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The Role of Endothelin 3 in Melanoma Progression and Metastasis

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THE ROLE OF ENDOTHELIN 3 IN MELANOMA PROGRESSION AND METASTASIS

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Nikeisha Latoya Chin

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To: Dean Michael R. Heithaus
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This dissertation, written by Nikeisha Latoya Chin, and entitled The Role of Endothelin 3 in Melanoma Progression and Metastasis, 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.

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DEDICATION

For the family I was born into, which helped shape me into the person I am, and for your enduring love, my father, Hubert, my mother, Gwenneth and my brothers Steven and Michael. Also to the family I have started, which make me strive to become an even better person every day, my husband Nicholas and our beautiful daughter Victoria.

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ABSTRACT OF THE DISSERTATION

THE ROLE OF ENDOTHELIN 3 IN MELANOMA PROGRESSION AND METASTASIS

by

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Professor Lidia Kos, Major Professor

Endothelin receptor b (Ednrb) and its ligand Endothelin 3 (Edn3) have been implicated in melanoma. Several studies have shown an upregulation of *EDNRB* and *EDN3* at both the protein and mRNA levels, as melanoma becomes more aggressive. This study investigated the putative role played by *Edn3* over-expression in melanoma progression and angiogenesis *in vivo*. We crossed *Tg*(*Grm1*)*Epv* transgenic mice that aberrantly express *metabotropic glutamate receptor1* under the *Dopachrome tautomerase* promoter, leading to spontaneous melanocytic lesions in the ears and tails that do not metastasize, with transgenics that overexpress *Edn3* under the *Keratin 5* promoter (*K5-Edn3*) or overexpress *Ednrb* in melanocytes (*Tg(Ednrb)1Lk*). In both the *Tg(Grm1)Epv/*K5-*Edn3 and Tg(Grm1)Epv/Tg(Ednrb)1Lk* mice, tumors appeared earlier and grew significantly larger and faster when compared to *Tg(Grm1)Epv* mice. Approximately eighty-one percent of *Tg(Grm1)Epv/ K5-Edn3* mice and 76% of *Tg(Grm1)Epv/Tg(Ednrb)1Lk* mice had pigmented lesions in distant organs such as the lung and brain. Real-Time PCR analysis showed higher expression levels

of genes involved in cell-cell and cell-matrix interactions and angiogenesis in lesions of *Tg(Grm1)Epv/*K5-*Edn3* when compared to controls. Considering the rapid tumor growth rate of in the *Tg(Grm1)Epv/*K5-*Edn3* mice, differences in the angiogenic response compared to control mice were investigated. Immunofluorescence analysis with the endothelial cell marker CD31 showed that there were more endothelial cells per tumor area in the *Tg(Grm1)Epv/*K5-*Edn3* mice than the controls. Proteome analysis showed that the *Dct-Grm1/K5-Edn3* mice had significant increases in other angiogenic related genes such as Angiogenin, CXCL 16 and Endoglin, when compared to controls, while real time PCR analysis of tail tumors also showed higher expression levels of angiogenic related genes such as *Hif-1α*. The results of this study showed that the EDNRB/EDN3 axis is sufficient to alter the kinetics of melanocytic tumors' progression, lead them to a fully malignant state, and increase the tumor angiogenic response.

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LIST OF ABREVIATIONS AND ACRONYMS

µM ..... Micrometer

64PP ..... 6-4 photoproduct

A ..... Adenine

ACTH ..... Adrenocorticotropic hormone

ARAF .....A-raf proto-oncogene serine/threonine protein kinase

ARF ..... Alternative reading frame

Arg ..... Argininine

BCA .....Bicinchoninic acid assay

BCL-2A1 .....B-cell lymphoma 2 related protein A1

bFGF ..... Basic Fibroblast Growth Factor

BGH .....bovine growth hormone?

b-HLH-Zip .....Basic helix-loop-helix leucine zipper

BMP .....Bone morphogenetic proteins

Bp ..... Base pair

BRAF .....B-raf proto-oncogene serine/threonine protein kinase

BSN ..... Bassoon Presynaptic Cytomatrix Protein

CA ..... California

Ca^{2+} .....Calcium Ionized

CAF ..... Cancer-associated-fibroblasts

CAM .....Cell-cell adhesion molecule

cAMP .....Cyclic adenosine monophosphate

CCN ... cysteine rich protein 61, connective tissue growth factor, Nephroblastoma Overexpressed

CD31 .....Cluster of differentiation 31

Cdc42 .....Cell division control protein 42 homolog

CDK4 or Cdk4 ..... Cyclin-dependent kinase 4

CDKN2A ..... Cyclin-dependent kinase inhibitor 2A

cDNA ..... complimentary DNA

COX-1 .....Cyclooxygenase 1

COX-2 .....Cyclooxygenase 2

CPD ..... Cyclobutane pyrimidine dimers

c-RAF1 ..... proto-oncogene serine/threonine-protein kinase

CREB .....Cyclic adenosine monophosphate response element-binding protein

CXCL1 .....Chemokine (C-X-C motif) ligand 1

CXCL16 .....Chemokine (C-X-C motif) ligand 16

CXCL2 .....Chemokine (C-X-C motif) ligand 2

DAG .....Diacyl-glycerol

DCT .....Dopachrome tautomerase

DiI ..... 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate

DP ..... Dimerization partners

E2F ..... E2F transcription factor

EC .....Endothelial cell

ECM .....Extracellular matrix

EDN1..... Endothelin 1

EDN2 ..... Endothelin 2

EDN3 or Edn3 ..... Endothelin 3

EDNRA ..... Endothelin receptor A

EDNRB ..... Endothelin receptor B

EMT ..... Epithelial to mesenchymal transition

Eph ..... Ephrin

ERK1 ..... Extracellular signal-regulated protein kinases 1

ERK2 ..... Extracellular signal-regulated protein kinases 2

FAMMM ..... Familial atypical multiple mole melanoma syndrome

FGF ..... Fibroblast growth factor

Flk ..... Mouse counterpart to kinase insert domain receptor

Flt ..... Fms-like tyrosine kinase-1

FVB ..... Friend leukemia virus B

G protein ..... G-protein coupled receptors

G protein ..... Guanine nucleotide protein

GAPDH ..... Glyceraldehyde 3-phosphate dehydrogenase

Glut 1 ..... Glucose transporter 1

GM-CSF ..... Granulocyte macrophage colony-stimulating factor

GPCR ..... Guanine nucleotide protein coupled receptor

GRM1 ..... Glutamate receptor 1

GTPases ..... Guanosine triphosphatases

HB-EGF ..... Heparin-binding Epidermal growth factor

HGF ..... Hepatocyte growth factor

HIF-1α .....Hypoxia-inducible factor- 1α

His .....Histidine

HRAS ..... Harvey rat sarcoma viral oncogene homolog

HRE ..... Hormone response element

HRP .....Horseradish peroxidase

IACUC .....Institutional Animal Care and Use Committee

IFN-γ ..... Interferon gamma

IGF-1 .....Insulin-like growth factor-1

IGFBP-2 .....Insulin-like growth factor-binding protein 2

IGFBP-3 .....Insulin-like growth factor-binding protein 3

IGF-β .....Insulin like growth factor-β

IHC .....Immunohistochemistry

IL-1 .....Interleukin 1

IL-10 .....Interleukin 10

IL-1β .....Interleukin 1β

IL-8 .....Interleukin 8

INHBA ..... Inhibin, beta A

INK4A .....Inhibitor of Cyclin-Dependent Kinase 4A

K5 .....Keratin 5

KDR .....Kinase insert domain receptor

KIT .....v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog

KRAS ..... Kirsten rat sarcoma viral oncogene homolog

Lle ..... Isoleucine

LOH .....Loss of heterozygosity

MA ..... Massachusetts

MAA .....Melanoma associated antigens

MAPK .....Mitogen-activated protein kinase

MC1R .....Melanocortin 1 receptor

MCP-1 .....Monocyte chemoattractant protein-1

MDM2 or Mdm2 ..... Mouse double minute 2 homolog

MITF ..... Micropthalmia-associated transcription factor

ml ..... Milliliter

MM ..... Metastatic melanoma

mm .....Millimeter

mM .....Millimolar

MMP .....Matrix metalloproteinases

MO .....Missouri

MSC .....Myeloid suppressor cells

MXI1 ..... MAX Interactor 1

MYC .....V-Myc Avian Myelocytomatosis Viral Oncogene Homolog

NC .....Neural Crest

NCC .....Neural Crest Cells

NGF .....Nerve growth factor

NK ..... Natural killer

NOS ..... Nitric oxide synthases

NOV .....Nephroblastoma Overexpressed

NRAS ..... Neuroblastoma RAS viral (v-ras) oncogene homolog

NY .....New York

OCT ..... Optimal cutting temperature

PARP-3 ..... Poly (ADP-Ribose) Polymerase 3

PBS .....Phosphate buffer solution

PCR ..... Polymerase chain reaction

PDGF .....Platelet-derived growth factor

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

PGE_2 .....Prostaglandin E_2

PH ..... Pleckstrin-homology

PHD1-3 ..... Prolyl hydroxylase domain protein 1-3

PI3K ..... Phosphatidylinositol 3-kinase

PIGF ..... Phosphatidylinositol-glycan biosynthesis class F protein

PIP3 .....Phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5) P 3)

PKB ..... Protein kinase B

PKC .....Protein kinase C

PTEN ..... Phosphatase and tensin homolog

Rac .....Ras-Related C3 Botulinum Toxin Substrate 1

Raf .....Raf proto-oncogene serine/threonine protein kinase

RAS .....Rat sarcoma

RB ..... Retinoblastoma

RET ..... ‘Rearranged during transfection’ proto oncogene

RGP .....Radial growth phase

RHC ..... Red hair color phenotype

Rho ..... Rhodopsin

ROS ..... Reactive oxygen species

rRNA ..... Ribosomal ribonucleic acid

RTK ..... Receptor Tyrosine Kinase

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

Ser ..... Serine

SF ..... Scatter factor

SNAI1 ..... Snail family zinc finger 1

Snail1 ..... Snail family zinc finger 1

SNAP21 ..... Synaptosomal-Associated Protein 21

SNAP91 ..... Synaptosomal-Associated Protein 91

SOX10 ..... Sex determining region Y-box 10

T ..... Thymine

Tg ..... Transgene

TGF-β ..... Transforming growth factor beta

TKO ..... Triple knockout mutant mice

TME ..... Tumor microenvironment

TNF-α ..... Tumor necrosis factor α

Trp ..... Tryptophan

TRP-1 ..... Tyrosinase related protein 1

TSC ..... Tumor stem cell

tTA ..... Tetracycline-sensitive transcriptional activator

t-test .....Test Statistic as per Student's t distribution

Tyr .....Tyrosinase

uPA ..... Urokinase plasminogen activator

uPAR ..... Urokinase plasminogen activator receptor

UV ..... Ultraviolet

UVA ..... Ultraviolet radiation A

UVB ..... Ultraviolet radiation B

UVC ..... Ultraviolet radiation C

Val ..... Valine

VE cadherin ..... Vascular endothelial cadherin

VEGF or Vegf ..... Vascular endothelial growth factor

VEGFB .....Vascular endothelial growth factor B

VEGFC .....Vascular endothelial growth factor C

VEGFD .....Vascular endothelial growth factor D

VEGFR-1 .....Vascular endothelial growth factor receptor type 1

VEGFR-2 .....Vascular endothelial growth factor receptor type 2

VEGFR-3 ..... Vascular endothelial growth factor 3

VGP .....Vertical growth phase

VM .....Vascular mimicry

vs .....versus

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

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

α ..... Alpha

α-MSH ..... Alpha-Melanocyte stimulating hormone

β .....Beta

CHAPTER I

INTRODUCTION

I. INTRODUCTION

1.1. The Skin

It is not unforeseen that skin cancer is the most prevalent of all human cancers, affecting approximately 1 million people in the United States each year, taking into consideration that the skin is the largest organ of the body. The skin forms the primary protective external barrier against foreign invaders and ultraviolet radiation (UV). There are three primary layers of the skin, the epidermis, the dermis with the basement membrane situated in between both, and the hypodermis or subcutis which is the deepest layer. Skin cancers develop primarily in the cells of the epidermis.

The epidermis is the outermost layer and thus the first level of protection. In the human skin this layer is ~0.1-0.2mm thick and consists of 5 separate strata. From outermost to innermost the sections are the stratum corneum, the stratum lucidum, the stratum granulosum, the stratum spinosum and the stratum basale. The four major types of cells found in the epidermal layer are keratinocytes, melanocytes, Merkel cells and Langerhans cells. Langerhans are antigen presenting immune cells found mainly in the stratum spinosum, while Merkel cells are receptor cells found in the stratum basal and are thought to be involved in somatosensory effects.

Keratinocytes are the most abundant type of cell in the epidermis. These cells begin as germinal cells in the stratum basal. Each of the other strata of the epidermis contain different maturation stages of the keratinocytes as they undergo cornification. Cornification is the process whereby these cells produce

the fibrous structural protein, keratin. As the cells accumulate keratin, the organelles and nuclei are lost, finally generating a protective layer of dead cells filled with keratin in the stratum corneum layer.

Melanocytes are the dendritic pigment producing cells of the body that are responsible for the actual skin color phenotype in human beings [1]. Embedded in the epidermal-dermal junction of the skin, melanocytes execute a complex process of synthesizing and storing the pigment melanin in lysosome related organelles called melanosomes [2]. Melanocytes produce two types of pigment, eumelanin which is brownish-black in color and pheomelanin which is reddish-yellow.

Melanosomes travel to the dendritic tips of the melanocytes where they are then released into the intercellular space, allowing endocytosis and phagocytosis of the melanosomes by the keratinocytes [2–5]. The melanin forms a cap over the nuclei of the keratinocytes, and protects the DNA from harmful UV by absorbing UVA (320-400nm) and UVB (260-320nm) rays [4,5]. One melanocyte distributes melanosomes to approximately 36 keratinocytes making up an 'epidermal melanin unit' [4]. This ratio of melanocytes to keratinocytes remains stable. Although keratinocytes have a high rate of growth and proliferation in the skin, melanocyte proliferation is strictly controlled by the keratinocytes. When melanocytes were co cultured with undifferentiated keratinocytes, the melanocytes were non-proliferative, however when the melanocytes were cultured with differentiated keratinocytes melanocytes proliferated at rates matching the keratinocytes and developed multiple dendrites [6]. During the few times melanocytes need to divide, the melanocyte

has to first detach from the basement membrane and keratinocytes and retract their dendrites before being able to proliferate. Afterwards, the melanocytes must adhere once again to the matrix and establish reconnection with the keratinocytes [4]. Cross-talk between melanocytes and keratinocytes is multifaceted involving a number of keratinocyte derived factors such as hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), prostaglandins and steel factor/KIT ligand [7,8]. This communication is very important in maintaining proper melanin production, and protection from UV damage. Dysregulation of any of these mechanisms can lead to various skin cancers, the most aggressive and terminal one being melanoma resulting from transformation of melanocytes.

1.2. Melanoma Skin Cancer

1.2.1. Statistics and Stages

Melanoma comprises less than 2% of all skin cancer cases, however it is responsible for most of the deaths caused by skin cancer. There have been reports that melanoma incidence has been considerably increasing over the past thirty years [9,10]. The American Cancer Society estimated that in 2015 approximately 74,000 individuals will be diagnosed with melanoma, about 43,000 of those diagnosed will be in men and 31,000 of those will be women. Although the risk of melanoma increases with age, it is one of the most common cancers seen in young adults [11]. There are several common elements that increase the lifetime risk of an individual being diagnosed with melanoma, the first, possessing acquired atypical moles/dysplastic nevi. Atypical moles are pigmented lesions that are usually benign, however if the moles change shape, color or size, they

may be potential sites of melanoma [12,13]. The second risk is known as ‘familial atypical multiple mole melanoma syndrome (FAMMM). This condition is inherited and is assessed when an individual has a number of dysplastic nevi and a least one family member that has had melanoma [14,15]. Hereditary melanoma accounts for about 10% of melanoma cases [15]. The third high risk element is ‘phototype’, an individual’s skin color and proneness to tan [16]. Caucasians are more than 20 times more likely to get melanoma than blacks [9,17]. Furthermore individuals with mutations in the melanocortin 1 receptor (MC1R) with very fair skin, red hair and light eyes are most at risk for acquiring melanoma[18]. The MC1R receptor is a seven transmembrane G-protein coupled receptor found on the plasma membrane of melanocytes. Stimulation of MC1R by its ligand α-melanocyte stimulating hormone (α-MSH) or other agonist adrenocorticotropic hormone (ACTH) by keratinocytes in response to UV, induces adenyl cyclase to produce cyclic AMP (cAMP) [19,20]. The increase in cAMP levels in turn phosphorylates and activates the cAMP responsive element binding protein (CREB) family of transcription factors. One important target of CREB is microphthalmia transcription factor (MITF), a basic helix-loop-helix leucine zipper (b-HLH-Zip) transcription regulator for genes encoding enzymes involved in melanin synthesis such as tyrosinase, Dopachrome tautomerase (DCT) and tyrosinase-related protein-1 (TRP-1) [20–24].

The *MC1R* gene is a major gene responsible for pigmentation phenotype [25]. There are a number of polymorphisms and allelic variants of the gene leading to differences in skin color and phototype in humans [26,27]. The *MC1R* variants

R151C, R160W and D294H are linked to the ‘red hair color phenotype’ (RHC) [28–30]. These single amino acid substitutions results in mutants unable to bind or activate adenyl cyclase which results in an increase in pheomelanin synthesis, which is more cytotoxic and less protective from irradiation than eumelanin [27–30].

The first stage of cutaneous melanoma is the common acquired and congenital nevi. This stage does not normally show any dysplastic changes [31,32]. The second stage is the formation of dysplastic nevi [31–33]. Dysplastic nevi have progressed to acquiring structural and architectural atypia. The radial growth phase (RGP) is the first malignant stage. At this stage the abnormal melanocytes start to spread laterally, but still remain close to the epidermis [31–34]. Although the tumor cells are locally invasive, they are not yet undergoing rapid growth. The RGP cells are still controlled by the exogenous growth factors of the keratinocytes. In the vertical growth phase (VGP) the melanoma cells are highly tumorigenic and clinically dangerous [31–34]. The tumor cells have gained metastatic potential and are now found in the basement membrane. The cells continue to invade both the upper and lower layer of the epidermis and dermis. The tumor cells in the area mostly form clusters of cells, with few cells breaking off and forming isolated regions. This stage of melanoma usually has poor clinical outcome, as the VGP melanomas increase their vascularization enabling them for the fifth stage [31–34]. At the metastasis stage the VGP cells are now completely free from keratinocyte signals, and have formed new interactions with other cells such as fibroblasts. The VGP’s now proliferate rapidly, overcoming anchorage constraints. The VGP’s are able to disperse to other organs in the

body [31–34]. It is important to note that these steps in melanomagenesis do not always occur sequentially. Malignancy may occur from melanocytes that do not go through the nevus stage [32]. Melanomagenesis is triggered by a combination of key genetic factors, UV- exposure and the tumor microenvironment.

1.2.2. Genetic Factors of Melanoma

The cyclin dependent kinase inhibitor 2A (*CDKN2A*) gene located on the 9p21 locus has been generally accepted as a familial melanoma susceptibility gene [33,35–37]. Several linkage and meta- analysis studies have identified loss of heterozygosity and germline mutations of *CDKN2A* in families with two or more individuals that were diagnosed with melanoma [36,38,39]. Approximately 25-40% of melanoma prone families exhibit *INK4A* mutations, however ~0.2-2% of individuals with sporadic melanoma have also been found to possess mutations in the gene [40]. The *CDKN2A* gene encodes two tumor suppressor proteins, Inhibitor of cyclin dependent kinase 4 (INK4A/$p16^{INK4A}$) and the Alternative reading frame product (ARF/$p14^{ARF}$) ($p19^{ARF}$ in mice) using two different promoters, 1α and 1β for INK4A and ARF, respectively, as well as different first exons. The two alternative transcripts share exons 2 and 3, however the open reading frames in exon 2 are distinct for INK4A and ARF resulting in the translation of two proteins with no common amino acid homology [41–43].

The protein INK4A inhibits cyclin dependent kinase 4, a regulator of cell cycle progression utilizing retinoblastoma protein (RB). RB is a tumor suppressor protein that binds to and obstructs transcription factors of the E2F family.

The E2F proteins along with their dimerization partners (DP) drive the cell cycle into the S phase. When RB binds to E2F forming a RB-E2F/DP complex, it inhibits excessive cell growth by resting the cell cycle in the G1 phase. When cell division needs to proceed, RB is hyper-phosphorylated by CDK4 which renders RB unable to bind to E2F and thus allowing the cell cycle to progress form G1 to the S phase. The *INK4A* gene is therefore a negative regulator of cell cycle progression as it blocks CDK4 from phosphorylating RB to induce cell division.

The importance of the INK4A/CDK4/RB- pathway is further evidenced by the finding of germline mutations for CDK4 in familial melanoma [44–47]. Alterations of CDK4 are attributed to Arg24Cys or Arg24His mutations that prevent inactivation of CDK4 by INK4A [48]. The CDK4 mutations are not paired with somatic INK4a activation, but both mutations can give rise to melanomas with similar incidence and progression mutually exclusive of each other [49]. Sotillo et al. (2001) reported that mice with a targeted 'knock in' mutation of *Cdk4* developed melanomas after exposure to carcinogens [50].

A number of familial melanoma cases [51], metastatic cell lines[52], as well as germline and sporadic deletions or mutations of 1β in the ARF coding sequence have been presented. These mutations of $p14^{ARF}$ are exclusive from *INK4A* mutations, suggesting an independent role for $p14^{ARF}$ in melanomagenesis. Although in mice *Ink4a* deficiency results in the development of tumors [53], when $p14^{ARF}$ haploinsufficiency was combined, the tumor phenotype was heightened [53,54].

In response to an increase in aberrant mitogenic and oncogenic signals $p14^{ARF}$ acts as an important tumor suppressor [55]. Overexpression of mitogens MYC and RAS stimulate the increase in *$p14^{ARF}$* transcription and its accumulation in the nucleus where $p14^{ARF}$ binds to and inhibits MDM2, a ubiquitin ligase of p53 [56,57]. Binding of $p14^{ARF}$ to MDM2 allows p53 to trigger cell growth arrest and apoptosis [57,58]. Loss of $p14^{ARF}$ function results in an increase in MDM2 and a concomitant loss of p53 mediated cell cycle control, resulting in cancer progression. The $p14^{ARF}$ protein can also inhibit proliferation independently of p53 by binding to nucleophosmin/B23, a ribosomal chaperone protein that prevents rRNA processing [59]. Triple 'knockout' mutant mice (TKO) nullizygous for *rf p53* and *Mdm2* acquired tumors significantly more than mice lacking only *p53* or *p53* and *Mdm2*. When ARF was restored in TKO embryo fibroblasts, Weber and colleagues found that ARF could arrest the cell cycle in the absence of MDM2, thus confirming that ARF can act through other mechanisms for cell cycle control [60].

Although mutations within the coding region of *p53* (*Tp53*) are often associated with most cancers, often p53 is not modified directly in human and murine melanomas. The mutation and subsequent targeted loss of $p14^{ARF}$ in melanoma may be the means for the p53 pathway to be disrupted.

Recently a link between the *CDNK2A* locus and *MC1R* has been established. A study on Australian families with individuals diagnosed with melanoma with CDKN2A mutations by Box and colleagues (2001) discovered that if a single MC1R polymorphism was present, the raw penetrance of the

CDKN2A mutation would significantly increase as well as earlier onset [61]. van der Velden and colleagues (2001) also studied Dutch families with FAMM, and described that the number of MC1R mutations in the individuals with CDNK2A mutations increased melanoma penetrance [62]. The MC1R mutations can therefore act as modifiers of melanoma susceptibility in individuals with CDKN2A mutations.

Over activation of the receptor tyrosine kinase (RTK) pathway, especially that involving one of its most important mediators, the mitogen activated protein kinase (MAPK) pathway is identified in a number of cancers including melanoma [63]. Receptor tyrosine kinase ligand binding activates the RAS family of GTPases (NRAS, HRAS, and KRAS). The protein RAS then activates the Raf family of serine threonine specific protein kinases (BRAF, ARAF and c-RAF1) which regulates the MAPK pathway to phosphorylate and activate extracellular signal regulated kinases 1 and 2 (ERK1 and ERK2) to promote transcription of genes participating in cell growth, differentiation, migration and senescence [64,65]. The majority of the *BRAF* constitutively active proto oncogene mutations, have been identified as a point mutation (T1799A), a T-A transversion (converts valine to glutamic acid) that encodes $BRAF^{V600E}$ [66–71]. Davies and colleagues (2002) genome wide cancer screening discovered activating BRAF mutations in approximately 60% of somatic melanoma cases [68]. Poynter and colleagues (2006) found the highest incidence of BRAF mutations in nevi (82%), followed by invasive melanomas (29%) with the least being in in-situ melanoma (5.6%) [72]. Mutation analysis on melanoma and nevi samples by Pollock and colleagues

(2003) as well as sequence analysis by Kumar and colleagues (2004) also detected BRAF mutations occurring mostly in benign or dysplastic nevi, thus suggesting that BRAF may be important in early transformation of melanocytes but other mechanisms may be necessary for complete melanoma progression [73,74]. Supporting this view are findings from Patton and colleagues (2005) that created a transgenic $BRAF^{V600E}$ mutant expressing zebrafish model. The fish acquired patches of ectopic melanocytes which only became invasive melanomas after the activated $BRAF^{V600E}$ was paired with p53 deficiency [75].

The $BRAF^{V600E}$ mutations occurrence in cutaneous melanomas have been correlated with UV exposure. Studies by Maldonado and colleagues (2003) and Curtin and colleagues (2005) examined cutaneous melanomas from different groups with different degrees of exposure to UV for alterations in BRAF. Both groups found that BRAF mutations were more common in melanomas that arose from skin exposed to intermittent high intensity UV exposure with very few BRAF mutations associated with melanomas from limited sun exposure such as mucosal melanomas and ones on the palms or soles of feet [66,76]. Although this would suggest a causal link for BRAF mutations by UV, the relationship is not as simple, as both groups also found that BRAF mutations were also rare in melanomas arising from chronic exposure to UV [66,76]. It has been suggested that BRAF mutations may be a result of cytotoxic, oxidizing or inflammatory response associated with intense sunlight exposure as opposed to being directly induced by UV [77].

Unlike BRAF mutations in melanoma NRAS mutations have been shown to manifest as a result of UV exposure. van Elsas and colleagues (1996) detected *NRAS* mutations in 26% of paraffin embedded DNA from melanoma samples exposed to chronic sunlight, with no *NRAS* mutations found in sun protected melanomas. The *NRAS* mutations occurred more frequently in individuals from the high sun exposed area, Australia, than individuals from North or Central Europe [78]. The *NRAS* point mutations are rarely found in dysplastic nevi, but are found in most stages of melanoma including congenital nevi, primary melanomas and metastatic melanoma indicating a progressive type role for NRAS in melanomagenesis [78–81]. The *HRAS* and *KRAS* mutations are seldom seen in melanoma [69,72,78,81]. In transgenic mice *Hras* mutations on an *Ink4a* deficient background developed non metastatic melanomas, however *Nras* mutations on a *Ink4a* deficient background led to those transgenic mice developing spontaneous cutaneous melanomas with high penetrance [82,83]. More studies are needed to fully understand the importance of RAS and RAF signaling in melanoma.

Hyperactivity of the phosphatidylinositol 3-kinase (PI3K)-AKT pathway is a common event in melanomagensis. Dai and colleagues (2005) examining phospho-Akt (p-Akt; Ser-473) in melanocytic lesions at different stages of progression found phospho-Akt expression increased as melanocytic lesions became more aggressive. Patient survival was also inversely correlated to pAKt expression [84]. The (PI3K)-AKT pathway can be activated by RTK's, RAS proteins, integrins or G-protein coupled receptors. Activation leads to production

of phosphatidylinositol (3,4,5) trisphosphates (PIP3), which serve as docking sites for other proteins with pleckstrin-homology (PH) domains to phosphorylate the chief effector of the PI3K pathway, protein kinase B (PKB) also known as AKT [85–87]. AKT phosphorylates targets that can initiate or inhibit cell growth, proliferation, survival and migration [85,88,89,89]. Loss of heterozygosity (LOH) or mutations of the tumor suppressor phosphate and tensin homolog (PTEN) is perhaps the most studied regulator of PIP3 in melanoma [84,89–92]. The protein PTEN acts as a negative regulator of the PIP3 pathway, thus it is often found mutated in a number of cancers [86,88,90,91]. Expression analysis studies show LOH of the PTEN and MYC antagonist MXI1 locus 10q24 develops in about 50% of sporadic melanomas [88,92–95]. Tsao and colleagues (2004) using mutated cell lines demonstrated BRAF activated mutations in conjunction with PTEN loss contribute to melanomagenesis [96]. Dankort and colleagues (2009) corroborated this phenomenon as their genetic altered mice expressing $Braf^{V600E}$ only developed few metastatic melanoma lesions. However when the $Braf^{V600E}$ mutation was combined with PTEN tumor suppressor gene silencing, the melanomas became 100% penetrant with short latency and formed distant metastatic lesions [67]. The aggressive nature of melanomas is in part due to the major mutations in these important tumor suppressor regulatory pathways. The intricacy of these pathways makes finding very effective therapeutic regimes targeting one of these players whether BRAF, PTEN, and others difficult.

1.2.3. Environmental Factors of Melanoma

Ultraviolet radiation is the most well-known, although not fully characterized, epidemiological factor for melanoma. Sunlight is divided into three Ultraviolet light spectrum regions, UVA (320-400nm), UVB (290-320nm) and UVC (200-290nm). Ultraviolet light in the C spectrum (UVC) is absorbed completely by the ozone with no effect on terrestrial life, however both Ultraviolet light in the A spectrum (UVA) and Ultraviolet light in the B (UVB) spectrum reach Earth. Ultraviolet B rays are short wavelengths of light and are deemed to be more carcinogenic as they are able to damage DNA in two different ways; either by causing cyclobutane pyrimidine dimers (CPD's) between adjacent thymine or cytosine residues, or forming 6-pyrimidine-4-pyrimidone photoproducts (64PP). These abnormalities create specific DNA mutations (C to T and CC to TT) [97–100]. Ultraviolet light in the A spectrum are longer wavelengths and although they penetrate deeper into the skin than UVB rays, they mutate DNA indirectly, by creating reactive oxygen species (ROS)[101,102]. The impact of UV mediated effects working with genetic factors to induce melanoma was demonstrated by Kannan and colleagues (2003) with murine melanoma mouse models with HRAS activation and $p19^{ARF}$ loss. UV exposed *Tyr*-RAS $p19^{ARF-/-}$ mice developed more melanocytic lesions with significantly shorter latency than *Tyr*-RAS $p19^{ARF-/-}$ mice not exposed to UV. Approximately, 50% of the tumors in the *Tyr*-RAS $p19^{ARF-/-}$ UV exposed mice also had amplification of cyclin dependent 6 (*cdk6*) implicating a UV induced mutagenic effect involving the Rb pathway [103]. Benaduce and colleagues (2014) using mice that overexpress EDN3 under the Keratin 5

promoter (K5-*tTA*; TRE-*Edn3-lacZ*) and XPA deficiency (*Xpa*$^{-/-}$ or *Xpa*$^{+/-}$), showed that a single neonatal dose of UV radiation, was sufficient to induce melanomagenesis in mice constitutively over expressing *End3,* independent of XPA deficiency [104]. *Xpa*$^{-/-}$ mice over expressing EDN3 however, displayed significantly higher penetrance and shorter tumor free survival than *Xpa*$^{+/-}$ mice also over expressing EDN3 [104]. The K5-*tTA*; TRE-*Edn3-lacZ* mice have an increased number of melanocytes in the epidermal-dermal junction which closely resembles their placement in the human skin, unlike wild type mice whose melanocytes are confined to the hair follicles. Therefore, it is still unknown whether it is the presence of EDN3 in the microenvironement or the location of the melanocytes that permits melanomagenesis upon UV exposure [104].

Melanin's photo-protective mechanism involves the absorption of UV photons that can otherwise be damaging to DNA, and the absorption of free radical species generated as a result of UV exposure that can cause oxidative stress and a cytotoxic environment [97]. In addition to these mechanisms, UV photons can directly cleave diacylglycerol (DAG) that activates the PKC pathway to increase melanin synthesis.

Epidemiological data such as the pooled data analysis conducted by Chang and colleagues (2009) find strong positive correlation between intense intermittent UV exposure and the risk of developing melanoma [102,105–108]. Berking and colleagues (2001 and 2004) demonstrated this link using both 'in vivo' and 'in vitro' experiments that UVB radiation was necessary in addition to the expression of growth factors basic Fibroblast Growth Factor (bFGF), Kit ligand

(SCF/KITL) and endothelin to fully transform melanocytes to metastatic melanomas[109 110]. A meta-analysis by Whiteman and colleagues (2001) found that sun exposure in children posed a greater risk for developing melanoma than sun exposure in adulthood. Noonan and colleagues (2001) investigated the correlation between the age of UV exposure and melanoma risk by creating transgenic mice overexpressing Hepatocyte growth factor/scatter factor (HGF/SF) under the metallothionein promoter [111]. The HGF/SF ligand binds to the receptor tyrosinse kinase MET receptor located on epithelial cells and melanocytes [112–116]. The HGF/SF protein is normally secreted by mesenchymal cells in the skin where it promotes activation of mitogenic and morphogenic signals for epithelial cells [112,114–118]. In melanoma an autocrine signaling loop of HGF/SF/Met promoting melanocyte proliferation and migration has been identified in several studies [113,119]. Upregulation of HGF/SF [112] and gain of function mutations of Met had been linked to mutations in melanoma [118,120]. The HGF/SF transgenic mice possess melanocytes located in the epidermal-dermal junction which more closely resembles the human skin phenotype as murine melanocytes are normally confined to the hair follicles [111]. Noonan and colleagues (2001) found that a single neonatal dose of UV radiation, 30 fold lower than that given in adult mice was sufficient to induce malignant melanomas with short latency, thus providing evidence that avoiding UV exposure in childhood is more important in preventing melanoma [111].

Some studies negate the positive relationship between childhood UV exposure and melanoma [98]. Pfahlberg and colleagues (2001) contradicted these

findings as their study on several melanoma case studies in Europe compiled with the history of sunburns in childhood and adulthood related to the cases, did not find a similar gradient for melanoma risk between childhood sunburns and those in adulthood. They did however find that more than five sunburns in life doubled the melanoma risk despite them being childhood or adulthood [121,122]. Whiteman and his group (2001) reviewing both case type studies and ecological studies that differed in this stance, stated that the way in which sun exposure was measured led to the contradictory findings and that it appears that the ecological type analysis studies were better and provided better quality evidence to support the importance of the threat of childhood sunburns [123].

Another relationship between UV and melanoma that still needs to be examined further is the speculation as to whether UVB or UVA differ in the effect in causing DNA damage and inducing melanoma [124]. There is some evidence to suggest the importance of UVB as highly tumor initiating. Probing this phenomena Fabo and colleagues (2004) irradiated HGF/SF transgenic mice with either UVA, UVB or combined wavebands of radiation. The HGF/SF mice irradiated with UVB acquired cutaneous malignant melanomas more readily and efficiently than the HGF/SF transgenic mice exposed to just UVA [124]. Human newborn skin grafted onto 158Rag-1 transgenic mice also acquired melanocytic lesions when exposed to chronic UVB radiation regardless of application of the tumor initiating carcinogen DMBA [105].

The tanning response in humans is a complex combined effect stemming from an increase in melanin production directed by melanocytes and mediated by

keratinocytes, fibroblasts, neurons and mast cells in the immediate environment[97,125]. These cells in response to UV stress increase the production and release of various paracrine factors including bFGF, nerve growth factor (NGF), α-MSH, and endothelin1 (Edn1). Other factors such as inflammatory responses trigger receptors on the melanocyte to promote melanin synthesis, dendricity as well as survival and proliferation of the cells [97,99,126]. Thus the tumor microenvironment (TME) is instrumental in inducing changes to transformed melanocytes.

1.3. Melanoma and the Tumor Microenvironment

The importance of the TME in melanoma progression becomes very clear, considering that when melanoma cells are removed and placed into cultures, they become increasingly vulnerable to targets or treatments. This behavior differs remarkably from when these cells are in their TME in patients where they are highly resistant to therapeutic regimens. The TME refers to the extracellular matrix (ECM) and the cellular environment in which the tumor is situated. The tumor milieu consists of, but not limited to, blood vessels, endothelial cells, pericytes, immune cells, fibroblasts and the surrounding ECM. The tumor and host environment are constantly communicating via a number of paracrine signals to promote tumor escape from the host immune system, formation of new vasculature and tumor heterogeneity which all facilitate growth, proliferation survival and migration of tumorigenic cells. The tumor host environment is primarily initiated by the tumorigenic cell, which then causes subsequent mutations and modifications in nearby cell types to promote malignancy, a

process known as co-opting. Cancer cells must circumvent restrictions and environmental pressures in their environment, so that they can grow, survive, and metastasize [127,128]. Microenvironmental pressures include lack of oxygen or nutrients, low pH, reactive oxygen species, and the inflammatory immune response of the body, all of which contributes to metastasis initiation and an increase in malignancy as the tumorigenic cells that can overcome these challenges end up being selected for and contribute to the aggressive phenotype [127]. Melanomagenesis occurs not only because of an accumulation of genetic alterations, but also because of a complex coordinated network of events involving keratinocytes, fibroblasts, endothelial cells, pericytes and infiltrating immunocytes.

1.3.1. Altered Cell-Cell Communication

The first critical event in melanomagenesis that transforms and shapes the tumor microenvironment involves the severance of the symbiotic relationship between keratinocytes and melanocytes. The separation allows melanocytes to undergo an epithelial to mesenchymal transition (EMT). The EMT is the process by which epithelial cells undergo morphological and molecular changes that cause the cells to lose their polarity and cell-cell adhesion to gain migratory and invasive plasticity associated with mesenchyme [127,129,130]. The EMT normally occurs at different stages of embryonic development, but is induced again by cancer cells with the intention to surmount the constraints of the microenvironment. To facilitate EMT various signaling pathways such as PIP3,TGF-β/MAPK [131] and growth factors such as HGF/SF/ Met [132] are activated,

as well as changes in the expression of cell-cell adhesion molecules (CAMs) such as cadherins and integrins [128].

Keratinocyte governance over melanocytes is principally maintained by contact-mediated regulatory mechanisms [133]. Melanocytes adhere to, and communicate with keratinocytes via Epithelial cadherin (E-cadherin) and desmoglein, both of which are expressed on the cell membranes of keratinocytes and melanocytes [134,135] . Co-culture experiments substantiate the control keratinocytes have on melanocyte behavior and phenotype. In mono-culture melanocytes are able to profusely proliferate, can exhibit bi-polar or tri-polar morphology unlike their normal multi dendritic morphology and begin to express proteases, growth factor receptors and melanoma associated antigens (MAA) that are not expressed by melanocytes 'in vivo' [6,136,137]. The protein E-cadherin is expressed by melanocytes but not melanoma cells [134,138]. Danen and colleagues (1996) conducted flow cytometry experiments with normal and melanoma cell lines and immunohistochemistry on frozen sections of melanoma lesions, found E-cadherin expression only on normal melanocytes and naevus cells but none on invasive metastatic melanoma cell lines [139]. Thus transformed melanocytes have to liberate themselves from keratinocyte-melanocyte adhesions by downregulating E-cadherin during malignancy [134]. Studies by Hsu and colleagues (2000) showed that when E-cadherin expression was functionally restored to melanoma cells by gene transfer techniques, a normal melanocyte phenotype was re-established and invasion related antigens were downregulated [133,140,141]. Cytogenic and molecular analysis do not detect mutations, methylation or

deletions in E-cadherin during melanomagenesis suggesting that downregulation of E-cadherin may a result of the effects of growth factors [142,143]. Because HGF/SF/ MET signaling stimulates the proliferation and motility of melanocytes [144] and Met expression is found in melanoma [145], Li and colleagues (2001) examined the effect of HGF/ MET on melanocyte transformation by inducing autocrine activation of HGF using an HGF-expressing adenovirus. The autocrine HGF signaling resulted in downregulation of both E-cadherin and desmoglein 1, promoting proliferation and invasiveness of the tumorigenic cells [146]. The downregulation of E-cadherin therefore involves cross talk between melanoma cells and HGF/ MET expressing cells such as fibroblasts. In addition to HGF, studies also show that the Snail family of transcription factors that repress E-cadherin during embryonic EMT are also expressed and directly correlate to the loss of E-cadherin in melanoma [143,147]. Moreover, Schneider and colleagues reported that UV-radiation of keratinocytes could induce paracrine release of EDN1, which can reduce E-cadherin expression in melanocytes [148]. In melanomas, down regulation of E-cadherin is concurrent with an increase in the mesenchymal protein neural cadherin (N-cadherin) [146,149].

The N-cadherin protein is normally expressed on endothelial, neuronal, and muscle cells as well as fibroblasts and endothelial cells [150]. The switch in cadherin expression from E-cadherin to N-cadherin on melanoma cells alters the binding partners of the transformed melanocytes from keratinocytes to fibroblasts- and endothelial cells allowing the tumorigenic cells to now communicate with these other cell types to prompt changes in the

microenvironment and incorporate and co-opt them as parts of the tumor, leading to tumor heterogeneity [150]. Wide global gene analysis techniques have demonstrated that aggressive melanoma cell lines express a number of factors that are affiliated with multiple cellular phenotypes such as fibroblasts, endothelial cells, and immune cells [151–154]. More importantly the transformed melanocytes can now respond to mitogenic signals and proliferate without the constraints of the keratinocytes and the upregulation of N-cadherin in melanoma lesions confers a fibroblastic phenotype making the cells more motile and invasive than normal melanocytes [149].

Additionally, transformed melanocytes display an upregulation of several integrins such as $\alpha_v\beta_3$ along with the cadherin switch to change intercellular signaling in the tumor microenvironment, as cadherins and integrins can regulate RTK signaling [155–157], β-catenin/Wnt signaling [158] and activators of several downstream targets such as small GTPases, Rho, Rac and Cdc42 [8,150]. These genetic adaptations facilitate the survival, growth and migratory capabilities of melanoma. $\alpha_v\beta_3$ Integrin expression in melanoma has also been linked to the progression from RGP to VGP [159].

The EMT in melanoma not only permits tumor cell invasion and altered cell communication partners by modifying cell-cell contacts, but also plays an important role in remodeling the ECM making it conducive for invasion and migration and creating what is coined ‘reactive stroma’ [160,161]. The adaptation of this stromal-cancer cell environment manifests even before the tumor is clearly noticeable [127,160]. The ‘reactive stroma’ consists of the tumor ‘*in situ*’, ECM and

mesenchymal cells, fibroblasts, vascular networks and associated cells, nerves and inflammatory cells resembling normal stroma when undergoing wound healing [160–162].

During wound healing there is a period of ischemia where blood supply is restricted accompanied by a shortage of oxygen and nutrients to the tissue, edema, followed by an influx of immune cells, and then a period of blood vessel growth and tissue repair [163,164]. Tumor progression for most cancers undergo all these phases of wound healing, generating the perception of cancers as 'wounds that do not heal' [160,165].

Fibroblasts are the first main group of cells to accumulate at sites of wound repair, where they present a scaffold for inflammatory and other cells that mediate scar and tissue formation [160]. The primary function of fibroblasts is aiding in the formation of the basement membrane and synthesizing components of the ECM, in which they are also embedded, such as collagen (I, III, V) and fibronectin [166,167]. Fibroblasts also secrete several important paracrine factors such as Insulin-like growth factor 1 (IGF-1), transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF) and bFGF to maintain ECM homeostasis and act in wound healing responses [159]. After tumorigenic cells undergo EMT, they can recruit and co-opt fibroblasts to secrete factors that only potentiate the tumor microenvironment and promote tumor progression [159].

A specific population of fibroblasts has been identified as being directly involved in cancer progression, they are known as cancer-associated-fibroblasts (CAFs) [160,168]. The CAFs are also termed 'reactive stromal fibroblasts' because

they begin to express α-smooth-muscle actin similar to myofibroblasts. Myofibroblasts are larger and more spindle-like in composition than regular fibroblasts; they also typically divide faster and produce more ECM [159,169–171]. Tumors can directly secrete signals such as growth factors and chemokines to dynamically recruit fibroblasts to the site of the tumor, while other fibroblasts may arrive at the tumor in response to the reactive stroma that mimics a wound. Melanomas can release a combination of bFGF, PDGF, and TGF-β signals to stimulate and induct fibroblasts [172,173]. When human melanoma cells (A2058) were co-cultured with primary human fibroblasts (HS-68), the fibroblasts gene expression was altered. The co-cultured fibroblasts began to significantly release more CXCL1 and CXCL2 chemokines associated with melanoma growth and migration. Meanwhile, the melanoma cells began to show a pro-inflammatory response indicated by down regulation of Interleukin-11 and DNA binding domain-1 in the cells [153]. In a 3D co-culture model Flach and colleagues (2011) found that melanoma spheroids could recruit and incorporate fibroblasts. The presence of the melanoma cell soluble factors that recruited the fibroblasts also increased the rate of wound closure as much as 38%, indicating an increase in the secretion of fibroblast associated factors [171].

The CAFS in turn enhance the expression of the same signals as well as release other factors such as IL-1, HGF, PDGF and Endothelin 3 (EDN3) to promote melanoma growth, migratory capability and neovascularization [31,34,172]. Fibroblasts elicit different effects on melanoma progression depending on the melanoma stage. Cornil and colleagues (1991) examined the effect dermal

fibroblasts had on melanoma cells taken from different stages of melanoma progression [174]. When early metastatic incompetent melanoma cells taken from RGP and VGP stages were co-cultured with fibroblasts, melanoma cell growth was repressed. However when 9 out of 11 cell lines from metastatic phases were co-cultured with fibroblasts, growth of the aggressive cell lines was stimulated [174]. It is speculated that the more aggressive tumor types may secrete factors that can induce and recruit fibroblasts to then release paracrine factors in favor of melanoma progression [159]. Thus fibroblast activation may be induced by more aggressive cell types within the tumor, moreover fibroblasts may aid in selection and enhancement of the very aggressive melanoma cells [174].

Recently fibroblasts have been recognized as important components of the body's immune response at wounds or sites of inflammation [175]. Fibroblasts can secrete various cytokines and chemokines to initiate chronic inflammation and regulate inflammatory infiltrates [160,176]. When fibroblasts are co-opted by melanoma cells, these inflammatory CAFs can maintain chronic inflammation that characterizes the tumor microenvironment and aid in the tumor's escape from the hosts immune surveillance which is a major hallmark in tumor progression [165,175,177].

1.3.2. Evading Immune Surveillance

Chronic inflammation in the TME creates tumor promoting effects as the microenvironment is teeming with mediators of inflammation such as cytokines, chemokines, growth factors, reactive oxygen species (ROS), nitrogen species (NOS) and prostaglandins produced by the either the tumor or associated stroma

cells [178179]. The abnormal growth of a tumor summons immune effector cells to the site of the tumor to deal with what is initially seen as a foreign invader, however the TME has been altered so much that the anti-tumor functions of the immune cells are downregulated, and instead tumor derived signals such as TGF-β, Interleukin 8 (IL-8), bFGF and tumor necrosis factor- α (TNF-α) successively recruit the inflammatory cells to support tumor progression [165].

Many of the inflammatory cells of both adaptive immunity such as T-lymphocytes, dendritic cells, and B cells and innate immunity including macrophages, leukocytes and natural killer (NK) cells can even infiltrate the tumor itself, favoring tumor survival and protection from subsequent immune reactions outside of the TME [165]. Another important group of immune cells found in the TME are myeloid suppressor cells (MSCs) [180]. Meyer and colleagues (2011) using the *ret* transgenic melanoma mouse model showed high numbers of myeloid suppressor cells infiltrating tumors and an increase in immune factors IL-1β, granulocyte macrophage colony- stimulating factor-1 (GM-CSF), and Interferon gamma (IFN-γ), in melanocytic lesions and lymph node metastases correlating with tumor progression [177]. Other studies demonstrated that, dysplastic nevi and RGP have considerable numbers of lymphocyte infiltrates. It is believed that melanoma cells can actively recruit lymphocytes by secreting several chemoattractants namely IL-8, GM-CSF and monocyte chemoattractant protein-1 (MCP-1) [31,181]. Melanomas are able to further escape tumor surveillance by promoting the release of immunosuppressants such as insulin like growth factor-β (IGF-β) and IL-10 into the microenvironment [31].

The recruitment of immune cells into the tumor microenvironment tricks the host's immune response into recognizing the tumor as 'self' rather than as a dangerous group of cells. Melanoma is well known for its ability to escape the host's immune surveillance. The tumor escape from the immune system is one of the features of the skin cancer that makes it very difficult to treat. The tumor response mechanisms are complex and not yet fully characterized or understood.

1.3.3. Inducing Neovascularization

Perhaps one of the most studied part of the TME response in tumor progression is tumor angiogenesis. As the tumor grows and co-opts various cell types into the tumor microenvironment and the tumor itself, oxygen and nutrients become limiting, thus creating an increasingly hypoxic and unfavorable tumor microenvironment [182]. The tumor and the TME respond by triggering angiogenesis, a mechanism described as the 'tumor angiogenic switch' [182–184]. Srivastava and colleagues (1988) found that the amount of staining for vessels could indicate the probability of metastasis [185].

Tumor angiogenesis is the formation of new blood vessels from pre-existing ones [186,187]. The process is one of the most significant events of tumor progression, as tumors cannot grow more than 2-3mm^3 without vascularization supplying the tumor with sufficient oxygen and nutrients as well as collecting wastes from the tumor [182,187,188]. Tumor angiogenesis follows several general sequential steps. Angiogenesis begins with modifications and degradation of the basement membrane by proteases, like matrix metalloproteinases (MMPs),

permitting the detachment of pericytes which are contractile cells that cover endothelial cells lining blood vessels [182,188,189]. The detachment of pericytes allows the directional migration of endothelial cells (ECs) into the interstitial space towards angiogenic signals secreted by the tumor and TME. The ECs proliferate and follow each other while forming attachments to each other, subsequently forming a lumen and vessel branches, a process known as sprouting [188,189]. To end with, the basement membrane re-forms and pericytes then re-attach to the newly formed vessels and blood flow begins [184,189,190].

Tumor blood vessel structure is uniquely different from those formed during normal angiogenesis [188 189]. The blood vessels of the tumor tend to be irregularly shaped, deflated, very twisted and circuitous with some having dead ends. Tumor blood vessels also lack definite organization into vein, arteries and capillaries, moreover the vessels are often characterized as ‘leaky’ or hemorrhagic, with poorly connected vessel walls, possibly due to the overproduction of the vascular permeability factor, vascular endothelial growth factor (VEGF). Blood flow is often irregular and slower in tumor vasculature than in normal blood vessels [188 189]. The abnormal properties of the tumor vessels aids in preventing proper administration of drug therapies to tumors.

The ‘angiogenic switch’ encompasses a delicate balance between the upregulation of positive angiogenic related factors such as VEGF family, bFGF, HGF and IL-8 negative regulators of angiogenesis including thrombospondin-1 and angiostatin [183,184]. The switch is prompted by tumor induction, hypoxic conditions and oncogenic pathways [186]. Tumor secreted autocrine growth factors

stimulates tumor growth which then stimulates paracrine pro-angiogenic factors that recruit stromal blood vessels [182,186]. Transformed melanocytes, especially those undergoing the VGP produce high amounts bFGF and VEGF both important angiogenic factors. The vascular endothelial growth factor family includes the key angiogenic regulator VEGF along with VEGFB, VEGFC, VEGFD and placental growth factor which bind with various affinities to the VEGF receptors Flt/VEGFR-1, Flk/KDR/VEGFR-2 and VEGFR-3. The VEGF receptors are found primarily on endothelial cells, but can be located on macrophages, bone marrow derived cells, vascular smooth muscle cells, hematopoietic cells and on some malignant cells[191–193]. The VEGF protein acts as a potent mitogen for endothelial cells (ECs) by activating phosphatidylinositol signal transduction pathways and the MAPK pathway in endothelial cells. The Vascular endothelial growth factor along with bFGF and other angiogenic factors, angiopoietins and their tie receptors also mediate chemotaxis of ECs and are responsible for EC survival, vessel sprouting and proper vessel formation [193–195]. Melanoma production of MMPs can release bFGF from the ECM, while cells in the TME such as fibroblasts can produce VEGF along with melanoma cells [196].

Other angiogenic factors, placental growth factor (PIGF) [197]and IL-8 [198] which aid in EC recruitment and vessel formation have also been found to be expressed by primary and metastatic melanomas to enhance tumor angiogenesis [199]. Secretion of IL-8 by endothelial cells in the TME can also promote melanoma cell migration [199,200]. Expression of urokinase plasminogen activator and its receptor (uPA/uPAR) and MMPs which promote melanoma cell

migration, invasion and metastasis can at the same time influence EC migration and neovascularization [201,202].

Tumors can initially co-opt pre-existing vasculature until hypoxic conditions trigger the secondary angiogenic response to create new vasculature [203]. Long diffusion distances in tumors creates chronic hypoxia and necrotic regions in tumors, leading to the up regulation of angiogenic regulators such as VEGF [186,188,195]. Under normoxic conditions, oxygen sensing prolyl hydroxylase domain proteins (PHD1-3) target the α-subunits of the hypoxic regulator, Hif-1α, for degradation via ubiquitination [182]. When conditions become hypoxic however the α-subunit is stabilized and translocated to the nucleus where it dimerizes with the β-subunit. The α/β complex then binds to hypoxia response elements (HRE) in the promoter regions to activate the transcription of angiogenic related genes such as VEGF, uPAR and MMP2 [204,205]. Hif-1α expression has been confirmed in melanoma by immunohistochemical and immunofluorescence of melanoma cells and biopsies and verified with western blots and PCR [206–208]. Increased expression of hypoxic inducible factors have also been shown in metastatic melanomas when compared to benign nevi [209]. Treviño-Villarreal and colleagues (2011) demonstrated that cells in the TME like stromal cells and pericytes expressed HIf-1α with the transformed melanocytes [210].

Hypoxic conditions in the TME promote melanoma malignancy in two ways; (1) stimulating tumor angiogenesis leading to increased vasculature in which melanoma cells can enter into the circulatory and lymphatic system to

invade and colonize distant organs and (2) provide an environment that selects for the more aggressive cells that can withstand increased hypoxic conditions [182]. Liu and colleagues (2011) reported that hypoxia could up-regulate *Snail1,* a mediator of decreased E-cadherin expression, thus aiding in EMT and melanoma migration [211]. Expression of Hif-1α is capable of actuating proto oncogenic effects through pathways such as HGF/SF [212]. Bedgoni and colleagues (2005) found that constitutive PKB activity resulting from loss in PTEN could transform melanocytes but only when exposed to hypoxic conditions. NRAS induced melanocyte transformation was also favored by hypoxia [213].

In aggressive and highly metastatic melanomas an interesting group of microcirculation patterns formed by the tumorigenic cells themselves has recently been described as vasculogenic mimicry (VM) [214]. The aggressive transformed melanocytes showing great tumor plasticity, form cell lined channels with the ECM to create their own tubular capillary-like network [152,214,215]. Probst and coworkers (2012) observing xenografts of human melanoma cell lines in nude mice, found that some of the endothelial cells from microvessels formed in the tumors were positive for human chromosome 17/Her-2, indicating that the endothelial cells were melanoma in origin [216]. The endothelial cell marker, CD31 and human Y chromosome ‘in situ’ and immunofluorescence labeling verified that the melanoma cells acquired the vascular endothelial like morphology and could contribute to creating neovascularization [216]. The presence of VM in melanomas is connected to poor prognosis and survival in patients [217,218]. The exact mechanism allowing VM is poorly understood. Vasculogenic mimicry modulating

genes are generally categorized into pathways associated with not only vascular signaling such as VE cadherin and VEGF but embryonic/stem cell signaling including NOTCH and hypoxia signaling as well [219].

1.4. Melanoma Heterogeneity and Malignancy

Tumor cell heterogeneity in the TME therefore not only defines the presence of different cell types such as immune cells, pericytes etc. but heterogeneity amongst the cancer cells themselves due to genetic and epigenetic variations [220]. Cancer tissues often consist of a sub-population of cells that have adopted a 'embryonic stem like' phenotype which makes it easier for them to adjust to the environment, and emit embryonic developmental factors to alter the environment around them [221]. The tumor stem cell (TSC) can self-renew and transdifferentiate to adopt the roles of neighboring stromal cells [221–223]. It was originally thought that during melanoma progression, changes in genetic expression of the tumor leading to TSCs or other melanoma cell phenotypes were unidirectional. Recently, new studies are challenging that idea as it appears that melanoma cells and its interaction with the TME allows for considerable plasticity of the tumorigenic cells defined by different transcription signatures. These interchangeable states alternate from a dominantly proliferative phenotype to an invasive state and possible other intermediate stages in between [224].

Hoeck and coworkers (2012) used expression profiling of various melanoma cell lines to identify transcription signatures for proliferative and invasive melanoma cell states [224]. Proliferative signatures include expression of melanocytic related genes *MITF*, *Tyrosinase* (TYR) and *Dopachrome*

Tautomerase (*DCT*), as well as neural crest factors associated with melanoblast development such as *SOX10* and *Endothelin receptor b* (*EDNRB*). The invasive signature states included the up-regulation of Serpine1 and inhibin beta A (INHBA) which are involved in TME modification [224]. Hoeck and colleagues (2012) then injected the DNA microarray characterized melanoma cell lines into immunocompromised mice, after which immunohistochemistry was performed on tumors to identify proliferative vs. invasive signatures. They showed that melanoma cells could switch back and forth between different genetic signatures as both proliferative and invasive cell types were found in the tumor despite what the original seed cell signature was [224].

The phenotype switching of melanoma cells may facilitate the special requirements of the cells in different melanoma states, from proliferative when growth is necessary, to invasive, then back again to proliferative when at distant metastatic sites. Phenotype switching may also explain why melanomas are resistant to cancer regimens and often re-appear. Tumorigenic cells that are not susceptible to chemotherapy may manipulate the TME to switch back to a proliferative state and initiate tumor progression again. The key to revolutionizing therapies for melanoma thus lies in understanding the dynamics of the tumor microenvironment and targeting the important signals that maintain the malignant cells.

The fact that malignant melanoma cells can alter their gene expression, and subsequently the gene expression of neighboring cells, proves their ability to adopt a ‘stem-cell likeness’, and become immortal, undifferentiated and invasive.

The plasticity makes it easy for the cells to leave their origin and occupy other regions. During melanomagenesis expression of genes involved with the development of their early embryonic progenitors, melanoblasts, has been observed. All melanocytes except for those found in the retina of the eye originate from the neural crest (NC) during embryonic development. Neural crest cells (NCC) are a transient and multipotent population of cells that are highly migratory [225,226]. The neural crest cells develop from the dorsal tip of the pseudostratified dorsal epithelium of the neural tube, giving rise to most cells of the peripheral nervous system forming neurons and glial cells, bones and craniofacial cartilage cells of the skeletal system, endocrine system derivatives for the adrenal and thyroid glands, adipose tissue, vascular smooth muscle tissues as well as mesenchymal dermal cells and melanocytes [227,228].

During neurulation, the NCCs undergo EMT whereby they separate from the neural tube and begin to stereotypically migrate extensively [229–231]. As the NCC migrate they are subjected to an interplay of signals including BMP, NOTCH, WNT and FGF from the non-neural ectoderm and mesoderm that not only aids in their migration but also helps determine their cell fate [228,232,233]. Neural crest cells that migrate dorsolaterally become melanoblasts, progenitor cells for melanocytes [227]. Melanoblast specification and differentiation depends on a complex system of signaling pathways including WNT, BMP, and EDN3. Proper melanoblast development does not take place in the absence of any of these signals [21,234,235]. Melanoblasts eventually migrate into the ectoderm where they become melanocytes and produce the pigment melanin.

Melanomas may be intrinsically prone to metastasize because of their EMT transformation and high migratory capability associated with their developmental program [236–239]. Bailey and colleagues (2012) transplanted aggressive human melanoma cells (c8161), poorly invasive human melanoma cells (c81-61) and human primary melanocytes into a chick embryonic NCC environment [237]. Laser capture microdissection and genetic analysis techniques were used to compare the gene and signaling pathways exploited by the c8161, c81-61 and primary melanocytes [237]. They reported that the malignant melanoma cells, c8161, responded more vigorously to the NCC environmental cues than the poorly invasive melanoma cells and primary melanocytes, indicating that receptors for NCC development were present and active in the c8161 cells [237]. The c8161 cells also commandeered the embryonic NCC invasion program to enhance their own invasiveness and plasticity [237]. A number of c8161 cells responded to the NCC microenvironment and migrated along NCC migration routes from the graft sites, while none of the non-invasive and primary melanocytes migrated [237]. The expression patterns of migrating c8161 cells did not exactly mirror that of the NCC suggesting that the tumor cells can manipulate the NCC development program to suit their own agenda [237]. Furthermore 40% of the analyzed NC-related genes were induced by the aggressive cell line compared to only 8% induction in the poorly invasive cells. The invasive cells also re-expressed approximately 12 NC-related genes not seen in the primary melanocytes such as SNAI1, ZEB1 and members of the Eph family and silenced others involved in differentiation[237].

Because of the dynamic regulation of both stemness and a highly complex microenvironment it is important to identify and fully characterize potential biomarkers for melanoma to improve diagnosis and treatment. One such target that has been implicated is EDNRB and its ligand EDN3.

1.5. Melanoma and Endothelin Signaling

Endothelins (EDN) are twenty one amino acid (aa) peptides that act as powerful vasoconstrictors and vasodilators [240–242]. Preproendothelin transcription yields the 203aa prepro-endothelins which are further modified by furin-like proteases to produce 37-41aa big-EDN intermediates which are biologically inactive. The big-EDNs are finally cleaved by membrane bound zinc metalloproteases known as endothelin converting enzymes (ECE) at the Trp-21-Val/lle-22 peptide bond to form the active peptides EDN1, Endothelin 2 (EDN2) and EDN3 [240–247]. The EDN1 protein is produced by vascular endothelial and smooth muscle tissue, as well as fibroblasts, cardiac myocytes and brain neurons, with EDN2 mainly secreted by ovaries and intestinal cells and Edn3 by endothelial cells, vascular smooth muscle cells, brain neurons, intestinal epithelial cells, hepatocytes and adventitial fibroblasts [241,242,248].

In humans, the EDNs bind to two different rhodopsin-type G-protein coupled receptors (GPCRs), Endothelin receptor A (EDNRA) and EDNRB with different affinities. Endothelin 1 and Endothelin 2 preferably bind to EDNRA, however EDNRB is non-selective and binds all three peptides with similar affinity [240,249]. Binding of EDN1 activates the heterotrimeric G proteins G_q/G_{11} and G_{12}/G_{13} while EDN3 triggers G_i/G_o and G_{13} to activate phospholipase Cβ and

signal through several different pathways including PKC and MAPK [249–254]. Endothelin receptor A and Endothelin receptor B can form homodimers or heterodimers which modifies not only ligand binding but receptor activation, and transmembrane signaling as well [250].

The structure, function and importance of the EDNRA/EDN1 axis in vasoconstriction, proliferation of melanocytes and as a melanogenic factor have been extensively characterized, however not as much is known about the role of the EDNRB/Edn3 axis. The human *EDNRB* gene is situated on chromosome 13, and has seven exons and introns. Approximately 64% of its sequence homology is shared with *EDNRA* [242]. Found mostly in ECs, EDNRB facilitates the release of NO and prostacycline, acting mainly as a vasodilator as opposed to EDNRA's vasoconstrictor effects in response to Ca^{2+} influx that results from stress on ECs during cardiovascular stress or hypoxia [248,255,256]. Endothein receptor B and EDN3 have a profound effect on melanocyte development and have been recently identified as a potential relevant factor in melanomagenesis.

Waardenburg syndrome IV is associated with mutations in *EDNRB* and *EDN3* that disrupt proper development of enteric nerves and melanocytes resulting in hearing loss, skin, eye, and hair color abnormalities as well as blockage of the intestines known as Hirschsprung disease [257–259]. Targeted mutations of *EDNRB* and *EDN3* in mice results in similar phenotypes such as aganglionic megacolon and pigmentation defects which corroborate the necessity for EDNRB and EDN3 signaling in proper melanoblast development [260–263,264].

Lee and colleagues (2003) used the tetracycline inducible system to control *EDNRB* expression to reveal that *EDNRB* is required during E10.5-E12 for melanoblast migration and proliferation [265]. Hou and colleagues (2004) used tissue re-combination experiments '*in vitro*' with NCC cultures and mouse embryos carrying a EDNRB null allele with the LacZ reporter gene to further show that EDNRB is required for melanoblast differentiation as it is needed for melanoblasts to express the melanogenic enzyme tyrosinase [265]. Endothelin receptor B acts in an autocrine manner as mammalian melanoblasts themselves begin to express EDNRB along the mediolateral pathway [265–267], while the indispensable role of EDN3 is believed to act in a paracrine fashion as EDN3 is expressed by the dermal mesenchyme and the ectoderm [265,268].

Studies carried out with avian NCCs show that EDN3 acts as a potent mitogen for both melanoblasts and differentiated melanocytes and promotes melanoblast proliferation and survival [269–271]. Absence of EDNRB and EDN3 in murine and avian NC cultures give rise to melanoblasts that are unable to differentiate properly as they lack the melanogenic markers involved in pigment production such as tyrosinase [272] or simply cannot produce pigment unless stimulated with EDNs [273]. Interestingly however, exposure of epidermal melanocytes to a strong treatment of EDN3 'in vitro' could stimulate the proliferation of not only pigmented melanocytes but a population of cells co-expressing melanin and glial markers and unpigmented cells expressing glial markers or early NC cell markers [271]. Thus continuous exposure of EDN3 can initiate transdifferentiation of differentiated melanocytes and maintain the

multipotent progeny [271]. The influence EDN3 has on melanocyte dedifferentiation may be important during melanomagenesis however more studies are needed to examine this relationship.

Endothelin receptor B signaling has been implicated as a melanoma progression marker. Although studies such as Kikuchi et al. (1996) and Eberle et al. (1999) communicated that *EDNRB* expression decreased in more malignant melanoma cell lines they examined, other studies such as that conducted by Bittner et al (2000) and Demunter et. al (2000) examining more melanoma cell lines found that *ENDRB* expression was instead upregulated [274–277]. Lahav and colleagues (1999) further explored the effect of ENDRB on melanoma progression 'in vitro' and 'in vivo'. The 'in vitro' experiments showed that exposure of 7 different melanoma cell cultures to the EDNRB antagonist BQ788, could inhibit cell growth and induce dendricity and increase pigmentation of the cells, thus conferring a phenotype more similar to mature melanocytes than melanoma cells [278]. When the A375 melanoma cell line was injected into nude mice, the resulting tumors were subjected to BQ788, after which the tumors showed either slowed tumor growth or slowed tumor growth with tumor shrinkage and complete growth arrest [278]. Later Lahav and colleagues (2004) used human melanoma cell lines from various stages of melanoma progression to support that EDNRB may be important in melanoma progression. Inhibition of EDNRB using BQ788 in the melanoma cell lines induced apoptosis and decreased cell viability of the metastatic melanoma cell lines more than that found in primary and cutaneous cell lines [279]. The inhibition of EDNRB may possibly trigger

apoptosis because of decreased expression of the survival factor, BCL-2A1and the PARP-3 enzyme which is aids in DNA repair [279]. Inhibition of the EDNRB axis both ‘in vivo’ and ‘in vitro’ in other studies likewise found reduction of melanoma growth [280].

Endothelin 3 activation of EDNRB may be the key factor in EDNRB mediated effects on melanoma growth as Nengxing et al. (2007) showed ‘in vitro’ that EDN3 prompted increased proliferation of A375 melanoma cells in a time and concentration dependent manner [281]. Cruz-Muñoz and colleagues (2012) used lentiviral vectors to upregulate EDNRB in stable variant human melanoma cell line models that spontaneously develop visceral metastasis (113/6-4L) and brain metastasis (131/4-5B1 and 131/4-5B2) [282]. Increased levels of EDNRB in both the visceral and brain metastatic cell lines boosted their metastatic potential leading to shorter median survival in those with the visceral metastasis and an increased number of brain metastases in the brain metastatic variant [282]. Cruz-Muñoz and colleagues (2012) identified EDN3, which is also highly expressed in the brain microenvironment, to be the major factor in the EDNRB mediated enhanced brain metastatic events [282].

Tang and colleagues (2008) gene expression profiling analysis comparing normal nevi biopsies to metastatic tissues identified abnormal expression of *EDN3* in melanoma [283]. Quantitative PCR analysis showed an up-regulation over 10 fold of *EDN3* in the metastatic melanoma biopsies than normal nevi biopsies [283]. Melanoma specific EDN3 immunohistochemistry (IHC) staining was also observed only in the metastatic tissues [283]. Six out of seven melanoma cell

lines, examined by Tang and his associates (2012), showed EDN3 expression while normal melanocyte cultures did not. Their data suggest that not only is the EDNRB/EDN3 axis involved in melanoma but also that the melanoma cells themselves may be the source of EDN3 [283].

The EDNRB/EDN3 axis may aid in melanoma progression by influencing mechanisms in the tumor microenvironment. Bagnato and colleagues (2004), Rosanò and colleagues (2004) and Jamal and colleagues (2002) used melanoma cell lines exposed to EDN1 and EDN3 to show that after treatment, the cell lines had decreased E-cadherin levels [148,284,285]. Bagnato and colleagues (2004) also showed a concomitant upregulation of N-cadherin and that mRNA of the EMT transcription factor, *Snail*, that negatively controls E-cadherin expression was observed in the primary melanoma cell line thus suggesting a potential role for EDNs in EMT [280]. Exposure of primary cell line (1007) and metastatic melanoma cell line (M10) to EDN1 and EDN3 resulted in the increased secretion of ECM proteases MMP-2 and MMP-9 and the integrins $\alpha_2\beta1$ and $\alpha_v\beta_3$ all important players in the tumor microenvironment [280].

The association of endothelins with endothelial cells, makes the EDNRB/EDN3 axis worth examining for any prospective roles in tumor angiogenesis. Endothelins are often secreted at sites of wounds or injured vessels [286]. Endothelin 1 and Endothelin 3 can stimulate production of NO and stimulate migration of endothelial cells [287], in addition, they are expressed by endothelial cells of lymphatic vessels, which is also important in cancer metastasis [288]. Spinella and colleagues (2007) research on primary melanoma

(1007) and metastatic melanoma (M10) cell lines conveyed that both EDN1 and EDN3 could increase Hif-1α mediated upregulation of VEGF and angiogenic factors, COX-1, COX-2 and PGE_2 in a concentration dependent manner [289]. The expression of COX-2, Hif-1α, VEGF and MMP-2 were also decreased in M10 tumor bearing mice in response to EDNRB inhibition with A-19262 [289]. Zbytec and colleagues (2013) used bioinformatics to also identify putative hypoxia response elements (HRE's) in *EDNRB* [209]. Spinella and colleagues (2013) further revealed that EDNRB/EDN1 axis could induce VEGF-3 signaling to activate MAPK and AKT to promote cell migration and vasculogenic mimicry of primary and metastatic melanoma cell lines [290]. Wren and colleagues (1993) showed that EDN3 could specifically support wound healing by stimulating endothelial cell proliferation without activation of either EDNRA or EDNRB in an 'in vitro' endothelial cell injury model, thus potentially calling into question other mediated mechanisms of EDN3 signaling [291].

Conversely, previous studies by Lahav and colleagues inferred negative effects of EDNRB on melanoma angiogenesis [279]. When they inhibited EDNRB in melanoma cell lines, microarray and real time RT-PCR analysis disclosed significant increased levels of *VEGF* and *Hif-1α* along with decreased levels of the angiogenic suppressor *gravin* [279]. A majority of the studies examining the relationship between the EDNRB axis and melanoma have been performed using 'in vitro' methods. However, it is well known that melanoma cells behave very differently in culture from when they are situated in their tumor microenvironment where they are affected by the induced changes in the host. It

is therefore important to examine the effects of EDNRB/EDN3 in an ‘in vivo’ model.

Mayuko and colleagues (2010) used a RET mouse model that form melanomas de novo (do not arise from pre-existing benign lesions) to study the status of EDNRB in melanoma progression [292]. They found a negative correlation between levels of EDNRB with tumor progression. Transgenic Ednrb(+/−);RET mice acquired their tumors significantly later but metastasized earlier and had shorter survival ratios when compared to Ednrb(+/+); RET mice [292]. Kumasaka and colleagues (2015) later described that the enhanced malignant phenotype in the Ednrb(+/−);RET mice was the result of reduced levels of Plexin C1 (PlxnC1) a protein normally involved in melanocyte adhesion that has been labeled as a melanoma suppressor protein [293].

Still, studies such as Soufir and colleagues (2005) who explored whether germline mutations in *EDNRB* associated with Hirschsprung disease could predispose individuals for malignant melanoma, found that fourteen out of fifteen malignant melanoma (MM) patients had *EDNRB* mutations found in Hirschsprung disease [294]. When the variables such as skin type and number of nevi were considered by multivariable logistic regression analysis, they still found a significant relationship between mutations in *EDNRB* and MM incidence [294]. Moreover, both ‘in vitro’ and clinical trials using the EDNRB antagonist bosentan to treat melanoma cell lines [295] and metastatic melanomas in patients [296] aided in apoptosis of melanoma cells and may have stabilization effects of metastatic disease [295–297].

1.6. Research Questions

Seemingly contradictory influences of the EDNRB/EDN3 axis on melanoma could be attributable to the use of 'in vitro' methods that expose the cells to unnatural and stressful tissue culture conditions as well as the lack of an 'in vivo' model that closely parallels melanoma progression in humans. Much is still unknown about the exact role played by EDNRB/EDN3 signaling in melanomagenesis. My overall hypothesis is that EDNRB/EDN3 signaling acts in a paracrine manner to promote melanoma growth and metastasis. I tested this hypothesis by asking three major questions.

Question 1: Is the up-regulation of the *Endothelin 3/Ednrb* signaling pathway sufficient to lead hyperproliferative melanocytic lesions to fully malignant metastasis?

Studies on the role that EDN3 plays in melanoma progression have mainly been conducted using melanoma biopsies and cells from different stages of melanoma progression. Although this has been instrumental in identifying EDN3 upregulation in more malignant melanoma stages, it is still not clear whether EDN3 signaling is a driving force in actual melanoma malignancy. In order to address this question in vivo I established a melanoma mouse model by crossing *Tg (Grm1) Epv* mice that aberrantly express metabotropic glutamate 1 (Grm1) exclusively in melanocytes under the regulation of the *Dopachrome tautomerase* promoter *with K5-tTA-Edn3* (here referred to as *K5-Edn3*) transgenic mice that over-express *Edn3* in the skin or *Tg(Ednrb)*1Lk (here referred to as Dct-*Ednrb*) transgenic mice that over-express *Ednrb* in melanocytes. The *Tg (Grm1) Epv* mice develop melanocytic hyperplasia with limited metastatic capability [298,299]. I

hypothesize that the presence of more Edn3 in the tumor microenvironment or *Ednrb* in melanocytes of the *Tg (Grm1) Epv* mice will lead hyperproliferative melanocytic lesions to fully malignant metastasis and contribute to melanoma progression.

Question 2: What are the gene expression changes caused by the over-expression of *Edn3* in the tumor microenvironment?

During embryogenesis, *Ednrb* and *Edn3* are involved in proper melanoblast development and migration. Melanoma cells share specific characteristics with melanoblasts that allow them to be malignant. These characteristics include high proliferative capabilities, a lack of differentiation and reduced intercellular adhesion. I hypothesize that the over-expression of *Edn3* in the tumor microenvironment in the *K5-Edn3/ Tg (Grm1) Epv* mice will alter gene expression of adhesion factors such as cadherins, integrins, and connexins and those involved in differentiation and proliferation such as *Mitf* and *HGF* that have been shown to occur '*in vitro*' during melanoma progression.

Question 3: Is the *Edn3* pathway involved in promoting changes in the tumor microenvironment that enhances melanoma malignancy?

Metastasis is a multistep process involving neighboring cells in the tumor microenvironment being reprogrammed by tumorigenic cells to express factors that aid in the tumor's survival, proliferation, immune escape and invasiveness. The angiogenic cascade is affected by multiple factors in the tumor microenvironment including tumor hypoxic conditions, and a host of bi-directional signaling among tumor and neighboring cells. Thus in order to investigate the true role of *EDNRB*/*EDN3* in

angiogenesis, it is best to do so in an 'in vivo' model where the *Edn3* axis can affect its specific targets or be influenced by other complement of factors that may be needed for it to mediate its effects. I hypothesize that the over-expression of *Edn3* will have a significant effect in melanoma angiogenic response.

1.7. References

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1.8. Figure Legends

Figure 1 Cutaneous melanoma may arise from precursor lesions, either a benign nevus or a dysplastic nevus. It is also believed that melanoma can develop directly from abnormal stem cells and not from an existing nevus. Melanomas can then progress to the radial growth phase characterized by proliferation of tumorigenic cells and invasion limited only to the epidermis. The vertical growth phase can then follow, in which the tumorigenic cells begin to grow vertically and invade the deeper tissue of the dermis. Once the melanoma invades the dermis, the tumorigenic cells and can enter into the lymphatic or circulatory system to colonize other organs leading to the metastatic phase of the skin cancer.

Figure 2 The tumor microenvironment includes various cells that have been recruited and inducted by tumorigenic cells such as carcinoma associated fibroblasts, immune cells such as neutrophils, macrophages and lymphocytes, cells that contribute to neovascularization such as endothelial cells and the modified extracellular matrix termed the ‘reactive stroma’. (A) The different cells in the tumor microenvironment are influenced by factors secreted by the tumorigenic cells, and in turn produce factors that contribute to the development, growth, invasion and metastasis of melanoma (B).

Figure 1 Melanoma Progression.

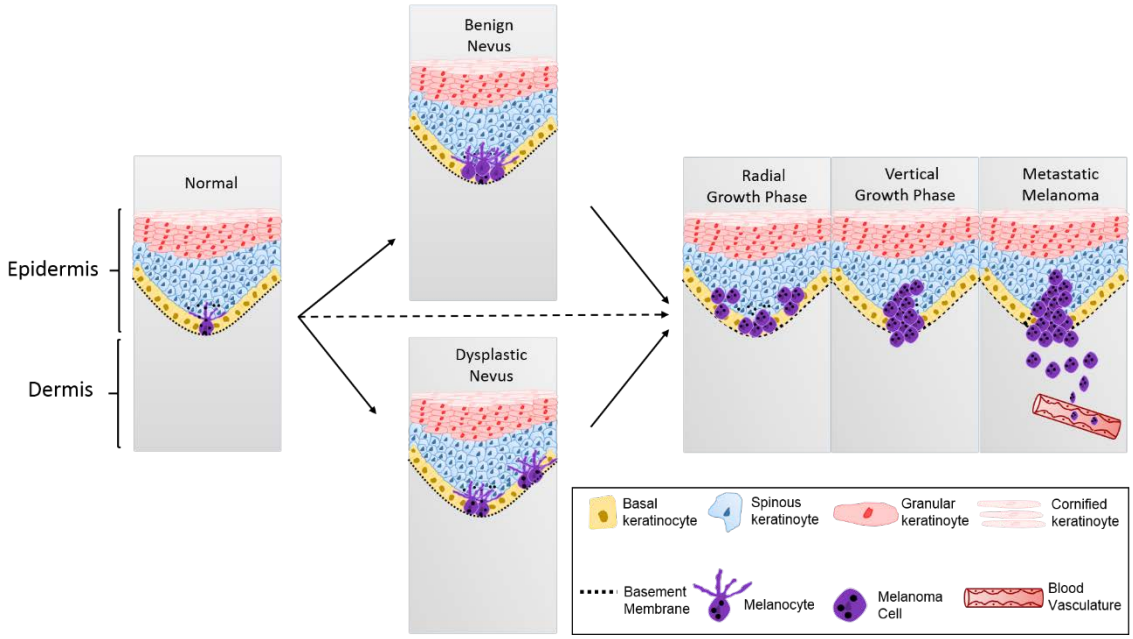
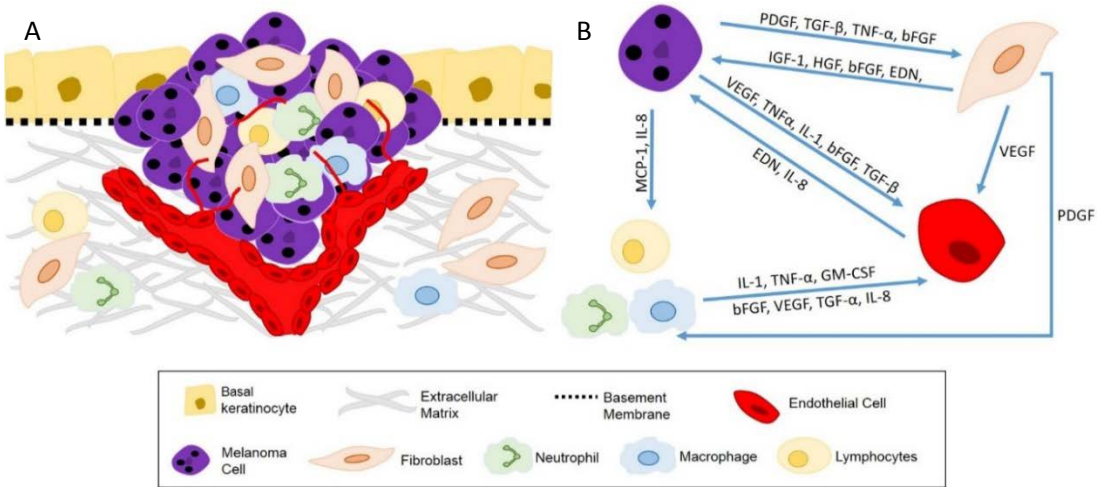


Figure 2 Melanoma Tumor Microenvironment.



CHAPTER II

ENDOTHELIN SIGNALING PROMOTES TUMOR PROGRESSION AND METASTASIS IN A MOUSE MODEL OF MELANOMA

II. ENDOTHELIN SIGNALING PROMOTES TUMOR PROGRESSION AND METASTASIS IN A MOUSE MODEL OF MELANOMA

2.1. Introduction

Tumors are comprised not only of transformed malignant cells but also of a complex system of stroma and various other cell types. This niche is commonly referred to as the 'tumor microenvironment', and each part is integral in promoting malignancy and metastasis [1, 2]. Melanoma results from the initial transformation of epithelial melanocytes and subsequent alteration in the associated stroma. Melanoma lesions encompass cells such as fibroblasts, keratinocytes, endothelial and immune cells, all of which inherit dysfunctional cytokine pathways that facilitate malignancy. The tumorigenic cells acquire the ability to reprogram the normal functions of these neighboring cells in the tumor microenvironment, and establish a number of paracrine and autocrine signaling loops to promote angiogenesis, epithelial to mesenchymal transformation and metastasis. The exact mechanisms of the signaling loops are not yet fully understood, but many of them are regulated by growth factors and G-protein coupled receptors (GPCRs) [3-5].

G Protein Coupled Receptors can become tumorigenic either by acquiring intrinsic mutations that make the receptors constitutively active or by being exposed to excessive amounts of their ligands [5-7]. Endothelin receptor b (EDNRB) is a GPCR that has been implicated in melanoma progression. Endothelin receptor B ligands, endothelins 1, 2, and 3 (EDN1, 2, 3) are primarily involved in vascularization, by acting as vasoconstrictors and vasodilators,

promoting endothelial cell survival and migration as well as in the mitogenesis of endothelial cells, vascular smooth muscle cells, fibroblasts, and pericytes [8, 9]. Additionally, during embryonic development EDNRB and EDN3 are critical for melanocyte proliferation and survival. Endothelin receptor B has also been implicated in melanocyte differentiation before the cells migrate into the epidermis, while EDN3 may be able to delay the differentiation of the melanocytes [10]. Mice carrying mutations of either *Ednrb* or *Edn3* exhibit pigmentation defects such as those seen in the spontaneous mouse mutants *piebald lethal* and *lethal spotting*, respectively [11-13].

In vitro studies using melanoma cell lines, along with gene expression analysis and immunohistochemistry on melanoma biopsies have found that *Ednrb* expression is progressively up-regulated from dysplastic nevi to metastatic melanomas [14-17]. Tang and colleagues found that *EDN3* was up-regulated more than 10-fold at the mRNA level in metastatic melanoma biopsies when compared to benign nevi, while *EDN1* and *EDN2* expression remained low. They also showed that six out of seven cultured melanoma cell lines expressed EDN3 while human epidermal melanocytes did not [18].

Treatment of melanoma cell lines with the EDNRB antagonist BQ788 resulted in decreased cell viability by inhibiting proliferation [14, 19] and sensitivity to the inhibitor was more intense in the metastatic malignant cells [20]. The antagonist BQ788 also induced signs of differentiation such as increased dendricity and pigmentation of cultured melanoma cells as well as reduced tumor growth in nude mice implanted with melanoma cell grafts [14]. Other in vitro studies suggest

that EDN3 may be relevant to specific tumor-host interactions, as treatment of melanoma cell lines with EDN1 and EDN3 resulted in decreased levels of the cell adhesion molecule E-cadherin and increased levels of N-cadherin, which allow the tumorigenic cells to interact with other cell types such as fibroblasts and endothelial cells [21].

The purpose of our study was to develop melanoma metastasis mouse models to characterize the EDN3/EDNRB axis in melanoma progression and the tumor-host relationships it is involved in to promote metastasis. Transgenic mice that present with spontaneous melanocytic lesions in regions such as the ears and tails with limited metastatic capability (*Tg(Grm1)Epv* (E) [22-24]) were crossed to transgenic mice that over-express EDN3 by keratinocytes (*K5-tTA;TRE-Edn3-lacZ* [25]), transgenic mice that over-express EDNRB in melanocytes (*Tg(Ednrb)1Lk*), or both. The over-expression of *Edn3* in the skin microenvironment or of *Ednrb* in melanocytes led to the formation of rapidly growing highly metastatic lesions allowing for the direct evaluation of the role played by EDN3 in the process of melanomagenesis in vivo.

2.2. Materials and Methods

2.2.1. Mice and Genotyping

The *K5-tTA;TRE-Edn3-lacZ* (*K5-Edn3* used here for simplicity) and *Tg(Ednrb)1Lk* mice (*Dct-Ednrb* used here for simplicity), which were generated in our laboratory [25] , were crossed with the *Tg(Grm1)Epv* (E) mice (*Dct-Grm1*used here for simplicity, a gift from Dr. William Pavan, NIH) [22]. Briefly, the *K5-Edn3* mice (C57BL/6XSJL/FVB/N) were generated using the tetracycline regulatory

inducible system and harbors the tTA activator in the regulatory region of the *Bos Taurus* gene for Keratin 5 to drive the constitutively active expression of *End3*. The *Dct-Ednrb* transgenic mice were generated by microinjection of a transgene containing the full length *Ednrb* cDNA (1958bp, genbank accession # U32329**)** under the regulation of 3431 bp of the mouse *Dct* promoter and the BGH poly adenylation signal into FVB F_1 zygotes. The founder line was propagated subsequently for over 10 generations by crosses with C57BL/6J mice. *Dct-Grm1* transgenic mice express the metabotropic glutamate receptor 1 under the regulation of the melanocyte specific *Dopachrome tautomerase* promoter.

Deoxyribonucleic acid (DNA) from tail biopsies were extracted using standard techniques from the progeny of the *Dct-Grm1*, *K5-Edn3*, and *Dct-Ednrb* matings, and Polymerase Chain Reaction (PCR) was used to genotype the mice using the following primers: *Dct-Grm1* 5'-CCGGGTCCGCATTAATCTTATCTA-3'and 5'-GGTAGCATACGGTTCCACGCA-3' which yield a 1700bp PCR product; the *K5-tTA* primers 5'-CCAGGTGGAGTCACAGGATT-3' and 5'-ACAGAGACTGTGGACCACCC-3'and the primers for the *tetO-Edn3* yield a 463 bp product and bind to the 3' end of the *Edn3* cDNA (5'-GGCCTGTGCACACTTCTGT-3') and to non-coding DNA (5'-TCCTTGTGAAACTGGAGCCT-3'); Dct-Ednrb 5'-ACAAGGAAGACTGGCGAGAA-3' and 5'-TCCTCCCCCTTGCTGTCCTGC-3' which produce a 2218 bp fragment flanking the *Dct* promoter. 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.

2.2.2. Tumor Measurements

After the genotypes of the mice were determined at approximately four weeks of age, mice from the experimental *Dct-Grm1/K5-Edn3* and *Dct-Grm1/Dct-Ednrb*, and control (*Dct-Grm1/ K5*, *Dct-Grm1/ Edn3* and *Dct-Grm1*) groups were monitored on a weekly basis for tumor development. The time of tumor appearance for each mouse was calculated as the time between the date of birth of the mouse and the date of the appearance of the first raised melanocytic lesion. The data was plotted as a Kaplan-Meier graph using the Statistical Package for the Social Sciences (SPSS) program Version 13.0 and a log rank test was used for statistical analysis.

Tumor growth of the experimental mice and their control counterparts were measured weekly for 16 weeks using calipers to calculate tumor volume using the equation: length X breadth^2 X 0.5 [14]. Student's t-test was used for the statistical comparisons. Differences were considered statistically significant at $p<0.05$.

2.2.3. Metastasis Analysis

At approximately 9-12 months of age, tumor burdened mice were examined for the presence of metastases in the lymph nodes as well as other common sites of melanoma metastasis such as the lungs and brain. Lymph nodes and organs considered to contain metastatic lesions were fixed overnight in 4% paraformaldehyde in phosphate buffered solution (PBS, pH 7.4), subjected

to 10% sucrose (in PBS, pH 7.4) for six hours, followed by 20% sucrose (in PBS, pH 7.4) overnight and finally embedded in OCT medium (Ted-Pella, CA) and stored at -80°C. Samples were sectioned and processed for immunofluorescence.

2.2.4. Immunofluorescence

For the analysis of proliferating tumor cells, cryosections (10µm thick) of tail tumors at three different stages of tumor development were obtained. The stages were classified as: Stage 1- when the tumor became a raised lesion at approximately 0.5mm in height, Stage 2- at approximately 25-30mm^3, and Stage 3- after the tumor developed for 9-12 months. The sections were bleached overnight in 10% hydrogen peroxide in PBS (pH 7.4) in order to decolorize melanin. After bleaching, the sections were washed with PBS (pH 7.4) three times for 15 minutes and then blocked for 1 hour and 15 minutes in a solution of 10% Goat Serum (Gibco), 0.1% Triton X-100 (Sigma, MO) in PBS (pH 7.4). For immunolabeling, the sections were incubated overnight at 4°C with Anti Ki-67 polyclonal antibody (1:100, Abcam, MA), for one hour with secondary antibody Alexa Flour 488 Goat Anti-Rabbit (1:200, Invitrogen, NY) and counterstained with Hoechst. The Ki-67 positive cells were counted in 10 different sections, 5 fields of view each section, per tumor, using Image J and shown as a percentage of the total number of cells identified by Hoechst.

To confirm the presence of metastatic lesions in lymph nodes and visceral organs, cryosections (10µm thick) were labeled with antibodies against the melanoma marker S-100 (1:200, Dako, CA) and the melanocyte marker Trp1

(1:200, polyclonal rabbit **antibody Pep1 provided by** Vincent Hearing, NIH). The secondary antibody used was Alexa Flour 488 Goat Anti-Rabbit (1:200, Invitrogen, NY) and the sections were counterstained with propidium iodide. Images were obtained and analyzed with a Leitz DMRB fluorescent microscope and photographed with a Leica DC 500 camera.

2.2.5. Real-time RT-PCR

RNA was extracted from tail tumors at stages 1, 2, and 3 of tumor development (n=5 for each stage). The RNA extraction and purification were conducted using TRIzol Reagent (Invitrogen, NY), the RNeasy Mini Kit (Qiagen, CA) and RNase Free DNase Set (Qiagen, CA). The RNA quality and quantity were assessed using absorbance readings at 260nm, 280nm and 230nm with the Nano Drop (ND-1000) spectrophotometer. First strand cDNA synthesis of RNA was performed using the Maxima Reverse Transcriptase Kit (Fermentas, MA).

Semiquantitative analysis of transcripts encoding α_v, α_2, β_1, β_3, VEGF-A, VEGF-B, Hif-1α, N-cadherin, E-Cadherin, EDNRB, MITF , MCAM, Galectin 3, and GAPDH was conducted by real-time Polymerase Chain Reaction using the Applied Biosystems 7300 Real-Time PCR System. All primer sequences are listed in Table 1 of the Supplementary Data. The real-time PCR reaction was performed in triplicates with SYBR Green I dye, for a total volume of 26µl per reaction which included 1µl of cDNA, and 0.44µM of each primer and 12.5µl of Maxima® SYBR Green/ROX qPCR Master Mix (Fermentas, MA). 'No template' controls were also used in the real time experiments. The threshold cycle (Ct)

values quantified for the target transcripts and the housekeeping gene, *Gapdh*, were used to derive the fold change for each transcript between the *Dct-Grm1/K5-Edn3* experimental group and *Dct-Grm1* controls.

2.3. Results

2.3.1. Edn3 Promotes Early and Rapid Melanoma Growth

The *Dct-Grm1* mice acquire melanocytic lesions that mature into overt melanomas on the tails and ears as a result of the aberrant glutamate signaling. However, the spontaneous acquired melanomas do not give rise to metastatic lesions in distant organs [22]. The melanoma mouse model was therefore a suitable candidate to assess a putative role for EDN3 in tumor progression and metastasis. The *Dct-Grm1* mice were crossed with the *K5-Edn3* mice to generate an experimental population of transgenic *Dct-Grm1/ K5-Edn3* animals as well as a population of control counterparts that lack the over-expression of EDN3 (*Dct-Grm1*, *Dct-Grm1/K5*, *Dct-Grm1/Edn3*). The *K5-Edn3* mice produce higher levels of Edn3 in the skin starting at around embryonic day 12.5 that persist into adulthood. The elevated levels of EDN3 lead to an increase in the number of melanocyte precursors and their maintenance in the adult skin resulting in a hyperpigmentation phenotype [25]. The experimental *Dct-Grm1/K5-Edn3* mice (n=25) and the control mice (*Dct-Grm1*, *Dct-Grm1/K5*, *Dct-Grm1/Edn3*) (n=25) were monitored weekly to observe differences in tumor development between the two groups. The time it took for the first melanocytic lesion to develop on the mice was calculated from the date of birth of the mice to the date when the first tumor appeared. The data plotted on a Kaplan Meier graph, revealed that the

Dct-Grm1/K5-Edn3 mice acquired their first melanocytic lesion significantly earlier ($p<0.001$) than the *Dct-Grm1* control mice (Fig.1A). The *Dct-Grm1/K5-Edn3* mice acquired their first tumor on average at about 23 weeks after birth, while the average time for the *Dct-Grm1* control mice to acquire their first tumor was at approximately 31 weeks after birth.

Once present, the tumors of the *Dct-Grm1/K5-Edn3* mice grew faster than the *Dct-Grm1* tumors. Ear tumors grew 13 times faster in the experimental mice (n=20) than in the control mice (n=20) (p=1.3864E-06). The size of the tumors at each week was also significantly different between experimental and control groups ($p \leq 0.009$) (Fig. 1B-D). The tail tumors of the *Dct-Grm1/K5-Edn3* mice grew 5 times faster than those of the *Dct-Grm1* mice. Over the 16 week period, the size of the *Dct-Grm1/K5-Edn3* tail tumors (n=20) were significantly larger (p=0.004) than the controls (n=20) (Supplementary Fig. S1).

In order to determine the underlying mechanism for the difference in tumor growth between the control and experimental animals, we counted the number of proliferating tumorigenic cells by labeling the lesions at Stages 1 and 2 with an antibody against Ki-67 (Fig. 2A-L). At both stages the *Dct-Grm1/K5-Edn3* mice had a higher percentage of Ki-67 positive cells than the *Dct-Grm1* controls (Fig. 2M, N), but the difference was only significantly greater for the *Dct-Grm1/ K5-Edn3* group at Stage 2 (p=0.01).

2.3.2. Edn3 Promotes the Formation of Dorsal Skin Tumors

Apart from the ear and tail tumors *Dct-Grm1/K5-Edn3* transgenic mice also acquired pigmented melanocytic lesions on their dorsal skin (Supplementary

Fig. S2), a condition not observed or previously described in the *Dct-Grm1* control mice. The dorsal skin lesions were observed in 75% of the *Dct-Grm1/K5-Edn3* mice (n=40) and could be detected as early as 12 weeks after the mice were born. Like the ear and tail tumors, the dorsal lesions first appeared as non-raised skin lesions and continued to form large overt tumors on the skin. The dorsal lesions stained positive for S100 and Trp1, thus confirming these lesions were of melanocytic origin and were spontaneous melanomas similar to the tail and ear tumors.

2.3.3. Edn3 Promotes Melanoma Metastasis to Lymph Nodes and Visceral Organs

The *Dct-Grm1/K5-Edn3* and the *Dct-Grm1* control mice were examined for signs of metastasis after the first melanocytic tumor had been present for approximately 6.5 to 9 months. Eight-one percent of the *Dct-Grm1/K5-Edn3* (n=37) mice presented with metastases in lymph nodes and distant organs while none of the *Dct-Grm1* control counterparts (n=35) did. The *Dct-Grm1* mice showed micrometastases, on average two per lung (n=3), but the areas where they were found were not labeled with antibodies against Trp-1 and S100. All experimental animals showed enlarged highly pigmented lymph nodes. Although some control animals also displayed enlarged lymph nodes, they never stained positive for Trp1 indicating the absence of cells of melanocytic origin (Supplementary Fig. 3A-F). The lymph nodes of the *Dct-Grm1/K5-Edn3* were significantly larger ($p \leq 0.001$) than those of the *Dct-Grm1* control mice (Supplementary Fig. 3G). The most common site where secondary lesions were

observed was the lungs (23/37) (Fig.3A-B). The lungs' pigmented lesions ranged from large overt metastatic tumors, smaller pigmented lesions that were clearly visible with the naked eye, to much smaller micrometastases that were only visible under a dissecting microscope. These lesions labeled positive for Trp1 and S100 confirming their melanoma nature (Figure 3C). The brain was the second most common site for melanoma metastasis (14/37) (Fig. 3D-F). Metastatic lesions were also found in the salivary glands (9/37), spleen (2/37) and kidney (1/37) of the *Dct-Grm1/ K5-Edn3 mice*. The lesions found in these organs were also positive for S100 or Trp1 (n=5). The *Dct-Grm1/K5-Edn3* mice often had metastatic lesions in multiple organs concomitantly, for example, in the brain and lungs (Supplementary Table 2).

2.3.4. Edn3 Alters the Expression of Genes Involved in Metastasis

Because the *Dct-Grm1/K5-Edn3* mice had a more aggressive metastatic phenotype than the *Dct-Grm1* controls we assessed for differences in the expression of genes implicated in melanoma metastasis that included cell adhesion and cell-matrix interaction proteins, angiogenic factors, and relevant melanocytic developmental genes. At Stage 1 there was significant up-regulation of α_v, α_2, β_3 integrin subunits, *VEGF-A*, both *E-cadherin* and *N-cadherin* as well as *Galectin 3* in the *Dct-Grm1/ K5-Edn3* tumors when compared to the controls (Fig. 4A). At Stage 2, only *VEGF-B* and *Hif-1α* were significantly up-regulated in the experimental tail tumors (Fig. 4B), while at Stage 3 the expression levels of *N-cadherin*, *E-cadherin*, *Galectin 3*, *Mitf* and *MCAM* were

found to be significantly higher in the *Dct-Grm1/ K5-Edn3* mice than the control group (Fig. 4C).

2.3.5. EDNRB Promotes Early and Rapid Melanoma Growth, and Strong Metastatic Properties

Since we did not observe much up-regulation of *Ednrb* in the lesions of *Dct-Grm1/ K5-Edn3* in comparison to those of control *Dct-Grm1* animals we investigated if forced *Ednrb* over-expression in melanocytes under the *Dct* promoter would also alter the dynamics of tumor growth and the metastatic potential of the tumorigenic cells. The *Dct-Grm1* mice were crossed with the *Dct-Ednrb* mice to generate an experimental population of transgenic *Dct-Grm1/ Dct-Ednrb* animals as well as a population of control counterparts that lack the over-expression of *Ednrb* (*Dct-Grm1*). As for the *Dct-Grm1/K5-Edn3* mice, *Dct-Grm1/ Dct-Ednrb* acquired their first melanocytic lesion significantly earlier (p=0.004) than the *Dct-Grm1* control mice (Fig.5A). The *Dct-Grm1/Dct-Ednrb* mice acquired their first tumor on average at about 26 weeks after birth, while the average time for the *Dct-Grm1* control mice to acquire their first tumor was at approximately 45 weeks after birth. The tumors of the *Dct-Grm1/Dct-Ednrb* mice grew faster than the *Dct-Grm1* tumors. Ear tumors grew 70 times faster in the experimental mice (n=11) than in the control mice (n=7) (p=3.365E-05). The size of the tumors at each week (except for week 1, 2 and 4) was also significantly different between experimental and control groups ($p \leq 0.04$) (Fig. 5B). The tail tumors of the *Dct-Grm1/Dct-Ednrb* mice grew 22 times faster than those of the *Dct-Grm1* mice. Over the 16 week period, the size of the *Dct-Grm1/Dct-Ednrb* tail tumors (n=15)

were significantly larger (p=0.001) than the controls (n=10) (Supplementary Fig. S4). Seventy six percent (n=25) of *Dct-Grm1/Dct-Ednrb* mice presented with metastasis by 1 year of age. Interestingly, the percentage of *Dct-Grm1/Dct-Ednrb* animals that had metastasis in the brain (60%, Supplementary Fig. S5) was almost double that of *Dct-Grm1/ K5-Edn3* animals (37%).

We also generated a small number of animals over-expressing both *Edn3* and *Ednrb* by crossing *Dct-Grm1*/*K5-Edn3* with *Dct-Grm1/Dct-Ednrb* animals. *Dct-Grm1/K5-Edn3/Dct-Ednrb* mice (n=19) acquired their first melanocytic lesion significantly earlier than the *Dct-Grm1-K5-Edn3* (n=16; p=0.001) and *Dct-Grm1/Dct-Ednrb* (n=4; p=0.006) mice (Supplementary Fig. S6). The *Dct-Grm1/K5-Edn3/Dct-Ednrb* mice acquired their first tumor on average at about 13 weeks after birth, while the average time for the *Dct-Grm1/K5-Edn3* mice to acquire their first tumor was at 22 weeks and for the *Dct-Grm1/Dct-Ednrb* was at 24 weeks after birth. All *Dct-Grm1/K5-Edn3/Dct-Ednrb* animals had metastasis in the lungs by 1 year of age. These results clearly demonstrate that the up-regulation of the *Edn3/Ednrb* signaling pathway in melanocytes accelerates the process of melanomagenesis and stimulates malignancy.

2.4. Discussion

In this study we created novel metastasis melanoma mouse models in order to evaluate the role of the *Ednrb* /*Edn3* axis in melanoma progression. The *Dct-Grm1/ K5-Edn3* mouse model showed that excessive Edn3 paracrine signaling from keratinocytes affects melanoma progression and metastasis. There have been very few genetically engineered mouse models of melanoma

with visceral metastasis reported [26]. To our knowledge, the *Dct-Grm1/ K5-Edn3* is the first spontaneous metastasis melanoma mouse model that is dependent on the presence of a signaling molecule exclusively in the tumor microenvironment. The *Dct-Grm1/ Dct-Ednrb* model further supports a role for the endothelin signaling pathway in melanoma progression and corroborates recent findings that over-expression of *Ednrb* may be an important factor driving melanoma brain metastases[27].

Previous studies using melanoma cell lines showed that inhibition of Ednrb resulted in the decrease of tumor growth [14, 21, 28]. Thus, EDN3 may be one of the critical mitogenic factors directly affecting melanoma growth as its up-regulation in the *Dct-Grm1/ K5-Edn3* mouse not only significantly shortened the length of time it took for the development of melanocytic lesions but also increased how fast both ear and tail tumors grew. Comparative immunofluorescence analysis of Ki-67 using two different stages of melanoma development also showed significantly higher amounts of proliferating cells in the tail tumors of the *Dct-Grm1/ K5-Edn3* mice. Thus, in vivo over-expression of *End3* alters melanoma progression kinetics leading to an increase in growth rate. Since endothelins are known to participate in angiogenesis, the effect of EDN3 in melanoma growth rate may be due to an increase in neovascularization that is necessary to provide the tumor with adequate supplies of nutrients and oxygen in order to survive and grow past 2mm [29].

Endothelin receptor B has been closely associated with the melanoma metastatic phenotype. Endothelin receptor B gene expression is higher in

metastatic melanoma cell lines and biopsies in comparison to nevi and primary melanomas [17, 20, 30]. It was suggested that Edn1 was the primary ligand acting on EDNRB to promote melanoma progression while EDN3 was not as vital since it was not found to be up-regulated by ultraviolet light [17, 31, 32]. Notwithstanding, Tang and colleagues (2008) found that EDN3, and not EDN1, was significantly up-regulated more than 10 fold in metastatic melanoma biopsies when compared to benign nevi and promoted melanoma cell survival [18]. Additionally, EDN3 was required along with other growth factors and ultraviolet light to transform human melanocytes into melanoma in vivo [33]. Combined with our results, these findings show that the presence of Edn3 in the tumor microenvironment contributes to melanoma progression.

To further characterize the effects of EDN3 on melanoma metastasis we examined whether its presence would enhance melanoma malignancy in the *Dct-Grm1/ K5-Edn3* mouse model when compared to the *Dct-Grm1* mice that have limited metastatic capability. It was recently reported that the *Dct-Grm1* mice have pigmented Grm1/Dct positive disseminated cells in the lymph nodes and non-pigmented Dct positive cells in the lung and liver [24]. Nevertheless, the *Dct-Grm1/K5-Edn3* mice have a considerable more aggressive melanoma phenotype than the *Dct-Grm1* control mice. Thus, EDN3 in the tumor microenvironment must lead to cellular and molecular changes required for the metastatic process. The occurrence of distant metastases in the *Dct-Grm1/Dct-Ednrb* mice, albeit with smaller penetrance when compared to the *Dct-Grm1/K5-Edn3* animals, indicates that the levels of ligand available in the skin and/or lesions is not

limiting for the changes required to enhance the metastatic potential of the tumorigenic cells.

Gene expression analysis of *Dct-Grm1/K5-Edn3* tail tumor development indicated that changes were stage specific and that the metastatic process was occurring as early as the lesions were noticeable. One of the most surprising findings was that the expression levels of *cadherins*, in any of the stages examined did not follow the E-cadherin to N-cadherin switch that has been widely accepted as a key component in melanomagenesis [34-36]. Except for the 2-3mm stage where *E-cadherin* was slightly down-regulated and *N-cadherin* was slightly up-regulated, both cadherins showed similar expression levels in the other two stages. One possible explanation for the failure to detect the cadherin switch is that we compared gene expression levels between two types of tumors and not between normal and transformed melanocytes where the switch normally occurs. Another possible explanation is that unlike human skin, mouse melanocytes are not found in the epidermis but reside primarily in hair follicles. Even though the melanocytes in the *Dct-Grm1/K5-Edn3* mice are localized around the epidermal-dermal junction, they may not be under the same type of control from the keratinocytes. Therefore *E-cadherin* levels may not need to be drastically reduced in these tumors.

The potential role of Edn3 in tumor angiogenesis is supported by the expression changes in *VEGF-A*, *VEGF-B* and *Hif-1α*. The observed up-regulation of *VEGF-A* in tumors at Stage 1 suggests its involvement in the recruitment, proliferation, and differentiation of endothelial cells that are important to the tumor

microenvironment [37]. More striking is the fact that the only two genes up-regulated at Stage 2, when the *Dct-Grm1/ K5-Edn3* tail tumors begin to grow faster than those in the *Dct-Grm1* controls, were *VEGF-B* and *Hif-1α*. These results corroborate previous studies that showed that Edn1 and Edn3 up-regulate the expression *of VEGF and Hif-1α* [38, 39].

The changes in the expression of cell-matrix and cell adhesion molecules such as integrin α_v, β_3 and α_2 subunits as well as *Galectin 3* in *Dct-Grm1/ K5-Edn3* lesions at stage 1 suggest that the tumorigenic cells gain migratory and metastatic properties very early in tumor development. The up-regulation of integrin β_3 subunit is normally used to characterize the melanoma vertical growth phase (VGP), is found on 80% of VGP's, 100% of metastatic melanomas [40], and correlates to the presence of lung metastases. Both the $\alpha_v \beta_3$ subunits and Galectin 3 function as receptors for elastins and elastin-derived peptides that have been shown to promote tumor progression and metastasis [41]. *Galectin 3* was also up-regulated at stage 3 along with *MCAM*, when melanoma lesions in distant organs were detected indicating that Edn3 may exert its effects throughout the metastatic process. A previous study showed that the silencing of *Galectin 3* in metastatic melanoma cells in nude mice significantly decreased the number of lung metastases [42]. Melanoma cell adhesion molecule gene expression progressively increases during melanoma progression until it is at its highest in metastatic melanoma cells [43-45], and MCAM aids in the later stages of metastasis including extravasation and establishing new foci of growth [46].

The other gene found to be up–regulated at stage 3 was the transcription factor *Mitf*. This result is in accordance with some but not all expression studies performed with human biopsies and cell lines. Microphthalmia associated transcription factor (*MITF*) was sometimes found to be highly expressed in benign nevi, and either absent or expressed at high levels in metastatic melanomas [47-49]. The expression of *MITF* in melanoma specimens has been inversely correlated to that of *Hif1-α* and the induction of *Hif1-α* in melanoma cell lines led to a decrease in the levels of *MITF* [50] . We observed the same relationship at all stages examined: when the expression of *Hif1-α* was high, *Mitf* levels were very low (stages 1 and 2) and vice-versa (stage 3).

Together, our findings show that increased signaling via Ednrb, either by higher levels of the receptor on melanocytes or the ligand in the microenvironment, promotes a more malignant melanoma phenotype, and can stimulate tumor growth and metastasis. The *Dct-Grm1/ K5-Edn3* and *Dct-Grm1/Dct-Ednrb* melanoma mouse models can be employed to further our understanding of changes that lead to melanoma progression and test strategies to prevent these changes in order to inhibit metastasis.

2.5. References

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2.6. Figure Legends

Figure 1 Time to tumor appearance and tumor growth rate in *Dct-Grm1/K5-Edn3* mice. (A) Kaplan–Meier curve of tumor-free survival. The tumor free-survival period of *Dct-Grm1/K5-Edn3* mice (n= 25) was significantly shorter (p=0.001, log-rank test) than that of *Dct-Grm1* controls (n=25). (B-C) *Dct-Grm1* and *Dct-Grm1/K5-Edn3* mice with same age ear tumors (arrows). (D) Weekly tumor size of *Dct-Grm1/K5-Edn3* (n=20) ear tumors is significantly larger (p= 1.3864E-06, T-test) than that of *Dct-Grm1* controls (n=20) Error bars, s.e.m. The sizes of the *Dct-Grm1/K5-Edn3* and *Dct-Grm1* control group ear tumors were significantly different at each week (*p ≤ 0.009, T-test).

Figure 2 Proliferation analysis of *Dct-Grm1/K5-Edn3* mouse tumors. (A-L) Representative sections of *Dct-Grm1/K5-Edn3* and *Dct-Grm1* tail tumors at Stage 1 and 2 labeled with Ki-67 antibody (green) and counterstained with Hoescht (blue). Scale bar: 50 μm. (M and N) Quantification of the ratio of Ki-67 positive cells to total number of cells (n=5). The number of Ki-67 positive cells was significantly higher (*p= 0.01) in the *Dct-Grm1/K5-Edn3* tumors when compared to those of *Dct-Grm1* controls only at Stage 2.

Figure 3 Metastatic lesions in *Dct-Grm1/K5-Edn3* mice. Metastases in lung (A) and brain (D). Scale bar: 2mm. Cryosections (10µm) of lung (B, C) and brain (E, F) metastases stained with Hematoxilin and Eosin (B, E) and labeled with Trp1 antibody (C, F). Scale bar: 2mm (A, D) 50µm (B, C, E, F).

Figure 4 Gene expression analysis of *Dct-Grm1/K5-Edn3* mouse tumors. Semi-quantitative real time RT-PCR analysis comparing tail tumors taken at three different stages of tumor progression from *Dct-Grm1/K5-Edn3* and *Dct-Grm1* controls. Expression levels determined to be significant when equal or larger than 2 fold.

Figure 5 Time to tumor appearance and tumor growth rate in *Dct-Grm1/Dct-Ednrb* mice. (A) Kaplan–Meier curve of tumor-free survival. The tumor free-survival period of *Dct-Grm1/Dct-Ednrb* mice (n= 18) was significantly shorter (p=0.004, log-rank test) than that of *Dct-Grm1*controls (n=18). (B) Weekly tumor size of *Dct-Grm1/Dct-Ednrb* (n=11) ear tumors is significantly larger (p= 3.365E-05, T-test) than that of *Dct-Grm1* controls (n=7) Error bars, s.e.m. The sizes of the *Dct-Grm1/Dct-Ednrb* and *Dct-Grm1* control group ear tumors were significantly different at each week (*p ≤ 0.04, T-test) except for week 1, 2 and 4.

Supplementary Figure 1 Tail tumor growth rate in *Dct-Grm1/K5-Edn3* mice. Weekly tumor size of *Dct-Grm1/K5-Edn3* (n=20) tail tumors is significantly faster (p= 0.004, T-test) than that of *Dct-Grm1* controls (n=20) Error bars, s.e.m. The sizes of the *Dct-Grm1/K5-Edn3* and *Dct-Grm1* control group tail tumors were significantly different at each week (*p ≤ 0.02, T-test).

Supplementary Figure 2 Dorsal tumors in *Dct-Grm1/K5-Edn3* mice. *Dct-Grm1/K5-Edn3* mice (n=30) developed tumors on the dorsal skin (A) that stained positive for the S100 melanoma marker (B). Scale bar: 50µm.

Supplementary Figure 3 Lymph node metastasis**.** (A-F) Representative sections of cervical lymph nodes labeled with Trp1 antibody (green). Scale bar: 50µm. (G) *Dct-Grm1/K5-Edn3* cervical (n= 8; *p= 8.99493E-06, T-test) and inguinal lymph nodes (n= 6; **p= 1.14E-04, T-test) were significantly larger than those of *Dct-Grm1* controls. Error bars, s.e.m.

Supplementary Figure 4 Tail tumor growth rate in *Dct-Grm1/Dct-Ednrb* mice. Weekly tumor size of *Dct-Grm1/K5-Edn3* (n=15) tail tumors is significantly faster (p= 0.001, T-test) than that of *Dct-Grm1* controls (n=10) Error bars, s.e.m. The sizes of the *Dct-Grm1/Dct-Ednrb* and *Dct-Grm1* control group tail tumors were significantly different at each week except for week 1(*p ≤ 0.01, T-test).

Supplementary Figure 5 Brain metastatic lesions in *Dct-Grm1/Dct-Ednrb* mice. (A) Metastases in the brain (arrows). (B) Hematoxilin and Eosin stained cryosection (10µm) brain. Scale bar: 2mm (A) 50µm (B).

Supplementary Figure 6 Time to tumor appearance in *Dct-Grm1/K5-Edn3/Dct-Ednrb* mice. Kaplan–Meier curve of tumor-free survival. The tumor free-survival period of *Dct-Grm1/K5-Edn3/Dct-Ednrb* mice (n=19) was significantly shorter than that of *Dct-Grm1/K5-Edn3* (n=16) and *Dct-Grm1/Dct-Ednrb* (n=4) mice (p=0.001 and p=0.006, respectively; log-rank test).

Supplementary Table 1: Location of metastases found in *Dct-Grm1/K5-Edn3* mice.

Supplementary Table 2: Primers used for Real Time PCR gene expression analysis

Figure 1 Time to tumor appearance and tumor growth rate in Dct-Grm1/K5-Edn3 mice.

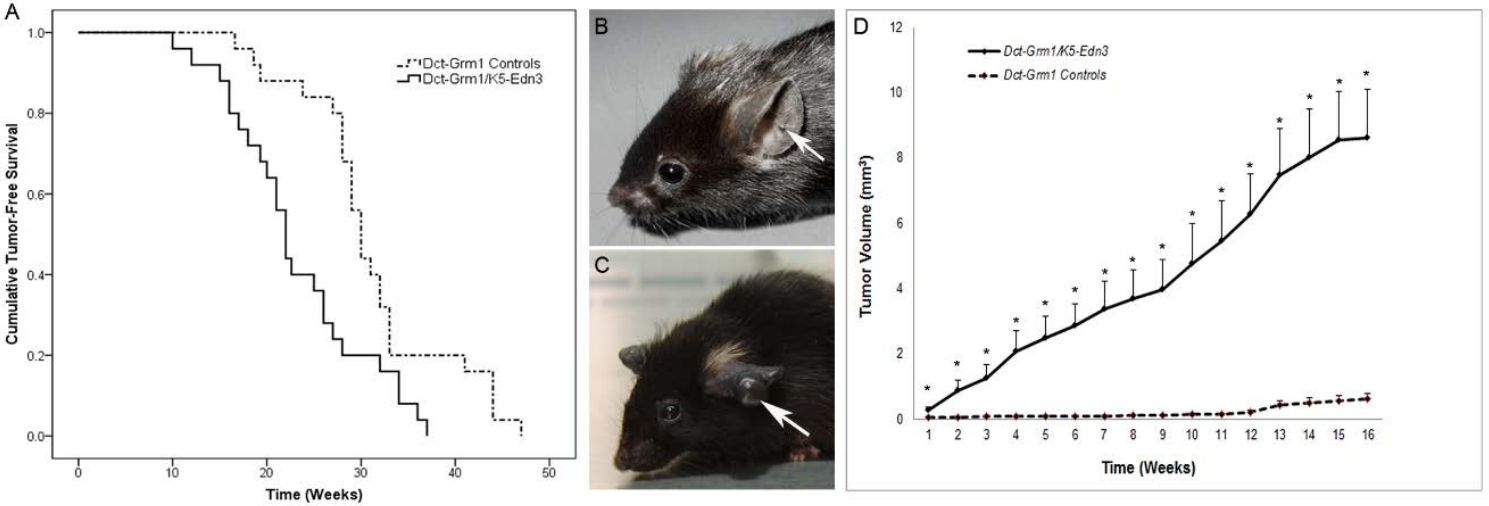


Figure 2 Proliferation analysis of *Dct-Grm1/K5-Edn3* mouse tumors.

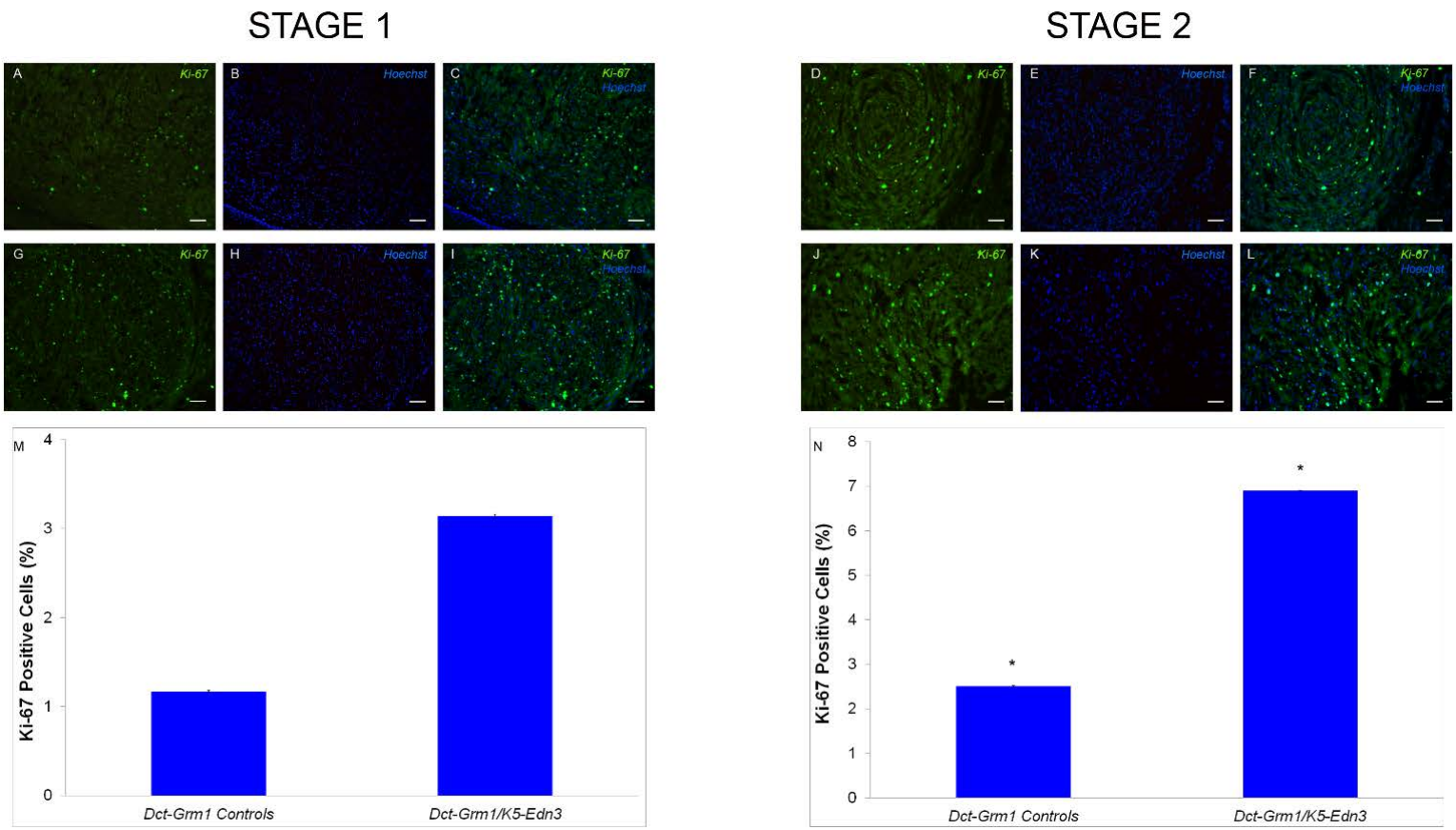


Figure 3 Metastatic lesions in *Dct-Grm1/K5-Edn3* mice.

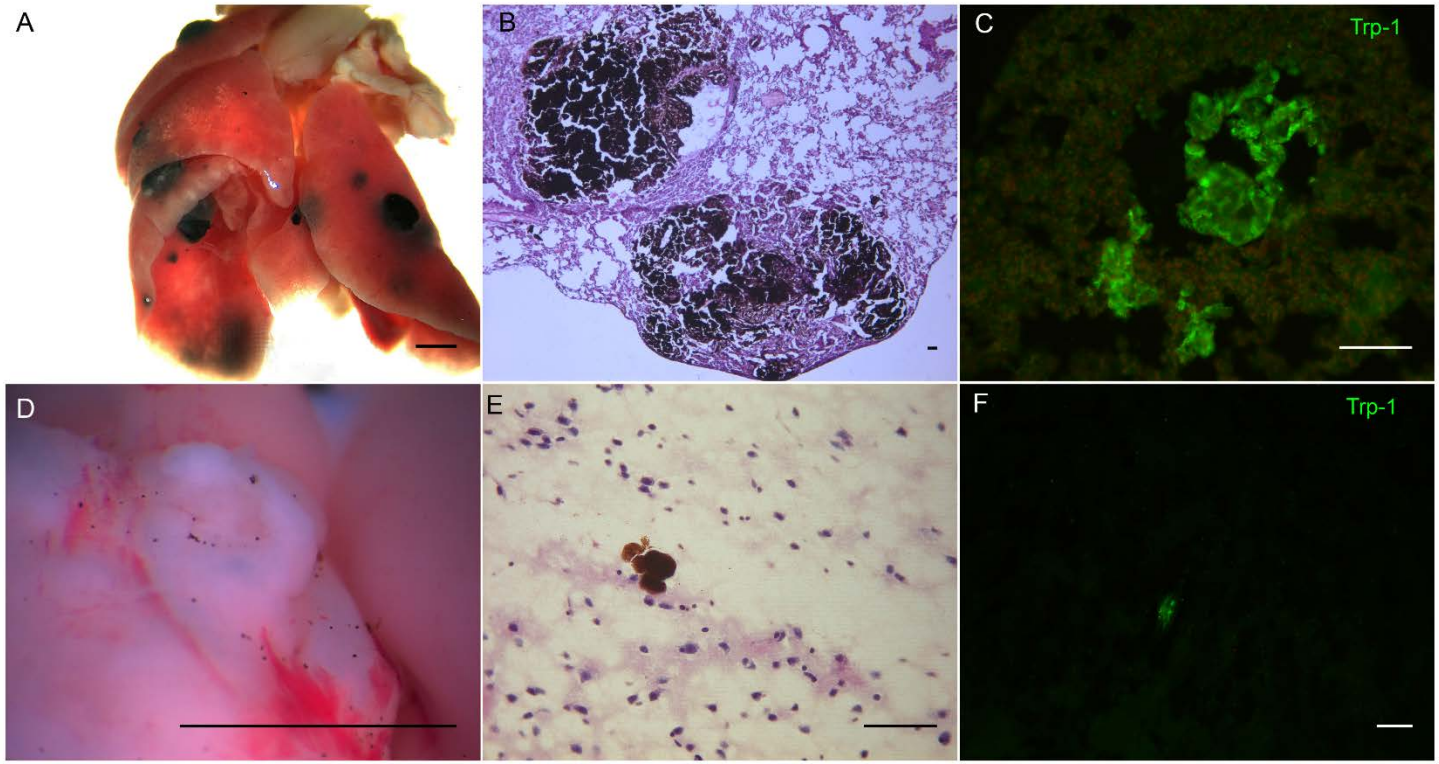
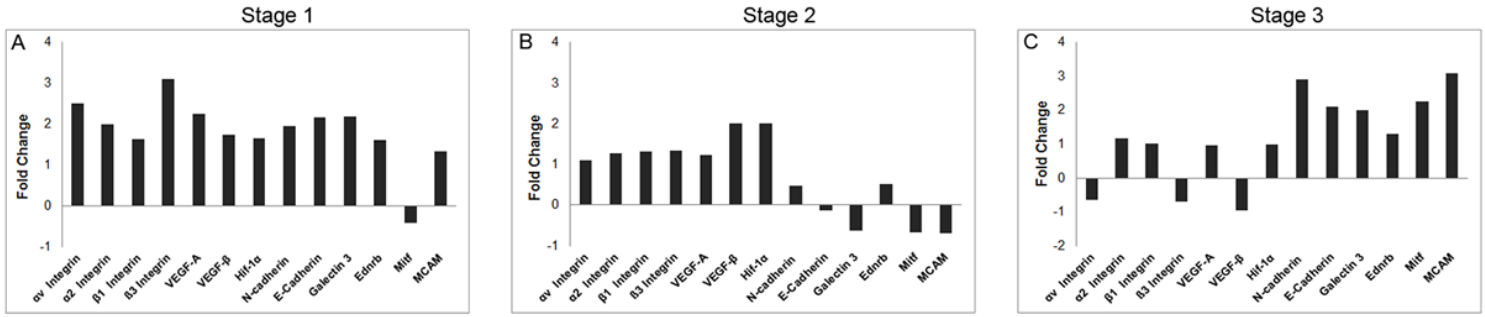
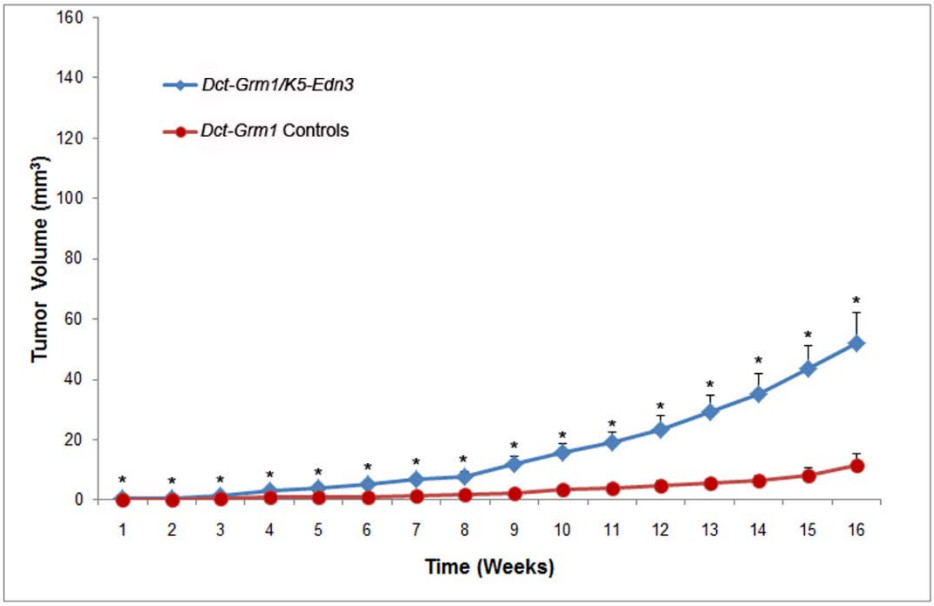


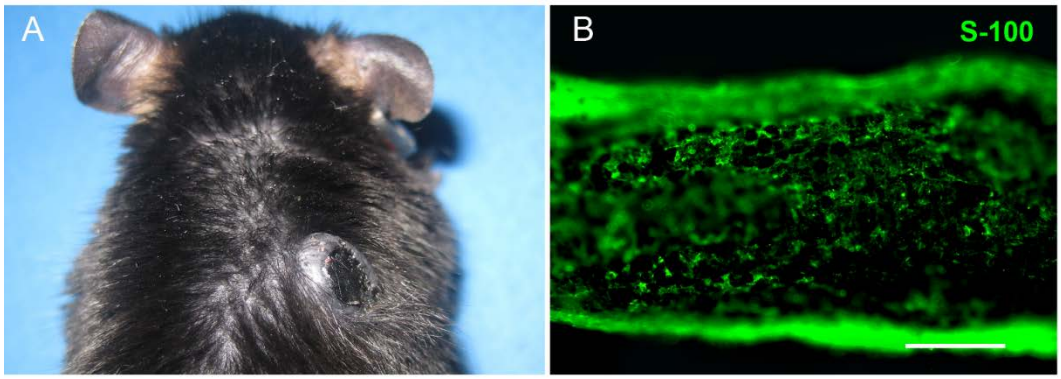
Figure 4 Gene expression analysis of *Dct-Grm1/K5-Edn3* mouse tumors.



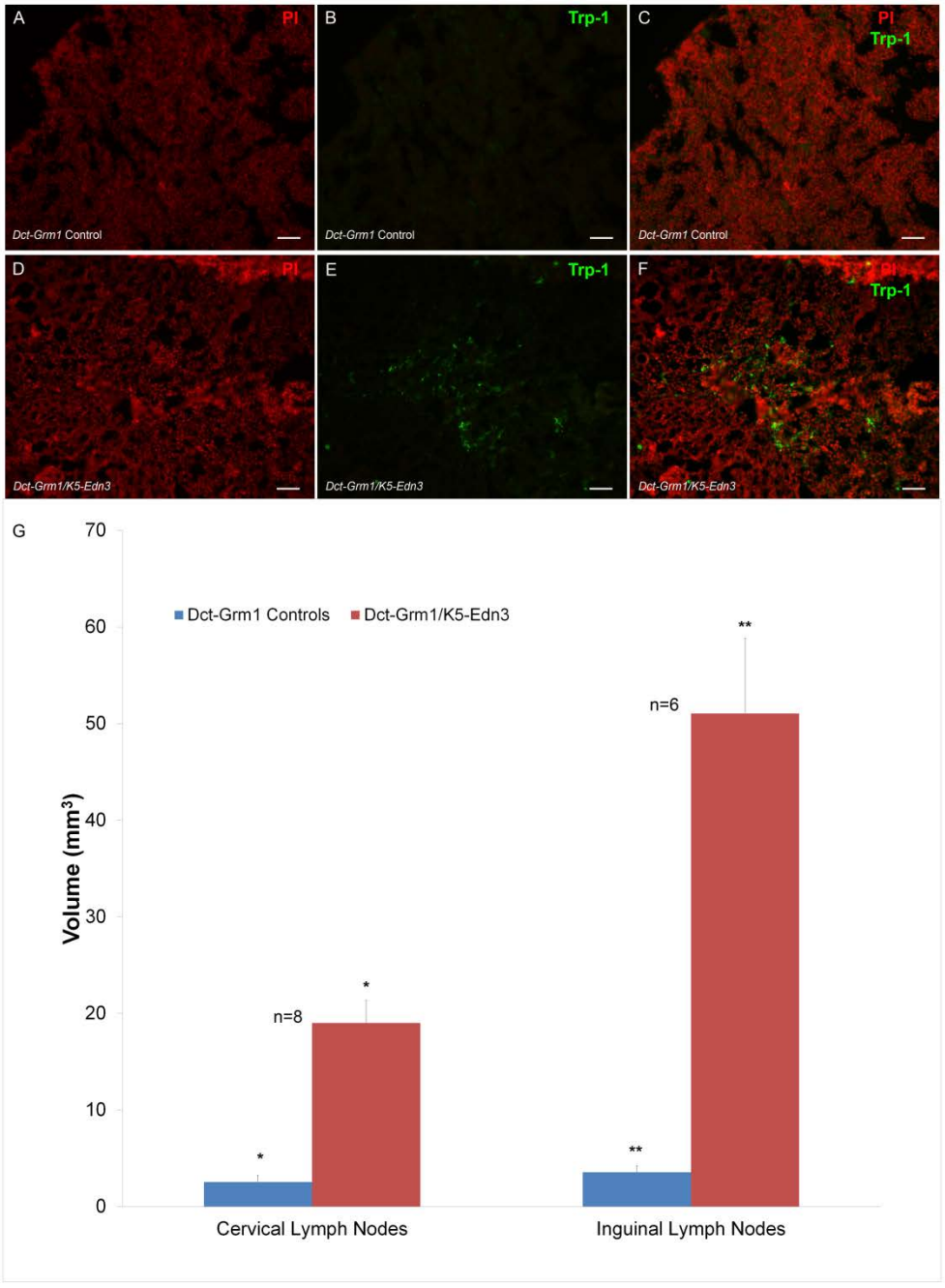
Supplementary Figure 1 Tail tumor growth rate in *Dct-Grm1/K5-Edn3* mice.



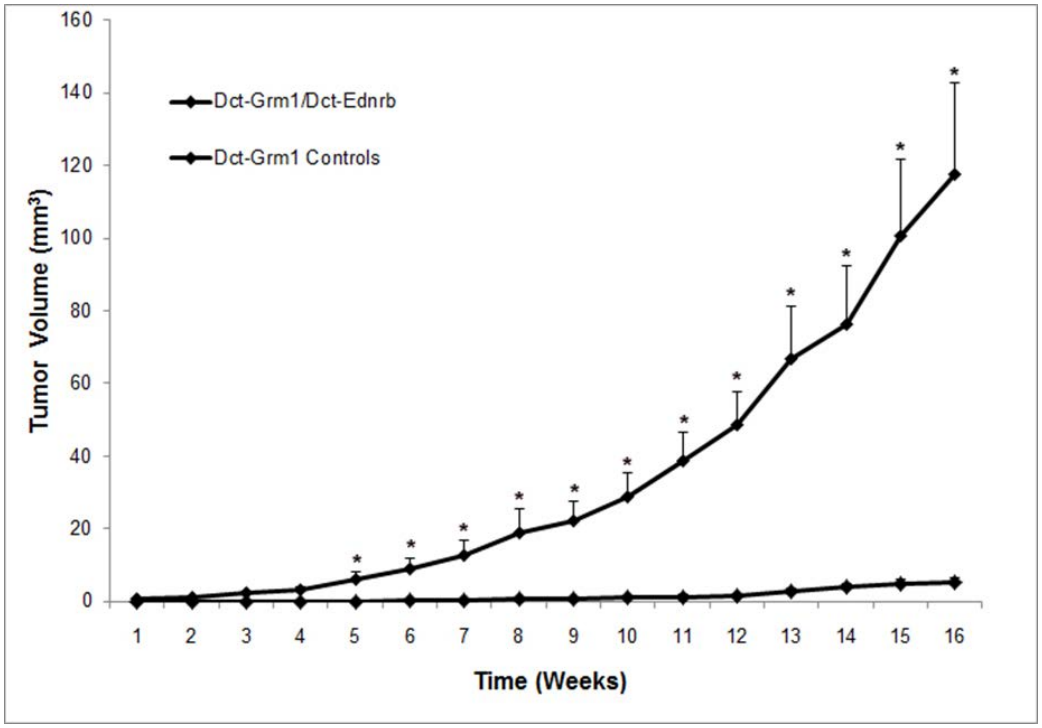
Supplementary Figure 2 Dorsal tumors in *Dct-Grm1/K5-Edn3* mice**.**



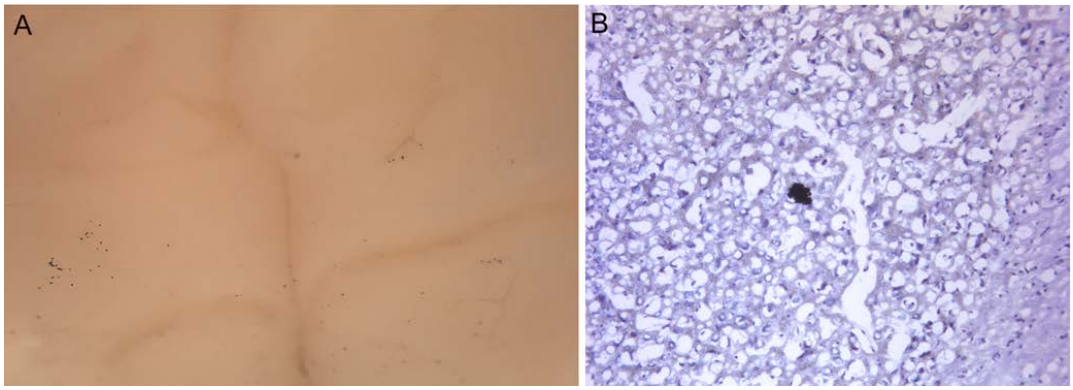
Supplementary Figure 3 Lymph node metastasis.



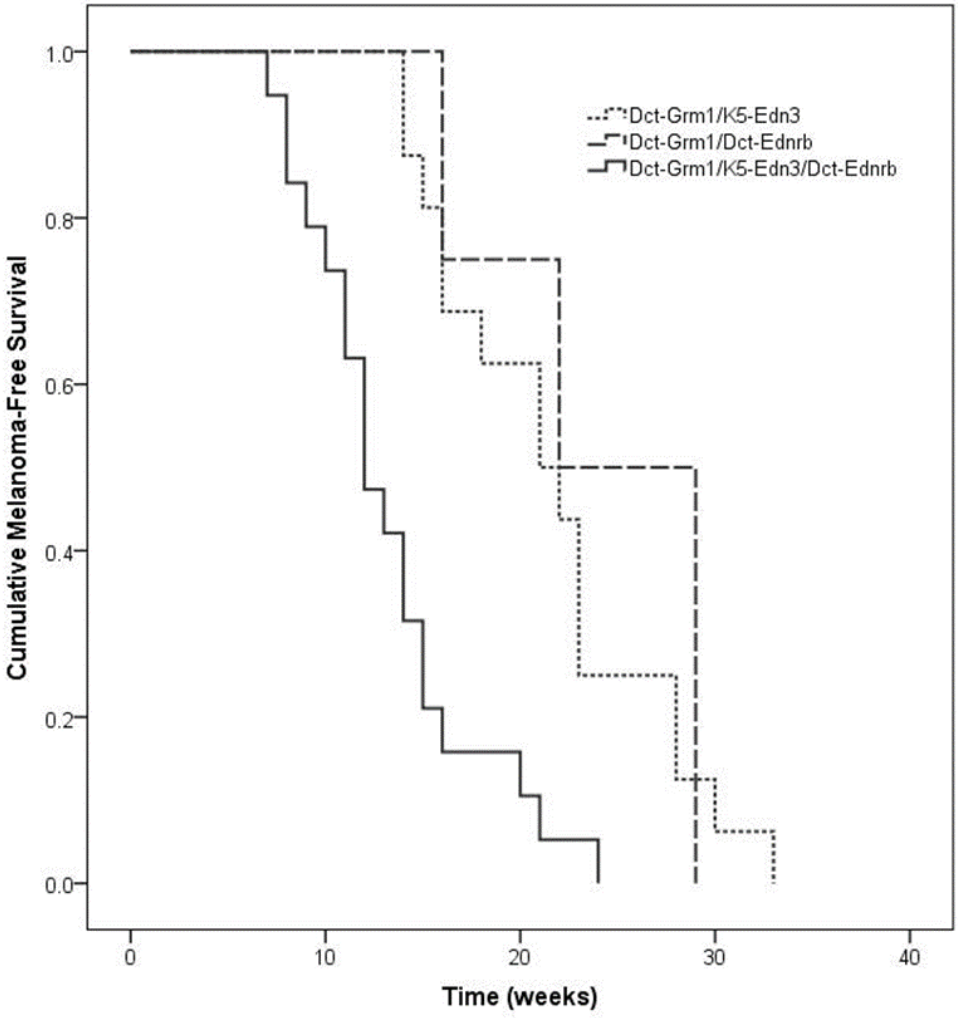
Supplementary Figure 4 Tail tumor growth rate in *Dct-Grm1/Dct-Ednrb* mice.



Supplementary Figure 5 Brain metastatic lesions in *Dct-Grm1/Dct-Ednrb* mice.



Supplementary Figure 6 Time to tumor appearance in *Dct-Grm1/K5-Edn3/Dct-Ednrb* mice.



Supplementary Table 1 Location of metastases in *Dct-Grm1/K5-Edn3* mice.

	Genotype	Lungs	Brain	Spleen	Salivary Gland	Liver	Kidney	Preputial Gland
1	*Dct-Grm1/K5-Edn3*	X			X			
2	*Dct-Grm1/K5-Edn3*				X			
3	*Dct-Grm1/K5-Edn3*				X			X
4	*Dct-Grm1/K5-Edn3*			X				
5	*Dct-Grm1/K5-Edn3*							
6	*Dct-Grm1/K5-Edn3*	X						
7	*Dct-Grm1/K5-Edn3*				X			
8	*Dct-Grm1/K5-Edn3*				X			
9	*Dct-Grm1/K5-Edn3*	X						
10	*Dct-Grm1/K5-Edn3*	X	X				X	
11	*Dct-Grm1/K5-Edn3*							
12	*Dct-Grm1/K5-Edn3*							
13	*Dct-Grm1/K5-Edn3*	X						
14	*Dct-Grm1/K5-Edn3*	X						
15	*Dct-Grm1/K5-Edn3*	X						
16	*Dct-Grm1/K5-Edn3*	X						
17	*Dct-Grm1/K5-Edn3*	X						
18	*Dct-Grm1/K5-Edn3*							
19	*Dct-Grm1/K5-Edn3*							
20	*Dct-Grm1/K5-Edn3*	X						
21	*Dct-Grm1/K5-Edn3*	X	X					
22	*Dct-Grm1/K5-Edn3*	X						
23	*Dct-Grm1/K5-Edn3*	X	X					
24	*Dct-Grm1/K5-Edn3*		X					
25	*Dct-Grm1/K5-Edn3*	X	X		X			
26	*Dct-Grm1/K5-Edn3*	X	X					
27	*Dct-Grm1/K5-Edn3*	X	X					
28	*Dct-Grm1/K5-Edn3*	X	X		X			
29	*Dct-Grm1/K5-Edn3*	X	X					
30	*Dct-Grm1/K5-Edn3*	X	X					
31	*Dct-Grm1/K5-Edn3*							
32	*Dct-Grm1/K5-Edn3*	X	X					
33	*Dct-Grm1/K5-Edn3*		X					
34	*Dct-Grm1/K5-Edn3*	X			X			
35	*Dct-Grm1/K5-Edn3*							
36	*Dct-Grm1/K5-Edn3*	X	X					
37	*Dct-Grm1/K5-Edn3*	X	X		X			

Supplementary Table 2 Primers used for Real-time PCR gene expression analysis.

	Sequence Name	Sequence 5' to 3'	Verified NCBI Reference Sequence	Reference
1	α_v sense	TTGGGGACGACAACCCTCTGACAC	NM_008402.2	J Clin Invest. 2011 Jan;121(1):226-37
	α_v anti-sense	TGCGGCGGGATAGAAACGATGAG		
2	α_2 sense	AGTATTCTGACAAAGGAGTTTC	NM_007743.2	J Clin Invest. 2011 Jan;121(1):226-37
	α_2 anti-sense	CCAGGAAGTCCAGGAGTC		
3	β_1 sense	TTCAGACTTCCGCATTGGCTTTGG	NM_010578.2	J Clin Invest. 2011 Jan;121(1):226-37
	β_1 anti-sense	TGGGCTGGTGCAGTTTTGTTCAC		
4	$ß_3$ sense	GAGCTAAGTGGGACACAGCA	NM_016780.2	Exp Eye Res. 2006 Jan;82(1):74-80
	$ß_3$ anti-sense	GTCCTCTGTAAACATAATGGCA		
5	VEGF-A sense	TTACTGCTGTACCTCCACCA	NM_001025257.3	Exp Eye Res. 2006 Jan;82(1):74-80
	VEGF-A anti-sense	ACAGGACGGCTTGAAGATGTA		
6	VEGF-β sense	TCTCGCCATCTTTTATCTCCCAG	NM_001185164.1	Invest Ophthalmol Vis Sci. 2009 Jul;50(7):3410-6
	VEGF-β anti-sense	CAGAACCCAAATCCCGTTATTG		
7	Hif-1α sense	TTGAACTAACTGGACACAGT	NM_010431.2	Designed using Primer Blast
	Hif-1α anti-sense	CTTCCACGTTGCTGACTTGATG		
8	N-cadherin sense	GGTGGAGGAGAAGAAGACCAG	NM_007664.4	World J Gastroenterol. 2009 Feb 14;15(6):697-704
	N-cadherin anti-sense	GGCATCAGGCTCCACAGT		
9	E-Cadherin sense	GTTCCGAGGTCTACACCTTC	NM_009864.2	Designed using Primer Blast
	E-Cadherin anti-sense	CAGCTCTGGGTTGGATTCAG		
10	Galectin 3 sense	ACTGCAGTAGGTGAGCATCGT	NM_010705.3	Designed using Primer Blast
	Galectin 3 anti-sense	GAGAGTGGCAAACCATTCAAA		
11	Ednrb sense	TGAAGTTTAAATTCCATGTGTTCAAC	NM_007904.3	Designed using Primer Blast
	Ednrb anti-sense	AAGTTGGTAGTATGCCTCCGAG		
12	Mitf sense	ATTTAACATAAACGACCGCATTAAG	NM_001178049.1	Designed using Primer Blast
	Mitf anti-sense	ATGGTACCGTCCGTGAGATCCAG		
13	MCAM sense	ACTCGTAAGAGTGAATTTGTAG	NM_023061.2	Oncogene. 2004 May 20;23(23):4060-7
	MCAM anti-sense	CGATGTATTTCTCTCCCTGGT		
14	Gapdh sense	ATTTGCAGTGGCAAAGTGGAGATTG	NM_008084.2	Designed using Primer Blast
	Gapdh anti-sense	TGGTTCACACCCATCACAAACATG		

CHAPTER III
ENDOTHELIN 3 ENHACES TUMOR ANGIOGENESIS IN AN EDN3 DEPENDENT MELANOMA MOUSE MODEL

III. ENDOTHELIN 3 ENHANCES TUMOR ANGIOGENESIS IN AN EDN3 DEPENDENT MELANOMA MOUSE MODEL

3.1. INTRODUCTION

Angiogenesis is a critical step triggered in response to hypoxic conditions, by the tumorigenic cells in melanoma malignancy [1–4]. The process encompasses the formation of new blood vasculature from pre-existing vessels located in close proximity to the tumors. The new vasculature can surround as well as enroot inside tumors, providing it with nutrients and oxygen to promote survival, growth and metastasis [5–7]. The melanoma cells initiate the tumor 'angiogenic switch' disrupting the balance between pro-angiogenic and anti-angiogenic factors, by secreting a number of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF) and transforming growth factors α and β (TGFα and TGFβ) [8–11]. These pro-angiogenic factors stimulate the proliferation, migration and survival of endothelial cells. Endothelial cells can then in turn secrete a number of autocrine and paracrine growth factors along with the tumor to recruit and activate pericytes, inflammatory cells and remodel the extracellular matrix to allow blood vessel formation [8–11]. One of the growth factors linked to angiogenesis in melanoma is Endothelin receptor B (EDNRB), a seven transmembrane G-protein coupled receptor [12,13].

Endothelin receptor b binds its three ligands endothelins 1, 2, 3 (EDN1, EDN2 and EDN3) with equal affinity [14,15]. Endothelins are important in melanocyte differentiation and survival as well as in endothelial localization and

vasoconstriction of blood vessels [16–18]. During melanomagenesis, EDNRB expression increases in malignant melanomas when compared to melanocytic nevi. Furthermore both ‘in vivo’ and ‘in vitro’ experiments blocking EDNRB with its antagonist BQ788 restricted melanoma growth and induced apoptosis of the tumorigenic cells [17,19,20]. Spinella and colleagues (2007) using both primary (1007) and metastatic (M10) cell lines of melanoma showed binding of EDN1 and EDN3 to EDNRB could enhance both *VEGF*, *Hif-1α* transcript levels and increase cyclooxygenase (COX)-1/COX-2 protein expression, COX-2 promoter activity and prostaglandin E2 (PGE2), all factors important in angiogenesis [21]. Although the EDNRB/EDN1 axis has been implicated in melanoma, little is known about the effect of the upregulation of EDN3 in the tumor microenvironment in melanoma progression. Tang and colleagues found that EDN3 was upregulated at both the mRNA and protein level almost 10 fold in malignant melanomas, while the other two ligands EDN1 and EDN2 did not show much change [22]. In Chapter 2 I described a novel EDN3 dependent melanoma mouse model, Dct-*Grm1- /*K5-*Edn3* mice, whose overexpression of EDN3 actuated melanocytic lesions with limited metastatic capability to a more aggressive form. The *Dct-Grm1/K5-Edn3* are hyperpigmented mice that constitutively overexpress *Edn3* under the *Keratin 5* promoter and have aberrant expression of *metabotropic glutatamate* receptor 1 (*Grm1*) under the *Dopachrome tautomerase* promoter. Real time RT-PCR studies on the melanocytic tumors of the *Dct-Grm1/K5-Edn3* mice revealed a significant fold increase in angiogenic related genes *Vegf*, *Hif-1α* and *integrins* $\alpha_v\beta_1$ *and* $\alpha_2\beta_3$ at

different stages of tumor progression when compared to the *Dct-Grm1* control group that do not overexpress EDN3. In this chapter I further examined the effects EDN3 plays in the angiogenic switch and tumor neovascularization, that may be contributing to the more characteristic aggressive phenotype of melanoma observed in the *Dct-Grm1/K5-Edn3* mice.

3.2. MATERIALS AND METHODS

3.2.1. Animal Model

The *Tg(Grm1)Epv/*K5-*Edn3* mice generated in our laboratory were crossed and genotyped. Deoxyribonucleic acid (DNA) from tail biopsies were extracted using standard techniques from the albino progeny of the matings, and Polymerase Chain Reaction (PCR) was used to genotype the mice using the following primers: Dct-*Grm1* 5'-CCGGGTCCGCATTAATCTTATCTA-3'and 5'-GGTAGCATACGGTTCCACGCA-3' which yields a 1700bp PCR product; the K5-tTA primers 5'-CCAGGTGGAGTCACAGGATT-3' and 5'-ACAGAGACTGTGGACCACCC-3'and the primers for the tet-O Edn3 yield a 463 bp product and bind to the 3' end of the *Edn3* cDNA (5'-GGCCTGTGCACACTTCTGT-3') and to non-coding DNA (5'-TCCTTGTGAAACTGGAGCCT-3'). The 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.

3.2.2. Immunofluorescence

Primary tail tumors (25-30mm^3) from *Dct-Grm1/K5-Edn3* mice (n=5) and *Dct-Grm1* control mice (n=5) were excised, fixed and embedded in OCT medium (Ted-Pella, CA) following the protocol previously described in Chapter 2. Cryosections (10µm thick) of the tail tumors were later decolorized of melanin by incubating overnight in 10% hydrogen peroxide in 1X PBS (pH 7.4). Ten sections from each tumor were immunolabeled with either the antibody Anti-CD31 (Abcam, MA) (1:50), an endothelial cell marker to visualize and quantify the vascular network of the tumor. Positive staining was identified with the secondary antibody Alexa Flour 488 Goat Anti-Rabbit antibody (Invitrogen, NY) (1:200) and the sections were counterstained with propidium iodide. A Leica Leitz DMRB florescent microscope and a Leica DC 500 camera was utilized to observe and take pictures of five fields of view at 200X of each of the ten sections for both the *Dct-Grm1/K5-Edn3* mice and the *Dct-Grm1* controls samples. The area of staining was analyzed using the pixel density feature of ImageJ software and compared between the experimental and control population using the Student T-test.

3.2.3. Angiogenesis Array

Proteome profiling using the 'Multianalyte Profiling Angiogenesis Kit' from R&D Systems (R&D Systems, MN) was used to analyze the relative expression of 53 mouse angiogenesis related proteins in tail tumors of *Dct-Grm1/K5-Edn3* experimental mice and their corresponding *Dct-Grm1* controls (*Dct-Grm1*, *Dct-Grm1/K5*, *Dct-Grm1/Edn3*). Primary tail tumors (25-30mm^3) were excised from *Dct-Grm1/K5-Edn3* mice (n=4) and *Dct-Grm1* controls (n=4) and homogenized in PBS with

protease inhibitors aprotinin (Sigma, MO), leupeptin (Sigma, MO), and Pepstatin (Sigma, MO). Protein was extracted from each of the samples and concentrations were quantified using the 'BCA Protein Assay Kit' (Thermo Scientific). Nitrocellulose membranes spotted with both control and capture antibodies for the mouse angiogenic proteins were incubated overnight, after which the membranes were washed. Streptavidin-HRP and chemiluminescent reagents were then used to detect the signal produced at each spot corresponding to the amount of bound protein, and the array data was developed onto an X-ray film for analysis using the Protein Array Analyzer program in Image J. Student's t-test was used for the statistical comparisons. Differences were considered statistically significant at $P<0.05$.

3.3. RESULTS

3.3.1. Edn3 Overexpression Increases the Vascular Density in Melanocytic Tumors

Tail tumor cryosections showed significantly ($p= 0.044$) higher levels of Anti-CD31 (Abcam, MA) staining in the *Dct-Grm1/K5-Edn3* mice (n=5) when compared to the *Dct-Grm1*mice (n=5) (Fig.1).

3.3.2. Edn3 Overexpression Affects the Levels of Expression of Angiogenic Proteins.

The Multianalyte Profiling Angiogenesis Kit' from R&D Systems (R&D Systems, MN) allowed for the parallel observation of the relative levels of 53 different mouse angiogenic proteins (Fig. 2) involved with neovascularization. When I compared protein extracts from primary tail tumors (25-30mm^3) of both *Dct-Grm1/K5-Edn3* mice (n=4) and *Dct-Grm1* control mice (n=4), I found ($p\leq0.05$) that the expression of the angiogenic

proteins Endoglin, Heparin-binding Epidermal growth factor (HB-EGF), Matrix metalloproteinase-9 (MMP-9), Angiogenin, Insulin-like growth factor-binding protein 2 and 3 (IGFBP-2 and IGFBP-3), CCN intercellular signaling protein 3 (CCN3), Edn-1 and Chemokine (C-X-C motif) ligand 16 (CXCL16) in the *Dct-Grm1/K5-Edn3* tumors was significantly higher than in the controls (Fig. 3).

3.4. DISCUSSION

During angiogenesis, endothelial cells are recruited by tumorigenic cells, whereby they dissever their normal cell-cell adhesions, migrate, proliferate, and form new cellular attachments to establish neovascularization. Platelet endothelial cell adhesion molecule (PECAM-1), also known as 'cluster of differentiation 31' (CD31) is a member of the Immunoglobin family that comprises most of endothelial intercellular junctions. The *Dct-Grm1/K5-Edn3* primary tail tumors showed significantly more CD31 immunofluorescence staining than primary tail tumors with the same average tumor volume of the *Dct-Grm1* control group. Greater levels of CD31 staining in the *Dct-Grm1/K5-Edn3* primary tail tumors suggest a greater degree of vascularization within tumors exposed to high levels of EDN3 in the tumor microenvironment. Endothelin signaling via EDNRB is reported as having direct effects on endothelial cell proliferation and neovascularization by enhancing Hif-1α mediated events [13,21]. It would be interesting to evaluate and compare the levels of hypoxia in the tail tumors of the *Dct-Grm1/K5-Edn3* mice and the *Dct-Grm1* control mice as tumorigenic cells found close to hypoxic regions have been observed to become more aggressive and metastatic [23].

The results obtained from the analysis of *Dct-Grm1/K5-Edn3* mice suggest one mechanism by which EDN3 in the tumor microenvironment increases melanoma malignancy. We profiled the expression of 53 mouse angiogenic related proteins and also found a significant increase in several angiogenic related factors, including MMP-9 and nephroblastoma overexpressed (NOV/CCN3) protein. Matrix metalloproteinases (MMP's), proteases are responsible for degrading and remodeling the ECM while matricellular proteins such as galectins, thrombospondin, osteopontin and nephroblastoma overexpressed (NOV/CCN) proteins regulate cell-matrix interaction as well as proliferation, differentiation and survival of particular cell types [24]. Both groups cooperate to facilitate the recruitment of cells involved in the neoangiogenic process as well as altering the migration of tumorigenic cells. Matrix metallopeptidase -9 along with Matrix metalloproteinase -2 are main degraders of collagen Type IV which comprises majority of basement membrane ECM [2526]. Matrix metallopeptidase -9 activity has been connected to melanomagenesis. Microarray gene chip analysis comparing cell lines of highly aggressive and poorly aggressive forms of melanoma performed by Seftor and colleagues (2001) found significant upregulation of MMP-9 [27]. Shellman and colleagues examined active secretions of normal melanocytes and cell lines of different melanoma stages using zymography techniques. They too positively correlated an increase in active MMP-9 secretions with malignancy. Matrix metallopeptidase -9 was only detected in the vertical growth phase and metastatic melanoma cell lines [28]. Matrix metallopeptidase -9 has also been associated with vascular mimicry in melanoma. Vascular mimicry is a process first described by Mantois et al. 1999, whereby tumorigenic cells of highly metastatic melanomas are able to form their own acellular microcirculatory channels,

founded of ECM and tumorigenic cells [29]. The vasculature does not include endothelial cells, instead mimic the neoangiogenic vessels formed during tumor angiogenesis. The vascular mimics connect to the host microcirculatory system and supply the tumor with nutrient rich oxygenated blood and a metastatic route [29,30]. Vascular mimicry is often linked to poor prognosis in melanoma patients [31]. When both endothelial cellular and vascular mimicry vessel number were decreased 'in vivo', MMP-9 gene and protein expression was also reduced, suggesting a role for MMP-9 in vascular mimicry and a more metastatic phenotype [31–34].

Studies on the role of matricellular protein CCN3 with respect to melanoma have been somewhat contradictory. CCN3 encoded by the *NOV* gene belongs to a family of ECM associated growth and differentiation regulators. NOV mediates its effects by binding to receptors such as integrin receptors and NOTCH [35,36]. In one study, gene and protein expression analysis revealed that co-culturing melanocytes with keratinocytes led to an increase of CCN3 levels, melanocyte proliferation was inhibited, as well as correct spatial localization of the melanocytes to the basement membrane was disrupted [37]. Melanocytes transduced with siRNA knocking down CCN3 showed decrease in their growth rates and attachment to Collagen IV when compared to cells transduced with control vectors. Later experiments using immunohistochemical analysis on different stages of melanoma progression found lower CCN3 expression levels in advanced melanomas. The '1205Lu' melanoma cell line overexpressing CCN3 became less proliferative and invasive on 3D skin reconstructs. However, no difference was observed in migration assays in the absence of Matrigel between

CCN3 overexpressing cells and controls. Matrix metalloproteinase -2 and matrix metallopeptidase -9 expression levels were also decreased in CCN3 overexpressing cells [38]. These results suggest an inverse relationship between CCN3 expression and melanoma progression.

Vallachi and colleagues (2008) however presented a positive function for CCN3 in fostering melanoma progression. A cDNA library constructed from primary and metastatic melanoma cell lines from the same patient was analyzed with Northern analysis and RT-PCR. Higher expression levels of CCN3 were found in the metastatic melanomas when compared to primary tumor cells [39]. The CCN3 protein expression was also higher in a large panel of visceral and nodal melanoma metastases of patients with short term survival or relapse patients compared to those patients with long term survival [39,40]. Immunocompromised mice transfected with melanoma cells expressing CCN3 further showed more visceral metastatic growth [39,40].

Several studies have established that the functions of CCN3 are dependent on the cellular and tissue context [24,37,41]. CCN3 is linked to prostate cancer progression [42], tumor differentiation in cartilage tumors [43] and yet has an anti-proliferative function in gliomas [44]. It is not surprising therefore that CCN3 activity in melanoma may be very complex depending on the stage of disease progression. The tumor microenvironment is also a very important facet when trying to characterize the importance or exact mechanism by which a particular factor might be involved in, especially one like CCN3 whose role has been inconsistent. In the ‘in vivo’ EDN3 dependent metastatic melanoma *Dct-Grm1/K5-*

Edn3 mouse model CCN3 was upregulated at the same stage when Hif-1α, MMP-9, and angiogenic related genes linked to melanoma such as angiogenin [45] and endoglin [46,47]. The angiogenic protein array also detected the upregulation of several Insulin-like growth factor binding proteins (IGFBPs). IGFPs are major regulators of the NOV family [39,40]. In the presence of EDN3 in the tumor microenvironment CCN3 may be elevated to enhance angiogenesis and malignancy in melanoma.

The heparin binding CXC chemokines are factors involved in inflammation and immunity that have also emerged as agents in tumorigenic angiogenesis for a number of cancers including melanoma [48]. The significance of CXCL 16, the ligand for the receptor CXCR6, has not yet been studied in melanoma or its relationship with endothelins. More research should be done to elucidate the importance of this molecule in melanoma progression.

Angiogenesis is a critical process in tumor survival and metastasis. Although it has been explored as a target of many cancer therapeutics [49], the tumor microenvironment along with the complexity of its mechanisms, that seem to vary depending on the type of cancer, diminishes or abolishes the effectiveness of treatments. A study conducted by Lahav and colleagues (2004) contradicts our findings, having reported that EDNRB signaling may have a negative effect on tumor angiogenesis as inhibition of EDNRB increased *VEGF* expression and suppressed angiogenic inhibitor *Gravin* [50]. The different results may be because Lahav and colleagues' study (2004) utilized human melanoma cell lines treated with the EDNRB antagonist BQ788 in culture, which differs from our methods that used an 'in vivo' melanoma model that also encompasses

changes EDN3 has on the tumor microenvironment and its subsequent effects on the tumorigenic cells. Melanocytic tumors in the *Dct-Grm1/K5-Edn3* melanoma mouse model tumors grow faster and display increased metastatic capability, hence EDN3 may be either directly or indirectly augmenting angiogenic related factors needed to not only increase vascularization but increase the metastatic capability of the melanocytic tumors in these mice when compared to the controls. Blocking EDN3 in the tumor microenviroement or *EDNRB* expression in the tumorigenic cells may decrease the angiogenic response in melanoma and help prevent the metastatic process. Thus the *Dct-Grm1/K5-Edn3* melanoma mouse model that has metastatic primary tumors with a strong angiogenic tumor response is a very useful tool to elucidate further the role EDNRB/EDN3 signaling plays in melanoma angiogenesis.

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3.6. Figure Legends

Figure 1 Immunofluorescence detection of Anti-CD31. 10µm sections of tail tumors (25-30mm^3) from *Dct-Grm1/K5-Edn3* (A) and *Dct-Grm1* control counterparts (B) were stained for the endothelial cell marker CD-31 and counterstained using propidium iodide. Image J pixel density analysis and the Students T-test determined that *Dct-Grm1/K5-Edn3* tail tumors had significantly more Anti-CD31 staining (p= 0.044) than the *Dct-Grm1* control tail tumors (C). Scale bar 50µm. (A, B).

Figure 3 Example of a labeled array demonstrating different levels of protein expression.

Figure 2 Proteome profiling of mouse angiogenic related proteins. Proteome profiling was used to analyze the relative expression of 53 mouse angiogenesis related proteins in primary tail tumors (25-30mm^3) of *Dct-Grm1/K5-Edn3* experimental mice and their corresponding *Dct-Grm1* controls. *Dct-Grm1/K5-Edn3* mice had significantly ($p \leq 0.05$) more expression of angiogenic proteins Endoglin, HB-EGF, MMP-9, Angiogenin, IGFBP-2, IGFBP-3, CCN3, Edn-1 and CXCL16.

Figure 1 Analysis of tumor vascular density in Dct-Grm1/K5-Edn3 mice.

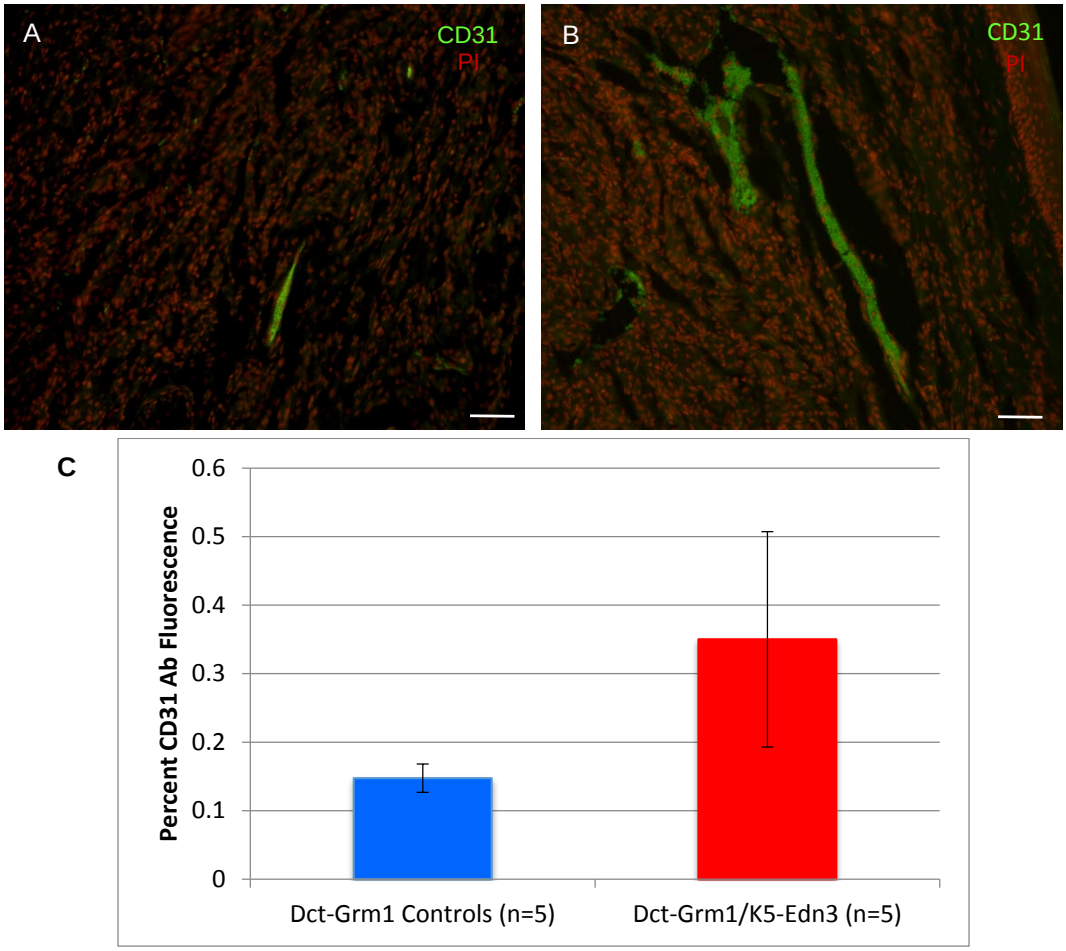
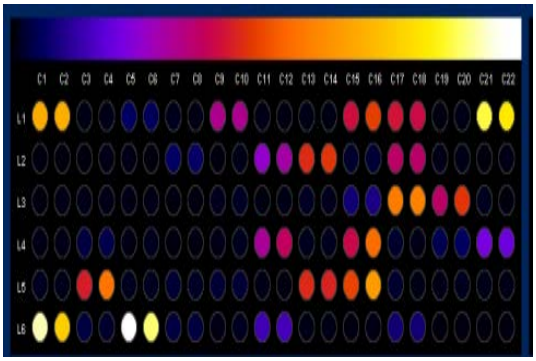
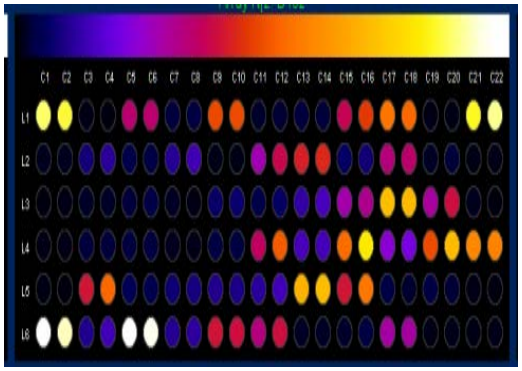


Figure 2 Example of angiogenic proteome profiling array.

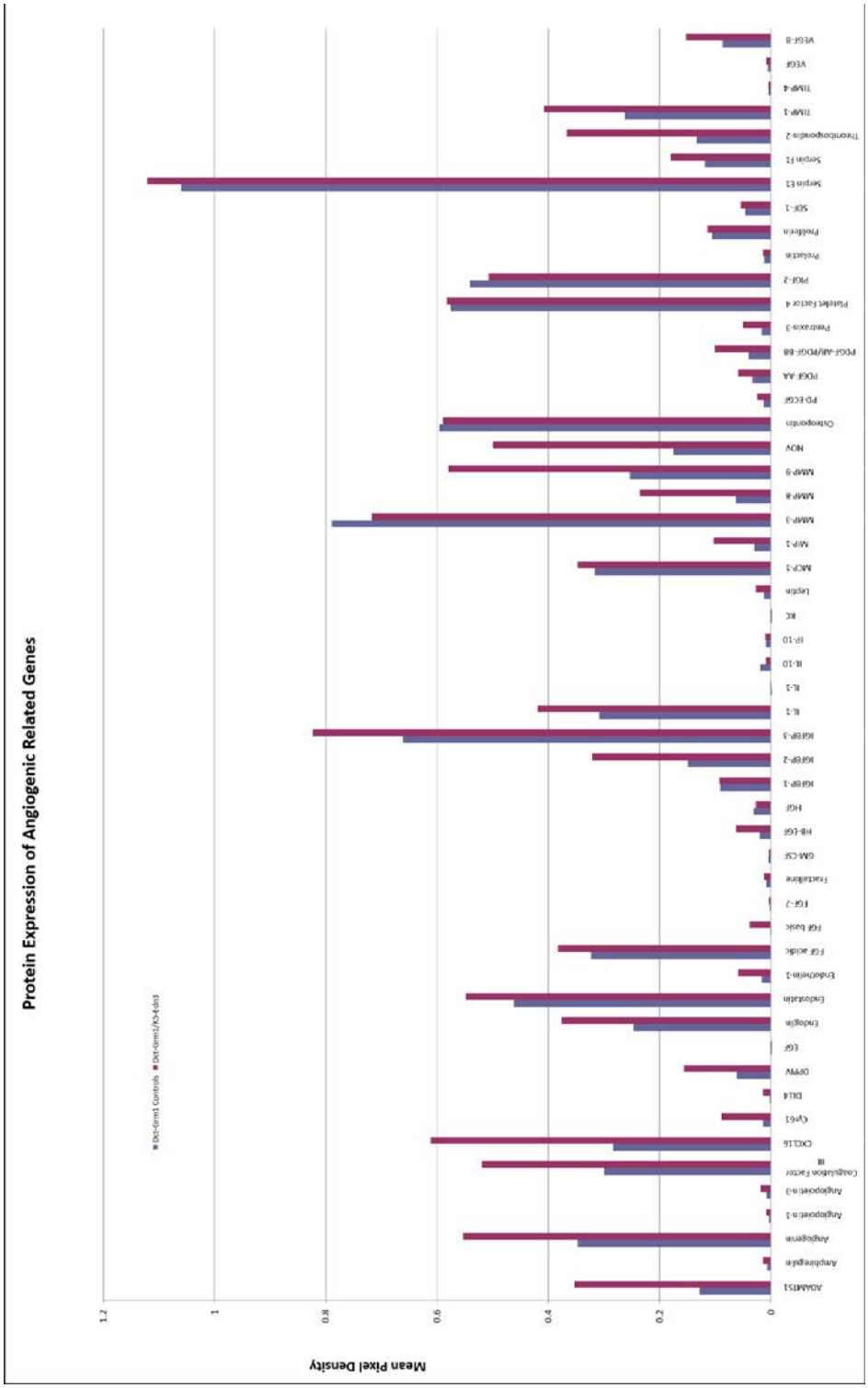


Dct-Grm1 Control



Dct-Grm1/K5-Edn3

Figure 3 Proteome profiling of mouse angiogenic related proteins.



CHAPTER IV

CONCLUSIONS, FUTURE DIRECTION AND IMPLICATIONS

IV. CONCLUSIONS, FUTURE DIRECTIONS AND IMPLICATIONS

4.1. Conclusions and Future Directions

This study provided evidence that upregulation of EDN3 in the tumor microenvironment or EDNRB in transformed melanocytes can affect tumor behavior leading to a more aggressive melanoma phenotype as well as alter expression of genes involved in cell adhesion, and the tumor angiogenic response.

In this study the over-expression of EDN3 in the skin was present prior to tumor formation and throughout its development. In order to further understand the relevance of EDN3 in tumor progression and evaluate the potential of targeting this pathway during the course of human disease, it will be important to identify the exact timing the effect of EDN3 is most critical. It will also be interesting to establish if the role EDN3 plays in tumor growth can be separated from that of its role in promoting metastasis. These questions can be addressed in the future utilizing the tetracycline inducible system in the *K5-Edn3* mouse model. EDN3 signaling can be switched off at different time points during melanoma tumor progression in *Dct-Grm1/ K5-Edn3* mice to determine when tumor appearance, growth and spread can be stopped.

My results showed that EDN3 affected the angiogenic response of melanoma by changing gene expression and an increase in the amount of tumor vascularization. Many cancer regimens focus on targeting tumor angiogenesis and tumor vasculature, a vital component in tumor survival, growth and metastasis. Anti-angiogenic therapies include targeting either factors involved in

angiogenesis such as VEGF or attempting to normalize the tumor vasculature. Some anti-cancer regimens are not effective because treatment cannot always be delivered to the entire tumor via its blood supply as irregular leaky vessels, associated with tumors, often do not reach all parts of the tumor[1]. The inadequate blood supply maintains hypoxic regions within the tumor and cancerous cells that can escape therapeutics. Often cancer patients may also exhibit anti-VEGF resistance. The mechanisms responsible for triggering resistance, and whether or not this is tumor type dependent within types of cancers has not been established.

Results from the proteome profiling array analysis showed that VEGF-β is expressed more than VEGF in the tumors (25-30 mm^3) of the *Dct-Grm1/ K5-Edn3* mice. We will investigate whether inhibition of both VEGF receptors (VEGFR-1 and VEGFR-2) using polyclonal IgG neutralizing antibodies to VEGFR-1 (R&D Systems; AF471) and VEGFR-2 (R&D Systems; AF471) at different tumor stages can significantly reduce tumor size, growth rate and prevent metastasis in the tumors of the *Dct-Grm1/ K5-Edn3* mice. These experiments will help us learn more about the importance of VEGF and potential VEGF therapeutics in melanoma treatment.

The tumors of *Dct-Grm1/ K5-Edn3* mice are an ideal system to evaluate the efficacy of novel non-invasive tools to measure blood flow and levels of oxygenation that may be used in patients to determine the status of certain types of cancer. For example, I have started to collaborate with a group from the Department of Biomedical Engineering at Florida International University to use diffuse reflectance spectroscopy and a novel spectral interpretation algorithm [2,3]

to analyze and compare regions of hemoglobin oxygenation in tail tumors of the *Dct-Grm1/ K5-Edn3* mice.

4.2. Implications

Although enlightening, studies on *EDNRB* in melanoma progression and metastasis were mostly conducted '*in vitro'* using cell lines or in 'pseudo-melanoma mouse models'. Therefore 'pro-tumor' effects of *EDNRB* are still incompletely characterized and more studies should be conducted in an '*in vivo'* model to fully understand the mechanisms involved in order to develop and test effective preclinical therapeutic regimes against melanoma metastasis. The *Dct-Grm1/ K5-Edn3* mouse model provides an 'in vivo' EDN3 dependent visceral metastatic melanoma model that does not require forced oncogene activation and in which EDN3 expression can be controlled using the tetracycline inducible system to examine melanomagenesis at various stages of progression associated with its tumor microenvironment. As a consequence of the nature of the *K5-Edn3* mice, the *Dct-Grm1/ K5-Edn3* mouse model has an increased number of melanocytes in the epidermal-dermal junction in the skin which more closely resembles the human skin phenotype which makes the model a prime candidate for investigating melanoma progression and correlating the findings to those found in humans [4].

The highly aggressive nature of melanoma renders it one of the most formidable cancers to treat. Some melanoma tumors less than 2mm in thickness have been diagnosed with metastatic potential, making it increasingly important to understand the molecular and genetic mechanisms of melanoma metastasis.

There are few mouse melanoma models that demonstrate such a robust metastatic phenotype as our *Dct-Grm1/ K5-Edn3* mouse model that can provide a valuable tool in understanding metastatic progression using 'in vivo' methods, as the microenvironment is indispensable when trying to fully elucidate metastasis. Park and colleagues (2011) using cross-species hybridization of microarray experiments to characterize the influence of the microenvironment on tumor cell behavior, showed that when melanoma cell line A375SM were xenografted into mouse brain organ sites, they acquired characteristics of neuronal lineage cells and began to express genes related to neurological signaling such as Synaptosomal-Associated Protein 21 (SNAP21), Synaptosomal-Associated Protein 91 (SNAP91) and Bassoon Presynaptic Cytomatrix Protein (*BSN*) [5]. These genes were not overexpressed by the A375SM cells when they were xenografted into other metastatic sites. Thus melanoma cells may not only adopt a more undifferentiated cell type, but may also undergo transdifferentiation due to epigenetic and transcriptome reprogramming to adopt similar characteristics of cells at the metastatic sites [5]. This process may explain the strong chemoresistance of melanoma cells. The Dct-Grm1/K5-Edn3 metastatic melanoma mouse model may therefore be an important tool for testing potential drug therapeutics.

4.3. References

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