype of metastatic cells associated with the pulmonary vasculature.

Question 4: What are the putative mechanisms underlying melanoma metastasis?

The GFP+ cells found in the lung were mostly associated with the vascular endothelium with an elongated shape. The mechanism and functional significance underlying the association between metastatic melanoma cells and vascular endothelial cells is not clear. I performed immunofluorescence staining of intravascular metastatic melanoma cells with endothelial cell specific markers and hypothesized that melanoma-endothelial transition in the primary tumors and endothelial-mesenchymal transition (EndMT) in the metastatic site are mechanisms underlying melanoma metastasis in the *Dct-Grm1/K5-Edn3* mice.

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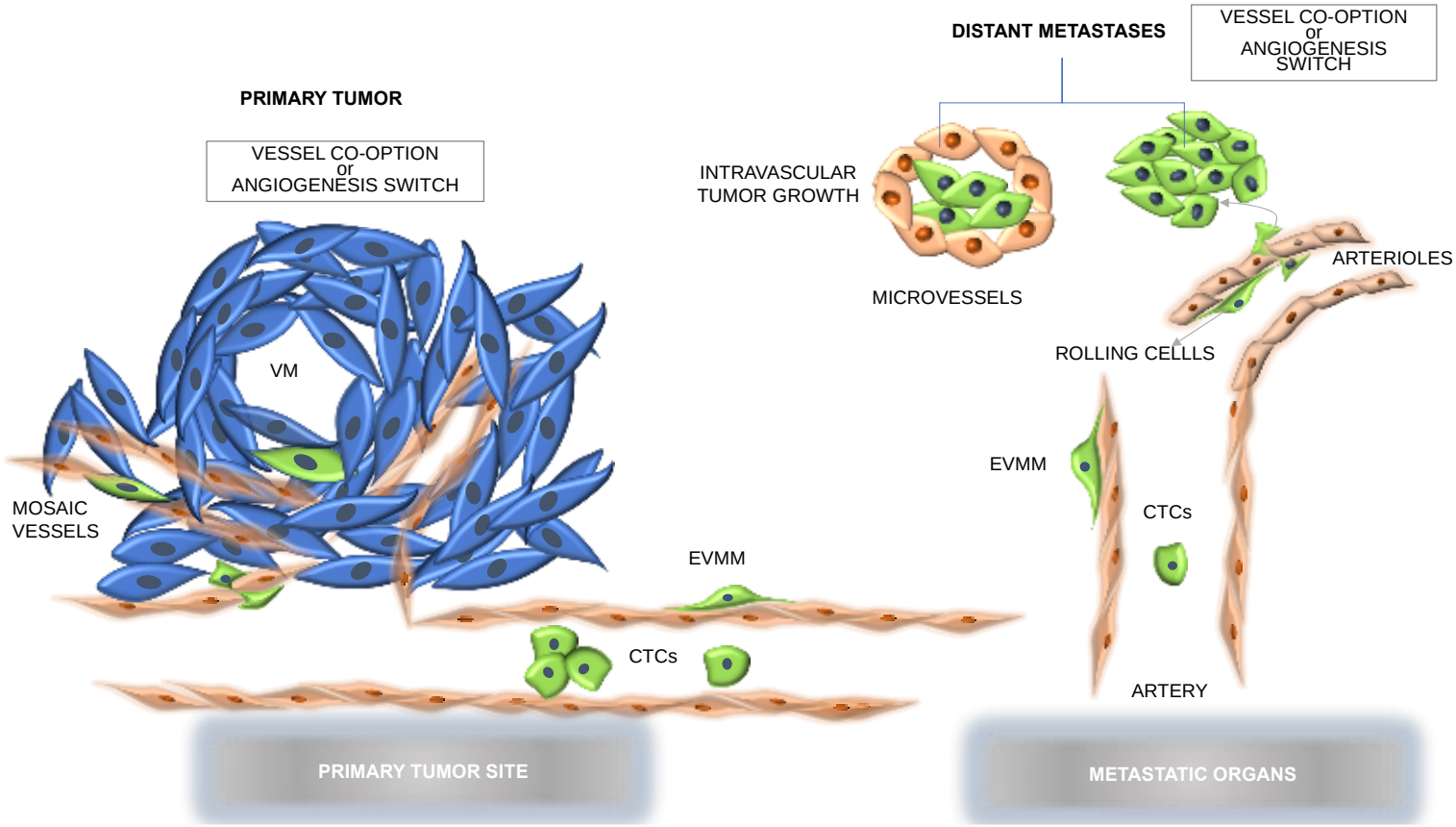
DISTANT METASTASES
VESSEL CO-OPTION
or
ANGIOGENESIS
SWITCH
PRIMARY TUMOR
VESSEL CO-OPTION
or
ANGIOGENESIS SWITCH
INTRAVASCULAR
TUMOR GROWTH
MICROVESSELS
ARTERIOLES
ROLLING CELLLS
VM
EVMM
MOSAIC
VESSELS
CTCs
EVMM
CTCs
ARTERY
PRIMARY TUMOR SITE
METASTATIC ORGANS

Figure 1.1 Diagram of cancer metastasis.

In primary tumors, angiogenesis, vessel co-option, VM and mosaic vessels are potential mechanisms underlying tumor vasculature. The vasculature in primary tumors not only provides oxygen and nutrients for tumor growth, but also provides a potential route for tumor dissemination. Metastatic tumor cells enter the circulation system as single CTCs or clusters through intravasation or other dissemination pathways or migrate along the abluminal vascular surfaces to nearby or distant sites as EVMM. CTCs arrest at the vascular endothelium in the metastatic organs and establish interactions with endothelial cells, roll along the endothelium to find the best sites for extravasation. Extravasated tumor cells proliferate and grow using angiogenesis or vessel co-option. EVMM is another potential source for metastases formation. Intravascular growth of metastatic cells in the microvessels with subsequent extravasation when intravascular micro-metastatic foci outgrow the vessels they are in can be another source.

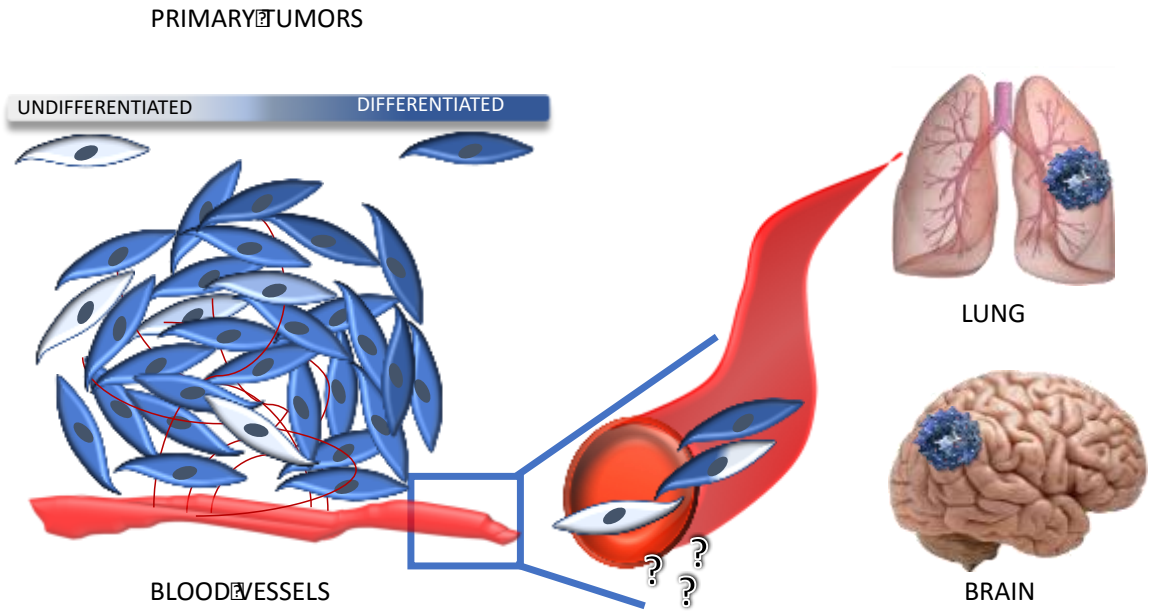

Figure 1.2 Identification of metastatic cell populations in melanoma.

Cells with varying differentiation levels are indicated by different colors in melanoma primary tumors. Certain melanoma cells can leave the primary tumor, enter the circulation system and reach the distal organs to seed metastases. The cell subpopulations that initiate melanoma distant metastases are still unclear and the phenotypic plasticity of the metastatic cell populations will be under investigation.

CHAPTER

2. LINEAGE TRACING AND CHARACTERIZATION OF THE METASTATIC CELL POPULATION IN A MOUSE MODEL OF MELANOMA

2.1 Introduction

Tumors are heterogeneous, involving not only the differences between various solid tumors but also between cell subpopulations within the same tumor. One of the important aspects of tumor heterogeneity that has recently gained increasing acceptance is the cancer stem cell model [1-3]. When it was first introduced in studies of human myeloid leukemia, it proposed that tumors are comprised of phenotypically diverse populations of cells, with only a rare, phenotypically distinct subset of cells having the capacity to significantly proliferate and form new tumors, while the bulk of cells within the tumor lose the tumorigenic capacity [4]. The cancer stem cell hypothesis has been extended to a great array of solid tumors. Using a model in which human breast cancer cells were grown in immunocompromised mice, Al-Hajj et al. showed that only a minority of breast cancer cells possessed the ability to form new tumors. Using the cell surface markers CD44+CD24−/low expression, they were able to distinguish the tumorigenic from the non-tumorigenic cancer cells within the breast cancer bulk [5]. Later, Singh et al. demonstrated that only the CD133+ brain tumor fraction contained cells capable of tumor initiation in NOD-SCID mouse brains and injection of as few as 100 CD133+ cells produced a tumor that could be serially transplanted and re-establish the heterogeneity of the parental tumor, whereas injection of 10^5 CD133- cells did not produce a tumor [6]. A similar study was carried out on prostate cancer. On the basis of the high surface expression of integrin $\alpha 2\beta 1$ and CD133, the authors reported the identification and characterization of a subset of cells from primary and metastatic prostate

tumors that had cancer stem cell characteristics, which was also able to regenerate the phenotypically mixed populations of nonclonogenic cells [7]. In a study of human colorectal cancer, O'Brien et al. identified a human colon-initiating cell subpopulation that was able to maintain themselves as well as differentiate and reestablish tumor heterogeneity upon serial transplantation using CD133 expression [8]. Subsequently, Dalerba et al. provided a more extensive surface marker profile, which better supported the tumor heterogeneity hypothesis, highlighting the epithelial cell adhesion molecule (EpCAM), also known as epithelial-specific antigen (ESA), and CD44 as more robust markers for colorectal cancer stem cells when compared to the previously reported marker CD133 [9]. Another study using a xenograft model identified a highly tumorigenic subpopulation of pancreatic cancer cells expressing the cell surface markers CD44, CD24, and ESA [10]. Using a similar approach as that for studies on breast cancer, Prince et al. identified a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma (HNSSC), which were CD44+ and typically comprised <10% of the cells in a HNSCC tumor [11].

The cancer stem cell model has also been proposed for melanoma [12] and several melanoma stem cell markers such as Nestin [13], CD133 [14, 15], CD271(also p75NTR) [16, 17], and JARID1B [18, 19] were demonstrated to select a subpopulation of melanoma cells with a higher tumor initiating capacity. However, the cancer stem cell model remains controversial in the melanoma field and several questions are still unanswered. For example, are the cancer stem cells a constant cell population within the hierarchically organized tumor? Or

does reversible phenotypic plasticity exist among these markers? Quintana et al. [20] pointed out that the frequency of tumorigenic melanoma cells that can be detected after xenotransplantation was highly assay-dependent. By making a series of changes in assay conditions, they increased the detectable frequency of tumorigenic melanoma cells after xenotransplantation into NOD/SCID IL2R**g**null (NSG) mice, proving that both CD133+ and CD133- fractions formed tumors that exhibited similar heterogeneity in CD133 expression. In a subsequent study [21] by the same group, neither ABCB5 nor CD271 expression defined a subpopulation of cells that had enriched tumorigenic potential since all injections formed tumors that were phenotypically similar to the parent tumors irrespective of whether the cells injected were marker positive or negative. Additionally, both CD271+ and CD271- cells formed tumors capable of metastasis. Combined, the results of Quintana et al. led them to the conclusion that tumorigenic cells appeared to undergo reversible changes in the expression of many markers in vivo. This conclusion is further supported by the study of Roesch et al. [19] demonstrating that the JARID1B phenotype was dynamic, in that JARID1B-negative cells also gave rise to a heterogeneous progeny containing JARID1B-positive cells, which suggested a new understanding of melanoma heterogeneity with tumor maintenance as a dynamic process mediated by a temporarily distinct subpopulation.

Overall, the two key components in the cancer stem cell scenario, the rarity and tumor initiating capacity, are still being questioned [22-24]. Xenotransplantation experiments were the basis to enrich tumorigenic cells from

non-tumorigenic cells in most of the cancer stem cell studies. Though widely used, as human tumor cells are being processed and transplanted, they are subjected to new environmental conditions that may alter their phenotype and consequently their tumor initiating capabilities [25]. Any of the conditions, such as the enzymatic dissociation conditions for human cells, mouse injection sites, the use of immunocompromised mice, as well as the patient's stage of disease can contribute to conflicting results. Civenni et al. [16] developed a protocol to avoid trypsin during melanoma cell fraction preparation and the results obtained were compatible with those of some earlier studies. Their study provided some more convincing data given that the injected cells most likely did not lose surface marker expression because of interactions with digestive enzymes. Roesch et al. [19] compared the different effects of cell culture conditions and found that human embryonic stem cell medium better supported the survival of cells with inherent self-renewal potential when compared with conventional culture conditions. The optimized xenotransplantation assay with a more highly immunocompromised NOD/SCID IL2R**γ**null mice increased the detection of tumorigenic melanoma cells, demonstrating that tumorigenic cells are common in some human cancers [20]. Furthermore, certain cancer cell subpopulations were shown to have reversible drug-tolerance capacity and lose their stem cell markers during this process [26]. It is thus reasonable to speculate that the injection of cells obtained from patients that have undergone particular medical treatments may result in the generation of stem cell marker negative cells with tumorigenic capacity.

Because of the complexity of the metastatic process and the conflicting results obtained from injection and xenotransplantation studies, I propose that the direct in vivo lineage tracing of tumorigenic cells in a spontaneous mouse model of melanoma is a more robust and powerful tool to understand how specific subpopulations of cells within a tumor acquire highly invasive properties and is responsible for the seeding of distant lesions. My study will provide significant value to the understanding of melanoma metastasis and offer new venues for therapy development of melanoma patients.

The use of lineage tracing in the cancer field has become more popular in recent years emphasizing the tracing of all progeny of one single cell. The basic foundations of lineage tracing and how data obtained from lineage tracing can be interpreted is reviewed by Blanpain and Simons with the focus on epithelial tissues [27]. Lineage tracing by genetic labeling is widely used for cell fate determination of stem and progenitor cells in the field of developmental biology, tissue maintenance and repair, and dynamics of tumor initiating capability of cancer cells. Shin and colleagues did fate mapping of Sonic hedhehog (Shh) expressing basal cells of the urothelium by lineage tracing and showed that Shh expressing cells can function as stem cells capable of generating all cell types to help maintain the epithelial integrity upon bacterial infection or chemical injury [28]. Another epithelial stem cell lineage tracing study performed in the murine small intestine showed that B lymphoma Mo-MLV insertion region 1 homolog (Bmi1)-expressing cells act as an alternative stem cell pool after the ablation of Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) expressing

stem cells [29]. In vivo lineage tracing and retracing by multicolor reporter mice gave the first strong evidence that Lgr5 expressing cells are stem cells in unperturbed primary tumors on a mouse model of intestinal adenomas [30]. A more recent clonal analysis using multicolor lineage tracing system on intestinal tumors further demonstrated the cancer stem cell role of Bmi1 and Lgr5 expressing cells during tumor propagation [31]. Another good example for lineage tracing was carried out by Zhu and colleagues on high grade brain tumors [32]. This study gave direct evidence that Tlx is a brain tumor stem cell marker in a mouse model using RCAS/tv-a system [33] for tumor induction and Cre/loxp system for in vivo lineage tracing. Although lineage tracing has been widely used in different fields and certain kinds of tumors, there is a substantial gap in the field of melanoma biology and little is known about the cellular origin of melanoma, especially in identification of the precursor cells of metastasis. This is most likely the result of the lack of a good metastatic melanoma mouse model that can be used together with Cre/loxp system. In contrast, lineage tracing of stromal or endothelial cells during melanoma formation is easier since melanoma tumors can be obtained through tumor engraftment and transgenic mice with stromal or endothelial cells labeled are available. A recent quantitative clonal analysis using multicolor lineage tracing by Corey et.al [34] showed that in an elaborate tumor microenvironment created by the injection of the murine melanoma cell line B16F10, blood vessels followed a pattern of dynamic clonal evolution accompanied by the loss of lymphocyte adhesion molecules favoring the immune escape of cancer cells. The establishment of an in vivo lineage

tracing system to provide a detailed characterization of the cellular origin of melanoma metastasis is needed and will be of great significance both to the field of basic cancer research as well as to future clinical applications.

2.2 Results

2.2.1 Tumor Heterogeneity Exists in Mouse Melanoma

To demonstrate cellular tumor heterogeneity in mouse melanoma, I studied the composition of tumors from Hepatocyte Growth Factor/Scatter Factor (HGF/SF) mice [35], Dct-Grm1/ K5-Edn3 mice and Dct-Grm1/K5-Edn3/Dct-Ednrb mice with spontaneously developed or UV induced lesions. Nestin [13], CD 271 (also known as p75) [16, 17] were chosen as the stem cell markers and tyrosinase (TYR) or tyrosinase related protein 1 (TRP1) were used as differentiation cell markers. All the three melanoma tumors showed cellular heterogeneity with non-overlapping cell subpopulations positive for various markers (Figure 2.1).

2.2.2 Mouse Model and Methodology Validation

The *Dct-Grm1/ K5-Edn3* mice were created in our laboratory [36, 37]. Depending on the aberrant expression of glutamate metabotropic receptor 1 in melanocytes and environmental Endothelin 3 (Edn3) expressed by keratinocytes, these mice not only spontaneously develop melanoma tumors on the ear, tail and dorsal skin, but also show metastases in the lung (Figure 2.2). Chin et al. previously showed that Edn3 signaling promotes melanoma progression in *Dct-Grm1/ K5-Edn3* mice resulting in 80% penetrant lung metastases compared with the *Dct-Grm1* controls that have limited metastatic capability [38]. On the basis of

the tumor heterogeneity scenario, this spontaneous mouse model of melanoma metastasis will be an ideal system to identify the intrinsic tumor initiating capacity of certain cell subpopulations within an unperturbed tumor environment. Given that the Nestin positive melanoma stem cells are rare in these tumors (Figure 2.1), the differentiated Tyr expressing cells which constitute the majority of the tumor is a better option for lineage tracing. Tyrosinase is a copper-containing enzyme that catalyzes the production of melanin and the substrates give the color of skin, hair and eyes. Tyr is expressed specifically in melanocytes, the pigment producing cells, and majority of melanoma cells.

Lineage tracing was performed using the Cre/loxp system with *TYR-CreER^{T2}/ mT/mG* mice. Briefly, in the *TYR-CreER^{T2}/ mT/mG* mice, prior to Cre recombination, cell membrane-localized tdTomato (mT) fluorescence is expressed in every single cell. After tamoxifen/4HT induction, Tyr positive Cre recombinase expressing cells (and future cell lineages derived from these cells) show cell membrane-localized GFP (mG) fluorescence expression replacing the red fluorescence (Figure 2.3A). In vivo lineage tracing of Tyr expressing cells in the primary tumors can be performed on *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice by topical 4HT application using a small painting brush on the primary tumor sites (Figure 2.3B).

The *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice obtained from the mating of *Dct-Grm1/ K5-Edn3* melanoma mice and *TYR-CreR^{T2}/ mT/mG* mice spontaneously develop primary and metastatic melanomas, and Cre inducible Tyr positive cells that can be labeled with GFP making them distinguishable from

the other red fluorescent labeled cells in the body. Because these mice were obtained through breeding of several transgenic mice, the copy numbers of transgenes essential for tumor development were dramatically diluted resulting in a considerable delay in tumor appearance when compared to what was initially reported in Chin, 2015 [38]. In *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice, the first primary tumors appeared at an average of 7.5 month (n=25), which is 2.5 month longer than previously reported for the *Dct-Grm1/ K5-Edn3* mice.

To validate the topical application methodology, dorsal skin of the *TYR-CreER^{T2}/ mT/mG* mice was topically painted and successful GFP labeling of melanocytes in the hair follicles was observed, while other cells in the skin retained the tdTomato red fluorescence color (Figure 2.3C). The same induction method was applied to *K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice, in which accumulation of melanocytes outside the hair follicles at the dermal-epidermal junction was expected [36]. Indeed, GFP+ green cells were found outside the hair follicles (Figure 2.3D). To establish if the TYR promoter can also drive Tyr expression in melanoma tumors in *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice, topical painting of 4HT was performed on the tail tumors of *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice. A mutually exclusive pattern of GFP and tdTomato colors was found inside the tumors (Figure 2.3E).

2.2.3 Labeling of the Tyr Expressing Cells in the Primary Tumors

At an average of 7.5 month of age when the lesion first appeared as a nevus (<1mm in height) on the *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice, I started 4HT topical administration. 4HT induction exclusively at an early stage

was not sufficient to label the majority of Tyr expressing cells in the primary tumors (Figure S1). To ensure the labeling of most Tyr expressing cells in the primary tumors, 4HT topical administration was repeated for 3~4 weeks during tumor growth until euthanasia. Numerous GFP+ cells were detected in cryosections of primary tumors and lung metastases (Figure 2.4). To establish if the GFP+ cells in the primary tumors expressed Tyr protein, cryosections of the tumor were prepared soon after 4HT application and labeled with Tyr antibody. GFP+ cells were found to be positive for Tyr immunoreactivity (data not shown).

2.2.4 Primary Tumor Derived Tyr Expressing Cells or Their Progeny Can Reach the Lung and Seed Metastasis

The appearance of metastases in distant organs was established 2 weeks to 7 months after the first 4HT painting of primary tumors. Micro-metastases, characterized as clusters of less than 10 cells, were detected in 86% of lungs examined (n=15). Each lung was sectioned and at least 20 cryosections per lung were examined for the presence of primary tumor derived GFP+ cells. In 50% of 4HT painted *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice, GFP+ cells were identified in various areas of the lung and other organs (Table S1). The number of GFP+ cells found in the lung varied considerably, from 2 to 20 or more cells per section. In most mice, the numbers were low, except for one mouse that presented with pepper like micro-metastases and another one with macro-metastases. No GFP+ cells were found in lungs of control mice (*Dct-Grm1/ K5-Edn3/ mT/mG*) that had tumors painted with 4HT (n=10). Additionally, no GFP+ cells were detected in the lungs of mice that did not bear tumors (*Dct-Grm1/ K5-*

Edn3/ TYR-CreER^{T2}/ mT/mG) and had their dorsum painted with 4HT (n=3). The location of the primary tumor affected the penetrance of GFP+ cells in the lung of 4HT painted *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice (Table S1). Only one out of seven mice (1/7, 14%) that had only tail tumors painted, in contrast to three out of four (3/4, 75%) mice that had only dorsal tumor painted, presented with GFP+ cells in the lung. The only mouse with a dorsal tumor that did not have GFP+ cells in the lung was painted only once at the nevus stage and no obvious tumor growth was observed upon euthanasia.

The GFP+ cells were distributed into three categories depending on their location in the lungs. The first category termed “inside the vessels” includes intravascular solitary cells with an elongated and spindle shape found in close association with the endothelial layer of the lung vasculature (Figure 2.5A). Cells in this category were detected consistently, even on metastasis-free lungs, providing support to the hematogenous dissemination of metastatic cells. The second category termed “outside the vessels” consists of clusters of GFP+ cells overlapping with the pigmented metastases (Figure 2.5B) with the possibility that some may be still inside the alveolar capillaries. The rarest third category consisted of solitary or tiny clusters (2~5) of cells outside the vasculature displaying no pigmentation. Immunostaining showed that the GFP+ cell populated metastatic lesions in the lung were positive for melanoma markers TYR and TRP1 (Figure 2.5C, D), indicating the tumor initiating capacity of the differentiated Tyr expressing cells or their progeny in the *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice. Of note, not all metastatic lesions in the lung

contained GFP+ cells. This finding was not unexpected given that the efficiency of the topical 4HT induction is not 100%, and that, primary tumor resident Tyr negative cells might also metastasize.

2.2.5 Presence of Metastatic Cells inside the Lung Vasculature

Melanoma metastasis has been suggested as an inefficient process, during which only a small number of cells can go through all the steps for a clinically significant metastasis [39, 40]. It has been generally thought that most metastatic cells are eliminated in the circulation [41]. In a small number of animals (n=4) that were not subjected to cardiac PBS perfusion, lungs were snap frozen immediately after euthanasia. In sections of these lungs, the blood vessels were filled with blood but very few circulating GFP+ cells were found inside the lumen of the vasculature (Figure S2E). Instead, we observed large numbers of metastatic GFP+ cells associated with the pulmonary vascular endothelium or invading the lung tissue. The number of GFP+ cells “inside the vessels” far exceeded the micro- or macro-metastatic lesions “outside the vessels”. To find out if GFP+ cells were proliferating, we performed Ki67 staining on the lung sections where GFP+ cells were found. Double labeled GFP and Ki67 cells were mostly confined to cell clusters “outside the vessels” (Figure 2.6E-H). In contrast, the solitary GFP+ cells “inside the vessels” were mostly Ki67 negative, although some Ki67+ cells were identified (Figure 2.6A-D). In the lungs of *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice (n=3), the percentage of Ki67+ intravascular GFP+ cells was less than 1% (Figure 2.7A). Disseminated cancer cells within the circulation are exposed to the mechanical sheer stress, as well as immune

surveillance, which may lead to rapid cell death [40, 42]. Most of the GFP+ cells were found inside the vasculature including the big pulmonary arteries. To determine if GFP+ cells had undergone apoptosis, we performed immunostaining with an antibody against Cleaved Caspase 3. We were only able to detect a small number of double labeled GFP + and Cleaved Caspase 3 + cells “inside the vessels” (Figure S3). In the lungs of *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice (n=3), the percentage of Caspase 3+ intravascular GFP+ cells was less than 3% (Figure 2.7B).

2.2.6 Presence of Perivascular Cellular Infiltrates in Metastatic Lungs

The detailed examination of lung cryosections of *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice where GFP+ cells were found, indicated the presence of perivascular infiltrates in close proximity to the GFP+ cells. Notably, the infiltrates were more obvious in lungs with micro-metastases (n=5) compared with that with macro-metastases (n=2). Hematoxylin-eosin (H&E) staining showed abnormal aggregates of cells surrounding the pulmonary vasculature, among which brown pigmented macrophages were noticed (Figure 2.8A, B). Small to no infiltrates were observed in GFP+ cell-free lungs. Fluorescence immunostaining of the lung cryosections showed that the infiltrates were mostly composed of CD45+ lymphocytes (Figure 2.8C), including CD3 positive T cells (Figure 2.8D). Most cells within the infiltrates showed high levels of proliferation as demonstrated by Ki67 immunoreactivity (Figure S4), suggesting the activation of an immune response to the arrival of GFP+ metastatic cancer cells inside the vasculature. These abnormal perivascular infiltrates found in lungs containing

foreign GFP+ cells may add another explanation for the dormancy of intravascular cancer cells.

2.2.7 GFP+ cells Lose Pigmentation upon Contact with Vascular Endothelium

In order to detect any changes in the differentiation status of metastatic cells, the pigmentation level of GFP+ cells was monitored as cells traveled from the primary tumor to the lungs. At the primary tumor site, tissues deep underneath the skin tumors containing blood vessels were collected and sectioned. GFP+ cells were identified both inside and outside the vasculature and interestingly, GFP+ cells inside the vasculature were not pigmented (Figure 2.9A, D, arrowhead) while GFP+ cells outside the vasculature were pigmented (Figure 2.9A, D, arrow). This same dynamic pattern of pigmentation levels was observed in the lung. Intravascular GFP+ cells in the lung vasculature were not pigmented (Figure 2.9B, E, arrowhead), but resumed pigmentation production while populating the lung metastases (Figure 2.9C, F, arrow).

2.2.8 Characterization of Metastatic GFP+ Cells Inside Pulmonary Vasculature

Given the loss of pigmentation by GFP+ cells as they entered the vasculature, further characterization was necessary to establish their level of differentiation. Immunostaining was performed on the lung cryosections with the neural crest specific marker SOX10 (Figure 2.10A-D) and differentiated melanocyte markers TYR and TRP1 (Figure 2. 10E-L). The GFP+ cells were negative for all of them. The testing of other melanocytic lineage markers such as

MITF and TRP2 also yielded negative results (data not shown). Based on the stem cell model, it is possible that the differentiated Tyr expressing cells or their progeny de-differentiated and transiently acquired stem cell properties. In previous studies, P75 and Nestin were shown to selectively label melanoma tumor initiating cells [13, 16, 17]. As such, we performed immunostaining with P75 and Nestin on the lung cryosections where GFP+ cells were found, and the results were also negative (Figure 2.10M-T). All fluorescence immunostaining was done with a positive control to validate the indicated antibodies used, and the staining results are representative of at least five mice.

2.2.9 Primary Tumor Derived Tyr Expressing Cells or Their Progeny Can Reach other organs and Seed Metastasis

Regional lymph node metastasis is regarded as an indicator of metastasis for many malignancies, including melanoma [43]. Although organ preferences of melanoma metastasis are observed, they can spread widely in the body. Most *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice with tumors showed enlarged and hyperpigmented lymph nodes. Cervical lymph nodes were collected from 4HT painted mice and examined for GFP+ cells. Fifty eight percent (7/12) of the mice with the presence of GFP+ cells in the lung also presented with GFP+ cells in the lymph nodes (Table S1 and Figure 2.11). The enlarged lymph nodes of *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice were rich with vessels (blood/lymphatic). The staining pattern of TYR in lymph nodes occupied by GFP+ cells was different from that in the lung. The GFP+ cells in lymph nodes were more heterogeneous, both TYR+ (Figure 2.11, arrowhead) and TYR- (Figure

2.11, arrow) GFP+ cells were found. Additionally, TYR+ cells that were not GFP+ were also observed (Figure 2.11, star) indicating either the insufficiency of 4HT topical induction or the presence of non-Tyr expressing cell subpopulations.

The most common sites of visceral metastases in the *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice were lungs, with only one mouse (1/15) showing evident kidney and spleen metastases. The GFP+ cells were found inside the metastatic foci of the kidney and spleen (Figure S2F-I), further demonstrating the tumor initiating potential of the primary tumor derived Tyr expressing cells or their progeny.

The *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice spontaneously develop multiple melanocytic tumors on the skin, all of which were presumed as primary tumors. However, it is plausible that cells from the first dorsal/tail tumor spread and seeded the secondary dorsal/tail tumor at different sites of the dorsum/tail. In three *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice, the dorsal tumors and only one tail tumor were subjected to topical 4HT painting. One tumor per mouse that appeared later and was not painted was checked for the presence of GFP+ cells. Interestingly, non-painted tumors from the three mice were mainly constituted by GFP+ cells (Figure S5). To exclude the inappropriate spread of 4HT from grooming behavior, the non-painted tumors examined were chosen as far as possible from the painted ones, for example, a non-painted tail tumor proximal to the body vs. a painted tail tumor distal to the body; a non-painted dorsal tumor on top of right hind leg vs. a painted dorsal tumor on left flank. Although we could not establish whether the tumorigenic GFP+ cells giving

rise to the non-painted tumor were dorsal tumor-derived or tail tumor-derived, the result presented here further proved the tumor initiating capacity of Tyr expressing melanoma cells or their progeny.

2.2.10 Metastatic Melanoma Cells Disseminate Early during Melanomagenesis

Late recurrence is fatal and has been shown clinically in melanoma patients who have gone through primary tumor resection at early diagnosis. Although greater than 85% cutaneous melanoma patients can be cured after the removal of the primary tumors, a small number of patients may show recurrence as late as 15-20 years [44]. This not only indicates the existence of metastasis dormancy but also suggests that metastatic melanoma cells may disseminate into the circulation system much earlier than previously expected. To test this, we grouped the *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice into three stages, before nevus stage (n=3, no noticeable nevus or tumor was observed), nevus stage (n=3, <1mm in height) and tumor stage (n=3). We painted 4HT on the nevus or tumor as described previously. For the before nevus stage group, painting of 4HT was performed 3-4cm from the end of the tail. The tails with the painted area were collected and sections containing blood vessels were analyzed for the presence of GFP+ cells. The GFP+ cells were found inside the tail vasculature at all three stages (Figure 2.12). The results indicate that tumorigenic cells acquire and maintain the ability to enter the circulation at all times during the process of tumorigenesis, even prior to the formation of a sizable tumor.

2.3 Discussion

Melanoma, the deadliest type of skin cancer, still imposes a great challenge for therapy because of its highly aggressive nature to metastasize to distant organs. As an important step of preclinical research, some melanoma murine models have been developed, including xenograft, syngeneic, and genetically engineered models, to help understand the mechanisms of melanoma progression or develop promising therapies to melanoma patients. It is generally believed that genetically engineered models are better options compared with xenograft or syngeneic models in regard to anticancer drug assessment [45]. Among the genetically engineered melanoma mouse models that have been developed, some require extra carcinogens to induce melanoma, such as UV exposure, which adds to the cost and labor-intensive interbreeding of multiple mouse strains. More important, few of the genetically engineered melanoma mouse models were reported to develop visceral metastases, which limits the study of melanoma metastasis. Of the several melanoma mouse models that show distant metastasis, some of them already employed Cre/loxp to induce tumors, preventing us from using the Cre/loxp system for lineage tracing [46, 47].

The *Dct-Grm1/ K5-Edn3* mice, to our knowledge, is the first spontaneous melanoma metastasis mouse model that is free of oncogenes and dependent exclusively on the presence of signaling pathways. The tumor metastasis process of these mice closely resembles that in human melanoma patients given the variability in time to tumor and appearance of metastasis. Although the *Dct-Grm1/ K5-Edn3* mice provide us with an optimum system for lineage tracing of

metastatic cells within an unperturbed tumor environment, the number of samples that can be obtained within a reasonable amount of time is limited. In the *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice where lung metastases are identified, the terminal fate of those labeled cells in the secondary organ can be determined and perhaps more interesting, the dynamic changes in genotype and phenotype during the metastatic process can also be investigated.

To spatially restrict Cre induction within the primary tumors, we topically applied 4HT on the tumors instead of systemic administration. 4-hydroxytamoxifen topical application has been successfully used in other studies for site specific Cre induction [48-50]. The fact that GFP+ cells were absent in some lungs with pigmented metastases attests to the spatial restriction of Cre induction, given that all melanocytic lesions should be labeled with GFP if systemic administration of 4HT through blood happened. In addition, the absence of GFP+ cells in some pigmented lesions, but not others, in the same lung further excluded the possibility of 4HT systemic administration. In other words, GFP+ cells found in the lung can only be derived from the dorsal/tail tumor that were 4HT painted. Although labeling of Tyr expressing cells may not be 100% efficient, this should not constitute a problem for this study considering the abundance of differentiated cells in the melanoma lesions. It is important to bear in mind that cancer is complex and dynamic. As tumors grow, they may recruit more melanocytes located in the dermal-epidermal junction, and it is also possible that stroma cells transdifferentiate into Tyr expressing melanocytes as it was shown

that a combination of three factors, MITF, SOX10 and PAX3, were sufficient to convert mouse and human fibroblasts to functional melanocytes in vitro [51].

The heterogeneity of tumors including melanoma has been recognized for decades pointing to the varied tumor initiating capacities of cell subpopulations within the solid tumors [52]. While some studies have confirmed that cells that express melanoma stem cell markers have tumor initiating capacity, they have mostly focused on primary tumor formation not metastasis. The robustness of the stem cell markers used in these studies is also controversial based on the use of xenotransplantion assays [13-17]. Here, using Cre/loxp system, we genetically labeled Tyr expressing cells within the primary tumors in the *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice and tracked the fate of the labeled cells by lineage tracing. We demonstrated that the more differentiated Tyr expressing cells can also be the progenitors of tumor initiating cells and seed distant metastases in various organs successfully in mice. However, what we have shown does not refute entirely the stem cell scenario in that gene expression profiles are dynamic, and it is possible that GFP+ cells de-differentiated to a more stem cell-like state after the labeling. This is also supported by the fact that GFP+ cells lost their pigmentation, as well as melanocytic lineage markers while in contact with the vascular endothelium, but, regained those features after seeding metastases. The fact that tumor cells are plastic increases the complexity of the melanomagenesis process, suggesting that the tumor initiating potential of melanoma cells may be underestimated and the spatial and timing elements

should be considered when a melanoma stem cell marker is defined or characterized.

Metastasis disease is the primary cause of death in cancer patients. Although prolonged survival occurs as a result of the development of newr therapies and better understanding of the metastatic mechanism, the early diagnosis of cancer metastases has always been a challenge. In general, human tumor metastasis consists of a series of biological processes including: metastatic cells leave the primary tumor, invade into surrounding tissues and enter the blood or lymphatic vessels, travel through the circulatory system, arrest at the distant organs and finally seed successful metastases [53, 54]. Most studies on melanoma metastasis were either focused on the first stage (primary tumors) or the last stage (metastatic tumors). Gene microarray data from different stages of melanoma progression were analyzed and shed light on the mechanism of metastasis [55-58]. Metastatic tumor cells in circulation have been long recognized, and CTCs in the blood stream have been successfully isolated from human blood [59-61]. An FDA approved detection system, Cellsearch, is available for the isolation of CTCs and has been successfully used for monitoring patients with metastatic breast, prostate and colon cancers [62]. Nonetheless, no consensus has been reached regarding melanoma CTCs because of sample scarcity, cohort heterogeneity, and the application of Cellsearch on melanoma patients seems not sensitive enough and needs further investigations [63]. The development of real-time imaging techniques, such as non-invasive methods or intravital microscopy, make it possible to study the dynamic process of cancer

metastasis [64-66], including melanoma [67, 68]. However, real time imaging cannot investigate the genetic diversity of the metastatic tumor cells, and most in vivo real time imaging has been based on cell injection, xenograft or syngeneic transplantations, bypassing the complexity of endogenous tumor-host interactions. In our study the use of a spontaneous melanoma mouse model depending on GRM1 and Edn3 signaling pathways allowed us to characterize the dynamic gene expression profiles of metastatic cells in relationship to their spatial distribution during the journey from primary tumor sites to secondary organs and represents a tremendous advantage in understanding mechanisms associated with melanoma metastasis.

It is noteworthy that the better studied CTCs are not necessarily the contributors of metastases or recurrence, instead, disseminating cells that survive after infiltrating distant organs, may be more dangerous since they can be dormant anywhere as invisible, undetectable solitary or cluster of cells. Studies about metastatic dormancy on a variety of cancers have been increasing [62, 64, 69], but little attention has been paid to metastatic dormancy of melanoma. During the discussion between members of the Melanoma Research Foundation (MRF) Scientific Advisory Council and Breakthrough Consortium in 2015, Merlino et al. highlighted melanoma dormancy as one of the four thematic areas and raised the issue that study of melanoma dormancy lagged significantly behind that of breast and prostate cancers and research about this area was required [70].

Although it has been proposed that metastatic dormancy of melanoma can be established directly at the target organ [70], in vivo evidence is missing. The best studied location of metastatic dormancy in cancers is the bone marrow [71-73], as has been shown in the only melanoma dormancy study with the Ret transgenic mouse model [74]. The data presented in this study suggested that the vascular endothelium in the target organ can act as a potential reservoir for metastatic melanoma cell dormancy. We found that the primary tumor derived metastatic melanoma cells associated with the pulmonary vascular endothelium did not proliferate or undergo apoptosis, recapitulating the state of metastatic dormancy. As a consequence of the limitation of our model, identification of the fate of each individual cell is impossible, as such, we cannot conclude that the dormant melanoma cells inside the pulmonary vasculature are the ones that exit and seed the overt metastases after reactivation. The CTCs may also be possible contributors [75].

Late recurrence of melanoma has been reported, and the recurrence site is commonly the lungs [76]. If the presence of putative dormant metastatic cells in the vasculature of the lung also occurs in human patients, stopping therapies in patients who are thought to be disease free may have dire consequences. The mechanism used by disseminated tumor cells to survive in immunocompetent individuals for an extended period of time is one of the biggest conundrums in tumor immunology. A possible explanation is what has been called tumor immunoediting: genetically and epigenetically unstable tumor cells can adapt to immune attack by either down regulating tumor associated antigens or shutting

down death signaling pathways [77]. The loss of the melanocytic/melanoma markers by the GFP+ cells may represent this concept of immunoediting. Although viable, the outgrowth of the dormant tumor cells is restrained by the adaptive immunity. Koebel et al. called this process “equilibrium” and they provided evidence that tumor cells in equilibrium were highly immunogenic whereas the outgrowing tumor cells were not [78]. This is in line with the results obtained with the Ret transgenic mouse model where it was shown that bone marrow melanoma-specific memory T Cells were detectable and functionally active in tumor free mice [74] and that CD8+ T cells could delay the visceral metastases in the mice [79, 80]. Stronger evidence of the tumor-immune “equilibrium” can be obtained from clinical reports of donor derived melanoma through transplantation surgeries [81-84]. In our study, the fact that lymphocytes in the perivascular infiltrates were found in the lungs where dormant melanoma cells were identified but not obvious in lungs with full-blown metastases can be explained by the immune equilibrium. Yet, further investigation is needed to elucidate the mechanisms underlying the interplay between immune cells and dormant metastatic cells.

Early dissemination of abnormal cells has been detected in murine models of various cancers [85-89]. Numerous cases of patients with metastatic melanoma free of detectable primary tumors have been reported clinically [90-95]. The presence of GFP+ cells inside the vasculature at all stages during the process of tumorigenesis, even prior to the formation of a sizable tumor indicated the early dissemination of melanocytes. Although there is a high chance that

these intravascular GFP+ cells disseminating early into tail vasculature are just dormant and not fully malignant, this still constitutes strong evidence that prospective metastatic melanoma cells may enter the circulation system before the formation of primary tumors. The study showing untransformed mouse mammary cells in the circulation could acquire malignant growth upon oncogene activation further argued the possibility that untransformed cells that disseminated early could account for the later metastases [87].

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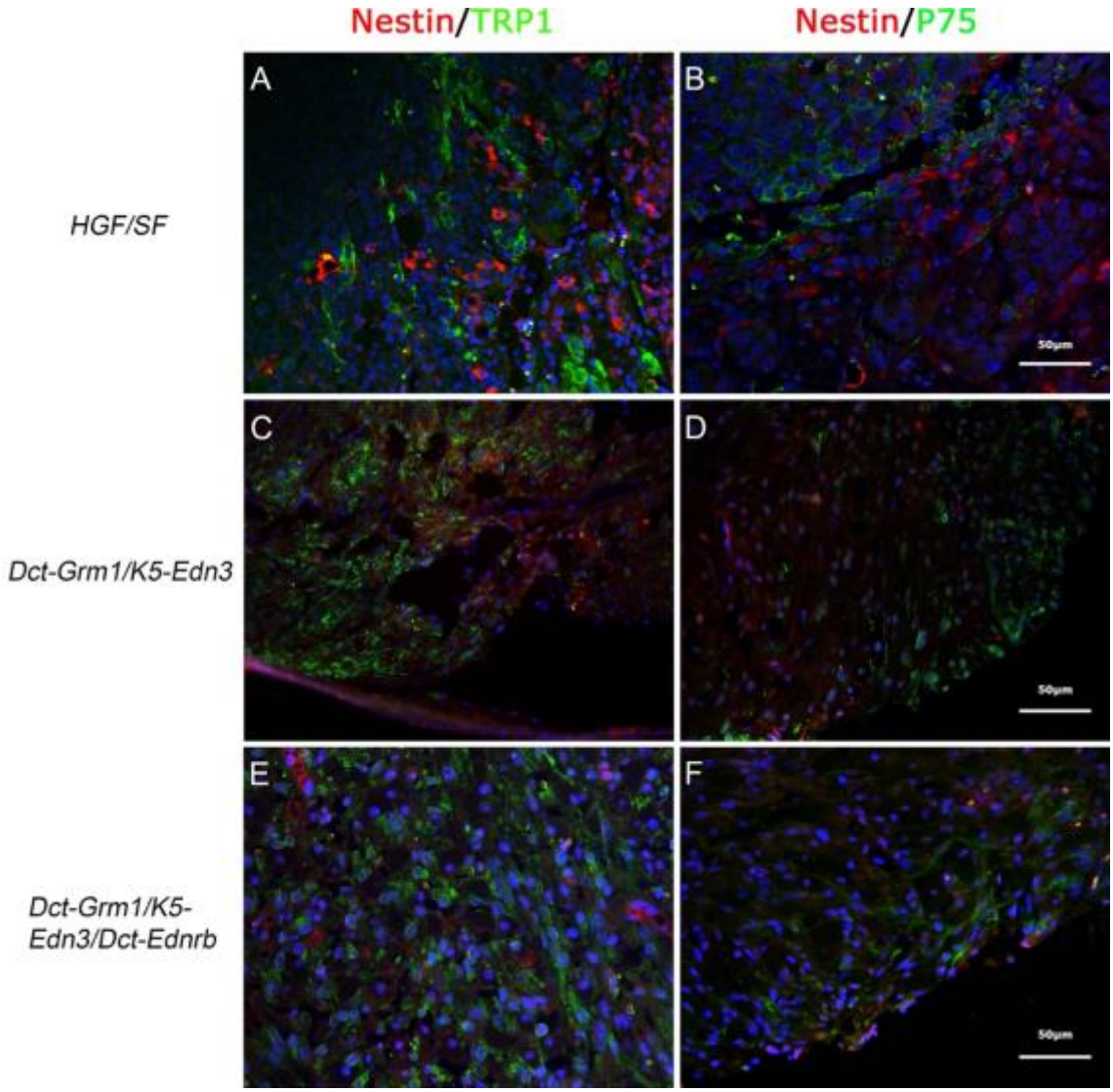


Figure 2.1 Heterogeneity of melanomas from different transgenic mice.

A and B, *HGF/SF* mouse; C and D, *Dct-Grm1/K5-Edn3* mouse; E and F, *Dct-Grm1/K5-Edn3/Dct-Ednrb* mouse. A, C and E indicate Nestin and TRP1 immunostaining of paraffin-sections. B, D and F indicate Nestin and P75 immunostaining of paraffin-sections. Note the distinct cell subpopulations in the same solid tumor.

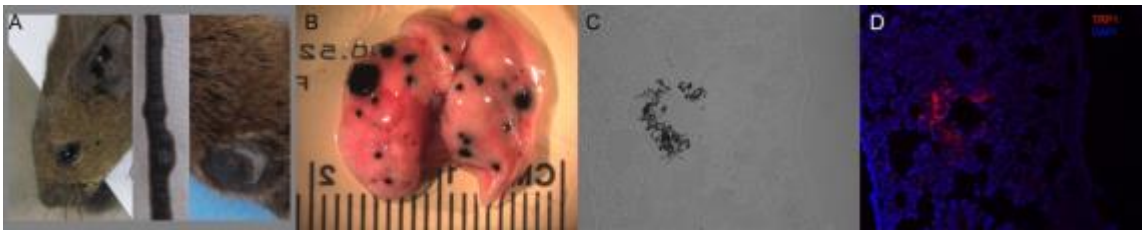
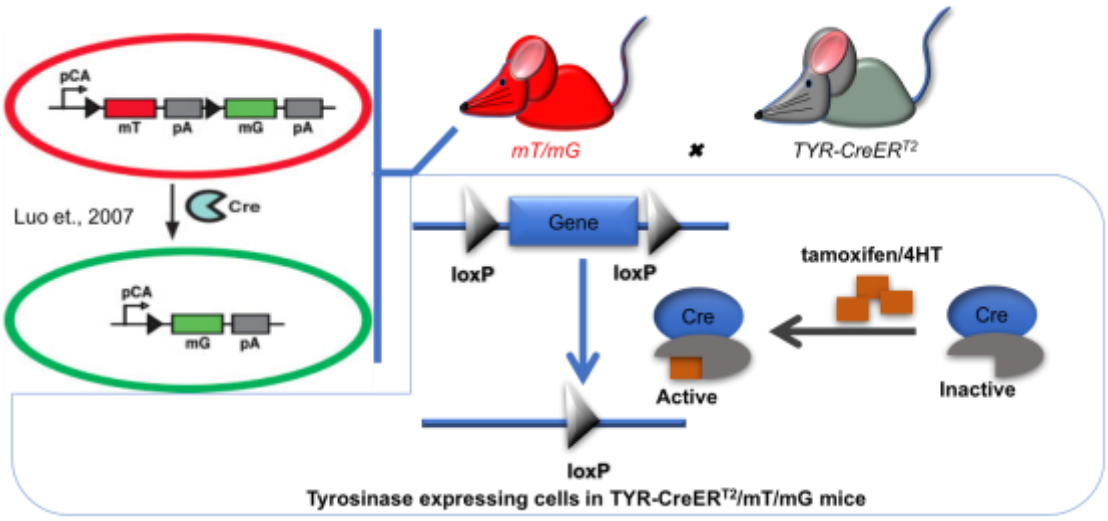


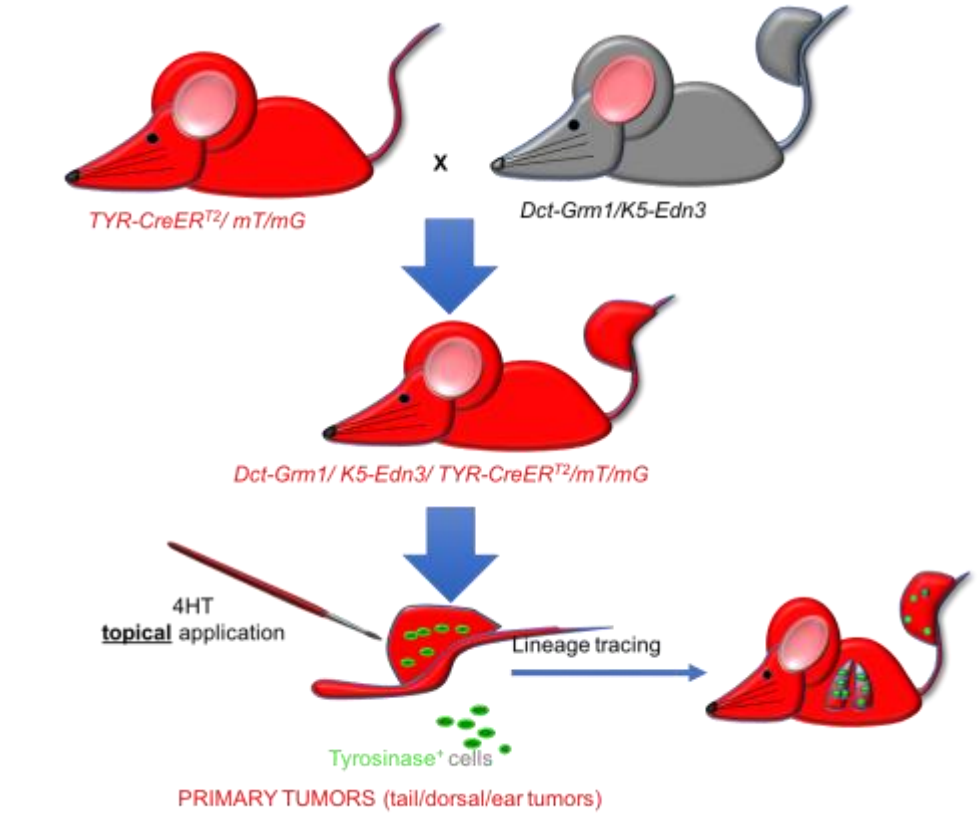
Figure 2.2 Mouse model of melanoma metastasis.

Dct-Grm1/ K5-Edn3 mice spontaneously develop melanocytic lesions on the ear, tail and dorsal skin (A) and metastases in the lung (B). The pigmented lesion in the lung is positive for melanoma marker TRP1 (C is the bright field of D).

A


pCA
mT
pA
mG
pA
Luo et., 2007
Cre
pCA
mG
pA
mT/mG
TYR-CreER^{T2}
Gene
loxP
loxP
tamoxifen/4HT
Cre
Active
Cre
Inactive
loxP
Tyrosinase expressing cells in TYR-CreER^{T2}/mT/mG mice

B


X
TYR-CreER^{T2}/ mT/mG
Dct-Grm1/K5-Edn3
Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/mT/mG
4HT
topical application
Lineage tracing
Tyrosinase^{+} cells
PRIMARY TUMORS (tail/dorsal/ear tumors)

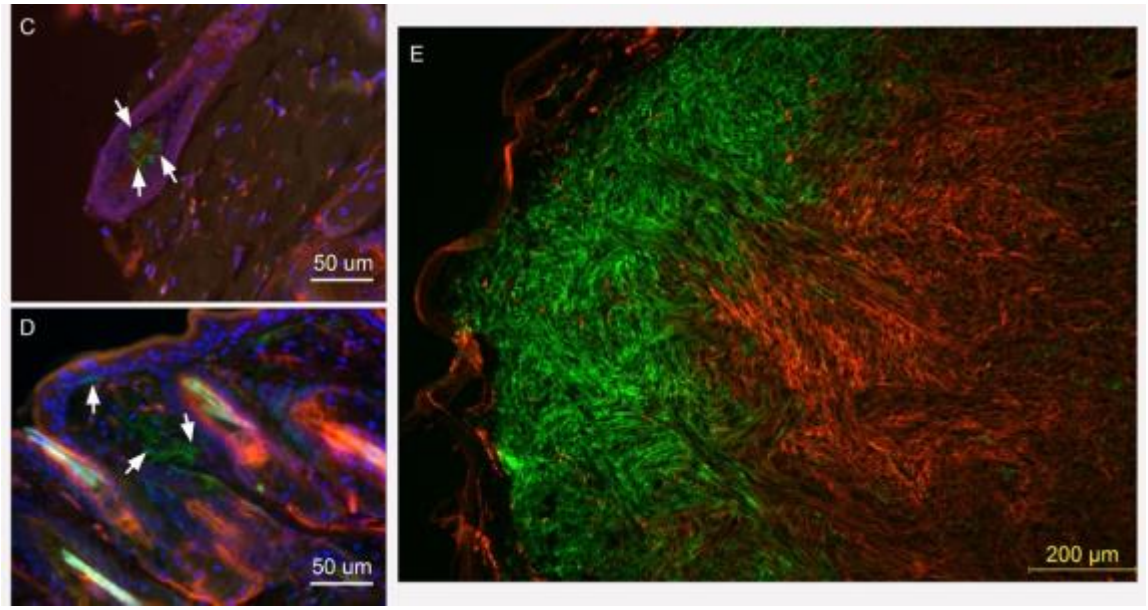


Figure 2.3 Mouse model of melanoma metastasis.

Diagram shows how Cre/loxp system was used with *mT/mG* loxp reporter mice and TYR promoter/enhancer controlled Cre expression in *TYR-CreER^{T2}* mice (A). An in vivo lineage tracing system can be established on the progeny mice carrying all the transgenes from *TYR-CreER^{T2}/mT/mG* and *Dct-Grm1/ K5-Edn3* mating. Instead of systemic administration, 4HT was topically applied to the primary tumor sites to exclusively label Tyr expressing cells inside the tumor, not the other sites. Lineage tracing of the GFP+ cells during melanoma metastasis is achievable (B). 4HT topical application was validated on both *TYR-CreER^{T2}/ mT/mG* mice, where melanocytes are located inside hair follicles (C, arrow), and K5-Edn3/ *TYR-CreER^{T2}/ mT/mG* mice, where melanocytes outside the hair follicles are also expected due to the abundant Edn 3 in the environment (D, arrow). This methodology was also valid in the melanoma mouse model *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG*. 4HT painted tail tumor cryosections were directly visualized under fluorescent microscope. Numerous green cells were found inside the tumor, consistent with the abundance of Tyr expressing differentiated cells in melanoma (E). Green, GFP+ melanocytes; Red, tdTomato, expressed in all non-recombined cells; Blue, nuclei counterstained with DAPI.

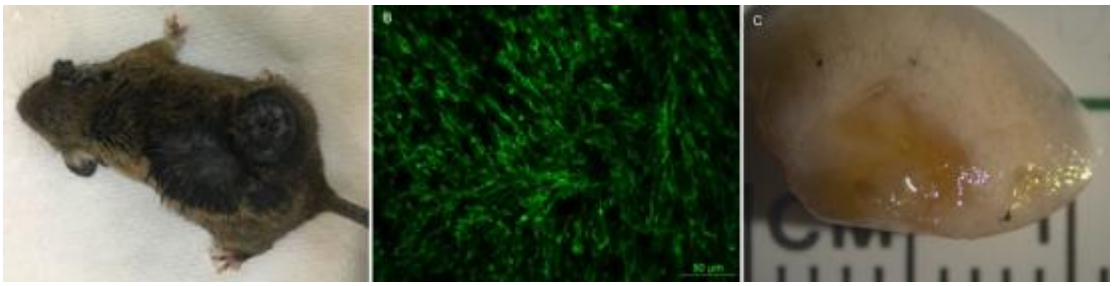


Figure 2.4 Labeling of the Tyr expressing cells in the primary tumors.

4HT was topically painted on the dorsal (A) or tail tumors of *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice, lung metastases with pigmentation were found after the mice were euthanized(C). Cryosections of the dorsal tumor were directly visualized under the fluorescence microscope and numerous GFP+ cells were found (B).

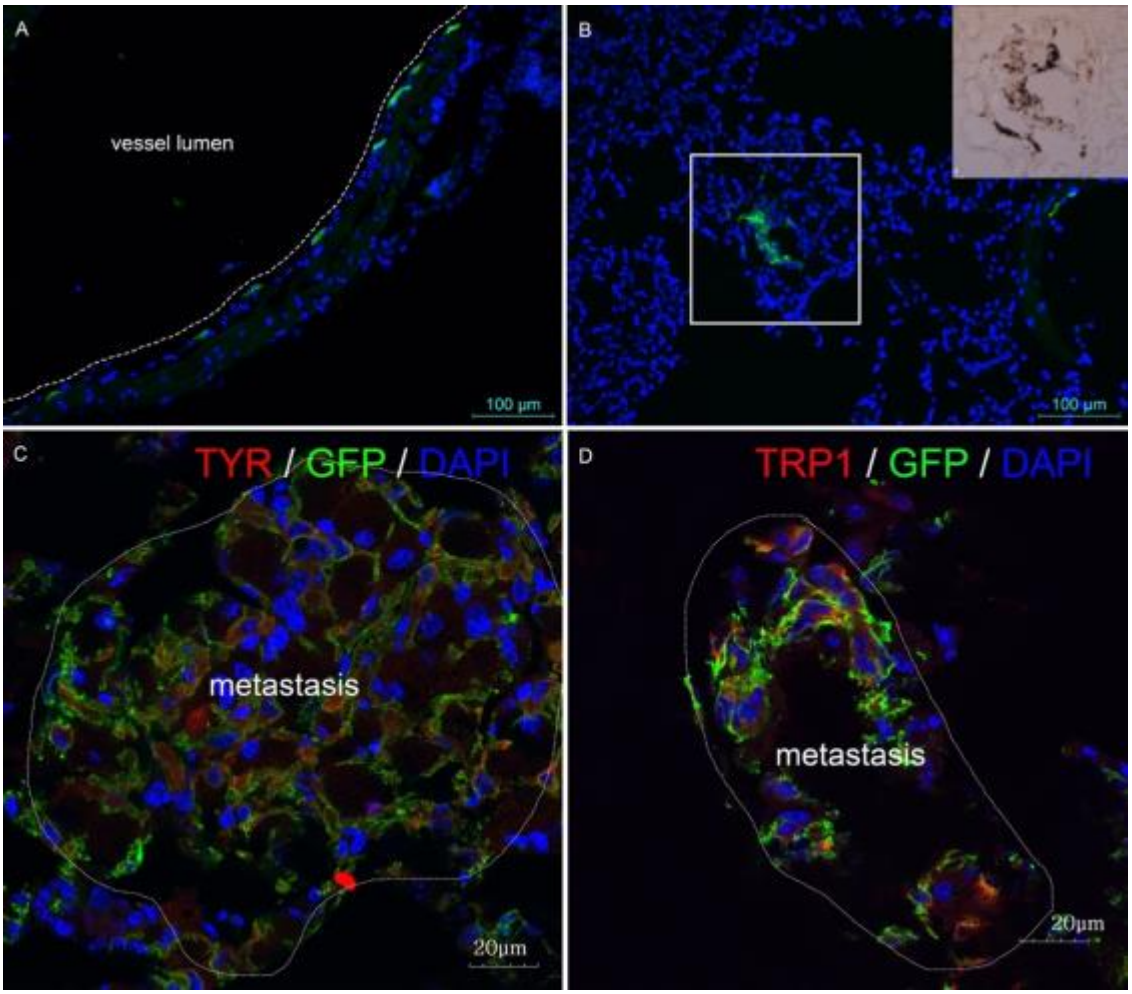


Figure 2.5 Lineage tracing of tumorigenic cells to the lung.

GFP+ cells were found in the lung, most of which were located at the inner wall of vasculature as single cells with an elongated, spindle shape (A), clusters of green cells were mainly seen in the lung metastatic foci (B, insert: bright field of the boxed area, showing green cells overlapped with pigment). GFP+ cell populated metastases in the lung were TYR and TRP1 positive showing the successful seeding of melanoma metastases (C, D).

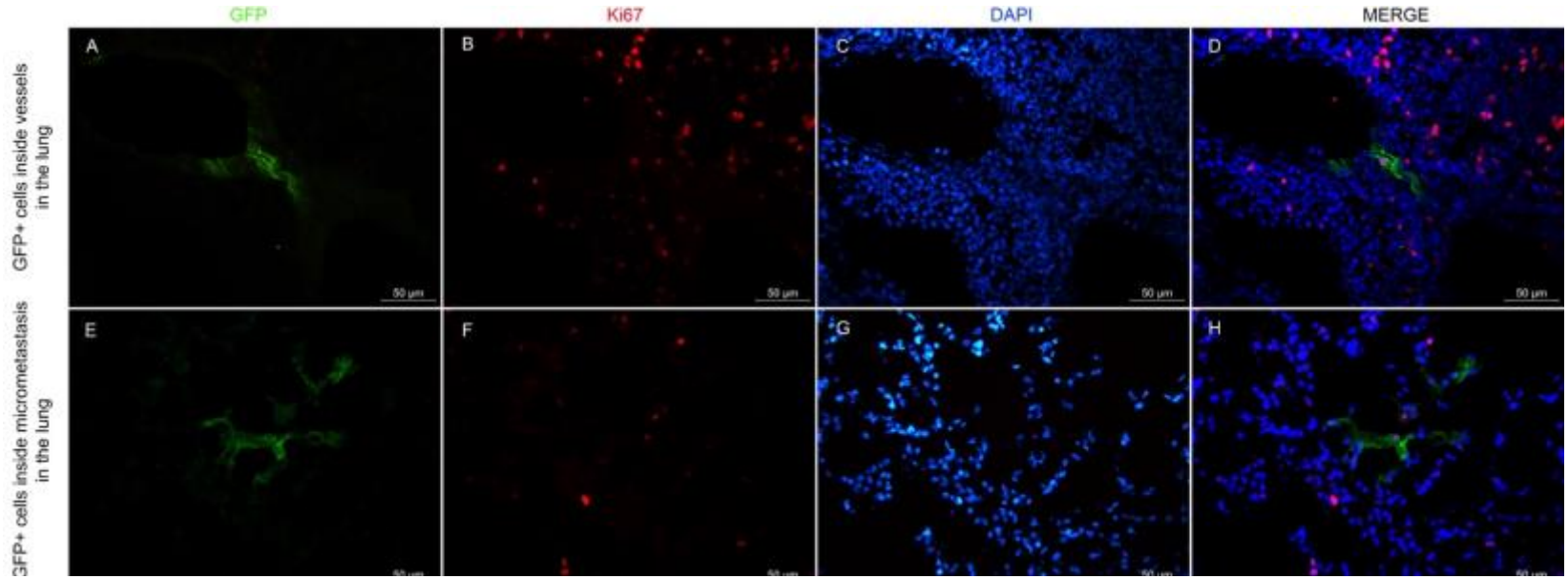
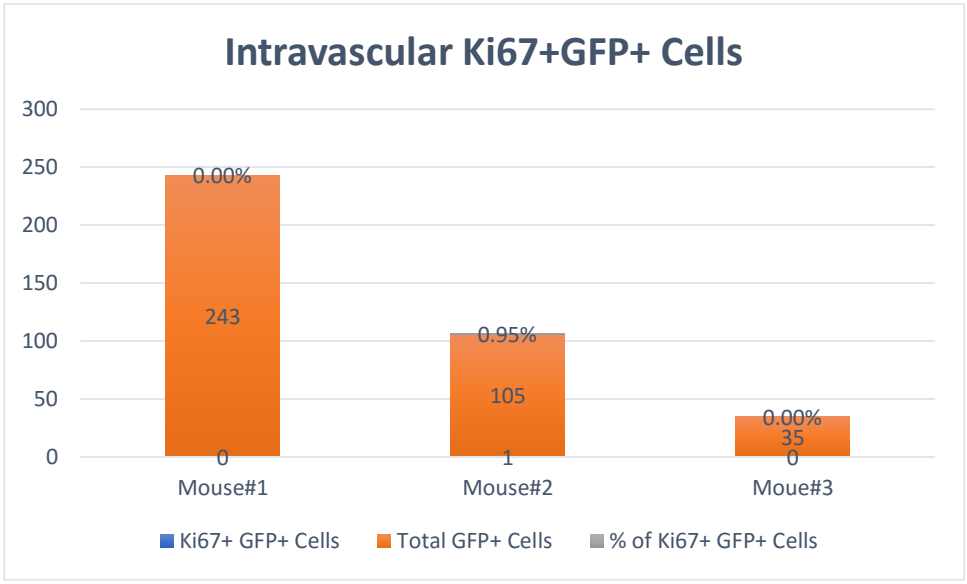


Figure 2.6 Primary tumor derived GFP+ cells proliferated in the lung.

Top panel (A-D) showed one GFP+ cell located at the inner layer of the lung blood vessel (the green cell was located at the longitudinal section part of the vasculature) was positive for Ki67. Lower panel (E-H) showed one GFP cell inside the pigmented micro-metastasis expressed Ki67. Red, Ki67; green, GFP from the Cre induction or anti-GFP antibody used to amplify the signal for immunostaining; blue, DAPI.

A



B

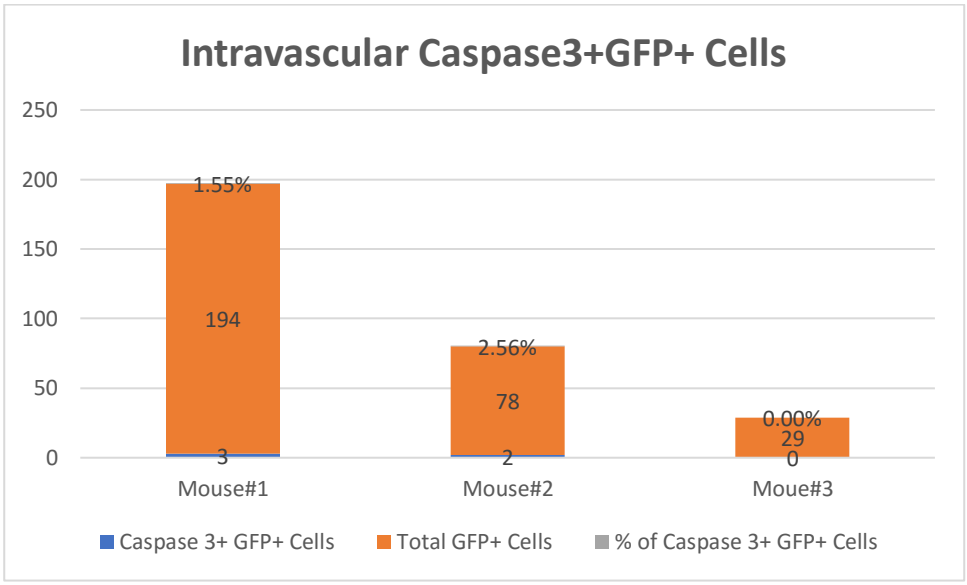


Figure 2.7 Numbers of Ki67 or Caspase3 staining of intravascular GFP+ cells in the lung.

Less than 1% Ki67+ and less than 3% Caspase3+ intravascular GFP+ cells were identified in the lung of *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice.

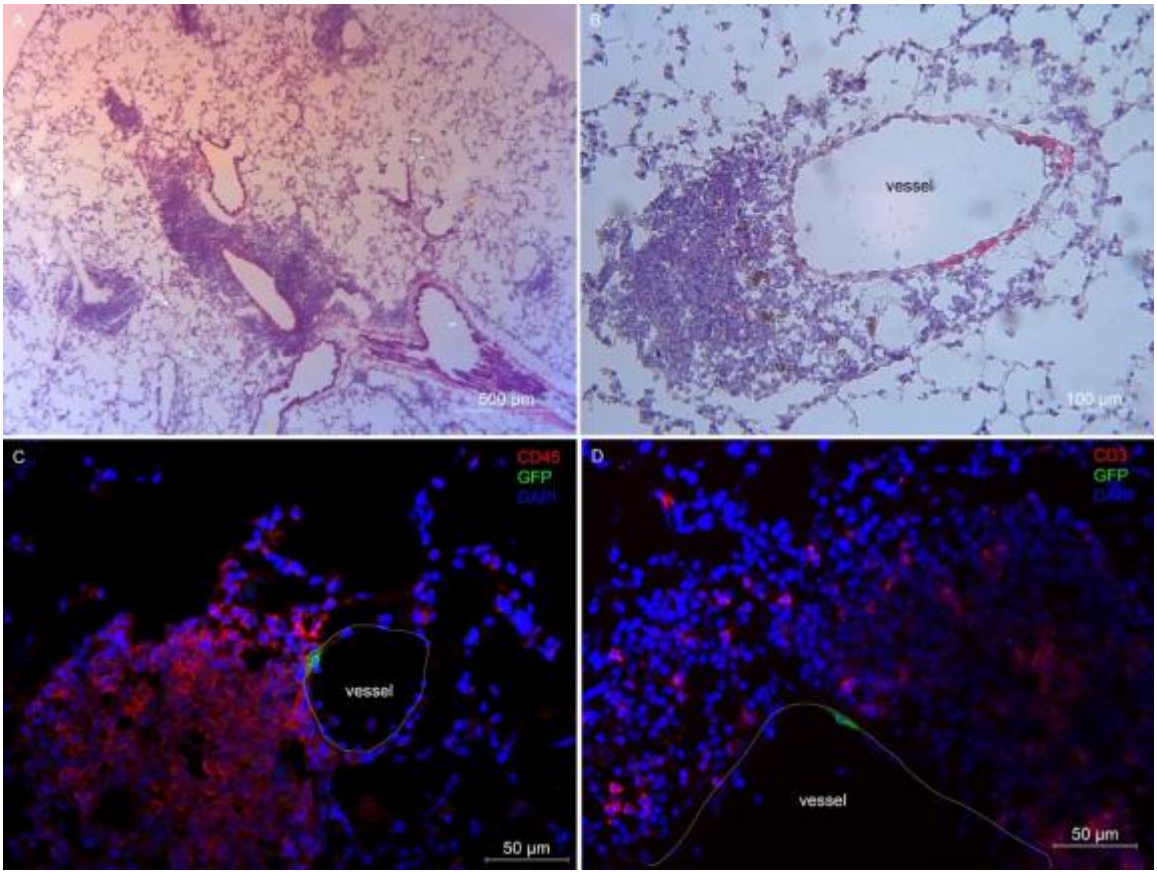


Figure 2.8 The presence of GFP+ cells in the lung was associated with perivascular infiltrates.

Hematoxylin-eosin (H&E) staining (A, B) of lung cryosections from *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice, of which primary tumor derived GFP+ cells were found. Perivascular cellular infiltrates were shown in the lung. B, a higher magnification of the cellular infiltrates, brown pigmented macrophages were seen inside the infiltrates. Immunostaining with indicated markers showed the perivascular infiltrates were mostly CD45 (lymphocyte common antigen) positive (C), and some were T cell marker CD3 positive (D). Note, one GFP+ cell was shown inside the vessels in C and D.

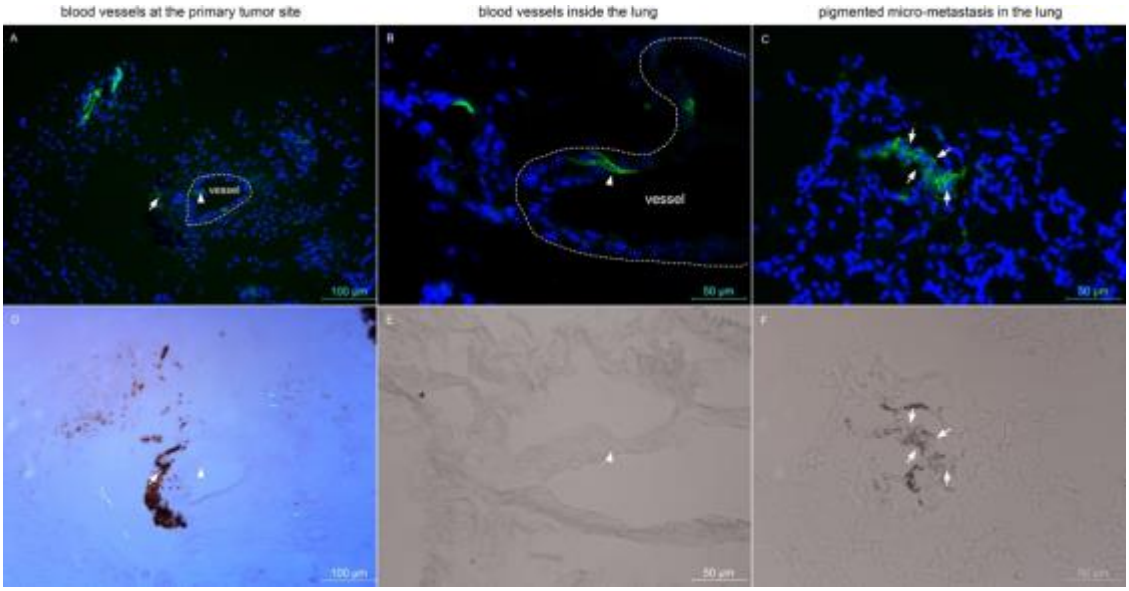


Figure 2.9 Pigmentation level of GFP+ cells during metastasis.

GFP+ cells lost pigmentation while they were inside the vasculature at the tail tumor site (A, D, arrowhead) and in the lung (B, E, arrowhead), but produced pigmentation when they were outside the vessels (A, D, arrow) or within the metastatic foci (C, F, arrows). D-F are bright fields of A-C, respectively. Cryosections were counterstained with DAPI.

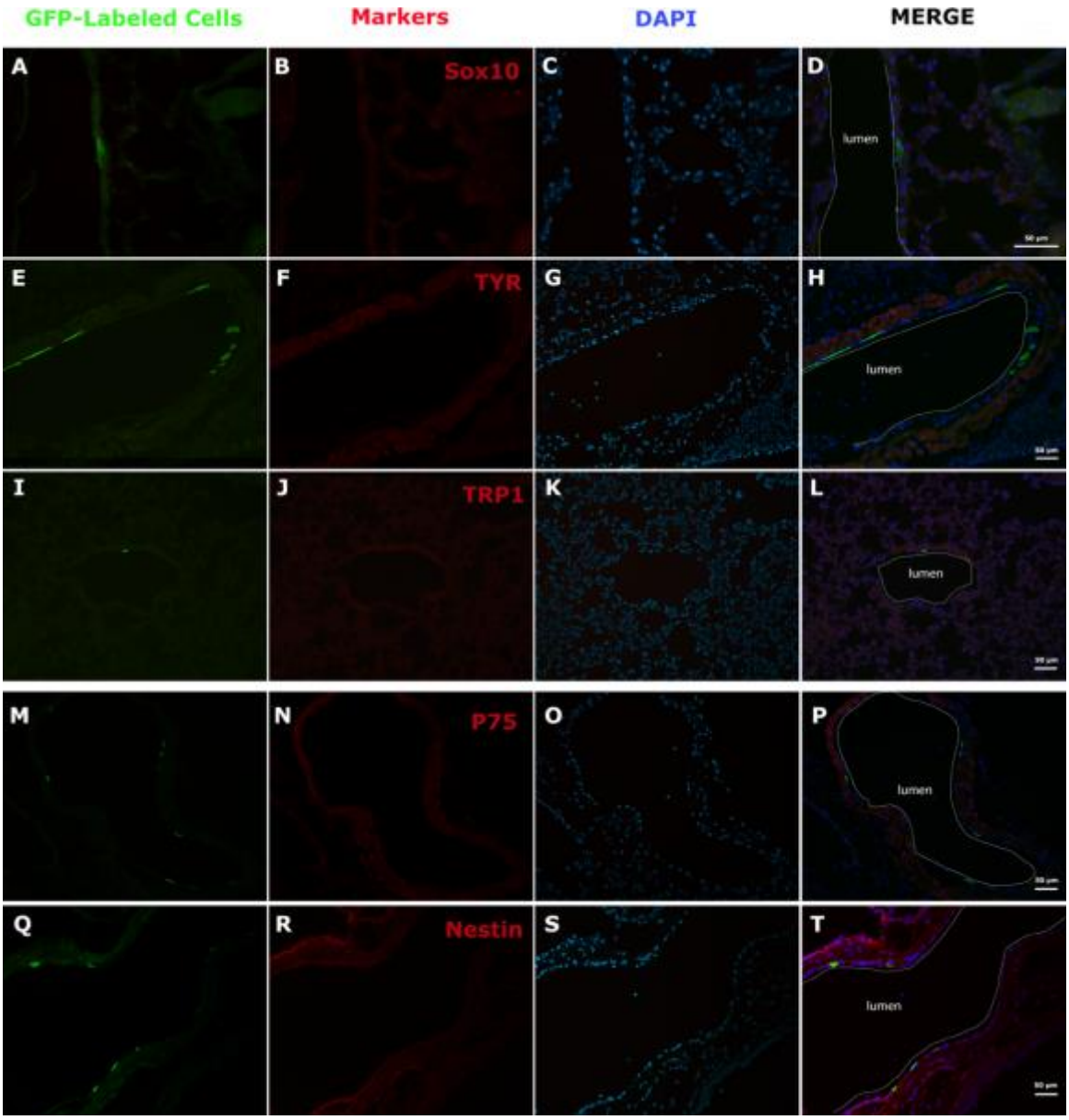


Figure 2.10 Immunostaining of GFP+ cells in the lung vasculature of *Dct-Grm1/ K5-Edn3/Tyr-CreER^{T2}/mT/mG* mice.

GFP+ cells in contact with lung vasculature (A, E, I, M, Q) did not express Sox10, TYR, TRP1 (B, F, J), P75, Nestin (N, R). Staining was performed on cryosections with appropriate positive controls to validate the indicated antibodies.

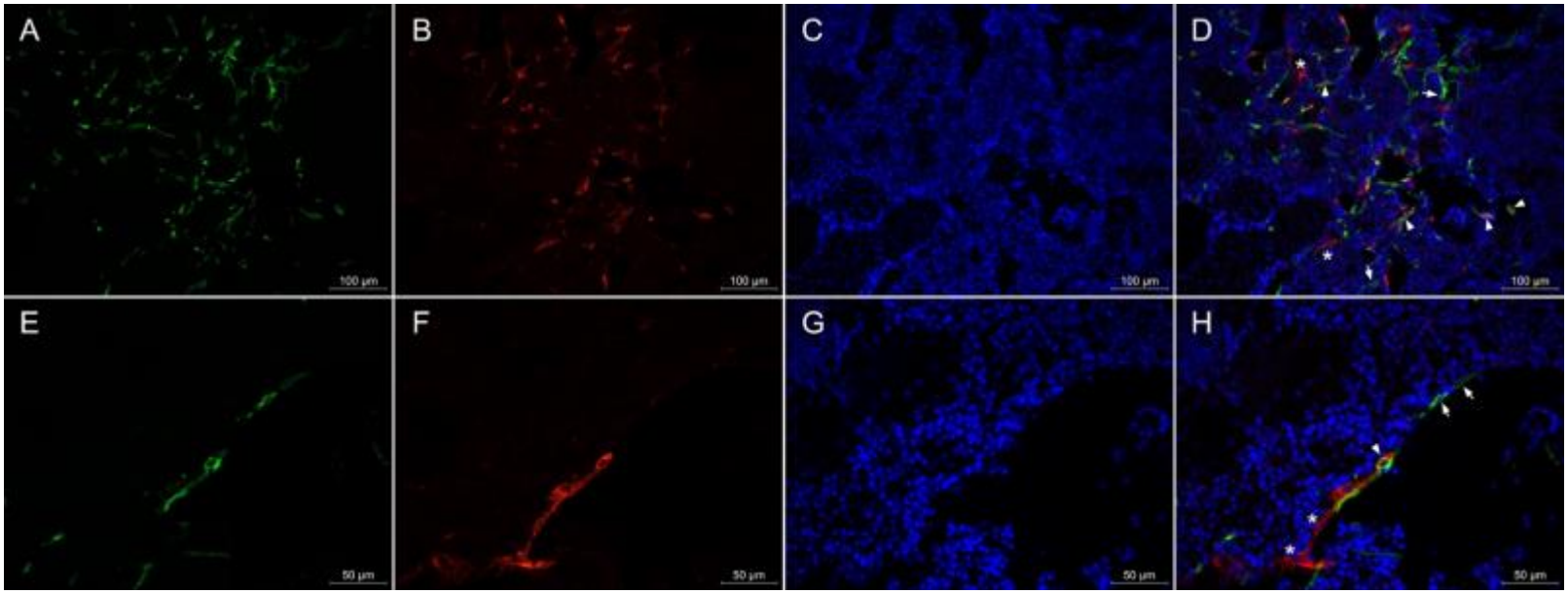


Figure 2.11 Characterization of GFP+ cells in lymph nodes.

TYR staining was done on the lymph node cryosections where GFP+ cells were found. Some GFP+ cells inside the lymph nodes expressed TYR (arrowhead), while some did not (arrow). A-D, a lower magnification; E-H, a higher magnification showed GFP+ cells inside the vasculature. Some TYR positive cells are not GFP+ (star).

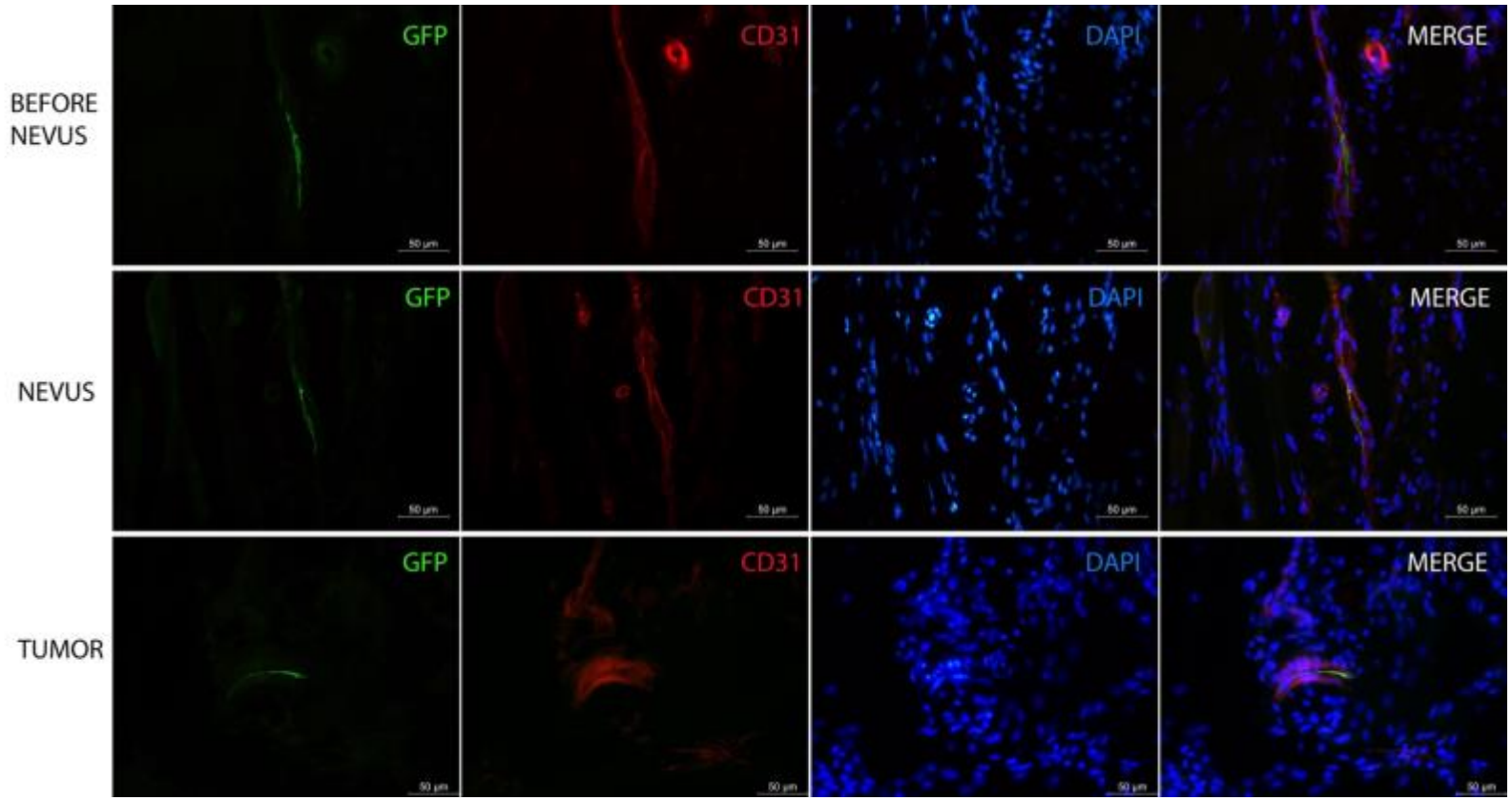


Figure 2.12 Timing of metastatic GFP+ cell dissemination.

4HT was painted on nevus or tumor or normal skin of the tail. Cryosections of the tail with the painted area were immunostained with CD31 to localize blood vessels underneath the tail skin. GFP+ cells were found inside the tail vasculature at all three stages. GFP, green; CD31, red; DAPI, blue. Only the vasculature underneath the painted area is shown, not the actual nevus/tumor/skin.

CHAPTER 3. PHENOTYPIC PLASTICITY OF METASTATIC MELANOMA CELLS

3.1 Introduction

The vasculature plays an essential role in cancer progression. Most studies that have implicated the vasculature as a key factor in tumorigenesis have focused on the process of angiogenesis [1]. However, there is some evidence that angiogenesis may not be required for metastasis and other mechanisms such as vessel co-option have been suggested as an alternative [2]. In vessel co-option, tumor cells hijack and migrate along preexisting vessels of the host organs. Vessel co-option has been observed in human primary tumors and metastases and is most frequently described in highly vascularized tissues such as brain, lung, and liver [2]. Vessel co-option was detected in melanoma, and proposed to be the process employed by tumors to continue growing after antiangiogenic therapies [3, 4].

Another alternative pattern of tumorigenic functional microcirculation that has received special attention is VM where tumor cells acquire the capacity to form endothelial-like vessels recapitulating embryonic vasculogenesis. Vasculogenic mimicry is a striking example of tumor plasticity where tumor cells can transdifferentiate into an endothelial-like phenotype and express vascular related molecules. Numerous VM studies in distinct cancers have been published since the first introduction of this concept in 1999 [5, 6]. Vasculogenic mimicry was first described in the context of human melanoma cells [6] and originally defined as the ability of aggressive melanoma cells to form CD31 negative but Periodic acid schiff positive vascular-like networks. Periodic acid schiff stains basement membranes including laminin, collage and glycogen and CD31 is an

endothelial cell specific marker. Later, the same group provided the first direct evidence that another endothelial-associated molecule, vascular endothelial (VE)-cadherin, was exclusively expressed by highly aggressive melanoma cells and dispensable for VM [7]. Signaling pathways underlying VM with a focus on VE-cadherin expression were recently reviewed [8]. Although absence of CD31 is a characteristic of VM in melanoma, CD31 expression was reported in VM capable cell lines from ovarian cancer [9], prostatic cancer [10] and breast cancer [11]. Distinct contributions of VE-cadherin and CD31 in vessel formation [12] and embryonic origins of the tumor cell types may provide some clues to the selective expression of these two genes, but further investigations are needed to elucidate their specific roles in VM [13].

Tumor cells in various cancers, including melanoma, that are capable of VM share the “stemness” feature [14-19]. Gene-expression profiling of more than 45 human cutaneous and uveal melanoma-cell lines showed that, compared with the poorly aggressive melanoma cells, highly aggressive melanoma cells down-regulated melanoma-specific markers including MITF, TYR, TRP1 and upregulated angiogenesis and vasculogenesis genes such as VE-cadherin, erythropoietin-producing hepatocellular carcinoma-A2 (EPHA2), and laminin 5 γ2-chain (LAMC2) [13]. The interplay between tumor cells acquiring endothelial-like and stem cell-like features may be what allows VM to participate in tumor metastasis or recurrence. The functional significance of VM is not well understood, but it may be involved in facilitating tumor perfusion and dissemination. Vasculogenic mimicry is positively related with tumor

aggressiveness and an overall shorter survival time [20-22]. Vascular endothelial-cadherin positive melanoma cells co-expressing the stem cell marker CD133 engaged in VM creating a perivascular niche essential for primary tumor growth in mouse xenografts [19]. Another study by Wagenblast et al., linked VM in primary tumors with distant metastases in a breast cancer tumor mouse model. They provided evidence that Serpine2 and Slpi not only played essential roles for VM, but, as anticoagulants, also promoted metastatic cell intravasation and ultimate metastases by maintaining perfusion of the tumor cell-lined networks at the vascular/extravascular interface [23].

Another special pattern of vasculature in tumors is mosaic vessels, where vessels contain both authentic endothelial cells and tumor cells. Mosaic vessels in tumors were first observed in colon carcinoma, featured by discontinuous endothelial marker CD31 expression in tumors [24]. Mosaic vessels in hepatocellular carcinoma were also reported [25]. One of the proposed potential mechanisms of mosaic vessel formation [26] lies in the plasticity of highly malignant tumor cells, suggesting tumor cells can mimic the endothelial cells thus displace endothelial cells of the vessel lining. Whereas mosaic vessel features the endothelial-like behavior of cancer cells, those cancer cells being part of the vasculature do not express the typical endothelial cell marker CD31.

Vessel co-option differs from VM and mosaic vessels in that it is a non-angiogenesis mechanism. Tumorigenic cells take advantage of the preexisting vasculature for tumor growth or migration. While vessel co-option has not been associated to the process of angiogenesis, VM and mosaic vessels have been

studied within its context. All studies about VM and mosaic vessels were carried out to demonstrate their relevance for primary tumor outgrowth and no correlation to the metastatic process has been established. Although some authors have hypothesized a functional role for VM and vessel co-option in facilitating tumor cell dissemination, no direct evidence is available. In the present study, I show a novel tumor-vasculature pattern observed in a spontaneous melanoma mouse model. In the new pattern, melanoma metastatic cells transdifferentiate into endothelial cells, hijack the existing vasculature at the metastatic sites and assemble an intravascular niche. I propose that in this niche metastatic cells are allowed to survive as dormant cells and re-acquire metastatic properties for latent spread.

3.2 Results

3.2.1 Vascular Endothelium Associated GFP+ Cells Express Endothelial Cell Markers

In the *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice, most metastatic GFP+ cells in the lung were located inside the pulmonary vasculature. To establish the exact intravascular location of GFP+ cells in the lung, we labeled the lung cryosections where GFP+ cells were found with an antibody against CD31 to identify the vascular endothelial cells. To our surprise, the average of intravascular GFP+ cells was CD31 staining was also observed in approximately 70% of the GFP+ cells associated with the vascular endothelium (Figure 3.1, top panel), including solitary GFP+ cells in alveolar capillaries (Figure S6). To further confirm the acquisition of an endothelial phenotype by the GFP+ cells, we used

another endothelial cell marker, VE-cadherin, a similar percentage of intravascular GFP+ cells also labeled with VE-cadherin demonstrating their endothelial nature (Figure3.1, bottom panel).

Since CD31 and VE-cadherin are expressed in the membrane at the cell-cell junction it is plausible that the staining detected was in fact associated with the vascular endothelial cells surrounding the GFP+ cells. In order to fully establish which cell type was immunolabeled with the antibodies against CD31 and VE-cadherin, small segments (2-3mm length) of pulmonary artery, pulmonary vein and aorta were obtained from 4HT painted mice and visualized directly by confocal microscopy. Since all cells in *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mouse express tdTomato red fluorescence protein except GFP+ Tyr expressing cells or their progeny, whole mount visualization of the vessels allowed us to detect the presence of GFP+ cells directly. As shown in Figure 3.2, primary tumor derived GFP+ cells were found in all three vessels. Of note, GFP+ cells were always located in association with or within the vascular endothelial layer, not the outer smooth muscle layer. The GFP+ cells embedded into the endothelium resembled the vascular endothelial cells morphologically. It is noteworthy that GFP+ cells changed their morphology according to the vascular endothelium where they were found (Figure 3.2, i.e., vein associated GFP+ cells showed a different shape compared with the artery associated ones), further indicating the plasticity of tumor cells. Whole mount staining of CD31 and VE-cadherin was carried out on the vessels and 3D reconstruction showed results consistent with the ones obtained from the lung cryosections. The GFP+

cells associated with the vasculature were clearly positive for CD31 and VE-cadherin (Figure 3.3).

3.2.2 Expression Pattern of Endothelial Cell Markers by GFP+ Cells is Dynamic

The phenotypic plasticity of GFP+ cells was demonstrated by characterizing the expression of endothelial cell markers during tumor progression. In the primary tumors, GFP+ cells did not present detectable CD31 expression (Figure 3.4A). In contrast, the majority of primary tumor resident GFP+ cells were VE-cadherin positive (Figure 3.4D), a feature of VM. In the lung vasculature, GFP+ cells associated with the vascular endothelium expressed both CD31 (Figure 3.4B) and VE-cadherin (Figure 3.4E). GFP+ cells populating the lung metastases lost the expression of both endothelial cell markers (Figure 3.4C, F). The fact that GFP+ cells lost CD31 expression when they seeded the metastases was expected given that they originally did not express it in the primary tumor. It was surprising that they lost VE-cadherin expression as well, in that they were VE-cadherin positive at the beginning of the metastatic journey.

As for what occurred in blood vessels, GFP+ cells also showed endothelial cell mimicry in lymphatic vessels. In the lymph nodes of *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice, GFP+ cells were found associated with the lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) positive lymphatic vessel cells. LYVE-1 is strongly expressed by endothelial cells of normal lymphatic vessels but not blood vessels [27]. 90% of the intravascular (LYVE-1+ cell-lined vessels) GFP+ cells were also LYVE-1 positive (Figure 3.5A). GFP+

cells that were associated to the lymph node blood vessels (LYVE1-/CD31+) expressed CD31 (Figure 3.5B) consistent with the results obtained from lung blood vessels. Although LYVE-1 can be used as a lymphatic vessel marker to discriminate lymphatic vessels from blood vessels in most tissues, it has been demonstrated to label various blood vessels in the mouse lung [28-30]. Indeed, we performed double immunostaining of CD31 and LYVE-1 on lung cryosections and found them to overlap considerably. In contrast, LYVE-1 only labeled the lymphatic vessels in lymph nodes though weak CD31 staining on some lymphatic vessels was also observed (data not shown). We found LYVE-1+/CD31+ intravascular GFP+ cells in the lung, but we cannot confirm the type of vessels they were associated with. However, GFP+ cells within the metastatic foci in the lung did not express LYVE-1 (Figure 3.5C).

3.2.3 EndMT as a Potential Mechanism Underlying Dormant Tumor Cell Awakening

Since the intravascular GFP+ cells in the metastatic sites acquired an endothelial cell phenotype, we wanted to find out if they could undergo EndMT as a way to regain the capacity to establish successful metastases. Endothelial-mesenchymal transition has been proposed as an important mechanism that underpins the role played by tumor-derived endothelial cells in tumor progression [31]. The hallmarks of EndMT are the acquisition of the mesenchymal markers VIMENTIN and α-smooth muscle actin (α-SMA). We performed VIMENTIN and α-SMA immunostaining on lung cryosections where GFP+ cells were found. VIMENTIN positive intravascular GFP+ cells were found in various areas of the

lung (Figure 3.6, arrowhead), but heterogeneity existed even between cells in close proximity (Figure 3.6, arrow). α-SMA+ GFP+ cells were also identified in the lung. Interestingly, some CD31+/α-SMA+ GFP+ cells were found inside alveolar capillaries (Figure 3.7, arrowhead), indicating an intermediate state of EndMT. Figure 3.8 shows the heterogeneity and plasticity of a small cluster of GFP+ cells located in an alveolar capillary with both CD31-/α-SMA+ (Figure 3.8, arrow) and CD31+/α-SMA+ (Figure 3.8, arrowhead) GFP+ cells. It is noteworthy that α-SMA+ GFP+ cells were only found in smaller vessels (arterioles, capillaries). In contrast, GFP+ cells associated with larger vessels (pulmonary arteries) were always α-SMA negative, and found wrapped around the α-SMA+ smooth muscle cells and vascular endothelial cells (Figure 3.9)

3.2.4 Plasticity and Heterogeneity of Metastatic GFP+ Cells

The phenotypic change of GFP+ cells as they progressed from the primary tumor to the site of metastasis may require an initial process of de-differentiation so they can acquire a novel fate. To evaluate if the intravascular GFP+ cells may also have gone through a stem-cell phenotype, immunostaining with the stem cell markers SOX2 and OCT4 was performed on lung cryosections of *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice. SOX2 and OCT4 positive metastatic melanoma cells were identified, but as for all the other markers tested, the staining pattern was quite heterogeneous (Figure 3.10).

Connexin43 (Cx43) was shown to be involved in tumor/vascular endothelial cell communication in highly metastatic melanoma cells [32] and

breast cancer cells [33]. Stoletov et al. showed that metastatic melanoma and breast cancer cells used Cx43 to initiate brain metastatic lesion formation in association with the vasculature [34]. Similarly to what they found, we detected a small population of GFP+ metastatic melanoma cells associated with the vascular endothelium that were Cx43 positive (Figure 3.10).

The heterogeneity and plasticity of primary tumor derived GFP+ cells were also observed in other metastatic organs. Immunostaining of VE-cadherin was performed on spleen and kidney cryosections from the *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mouse where visible metastases were found. It is intriguing that no detectable or weak signal of VE-cadherin immunoreactivity was found on GFP+ cells scattered in the spleen and kidney, whereas GFP+ cells within the metastatic foci in the spleen were positive for VE-cadherin (Figure 3.11A). S100 is used clinically for melanoma diagnosis, and we performed S100 immunostaining on the lymph node cryosections where GFP+ cells were found. S100 was highly expressed in the metastatic lymph node but intravascular and circulating GFP+ cells did not show S100 expression (Figure 3.11B).

3.3 Discussion

In our study, we found that primary tumor derived Tyr expressing cells or their progeny mimicked endothelial cells when in contact with blood or lymphatic vascular endothelium during metastasis but lost the endothelial phenotype in the lung metastatic foci. The strong association between vascular endothelial cells and GFP+ tumor cells observed in the spontaneous mouse melanoma model *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* is reminiscent of VM and mosaic

vessels. However, it is obviously distinct from these two mechanisms in that 1) the association was observed in metastatic sites not primary tumors, 2) GFP+ cell associated with original vasculature of the circulatory system, not newly formed vessels, 3) the metastatic GFP+ melanoma cells associated with the vascular endothelium were CD31 positive. Despite of these distinctions, similarity is evident because all these previously described mechanisms underlie the phenotypic plasticity of cancer cells by mimicking vascular endothelial cell properties. The possibility that metastatic GFP+ cells associated with the vasculature may achieve long-term survival because of the easy access to oxygen and nutrients also mirrors another tumor-vasculature pattern, vessel co-option. But then again, the intravascular location of the GFP+ cells is different from that where tumor derived cells are found in vessel co-option.

A recent review by Sun et al. suggested that a connection exists between VM and epithelial-to-endothelial transition (EET). They identified hypoxia, high interstitial fluid pressure and ECM remodeling as the likely factors that promote EET [35]. On the basis of the data provided in this study, we propose a melanoma (which is better characterized as a mesenchymal cell)-to-endothelial transition or transdifferentiation. This is not the first time that such a transition is suggested in the context of cancer. Tumor-derived endothelial cells were recognized decades ago in human B-cell lymphoma and human neuroblastoma when tumor specific chromosomal alterations were identified in tumor endothelial cells through fluorescence in situ hybridization [36, 37]. Notwithstanding, the transdifferentiation of tumorigenic cells into endothelial cells was only recently

characterized in glioblastoma (GBM) using in vitro and in vivo evidence. In vitro, human glioma stem progenitor cells transdifferentiated into endothelial cells and formed tubular-like structures when cultured in endothelial differentiation medium. The expression of the endothelial markers CD31, CD34 and Von Willebrand factor (vWF) in glioma cells was induced by hypoxia or oxygen–glucose deprivation [38]. The in vivo evidence showing that GBM tumor cells transdifferentiated into CD31+ endothelial cells was first demonstrated through xenotransplantation. The authors found a GBM stem cell CD133+ subpopulation that had the capacity to differentiate along both tumor and endothelial cell lineages, and implicated VEGF and Notch pathways as important in this process [39]. These observations were corroborated by another group, who detected that a variable number (range 20–90%) of endothelial cells in human glioblastoma carried the same genomic alteration as tumor cells [40]. Glioblastoma cell to endothelial transdifferentiation was also reported in a syngeneic C6 glioma rat model [41] and xenotransplantation assay by Shaifer's group [42]. Stronger evidence emerged from a study on a novel GBM mouse model using Cre/loxp-controlled lentiviral vectors for tumor formation [43]. Tumor cells were labeled with GFP when lentiviral induced tumor formation happened and, notably, GFP+ tumor cells that associated with the vasculature expressed vWF, CD31, CD34 and CD144. Further in vitro differentiation experiments revealed that hypoxia and independence of VEGF underlie the mechanism responsible for the cancer cell into endothelial cell transdifferentiation [44]. The latter was also shown by Seno's group using miPS-LLCcm cells (a representative mouse induced pluripotent stem

cells, miPS, converted with culture medium from Lewis lung carcinoma cells) both in vitro [45-47] and in vivo [18].

But why do tumor cells masquerade as endothelial cells? We showed in Chapter 2 that intravascular GFP+ cells in the lung of *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice lost melanocytic lineage markers and recapitulated the state of dormancy. If the transdifferentiation of melanoma cells into endothelial cells is the next step in the process, then endothelial cell mimicry may serve as a mechanism underlying metastatic dormancy to help tumor cell survival or escape immune surveillance.

Although endothelial cell markers CD31 and CD34 were detected in histological sections of uveal melanoma [48] and cutaneous melanoma [49], their presence was not connected to tube-like structures and the origin of the endothelial marker positive cells was not defined. Dudley's group described a CD31 positive cutaneous melanoma cell population that was also positive for the melanocytic marker Tyr. These CD31+/Tyr+ VM-competent tumor cells existed as stable, yet hidden subpopulations in heterogeneous melanomas [50]. The population of CD31+ cells described in this study is different in that they transitioned from expressing Tyr to expressing CD31 during the dissemination process and reverted back to expressing Tyr upon successful metastasis formation. VE-cadherin is a well-recognized marker for VM, GFP+ cells within the primary tumors of *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice were positive for VE-cadherin and maintained VE-cadherin expression during the metastatic process. However, GFP+ cells lost VE-cadherin expression in the metastatic foci

in the lung. Given the rich vasculature and gas exchange function of the lung tissue, this result could be interpreted with the assumption that VM is not necessary for the growth of metastases. Nonetheless, the internal or external factors required for triggering the dynamic gene expression changes of metastatic melanoma cells in *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice are not clear. The study led by Kaessmeyer et al. demonstrated that the co-culture of lung endothelial cells and squamous or adenocarcinoma lung cancer cells resulted in a contact dependent “CD31 staining switch”, shown by the acquisition of CD31 expression of the tumor cells in direct contact with endothelial cells. This study suggested that tumor plasticity might be influenced by the endothelial microenvironment and by direct interactions with endothelial cells [51].

Endothelial-mesenchymal transition featured by the loss of endothelial marker expression, such as CD31, with the concomitant increase in mesenchymal marker expression, such as α-SMA / VIMENTIN / FSP1, was originally observed during heart development where a population of endocardial cells differentiated into mesenchymal heart cushion cells forming cardiac septa and valves [52]. Later on, this phenotypic transition was shown to contribute to cardiac fibrosis [53] and carcinoma associated fibroblasts [54] in mouse models [55]. Transforming growth factor-beta (TGF-β) and bone morphogenetic protein (BMP) family of growth factors are key players in EndMT [56, 57]. Generation of mesenchymal cells from endothelium is a crucial step in the multipotent differentiation of endothelial cells and acquisition of a stem-like phenotype was

observed in endothelium during EndMT [57, 58]. The role of EndMT in cancer, especially during metastasis, has only recently been recognized. Endothelial-mesenchymal transition was observed in endoglin-deficient vasculature and this process facilitated tumor cell intravasation in multiple mouse models [59]. The observation that cancer cells can acquire an endothelial-like phenotype and be incorporated into vessels suggested an extra role for EndMT in tumor progression. Selek et al. proposed that through EndMT, endothelial-like cancer cells can transdifferentiate into cancer associated fibroblasts or mesenchymal cancer stem cells (MCCs), and that either one can contribute to tumor progression. Additionally, disruption of tumor vasculature by antiangiogenic therapies may result in the dissemination of MCCs responsible for latent metastasis[31].

Despite of substantial evidence supporting the existence of tumor derived endothelial cells and the role played by EndMT in tumor progression, a robust connection between them and tumorigenesis is still missing, particularly in melanoma. The results presented here showed that in vivo metastatic melanoma cells can transdifferentiate into endothelial cells during metastasis and the subsequent transition into a mesenchymal phenotype may contribute to latent metastasis in the lung. We hope these findings will fill in the gaps in melanoma to endothelial transition, and offer a possible new mechanism underlying melanoma latent metastasis.

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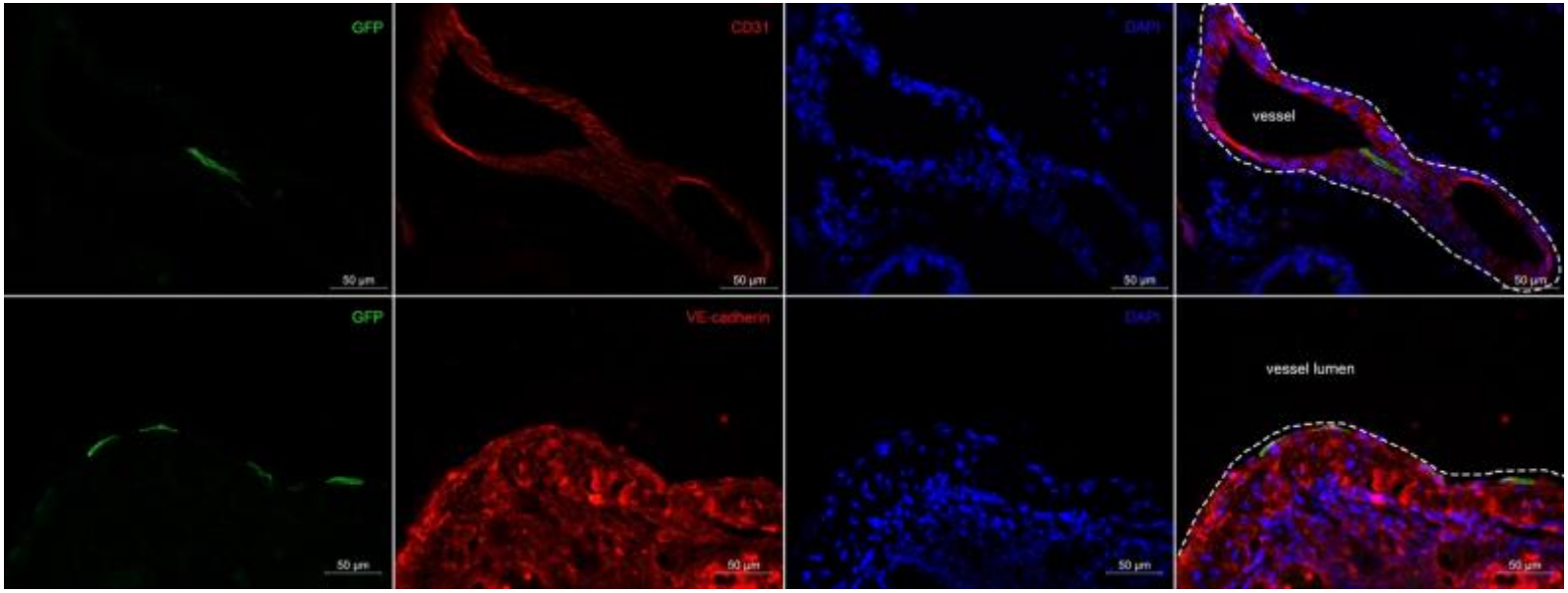

Figure 3.1 GFP+ cells inside the pulmonary vasculature expressed endothelial cell markers.

Immunostaining on lung cryosections indicated the primary tumor derived GFP+ cells were positive for endothelial cell specific markers CD31 (A-D) and VE-cadherin (E-H).

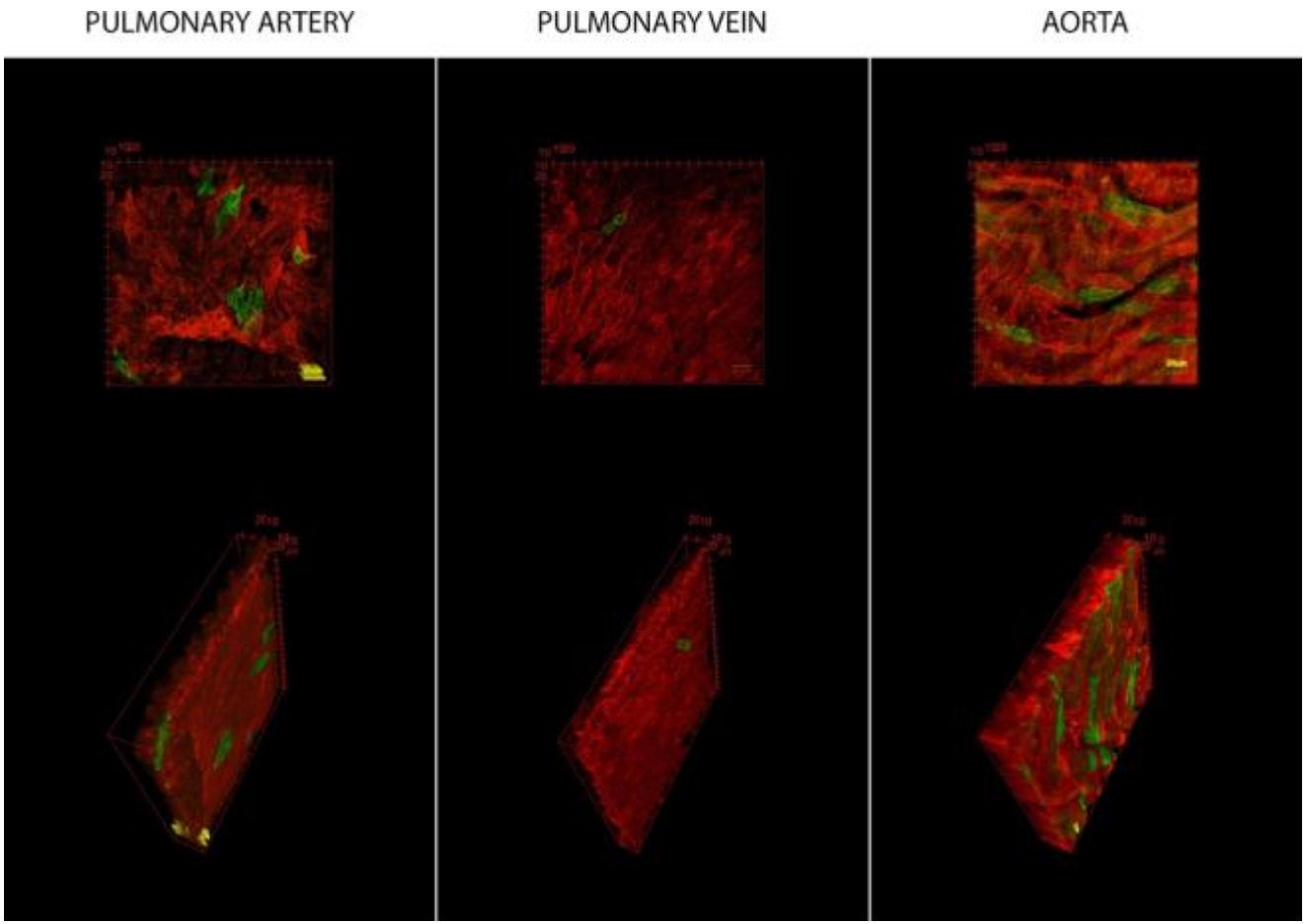

Figure 3.2 Confocal and 3D reconstruction of blood vessels.

2-3mm segments of pulmonary artery, pulmonary vein and aorta were dissected and directly visualized under the confocal microscope. GFP+ tumor cells were found within the endothelial layer of all three types of vessels. GFP+ cells exhibited different morphologies that corresponded to the type of blood vessel they were located.

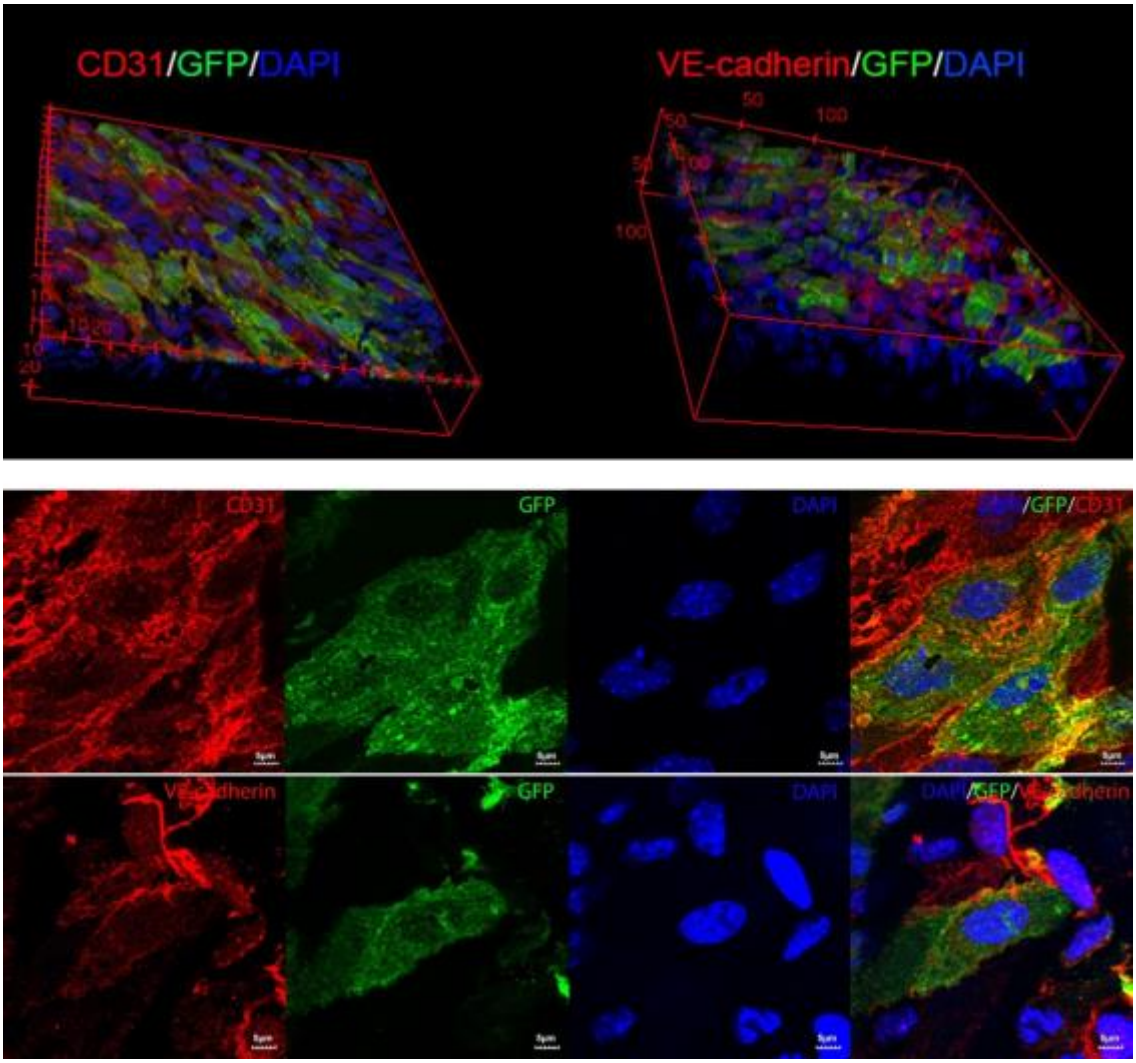

Figure 3.3 Whole mount staining and 3D confocal reconstruction of arteries where GFP+ cells were found.

Top panel 3D reconstruction of the whole mount staining showed that GFP+ cells integrated into the vascular endothelium and expressed CD31 and VE-cadherin (top surface is the endothelium of the vasculature, bottom is the smooth muscle cell layer negative for endothelial cell markers). Lower panel is one section from the 3D reconstruction confirming GFP+ cells are positive for endothelial markers CD31 and VE-cadherin.

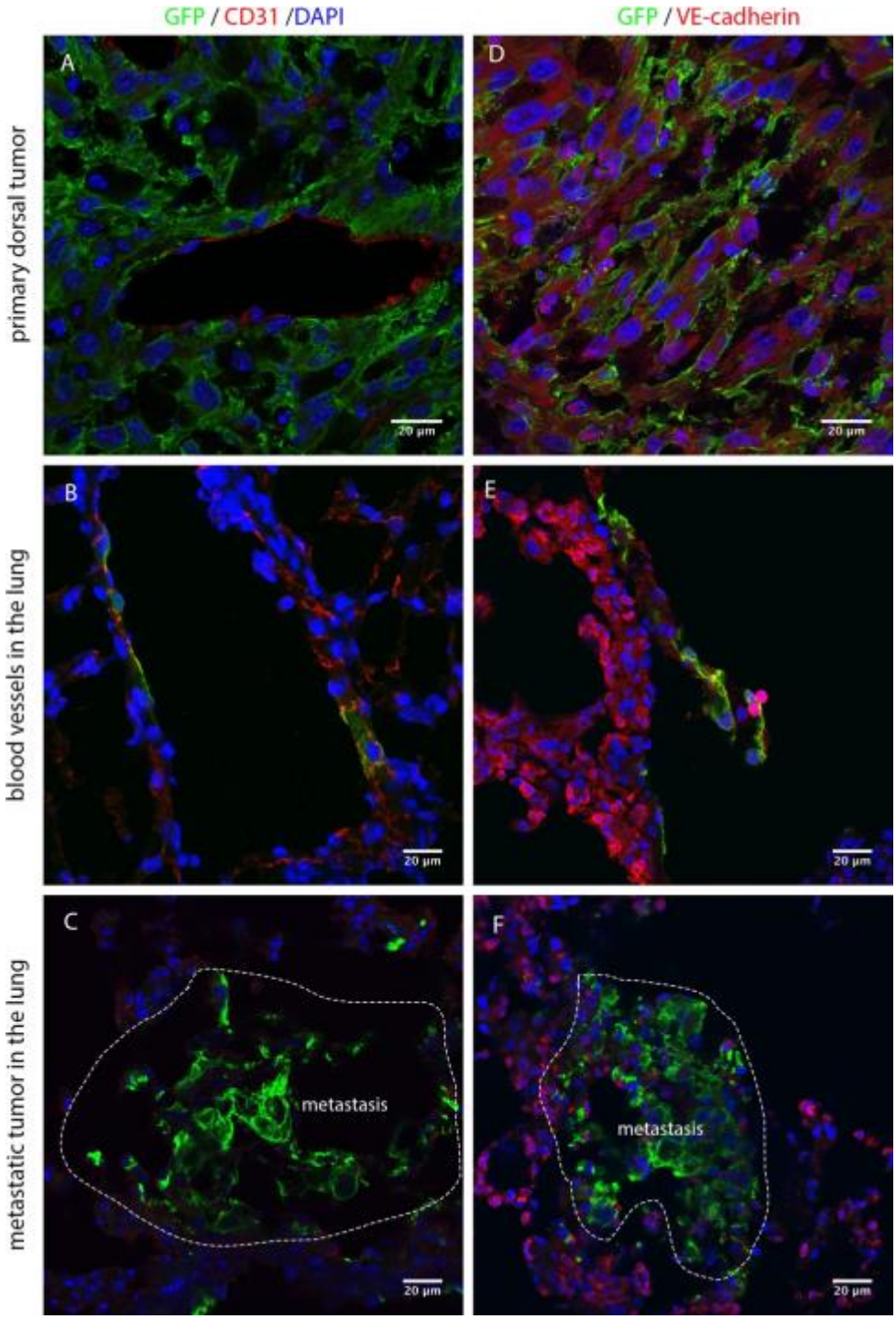

Figure 3.4 Plasticity of GFP+ cells from primary tumor sites to lung metastases.

GFP+ cells did not express endothelial cell marker CD31 in the primary tumors (A, only endothelial cells in the blood vessels were CD31+) but were VE-cadherin positive (D). GFP+ cells were positive for both CD31 and VE-cadherin in the lung vasculature (B, E), but lost the endothelial cell markers in lung metastases (C, F).

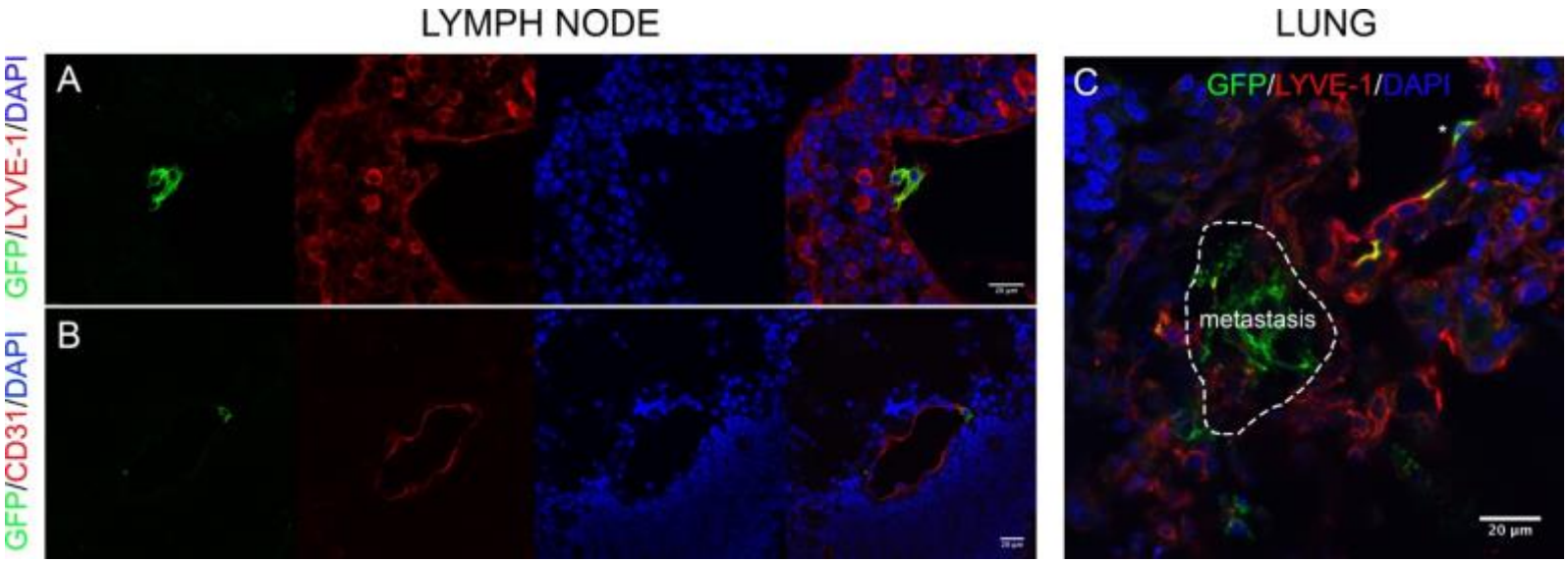

Figure 3.5 Endothelial cell mimicry in lymph nodes.

Primary tumor derived GPF+ cells in the lymph nodes expressed LYVE-1 inside lymphatic vessels (A) and CD31 inside the blood vessels (B) and were strongly associated with the vascular endothelium no matter which type of vessels they were in. GFP+ cells lost the lymphatic endothelial marker LYVE-1 inside the lung metastatic foci (C). Note, one GFP+ cell outside the metastatic foci in the lung was LYVE-1 positive (C, star), although we cannot identify whether this is a lymphatic vessel or blood vessel.

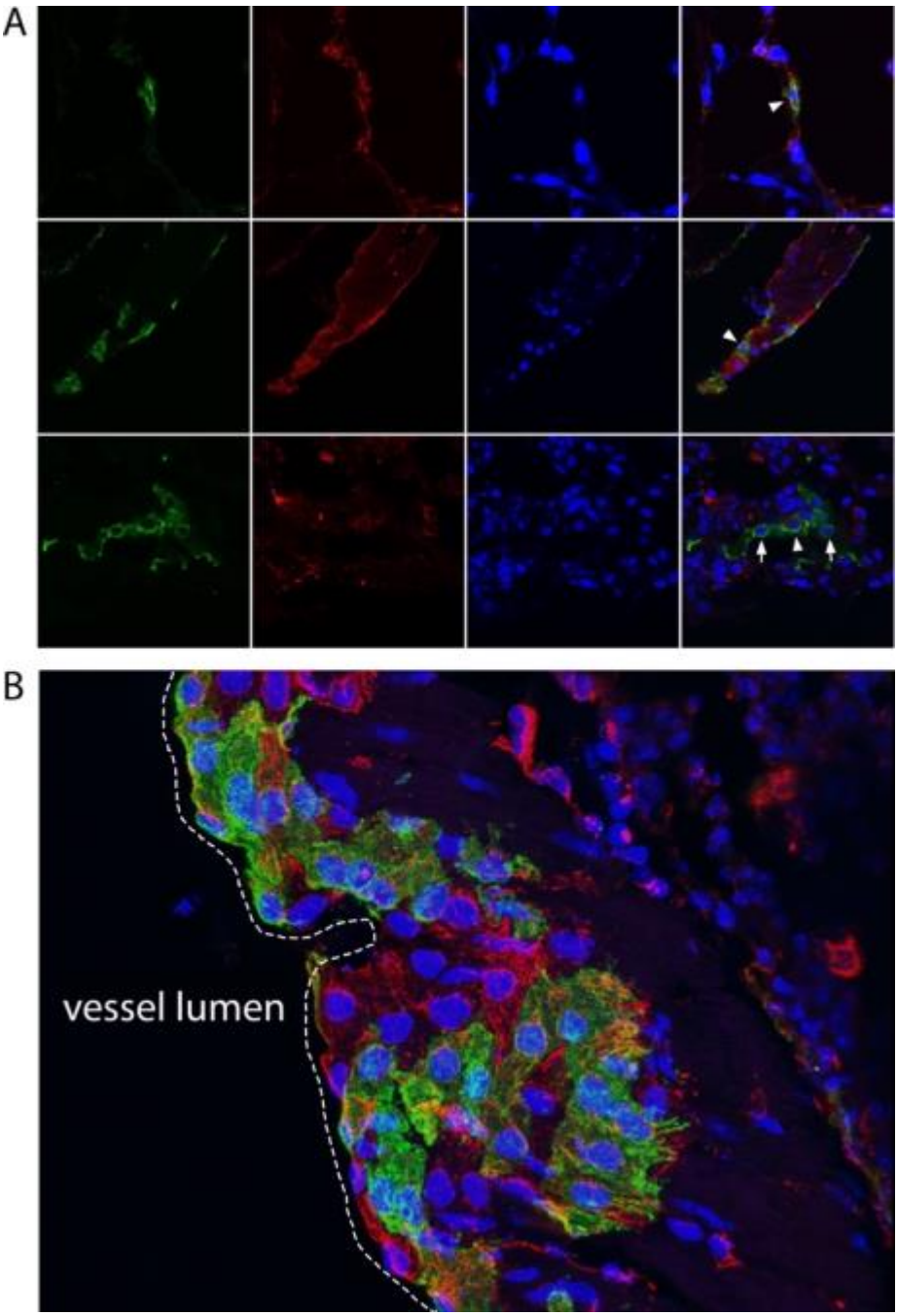

Figure 3.6 Immunostaining of VIMENTIN on lung cryosections.

VIMENTIN positive (arrowhead) and negative (arrow) GFP+ cells were identified at various locations in the lung (A), 3D projection from confocal z-stack images (B) confirmed that primary tumor derived GFP+ metastatic melanoma cells expressed mesenchymal cell marker VIMENTIN. Green, GFP; red, VIMENTIN; blue, DAPI.

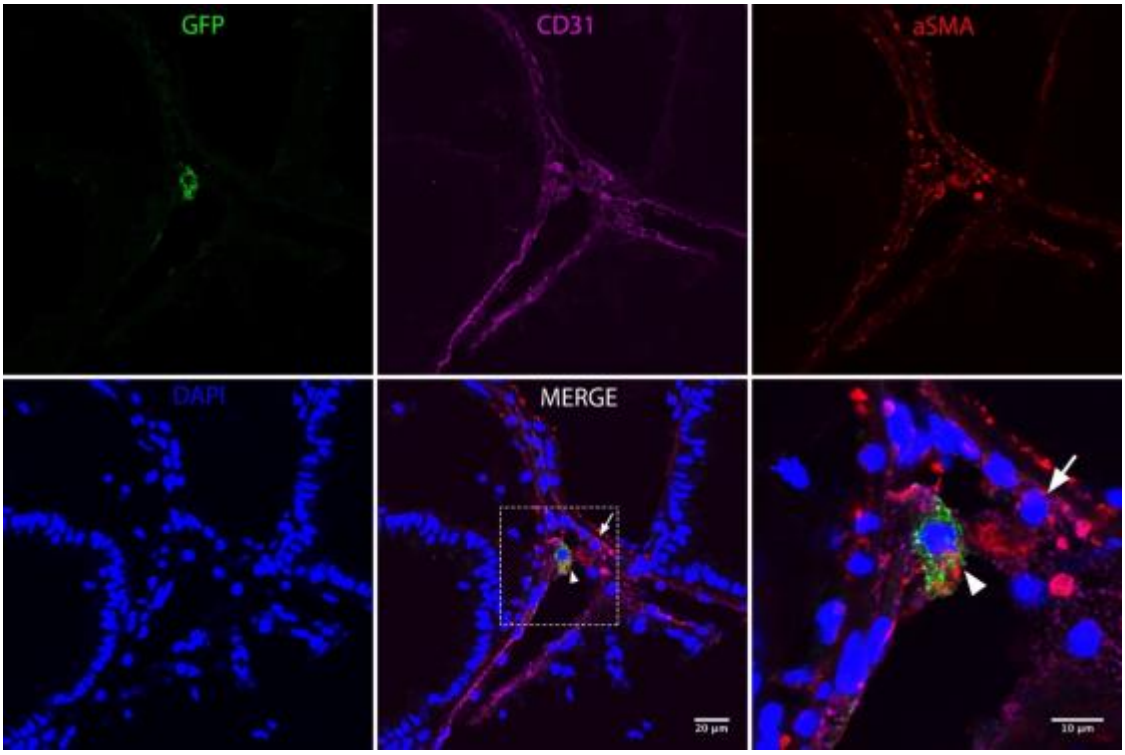

Figure 3.7 GFP+ metastatic tumor cell acquired mesenchymal marker α-SMA expression in the lung.

GFP+ cell (arrowhead) maintained endothelial cell marker CD31 and acquired the expression of mesenchymal cell marker α-SMA, indicating an intermediate state of EndMT. A CD31+/α-SMA+/ GFP- cell (arrow) in close proximity to the GFP+ cell shows the same phenotype. It is possible that this is an authentic vascular endothelial cell undergoing EndMT, or a metastatic cell that was not labeled with GFP. Lower right picture shows a higher magnification of the boxed area. Green, GFP; red, α-SMA; blue, DAPI; magenta, CD31.

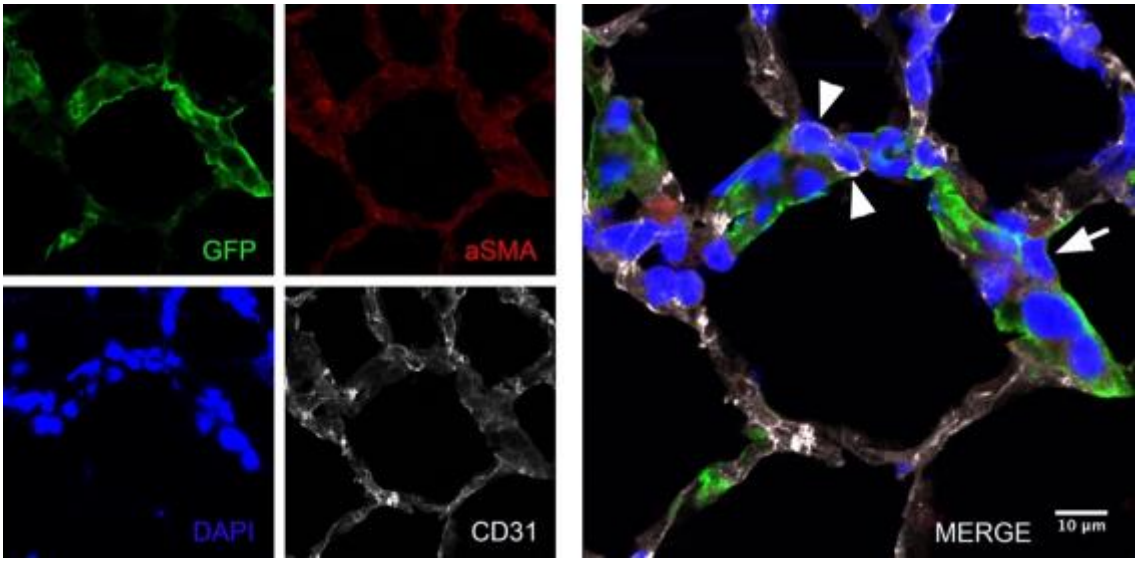

Figure 3.8 α-SMA and CD31 immunostaining on lung sections.

A cluster of GFP+ cells located at the alveolar capillary. Both CD31-/α-SMA+ (arrow) and CD31+/α-SMA+ (arrowhead) GFP+ cells were found. No cell with strong CD31+/α-SMA- immunoreactivity was found in this view. Green, GFP; red, α-SMA; blue, DAPI; gray, CD31.

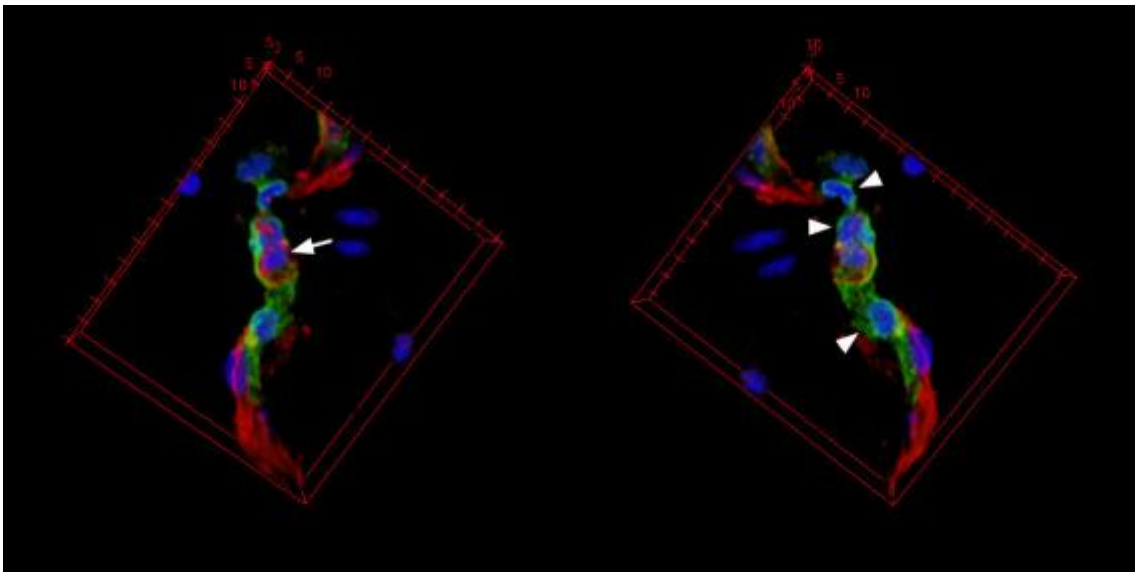

Figure 3.9 3D reconstruction of GFP+ cells located in the pulmonary artery.

3D reconstruction of GFP+ cells in lung cryosection showed three α-SMA negative GFP+ cells (arrowhead) wrapped around one α-SMA positive smooth muscle cell (arrow). GFP, green; red, α-SMA; blue, DAPI.

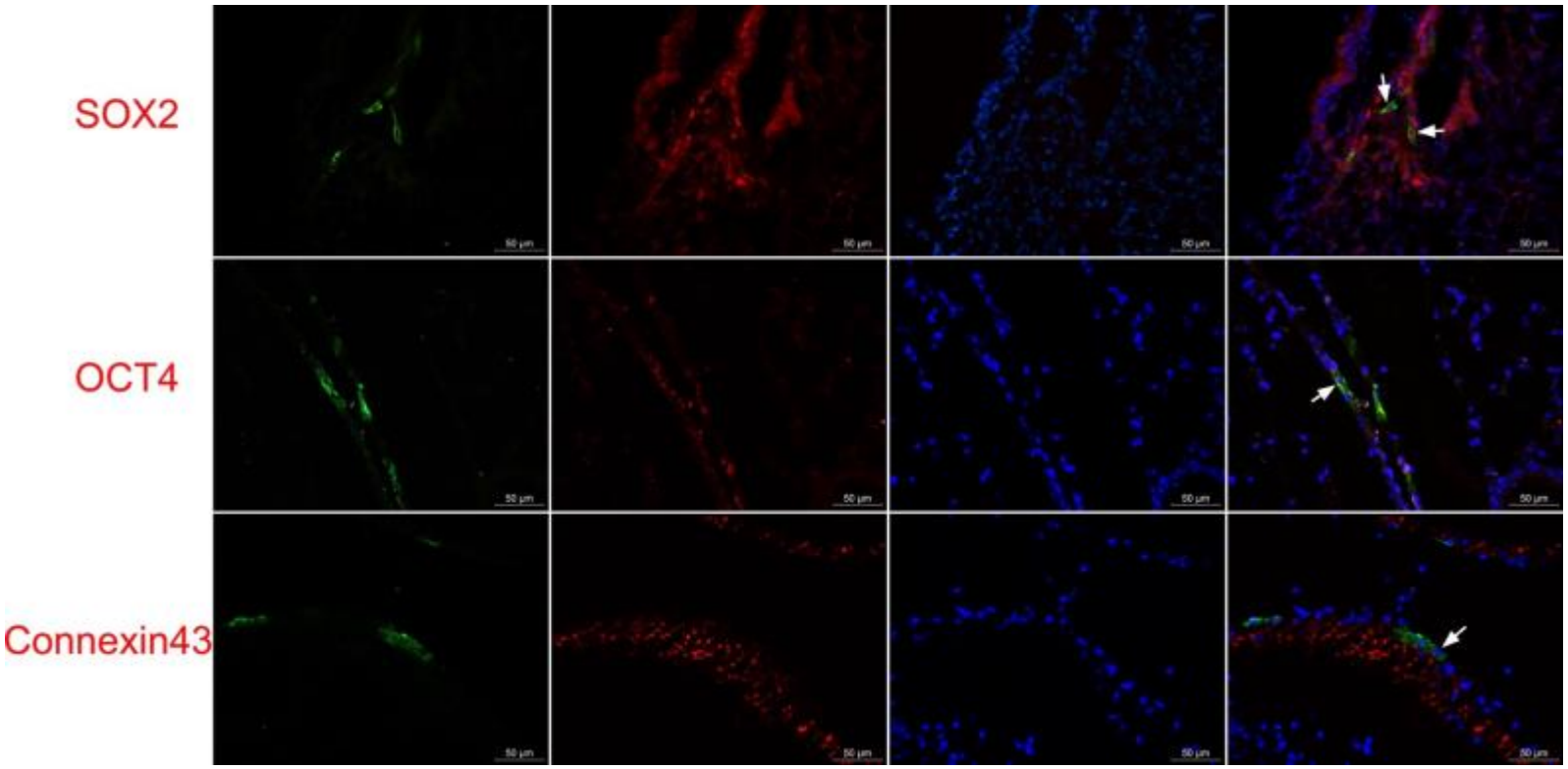

Figure 3.10 Plasticity of GFP+ cells associated with the lung vasculature.

Immunostaining on lung cryosections was performed. Intravascular GFP+ cells showed stem cell marker SOX2 and OCT4 immunoreactivity. A subpopulation of GFP+ cells expressed gap junction protein connexin43. Arrows indicate the immunoreactive GFP+ cells.

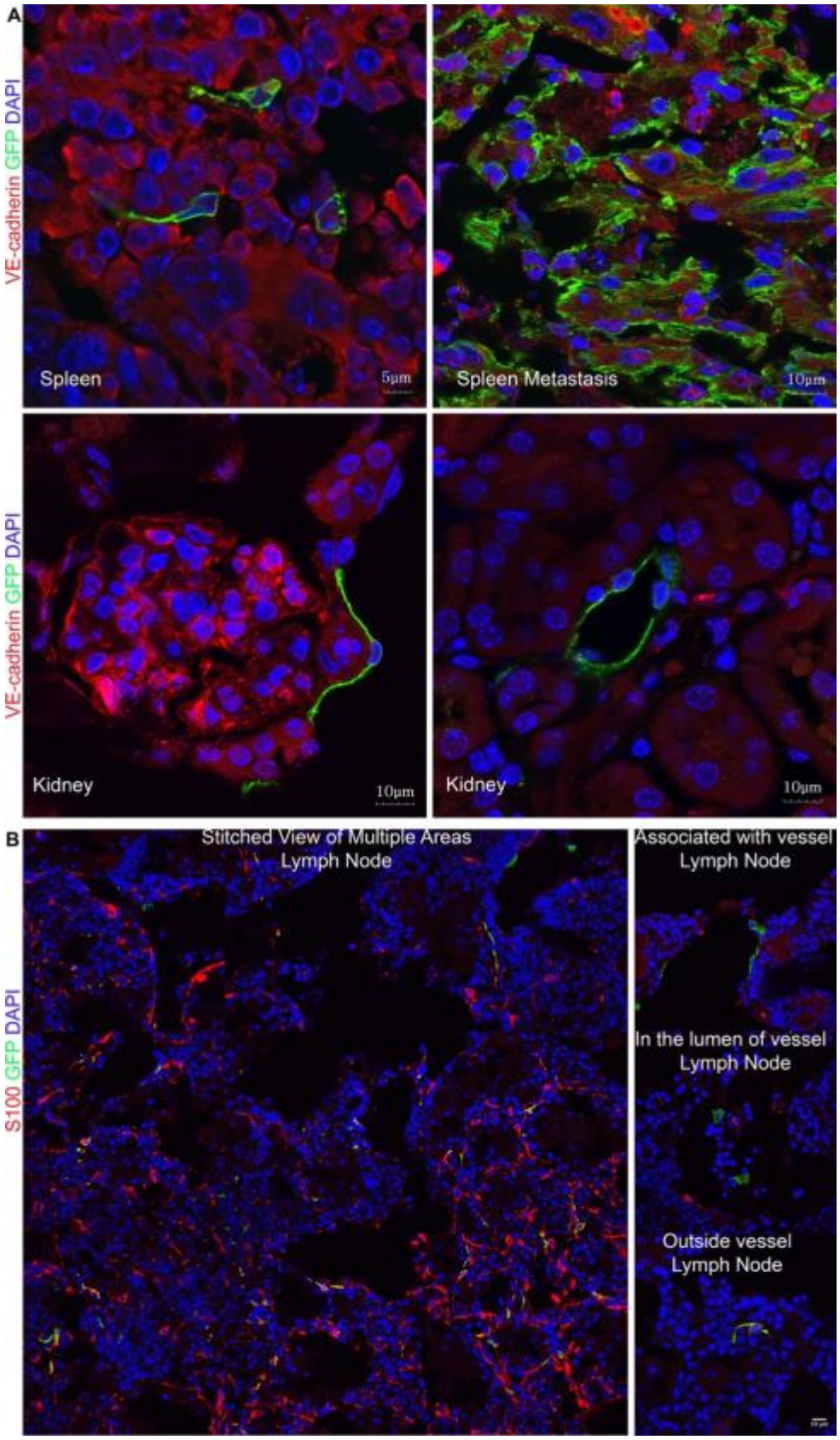
A
VE-cadherin GFP DAPI
Spleen
5μm
Spleen Metastasis
10μm
VE-cadherin GFP DAPI
Kidney
10μm
Kidney
10μm
B
S100 GFP DAPI
Stitched View of Multiple Areas
Lymph Node
Associated with vessel
Lymph Node
In the lumen of vessel
Lymph Node
Outside vessel
Lymph Node

Figure 3.11 Plasticity and heterogeneity of melanoma metastatic cells in other organs.

Immunostaining of various markers in spleen, kidney and lymph nodes from *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice. Primary tumor derived GFP+ cells scattered in the spleen had undetectable VE-cadherin expression while GFP+ cells inside the metastatic foci in the spleen showed strong VE-cadherin expression (A, top panel). In the kidneys, no detectable or weak signal was detected on GFP+ cell (A, lower panel). S100 staining was performed on lymph node cryosections where GFP+ cells were found. GFP+ cells associated with the vascular endothelium or within the lumen were negative for S100 (B).

CHAPTER 4. CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Conclusions

Melanoma is not the most common type of skin cancer, but it causes the most deaths. It is notorious for the high propensity to metastasize and poor response to therapies. Numerous questions remain unanswered regarding the process of melanoma metastasis, which is partially due to the lack of proper melanoma metastasis mouse models. The *Dct-Grm1/ K5-Edn3* mice not only spontaneously develop melanoma tumors on the ear, tail and dorsal skin, but also show metastases in the lung, providing an ideal system to identify the intrinsic tumor initiating capacity of specific cell subpopulations within an unperturbed tumor environment.

Using Cre/loxp system, I was able to label the primary tumor resident Tyr expressing cells with GFP and perform lineage tracing of the GFP+ cells during metastasis in the *Dct-Grm1/ K5-Edn3* mice. I showed that the primary tumor-derived differentiated Tyr expressing cell subpopulation or their progeny possessed metastasis-initiating capacity and exhibited phenotypic plasticity during the metastatic process. The GFP+ metastatic melanoma cells could reach the distant organs and seed metastases successfully. Numerous GFP+ cells were detected inside the vasculature and established strong association with the vascular endothelium in the organs where metastases were identified. Interestingly, GFP+ cells lost the pigmentation as well as melanocytic lineage markers upon contact with the vascular endothelium was established. Vasculature-associated GFP+ cells did not express melanoma stem cell markers,

however, they mimicked vascular endothelial cells by expressing CD31 and VE-cadherin. Three-dimensional reconstruction by confocal microscopy further identified the endothelial cell mimicry of GFP+ cells. The phenomenon was also observed in the lymphatic vessels of lymph nodes. The phenotypic plasticity of metastatic GFP+ cells was also corroborated by the fact that GFP+ cells lost the endothelial cell marker expression and resumed pigment production when populating the metastatic foci. Furthermore, the morphology and distinct phenotypic entities of GFP+ cells in different metastatic organs substantiated the plasticity of metastatic melanoma cells.

GFP+ cells in the lung vasculature were found positive for mesenchymal markers α-SMA or VIMENTIN. Assuming that the intravascular GFP+ cells were the potential metastasis-initiating cells in the lung of *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice, I propose a two-step mechanism underlying melanoma metastasis that involves melanoma-endothelial cell transition at the primary tumor site and EndMT at the metastatic site. The proposed model based on the experimental observations and implied suggestions for the process of melanoma metastasis in the *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mouse is summarized in Figure 4.1.

In conclusion, I have provided direct evidence that a specific cell subpopulation within the primary tumor possesses tumor initiating capacity in the context of metastasis. Lineage tracing of the metastatic melanoma cells in vivo indicated the phenotypic plasticity of cancer cells. My results will not only enhance our understanding of melanoma metastasis, but also provide insights

for the diagnostics and re-examination of melanoma patients aiming at the detection of “vascular hidden” metastatic melanoma cells.

4.2 Future Directions

The heterogeneity of tumors including melanoma has been recognized for decades addressing the varied tumor initiating capacities of cell subpopulations within the same solid tumors [1]. Although the cancer stem cell model has been proposed for melanoma [2], it remains controversial and several questions are still unanswered. In this study, I have shown that the more differentiated Tyr expressing cells or their progeny (GFP+) possessed metastasis-initiating capacities and seeded successful metastases in various distant organs of the *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice. However, the exact subpopulation of the primary tumor derived GFP+ cells that initiated the distant metastasis is not clear. Considering this, it would be of great interest to isolate the circulating GFP+ cells and the lung vasculature-associated GFP+ cells in the *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice and characterize the tumorigenic capacities of these two populations. High deformability of Sox2-expressing melanoma cells grown in 3D soft fibrin gels has recently been shown to facilitate the extravasation of metastatic cells [3]. Given the morphological plasticity of metastatic GFP+ cells I observed in the established lineage tracing system, it is worthwhile to test the mechanical stiffness of the two GFP+ cell subpopulations. RNA-sequencing would be another approach to further identify the mechanism underlying the distinct tumorigenic capacities, if any, between the circulating GFP+ cells and the lung vasculature-associated GFP+ cells.

I proposed the melanoma-endothelial cell transition as the first step for Tyr expressing cells to successfully metastasize based on the finding that CD31+ GFP+ cells were identified inside the vasculature at the primary tumor site. Most of the studies focusing on characterizing tumor derived endothelial cells have been carried out in the context of glioma. Hypoxia has been suggested as a promoter of glioma cell transdifferentiation [4, 5]. Although melanoma tumors from the *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice were suggested to display hypoxic areas [6], I did not observe sufficient CD31+ GFP+ cells within the primary tumor mass. One possibility to explain this finding is that it was very difficult to detect single cells due to the extensive amount of pigmentation in the tumor sections. However, alternative factors other than hypoxia may be the trigger of melanoma to endothelial cell transdifferentiation. For example, lung carcinoma cells co-cultured with human microvascular lung endothelial cells became strongly positive for CD31 as a non-angiogenic process for blood supply [7]. To characterize the potential effects of tumor-endothelial cell contact in melanoma, it would be interesting to perform similar co-culture of human microvascular lung endothelial cells with human melanoma cells. To better replicate the context of my in vivo experiments, human melanoma cells overexpressing Grm1 would be the ideal cells to employ.

Furthermore, in the *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice, primary tumor derived GFP+ cells associated with lung vasculature were not proliferating or dying and the number of those intravascular cells far exceeded the number of metastatic foci in the lung. The above observations imply the

features of metastatic dormancy. The presence of lung infiltrates surrounding the GFP+ cell-containing vessels raises the question of why GFP+ cells were found embedded in the vascular endothelium. Suppression of the immune system in *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice will allow us to identify whether lymphocyte infiltrates are the main suppressor of extravasation or EndMT of GFP+ cells. Alternatively, the immune response observed is possibly a general phenomenon of metastasis, and the intravascular GFP+ cells are intrinsically programmed for remaining in a dormant state and can only be re-awaken upon exposure to as yet unidentified environmental factors. Further studies to determine both the intrinsic and extrinsic cues underlying entry into and exit from dormancy state would be very valuable.

Late recurrence has been observed in human melanoma patients [8], indicating the presence of residual cancer cells that escape the detection of available techniques. Based on my observations in the mouse model, it is highly possible that metastatic melanoma cells cease to express most, if not all, melanocytic features and masquerade themselves as other cell types and become clinically invisible. Labeling of primary tumor cells is impossible in human patients and the loss of pigmentation of the intravascular metastatic melanoma cells challenges their detection in metastatic organs. Other than the genetic tracing of tumor cells in mouse models, analysis of tumor specific chromosomal alterations[9-11] was used to identify the tumor derived endothelial cells in human samples. BRAF mutations are found in approximately 60% of somatic melanoma cases [12]. An antibody against BRAF V600E, the mutated form of

BRAF, is commercially available. One possible alternative for detecting these "invisible" metastatic cells in human melanoma samples is to use immunostaining or flow cytometry with Anti-BRAF V600E and endothelial cell specific markers. If a population of double positive cells in the primary or metastasis melanoma samples can in fact be revealed, current strategies for melanoma metastasis diagnostics and metastasis-targeted therapies need to be enhanced taking the existence of these cells into consideration.

4.3 References

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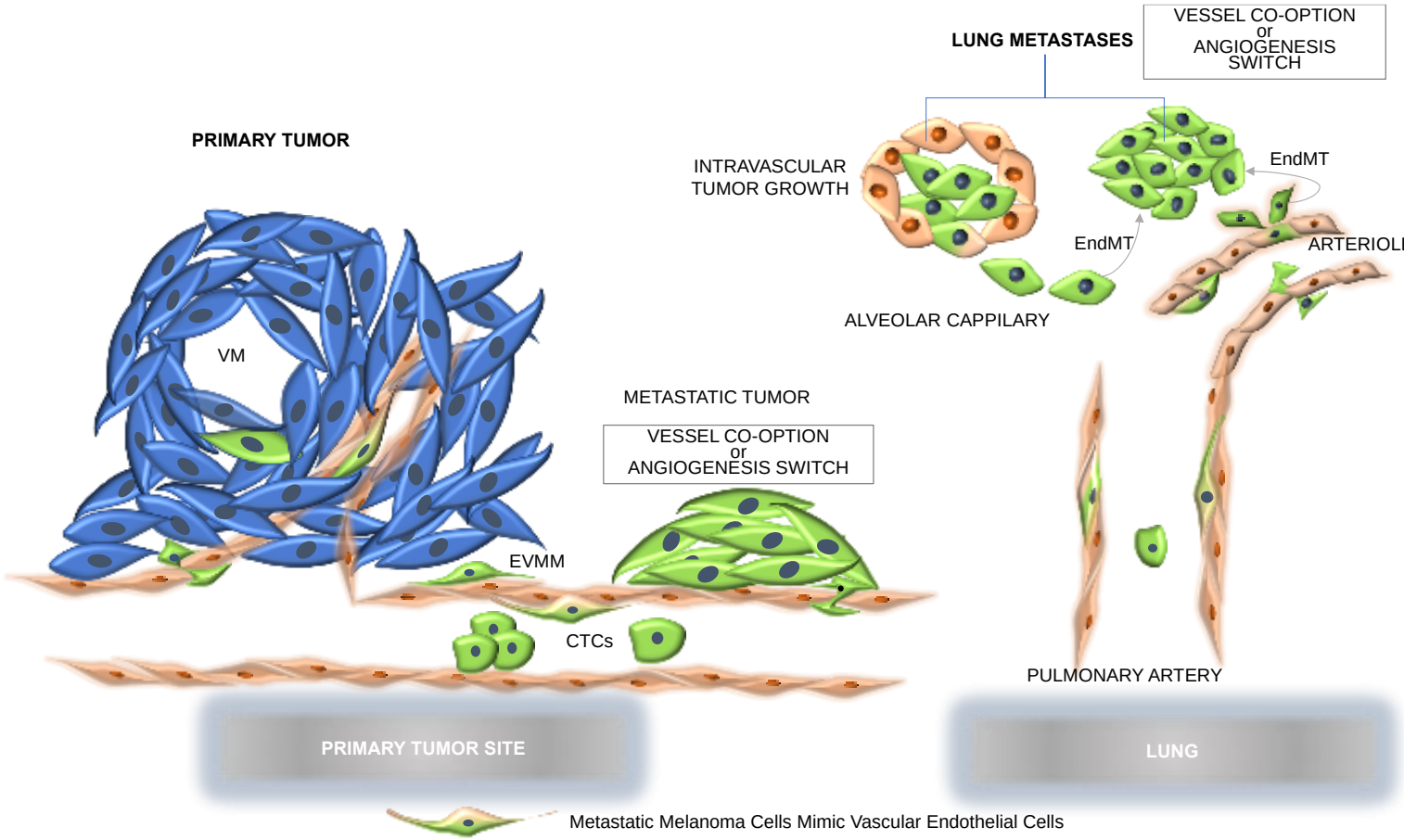
LUNG METASTASES
VESSEL CO-OPTION
or
ANGIOGENESIS
SWITCH
PRIMARY TUMOR
INTRAVASCULAR
TUMOR GROWTH
EndMT
EndMT
ARTERIOLES
ALVEOLAR CAPPILARY
VM
METASTATIC TUMOR
VESSEL CO-OPTION
or
ANGIOGENESIS SWITCH
EVMM
CTCs
PULMONARY ARTERY
PRIMARY TUMOR SITE
LUNG
Metastatic Melanoma Cells Mimic Vascular Endothelial Cells

Figure 4.1 Diagram of melanoma metastasis in the Dct-Grm1/ K5-Edn3/ *TYR-CreER^{T2}/ mT/mG* mice.

Primary tumor resident Tyr expressing cells express VE-cadherin, indicating VM. Metastatic Tyr expressing cells or their progeny (GFP+, green color cells in diagram) enter the vasculature through extravasation or transdifferentiate into CD31+ endothelial cells establishing associations with the vascular endothelium. Intravascular GFP+ cells move towards the metastatic site as circulating tumor cells (CTCs) or may roll over the vascular endothelium during endothelial mimicry. EVMM was only observed in metastatic tumor at the primary tumor site, not distant organs in the *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice. In the lung, intravascular GFP+ cells form metastases through extravasation and grow using angiogenesis or vessel co-option. A subset of CD31+ GFP+ cells in smaller pulmonary vessels went through EndMT, suggesting a potential mechanism for the metastasis-initiating capability of vasculature-associated metastatic cells. Intravascular growth of GFP+ cells in the pulmonary capillaries with subsequent extravasation when intravascular micro-metastatic foci outgrow the vessels they are in can be another source for metastases formation.

MATERIALS AND METHODS

Mice and Genotyping

The *K5-tTA;TRE-Edn3-lacZ* (*K5-Edn3*, for simplicity) mice, which overexpress Edn3 under the keratinocyte promoter Keratin 5 resulting in a hyperpigmentation phenotype due to the accumulation of melanocytes at the dermal-epidermal junction, were generated in our laboratory[1]. The *Tg(Grm1) Epv (E) mice* (*Dct-Grm1*, for simplicity, a gift from Dr. William Pavan, NIH) [2]express the metabotropic glutamate receptor 1 under the regulation of the melanocyte specific Dopachrome tautomerase promoter and develop spontaneous melanocytic tumors on the ear and tail, but rarely metastasize. The progeny from the *K5-Edn3* mice and *Dct-Grm1* mice mating carrying all transgenes, *Dct-Grm1/ K5-Edn3*, were used in this study as a melanoma mouse model. The *Dct-Grm1/ K5-Edn3* mice not only spontaneously develop melanoma on the ear, tail and dorsal skin, but also metastasize to the lung with 80% penetrance.

To genetically label Tyrosinase expressing cells in the *Dct-Grm1/ K5-Edn3* mice and establish an in vivo lineage tracing system, Cre/loxp system was used in this study. *TYR-CreER^{T2}* mice are widely used for melanoma or melanocyte related studies. These mice have expression of CreER^{T2} fusion protein directed by mouse Tyr promoter/enhancer regions. When bred with mice containing loxp-flanked sequences, tamoxifen/4HT-inducible Cre-mediated recombination results in the deletion of the floxed sequences in the Tyr-expressing cells of the offspring. For the Cre-reporter mice, the bifluorescent mice *mT/mG* [3] was selected due to the following reasons. First, it not only labels cells after Cre-mediated

recombination, but also labels non-recombined cells. Second, the double fluorescent reporter genes in these mice simplify the visualization of recombined and non-recombined cells without the addition of an exogenous enzymatic substrate like β-Galactosidase used in LacZ reporter mice. Third, the membrane targeted tdTomato and GFP fluorescent proteins in *mT/mG* mice allows the labeling to be visualized at single cell resolution which is important for lineage tracing. The Cre/loxp system in this study using *TYR-CreER^{T2}* and *mT/mG* mice will be established through the mating of those two mouse strains resulting in the offspring *TYR-CreER^{T2}/mT/mG* mice.

The genotyping of the *Dct-Grm1/K5-Edn3/TYR-CreER^{T2}/mT/mG* mice was carried out by DNA extraction from a small piece of the tail using standard procedures and followed by PCR using the appropriate primers (Table S2) for each transgene ([1, 2], The Jackson Laboratory). 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 and all Institutional Animal Care and Use Committee regulations were followed.

4HT Topical Painting on Tumors

Topical administration of 4HT was performed by preparing a 50mg/ml solution of 4HT (Sigma, H6278) in DMSO and applying enough solution to wet the tumors with a small paint brush. *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice were monitored for tumor appearance on the tail and dorsum. As soon as the first tumor appeared, we topically painted 4HT on the primary tumors twice a

day for three consecutive days during 3~4 weeks to ensure the labeling of most Tyr expressing cells within the primary tumors. Mice were monitored for primary tumor growth until 30 weeks, unless euthanasia was necessary due to poor health. As such, mice were euthanized at different times after the first painting that varied from 2 weeks to 30 weeks. After euthanasia, tumor burdened mice were examined for the presence of metastases in the lungs as well as other organs under the dissecting microscope. Pigmented lesions in the lung ranged from large macro-metastatic tumors (>1mm in diameter) to smaller pigmented lesions that were clearly visible with the naked eye (pepper-like micro-metastases) or much smaller micro-metastases that were only visible under a dissecting microscope.

Immunofluorescence Staining

Harvested organs were fixed in 4% paraformaldehyde (PFA) overnight at 4° C and washed in 1X PBS. Organs were cryo-protected by sequential immersion in 15% and 30% sucrose and then embedded with OCT. 10μm sections were obtained for immunofluorescence staining. Sections were incubated in blocking buffer (10% normal serum, 0.3% Triton X-100, 1%BSA power) at room temperature for 1 hour and followed by primary antibody incubation at room temperature for 1 hour or at 4° C overnight. Primary antibodies were diluted in dilution buffer (1% BSA, 1% normal serum, 0.3% Triton X-100). Sections were thoroughly washed before incubation in secondary antibodies for 1 hour at room temperature. Sections were mounted with fluoroshield mounting medium with DAPI (Abcam, ab104139) and visualized on a

Leica Leitz DMRB fluorescent microscope or Olympus Confocal BX61 microscope. For pigment bleaching on primary tumor sections, slides were incubated in 10% H_2O_2 in PBS at room temperature for about 16 hours followed by regular immunostaining. Where necessary, tissue GFP and tdTomato fluorescence from the mT/mG allele were photobleached by treatment with 3% H_2O_2 in methanol and illumination on a fluorescent light box for 4 hours to overnight at 4°C. GFP signal was amplified by anti-GFP antibody if necessary.

Primary antibodies used: GFP (1:500, Aves Labs, GFP-1020), Ki67 (1:200, Abcam, ab15580), Cleaved Caspase 3 (1:200, CST, 9664s), Rat CD31 (1:200, eBioscience, 14-0311-82), Rabbit CD31 (1:200, Abcam, ab28364), CD45 (1:200, Biolegend, 103101), CD3 (1:200, Novusbio, NB600-1441), P75 (1:200, Millipore, ab1554), Nestin (1:50, Millipore, MAB353), SOX10 (1:50, Santa cruz, sc17342), TYR/TRP1 (1:200, gift from Dr. Vincent Hearing), LYVE-1 (1:200, eBioscience, 14-0443-80), VE-cadherin (1:200, Abcam, ab33168), SOX2 (1:200, Abcam, ab97959), OCT4 (1:200, Abcam, ab18976), Connexin43 (1:200, CST, 3512), S100 (1:200, Sigma, S2644). Secondary antibodies used: anti-chicken 488 (1:500, Abcam, ab150169), anti-rabbit 594 (1:400, Invitrogen, A11012), anti-rabbit 647 (1:400, Abcam, ab150075), anti-rat 647 (1:400, Abcam, ab150159).

H&E Staining of Lung Cryosections

Cardiac perfusion with PBS was performed immediately after euthanasia. After the PBS perfusion, lungs were infiltrated with OCT through the trachea by a 18G needle. Sections were subjected to H&E staining as follows: dH2O for 30 secs; Gill's Hematoxylin 3 (VWR, 87001-924) for 5 mins; running tap water for 1

min; Clarifier (VWR, 89237-218) for 15 secs; dH2O for 30 secs; Bluing Agent (VWR, 95057-852) for 45 secs; dH2O for 30 secs; 95% ethanol for 15 secs; Eosin (poly scientific R&D Crop, s2186) for 45 secs; 95% ethanol for 30 secs; 100% ethanol for 10 secs; Xylene for 15 secs; Xylene for 1 min; dry slides and mount with cytoseal (xylene-based medium).

Counting of Ki67+ or Caspase 3+ Intravascular GFP+ Cells in the Lung

15 non-parallel lung cryosections per *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mouse (n=3) were immunostained for Ki67 or Caspase 3 together with GFP. Total intravascular GFP+ cells were counted for each mouse. Among the intravascular GFP+ cells counted, double positive (Ki67+GFP+ or Caspase 3+GFP+) cells were counted. Total number of intravascular GFP+ cells varied from 29 to 243 per mouse. Percentage of Ki67+ or Caspase 3+ intravascular cells was obtained through double positive intravascular cells/total intravascular GFP+ cells.

Timing of Metastatic Cell Dissemination

Adult *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice were separated into three groups based on the stage of tumor development on the tail: 1) before nevus stage, where no noticeable tumor or nevus was observed; 2) nevus stage, where the lesion was less than 1mm in height; 3) tumor stage, where a clearly detectable tumor had formed. 4-HT was topically applied to the nevus/tumor site by a small painting brush twice a day for three consecutive days. For the before nevus stage, 4-HT was applied to the tail of *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mice that were 8-month-old yet without detectable tumor or nevus. The

proximal 3-4 cm of the tail where lesions generally develop was 4HT painted twice a day for three consecutive days.

Whole Mount Confocal Imagining and Immunofluorescence Staining of Blood Vessels

A small segment (2-3mm length) of pulmonary artery, pulmonary vein, thoracic aorta or abdominal aorta was used for whole mount imaging or staining. The vessel segments were dissected and fixed in fresh 4% PFA in PBS at 4°C overnight. For confocal imaging, the vessels were cut open and mounted flat on glass slides with fluoroshield mounting medium with DAPI (Abcam, ab104139). For immunofluorescence staining, vessels were cut open and washed in 1% Triton X-100 in PBS for 40 minutes and blocked in 1% Triton X-100, 10% FBS in PBS for 90 minutes at room temperature. Primary antibodies were diluted in blocking buffer as described previously; vessels were incubated with primary antibodies in 0.5mL PCR tube at 4°C for 20 hours with shaking. After PBS washing for 30 minutes twice, secondary antibodies were applied in blocking buffer for 2 hours. Vessels were washed in PBS thoroughly and mounted with fluoroshield mounting medium with DAPI. Z-stack images were taken from the top layer (endothelial layer) to the bottom layer (smooth muscle layer) at a step size 0.5 to 1μm, 30 to 60 z-slice images were taken with Olympus Confocal BX61 microscope. 3D reconstruction or 3D projection of the z-stack images was performed with Fiji-ImageJ software.

References

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2. Pollock, P.M., et al., *Melanoma mouse model implicates metabotropic glutamate signaling in melanocytic neoplasia.* Nat Genet, 2003. **34**(1): p. 108-12.

3. Muzumdar, M.D., et al., *A global double-fluorescent Cre reporter mouse.* Genesis, 2007. **45**(9): p. 593-605.

SUPPLEMENTARY FIGURES AND TABLES

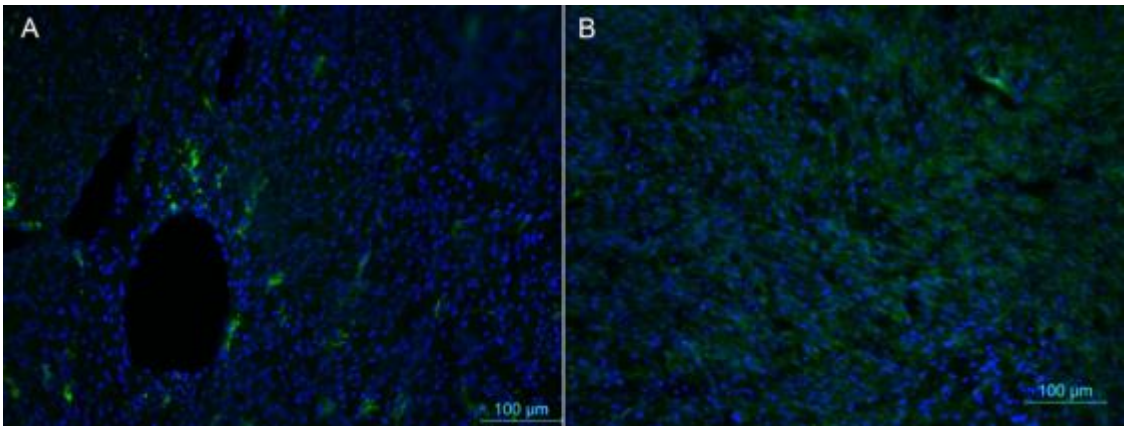

Figure S 1. GFP+ cells in the tail tumors after 4HT induction.

Primary tumor cryosections 7 months after the first 4HT painting. 4HT topical painting was performed once (twice a day for three consecutive days) when the tail tumor was at the nevus stage. Few GFP+ cells were identified in the tumor (A). 4HT topical painting was performed for 4 weeks since the nevus stage. Numerous GFP+ cells were found in the tumor (B). Sections were counterstained with DAPI (blue).

Ear Tag# &Genotype	4HT Painting Site	Painting Times	Lung Metastasis	GFP+ Cells in Lung	GFP+ Cells in Lymph Nodes	GFP+ cells in Other Organs
#298*	tail tumor	2	micro-metastasis	no	no	NA
#875*	tail tumor	5	micro-metastasis	no	no	NA
#300*	tail tumor	5	micro-metastasis	yes	no	NA
#396*	dorsal tumor with 2-3 mm in height	3	micro-metastasis	yes	no	NA
#471*	tail tumor	4	no	no	NA	NA
#231*	tail tumor	8	micro-metastasis	no	yes	NA
#365*	tail tumor	5	micro-metastasis	no	no	NA
#946*	tail & dorsal tumor with 2-3 mm in height	tail 4, dorsal 1	micro-metastasis	yes	yes	NA
#997*	tail & dorsal tumor	tail 1, dorsal 5	micro-metastasis	yes	yes	NA
#369*	tail & dorsal tumor	tail 4, dorsal 7	pepper like micro-metastasis	yes	yes	NA
#443*	dorsal tumor	4	no	yes	yes	NA
#476*	dorsal tumor	1	micro-metastasis	no	yes	NA
#451*	tail & dorsal tumor	tail 3, dorsal 5	micro-metastasis	yes	NA	NA
#479*	dorsal tumor	10	macro-metastasis	yes	yes	spleen, kidney
#425*	tail tumor	8	micro-metastasis	no	NA	
Control (n=10) *Dct-Grm1/K5-Edn3/mT/mG*		1~8	yes	no	NA	NA
Control (n=3) *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG*	dorsum (before nevus)	1	no	no	NA	NA

Table S 1. 4HT induction of GFP expression and lung metastasis.

Macroscopic metastases in other organs were only observed in mouse#479, and GFP+ cells were found in those organs. When macroscopic metastases were not found, organs were not checked for GFP+ cells (NA), except brains (no GFP+ cells in any of the mice). Painting time “1” means 4HT topical application twice a day for three consecutive days. Every painting was performed for a duration of three or four weeks. All paintings started from nevus stage, unless specified, and lasted until the mouse was euthanized. Painting of dorsal tumors may have started post nevus stage because of the difficulty in detecting the nevi under the coat. For genotype, “*” indicates *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* experimental mice, genotypes of control mice were specified in table.

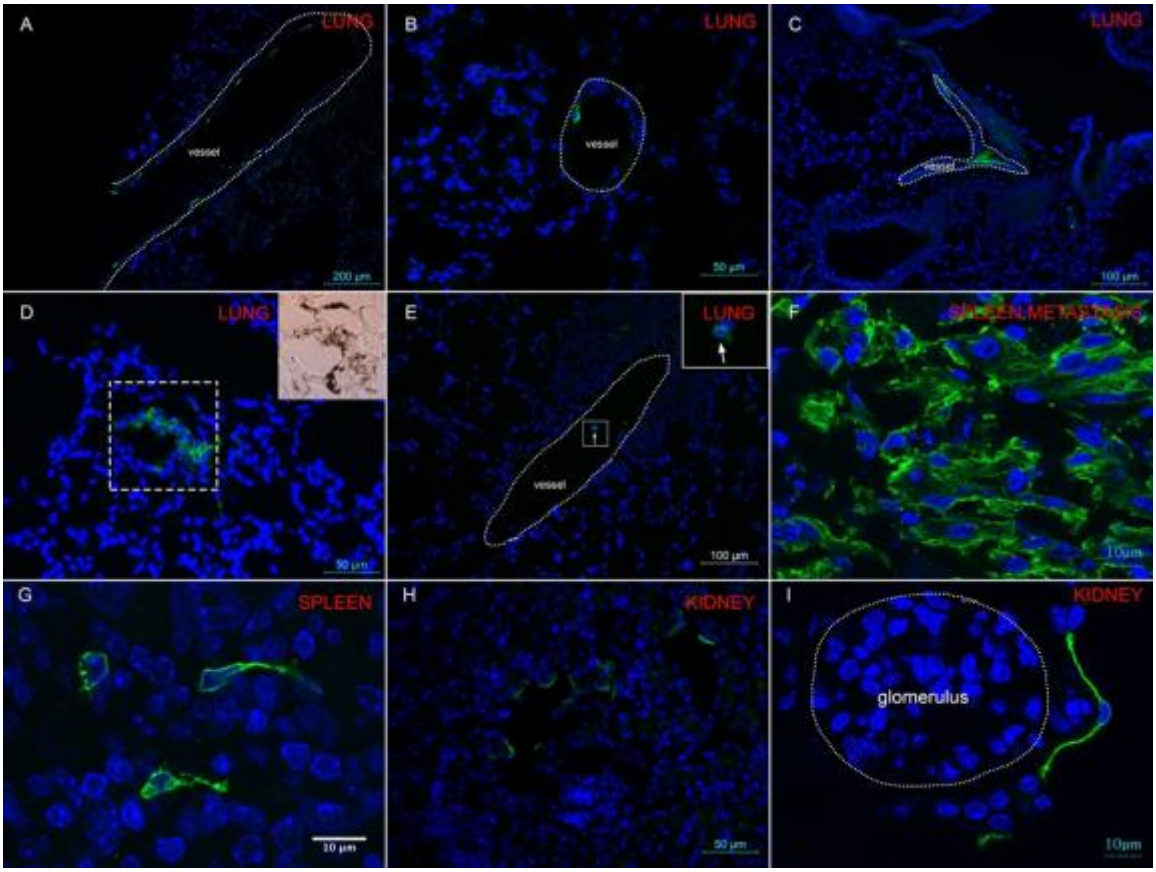

Figure S 2.Primary tumor derived GFP+ green cells were found in different organs.

A-E, lung; F-G, spleen; H-I, kidney. Note, most of the green cells were single cells with an elongated shape; clusters of cells were mostly confined to micro-metastasis (D) or macro-metastasis (F, pigmented lesion in the spleen). Most GFP+ cells found in the lung vasculature were in close association with the vascular endothelium (A-C) with rare cases in the lumen of the vasculature corresponding to the circulating cells (E). Scattered single GFP+ cells were detected in the spleen (G), between the renal tubules (H) and adjacent to glomeruli (I).

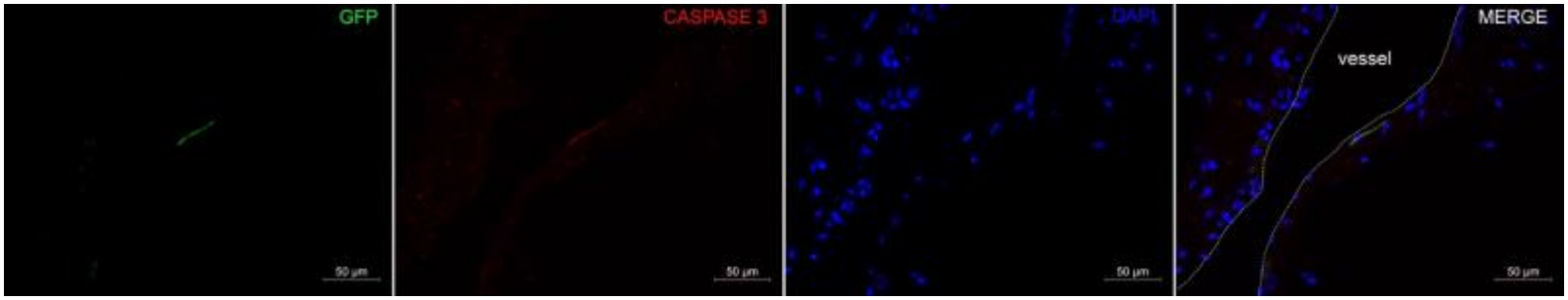

Figure S 3. Cleaved Caspase 3 staining on the lung.

One GFP+ cell in contact with the pulmonary vascular endothelium was positive for Caspase 3, indicating this cell underwent apoptosis.

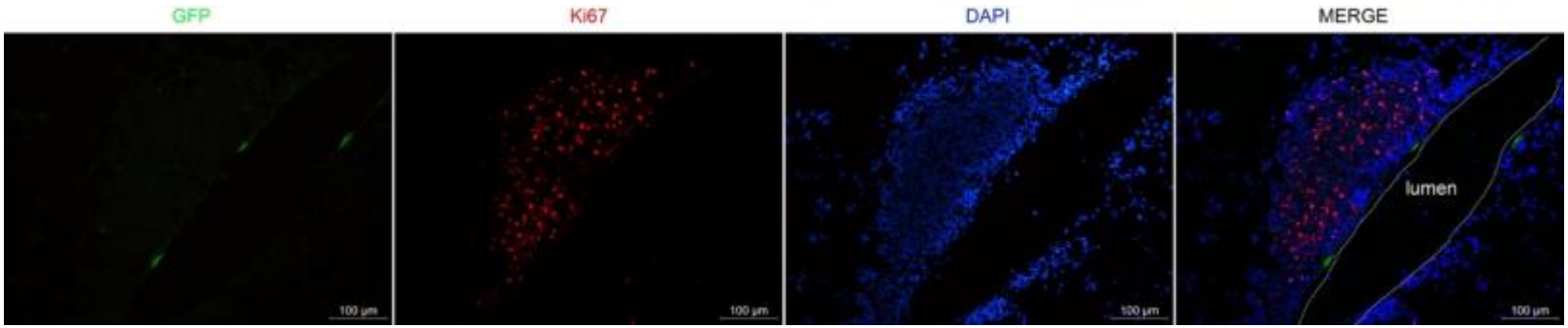

Figure S 4. Ki67 staining on lung perivascular infiltrates.

Most lymphocytes inside the infiltrates were Ki67 positive indicating the activation of immune response towards the emergence of GFP+ cells inside the vessels.

Figure S 5. Metastatic tumors on the skin.

4HT was applied to the dorsal tumor and distal tail tumor (arrowhead) of the *Dct-Grm1/ K5-Edn3/ TYR-CreER^{T2}/ mT/mG* mouse shown here. Proximal tail tumor (boxed area) was mostly constituted by GFP+ cells indicating it was a metastasis derived from the Tyr+ cells from the distal tail or dorsal tumor.

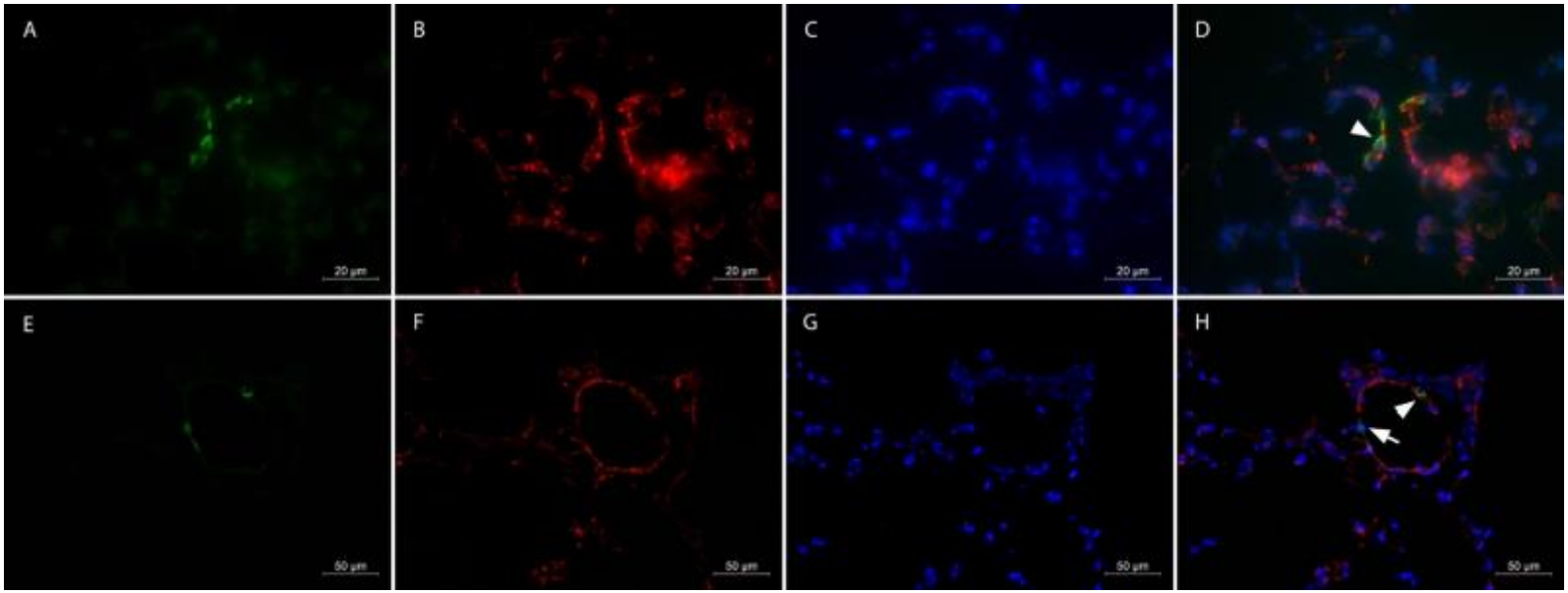

Figure S 6. GFP+ cells inside the alveolar capillaries were CD31 positive.

Lung cryosections (A-H) depicting GFP +cells (A, E) inside alveolar capillaries, were labeled with CD31 antibody (B, F) and counterstained with DAPI (C, D). Some GFP+ cells were CD31 positive (D, H, arrowhead) and some were not (H, arrow) indicating the heterogeneity of metastatic tumor cells.

Gene	**Sequence 5' --> 3'**
TYR-CreER^{T2}	CTC TGC TGC CTC CTG GCT TCT
	CGA GGC GGA TCA CAA GCA ATA
	TCA ATG GGC GGG GGT CGT T
mT/mG	GCG GTC TGG CAG TAA AAA CTA TC
	GTG AAA CAG CAT TGC TGT CAC TT
	CTA GGC CAC AGA ATT GAA AGA TCT
	GTA GGT GGA AAT TCT AGC ATC ATC C
Dct-Grm1	CCGGGTCCGCATTAATCTTATCTA
	GGTAGCATACGGTTCCACGCA
K5-Edn3	CCAGGTGGAGTCACAGGATT
	ACAGAGACTGTGGACCACCC
	GGCCTGTGCACACTTCTGT
	TCCTTGTGAAACTGGAGCCT

Table S 2. Primers for mouse genotyping.

VITA

XIAOSHUANG LI

2006-2010	Bachelor's Degree Biology Science-Animal & Developmental Biology Shandong University, Jinan, Shandong, China
2009	Research Trainee Institute of Development and Molecular Medicine Fudan University, Shanghai, China
2010-2011	Research Assistant Laboratory Animal Research Center Institute of Biophysics, Chinese Academy of Science (CAS), Beijing, China
2011-2015	Graduate Teaching Assistant Department of Biological Sciences Florida International University, Miami, FL, USA
2015-2017	Graduate Research Assistant Department of Biological Sciences Florida International University, Miami, FL, USA
2011-2017	PhD candidate Department of Biological Sciences Florida International University, Miami, FL, USA
2014	BRI Summer Research Award Florida International University, Miami, FL, USA
2015	BRI Summer Research Award Florida International University, Miami, FL, USA
2016	BRI Summer Research Award Florida International University, Miami, FL, USA
2017	AACR WICR Scholar Award, Women in Cancer Research American Association for Cancer Research (AACR) Annual Meeting 2017, Washington, D.C., USA

SELECTED PUBLICATIONS AND PRESENTATIONS

Li, X., Kos L (2015). Cellular origin of melanoma metastasis. 17th Biomedical & Comparative Immunology Symposium, Florida International University, Miami, FL, USA (Oral Presentation)

Li, X., Kos L (2015). Identification of the metastatic cell populations in a mouse model of melanoma. Society for Melanoma Research 2015 Congress, San Francisco, California, USA (Poster Presentation)

Li, X., Torres, R., Kos L (2016). Identification of the metastatic cell populations in a mouse model of melanoma. 18th Biomedical & Comparative Immunology Symposium, Florida International University, Miami, FL (Oral Presentation)

Li, X., Torres, R., Kos L (2017). Identification of the metastatic cell populations in a mouse model of melanoma. AACR Annual Meeting 2017, Washington, D.C., USA (Poster Presentation)