DIFFERENTIAL EXPRESSION OF MICRORNAS IN MELANOMA

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
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Expression of microRNAs (miRs) has been shown to be altered in lung, breast, stomach, prostate, colon, and pancreatic tumors but has not been fully explored in melanoma. The malignant potential of some melanocytic lesions is difficult to predict. We hypothesized that characterization of miR expression in indeterminate/borderline melanoma tumors would lead to the identification of a unique molecular profile for lesions with high malignant potential and that expression of these miRs would contribute to the malignant phenotype of melanoma cells. In Chapter 2, we evaluated the miR expression profile of melanocytic lesions including dermal nevi (n=7), malignant melanoma (n=28), and indeterminate melanocytic tissues (n=49) by Real-Time PCR and in situ hybridization. MicroRNAs differentially expressed in melanoma compared to cultured melanocytes were identified by microarray analysis. PCR analysis revealed that primary cutaneous melanomas had a 7.6-fold over-expression of miR-21, a 13.3-fold over-expression of miR-155, and a 0.35-fold reduction in miR-211 expression as compared to benign dermal nevi (p<0.0001), whereas there was no significant difference in levels of miR-17-5p, miR-34b, miR-107, miR-130a, miR-145, miR-181b, miR-221, and miR-373. These results were confirmed by in situ hybridization. miR-21 and miR-155 were variably expressed within indeterminate lesions; however, when these lesions were categorized by mitotic activity and Breslow thickness, miR-155 levels correlated with respect to both
variables while miR-21 correlated with mitotic activity only (p<0.05). Indeterminate/borderline lesions from fourteen patients with a positive sentinel lymph node biopsy also had higher miR-21 and miR-155 expression as compared to lesions from node negative patients. We concluded that levels of miR-21 and miR-155 are elevated in melanoma lesions and in high risk indeterminate/borderline lesions, indicating that these miRs may be involved in the malignant progression of melanoma and with further study may represent a novel molecular marker of lesions likely to progress to metastasis.

In Chapter 3, we employed synthetic pre-miRs to increase the expression of miR-21 in melanoma cell lines derived from melanomas at differing stages of melanoma. Increased miR-21 did not change proliferation as measured by MTT assay and doubling time or the rate of wound closure in melanoma cell lines. However, in Boyden chamber assays, increased miR-21 expression was associated with a significant increase in invasive capacity. Upon examination of protein expression of several confirmed and putative targets of miR-21 by immunoblot, TIMP3 protein was decreased in cell lysates derived from miR-21 transfected cells. Luciferase activity confirmed that the TIMP3 3’UTR is a direct target of miR-21 in melanoma cells lines. This decrease was Depletion of TIMP3 protein with TIMP3-specific siRNA also resulted in an increase in invasion, indicating this may be one of the mechanisms by which miR-21 may be mediating its invasive effects.
To my loving parents.

For when I told them my dreams, their answer was always, “What can we do to help?”
ACKNOWLEDGMENTS

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I heartily thank the members of the Carson lab, past and present. Special thanks to Dr. Jason Zimmerer for laying the groundwork for the beginning of my project and Dr. Gregory Lesinski for support, suggestions, and assistance throughout my years in the lab.

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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>3’</td>
<td>three prime</td>
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<tr>
<td>5’</td>
<td>five prime</td>
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<tr>
<td>°C</td>
<td>degrees Celsius</td>
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<tr>
<td>Ago1</td>
<td>argonaute-1</td>
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<tr>
<td>Ago2</td>
<td>argonaute-2</td>
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<tr>
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<td>argonaute-3</td>
</tr>
<tr>
<td>Ago4</td>
<td>argonaute-4</td>
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<tr>
<td>AID</td>
<td>activation-induced cytokine deaminase</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
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<tr>
<td>APAF-1</td>
<td>apoptotic peptidase activating factor 1</td>
</tr>
<tr>
<td>ARF</td>
<td>alternative reading frame product</td>
</tr>
<tr>
<td>ARPC2</td>
<td>actin-related protein 2/3 complex subunit 2</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine tri-phosphate</td>
</tr>
<tr>
<td>b-Raf</td>
<td>v-raf murine sarcoma viral oncogene homolog B1</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B cell lymphoma-2</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<tr>
<td>BMP-2</td>
<td>bone morphogenetic protein 2</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>BMP-4</td>
<td>bone morphogenetic protein 4</td>
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<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>βTrCP1</td>
<td>β-transducin repeat-containing protein 1</td>
</tr>
<tr>
<td>c-Met</td>
<td>cellular mesenchymal-epithelial transition factor</td>
</tr>
<tr>
<td>C/EBP-α</td>
<td>CAAT enhancer binding protein α</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>CDK4</td>
<td>cyclin-dependent kinase 4</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>cyclin-dependent kinase inhibitor 2 A</td>
</tr>
<tr>
<td>CLL</td>
<td>chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>C&lt;sub&gt;i&lt;/sub&gt;</td>
<td>cycle threshold</td>
</tr>
<tr>
<td>DGCR8</td>
<td>DiGeorge syndrome critical region 8</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-dimethylbenz(a)anthracene</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double stranded ribonucleic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>eiF4E</td>
<td>eukaryotic translation initiation factor 4E</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-related kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FN1</td>
<td>fibronectin 1</td>
</tr>
<tr>
<td>FOXO3</td>
<td>forkhead box O3</td>
</tr>
<tr>
<td>HER2/neu</td>
<td>human epithelial growth factor receptor 2</td>
</tr>
<tr>
<td>HIF1α</td>
<td>hypoxia-inducible factor 1 α</td>
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</table>
HOXD3: homeobox D3
HPF: high power field
hr: hour
hTERT: telomerase holoenzyme
ICAM-1: intercellular adhesion molecule 1
IFN-β: interferon-β
IL-2: interleukin-2
INK4A: inhibitor of cyclin-dependent kinases 4 A
IRB: institutional review board
IRES: internal ribosome entry site
Ki-67: antigen recognized by monoclonal antibody Ki-67
LCM: laser capture microdissection
Let-7: lethal-7
Lin-4: lineage-abnormal-4
Lin-14: lineage-abnormal-14
LCM: laser capture microdissection
m^7GTP: 7-methylguanosine triphosphate
MAPK: mitogen activated protein kinase
MARCKS: myristoylated alanine-rich C-kinase substrate
Mcl-1: myeloid leukemia-1
MDM2: murine double minute 2
MICA: major histocompatibility complex class-I related chain A
MICB: major histocompatibility complex class-I related chain B
miPPR-21: miR-21 promoter region
miR: microRNA
miRNA: microRNA
MITF: microphthalmia-associated transcription factor
mm: millimeter
MMP-2: matrix metalloproteinase-2
MMP-9: matrix metalloproteinase-9
MNT: max-binding protein
mRNA: messenger ribonucleic acid
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCI: national cancer institute
NF45: nuclear factor 45
NF90: nuclear factor 90
NFIB: nuclear factor I/B
NFκB: nuclear factor κB
NK cell: natural killer cell
NKG2D: natural killer group 2D
nt: nucleotide
p21: cyclin-dependent kinase inhibitor 1A
p53: protein 53
PACT: protein kinase, interferon-inducible double stranded RNA dependent activator
PCR: polymerase chain reaction
PDCD4: programmed cell death protein 4
PE: phycoerythrin
PKCε: protein kinase Cε
pre-miR: precursor microRNA transcript
pri-miRNA: primary microRNA transcript
PTEN: phosphatase and tensin homolog
Ras: rat sarcoma
RB: retinoblastoma protein
RECK: reversion-inducing cysteine-rich protein with Kazal motifs
RGP: radial growth phase
RGS1: regulator of G-protein signaling 1
RISC: ribonucleic acid-induced silencing complex
RNA: ribonucleic acid
siRNA: small interfering ribonucleic acid
SLNB: sentinel lymph node biopsy
SOCS1: suppressors of cytokine signaling 1
SPP1: secreted phosphoprotein 1
SRF: serum responsive factor
STAT3: signal transducer and activator of transcription 3
stRNA: small temporal ribonucleic acid
SV40ER: simian virus 40 early region
TGF-β: transforming growth factor-β
TIMP3: tissue inhibitor of metalloproteinase 3
TM1: tropomyosin 1

TNF-α: tumor necrosis factor α

TP53/NP1: tumor protein 53-induced nuclear protein

TPM1: tropomyosin-1

TRAIL: tumor necrosis factor-related apoptosis-inducing ligand

TRBP: TAR ribonucleic acid binding protein

UTR: untranslated region

UV: ultraviolet

VEGF: vascular endothelial growth factor

VEGFR2: vascular endothelial growth factor receptor 2

VGP: vertical growth phase

VNTR: variable nucleotide tandem repeat

WNT2: wingless-type MMTV integration site family, member 2
CHAPTER 1: INTRODUCTION

1.1. Skin

The skin is the largest organ of the human body with an average surface area of 21 square feet and average weight of 7 lbs. With its size come numerous functions. First and foremost, it acts as a mechanical barrier through which microorganisms, harmful substances, and ultraviolet light cannot easily penetrate and vital substances (i.e. water) cannot escape. Additionally, the skin participates in heat regulation, sensation, and the synthesis and storage of vitamin D. A myriad of specialized cells mediate these effects. Keratinocytes release keratin as they mature and move upwards in the layers of stratified squamous epithelium to form a water-tight barrier. Merkel cells participate in detection of light touch. Langerhans cells uptake antigens found in the skin and present them to other immune cells. Lastly, melanocytes produce pigments to protect the surrounding cells from the harmful effects of ultraviolet (UV) light.

Melanocytes are derived from melanoblasts which migrate over long distances from the neuroectoderm through the dermis to populate the epidermis and associated hair follicles. These cells produce two forms of melanin in vesicles termed melanosomes and deliver these pigments through cytoplasmic processes that fuse with surrounding
keratinocytes. In keratinocytes, melanin forms a protective cap that sits above the nucleus.¹ Skin color is determined by a variety of factors including the distance the cytoplasmic processes extend through the epidermis as well as the maturity of the melanosomes delivered through these processes.¹ As more mature melanosomes contain a greater quantity of melanin, keratinocytes in dark skin possess the ability to dissipate more UV rays.

1.2. Melanoma

Melanoma is a malignancy of transformed melanocytes and the deadliest form of skin cancer. In terms of incidence, it is the fastest growing cancer in the world, an increase that cannot simply be attributed to increased awareness and screening.⁵ The lifetime risk of melanoma diagnosis is currently estimated at 1 in 75 individuals. More than 68,000 new cases and 8,000 deaths due to melanoma are expected this year in the United States alone.⁶ Five-year adjusted survival rates across all stages of melanoma are approximately 91%. However, the five-year survival rate for those patients that have Stage IV melanoma, melanoma that has spread beyond the primary lesion, is approximately 18%.⁷ Below, the pathogenesis, diagnosis, classification, treatment, and prognosis of this malignancy will be further discussed.

Risk factors

Five to twelve percent of all cases of melanoma are found in patients with a positive family history.⁸⁻⁹ In fact, having a first degree relative diagnosed with melanoma
portends a 2.24-fold increase in lifetime risk of melanoma. Genetic mapping of families with multiple members having melanoma has revealed two loci associated with melanoma development, the most common being in the CDKN2A gene. Alternative promoters within the CDKN2A gene yield two different proteins, INK4A and ARF, which function in different pathways. INK4A possesses inhibitory activity against CDK4, a G1 cyclin-dependent kinase. Unhindered, CDK4 phosphorylates retinoblastoma protein (RB), resulting in the inactivation of RB and the release of factors that facilitate the transition of the cell from G1 to S phase. INK4A expression therefore acts as a brake that slows or prohibits the cell from progressing through the cell cycle and proliferating. ARF, on the other hand, functions to inhibit the activity of MDM2 which would otherwise participate in promoting the degradation of p53. ARF thereby stabilizes p53 to allow for a halt in cell cycle progression so that DNA damage may be repaired. Decreased activity of INK4A and ARF would thus lead to unrestrained progression through the cell cycle and the inactivation of the key genomic surveillance pathway. Germline point mutations in CDKN2A and deletions within exon 2, the exon shared but translated in different reading frames by INK4A and ARF, have been identified in melanoma families. It has been noted, however, that these mutations and deletions often preferentially detrimentally affect the INK4A product and spare the ARF product with synonymous mutations. However, there is evidence for independent tumor suppressor activity of both INK4A and ARF. Germline mutations at the site encoding the region of CDK4 that allows for the inhibitory interaction of INK4A have also been identified in melanoma kindreds. Mutations such as these have been seen in sporadic melanoma, as well, and occur exclusive from inactivating mutations in INK4A
as such events would be considered molecularly redundant.\textsuperscript{17} Similarly, patients with hereditary retinoblastoma, characterized by inactivation of RB1, have been shown to be predisposed to the development of melanoma with lifetime risk increased anywhere from 4- to 80-fold,\textsuperscript{18-19} highlighting the importance of the interaction of INK4A, CDK4, and RB in this malignancy.

Excessive exposure to sunlight has been epidemiologically linked to melanoma.\textsuperscript{20-22} Several studies have suggested that intense and intermittent exposure to the sun has a greater association with melanoma incidence than cumulative exposure.\textsuperscript{23} In fact, individuals who work indoors have higher rates of melanoma than those who work outdoors.\textsuperscript{1} Additionally, sun exposure sufficient to induce a sunburn in early childhood has been connected to an increase in lifetime melanoma risk.\textsuperscript{24-26} Experimental evidence supports this link in that immunodeficient mice receiving grafts of neonatal foreskin more readily form melanocytic proliferations following exposure to UV light in comparison to adult skin grafts.\textsuperscript{27-28} Still, a causal link between UV and melanoma has yet to be established. In addition to the development of melanoma at internal primary sites (i.e., sites that have no exposure to the sun), little evidence of UV signature DNA damage has been found upon genetic analysis of primary melanomas arising in sun-exposed skin.\textsuperscript{29} An example of DNA damage characteristic of UV is that of 6-4 photo products and pyrimidine dimers, lesions that form at the site of two adjacent pyrimidine bases. DNA mutations at dipyrimidine sites have been found in the CDKN2A locus, however, these same mutations have been found in other cancers of internal organs.\textsuperscript{30} Yet, when the incidence of melanoma in individuals who harbor a single non-
functional allele of the CDKN2A locus is analyzed on the basis of UV exposure as determined by residential climate (Europe v. Australia), a heightened rate of melanoma is found in those heterozygotes residing in Australia. Additionally, while ultraviolet light alone is not sufficient to generate melanoma in mice, it is capable of doing so in several transgenic models as well as when applied in combination with chemical carcinogens such as 7,12-dimethybenz(a)anthracene (DMBA). The role UV light plays in the pathogenesis of melanoma remains to be completely understood, but strong epidemiological evidence warrants the consideration of UV light as a significant risk factor.

Race and skin type are also associated with melanoma risk. Hereditary family traits such as fair skin that freckles or burns easily without tanning and/or possessing red or blonde hair, regardless of family history, are considered risk factors. Beyond the concept of just possessing fair skin, the incidence of melanoma is highest in Caucasians. People of races characterized by darker skin and thus more protective melanin such as African Americans, Asians, and Hispanics seem generally less susceptible to melanoma. However, in those populations, melanoma tends to be diagnosed later and in areas with little or no sun exposure. These epidemiological associations also lend weight to the belief that UV light plays a role in melanoma sensitivity but cannot account for all instances.

Other factors that have been associated with an elevated risk of developing melanoma include a heavy burden of moles. With congenital nevi, moles present at birth
or appearing shortly thereafter, the volume of the lesion shows a positive correlation with melanoma progression. However, a malignancy arising within such lesions generally occurs during childhood. It is important to note that only roughly a quarter of melanoma primaries have histological evidence of arising from a nevi and only half of those nevi have dysplastic features. Still, the risk of developing melanoma in skin unaffected by nevi is increased in patients with acquired nevi. One potential explanation for this posed by Bataille et al. stems from the finding that leukocytes acquired from patients with multiple nevi have longer telomeres than those acquired from normal donors, suggesting a greater propensity for proliferation of all cells in these patients’ bodies.

**Melanoma progression**

A step-wise progression from normal melanocyte to metastatic melanoma has been proposed to explain melanoma development. Hypothetically, a common acquired nevus forms from multiple melanocytes proliferating together rather than existing singly as normally seen in the epidermis. This common nevus may then gain cytologic and architectural atypia and thus present as a dysplastic nevus. Additional mutational events then transform this dysplastic nevus to a melanoma lesion in which lesion growth is confined to horizontal expansion. Such radial growth phase (RGP) melanomas lack invasive characteristics and metastatic capabilities. The onset of invasive features defines a transition to a vertical growth phase (VGP) melanoma. The appearance of new lesions in other areas of the body then marks metastatic melanoma. Melanoma metastases have been found in lymph nodes, brain, liver, bowel, bone, and
previously unaffected skin. However, this model of progression cannot account for all cases of melanoma. Metastases that arise without a recognized primary lesion account for 8% of metastatic melanoma cases.\textsuperscript{40-41}

Another model for tumor progression suggests the following changes are necessary for melanoma development from melanocytes: an event that results in proliferation and expansion of the melanocyte population, one that overcomes the senescent program of the melanocyte, and one that inhibits the induction of pro-apoptotic pathways.\textsuperscript{42} The most common activating event in melanoma results from gain-of-function mutations in B-Raf found in 50-70% of sporadic melanomas.\textsuperscript{43} Such an event increases signaling through the MAPK pathway and transforms melanocytes.\textsuperscript{44} Mutations in the Ras family of proteins have also been detected in melanomas with a result similar to B-Raf mutations. These mutations are not exclusive to malignant melanocytic lesions and have been found in benign nevi as well.\textsuperscript{45-46} In fact, hyperactivity of this signaling pathway most often leads to the activation of senescent pathways in the transformed melanocyte. Senescence is the loss of the ability of cells to divide and normally occurs after 50-100 doublings \textit{in vitro} and can be stimulated by the shortening of telomeres, DNA damage as a result of increased production of reactive oxygen species, and other mechanisms of oncogenic stress.\textsuperscript{47} In melanoma, overcoming oncogenic stress is often accomplished through some mutation in the INK4A/CDK4/Rb pathway by directly affecting the partners previously described or through stabilization of $\beta$-catenin and constitutive activation of Wnt signaling.\textsuperscript{48} Bypassing the DNA damage checkpoints does not necessitate the inactivation of p53 in melanomas as p53 is rarely
altered in primary lesions. Although the function of the other CDKN2A gene product ARF was discussed in the context of its effects on p53 activity, a decrease in ARF expression can bypass the induction of senescence in a p53-independent manner yet to be fully determined. Increased expression in telomerase activity has also been reported as melanoma lesions evolve. PTEN loss results in an increase in AKT signaling and NFκB activation, a common way in melanoma to upregulate a multitude of anti-apoptotic proteins. Interestingly, loss of PTEN tends to be noted only in melanomas that have activated B-Raf. Decreases in the pro-apoptotic protein APAF-1 and increases in anti-apoptotic elements such as Mcl-1 are commonly noted in primary tumors, as well. Other notable genetic events resulting in increased proliferative activity include increased c-met expression found largely in metastatic lesions and up-regulation of epidermal growth factor receptor (EGFR) often driven by and required for Ras transformation.

The differences between RGP and VGP have been studied extensively in the hope to gain greater understanding as to the molecular events that lead to the metastatic dissemination of melanoma cells. Antigen analysis has found higher expression of such adhesion-related proteins as the vitronectin receptor, ICAM-1, tenascin, and ganglioside GD2 in VGP and metastases in comparison to expression in RGP. Cell lines have been established from primary VGP melanomas with a success rate ranging from 30-70%, while RGP melanomas do not propagate as readily. Those cell lines that have been derived from RGP form few if any colonies when cultured in soft agar, require additional growth factors such as insulin and bFGF for survival, and either do not form tumors when implanted into immunocompromised mice or form very small ones with a long latency.
period. VGP cell lines, however, are capable of forming many more colonies in soft agar, can be adapted to growing conditions lacking serum, and form tumors upon injection into immunocompromised mice. While it has been noted that VGP have more cytogenetic abnormalities than RGP, the precise changes that lead to a transition from RGP to VGP have not been firmly established.

Melanoma is a particularly aggressive malignancy, evidenced by its propensity for widespread metastasis. Furthermore, the transplant of a single cell isolated from a patient’s tumor is sufficient to form a palpable tumor in highly immunocompromised mice 27% of the time.\(^{57}\) Melanocytes may be particularly predisposed to respond more aggressively to neoplastic transformation as primary melanocytes engineered to express the Simian Virus 40 early region (SV40ER), hTERT, and either a constitutively active Ras or c-met receptor readily metastasized to lymph nodes and visceral organs when orthotopically implanted in nude mice.\(^{58}\) Such behavior was a characteristic not shared with primary epithelial and fibroblast cells transformed in the same manner. Many efforts have been made to analyze gene signatures that correlate with a more aggressive melanoma phenotype. One study serially passaged cell lines through immunodeficient mice to select for more metastatic variants and compared mRNA expression signatures of the resulting cell lines to each other, human metastases, and primary melanomas.\(^{59}\) Interestingly, the resulting “aggressive” signature was enriched for membrane associated and secreted proteins, suggesting increased interaction with the tumor microenvironment.
**Melanoma diagnosis**

The first step to diagnosing a melanocytic lesion as melanoma is a moderate degree of clinical suspicion. Pigmented lesions are visually evaluated by the ABCD criteria. A stands for asymmetry. Benign nevi are most often symmetrical. B stands for border. While benign lesions have clearly demarcated borders, melanocytic lesions of concern often have irregular borders that may fade into the skin. C stands for color. Rather than have a uniform color of a shade of brown, potentially malignant lesions may have a mixture of pigments from brown to black to red. D is for diameter. A lesion greater than 6 mm warrants further examination. Recently, the addition of the letter E, standing for evolution, has been proposed. A change observed in an existing mole is reason for concern.

Excisional biopsies are necessary to establish a lesion as melanoma as the gold standard of melanoma diagnosis is histopathological analysis. If an excisional biopsy is not feasible as the lesion in question is large or is located in an area in which removal of the entire mole might be disfiguring, an incisional biopsy may be made in the nodular or darkest region of the lesion. It is critical to remove an adequate sample of the lesion to assess how deeply it has penetrated the skin as diagnosis and prognosis are often dependent upon this criterion.

Histopathological analysis is performed by examining paraffin-embedded specimens stained with hematoxylin and eosin (H&E) under a light microscope. Cells that would be considered atypical appear with enlarged nuclei containing variable shapes
and chromatin patterns and sometimes large nucleoli. A thick nuclear membrane with irregular contours is often also present. The cytoplasm is typically abundant, pink, and granular. Beyond the presence of atypical cells, other factors considered in diagnosis include effacement of epidermal rete ridges, pagetoid spread, the distribution of abnormal melanocytes within the lesion, definition of lesion margins, the presence of nests versus single melanocytes, lesion depth, degree of invasion, mitotic activity, inflammatory infiltrate, fibrosis, and ulceration. The aforementioned are by no means an exhaustive list of concerns. Due to the numerous subtypes of melanoma and melanocytic lesions, a full description of histologic criteria is beyond the scope of this manuscript. Instead, it should be noted that elements such as pagetoid spread, the movement of melanocytes up into more superficial layers of the epidermis, are most often considered to be associated with melanoma but can also be found in benign nevi.

As illustrated above, accurately diagnosing malignant melanoma can be a challenge for even experienced clinicians and dermatopathologists, an issue that has not been resolved with the development of new technology. A study published in 1975 reported that physicians working in the oncology section of a skin and cancer ward made a correct diagnosis of melanoma 64% of the time. A later study explored the concordance of eight melanoma experts given a panel of melanocytic lesions that had been submitted for their “classic” presentation. Each expert was given the same lesion section on the same slide and was asked to make a judgment as to the biological significance of the lesion, namely whether the lesion was benign, indeterminate, or malignant. A unanimous conclusion was made for only 13 of the 37 specimens. Ten of
the specimens had one dissenting opinion. Statistical analysis of these results concluded that even among experts, only moderate concordance in diagnosis is seen. Confounding the difficulty of consistent interpretation of visual observations are the numerous elements requiring consideration, the often conflicting and changing criteria by which melanocytic lesions are categorized, and the sheer number of sub-categories often employed.

**Prognosis**

The most significant prognostic indicators of melanoma are Breslow tumor thickness and Clark’s anatomic level. Breslow thickness is measured from the top of the granular layer of the epidermis to the deepest foci of invasion. An increase in tumor thickness in increments of 1 mm is associated with positive sentinel lymph node biopsy and decreased survival beyond 10 years. The five levels of Clark’s invasion are I - intraepidermal melanoma; II – malignant cells in the papillary dermis; III – malignant cells extending to where the papillary and reticular dermis meet; IV – malignant cells in the reticular dermis; and V – malignant cells in the subcutaneous tissue. These levels correlate negatively with outcome as one study reported 5-year survival for patients with level II lesions at 95%, level III at 81%, level IV at 68%, and level V at 47%. Employing the described invasion scheme is one way to aid in the identification of thin melanoma lesions that progress to metastasis. Additionally, the presence of a VGP in melanomas with a depth of less than 1 mm indicates a 42-fold risk of metastasis. Few studies have also assessed the significance of tumor volume and found associations with
survival. However, the methods employed to determine tumor volume were both varied and cumbersome, limiting the utility of this potential predictor.

Other predictors observed upon histological examination include ulceration, mitotic activity, regression, inflammation, and cellular atypia. Ulceration is an interruption of the epithelium and may be due to ischemia from a rapidly growing tumor. Accordingly, the presence of ulceration has been linked to decreased overall survival. Mitotic rate is often expressed as the number of mitoses found per 10 high power fields with the fields beginning in the portion of the tumor with the highest number of mitoses. Gimotty et al. reported a 7.7-fold increase in the risk for metastasis in those patients whose lesions had a mitotic rate greater than zero. Another study found that as the calculated mitotic rate of melanoma primaries increased, 10-year survival rates decreased. Regression of a melanoma lesion as indicated by a replacement of tumor with fibrosis is also a negative indicator, presumably because it represents the selection of resistant and aggressive melanoma cells by immune elimination of the less hardy cells. On the contrary, the presence of an inflammatory infiltrate is a positive sign.

The age of melanoma diagnosis, the location of the growth, and the sex of the patient are variable prognostic indicators. Generally, patients older than 60-years old have a lower 5-year survival rate than younger adult patients. However, melanomas found on older patients also tend to be thicker and ulcerated. The role of age in children with melanoma is reversed. Melanomas from younger patients tend to have more advanced characteristics but younger children have a higher 5-year event-free survival.
Women also often fare better than men, which may be partially due to presentation of disease at an earlier age.\textsuperscript{76} Also, women have a greater proportion of lesions found on the extremities as compared to the trunk, which has been suggested to be a predictor of disease-free survival.\textsuperscript{82} Tumors found on the scalp, however, have a more negative association with a 5-year survival rate of 40\% in one study.\textsuperscript{83}

**Treatment**

Surgery remains the first-line and most successful method of melanoma treatment. A wide excision of the melanoma remaining after the initial biopsy with sufficient margins is necessary. The exact width of the margins remains a source of debate, and melanomas of the face, hands, and feet often require a deviation from the recommended margins.\textsuperscript{84} Sentinel lymph node biopsies are performed on patients whose primary lesions have a depth greater than 0.75 mm or have otherwise worrisome features such as ulceration and a high Clark’s level of invasion. Regional node dissections are limited to patients with evidence of melanoma in one or more sentinel nodes.\textsuperscript{60} While the benefit of sentinel lymph node biopsies on survival is hotly debated, staging information gained from this procedure aids clinical decision-making in terms of the necessity of adjuvant therapy. The current recommended therapy for patients at risk for recurrence is high dose interferon-\(\alpha\)-2b. Unfortunately, the high dose of this hormone is associated with toxicities that often make completion of one year at the recommended dose impossible.
Unfortunately, treatment options for patients with evidence of visceral or skin metastases are limited. Regimens of high dose IL-2 have resulted in complete and durable responses in a subset of patients. However, IL-2 therapy comes with serious side effects that require hospital admission for observation during treatment and identification of the subset of patients that ultimately benefit from treatment remains elusive. The standard chemotherapeutic agent used against metastatic melanoma is dacarbazine, however clinical responses have only been observed in 15-20% of patients and the median duration of these responses is only 4 months. Combinatorial therapy with other agents such as carmustine, lomustine, semustine, cisplatin, carboplatin, vincristine, binblastine, paclitaxel and docetaxel are currently being explored. Targeted agents, such as sorafenib that was designed to interfere with the constitutively active B-Raf, have not proven to be more effective than dacarbazine or interferon-α. Clinical trials exploring the utility of cancer vaccines designed to stimulate the immune system against antigens commonly expressed by melanomas are also underway. Sadly, the benefits ascribed to the majority of the aforementioned therapies are expressed in months. The lack of effective treatment for melanoma that has already disseminated highlights the importance of early detection and accurate diagnosis.

1.3. MicroRNAs

MicroRNAs (miRs) are a relatively new class of small, non-coding RNAs discovered in the current decade that add another layer of complexity to gene regulation. Initially, small 22-nucleotide (nt) transcripts were found to be encoded by the genes lin-4
and let-7, previously identified as being responsible for the control of transitions in the early stages of *Caenorhabditis elegans* larval development.\(^{88-89}\) Lin-4 had been shown to decrease lin-14 protein expression through an unknown mechanism. Discovery of a 22-nt RNA product of lin-4 led to the observation that there were sequences in lin-4 that were homologous to sequences in the 3’ untranslated region (3’ UTR) of lin-14 mRNA.\(^{88}\) Mutation of that region of the 3’UTR abolished lin-4 regulatory activity, and linking the lin-14 3’ UTR to a reporter rendered the reporter subject to regulation by lin-4.\(^{90}\) Additionally, the repression occurred without a change in the amount of lin-14 mRNA.\(^{91}\) These observations provided the first suggestions that a small, endogenously encoded RNA can act as a negative regulator of translation and formed the basis of future understanding of microRNA activity.

Because of their role in development, these small RNAs were dubbed small temporal RNAs or stRNAs. Due to the highly conserved nature of the let-7 sequence it was hypothesized that other stRNAs existed,\(^{92}\) but it wasn’t until cloning methods designed to capture siRNA products of exogenous dsRNA were employed in *Drosophila melanogaster* that new endogenous small RNAs were detected in other species.\(^{93}\) The existence of microRNAs had likely escaped the attention of the scientific community as previous cloning efforts had precluded the population of small, less than 25-nt RNAs and genomic screening had largely been focused on the identification and characterization of open reading frames. Since the release of three seminal studies that first characterized and generalized microRNA expression in September 2001, over 6,300 original articles
and reviews have been published. Yet with over 600 and climbing human microRNAs identified, our knowledge of these tiny regulators is far from complete.

**Biogenesis**

While the functional microRNA unit is approximately 19-24-nucleotides in length, production of this mature form begins with the transcription of an intermediate kilobases in length. Similar to that of mRNA, microRNA genes are transcribed by RNA polymerase II and possess polyadenylated tails and 5’ caps.\(^{94-95}\) This directly transcribed microRNA intermediate has been termed the pri-miRNA. While traditionally the eukaryotic genome is arranged such that one gene is transcribed into one mRNA, groups of microRNAs, such as the miR-23~27~24-2 cluster, have been discovered to be under the control of one promoter and exhibit polycistronic transcription, resulting in a pri-miRNA encoding multiple microRNAs.\(^ {96}\) Recently, a database has been constructed containing genomic and transcriptional information of microRNAs, including putative transcription factor binding sites and sequences.\(^ {97}\)

Pri-miRNA transcripts localize to the cell nucleus and are processed there into the second intermediate form of microRNAs, the pre-miR which has been found in both the nucleus and the cytoplasm.\(^ {96}\) Pre-miRs possess hallmarks of RNAse III digestion with a 5’ phosphate, a 3’ hydroxyl group, and a 1-4 nt 3’ overhang.\(^ {98}\) This finding led to the identification of the protein Drosha as the RNAse III enzyme responsible for the cleavage of the several kilobase long pri-miRNA to the 60-70 nt pre-miR.\(^ {99}\) Drosha partners with DGCR8 to form the microprocessor complex. DGCR8 helps position the complex by
recognizing the approximately 30 base pair (bp) stem loop in the pri-miRNA and the flanking single-stranded segments. Drosha then cleaves the pri-miRNA approximately 11 bp from where the transcript switches from double-stranded to single-stranded.\textsuperscript{100-101} Interestingly, Drosha localizes to sites of transcription and has been shown to cleave pre-miRs from within introns of protein-coding genes prior to splicing of the introns.\textsuperscript{102} The proximity of the microprocessor complex with sites of active transcription may explain why pri-miRs are generally found at such low levels in cells as pri-miR processing appears to occur concurrently with transcription. Following processing by the microprocessor complex, the pre-miR binds the export receptor exportin-5 in a Ran-GTP dependent manner and exits the nucleus for further processing in the cytoplasm.\textsuperscript{103}

Cleavage of the pre-miR into 22 nucleotide double-stranded RNAs is accomplished by another RNAse III enzyme called Dicer. Dicer is dependent on the presence of magnesium ions for its catalytic activity but not for binding to double-stranded RNA. However, Dicer does not require ATP for cleavage.\textsuperscript{104-105} Dicer is highly conserved across mammals and represents a convergence of the microRNA processing pathway and that of siRNA. Dicer has also been shown to associate with other RNA binding proteins such as TRBP and PACT as well as one of the members of the family of argonaute proteins, Ago1, Ago2, Ago3, and Ago 4, forming the RNA-induced silencing complex (RISC) loading complex.\textsuperscript{106-107} It is believed that following cleavage into the double-stranded mature microRNA, TRBP remains bound to the stable end of the microRNA and the Argonaute protein binds to the less stable end.\textsuperscript{108} Stability of the nucleotide interactions is the most important factor determining which strand of the
microRNA is selected for participation in the RISC. The 5' end of the complex requiring the least energy to disrupt base pairing is most often preferentially loaded into the RISC, although the opposite strand may sometimes be used.\textsuperscript{109} The more abundant strand of a microRNA is designated with the prefix miR followed by a number. The less commonly utilized strand is characterized with the same prefix and number followed by an asterisk. Central mismatches within the double-stranded microRNA also help facilitate unwinding of the complex but are less likely to affect strand selection. Some evidence exists to suggest that the argonaute proteins Ago1 and Ago2 possess helicase activity and can separate the double-strand mature miR; however it is believed that other helicases may be recruited to the RISC loading complex to facilitate this separation.\textsuperscript{110}

**Biological activity of microRNAs**

MicroRNAs mediate their role in gene regulation through the association of the microRNA-loaded RISC and complementary sequences in target mRNAs by one or more mechanisms that remain unclear. The observations that mature microRNAs in *C. elegans* co-sedimented with polysomes following fractionation by sucrose gradient and were found in concert with messages being actively translated led to the hypothesis that microRNAs exert their translational repression following the initiation of translation.\textsuperscript{111-114} It has been postulated that microRNAs cause the premature removal of translating ribosomes from target mRNA as in the presence of inhibitors of initiation, ribosomes dissociate more rapidly from mRNA bound by specific microRNAs than mRNAs incubated with non-complementary oligonucleotides.\textsuperscript{115} The absence of truncated protein products from targeted mRNAs in the presence of actively translating
ribosomes has resulted in speculation that microRNAs may direct degradation of target mRNA products;\textsuperscript{116} however, the source and presence of such proteolytic activity has yet to be identified. Other studies in mammalian systems have co-sedimented microRNAs with target mRNAs not associated with ribosomes,\textsuperscript{117} indicating that active translation may not be necessary for translational repression by microRNAs. In fact, Ago2 has been shown to efficiently bind $\text{m}^7\text{GTP}$ in its central domain which shares homology with eIF4E, a cap-binding protein.\textsuperscript{118} This suggests that Ago2 may mediate microRNA translation repression by blocking access of translation initiating factors to the 5’ cap of target mRNA. Other studies indicated that the RISC complex may interfere with translation intiation by preventing the binding of the small 40S ribosomal subunit to the large 60S subunit.\textsuperscript{119} microRNAs may also result in the depletion of target mRNA. This is less likely due to directed cleavage by Argonaute proteins as seen in plants and more likely to be the result of the activation of traditional mRNA degradation pathways. Supporting this theory are data showing increased deadenylation and cap removal in mRNAs targeted by microRNAs.\textsuperscript{120-122} Argonaute proteins, microRNAs, and target mRNAs have also found to be associated with P bodies, which implicates sequestration of mRNA from access by ribosomal components as yet another potential mechanism for microRNA action. However, microRNA-directed repression can still be found in cells lacking P bodies.\textsuperscript{123} Whatever the mechanism, the most common result of microRNA expression is translational repression of its target mRNAs.

Several factors have been postulated to play a role in both the interaction of microRNAs with target mRNAs and the degree and mechanism of repression effected by
it. Binding of microRNAs to mRNA appears to be most affected by the nucleotides in the positions 2-7 of the mature microRNA. Mutations at these locations decrease repressive activity, while mutations introduced further toward the 3’ end of the mature microRNA have no effect on repression of target expression. Additionally, the introduction of a G:U wobble pairing in this critical stretch of nucleotides, while normally conferring RNA interaction stability, decreases the actions of microRNAs.

The target mRNA appears to be the moiety that dictates whether translation is disrupted by mRNA decay or direct repression. As one microRNA can mediate the reduction in target expression through different mechanisms on different targets depending on the degree of complementarity between the microRNA and the target and target secondary structure. The placement of the microRNA-binding site within the target mRNA does not appear to limit the effectiveness of microRNA repression, however. Binding sites artificially inserted into the 5’ UTR of reporter mRNAs still exhibited specific repression. In fact, a functional miR binding site in the coding region of the βTrCP1 mRNA has been identified. Still, the majority of predicted and verified microRNA binding sites have been located in the 3’ UTR.

Functions aside from translation repression and mRNA degradation have been ascribed to microRNAs. Several studies have observed that siRNA can guide heterochromatin formation and silence transcription of affected genes. Gonzalez et al. demonstrated microRNAs such as miR-17-5p and miR-20 can induce similar chromatin states. Confirmation of such actions indicates yet another negative regulatory function for microRNAs. Positive regulation by the same microRNAs that mediate translational
repression have been detected upon changes in cell cycling. Up-regulation of translation of the TNF-α transcript was observed upon cell cycle arrest due to serum starvation and found to be dependent on association with AGO2, fragile X mental retardation-related protein 1 (FXR1), and miR-369-3.\textsuperscript{129} Additionally, the activity of a microRNA mimic oscillated between repression and activation of translation depending on the presence or absence of serum. Another notable observation from this study was that synchronization of the cell cycle of cultured cells allowed for the detection of greater levels of suppression. It is possible that utilization of an asynchronous population of cells results in the underestimation of the repressive activity of microRNAs. Recently, it has been suggested that regardless of cell cycle status, some small dsRNAs can act as activators of target expression. Such an increase in target transcript was shown to be dependent on Ago2 and associated with the demethylation of histones at the target sites upon introduction of exogenous small RNAs.\textsuperscript{130} Place \textit{et al.} proposed such activity for microRNAs by demonstrating that mutation of miR-373 complementary sequences in the E-cadherin promoter abrogated a miR-373-specific increase in transcription of E-cadherin mRNA.\textsuperscript{131} Factors that influence this activating mechanism remain to be elucidated.

**Regulation of microRNAs**

Because of the many functions of microRNAs and their often tissue specific expression, these tiny regulators must be regulated as well. At the level of transcription, transcription factors and epigenetic changes can control the expression of microRNAs. For example, binding of AP-1 and δEF1 to the promoter region of miR-21 has been
shown to activate transcription of pri-miR-21. As miR-21 negatively regulates a negative regulator of AP-1, an amplification loop can also be established to further increase miR-21 expression. Tissue-specific expression of microRNAs exclusive to the placenta is maintained by methylation of upstream CpG rich regions in all other tissues.

Rapid changes in levels of microRNAs in response to external signals are not likely to be the result of transcriptional regulation. Instead, association of signal transducers such as SMAD proteins have been shown to be recruited to specific pri-miRNAs to quickly produce pre-miRs and mature miRs to mediate the effects of an external signal. Stabilization of RISC loading complexes by the phosphorylation of TRBP by activated ERK enhances the conversion of pre-miRs into mature microRNAs involved in cell division and growth while decreasing the mature form of microRNAs that oppose these activities. On the contrary, the complex of NF90 and NF45 proteins negatively regulates the production of mature microRNAs by binding pri-miRNAs and preventing the binding of the microprocessor complex. This complex has a variable affinity for different microRNAs and may thereby coordinate the activity of microRNAs associated with a common process. Hormonal control by estrogen has been shown to be due to the association of estrogen-bound estrogen receptor α with the microprocessor complex, resulting in a decrease in the processing of pri-miRNAs into pre-miRs. Additionally, mature microRNAs not bound to a RISC are degraded by the 5’ to 3’ exoribonuclease XRN-2. This activity maintains turnover of excess microRNAs and
also explains why the strand not selected for loading into the RISC is most often undetectable in the cytoplasm.

In the absence of direct cleavage, the effects of microRNAs are immediate, reversible, and dose-dependent.\textsuperscript{124} Such regulation is important for rapid cellular responses to changing stimuli and critical to normal physiology. Presently, microRNAs have been shown to participate in such diverse processes as hematopoiesis, innate and adaptive immune responses, maintenance of a contractile phenotype, insulin sensitivity, and fat metabolism.\textsuperscript{140-144} Therefore it would stand to reason that abnormal expression of microRNAs would lead to disease pathology. Loss of expression of either Dicer or Drosha and thus loss of the expression of mature microRNAs in the T cell compartment of mice resulted in rampant inflammation and autoimmunity.\textsuperscript{145-147} Elevated levels of miR-29 were found in the muscle, fat, and liver of diabetic rats, and over-expression of miR-29 paralogs decreased the uptake of glucose by adipocytes in response to insulin.\textsuperscript{144} Expression of miR-21 has been associated with cardiac pathology.\textsuperscript{148-149} These studies highlight the pathological impact of dysregulation of microRNAs and the importance of tight regulation of microRNA expression.

**MicroRNAs and cancer**

The discovery of microRNAs as novel regulators coupled with the critical role in development the first identified microRNAs play fueled scientific curiosity into the possibility of microRNA involvement in human malignancies. Initial studies mainly focused on identifying those microRNAs that were either up-regulated or down-regulated
in malignant tissues as compared to normal tissue. The first such study identified microRNA genes located at a locus commonly deleted in chronic lymphocytic leukemia (CLL). Calin et al. observed a decrease in expression of these microRNAs, miR-15 and miR-16, in primary B cell CLL cells in both the presence of the deletion and its absence, indicating that down-regulation of miR-15 and miR-16 may be an important event in CLL pathogenesis. Another report by the same group found that over half of the microRNA genes known at the time the study was conducted are located at loci frequently altered in cancer. These include sites of fragility where rearrangements and viral integration may occur, minimal regions of amplifications, breakpoint regions, and minimal regions of loss of heterozygosity. A signature of solid tumors was developed through the comparison of microRNA expression in over 540 lung, breast, stomach, prostate, colon, and pancreatic tumors. miR-17-5p, miR-20a, miR-21, miR-92, miR-106a, and miR-155 were found to be highly expressed in each tumor type tested as compared to matching tumor-free tissue. Several later studies, some discussed below in this chapter, have confirmed roles for the aforementioned microRNAs in solid tumors. The finding of such commonality across a diverse set of malignancies underscores the potential power of microRNAs in driving various tumor-promoting pathways. These were the first sets of studies that surmised that microRNAs may act as either tumor suppressors by repressing the expression of oncogenes or as oncogenes by repressing the expression of tumor suppressors.

Comparison of expression of microRNAs in normal tissues and tumors have aided in the identification of changes associated with the development of cancer. MicroRNA
signatures have also been helpful in identifying inherent differences between types of cancer developing in the same tissues and between aggressive and tamer tumors, allowing for the development of microRNA signatures with prognostic value. Decreased expression of let-7 microRNAs in adenocarcinomas of the lung was linked with significantly shorter post-surgical survival times.\textsuperscript{153} High miR-21 expression was associated with decreased overall survival and poor therapeutic outcome in colon adenocarcinoma.\textsuperscript{154} Other prognostic signatures have been found in hepatocellular carcinoma, acute myeloid leukemia, esophageal squamous cell carcinoma, colorectal cancer, breast cancer, gastric cancer, oral carcinoma, head and neck squamous cell carcinoma, and ovarian cancer.\textsuperscript{155-165} A similar prognostic signature for melanoma has yet to be developed. Further highlighting the general impact of microRNA expression on homeostasis are the findings that reduction of Dicer expression was associated with shorter post-surgical survival times in non-small cell lung cancer and higher expression of Dicer and Drosha in ovarian cancer was associated with an increase in median survival.\textsuperscript{166-167}

While tumors in different tissues and even tumors arising in the same tissues often have different genetic mechanisms driving progression, Drs. Hanahan and Weinberg proposed six properties of cancer that all tumors must address to become malignant. These hallmarks include evasion of apoptosis, self-driven growth, resistance to anti-growth signals, tumor-driven angiogenesis to sustain growth, the ability to divide without limits, and invasion and metastasis.\textsuperscript{168} As microRNAs have been shown to be dysregulated in human malignancies, it stands to reason that altered expression of these
molecules plays a role in tumor progression and acquisition of malignant properties. Elevated miR-221 and miR-222 expression was associated with androgen-independent prostate cancer and was sufficient to eliminate the need for androgen stimulation in previously androgen-dependent cell lines. These microRNAs were also shown to down-regulate expression of p27 and p57 and when elevated signal entry into S phase regardless of the presence of growth factors. However, miR-221 and miR-222 can only overcome quiescence in this manner if induction of the apoptotic pathway is blocked in some way. Ebi et al. demonstrated a role for increased expression of the miR-17-92 cluster in decreasing oncogene-induced DNA damage by reactive oxygen species, thus bypassing one senescence pathway and increasing replicative potential. miR-126 was observed to be down-regulated in lung cancer cell lines and restoration of expression resulted in a decrease in VEGF production, suggesting that decreased expression of miR-126 may play a role in stimulating angiogenesis in lung cancer tumors. miR-210 production is stimulated by HIF1α and normally functions to suppress genes that are only required under normoxic conditions. While it has been shown that miR-210 represses initial tumor growth, expression upon tumor establishment may contribute to the development of new blood vessels to allow the tumor to expand. To this end, in a variety of tumor cell lines expression of miR-210 is induced by hypoxia and down-regulates the expression of MNT, an antagonist of MYC. This induction rescued the cell lines from cell cycle arrest that would otherwise occur in response to hypoxia. Recently, it has been suggested that evasion of the immune system is another requirement for successful tumoral growth. MicroRNAs can contribute to this phenomenon as well. Various tumors have been shown to exploit microRNA control of the NK cell NKG2D ligands MICA and
MICB to down-regulate these markers that would otherwise target the stressed cells for NK cell-mediated destruction.\textsuperscript{142}

A myriad of microRNAs have been associated with the avoidance of apoptosis. miR-221 has been shown to target the mRNA of Bmf, a pro-apoptotic protein, in hepatocellular carcinoma, and decreasing the expression of miR-221 \textit{in vitro} resulted in an increase in cell death following matrix detachment.\textsuperscript{175} miR-221 and miR-222 have also been shown to produce resistance to induction of apoptosis by TRAIL in lung, liver and bladder cancers.\textsuperscript{176-177} Decreased expression of miR-29 has been observed in hepatocellular carcinoma and was subsequently found to directly target the mRNA of anti-apoptotic factors Bcl-2 and Mcl-1. Additionally, enforced expression of miR-29 in hepatocellular carcinoma cell lines resulted in sensitization to the induction of apoptosis by serum starvation, hypoxia, and chemotherapeutics.\textsuperscript{178} Decreased expression of miR-34a is also associated with resistance to apoptosis and chemotherapy as well as an impaired response to DNA damage in CLL.\textsuperscript{179}

A great deal of literature has been published on the effects of microRNAs on metastasis and invasion. Several groups have evaluated metastatic/aggressive microRNA signatures by comparing primary tumors and metastases in lymph nodes, comparing microRNA methylation in normal tissues and lymph node metastases, and by comparing node-negative tumors that eventually metastasized to those that did not.\textsuperscript{180-182} These studies led to the postulation of the involvement of miR-7 and miR-516-3p in cell cycle progression and increased chromosomal instability and miR-128a in cytokine signaling.
Up-regulation of miR-21, miR-182, and miR-27b in various malignancies has also been associated with an increase in invasive activity.\textsuperscript{183-186} On the other hand, miR-34b, miR-34c, and miR-199a\textsuperscript{*} have been shown to decrease MET expression and thereby decrease cell motility and invasiveness \textit{in vitro}.\textsuperscript{187} miR-146 re-expression in breast cancer cell lines decreases invasion and migration due to repression of NF-B activity.\textsuperscript{188} Those microRNAs associated with decreased motility, invasion, and metastatic capabilities are often suppressed by hypermethylation.\textsuperscript{181} Other microRNAs have been implicated in controlling epithelial to mesenchymal transition (EMT), a precursor to metastasis characterized by a decrease in cellular adhesion and an increase in motility. miR-205 is one such microRNA as enforced expression in prostate tumor cells results a reversal of EMT through the down-regulation of protein kinase C\textit{e}.\textsuperscript{189} Up-regulation of miR-200 and let-7 has also been shown to reverse the EMT phenotype in pancreatic cancer.\textsuperscript{190} Previous EMT-inducers have also been tied to microRNA regulation. Expression of miR-155 following stimulation with TGF-\(\beta\) was found to be necessary for TGF-\(\beta\) induced EMT as knockdown of miR-155 in epithelial cells with concomitant TGF-\(\beta\) treatment failed to exhibit an EMT phenotype.\textsuperscript{191} Over-expression of miR-10b, miR-373, and miR-520 and decreased expression of miR-31, miR-335, miR-206, and miR-146 have also been associated with an increase in metastatic burden in animal models and human patients.\textsuperscript{192-196}

The growing evidence of microRNA involvement in cancer progression and initiation prompted us to explore differentially expressed microRNAs in melanoma. The following chapters represent our efforts. In chapter 2, studies examining the expression
of previously identified microRNAs in benign nevi, primary melanoma tissues, and melanocytic lesions with an indeterminate biological significance are explained. *In vitro* studies evaluate the effects of enforced expression of miR-21 on the phenotype of melanoma cell lines and are described in chapter 3.
CHAPTER 2: MICRORNA-21 AND MICRORNA 155 ARE UP-REGULATED IN MELANOMA AND ARE ASSOCIATED WITH LESION DEPTH AND MITOTIC ACTIVITY OF INDETERMINATE/BORDERLINE MELANOCYTIC LESIONS

Introduction

MicroRNAs (miRs) are a recently discovered class of small non-coding RNAs. The binding of miRs to target mRNAs results in reduced expression of that gene. miRs are synthesized as long primary transcripts in the nucleus by the RNA polymerase II complex and cleaved by the RNase III enzyme Drosha to produce RNA precursor molecules of ~70 nucleotides in length (pre-miRs) that assume a stem-loop structure. The pre-miRs are then exported to the cytoplasm where a second RNase III enzyme Dicer processes them to give a double-stranded RNA that measures 21-23 nucleotides in length. One strand of the duplex is selectively stabilized and incorporated into a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC). After integration into the active RISC complex, miRs base pair with their complementary mRNA molecules and induce mRNA degradation by Argonaute proteins, the catalytically active component of the RISC complex. Alternatively, some miRs inhibit the initiation of translation or interfere with translation already in progress by causing the ribosome to disengage from the target mRNA. Each miR is believed to regulate multiple genes,
and because hundreds of miR genes have been identified in humans, the regulatory potential of miRs is enormous.\textsuperscript{200} Several groups have provided evidence that miRs may act as key regulators of multiple cellular processes. It has been documented that miRs are over-expressed in most forms of cancer and play a role in maintaining the malignant phenotype via alterations in processes such as cellular adhesion, angiogenesis, cell cycle control, cell signaling, and apoptosis.\textsuperscript{201-202}

Melanocytic proliferations are among the most diversified of cutaneous lesions ranging from completely benign nevi such as the junctional nevus, congenital nevus and blue nevus to those with overtly malignant features. However, there are other lesions which present a diagnostic conundrum for dermatopathologists. This group of proliferations is nebulous both clinically and morphologically. These indeterminate/borderline melanocytic tumors manifest architectural features and cytologic atypia which exceeds that encountered in nevi. In such cases the potential for an aggressive biological course arises. A categorical approach to the indeterminate/borderline melanocytic proliferation has been proposed by Magro, Crowson and Mihm wherein they recognized four main categories of indeterminate/borderline melanocytic proliferations; 1) borderline melanocytic proliferations arising in association with a deep penetrating nevus, 2) those exhibiting borderline features of nevoid melanoma, 3) atypical Spitz tumors, and 4) pigmented epithelioid melanocytoma formerly designated animal type melanoma.\textsuperscript{203} In addition, dysplastic nevi may also pose a diagnostic dilemma to clinicians.\textsuperscript{204}
Indeterminate/borderline lesions pose a therapeutic dilemma for pathologists, oncologists, and surgeons, as a false negative reading of a benign nevus could lead the clinician to recommend against further therapy. Under-treatment of a melanocytic lesion with malignant potential could thus adversely impact survival. Conversely, over-treatment of a false positive reading of malignancy could lead to needless morbidity from surgery or adjuvant therapy. Therefore we sought to identify molecular markers that could be used to further characterize these lesions and predict their clinical behavior. Current prognostic indicators for primary melanoma lesions include Breslow depth and ulceration. These factors and mitotic activity are often considered in the diagnosis of indeterminate/borderline lesions but do not serve to define such lesions as malignant or benign. We hypothesized that the analysis of miR expression in melanoma tumors would lead to the identification of a molecular profile that could be used to differentiate lesions with high or low malignant potential. This information could also provide further insight into the role played by miRs in the development and progression of malignant melanoma.

**Materials and Methods**

**Patient Samples.** A series of indeterminate/borderline melanocytic lesions, dysplastic nevi, benign dermal nevi, and melanomas were evaluated and diagnosed by one of the authors of this paper (CMM). The melanomas were classified according to established criteria with recognition of the presence or absence of the radial growth phase and/or vertical growth phase. The cases were derived retrospectively and reflect consecutive encounters from the routine and consultative dermatopathology practice of
the Ohio State University Medical Center’s Department of Pathology. The cases were encountered between 2000 and 2006 and selected for further analysis (IRB No. 2007 C0015). For RNA isolation, four to eight unstained 20 micron sections were cut from archived formalin-fixed, paraffin-embedded tissue derived from punch biopsies.

**Cell Lines.** The HT144, A375, and Hs294T human melanoma cell lines were obtained from the American Type Culture Collection (Manassas, VA). The 1259 MEL, 18105 MEL, 1074 MEL, 1106 MEL, MEL 39, 1174 MEL, and FO1 human melanoma cell lines were a gift from Dr. Soldano Ferrone (Roswell Park Cancer Institute, Buffalo, NY).

**Isolation of Total RNA.** Paraffin-embedded tissue was harvested utilizing the RecoverAll™ Total Nucleic Acid Isolation Kit as per the manufacturer’s recommendations (Ambion, Foster City, CA). Nuclease-free water was used to elute the RNA.

**Real-Time PCR.** Single tube TaqMan MicroRNA Assays were used to detect and quantify mature miRs. All reagents, primers and probes were obtained from Applied Biosystems (Foster City, CA). PCR for RNU6B, a small ubiquitous RNA, was used to normalize all RNA samples. Reverse Transcriptase reactions and Real-Time PCR were performed according to the manufacturer’s protocols. Gene expression levels were quantified using the ABI Prism 7900HT Sequence detection system (Applied Biosystems). Comparative Real-Time PCR was performed in triplicate, including
no-template controls. Relative expression was calculated using the comparative \( C_t \) method.\(^{205}\)

**In Situ Hybridization.** The protocol for detection of miRs in paraffin-embedded tissue by in situ hybridization has been previously published.\(^{206}\) The sequence of the locked nucleic acid modified cDNA probes were: miR-155 = ACCCCTATCAGATTAGCATTAA and miR-21 = TCAACAGTCAGTCTGATAAGCTA. The probes were labeled with the 3’oligonucleotide tailing kit using biotin as the reporter (Enzo Diagnostics, Farmingdale, NY). Negative controls were omission of the probe, use of a scrambled probe, and internal controls present in the tissue.

**REMARK Considerations.** In view of our hypothesis that the analysis of miR expression in melanoma tumors would lead to the identification of a molecular profile that could be used to differentiate lesions with high or low malignant potential, we proceeded to apply the 20 distinct REMARK criteria to this study, referred to here as R1-20.\(^{207}\) The tissue examined for this study was taken from primary cutaneous lesions, prior to the initiation of therapy from patients with benign nevi and primary melanomas (R-1-3). The type of tissue, preservation, and method of case selection are outlined above under Patient Samples (R-4,6). Detailed descriptions of the assays used are presented under the headings Isolation of RNA, Real-Time PCR, and In Situ Hybridization (R-5). Workers performing these assays were blinded to the tissue origin and tissue characteristics (R-5). Outcome determination was not a primary aim of this.
study. Follow-up beyond the only clinical endpoint examined (sentinel lymph node biopsy) was not pursued as preliminary analysis was necessary to obtain IRB approval for further data gathering (R-6,7). During analysis, the lesions were stratified by lesion type but not age or stage of disease (R-6). Growth phase (radial vs. vertical) was initially considered for analysis with miR-21 and miR-155 expression, in addition to mitotic activity, primary lesion depth, and results of sentinel lymph node biopsy (R-8). The sample size was dependent upon the availability of indeterminate/borderline lesions (R-9). The statistical approach is described below under the heading Statistical Analysis (R-10,11). The number of patients included at each stage of analysis is included within the respective results section (R-12). Demographic characteristics of the patient population can be found in Table 2.1 (R-13). As this study examined the association of miRs with standard prognostic variables (mitotic index, sentinel lymph node biopsy, lesion depth) and not recurrence or time to a clinical event, multivariable analyses and hazard ratios were not performed (R-14-18). An evaluation of study limitations and implications for future research are presented in the discussion (R-19,20).

**Statistical Analysis.** Statistical analyses were assessed by analysis of variance (ANOVA). Pairwise comparisons were performed using a two-sided \( \alpha=0.05 \) level of significance. All analyses were performed using SAS v9.1 (SAS Institute, Cary, NC) and utilized the raw, non-transformed data. The analysis of the association between miR expression and lesion depth/mitotic activity was performed in those lesions for which sufficient information was available. No cutpoints were used in the analysis.
Table 2.1 Lesion demographics

<table>
<thead>
<tr>
<th>Type of nevomelanocytic proliferation</th>
<th># in study</th>
<th>w/ SLN biopsy</th>
<th>Follow-up available</th>
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</thead>
<tbody>
<tr>
<td>Dermal nevus</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dysplastic nevus</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nevoid borderline</td>
<td>13</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Atypical Spitz tumor</td>
<td>22</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Pigmented epithelioid melanocytoma</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Deep penetrating nevus or overlap features with deep penetrating nevus</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Malignant melanoma</td>
<td>28</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>
Results

Comparison of miR-21 and miR-155 expression levels in benign nevi and malignant melanoma.

Our group evaluated the expression of 224 mature miRs by microarray in cultured melanocytes, keratinocytes, melanoma cell lines and in normal epidermal skin or matched primary/metastatic melanoma tumors. Using this approach, several miRs (miR-17-5p, miR-21, miR-107, miR-130a, miR-155, miR-181b, and miR-221) were found to be preferentially up-regulated in primary melanoma tumors when compared to miR expression in cultured melanocytes. Total RNA was isolated from paraffin-embedded tissue and tested for the expression of these miRs by Real-Time PCR (Figure 2.1A, B). miR-21 was expressed to a significantly greater level in malignant melanoma samples as compared to benign nevi (mean fold increase = 8.5 ± 7.4, p < 0.0001). miR-155 was also expressed to a significantly greater extent in malignant melanoma samples (mean fold increase = 12.9 ± 16.3, p < 0.0001). There was also elevated expression of four of the other five melanoma-specific miRs previously identified by microarray (mean fold increase = 1.87 ± 1.24, 2.18 ± 2.51, 1.27 ± 0.85, 1.89 ± 1.37, and 0.26 ± 0.14 for miR-17-5p, miR-107, miR-130a, miR-181b, and miR-221, respectively. However, the up-regulation of these miRs in melanoma was modest and did not reach statistical significance (p’s > 0.11, data not shown). Therefore, expression of these miRs in melanoma tumors was not pursued any further. Other miRs identified by microarray as being down-regulated in primary melanomas as compared to
Figure 2.1. **Expression of microRNAs in benign nevi and malignant melanoma.**

Real-Time PCR was used to determine the expression of (A) miR-21, (B) miR-155, and (C) miR-211 in benign nevi (n=7) and malignant melanoma (n=28). Data were expressed as the mean fold increase relative to benign nevi and normalized to the level of RNU6B.
Figure 2.1 continued

B

Fold change v. benign nevi

miR-155

Benign nevi  Malignant melanoma

C

Fold change v. benign nevi

miR-211

Benign nevi  Malignant melanoma
cultured melanocytes were also down-regulated in our data set (mean fold increase of 0.35 ± 0.72 and 0.76 ± 0.76 for miR-211 and miR-373, respectively). Of these miRs, only miR-211 was down-regulated to a statistically significant degree as compared to benign nevi (p = 0.012) (Figure 2.1C). Examination of miR-21 and miR-155 expression in melanomas with differing growth phases revealed a non-significant trend towards increased miR-155 and miR-21 expression in melanomas exhibiting an incipient or fully evolved vertical growth phase as compared to melanomas with only a radial growth phase (data not shown).

**In situ hybridization demonstrates the presence of miR-21 and miR-155 within melanoma tumors.**

In situ hybridization was used to evaluate the expression of miR-21 and miR-155 in normal skin, benign nevi, and malignant melanoma. miR-21 and miR-155 expression was not detected in normal skin (Figure 2.2A, B) or benign dermal nevi (Figure 2.2C, D). However, miR-21 and miR-155 signals were abundant in malignant melanoma samples (Figure 2.2E, F). Notably, staining for these miRs was confined to malignant cells, while surrounding normal cells showed no evidence of miR-21 or miR-155 expression. Real-Time PCR performed on RNA isolated from the identical tissue samples validated the expression of these miRs (Figure 2.2A-F inset).

**Melanoma cell lines show variable expression of miR-21, miR-155, and miR-211.**

The expression of miRs was next examined in human melanoma cell lines. Total RNA was harvested from a panel of ten melanoma cell lines after they had reached
Figure 2.2. **In situ hybridization demonstrates the presence of miR-21 and miR-155 within melanoma tumors.** In situ hybridization for miR-21 and miR-155 in (A,B) normal skin (200X), (C,D) benign nevi (400X), and (E,F) malignant melanoma (400X). Specific staining is blue, and counterstain is pink. miR-21 and miR-155 expression determined by Real-Time PCR is indicated in the corner of each panel.
70-80% confluence (Table 2.2). The RNA was analyzed by Real-Time PCR for miR expression. miR-21 was found to be consistently up-regulated in human melanoma cell lines with expression levels ranging from a 5.5-fold increase in 1259 MEL to a 321-fold increase in Hs294t (Figure 2.3A). Elevated expression of miR-155 was seen only in HT144 (Figure 2.3B). Reduced expression of miR-211 was found in the majority of cell lines (Figure 2.3C).

Levels of miR-21 and miR-155 in dysplastic nevi and indeterminate/borderline melanocytic tumors.

To characterize the expression of miR-21, miR-155, and miR-211 in indeterminate lesions, total RNA was harvested from 49 indeterminate melanocytic tumors and dysplastic nevi and analyzed for miR expression by Real-Time PCR. miR expression values were calculated relative to those for benign nevi. miR-21 and miR-155 expression levels were highly variable among individual specimens (range = 0.1 to 45-fold increase for miR-21 and range = 0.2 to 21-fold increase for miR-155; Figure 2.4A, B). miR-211 expression was also variable (range = 0.03 to 11-fold increase; Figure 2.4C). A statistically significant positive correlation between the expression of miR-21 and the expression of miR-155 was observed in indeterminate melanocytic tumors (p = 0.0003, R = 0.53, Figure 2.4D). This relationship was not observed in primary melanoma tumors or between miR-211 and the expression of miR-21 or miR-155 (data not shown).
Table 2.2 *Origins of cell lines utilized*

<table>
<thead>
<tr>
<th>Tumor Line</th>
<th>Site of Origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1074 MEL</td>
<td>Subcutaneous</td>
<td>Restifo et al., 1996</td>
</tr>
<tr>
<td>1106 MEL</td>
<td>Subcutaneous</td>
<td>Restifo et al., 1996</td>
</tr>
<tr>
<td>1174 MEL</td>
<td>Lymph Node</td>
<td>Restifo et al., 1996</td>
</tr>
<tr>
<td>1259 MEL</td>
<td>Subcutaneous</td>
<td>Restifo et al., 1996</td>
</tr>
<tr>
<td>18105 MEL</td>
<td>Unspecified Metastatic Lesion</td>
<td>Hicklin et al., 1998</td>
</tr>
<tr>
<td>A375</td>
<td>Skin</td>
<td>Giard et al., 1973</td>
</tr>
<tr>
<td>FO1</td>
<td>Metastasis from Iliac Node</td>
<td>Giovanella et al., 1972</td>
</tr>
<tr>
<td>Hs294t</td>
<td>Lymph Node Metastasis</td>
<td>Gershwin et al., 1977</td>
</tr>
<tr>
<td>HT144</td>
<td>Subcutaneous</td>
<td>Fogh et al., 1977</td>
</tr>
<tr>
<td>MEL 39</td>
<td>Unspecified Metastatic Lesion</td>
<td>Ferrone, S. Personal Communication</td>
</tr>
</tbody>
</table>
Figure 2.3  Melanoma cell lines show variable expression of miR-21, miR-155, and miR-211. Real-Time PCR was used to determine the expression of (A) miR-21, (B) miR-155, and (C) miR-211 in 10 melanoma cell lines. Data were expressed as the mean fold increase relative to benign nevi. All Real-Time PCR data were normalized to the level of RNU6B.
Figure 2.3 continued
Figure 2.3 continued

C

miR-211

Fold change v. benign nevi

1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00

1074 MEL
1106 MEL
1174 MEL
1259 MEL
18105 MEL
A375
FOI
Hs294t HT144 MEL 39
Figure 2.4  **Levels of miR-21, miR-155, and miR-211 in dysplastic nevi and indeterminate melanocytic tumors.** Real-Time PCR was used to determine the expression of (A) miR-21, (B) miR-155, and (C) miR-211 in indeterminate melanocytic lesions. Data were expressed as the mean fold increase relative to benign nevi and normalized to the level of RNU6B. miR-21 and miR-155 expression levels within individual lesions were plotted against each other (D).
Figure 2.4 continued

B

miR-155

Fold change v. benign nevi

Deep-penetrating nevi
Dyplastic Nevi
Nevoid borderline
Atypical Pigment Synthesizing
Atypical Spitz

C

miR-211

Fold change v. benign nevi

Deep-penetrating nevi
Dyplastic Nevi
Nevoid borderline
Atypical Pigment Synthesizing
Atypical Spitz

continued...
The expression of miR-21 and miR-155 in indeterminate lesions correlates with mitotic activity and lesion depth.

Expression of miR-21, miR-155, and miR-211 in pathologically indeterminate melanocytic lesions was evaluated in the context of two markers of malignant potential, mitotic activity and depth of invasion. Melanocytic lesions with \( \geq 1 \) mitosis per 10 high power field (n=15) expressed significantly higher levels of both miR-21 and miR-155 as compared to lesions with no mitotic activity (n=19) (\( p = 0.013 \) and 0.049, respectively; Figure 2.5A, B). miR-211 expression did not correlate with mitotic activity.

Indeterminate melanocytic lesions with a depth \( \geq 1 \) mm (n=17, range: 1.18–3.1 mm; median: 1.68 mm) had significantly higher levels of miR-155 expression as compared to lesions with a depth <1 mm (n=17, range: 0.2-0.94 mm; median: 0.62 mm) (\( p = 0.0058 \);
Figure 2.5C). No statistically significant relationship was evident between miR-21 or miR-211 expression and lesion depth (p = 0.22 and 0.20, respectively).

Figure 2.5 **Association of miR-21 and miR-155 expression with mitotic activity and lesion depth.** Melanocytic lesions with a mitotic activity >1 in 10 HPF had significantly higher levels of both (A) miR-21 and (B) miR-155 expression levels as compared to lesions with no mitotic activity. Melanocytic lesions with a depth ≥1 mm had greater expression of (C) miR-155 as compared to lesions with a depth <1 mm.
Figure 2.5 continued

B

Fold change v. benign nevi

miR-155

Mitotic Activity per 10 x HPF

C

Fold change v. benign nevi

miR-155

Lesion Depth (mm)
Expression of miR-21 and miR-155 according to lesion type.

miR expression was further analyzed according to lesion type (borderline tumors in deep penetrating nevi, nevoid borderline, atypical Spitz tumor, pigmented epithelioid melanocytoma, and dysplastic nevi). Significant increases in miR-155 and miR-21 were seen in the setting of the borderline tumors arising in deep penetrating nevi as compared to benign nevi (n=3, mean fold increase 6.93 ± 4.22, p < 0.01 and 20.91 ± 10.40, p < 0.001 for miR-155 and miR-21, respectively). The nevoid borderline lesions (n=13) manifested significantly increased levels of miR-155 (mean fold increase 3.70 ± 3.17, p = 0.04) and miR-21 (mean fold increase 4.23 ± 3.31, p = 0.02) as well as significantly decreased miR-211 expression (mean fold increase 0.54 ± 0.36, p = 0.03) as compared to benign nevi. For the atypical Spitz tumor lesions (n=22), miR-211 expression exhibited a significant decrease (mean fold increase 0.24 ± 0.28, p < 0.001) whereas expression of miR-21 and miR-155 was variable. Additionally, miR-155 expression was found to be moderately elevated in atypical Spitz tumors when compared to primary typical Spitz lesions (mean fold increase 3.55 ± 4.83 v. 1.58 ± 1.18, respectively). As for dysplastic nevi (n=8) and pigmented epithelioid melanocytomas (n=3), while tissue miR-21 and miR-155 expression was increased as compared to benign nevi, the increase did not meet statistical significance. When analysis was extended to each indeterminate lesion subtype according to lesion depth, significantly greater miR-155 expression was seen in atypical Spitz lesions with a depth ≥1 mm (n=6) as compared to atypical Spitz lesions with a depth <1 mm (n=12) (p = 0.013). There was no obvious correlation between miR profiles and the degree of atypia within any of these tumors.
miR-21 and miR-155 expression levels in specimens from patients who underwent sentinel lymph node biopsies.

It is difficult to make a definitive determination of malignant potential in some patients with indeterminate melanocytic proliferations, and in some cases the regional lymph nodes will be assessed via sentinel lymph node biopsy (SLNB). We examined the expression of miR-21, miR-155, and miR-211 in 14 patients who had undergone a sentinel lymph node biopsy after being diagnosed with an indeterminate melanocytic lesion. Patients with a positive SLNB were found to have higher levels of miR-21 and miR-155 expression in the primary lesion as compared to lesions from patients who had a negative SLNB. For miR-21 the mean fold increase as compared to benign nevi was 5.9 ± 3.1 in SLNB positive patients vs. 1.8 ± 1.4 in SLNB negative patients. For miR-155 the mean fold increase was 8.3 ± 8.8 in SLNB positive patients vs. 4.0 ± 4.5 in SLNB negative patients (Table 2.3). As predicted, miR-211 expression was decreased in lesions from patients with a positive SLB as compared to expression in lesions from patients who had a negative SLNB (mean fold increase = 0.28 ± 0.18 vs. 0.69 ± 0.84). The expression of miR-21, miR-155, and miR-211 is now being evaluated prospectively by our group in patients that undergo SLB for indeterminate lesions in order to evaluate the statistical significance of this finding.
Table 2.3 miR expression in primary lesions from patients with SNLB

<table>
<thead>
<tr>
<th></th>
<th>miR-21</th>
<th>miR-155</th>
<th>miR-211</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative lymph node biopsy</td>
<td>1.91</td>
<td>2.71</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td>0.69</td>
<td>0.85</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>3.11</td>
<td>1.82</td>
<td>0.81</td>
</tr>
<tr>
<td>miR-21 avg = 1.80</td>
<td>0.13</td>
<td>0.20</td>
<td>1.45</td>
</tr>
<tr>
<td>miR-155 avg = 3.99</td>
<td>2.52</td>
<td>9.39</td>
<td>2.40</td>
</tr>
<tr>
<td>miR-211 avg = 0.69</td>
<td>4.30</td>
<td>12.76</td>
<td>0.04</td>
</tr>
<tr>
<td>Positive lymph node biopsy</td>
<td>0.66</td>
<td>3.36</td>
<td>0.13</td>
</tr>
<tr>
<td>miR-21 avg = 5.91</td>
<td>1.10</td>
<td>0.79</td>
<td>0.05</td>
</tr>
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</table>

Discussion

The present study was conducted to determine if there was a relationship between the expression of miRs identified by microarray and factors that contribute to a malignant phenotype in melanocytic lesions. The following observations were made: First, the expression of miR-21 and miR-155 was significantly higher in primary melanoma lesions as compared to benign nevi while expression of miR-211 was significantly lower;
second, miR-21, miR-155, and miR-211 expression levels were highly variable within indeterminate lesions derived from individual patients; and third, the expression of miR-21 and miR-155 in indeterminate melanocytic lesions correlated with mitotic activity, while miR-155 expression correlated positively with tumor depth.

Several studies have examined miR expression in malignant melanoma. Using high-resolution array-based comparative genomic hybridization, Zhang et al. showed that 12 of 45 melanoma cell lines had an increased copy number of miR-21 and 7 of 45 melanoma cell lines had an increased copy number of miR-155. They also showed that miR-1-1, miR-96, and miR-296 exhibited an increased copy number in these cell lines; however, these three miRs were not up-regulated in our panel of melanoma tumors as determined by Real-Time PCR analysis (data not shown). To identify the miRs that were uniquely expressed in specific tumor types, Gaur et al. characterized the miR profiles of tumors represented in the NCI-60 panel which contains hematologic, colon, CNS and melanoma cell lines (n=8). Using a highly sensitive PCR-based method, they found that miR-146, miR-204, and miR-211 were uniquely expressed in these melanoma cell lines as compared to normal tissues and the other 51 cell lines in the panel. In contrast, we had found that miR-211 is down-regulated in melanoma cell lines and primary tumors. Also, further analysis of our panel of melanoma primaries by Real-Time PCR revealed no significant increase in the expression of miR-146 or miR-204 as compared to benign nevi. The discrepancy between the two studies can likely be ascribed to the unique make-up of the NCI-60 panel. Schultz et al. compared the expression of 157 mature miRs in a pooled sample of ten benign melanocytic nevi and a pooled sample of ten primary melanomas. While they observed a decrease in the
expression of members of the let-7 family, an increase in miR-21 or miR-155 expression in the melanoma sample was not observed. This result contrasts with that of Felicetti et al. who demonstrated a dramatic over-expression of miR-221 and miR-222 in melanoma cell lines as well as within melanoma lung and lymph node metastases. Of note, decreased expression of let-7 family members and increased expression of miR-221 and miR-222 were not observed in our panel of primary melanoma tumors (data not shown). Taken together, these results underscore the fact that miR expression profiles will vary according to the technique employed to measure miR expression and the tissue being examined. Given that over-expression of miR-21 and miR-155 in melanoma primary tumors was confirmed using three distinct assays, we feel that further analysis of these miRs in the setting of melanoma is warranted.

Histopathological evaluation is the currently the standard method for the classification of indeterminate/borderline lesions. Few studies have examined methods to characterize the malignant potential of indeterminate melanocytic lesions. Kashani-Sabet et al. used a panel of five immunohistochemical markers (ARPC2, FN1, RGS1, SPP1, and WNT2) to distinguish melanoma tumors from benign nevi. In a set of 24 pigmented lesions that were originally misdiagnosed as being either benign or malignant, the pattern of staining of these five markers correctly classified 18 of the 24 lesions (75%). It has been proposed that atypical Spitz tumors represent a distinct subtype of melanocytic lesion, but reliable methods to differentiate between those lesions likely to metastasize and those that will exhibit a benign course have yet to be verified. Immunohistochemical analysis of benign Spitz tumors, atypical Spitz tumors, and
malignant melanomas for Ki67 (a marker of proliferation), p21 (cell cycle regulator), and fatty acid synthase (metabolic marker) revealed that the atypical lesions represent a true intermediate stage. CD99 was identified by King et al. as being present by immunohistochemistry in 15 of 27 (56%) definitively malignant Spitzoid melanomas and only 3 of 58 (3%) benign Spitz tumors. However, the expression of this marker was not explored within indeterminate atypical Spitz tumors. Given the ability of miRs to correctly classify many other types of cancers, we believe that their expression in borderline/atypical melanocytic lesions deserves further study.

Several potential limiting factors in this study must be noted. Our choice of normalization strategy (comparison to benign nevi) makes intuitive and clinical sense, but other tissues, such as melanocytes or normal skin, might also be employed. Another potential limiting factor was the decision to collect samples from only our institution, because this restricted the numbers of indeterminate lesions that could be examined. A further concern is that miR analysis was performed on lesions that had been obtained via punch biopsy. Although these specimens contained very little if any surrounding normal tissue, it might be desirable in future studies to employ laser capture microdissection in order to exclude the potential contribution of miR expression within normal tissues. Importantly, in situ hybridization showed conclusively that surrounding normal stroma and normal skin have low expression of the tested miRs. Despite the aforementioned limitations, the strategy of analysis employed in this study was sufficient to accurately detect differential microRNA expression and serves as a necessary first step in the characterization of miR expression in indeterminate lesions.
miR-21 has been shown to be up-regulated in a variety of solid malignancies including cancers of the brain (glioblastoma), breast, colon, esophagus, lung, pancreas, prostate, stomach, head and neck, cervix, ovary, uterus, and hematolymphoid tissues, while increased miR-155 expression has been observed in cancers of the thyroid, lung, breast, colon, pancreas and hematolymphoid tissues.\textsuperscript{152,222-233} miR-211 has recently been shown to be elevated in oral carcinomas with a poor prognosis, but no other studies have shown an association between miR-211 and cancer.\textsuperscript{162} The available data suggest that miRs may function to maintain a malignant phenotype by modulating cell proliferation or the expression of proteins that promote cell survival. Indeed, investigation of primary colorectal cancer lesions from human patients revealed that high miR-21 expression correlated with lymph node metastases, the development of distant metastases, poor survival and poor therapeutic outcome.\textsuperscript{154,234} Elevated expression of miR-155 in non-small cell lung cancer has also been found to negatively correlate with survival.\textsuperscript{233} The genes that have been identified as targets of miR-21 include the tumor suppressors tropomyosin 1 (TPM1), PTEN, programmed cell death 4 (PDCD4), and Maspin.\textsuperscript{235-239} Verified targets of miR-155 include the tumor protein 53-induced nuclear protein 1 (TP53/NP1), suppressors of cytokine signaling 1 (SOCS1) and activation-induced cytidine deaminase (AID).\textsuperscript{240-244} Other putative targets continue to be investigated. Functional studies have demonstrated that inhibition of miR-21 in human glioblastoma and breast cancer cell lines led to decreased proliferation and increased apoptosis.\textsuperscript{245-246} Other \textit{in vitro} studies have shown that increased miR-21 expression promoted invasion and metastasis and could modulate chemosensitivity.\textsuperscript{235,246-247} Aberrant expression of miR-155 under the control of the heavy chain promoter in B cells in a transgenic mouse
was sufficient to drive the development of a B cell malignancy.\textsuperscript{248} The pathways by which mature miR-21 and miR-155 become over-expressed in melanoma and the contributions of this event to disease progression are just now being elucidated.\textsuperscript{249-252}

The present study has explored a potential new approach to the characterization of indeterminate melanocytic lesions. These results are exciting yet preliminary and must be confirmed within a larger population. We have demonstrated that miR-21 and miR-155 correlate with mitotic activity and miR-155 correlates with lesion depth of indeterminate lesions. Expression of miR-155 by indeterminate melanocytic proliferations may be indicative of a more aggressive lesion because increasing depth is associated with a poor prognosis in melanoma. In the most aggressive form of indeterminate tumors, namely those arising in association with deep penetrating nevi or within the borderline nevoid tumors, significant increases in the expression of miR-21 and miR-155 were observed. Although node positive indeterminate tumors showed higher expression of miR-21 and miR-155, additional cases must be examined before any firm conclusions can be drawn. In the future, miR profiles may be utilized to help differentiate between lesions, establish prognosis, and prompt a more thorough search for foci of invasion.
CHAPTER 3: INCREASED MIR-21 ACTIVITY ENHANCES INVASION AND DIRECTLY TARGETS TIMP3 EXPRESSION IN MELANOMA

Introduction

In terms of incidence, melanoma is the fastest growing cancer in the United States. Surgery can be curative for early stage lesions with 5-year survival rates of 92-99% for Stage 1A/B melanoma. However, there is no consistently effective chemotherapy to treat patients in whom the primary melanoma has spread to distant organs. Understanding the pathogenesis of this disease is critical to identifying new targets for the development of better therapies as well as new diagnostic techniques.

MicroRNAs (miRs) are a class of small, non-coding RNAs, the mature form of which is approximately 19-23 nucleotides in size. These RNAs exert their effects by binding to target mRNAs and altering the translation of the mRNA. Multiple studies have shown miRs to be differentially expressed in solid and hematologic malignancies, including in melanoma. miRs affect multiple tumorigenic processes including angiogenesis, cell cycle control, cellular adhesion, cell signaling, and apoptosis.

Our group previously identified miR-21 as being over-expressed in primary cutaneous melanomas as compared to benign nevi, suggesting that miR-21 may play a
role in melanoma pathogenesis. However, the function of this microRNA in melanoma has yet to be elucidated. In this report, we evaluated the effect of increased miR-21 expression on melanoma cell line behavior in vitro. The cell lines derived from multiple stages of melanoma development exhibited increased invasion and decreased TIMP3 protein expression with increased miR-21 activity. Decreased TIMP3 expression recapitulated this increase in melanoma cell invasion. Additionally, luciferase activity assays revealed that miR-21 directly targets miR-21 binding sites in the TIMP3 3’UTR.

**Materials and Methods**

**Cell Lines and Transfection.** The human radial growth melanoma cell line WM 1552c and the human vertical growth melanoma cell line WM 793b were provided by Dr. M. Herlyn (Wistar Institute, Philadelphia, PA) and cultured as previously described. The human metastatic melanoma cell line MEL 39 was a gift from Soldano Ferrone (Roswell Park Cancer Institute, Buffalo, NY) and cultured as previously described. A375 human metastatic melanoma cell line was obtained from American Type Cell Culture Collection (ATCC, Manassas, VA). Cells were transfected with pre-miR-21, pre-miR control, (Ambion, Austin, TX) or TransIT TKO transfection reagent alone (Mirus Bio, Madison, WI) 24 hours prior to use in all assays. Real-time PCR was performed on RNA isolated from all transfection reactions to confirm mature miR-21 expression increases.

**Reagents.** Tissue inhibitor of metalloproteinase 3 (TIMP3)-specific small-interfering RNA (siRNA) and negative control constructs were purchased from Santa
Cruz Biotechnology, Inc (Santa Cruz, CA). The pMir-Report vector was obtained from Ambion and the pMir-Report-3’TIMP3-wt and pMiR-Report 3’TIMP3-mut were provided by Dr. A. Krischevsky (Harvard University).\textsuperscript{184}

**Proliferation Assay.** Cell proliferation was measured as absorbance at 570 nm using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Cell Proliferation Assay kit according to the manufacturer's instructions (ATCC). All assays were performed in triplicate as previously described.\textsuperscript{258}

**Doubling Time Assay.** Twenty-four hours following transfection with pre-miRs, 5 x 10\textsuperscript{3} cells were serially plated in six well plates. Total cell number was determined through cell counting with a hemocytometer at 72, 96, 120, and 144 hours. Doubling time for each experiment was determined to be equal to 1/slope of the line of best fit. All experiments were performed in duplicate.

**Scratch Test.** Cells were plated overnight in 6-well plates at a density of 4 x 10\textsuperscript{5} cells per well. When the cells were 70-80\% confluent, they were transfected with pre-miRs. Twenty-four hours post-transfection, a pipette tip was used to create a scratch in the monolayer. Photos of three different places along the wound were taken immediately and 18 hours later. The width of the scratch was measured at 5 points within each photo. Percent wound closure was determined by calculating the ratio of the wound width at 18 hours to the width at 0 hours.
**Invasion Assays.** Matrigel invasion assays were conducted according to manufacturer’s instructions (BD Biosciences, San Jose, CA). Briefly, 2.5 x 10⁴ transfected cells in media containing 2% FBS were plated in duplicate on Transwell filters coated with or without matrigel. The lower compartments of the invasion chambers contained media with 10% FBS. After a 22 hr incubation at 37°C, cells remaining on the upper surface of the filter were removed, and the cells that migrated through the filter were stained with toluidine blue. Photographs were taken of 5-10X fields for each filter and cell numbers were enumerated from the images. Data is expressed as the percent invasion through the Matrigel matrix by calculating the ratio of the mean number of cells that invaded through the matrigel matrix to the mean number of cells that migrated through the control insert.

**Immunoblot Analysis.** Immunoblots were prepared as previously described and probed with antibodies specific for TIMP3, programmed cell death protein 4 (PDCD4), tropomyosin-1 (TM1), (Santa Cruz Biotechnology), phophatase and tensin homolog (PTEN) (Cell Signaling Technology), or β-actin (Sigma).259 Following incubation with the appropriate horseradish peroxidase-conjugated secondary Ab, immune complexes were detected using the ECL Plus detection kit (Amersham Biosciences, Ayesbury, UK).

**Luciferase miRNA targeting assay.** Melanoma cell lines were stably transfected with pMiR-Report, pmiR-Report-3’TIMP3-wt, or pMir-Report-3’TIMP3-mut. Cells were later transfected with pre-miRs as described above. Luciferase activity was measured 24 hours following miR transfection.
Statistical Analysis. Statistical significance of differences between groups was analyzed by unpaired Student’s t test, and p ≤ 0.05 was considered to be statistically significant.

Results

Increased miR-21 activity does not affect proliferation.

The observation that miR-21 expression is increased in primary malignant melanoma tumors led us to explore the function of miR-21 in human melanoma cell lines (WM 1552c, WM 793b, A375, and MEL 39). In order to examine the contribution of increased miR-21 expression to an aggressive melanoma phenotype, we transfected four melanoma cell lines with a non-specific control pre-miR or pre-miR-21. Sham-transfected cells were used as an additional control. Mature miR-21 expression was significantly and consistently over-expressed in each cell line transfected with pre-miR-21 as compared to sham-transfected cell lines (WM 1552c: 341 ± 60 fold increase; WM 793b: 242 ± 96 fold increase; A375: 50 ± 19 fold increase; MEL 39 – 13 ± 3.9 fold increase) (Figure 3.1A).

Potential changes in the proliferative capacity of cells expressing elevated amounts of miR-21 were evaluated through mitochondrial reduction of yellow MTT to purple formazan seventy-two hours following the plating of transfected cells. Relative growth of pre-miR-21 as compared to sham-transfected cells failed to produce an
increase or decrease of greater than 6.3% in each of the four melanoma cell lines (Figure 3.1B). A lack of a difference in proliferation was confirmed upon failure to detect a change in doubling time between cells transfected with pre-miR-21 and those cells sham-transfected or transfected with the control pre-miR (Table 3.1). While there was an average increase of 14.9% or 3.8 hours in the pre-miR-21 transfected WM 793b cell line as compared to sham-transfected cells, this increase was matched by that of the control pre-miR transfected cells. This indicated that the increased proliferative activity was not specific to the presence of increased miR-21.

**Increased miR-21 activity leads to increased invasion but not migration in vitro.**

For metastasis to occur, primary melanoma cells must degrade the basement membrane and extracellular matrix and migrate through the stroma. In order to assess the effects of elevated miR-21 expression on melanoma cell movement, uniform wounds were created in transfected monolayers and changes in lesion width was monitored after 18 hours (Figure 3.2A). The difference in wound closure between control pre-miR-transfected and pre-miR-21-transfected monolayers was unremarkable in each of the cell lines except one. Transfection with pre-miR-21 resulted in a non-specific increase in WM 1552c melanoma cell movement into the wound by 36 ± 15% (Figure 3.2B). Invasive potential was examined using the Boyden chamber assay. All melanoma cell
Figure 3.1. **Effects of miR-21 on proliferation.** Real-time PCR was used to determine the expression of mature miR-21 in four human melanoma cell lines 24 hrs post-transfection with transfection reagent, control pre-miR, or pre-miR-21 (A). Proliferation was measured by MTT assay 72 hours following the plating of transfected cells described above (B). Error bars represent standard error from at least five independent experiments. * p < 0.05; *** p < 0.001
Table 3.1  Doubling time of transfected cell lines expressed in hours.

<table>
<thead>
<tr>
<th></th>
<th>WM 1552c</th>
<th>WM 793b</th>
<th>A375</th>
<th>MEL 39</th>
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<tbody>
<tr>
<td>sham</td>
<td>22.2 ± 1.5</td>
<td>25.2 ± 0.8</td>
<td>21.8 ± 3.0</td>
<td>22.1 ± 2.7</td>
</tr>
<tr>
<td>control</td>
<td>20.2 ± 1.1</td>
<td>28.9 ± 2.4</td>
<td>24.2 ± 4.6</td>
<td>22.5 ± 1.0</td>
</tr>
<tr>
<td>pre-miR-21</td>
<td>22.4 ± 1.8</td>
<td>29.0 ± 1.0</td>
<td>23.0 ± 3.6</td>
<td>22.5 ± 2.3</td>
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lines tested exhibited increased invasion upon transfection with pre-miR-21 as compared to sham-transfected cells (WM 1552c: 13.9% ± 4.8 v. 43.6% ± 4.1; WM 793B: 23.8% ± 5.4 v. 47.2% ± 6.3; A375: 7.4% ± 1.9 v. 17.8% ± 3.9; MEL 39: 20.8% ± 9.4 v. 33.5% ± 11.0) (Figure 3.2C). This increase was significant in WM 1552c, A375, and MEL 39 (p < 0.05) and approached significance in WM 793b.

**Changes in TIMP3 protein expression is associated with miR-21 expression.**

Several mRNA targets for miR-21 have been recently verified, including phophatase and tensin homolog (PTEN), tropomyosin-1 (TM1), and programmed cell death protein 4 (PDCD4).235-237,260 Additionally, tissue inhibitor of metalloproteinases-3 (TIMP3) has been identified as a putative target and has been shown to be decreased in response to miR-21.184,261 Immunoblots of cell lysates obtained 24 hours post-transfection revealed a decrease in the TIMP3 protein in melanoma cell lines transfected with pre-miR-21 (Figure 3.3A). There were no reproducible trends in the expression of TM1 and PDCD4. Interestingly, an increase in PTEN protein was observed in the WM 1552c, WM 793b, and MEL 39 cell lines in response to pre-miR-21 transfection. Because miRs can achieve a decrease in protein expression by causing the degradation of target mRNAs and by inhibiting translation of mRNA, Real-time PCR was performed to evaluate changes in TIMP3 mRNA expression in response to increased miR-21 (Figure 3.3B). TIMP3 mRNA expression was highly variable both between transfection conditions and experiments and could not be associated with the consistent observed decrease in TIMP3 protein.
Figure 3.2 *Effects of miR-21 expression on migration and invasion.* Following sham transfection or transfection with control pre-miR or pre-miR-21, a wound was created in
the cell monolayers. Photographs were taken of the wound immediately following wound initiation and 18 hours later. Representative experiments for each cell line are shown (A). Migration was measured as the percent wound closure (n = 4) (B). Boyden chamber assays were used to evaluate invasive activity of melanoma cells. Data are represented as the ratio of the average number of cells migrating through control inserts to the average number of cells invading through inserts layered with matrigel (C). Error bars represent the standard error of at least four experiments. * = p < 0.05
Figure 3.2 continued

B

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continued...
Figure 3.2 continued

B

% wound closure

- sham
- control
- pre-miR-21

WM 1552c  WM 793b  A375  MEL 39

C

% Invasion

- sham
- control
- pre-miR-21

WM 1552C  WM 793b  A375  MEL 39
Figure 3.3 Effects of miR-21 expression on levels of target proteins. Cells were collected 24 hours post-transfection for lysis and total RNA isolation. Twenty μg of protein were loaded and subjected to immunoblotting for the proteins TM1, TIMP3,
PDCD4, and PTEN (A). Immunobloting for β-actin was used as the loading control. The RNA was converted to cDNA and Real-time PCR for TIMP3 was performed (B). Induction of expression was calculated relative to β-actin and compared to sham-transfected cells.
Figure 3.3 continued

**Decreased TIMP3 expression increases melanoma invasiveness.**

The finding that TIMP3 expression was decreased in melanoma cells with elevated miR-21 and increased invasiveness prompted an exploration into the influence of TIMP3 on invasion. Invasion through matrigel was significantly enhanced in melanoma cell lines in which TIMP3 expression was down-regulated by siRNA. (Figure 3.4A). Reduction of TIMP3 protein expression was confirmed by immunoblot (Figure 3.4B).
Figure 3.4  **Effect of TIMP3 down-regulation on melanoma invasion.** Immunoblot performed on lysates collected from cells 24 hours post-transfection with TIMP3-specific siRNA confirmed down-regulation of TIMP3 expression (A). Immunoblotting for β-actin was used as the loading control. Boyden chamber assays were used to evaluate invasive activity of melanoma cells transfected with TIMP3 siRNA. Data are represented as the ratio of the average number of cells migrating through control inserts to the average number of cells invading through inserts layered with matrigel (B). Error bars represent standard error of four experiments. * = p < 0.05
miR-21 directly targets the 3’UTR of TIMP3.

To determine whether miR-21 mediated repression of TIMP3 was a direct result of mature miR-21 binding to bioinformatically predicted sites on the 3’UTR of TIMP3, we obtained luciferase constructs under the control of the wild-type TIMP3 3’UTR (TIMP3 3’UTR), or the TIMP3 3’UTR in which the miR-21 binding sites were mutated (TIMP3 3’UTR-mut), and the wild-type luciferase 3’UTR (Empty) (Figure 3.5A). Melanoma cell lines were stably transfected with these constructs and then transfected with pre-miR-21. Luciferase assays performed 24 hours post-transfection revealed a significant reduction of 25% in luciferase activity in cells co-transfected with pre-miR-21 and TIMP3 3’UTR as compared to sham transfection (p’s < 0.05) (Figure 5.5B). This
reduction was not observed upon transfection with pre-miR-21 in melanoma cell lines expressing the other luciferase constructs. These results indicate that miR-21 acts directly on the TIMP3 3’UTR in melanoma cell lines and that its activity is mediated by the predicted binding sites.

Figure 3.5. **Effects of miR-21 on TIMP3 3’ UTR.** Schematic of luciferase expression vectors (A). Luciferase assays were performed on melanoma cell lines WM 1552c (B), WM 793b (C), A375 (D), and MEL 39 (E) that had been stably transfected with luciferase expression vectors and then transfected with pre-miR-21. Data represent the average of four experiments. Error bars represent standard error.
Figure 3.5 continued

**B**

![Graph for WM 1552c](image)

**C**

![Graph for WM 793b](image)

continued...
Figure 3.5 continued

D

![Graph showing relative luciferase activity for different conditions.](image)

E

![Graph showing relative luciferase activity for different conditions.](image)
Discussion

Our results demonstrate that increased miR-21 expression enhances invasion but not proliferation or migration in melanoma cell lines. Increasing the expression of miR-21 resulted in a decrease in TIMP3 protein expression but did not alter that of other recognized targets of miR-21. Subsequent silencing of TIMP3 expression phenocopied the results of increased miR-21 in melanoma. The effects of miR-21 thus appear to be mediated by down-regulation of TIMP3 through direct action of miR-21 on the TIMP3 3’UTR.

TIMP3 is a member of the tissue inhibitor of metalloproteinases family which bind metalloproteinases and other proteolytic enzymes to reduce their activity. Unlike other TIMPs, TIMP3 has decreased solubility and is found in association with the extracellular matrix and basement membrane and not in cell culture supernatants.\textsuperscript{262-264} Decreases in TIMP3 expression have been observed in a variety of malignancies and correlated with aggressiveness in cancers arising in the thyroid, breast, prostate and lung, supporting a role for TIMP3 as a tumor suppressor.\textsuperscript{265-269} Expression of TIMP3 has been associated with decreases in invasion due to decreased extracellular matrix degradation, decreased angiogenesis due to the prevention of VEGF binding to VEGFR2, and increased apoptosis. An increased sensitivity to serum starvation and sensitization to TNF-\(\alpha\), anti-Fas-antibody, and TRAIL has been ascribed to an inhibition of the shedding of surface proteins by TIMP3.\textsuperscript{270} While over-expression of TIMP3 in cell lines derived metastases from cutaneous melanoma has been shown to decrease invasion \textit{in vitro}.\textsuperscript{271}
our results represent the first time a decrease in TIMP3 has been shown to confer an invasive phenotype on a cell line originating from a primary radial growth melanoma, a form of melanoma that lacks the capacity to invade and metastasize. This implicates a decrease in TIMP3 expression as a potential mechanism by which melanomas exhibiting only the radial growth phase may transition to the more aggressive vertical growth phase.

Little is known about how TIMP3 expression is regulated in melanoma pathogenesis. Promoter methylation in primary cutaneous melanomas and metastatic lesions was shown to be a low frequency event,\textsuperscript{272} suggesting that its decreased expression in melanoma must be accomplished through another mechanism of regulation. Interestingly, a study of uveal melanomas observed high levels of TIMP3 mRNA transcripts in association with little to no expression of TIMP3 protein.\textsuperscript{273} This supports the hypothesis that post-transcriptional regulation of TIMP3 is critical in melanoma. The findings that the TIMP3 3’ UTR is sensitive to miR-21-directed suppression (\textbf{Figure 3.5}) in melanoma and that miR-21 expression is elevated in primary melanoma tumors (\textbf{Figure 2.1}) implicates a role for miR-21 silencing of TIMP3 in the progression of melanoma.

While miR-21 has been identified as a microRNA commonly expressed by a multitude of solid tumors\textsuperscript{152}, this study represents the first time a function has been ascribed to miR-21 in the context of melanoma. miR-21 expression has been previously associated with an invasive phenotype in several malignancies due to its ability to target multiple pathways affecting invasion. Meng \textit{et al.} found miR-21-mediated increases in
hepatocellular carcinoma invasion were due to the direct targeting of PTEN mRNA.\textsuperscript{237} The resulting decrease in PTEN protein was associated with enhanced expression of MMP-2 and MMP-9, two matrix proteases that are also implicated in melanoma pathogenesis. A role for miR-21-associated down-regulation of PDCD4 in colon cancer and TM1 and PDCD4 in breast cancer has also been confirmed.\textsuperscript{235,246} In the current study, however, increased miR-21 expression was not found to decrease PTEN, TM1, or PDCD4 protein 24 hours post-transfection, suggesting that these pathways do not play a role in miR-21-initiated invasion of melanoma. Other newly identified targets of miR-21, including Maspin, MARCKS, and RECK, have been shown to participate in decreasing invasion through matrigel and therefore warrant some exploration into their ability to do so in melanoma as collaboration of factors likely causes this effect of miR-21.\textsuperscript{183-184,246,274}

The observation of a greater invasive potential without a concomitant increase in migration in miR-21-transfected cells suggested that miR-21 was acting on one or more factors affecting the cell’s ability to degrade the extracellular matrix in cells that already had mobility attributes. The identification of TIMP3 as a functional target of miR-21 supports our phenotypic findings. Decreased expression of TIMP3 in response to increased miR-21 was first described in cholangiocarcinoma and glioma,\textsuperscript{184,261} however, this study is the first to identify TIMP3 as a direct target of miR-21 in the context of melanoma.
CHAPTER 4: DISCUSSION

Melanoma continues to be a poorly understood malignancy. Animal modeling systems are limited due to the unique distribution of melanocytes in the basal layer of the epidermis in humans as opposed to the dermal residence of melanocytes in mice and other species. With few effective treatments for residual disease after surgical resection, there is a significant need for both greater insights into melanoma pathogenesis and diagnostic tools that can aid in the identification of patients at an increased risk for metastasis so that aggressive treatment may be pursued. The studies described in chapter 2 attempted to address these issues by evaluating the expression of microRNAs in melanoma. miR-21, miR-155, and miR-211 were identified as being differentially expressed when benign nevi and primary melanomas were compared. These microRNAs were found to be variably expressed in primary melanocytic lesions that could not be classified as benign or malignant. miR-21 and miR-155 expression in these lesions were also associated with mitotic activity, and miR-155 was associated with increased depth, both being established prognostic factors. These results coupled with the findings that miR-21 and miR-155 levels were elevated in primary indeterminate melanocytic lesions excised from patients in which melanocytic cells had been detected in the sentinel lymph nodes indicate that miR-21 and miR-155 may be useful in classifying melanocytic disease.
One potential limitation of the study conducted in chapter 2 is the use of whole sections of melanocytic lesions rather than laser capture microdissection (LCM) to isolate the melanocytic cells prior to total RNA extraction. As miR-155 has been identified as critical to immune responsiveness and is elevated in macrophages, dendritic cells, T cells, and B cells following stimulation with antigen, TNF-α, IFN-β, poly(I:C), and other pro-inflammatory cytokines, elevated miR-155 in primary melanomas may reflect an expression in an increased immune infiltrate. However, in situ hybridization showed that miR-155 expression in melanoma tumors localized to the malignant cells rather than the surrounding stroma (Figure 2.2). As the focus of our evaluation of microRNA expression was to examine their potential for use in melanoma diagnosis, LCM would be cumbersome to diagnosticians. In addition to representing an additional step in processing, LCM would likely require more tissue for analysis. As discussed in chapter 1, melanoma tumors are small, on the order of mm, and adequate tissue must be reserved for histological examination. This leaves a small amount of lesion left over for further evaluation. Based on the highly variable expression of the microRNAs discussed in chapter 2 (Figure 2.1), it is also highly unlikely that levels of one microRNA would be able to firmly distinguish between tumors with a malignant future and tumors without one. Therefore, enough RNA must be isolated to allow for the testing of multiple microRNAs, and LCM represents an approach that likely would be unable to meet such demands. As was accomplished in chapter 2, ensuring that RNA would be isolated from samples that contain as little of normal surrounding tissue as possible would allow for sufficient RNA recovery.
Expression of microRNAs in patient plasma is another potential source of diagnostic and prognostic information. Circulating microRNAs have been shown to be remarkably stable at room temperature and in response to treatment with RNases and repeated freeze/thaw cycles.\textsuperscript{278} The stability is likely due to their containment in microvesicles. In normal healthy donors, the microRNAs isolated from peripheral blood microvesicles were similar to the microRNAs isolated from circulating cells. In patients with lung or colorectal cancer, however, the overlap in microRNA expression is less with several unique microRNAs found in the serum that are not found in normal serum or circulating cells.\textsuperscript{279} The finding that human microRNAs could be found circulating in plasma taken from mice xenografted with human prostate cancer confirmed that tumors do in fact shed microRNAs into the peripheral circulation.\textsuperscript{278} Since this discovery, much effort has been put into finding a suitable small RNA to be used for normalization, although the search has yielded conflicting results. A consensus or at the very least a preponderance of opinion must be reached for testing of circulating microRNAs to become an acceptable practice. Still, serum levels of miR-21 and miR-155 may someday be useful in measuring melanoma progression.

While microRNA profiling of tumor tissues was initially a time-consuming and expensive process, the development of bead-based flow cytometry detection systems have made such profiling more feasible in a diagnostic setting. Originally described by Lu et al., beads colored with a mixture of two dyes and conjugated to oligonucleotides for specific microRNAs are used for hybridization to microRNAs from test tissues
following their amplification and tagging with biotin. The presence of microRNAs is then distinguished by the color of the beads and the amount of the specific microRNA is determined by the intensity of staining with strepavidin-labeled PE. Such a high through-put means of quantitatively measuring microRNA is ideal for diagnostics.

Previous studies classifying poorly differentiated tumors or tumors of unknown origins have relied on the expression of multiple microRNAs. It is thus reasonable to believe that miR-21 and miR-155 in combination with other microRNAs that have been found to be dysregulated in melanoma have the potential to define a more robust signature for the identification of malignant melanocytic tumors. Down-regulation of let-7a and miR-34a and up-regulation of miR-182 and miR-15b have been independently shown to be increased in melanoma and are implicated in invasion. Expression of let-7a in melanoma cell lines led to a decrease in matrigel invasion due to the direct targeting of integrin β3 transcripts. Increased integrin β3 expression had been previously revealed to be associated with the onset of malignant and invasive phenotypes in melanoma, making its microRNA regulator an excellent candidate for the identification of invasive components in melanocytic tumors. The miR-34a locus has shown to be methylated in a myriad of malignancies including melanoma. In uveal melanoma, miR-34a expression was found to decrease cell proliferation and migration through a decrease in c-Met expression. Through the evaluation of microRNA genes located in genomic regions amplified in melanoma, miR-182 expression was found to decrease MITF and FOXO3 protein expression and thereby promote migration in vitro and metastasis in vivo. Interestingly, miR-137 has also been shown to target MITF but
was often shown to be decreased in melanoma cell lines due to the presence of a variable nucleotide tandem repeat (VNTR) in the pri-miRNA form. This VNTR altered the secondary structure of pri-miR-137 and resulted in a decrease in the expression of mature miR-137. It has been previously postulated that MITF expression may oscillate during progression as its expression can promote proliferation but also differentiation. Additionally, loss of MITF expression in cell lines has been associated with a more invasive phenotype. Gain of MITF expression may thus represent an early event in melanoma and loss may be indicative of advancement of the malignancy, making miR-182 a more interesting marker of melanoma progression. The utility of miR-15b as a prognostic indicator has already been shown by Satzger et al. Their group observed an up-regulation of miR-15b expression in cultured and primary melanomas and increased expression in primary melanoma tumors was associated with poor recurrence free survival and overall survival. Further investigation into how the expression of the aforementioned microRNAs, miR-21, and miR-155 relates represents the next step in this endeavor.

Other microRNAs have also been identified as potentially involved in melanoma pathogenesis. Increased expression of miR-532-5p was first posited to be highly expressed in melanoma due to the finding of low expression of its target RUNX3. RUNX3 is a tumor suppressor that has recently been shown to be decreased in primary and metastatic melanomas in comparison to normal skin. In parallel, miR-532-5p was also found to be highly expressed in metastatic lesions as compared to the primary lesions and cultured melanocytes. However, miR-532-5p would not be suitable for the
development of a diagnostic signature with a purpose of identifying the malignant potential of the primary melanocytic lesion as the greatest increase in expression is seen in the metastatic lesions. The same argument can be made for miR-221 and miR-222 as in situ hybridization revealed the most robust staining in visceral metastases with a sparser distribution in primary melanomas.\textsuperscript{218} On the other side of the spectrum, let-7b has been found to be decreased in primary melanomas as compared to benign nevi and is thought to suppress growth by affecting expression of cyclin D1.\textsuperscript{217} However, this likely represents an event early in melanoma pathogenesis and is thereby likely to be altered in borderline melanocytic lesions.

Current studies in melanoma have utilized several different strategies to identify microRNAs that may be involved in malignant pathogenesis. miR-182 was found due to its association with a region of genomic amplification.\textsuperscript{185} Investigation of an area associated with loss of heterozygosity led to miR-137.\textsuperscript{254} The importance of miR-221 and miR-222 was first postulated due to their ability to target a protein often down-regulated in melanoma, the c-kit receptor.\textsuperscript{218,255} However, much of the investigation into microRNAs differentially expressed in melanoma has focused on the differences in expression between melanoma cell lines and cultured melanocytes followed by exploration in primary lesions. Limiting the initial scope to the culture dish, though, leads to a failure to observe other critical differences found in vivo. For example, when only cultured cells were considered, miR-21 and miR-155 were not identified as differentially expressed, even when compared in cell lines derived from a wide spectrum of primary lesions.\textsuperscript{255-256,287} This is likely due to the highly variable expression of miR-
21 in the melanoma cell lines derived from metastatic lesions evaluated in chapter 2 and the low expression of miR-155 in the same cell lines (Figure 2.3). In fact, with few exceptions levels of miR-155 in these cell lines were less than half that observed in benign nevi. Corroborating this is one report in which miR-155 expression was elevated in cultured melanocytes and primary tumors but low in cultures of melanoma cell lines and their more metastatic derivatives.\(^{256}\) As shown in chapter 2, evaluation of the expression of these microRNAs has great potential for diagnosis and in chapter 3, expression of miR-21 affects the activity of melanoma cells. Filtering candidate microRNAs through cultured expression misses this. More studies examining differences in tissues derived in vivo would result in the identification of more microRNAs of potential importance to melanoma progression.

As the culture environment discounts the effects of stromal cells and inflammatory infiltrates, the lack of miR-155 expression in cell lines suggests that miR-155 expression is more critical in vivo than in vitro or its production is stimulated by interactions with the microenvironment. TNF-\(\alpha\) and TGF-\(\beta\) have been found to up-regulate miR-155 in this way in lymphomas and epithelial cells.\(^{191,288}\) Neutralization of TNF-\(\alpha\) with commercially available pharmaceuticals decreased miR-155 expression in lymphomas and lessened the tumor burden of a lymphoma mouse model.\(^{288}\) Also, miR-155 expression is critical to the induction of EMT by TGF-\(\beta\) as knockdown of miR-155 results in suppression of cell migration and invasion.\(^ {191}\) In vitro, melanoma cell lines treated with TGF-\(\beta\) and TNF-\(\alpha\) exhibited decreased proliferation.\(^ {289}\) In vivo, however, expression of TGF-\(\beta\) and TNF-\(\alpha\) by melanoma cells was increased in thick
melanomas as compared to those less than 1 mm in depth or classified as benign nevi.\textsuperscript{290} Additionally, increasing the expression of miR-155 in melanoma cell lines was shown to inhibit proliferation.\textsuperscript{291} These findings highlight how the microenvironment context can alter cellular responses and that exploration of the function of miR-155 should be performed in that context.

While chapter 2 establishes that miR-21 is over-expressed in melanomas, it does not explore the mechanism of the over-expression. In fact, little is known about how miR-21 is up-regulated in tumor cells. While located on chromosome 17q near a region that is amplified in many malignancies, the copy number of the miR-21 gene does not appear to be increased.\textsuperscript{292-293} Detailed studies of the miR-21 promoter region (miPPR-21) have revealed highly conserved binding sites for the transcription factors AP-1, Ets/PU.1, C/EBP-\(\alpha\), nuclear factor I, SRF, p53, and STAT3.\textsuperscript{249} NFIB and C/EBP-\(\alpha\) binding was found to regulate the basal transcription of miPPR-21 and dissociate to allow for binding of transcriptional activators in response to a stimulus. Activation of the MAPK pathway through the epidermal growth factor receptor, HER2/neu, or Ras can provide such a stimulus and up-regulates transcription of miR-21 in a manner dependent on AP-1 induction and partially dependent on the phosphorylation of ERK.\textsuperscript{132,294-295} Interestingly, transcription of miR-155 has also been shown to increase in response to activation of the MAPK pathway.\textsuperscript{251,296} As noted in chapter 1, activation of B-raf, a protein upstream of ERK in the MAPK pathway, is a common event in the development of melanoma. This distinction would lead one to hypothesize that the up-regulation of miR-21 and miR-155 observed in melanoma would be a consequence of B-raf activation. However, B-raf
mutations are common in benign nevi and the findings in chapter 2 show that both microRNAs are elevated to a greater extent in melanoma. These data suggest that another mechanism must be responsible for the increase in expression over benign nevi. Evidence for STAT3 regulation of miR-21 was shown in myeloma cells as IL-6-mediated phosphorylation of STAT3 increased the levels of miR-21. As STAT3 has been shown to be activated in both melanoma cell lines and primary melanoma specimens but not benign nevi, constitutive STAT3 phosphorylation is one potential explanation for increased miR-21 expression in melanoma tumors. Other known activators of miR-21 expression include the binding of the p65 subunit of NFκB, δEF1, and the androgen receptor. Alterations in the rate of processing of pri-miR-21 in melanoma could also affect levels of the mature microRNA. Stimulation of vascular smooth muscle cells with TGF-β, BMP-2, or BMP-4 led to an increase in mature miR-21 due to the association of SMAD proteins with pri-miR-21. This association was hypothesized to stabilize the Drosha/p68 complex for more efficient processing. In breast cancer cells, the activation of PKCε and subsequent phosphorylation of Nanog led to an association of Nanog with Drosha and p68 that also increased levels of mature miR-21. An increase in processing by a mechanism similar to those described coupled with B-raf activation poses a plausible scenario by which miR-21 expression increases in the transition from benign melanocytic lesion to malignant melanoma.

miR-21 mediates its physiologic and pathologic effects by targeting a variety of proteins (Table 4.1) with the down-regulation of some contributing to a more invasive phenotype. In chapter 3, miR-21 was found to increase the invasiveness of melanoma
Table 4.1 **Confirmed targets of miR-21**

<table>
<thead>
<tr>
<th>Target</th>
<th>Normal function of target</th>
<th>Target confirmation tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMPRII</td>
<td>TGF-β superfamily signal transduction</td>
<td>prostate cancer&lt;sup&gt;303&lt;/sup&gt;</td>
</tr>
<tr>
<td>BTG2</td>
<td>cell cycle regulation</td>
<td>laryngeal cancer&lt;sup&gt;304&lt;/sup&gt;</td>
</tr>
<tr>
<td>CDC25A</td>
<td>cell cycle regulation</td>
<td>colorectal cancer&lt;sup&gt;305&lt;/sup&gt;</td>
</tr>
<tr>
<td>HNRPK</td>
<td>cell cycle regulation</td>
<td>glioblastoma&lt;sup&gt;306&lt;/sup&gt;</td>
</tr>
<tr>
<td>IL12p35</td>
<td>inflammation</td>
<td>lung&lt;sup&gt;307&lt;/sup&gt;</td>
</tr>
<tr>
<td>JAG1</td>
<td>development and differentiation</td>
<td>immune cells&lt;sup&gt;308&lt;/sup&gt;</td>
</tr>
<tr>
<td>LRRFIP1</td>
<td>transcriptional repression</td>
<td>glioblastoma&lt;sup&gt;309&lt;/sup&gt;</td>
</tr>
<tr>
<td>MARCKS</td>
<td>cell adhesion and motility</td>
<td>prostate cancer&lt;sup&gt;183&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maspin</td>
<td>inhibition of invasion</td>
<td>breast cancer&lt;sup&gt;246&lt;/sup&gt;</td>
</tr>
<tr>
<td>NFIB</td>
<td>development</td>
<td>leukemic cells&lt;sup&gt;249&lt;/sup&gt;</td>
</tr>
<tr>
<td>PDCD4</td>
<td>translation regulation</td>
<td>breast cancer&lt;sup&gt;246,260&lt;/sup&gt;, cervical cancer&lt;sup&gt;310&lt;/sup&gt;, colorectal cancer&lt;sup&gt;235&lt;/sup&gt;, glioblastoma&lt;sup&gt;311&lt;/sup&gt;, peripheral blood mononuclear cells&lt;sup&gt;312&lt;/sup&gt;, prostate cancer&lt;sup&gt;183&lt;/sup&gt;, vascular smooth muscle&lt;sup&gt;313&lt;/sup&gt;</td>
</tr>
<tr>
<td>PTEN</td>
<td>cell cycle regulation</td>
<td>breast cancer&lt;sup&gt;246&lt;/sup&gt;, cholangiocarcinoma&lt;sup&gt;260&lt;/sup&gt;, fibroblasts&lt;sup&gt;148&lt;/sup&gt;, glomerulus&lt;sup&gt;315&lt;/sup&gt;, liver cancer&lt;sup&gt;237&lt;/sup&gt;</td>
</tr>
<tr>
<td>RECK</td>
<td>regulation of matrix metalloproteinases</td>
<td>gastric cancer&lt;sup&gt;274&lt;/sup&gt;, glioblastoma&lt;sup&gt;184&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sprouty1</td>
<td>regulation of angiogenesis</td>
<td>cardiomyocytes&lt;sup&gt;149&lt;/sup&gt;</td>
</tr>
<tr>
<td>Sprouty2</td>
<td>regulation of angiogenesis</td>
<td>cardiomyocytes&lt;sup&gt;238&lt;/sup&gt;, colorectal cancer&lt;sup&gt;238&lt;/sup&gt;</td>
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<tr>
<td>TAp63</td>
<td>apoptosis</td>
<td>glioblastoma&lt;sup&gt;306&lt;/sup&gt;</td>
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<tr>
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<tr>
<td>WNT1</td>
<td>development</td>
<td>immune cells&lt;sup&gt;308&lt;/sup&gt;</td>
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</tbody>
</table>
cells by directly binding to the 3' UTR of the TIMP3 transcript but did not affect the expression of PTEN, PDCD4, and TM1 which had previously been implicated in the invasion of extracellular matrix by cells derived from colorectal, breast, prostate, and liver cancers.\textsuperscript{183,235,237,246} An association between increased miR-21, decreased TIMP3 protein, and increased invasion was initially observed in glioma cells and cholangiocarcinoma, but an interaction between miR-21 and TIMP3 mRNA was not observed in these malignancies.\textsuperscript{184,261} These studies and our observations in Chapter 3 of direct targeting of TIMP3 by miR-21 and a lack of down-regulation of other previously described targets underscore the complexity of gene regulation by microRNAs. As microRNA repression has been shown to be dose-dependent, one might consider the abundance of mRNA targets as one factor that may determine which target is suppressed. In the case of melanoma cells, PTEN expression has been found to be reduced due to loss of chromosome 10, intragenic deletions, and promoter methylation in approximately 77% of melanoma tumors studied.\textsuperscript{316-319} Therefore, the TIMP3 mRNA may provide a greater abundance of miR-21 target sites than PTEN and thereby effectively reduce the pool of miR-21 available for further PTEN down-regulation. Currently, there are no reports describing PDCD4 expression in melanoma and changes in TM1 in melanoma have only been observed to occur in association with HOXD3-stimulated invasiveness.\textsuperscript{320}

In summary, we provide strong evidence suggesting that miR-21 and miR-155 expression is increased during the progression of melanoma. We propose that this increase can help define a microRNA signature that differentiates indeterminate melanocytic lesions based on features associated with a more malignant phenotype.
Furthermore, we identified increased invasion as a function of miR-21 up-regulation in melanoma and showed that it mediates this activity at least in part by the down-regulation of TIMP3. This occurs on a post-transcriptional level and without a change in TIMP3 mRNA. These findings contribute to a further understanding of melanoma progression and behavior and hopefully will be used to aid in the development of new diagnostic methods and treatments.
References


