January 2012

The Effect Of E-Cadherin Loss On Melanoma Formation And Metastasis

Lara Rosenbaum

Follow this and additional works at: http://elischolar.library.yale.edu/ymtdl

Recommended Citation
http://elischolar.library.yale.edu/ymtdl/1755
The effect of E-cadherin loss on melanoma formation and metastasis

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degrees of Doctor of Medicine & Master of Health Science

by
Lara Elise Rosenbaum
2012
Abstract

THE EFFECT OF E-CADHERIN LOSS ON MELANOMA FORMATION AND METASTASIS.

Lara E. Rosenbaum, William Damsky, Katrina Meeth, and Marcus Bosenberg. Department of Dermatology, Yale University School of Medicine, New Haven, CT.

Melanoma is the most lethal form of skin cancer and accounts for the majority of skin cancer related mortality. Early metastases are characteristic of melanoma and are an ominous sign, as current therapeutic interventions have little effect on overall survival. The lack of accurate prognostic indicators and effective therapies emphasize the need for a better understanding of the genetic and phenotypic changes in melanoma formation and progression. One such change is the loss of E-cadherin, which normally plays a role in cell-cell adhesion and is thought to be a central feature of epithelial-mesenchymal transition (EMT). Maintenance of E-cadherin in melanoma may function to restrict melanoma invasion, and loss of E-cadherin expression is associated with mesenchymal features and melanoma metastasis. While several studies have suggested that loss of E-cadherin is associated with cancer progression, functional evaluation of the effects of E-cadherin loss on metastasis has not been well established.
In order to evaluate these important issues on the role of E-cadherin in melanoma formation and metastasis, we have utilized a well-characterized conditional mouse model combining the activating Braf V600E mutation with Pt en tumor suppressor loss (Braf/Pten tumor model). This model allows us to alter these genes in mouse melanocytes in a spatially and temporally restricted manner, producing melanoma specifically and reproducibly. We have combined this model with conditional E-cadherin inactivation in order to evaluate the role of E-cadherin loss on melanoma formation, progression and metastasis as well as changes in cell morphology and signaling. Loss of E-cadherin significantly decreases survival of mice in the Braf/Pten tumor model (p=0.024) and alters the expression of markers of EMT as demonstrated by RNA microarray analysis. Surprisingly, no difference in metastases to either the lymph nodes (p=0.28) or lungs (p=0.39) are seen with loss of E-cadherin.

Additionally, Braf activation in combination with E-cadherin loss (Braf/Ecad model) leads to melanoma in 100% of mice with tumor death occurring after an average of 302 days (range: 199 – 395 days) or approximately 10 months. This novel model is in striking contrast to the Braf/Pten tumor model, which has an average lifespan of 40 days. No
tumors develop in mice with Braf activation alone or E-cadherin loss alone nor in mice with both loss of E-cadherin and loss of Pten. The Braf/Ecad tumor model shows activation of mammalian target of rapamycin (mTOR) signaling, a commonly dysregulated protein in human cancers, as well as activity in the P13K/Akt pathway which regulates cellular proliferation and survival. Some markers of EMT are also upregulated in the Braf/Ecad tumor model.

These findings functionally delineate the role of E-cadherin loss in melanoma formation, metastasis and EMT. Additional evidence is garnered which supports the role of E-cadherin as a tumor suppressor gene and that its loss is functionally important in altering key pathways of cellular growth and survival. With better understanding of melanoma, new treatments will be developed to help manage this devastating cancer.
Acknowledgements

I would like to acknowledge Marcus Bosenberg, my thesis advisor and mentor, for his unwavering support and dedication to my success both in research and in my medical career. He is a constant source of inspiration. Marcus is a mentor who not only wanted the very best for me, but also supported me wholeheartedly in my goals. His office was always open to me and I have grown leaps and bounds as a scientist and physician under his watch. I am truly grateful for the amazing research year that I had in Marcus’s lab and will always carry what I learned from Marcus with me.

I would also like to thank Bill Damsky, who, as an MD/PhD student in Marcus’s lab, helped enormously to make my research year a success. This project was started by Bill and he generously allowed me to take it over and make it my own. Bill taught me a huge amount and has become a close friend. I know that Bill will be incredibly successful in his career as a physician-scientist and I count myself as lucky to have had the opportunity to work with him.

Additionally, I would like to thank Katie Meeth, a PhD student and recent addition to Marcus’s lab. I had a great deal of fun introducing Katie to
the lab when she first rotated through and was so excited when she chose to join for her PhD. Katie was instrumental in putting the finishing touches on this project when I returned to medical rotations and was no longer able to work full-time on this project. This thesis could not have been completed without Katie’s incredible dedication to seeing it through to the end. For this, I am so grateful.

Furthermore, I would like to thank the entirety of the Bosenberg lab, both past and present, for their support throughout my research year. It was an amazing environment in which to work and I learned an enormous amount. Thank you for welcoming me into the group.

I would also like to acknowledge my thesis committee, Drs. Michael Caplan and Richard Edelson. Their support and constructive critiques have made this project even better, and they have pushed me and challenged me to be a better scientist. Dr. Caplan has been a mentor from day one of medical school when he first introduced me to medical physiology. I am fortunate to have had the opportunity to learn from him both in the classroom and through this research. Dr. Edelson is an amazing leader and mentor. He has supported me both in research and at the start to my medical career. I thank him for his insightful advice and inspiring stories.
Additionally, I have to give thanks to Dr. Robert Tigelaar, who has not only guided me over the last five years, but who pointed me in all the right directions by suggesting that I complete a research year and look closely at the lab of Marcus Bosenberg for my project. Dr. Tigelaar has supported me professionally, scientifically and personally, and I am enormously grateful.

I would also like to thank the Howard Hughes Medical Institute (HHMI), which funded my year of research. They saw potential in me and gave me the financial support I needed to be successful. HHMI generously funded the many national and international conferences that I attended and supported local and regional HHMI events, and I am thrilled to be part of the HHMI family.

Finally, I would like to express my deepest gratitude to my parents. They have loved and supported me through all these years and are thrilled that I am finally done accruing large tuition bills. I could not be as successful as I am without them. Thank you for everything.
# Table of Contents

Abstract .................................................................................................................. ii

Acknowledgements ................................................................................................. v

Introduction .............................................................................................................. 10

Melanoma ............................................................................................................... 10
  Importance of Melanoma ......................................................................................... 10
  Therapeutic Advances ............................................................................................. 11
  Melanoma Genetics ................................................................................................. 12

E-cadherin .............................................................................................................. 13
  E-cadherin Function ............................................................................................... 13
  Importance of E-cadherin in Cancer Biology .......................................................... 14
  E-cadherin as Tumor Suppressor: Activation of the PI3K/Akt Pathway ............... 15

Epithelial-Mesenchymal Transition (EMT) .............................................................. 17
  History of EMT ....................................................................................................... 17
  EMT in Cancer Biology ............................................................................................ 18

A Mouse Model of Melanoma .................................................................................. 21

Statement of Purpose ............................................................................................... 25
  Hypothesis ............................................................................................................... 25
  Specific Aim 1 ......................................................................................................... 25
  Specific Aim 2 ......................................................................................................... 26
  Specific Aim 3 ......................................................................................................... 27

Methods .................................................................................................................... 29

Results ....................................................................................................................... 34
  Loss of E-cadherin in the Braf/Pten Tumor Model .................................................... 34
    Decreased Survival with Loss of E-cadherin.......................................................... 35
    No Difference in Gross Phenotype ........................................................................ 37
    Metastasis to the Lymph Nodes and Lungs ............................................................ 39
Assessment of mTOR Activity ................................................................. 43
Markers of EMT.................................................................................................. 45
A Novel Mouse Model: Braf Activation and Loss of E-cadherin ............ 47
Braf Activation with Loss of E-cadherin Leads to Melanoma Formation
......................................................................................................................... 47
Assessment of mTOR Activity and Markers of EMT ......................... 50
Microarray Analysis of EMT ........................................................................... 58
Discussion .................................................................................................................. 59
References .................................................................................................................. 64
Introduction

**MELANOMA**

*Importance of Melanoma*

Melanoma is a common cancer arising from melanocytes, the pigment-forming cells of the skin. Given its aggressive nature and increasing incidence, research to further understand melanoma formation and metastasis is important for improving human health. It is estimated that 76,250 cases of melanoma and 9,180 deaths due to melanoma will occur in 2012, making it the fifth and sixth most common cancer diagnosed in men and women, respectively (1). In fact, 1 in 36 men and 1 in 55 women will develop melanoma during the course of a lifetime (1). Unfortunately, as the incidence rate is increasing by >2% per year in the white population, melanoma continues to be of significant concern (2).

If melanoma is recognized and diagnosed early, treatment may involve simple local excision with greater than 90% survival at 10 years for patients with melanomas less than 1.00 mm thick (3). However, once melanoma has metastasized, it is challenging to treat and prognosis is poor. At best, one-year survival in this group is 62% (based on location of melanoma spread); at worst, one-year survival can be as low as 33% (3). Despite the increasing incidence of melanoma, the death rate due to melanoma has remained essentially stable over the last 20 years (4).
Recent advances in immunomodulatory therapies and targeted therapies has led to Food and Drug Administration (FDA) approval of two new treatments for melanoma in 2011. These novel therapies are the first to be approved for melanoma treatment in the last 13 years.

The first, ipilimumab, is a monoclonal antibody that blocks cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4). Blocking of CTLA-4 leads to enhancement of antitumor activity. While survival continues to be poor in patients with metastatic melanoma, treatment with ipilimumab can lead to prolonged survival in approximately 20% of patients (compared to <10% without ipilimumab) (5) (6).

The second new treatment is vemurafenib (also known as PLX4032), a small molecule inhibitor of mutant Braf. Braf, an intracellular protein important in pathways affecting cell division, is mutated in approximately 50% of human melanomas (7) (see next section: Melanoma Genetics). In patients treated with vemurafenib, there is a 6-month survival of 84%, compared to 64% at 6 months in patients treated with dacarbazine (a standard chemotherapeutic agent) (7). Though both of these therapies show exceptional promise and are making a clear impact on patient
lives, more work is needed to both better understand and treat this lethal disease.

**Melanoma Genetics**

Much work has been completed in recent years to assess commonly mutated genes in melanoma with the hopes of better understanding this deadly disease and developing improved therapeutics. As discussed above (see previous section: Therapeutic advances), Braf is mutated in about 50% of human melanomas (7) leading to constitutive activation of the mitogen activated protein kinase (MAPK) pathway. Activation of this pathway leads to increased cellular proliferation. Confirmation of mutant Braf as an activating oncogenic mutation has been confirmed in several studies, including in a mouse model developed by the Bosenberg lab (8).

Also commonly deregulated in melanoma are proteins in the phosphoinositide 3-kinase (PI3K) / Akt pathway, which also regulates cellular proliferation and survival. Within this pathway is the phosphatase and tensin homologue (Pten), which inhibits activation of the PI3K/Akt pathway. Inversely, loss of the tumor suppressor Pten leads to activation of the pathway. Unlike Braf, Pten is only mutated in approximately 5% of melanomas; however, expression is lost in up to 50% of melanomas (9) (10). Like Braf, this mutation has been shown to be functionally important
in melanoma formation and progression by the Bosenberg lab (8). Additionally, activation of the PI3K/Akt pathway leads to activation of mammalian target of rapamycin (mTOR) signaling, which consists of two protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTOR signaling is important for a myriad of processes related to cellular metabolism and is commonly dysregulated in cancer (11).

**E-CADHERIN**

**E-cadherin Function**

E-cadherin is a cell-cell adhesion protein that is part of a family of proteins called cadherins. The classic cadherins, which includes E-cadherin, are calcium-dependent homophilic molecules that can be found throughout the human body (12). E-cadherin was named due to its predominance in epithelial tissues in which it forms the adherens junction. It is expressed on keratinocytes as well as melanocytes and is functionally important in the interaction of these cells (13). Other cadherins include N-cadherin (neural cadherin), P-cadherin (placental cadherin) and R-cadherin (retinal cadherin), though many other classical cadherins have also been identified.

E-cadherin is a single pass transmembrane protein. The extracellular domain consists of five cadherin-type repeats that in conjunction with
calcium form a rod-like structure. The 3-D configuration of these extracellular domains within an adhesive cell-cell homophilic bond remains unclear, though several models have been proposed (12). The intracellular interactions and functions of E-cadherin have also been well studied. E-cadherin is attached to the actin cytoskeleton through an interaction with α-catenin and β-catenin. Additional proteins (p120, formin-1, vinculin and α-actinin) also interact with E-cadherin intracellularly and may play a role in actin-binding or other cell-signaling functions (12).

**Importance of E-cadherin in Cancer Biology**

Germline mutations in E-cadherin lead to hereditary diffuse gastric cancer (HDGC), an autosomal dominant cancer syndrome. In patients with E-cadherin mutations, >80% will develop advanced gastric cancer by 80 years of age; thus, prophylactic gastrectomy is typically offered at age 20 when the risk of cancer development is still quite low (14). While no dominant mutation has been identified in CDH1 (the gene for E-cadherin), over 100 mutations have been identified in unrelated families of varying countries of origin (15). Diffuse gastric cancer begins to develop in these patients when the second (normal) copy of E-cadherin is either inactivated, downregulated (often through promoter hypermethylation) or lost (15), suggesting a role for E-cadherin as a tumor suppressor (see next section: E-cadherin as Tumor Suppressor). Additionally, women with this
mutation have a significantly increased risk of developing lobular breast
cancer (60% by age 80) and screening for breast cancer typically begins
by age 35 (14).

Apart from HDGC, E-cadherin is also lost or downregulated in numerous
human cancers in individuals with normal E-cadherin in the rest of their
tissues. E-cadherin has shown to be downregulated in many cancers,
including head and neck squamous cell carcinomas, lobular breast
carcinoma, colorectal carcinoma, gastric carcinomas, prostate
carcinoma, lung carcinoma, thyroid carcinoma, hepatocellular
carcinoma, bladder carcinoma, and melanoma (16), (17). While for
many of these cancers, it is thought that loss of E-cadherin leads to a
poorer prognosis (compared to intact E-cadherin expression), this
relationship has sometimes been difficult to conclusively establish in many
of these cancers, including in melanoma (17).

**E-cadherin as Tumor Suppressor: Activation of the PI3K/Akt Pathway**

As discussed in the previous section, one of the strongest suggestions that
E-cadherin functions as a tumor suppressor is the discovery that germline
mutations lead to an autosomal dominant cancer syndrome. Indirectly,
loss of E-cadherin, either through mutations, loss of heterozygosity, or
hypermethylation, is noted in many human cancers, implicating a key role in cancer development (18).

Many recent studies have utilized cancer cell lines to demonstrate a relationship between loss of E-cadherin and activation of the PI3/Akt pathway (19) (20) (21). In Lau et al., the authors used ovarian cancer cell lines and were able to show that loss of E-cadherin leads to phosphorylation (and thus activation) of Akt as well as increased cellular growth. They showed some evidence that this activation was due to β-catenin inhibiting early growth response gene 1 (Egr1), which in turn downregulated Pten (19).

Not all studies have implicated β-catenin as the key mediator of Pten downregulation by E-cadherin. In Li et al., researchers used breast carcinoma cell lines to investigate the relationship between E-cadherin and Pten. They were able to show that both loss of E-cadherin and disruption of E-cadherin cell-cell adhesion leads to decreased levels of intracellular Pten (21). These findings suggest that activation of the PI3K/Akt pathway due to loss of E-cadherin is not due exclusively to β-catenin.
Finally, Fournier et al. were able to show that loss of E-cadherin activated the PI3K/Akt pathway in normal breast epithelial cell lines also via downregulation of Pten. By treating these cell lines with an E-cadherin blocking antibody, these researchers were able to show an approximately 30% decrease in intracellular Pten. This finding correlated with increased, disorganized cell growth (20).

EPITHELIAL-MESENCHYMAL TRANSITION (EMT)

History of EMT

In order to understand EMT, it is important to first understand the tissue types being discussed. An epithelium consists of cells with apical-basal polarity, strong cell-cell contacts, low motile capacity and which typically perform a secretory or barrier role. Mesenchymal cells, on the other hand, have front-back polarity or no polarity, are spindle-shaped, have few cell-cell contacts, are highly motile and may perform scaffolding or anchoring roles (22), (23). The concept of EMT was first described by RL Trelstad, JP Revel and ED Hay in 1966 while investigating chick embryogenesis to explain the formation of mesoderm (mesenchymal tissue) from ectoderm (an epithelium) in the embryo (24) (25). EMT occurs most clearly during gastrulation and neural crest cell formation. In gastrulation, some cells of the ectoderm begin to ingress and take on more mesenchymal features, eventually becoming the mesoderm (23). Further in embryogenesis, the
neural tube will form and begin to close. At the time of closing or just after, cells from the neural ectoderm undergo an EMT to form neural crest cells (26). Neural crest cells give rise to many cell types in the complete embryo, including neurons, Schwann cells and melanocytes (26).

Expression of many proteins is altered during the course of an EMT in embryogenesis. Studies have shown that E-cadherin, which is highly expressed in epithelial tissues, is lost and N-cadherin is upregulated (26). This phenomenon is frequently termed “cadherin switching.” Vimentin, a cytoskeletal protein, and fibronectin, an extracellular glycoprotein, are also upregulated (27), (28). Snail, Slug, Twist and Zeb1, transcription factors that repress transcription of the gene for E-cadherin, are upregulated (26), (27). Additional markers of EMT have also been described (28).

**EMT in Cancer Biology**

The acquisition of mesenchymal traits by epithelial cancer cells during the process of tumor invasion and metastasis has been a subject of intense interest recently. This process has several features of epithelial mesenchymal transitions (EMT) described in developmental biology studies (29). Loss of E-cadherin expression and increased expression of members of the Twist family of zinc finger transcription factors are key
features of EMT. While several studies have demonstrated that expression of mesenchymal markers is associated with progression of many cancers, functional evaluation of the effects of altering the characteristic features of EMT on metastasis has not been well established.

The majority of studies establishing the existence of EMT in cancer biology have used in vitro assays (28). These assays have demonstrated changes in cell morphology, a loss of cell polarization, increased cell matrix interactions, increased motility, and resistance to anoikis (a form of programmed cell death), which all suggest a loss of epithelial features and a gain of mesenchymal cell characteristics. These assays suggest an EMT has occurred and are consistent with acquisition of increased invasive capabilities.

Despite clear in vitro evidence, the in vivo evidence for EMT has been somewhat more controversial (30). On one hand, a xenograft study using human cell lines in immunocompromised mice showed that loss of E-cadherin in immortalized human breast epithelial cells resulted in increased mesenchymal features and a more invasive and metastatic phenotype when subcutaneously injected into mice (31). Other studies in mouse models have found that when metastases of cells expected to have undergone EMT were analyzed, they resembled epithelial cells more
than mesenchymal cells. This suggests that if an EMT had occurred it must have been followed by a mesenchymal to epithelial cell transition (MET) once the cells seeded the target organ (32). One hypothesis to explain this phenomenon is that in the absence of a microenvironment inducing EMT, the cells revert to their baseline epithelial state (33). Still other studies have suggested that cells in vivo may actually exhibit a more hybrid epithelial-mesenchymal phenotype compared to the more complete EMT seen in vitro (28). As stated above, the most convincing evidence for EMT has been established in an in vitro environment. This environment is highly artificial, lacking the complex cell-cell interactions in a three-dimensional space with no vascular, neurologic, or endocrine input. Additionally, if EMT were so critical to cancer metastasis, one would expect this to be readily apparent on histopathologic examination of human cancers sent for diagnosis. This, however, is not typically seen, and in fact, most invasive carcinomas are readily recognizable as of epithelial lineage (30).

Despite these controversies in the literature about EMT, many feel that E-cadherin loss is a central feature of EMT and is associated with cancer progression (34). E-cadherin loss has also been documented in melanoma and is frequently associated with increased N-cadherin expression (35). Downregulation of E-cadherin in human melanoma may occur by several mechanisms, including promoter hypermethylation (36),
or repression by Snail, Slug, Zeb-1, or Tbx3 transcription factors (28). While the correlation of E-cadherin downregulation in cancer has been established, it is not clear how much of the EMT phenotype is due to E-cadherin loss and what the effect of E-cadherin loss is on metastasis.

**A MOUSE MODEL OF MELANOMA**

In order to address important questions in melanoma formation and metastasis, a novel mouse model has been developed to investigate specific genetic hits within the melanocytes of mice. These novel mouse models are based on a transgenic mouse line that was previously generated and characterized in which expression of a Cre recombinase-estrogen receptor fusion protein is expressed under the melanocyte-specific tyrosinase promoter (Tyr::CreER<sup>T</sup>) (37). In brief, inducible recombination of target genes containing lox sites that flank a DNA sequence to be excised is efficiently induced by topical 4-hydroxytamoxifen (4-HT) application in a spatially and temporally restricted manner. Following the demonstration of functionality of the Tyr::CreER<sup>T</sup> lines, lox knock-in lines of genes relevant to human melanoma were acquired including Braf, Pten, and E-cadherin.

The Braf ser/thr kinase is the most commonly mutated gene known in human melanoma, with about 60% of the most common forms of
melanoma containing activating mutations of Braf (usually Braf V600E) (38). Martin McMahon's laboratory at UCSF has generated a knock-in allele of activated Braf (BrafCA) that is phenotypically wild type until Cre-mediated recombination produces an allele (Braf V600E) that contains the V600E point mutation and retains a single intronic 34bp loxP site, but otherwise resembles the wild type chromosome exactly (8).

The Pten tumor suppressor undergoes promoter hypermethylation and reduction of expression in roughly 30% of melanomas, but is only mutated in 5% of melanomas (10). We acquired a Pten allele from Ron DePinho and James You in which exon 5 is flanked by lox sites (8).

E-cadherin is commonly downregulated in human melanoma with variable levels of expression. While difficult to quantify, there is a trend towards poorer prognosis in those melanomas with less expression of E-cadherin (17). The Bosenberg lab has acquired an E-cadherin allele with lox sites that upon recombination leads to loss of exons 6 – 10 (39).

The Bosenberg lab has generated and analyzed 4-HT-treated cohorts of the genetic hits individually and in many combinations. All of the inductions of genetic recombination in these experiments were performed with a relatively high concentration of topical 4-HT (50 mg/ml in DMSO)
applied to the mouse on days 3, 5, and 7 of life and resulted in
generalized/systemic recombination of lox-containing alleles specifically
in melanocytes. Activation of Braf in melanocytes results in a mild
melanocytic hyperplasia/nevus formation (8), although tumor formation
has been noted using a similar mouse model (40). None of the other
individual genetic hits listed above have a clearly altered melanocytic
phenotype. In contrast, combinations of particular genetic hits have
produced some of the most interesting tumorigenic and metastatic
phenotypes described in any mouse model to date.

Activation of Braf combined with loss of Pten produces a dramatic
phenotype (Braf/Pten tumor model). Following induction of
recombination on days 3, 5, and 7 of life, widespread pigmented
melanocytic proliferations are evident within days; pups are uniformly
darkly pigmented within 12 days; nodular growths are evident within 15
days, and the mice need to be euthanized shortly after weaning (~30
days) secondary to decreased motility, failure to thrive, and the
observation of occasional death of littermates with similar behavioral
changes (8). All of the mice have multiple lymph node metastases, many
of which have shown evidence of growth within the lymph node with
partial to near-complete lymph node replacement. In addition, several
lung metastases are also typically noted within the two to three week
span of this experiment (8). The Bosenberg lab has also optimized local induction protocols in Tyr::CreER\textsuperscript{T} Braf/Pten mice that result in melanoma formation at the site of application that can be resected to allow for longer follow-up of metastatic lesions (8).
Statement of Purpose

Hypothesis
Loss of E-cadherin plays an important role in melanoma formation and metastasis.

**Specific Aim 1: Determine the role of E-cadherin in melanoma formation.**
We will utilize the Tyr::CreERT mice that we generated and E-cadherinlox mice (39) to conditionally inactivate E-cadherin following 4-HT-induced Cre-mediated recombination. We will inactivate E-cadherin specifically in melanocytes in both newborn and adult mice and evaluate effects on melanocyte proliferation and survival at several time points (0, 3, 7, 21, 60, and 360 days following recombination). 4-HT-treated Tyr::CreERT mice will be used as controls for these experiments. We will determine if loss of E-cadherin in melanocytes is sufficient to induce melanoma by following a cohort of 10 mice for 1 year that have undergone E-cadherin recombination as newborns. These mice will be monitored daily for tumor growth. Based on experience with other mouse models of melanoma, it is likely that additional genetic hits will be required to determine if E-cadherin loss can contribute to melanoma formation. We will cross mice to generate the following treated cohorts: E-cadherin loss with Braf activation and E-cadherin loss with Pten loss.
Specific Aim 2: Determine the effect of E-cadherin loss on epithelial-mesenchymal transition (EMT).

We will evaluate the effect of inactivation of E-cadherin on cellular morphology, migration, and expression of markers of epithelial-mesenchymal transition in vivo. E-cadherin has been proposed to be a downstream effector in the process of EMT; however, a reciprocal relationship has been demonstrated between E-cadherin re-expression in melanoma cell lines and subsequent down regulation of N-cadherin (41). We will examine whether loss of E-cadherin is sufficient to induce additional markers of EMT. In particular, we will determine protein levels on the skin tissues generated in Specific Aim 1 by western blotting of E-cadherin, N-cadherin, Vimentin, and Slug in lysates. In addition, we will perform unbiased gene expression analyses (Affymetrix GeneChip Mouse Gene 1.0 ST Arrays) in order to determine the gene set that is upregulated and downregulated by inactivation of E-cadherin and specifically examine markers associated with EMT.
Specific Aim 3: Determine the effect of E-cadherin loss on melanoma metastasis.

In order to determine the effect of E-cadherin on melanoma metastasis, we will induce E-cadherin loss in the setting of Braf activation, Pten loss, and Braf activation and Pten loss. These models have different endogenous propensities for tumor formation and metastasis that may be uniquely modified by E-cadherin loss. From previously published work by the Bosenberg lab, we know that Braf activation with Pten loss results in exuberant tumor formation without a clear latency and with bulky lymph node metastases within weeks of induction. Isolated tumor cells are present in the lung (8). We will carefully evaluate mice in these cohorts for extent and character of metastatic disease and any alterations in these phenotypes that E-cadherin loss causes. In addition to these "metastasis" cohorts, we will carefully evaluate any mice for metastases from Specific Aim 1 that form tumors following inactivation of E-cadherin and Braf activation or inactivation of E-cadherin and Pten.

We will evaluate the histology of primary and metastatic melanomas carefully to determine if there is an increase in the mesenchymal characteristics of tumorigenic cells, including a more spindled appearance, greater extent of invasion at the leading edge of the tumor, and increases in local lymphovascular invasion. We expect that E-
cadherin will alter the characteristics of both the primary melanomas and metastases; however, it is unclear if the extent of metastasis will be altered or if the sites will be altered.
Methods

Work was completed by the following investigators:

Lara E. Rosenbaum (LER), William Damsky (WD), Katrina Meeth (KM)

Mouse strains and activation of Tyr-CreER

Responsible investigators: Breeding, genotyping and general maintenance of the mouse colony was completed by WD and LER, with WD being primarily responsible for the Braf E-cadherin cohort and E-cadherin cohort and LER primarily responsible for the Braf Pten, Pten E-cadherin and Braf Pten E-cadherin cohorts presented here.

The Tyr-CreER, Braf, Pten and E-cadherin mouse strains were genotyped and assayed for recombination as previously described (37), (42), (43), and (39). All strains were on a mixed background of C57BL/6, FVB and 129 mice. For tumor inductions, 4-hydroxytamoxifen (4-HT) (#H6278, 70% Z-isomer from Sigma-Aldrich) was dissolved in dimethylsulfoxide (DMSO) to a concentration of 50 mg/ml. For perinatal inductions, this 4-HT solution was applied with a small paintbrush (enough to wet the skin) to the belly of the mouse on postnatal days 3, 5 and 7. For localized tumor inductions, a mouse was selected at postnatal day 21 and the hair between the shoulder blades was removed with a topical depilatory. Next, 1 µl of 4-HT
at 8.3 mg/ml (dissolved in 1 part DMSO and 5 parts Ethanol) was directly applied to the skin of the mouse in the hairless area. All experiments involving animals were reviewed and approved by the Yale Institutional Animal Care and Use Committee (IACUC).

**Immunohistochemistry and western blotting**

**Responsible investigators:** Immunohistochemistry was completed by KM. Western blotting was completed by LER and KM.

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tumor sections. Slides were processed using Vectastain peroxidase kit (Vector, #PK-4000) and developed with a 3, 3'-diaminobenzidine (DAB) substrate kit (Vector, #PK-4100), according to the manufacturer’s instructions.

Western blotting was performed using standard methods on uncultured, macrodissected tumor protein lysates.

The protein detection in the immunohistochemistry and western blotting was accomplished with the following purchased antibodies:

From Cell Signaling Technology: phospho-4E-BP1 (Thr37/46) (#2855), Actin
(#4970), Total Akt (#9272), phospho-Akt (Thr308) (#2965), phospho-Akt (Thr450) (#9267), phospho-Akt (Ser473) (#4060), E-cadherin (#3195), phospho-p70 S6 kinase (Thr389) (#9205), Total S6 ribosomal protein (#2217), phospho-S6 (Ser235/236) (#4858), and Slug (#9585).

From Abcam: phospho-SGK1 (Ser422) (#ab55281) and Vimentin (#ab92547).

From Millipore: N-cadherin (#04-1126).

**RNA purification and expression analysis**

*Responsible investigator:* RNA purification and expression analysis were completed by LER.

Total RNA was extracted from homogenous portions of macrodissected uncultured tumors using an RNeasy Mini kit (Qiagen) and eluted in the presence of Rnase inhibitor (Promega). RNA was quantified and prepared per the University of Vermont Cancer Center Microarray Facility standards. Quality was assessed by the facility with an Agilent 2100 Bioanalyzer. Samples were hybridized to GeneChip Mouse Gene 1.0 ST Arrays (Affymetrix).
Analysis of lymph node metastases

Responsible investigator: Data collection and analysis performed by LER.

Lymph nodes (cervical, axillary, brachial, and inguinal) were formalin-fixed, paraffin-embedded and sections were stained with hematoxylin and eosin (H&E). Images of each lymph node were digitally captured and manually analyzed using Adobe Photoshop Creative Suite 5.

Quantification of lung metastases

Responsible investigator: Method development, data collection and analysis were performed by LER.

Lung lobes were individually separated and rinsed in PBS at room temperature. Each lobe was mounted to a glass slide with Permount and coverslip and allowed to dry for 24 hours. Low and high power microscopic images were obtained with a Vectra microscope from Cambridge Research & Instrumentation (CRI) and digitally captured with inForm software. Each high power image was manually assessed for the presence or absence of melanoma metastases. This method was developed (by LER) to be able to assess metastases not visible under a dissecting microscopic and to allow for a more rigorous assessment of each lung lobe.
**Statistical analyses**

*Responsible investigator: Statistical analyses were completed by LER.*

Prism statistical analysis software was used for all analyses. Kaplan-Meier survival curves were constructed and significance was determined using the Log-rank (Mantel-Cox) test. For comparison of pooled data with two groups, unpaired t tests were used to determine significance.
Results

LOSS OF E-CADHERIN IN THE BRAF/PTEN TUMOR MODEL

In order to assess the role of E-cadherin in melanoma progression and metastasis, we first examined its loss in the context of the well-characterized Braf/Pten tumor model. This model, previously published in Nature Genetics in 2009, utilizes the Tyrosinase-CreER\(^T\) system and engineered loxP sites in the genes for Braf and Pten (8). When 4-hydroxytamoxifen (4-HT) is applied to the mouse, these genes are altered specifically in melanocytes, resulting in activation of Braf and loss of Pten. These genetic changes result in melanoma in 100% of mice that is lethal in approximately 30-40 days with metastases to the lymph nodes and lungs.

Two different modes of tumor induction have been developed. The first, henceforth referred to as perinatal induction, involves applying 4-HT topically on days 3, 5, and 7 of life leading to systemic absorption and genetic recombination in effectively all melanocytes on the mouse. The second method of tumor induction, called local induction, requires a small volume of 4-HT applied once to a small area of skin on the mouse, leading to genetic recombination in just that local area. This method of local induction allows for a single tumor nodule to grow. Per the approved animal protocol utilized here, mice were euthanized when the tumor reached 1 cm\(^3\) in size.
**Decreased Survival with Loss of E-cadherin**

These two methods were utilized to assess tumor growth and development in the context of loss of E-caderin. The comparison groups focused on in this section are the Braf/Pten inducible mice versus Braf/Pten mice with inducible, melanocyte-specific loss of E-cadherin (Braf/Pten/Ecad model) (Figure 1). As shown here, comparisons with both the perinatal induction (Figure 1A) and the local induction (Figure 1B) show a quicker progression to lethality in the Braf/Pten/Ecad group compared to the Braf/Pten group (p=0.024 [perinatal induction] and p=0.043 [local induction]).
Figure 1. Kaplan-Meier survival curves for Braf/Pten/Ecad cohorts vs. Braf/Pten cohorts. (A) Perinatal induction protocol and (B) Local induction protocol.
**No Difference in Gross Phenotype**

By inspection, there are no gross differences in the melanomas formed in the Braf/Pten/Ecad tumor model compared to the Braf/Pten tumor model (Figure 2). In both cases, the mice become covered in darkly pigmented confluent melanoma (perinatal induction) (Figure 2A), including over their peri-oral skin (Figure 2B) and footpads (Figure 2C).
<table>
<thead>
<tr>
<th>A</th>
<th>Braf / Pten / Ecad</th>
<th>Braf / Pten</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>B</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
</tr>
<tr>
<td>C</td>
<td><img src="image6.png" alt="Image" /></td>
<td><img src="image7.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 2. Gross phenotype (perinatal induction) of representative mice.**
(A) Whole mouse, (B) Peri-oral skin, and (C) Footpads.
Metastasis to the Lymph Nodes and Lungs

The next important assessment was to determine the pattern of metastases in the Braf/Pten/Ecad cohort compared to the Braf/Pten cohort. In both cohorts, metastases to the lymph nodes were assessed and quantified from perinatally induced mice (Figure 3). Both cohorts showed clear metastases to the lymph nodes both macroscopically and histologically (Figure 3A and 3B). When the area of lymph node involved with melanoma was quantified from the hematoxylin and eosin (H&E) slides, there was no difference in the amount of melanoma in each lymph node (p=0.28) (Figure 3C).
Figure 3. Metastases to the Lymph Nodes. Metastases to the lymph nodes can be seen (A) macroscopically and (B) histologically, stained by hematoxylin and eosin (H&E). (C) Quantification of the area of lymph node involved with melanoma.
Additionally, metastases to the lungs were assessed in the two perinatally induced cohorts (Figure 4). The number of lung metastases was quantified by a newly developed pressed lung technique. This technique allowed for visualization of microscopic metastases that are not easily detected by other means (Figure 4A). These metastases were quantified per lobe of lung and there was no difference between the two cohorts (p=0.39) (Figure 4B).
Figure 4. Metastases to the Lungs. Entire lung lobes were digitally captured under a light microscope and assessed for the presence of metastases. (A) Representative lung metastasis. (B) Quantification of lung metastases per lobe in each cohort.
Assessment of mTOR Activity

While both cohorts were expected to show significant mTOR activity due to the engineered loss of Pten, it was important to assess for any changes in activity of either mTORC1 or mTORC2 with concomitant loss of E-cadherin. This goal was accomplished through western blotting of tumor protein lysates from representative samples of the two cohorts (Figure 5). Several downstream targets of mTOR were assessed. For mTORC1 activity, levels of phospho-4E-BP1 (Thr 37/46) and phospho-S6 ribosomal protein (Ser235/236) were assessed along with total levels of S6 ribosomal protein for comparison (Figure 5A). These threonine and serine residues on 4E-BP1 and S6, respectively, are known targets of mTORC1 and thus serve as a proxy for the level of mTORC1 activity in these tumors. For mTORC2 activity, levels of phospho-SGK1 (Ser422) and phosphor-Akt (Ser473) were assessed along with total Akt levels (Figure 5B). These serine residues are known targets for mTORC2 and thus correlate with mTORC2 activity. From this data there are no clear differences in either mTORC1 or mTORC2 activity in the Braf/Pten/Ecad tumors compared to the Braf/Pten tumors.
**Figure 5. Assessment of mTOR activity.** Western blotting was performed on Braf/Pten/Ecad and Braf/Pten tumor lysates as well as control skin lysate (Skin) and melanocyte lysate (Mel). (A) mTORC1 activity was assessed by probing for phospo-4E-BP1 (Thr37/46) and phospho-S6 ribosomal protein (Ser235/236), with total S6 ribosomal protein and actin controls. (B) mTORC2 activity was assessed by probing for phospo-SGK1 (Ser422) and phospho-Akt (Ser473), with total Akt and actin controls.
Markers of EMT

Finally, loss of E-cadherin is predicted to allow for EMT to occur. To assess for markers of EMT, RNA extracted from each of the tumor cohorts was probed and quantified using a mouse gene microarray. This data was mined for markers of EMT, and RNA expression levels of these markers for the Braf/Pten/Ecad were compared to expression levels in the Braf/Pten cohort (Table 1). These markers fell into two broad categories: transcription factors and structural proteins. While several of the transcription factors and structural proteins that are predicted to be upregulated following an EMT were indeed upregulated (Slug, Twist2, Zeb1, N-cadherin and Fibronectin), others were equally expressed in the two tumor cohorts (Snail, Twist1, Zeb2, Hif1α and Vimentin). Notably, one protein expected to decrease following an EMT (Claudin 1) was significantly upregulated in the Braf/Pten/Ecad cohort.
<table>
<thead>
<tr>
<th>Transcription Factors</th>
<th></th>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>Protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snai1</td>
<td>Snail</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>Snai2</td>
<td>Slug</td>
<td>5.6</td>
<td>9.6</td>
</tr>
<tr>
<td>Twist1</td>
<td>Twist1</td>
<td>1.3</td>
<td>1.8</td>
</tr>
<tr>
<td>Twist2</td>
<td>Twist2</td>
<td>3.1</td>
<td>2.3</td>
</tr>
<tr>
<td>Zeb1</td>
<td>Zeb1</td>
<td>10.8</td>
<td>8.8</td>
</tr>
<tr>
<td>Zeb2</td>
<td>Zeb2</td>
<td>1.6</td>
<td>1.7</td>
</tr>
<tr>
<td>Hif1α</td>
<td>Hif1α</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Structural Proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdh2</td>
<td>N-cadherin</td>
<td>4.9</td>
<td>8.6</td>
</tr>
<tr>
<td>Vim</td>
<td>Vimentin</td>
<td>1.2</td>
<td>1.3</td>
</tr>
<tr>
<td>Fn1</td>
<td>Fibronectin</td>
<td>4.4</td>
<td>5.4</td>
</tr>
<tr>
<td>Cldn1</td>
<td>Claudin 1</td>
<td>14.5</td>
<td>24.7</td>
</tr>
</tbody>
</table>

Table 1. Microarray RNA expression levels of markers of EMT.  
Fold-change in expression of RNA transcripts within Braf/Pten/Ecad tumors (Samples 1 and 2) relative to Braf/Pten tumors.
A NOVEL MOUSE MODEL: BRAF ACTIVATION AND LOSS OF E-CADHERIN

While the Braf/Pten/Ecad melanoma model allowed for analysis of metastasis and markers of EMT compared to the previously characterized Braf/Pten model (8), it was important to evaluate E-cadherin loss in a system with fewer genetic changes to assess for its role in melanoma formation. With that goal in mind, cohorts of mice were generated with the same inducible melanocyte-specific system using the perinatal induction protocol. The cohorts generated included loss of E-cadherin only (Ecad model), Braf activation and loss of E-cadherin (Braf/Ecad model), and loss of E-cadherin and loss of Pten (Pten/Ecad model). From the previously published study on the Braf/Pten model, we also have data for a cohort with Braf activation only (Braf model), a cohort with Pten loss only (Pten model) and of course the data from the Braf/Pten cohort (8). Together, these cohorts represent all possible permutations of these three genetic changes (except for the Braf/Pten/Ecad tumor model described in the previous section).

Braf Activation with Loss of E-cadherin Leads to Melanoma Formation

The Kaplan-Meier survival curves for all of the above-mentioned cohorts were generated and followed until death or at least 1.5 years (Figure 6). Examining these cohorts led to the discovery that Braf activation in combination with E-cadherin loss leads to melanoma formation in 100% of
the mice with tumor death occurring after an average of 302 days (range: 199 – 395 days) or approximately 10 months. This finding is in striking contrast to the Braf/Pten tumor model, which has an average lifespan of 40 days (Figure 1, perinatal induction). Mice with loss of E-cadherin only, loss of Pten only or Braf activation only show no tumor formation for 1.5 or more years. Additionally, the Pten/Ecad model shows no tumor formation for an average of 720 days (range: 641 – 884 days) or approximately 2 years. The Braf/Ecad tumor model when compared to the other tumor models individually (none of which develop tumors) is statistically significant with p<0.0001 for all comparisons. Additionally, the Braf/Ecad tumor model is statistically different from the Braf/Pten tumor model (p<0.0001).
Figure 6. Kaplan-Meier survival curves for Braf/Ecad cohorts vs. Braf, Pten, Ecad and Pten/Ecad cohorts. There is a statistically significant difference (p<0.0001) between the Braf/Ecad cohort and all other cohorts shown here.
Assessment of mTOR Activity and Markers of EMT

In order to assess the functional changes that occurred within the Braf/Ecad tumor model, western blotting was performed to investigate mTOR activity as well as markers of EMT (Figure 7). First, mTORC1 activity was assessed by looking at downstream targets, including phospho-4E-BP1 (Thr 37/46), phospho-S6 ribosomal protein (Ser235/236), and phospho-p70 S6 kinase (Thr389) (Figure 7A). From this data, there is evidence of mTORC1 activity in the Braf/Ecad tumors, though somewhat decreased compared to the Braf/Pten tumors. While there is less phosphorylation of S6 ribosomal protein in just one out of three Braf/Ecad tumors, there is decreased phosphorylation of both p70 S6 kinase and 4E-BP1 in all Braf/Ecad tumors compared to Braf/Pten tumors.

Next, mTORC2 activity was assessed by looking at levels of phospho-SGK1 (Ser422) and phospho-Akt (Ser473) (Figure 7B). Additional phosphorylation sites on Akt (Thr450 and Thr308) were also examined (Figure 7B). These three Akt phosphorylation sites (Thr308, Ser473, and Thr450) all correspond to activation of Akt. While one of the Braf/Ecad tumors has elevated levels of phospho-SGK1 (Ser422) suggesting the possibility of elevated mTORC2 activity, this is not consistent across the other two Braf/Ecad tumors nor is it consistent when looking at phospho-Akt (Ser473), another marker of mTORC2 activity. By examining the three phospho-Akt blots,
there is a trend toward decreased, though present, phosphorylation of Akt in the Braf/Ecad tumors compared to the Braf/Pten tumors. This result suggests that Akt is active in the Braf/Ecad tumors, but less so than in Braf/Pten tumors.

Finally, it is important to investigate whether or not an EMT has occurred within the Braf/Ecad tumors. To assess this, tumor lysates were probed for E-cadherin, N-cadherin, Slug, and Vimentin (Figure 7C). If an EMT has occurred, one would predict decreased or no E-cadherin, increased N-cadherin (termed “cadherin-switching” to explain the “switch” from E-cadherin to N-cadherin), increased Vimentin (an intermediate filament found in mesenchymal cells) and increased Slug (a transcription factor that downregulates expression of E-cadherin). In the Braf/Ecad tumor lysates the first important aspect to note is that two of the lysates contain E-cadherin. This finding is due to contamination of the tumor lysate from overlying skin, which strongly expresses E-cadherin (as can clearly be seen in the skin lysate control) and not due to expression of E-cadherin in the tumors (which have been genetically-engineered to lack E-cadherin). Looking at the other structural proteins (N-cadherin and Vimentin), both the Braf/Ecad and Braf/Pten tumors express similar levels of these proteins. Interestingly, differences are found when examining expression levels of Slug. There are wide variations in expression level when looking at the
three Braf/Ecad tumors, from none to slight to high, while the Braf/Pten tumors consistently show moderate expression of Slug. The variation in expression levels in the Braf/Ecad tumors is not entirely surprising, as these tumors have a long latency period with a wide range, suggesting there might be stochastic variation within these tumors. This observation is in contrast to the Braf/Pten tumor model, which has a short latency period for tumor development and thus, little variability in protein expression.
See next page for figure legend.
See previous page for figure.

**Figure 7. mTORC1/2 activity, Akt activity and markers of EMT.** Western blotting was performed on Braf/Ecad and Braf/Pten tumor lysates as well as control skin lysate (Skin) and melanocyte lysate (Mel). (A) mTORC1 activity was assessed by probing for phospo-4E-BP1 (Thr37/46), phospho-p70 S6 kinase (Thr389) and phospho-S6 ribosomal protein (Ser235/236), with total S6 ribosomal protein and actin controls. (B) mTORC2 activity was assessed by probing for phospho-SGK1 (Ser422) and phospho-Akt (Ser473). Akt activity was additionally assessed with phospho-Akt (Thr450) and phospho-Akt (Thr308). Total Akt and actin controls are shown. (C) Markers of EMT include E-cadherin, N-caderin, Slug, and Vimentin, with an actin control.
As was previously established by the Bosenberg lab (8), activation of Braf alone in melanocytes does not lead to melanoma formation but does lead to nevi formation in 100% of mice. To further understand the formation of tumors in the Braf/Ecad cohort, immunohistochemistry (IHC) was performed on Braf/Ecad tumors and compared to IHC performed on Braf/Pten tumors as well as these Braf-activated nevi (Figure 8). Braf/Ecad tumors were probed for E-cadherin (to definitively show the genetically engineered loss) as well as Vimentin (Figure 8A). A representative hematoxylin and eosin (H&E) slide of a Braf/Ecad tumor is also shown. It is worth noting that the Braf/Ecad tumor contains no E-cadherin, as expected, but that the overlying epidermis shows clear, strong E-cadherin staining. Vimentin is strongly expressed in the Braf/Ecad tumor.

To further establish mTORC1 activity in the Braf/Ecad tumors, IHC was performed with phospho-S6 ribosomal protein (Ser235/236) antibody (Figure 8B). This conclusively shows that mTORC1 is active in the Braf/Ecad tumors at a similar level to that of Braf/Pten tumors and significantly increased from Braf nevi. Additionally, the intensity of immunohistochemical staining for mTORC2 substrates, including phospho-Akt (Ser473) and phospho-SGK1 (Ser422), was moderate and essentially
equal in Braf/Ecad tumors and Braf/Pten tumors and was greater than that seen in Braf nevi (data not shown).
Figure 8. Markers of EMT and mTORC1 activity in Braf/Ecad tumors. Immunohistochemistry was performed on Braf/Ecad tumors, Braf/Pten tumors and nevi from mice with Braf activation alone. (A) Braf/Ecad tumors were stained with H&E and also probed with antibodies to E-cadherin and Vimentin. (B) Braf/Ecad tumors, Braf/Pten tumors and Braf nevi were probed for phospho-S6 ribosomal protein (Ser235/236) (a marker of mTORC1 activity).
**Microarray Analysis of EMT**

Finally, RNA microarray analysis was used to assess for markers of EMT within the Braf/Ecad tumors and compared to the Braf/Pten tumors (Table 2). As discussed previously, these markers fell into two broad categories: transcription factors and structural proteins. While several of the transcription factors and structural proteins that are predicted to be upregulated following an EMT were indeed up-regulated (Slug, Zeb1, N-cadherin and Fibronectin), others were equally expressed in the two tumor cohorts (Snail, Twist1, Twist2, Zeb2, Hif1α and Vimentin). Notably, one protein expected to decrease following an EMT (Claudin 1) was significantly upregulated in the Braf/Ecad cohort. These data are quite similar to that seen the Braf/Pten/Ecad cohort (Table 1).
Table 2. Microarray RNA expression levels of markers of EMT.
Fold-change in expression of RNA transcripts within Braf/Ecad tumors (Samples 1 and 2) relative to Braf/Pten tumors.

<table>
<thead>
<tr>
<th>Transcription Factors</th>
<th>Gene</th>
<th>Protein</th>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snai1</td>
<td>Snail</td>
<td>1.0</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Snai2</td>
<td>Slug</td>
<td>14.9</td>
<td>12.4</td>
<td></td>
</tr>
<tr>
<td>Twist1</td>
<td>Twist1</td>
<td>1.2</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>Twist2</td>
<td>Twist2</td>
<td>0.9</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>Zeb1</td>
<td>Zeb1</td>
<td>12.8</td>
<td>11.5</td>
<td></td>
</tr>
<tr>
<td>Zeb2</td>
<td>Zeb2</td>
<td>1.5</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Hif1α</td>
<td>Hif1α</td>
<td>0.4</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Structural Proteins</th>
<th>Gene</th>
<th>Protein</th>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdh2</td>
<td>N-cadherin</td>
<td>25.3</td>
<td>18.1</td>
<td></td>
</tr>
<tr>
<td>Vim</td>
<td>Vimentin</td>
<td>1.5</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>Fn1</td>
<td>Fibronectin</td>
<td>10.9</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td>Cldn1</td>
<td>Claudin 1</td>
<td>49.5</td>
<td>10.5</td>
<td></td>
</tr>
</tbody>
</table>
Discussion

This work investigates the role of E-cadherin loss in melanoma formation and metastasis and its role in EMT. Few investigators have assessed E-cadherin in such a controlled manner in an *in vivo* immunocompetent model. Two primary models were assessed here, that of Braf/Pten/Ecad and the newly discovered Braf/Ecad model, but other genetic contexts utilizing this melanocyte-specific inducible loss of E-cadherin could also be developed in future work.

Interestingly, in the Braf/Pten/Ecad tumor model one would have predicted an increase in metastases with possible concomitant increase in mortality. While the Braf/Pten/Ecad tumor model does show a decreased lifespan compared to the Braf/Pten tumor model, there are no clear phenotypic differences between the two models and there is no change in the metastatic potential. This finding suggests that if loss of E-cadherin leads to increased metastasis, it is contingent on genetic context. While it is not clear why there is a decreased lifespan in this cohort, changes in mTORC1/2 do not account for any differences between these two models. Future investigation into tumor growth rates and other mechanistic changes may shed light on these questions.
The discovery that Braf activation in combination with loss of E-cadherin was sufficient to produce melanoma in 100% of mice is somewhat surprising, but may have been predictable due to the suggested role of E-cadherin as a tumor suppressor and increasing evidence that loss of E-cadherin leads to activation of the PI3K/Akt pathway (19) (20) (21). This relationship, however, is challenging to so clearly illustrate in epidemiologic or in vitro studies but is readily apparent in the melanoma tumor model developed here. Mechanistic studies investigating the Braf/Ecad tumors reveal activation of Akt, as well as both mTORC1 and mTORC2, supporting the idea that loss of E-cadherin leads to activation of the PI3K/Akt pathway in an in vivo immunocompetent cancer model. As shown previously with the Braf/Pten tumor model (8), Braf activation with activation of the PI3K/Akt pathway (in this model due to loss of Pten) is synergistic and sufficient for melanoma formation in 100% of mice. The same mechanism appears to be important in the development of tumors in the Braf/Ecad model, though stochastic changes are likely to also play a significant role given the long latency period of tumor development.

Finally, it is important to address the question of whether or not E-cadherin induces an EMT. There were some markers of EMT that were upregulated in both the Braf/Pten/Ecad cohort and the Braf/Ecad cohort relative to Braf/Pten tumors. However, RNA levels of other markers were unchanged.
Importantly though, loss of E-cadherin did not lead to wildly enhanced metastasis (in either genetic context), suggesting that while some events characteristic of an EMT did occur (as shown by the microarray data), these events were not sufficient for increased metastasis. In reality, there is likely a hybrid state that occurs in vivo, in so far as both mesenchymal and epithelial markers are expressed in the highly abnormal dysregulated cancer cell.

Within the study of E-cadherin and tumor metastasis, some investigators believe that release of β-catenin is the key downstream mechanism leading to the molecular changes and metastatic potential of cells lacking E-cadherin. While β-catenin was not directly addressed in this study, it has been definitively addressed by Damsky et al. with a mouse model also developed in the Bosenberg lab (44). In this study, β-catenin was both stabilized and knocked-out in an inducible manner and specific to melanocytes in the context of Braf activation and loss of Pten. The model that included β-catenin stabilization, Braf activation and Pten loss (Braf/Pten/β-cat-STA) showed a remarkable increase in the number of metastases to the lungs as well as to the spleen and bowel. This phenotype is in stark contrast to that of the Braf/Pten/Ecad and Braf/Ecad models and supports the idea that the functional changes within the cells of these tumors from loss of E-cadherin are not simply due to changes of
expression of β-catenin. Additionally, the tumors formed in the Braf/Pten/β-cat-STA tumor model, despite being highly metastatic, actually showed an increased level of E-cadherin expression.

While the work completed here further enhances the understanding of the role of E-cadherin in melanoma formation and metastasis, more work will need to be done to further understand the complexities of tumor formation and spread. While loss of E-cadherin can play a substantial role in melanoma formation as shown in the Braf/Ecad tumor model, its role in EMT and metastasis is more complex and likely highly dependent on genetic context. The Braf/Pten/Ecad tumor model did not show an increase in metastases and the Braf/Pten/β-cat-STA model (44) shows a remarkable increase in metastases but also with increased E-cadherin expression. Additional genetic combinations will need to be investigated to further understand the role of E-cadherin in melanoma formation and metastasis. With better understanding, new treatments will be developed to help manage this devastating cancer.
References


