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Obesity Promotes B16BL6 Melanoma Cell Invasiveness and Snai1 Expression

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Obesity Promotes B16BL6 Melanoma Cell Invasiveness and Snai1 Expression

BY

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Thesis

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Master of Arts

The University of Texas at Austin

May 2012

DEDICATION

This thesis is dedicated to my family - loving parents and awesome brothers - who have always trusted and encouraged me in all that I do. I would also like to dedicate this work to my dear husband and his wonderful family who fully supported me with their love and patience.

ACKNOWLEDGEMENTS

I would like to thank Dr. Nomelí P. Núñez for being my PI and giving me a place in his lab to pursue my degree. I would also like to thank Dr. Stephen D. Hursting for his expertise and advice. I would further like to thank my lab mates Renee E. Stubbins, Amy W. Wong, Dr. Jina Hong, post-doctorate fellow Dr. Valerie B. Holcomb, and undergraduate students for making lab time a great experience. Lastly, I would like to thank the great friends I've made in Austin who kept me grounded throughout this process. I sincerely thank everyone for being a part of my life as they have helped me become the happy person I am today!

ABSTRACT

Obesity Promotes B16BL6 Melanoma Cell Invasiveness and Snai1 Expression

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Malignant melanoma is cancer arising from melanocytes that have acquired the ability to metastasize and colonize secondary organs such as the lungs, liver, and brain. According to the Melanoma Research Foundation, malignant melanoma is the most rapidly increasing type of cancer with an annual incidence increase of ~ 4% despite the therapeutic and medical breakthroughs in cancer treatment. Melanoma is the most common cancer in young adults ages 20-30, and it is the leading cause of cancer death in females ages 25-30. Non-modifiable risk factors include age, gender, and inherited predisposition to moles. As for modifiable risk factors, exposure to UV rays from the sun is well-established, but obesity has recently emerged as a factor through recent epidemiological and animal studies. Our results showed that obesity modulates the expression of the transcription factor Snai1, which has been shown to be a key gene in the regulation of the Epithelial-to-Mesenchymal Transition (EMT). Serum from obese

ob/ob mice, as well as conditioned media from 3T3L1 adipocytes, increased the invasive ability of melanoma cells and the expression of the transcription factor Snai1. Yet, the cytokine IL-6 may not be a critical component of obesity-mediated B16BL6 melanoma cell invasiveness.

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CHAPTER 1: INTRODUCTION

Malignant melanoma accounts for 75% of deaths associated with skin cancer due to its aggressive metastasis to vital organs such as the brain and lungs. Obesity has recently been suggested to be a factor that modulates the metastatic ability of melanoma cells, though the mechanism is still unclear. Thus, in order to better understand the biology by which melanomas metastasize, we investigated obesity's effect on the metastatic cell phenotype and on the expression of key genes known to affect the Epithelial-to-Mesenchymal Transition (EMT), such as Snai1.

CANCER

The disease of cancer was first described back in 3000 BC in the Edwin Smith Papyrus that contained descriptions of patient cases regarding tumors or ulcers of the breast that exhibited "crab-like" projections (1). Compared to the past where the disease was stated to "[have] no treatment", we have come a long way in the field of cancer therapy. However, even though we have made great advances in cancer research that have led to effective preventative and clinical interventions, much remains to be done in order to eradicate cancer.

According to the National Cancer Institute, cancer is a disease that results from the accumulation of various genetic mutations in a cell that gives the cell the ability to proliferate and grow uncontrollably (2). According to the World Health Organization (WHO), despite the vast improvement in early detection and effective treatments for several cancers, cancer is still the leading cause of human mortality with about 7.6

million deaths a year; this translates to about 13% of all causes of deaths globally (3). Quite daunting is the projection that the global cancer-related death count will increase to over 11 million worldwide by 2030 if we maintain this trajectory. Similarly, medical costs associated with cancer have nearly doubled from the $24.7 billion/yr back in 1987 to $48.1 billion/yr during the years of 2001-2005 in the United States (4). According to WHO, about 30% of cancers can be prevented with dietary interventions, proper physical activity, and less use of tobacco (3).

SKIN

Averaging a surface area of 21 square feet, the human body is surrounded by a protective barrier called the skin. The skin is indeed the body's largest organ that is composed of numerous cell types that regulate important body functions such as heat regulation. Its primary function, however, is to act as a barrier against harmful substances such as microorganisms, chemicals, and ultraviolet light (5). As a consequence, the skin is constantly exposed to numerous agents making skin cancer the most common form of human cancer (6-8).

The skin is composed of many cells and many layers. One type of cell that makes up the outer-most layer of the skin called the epidermis is the melanocyte that produces the pigment melanin to protect the skin from the damaging effects of UV light (**Figure 1.1**) (9). Melanocytes originate from melanoblasts that arise from the neuroectoderm to colonize and populate the surface ectoderm of the skin (10). These cells produce melanin in vesicles that can be deposited into surrounding cells such as keratinocytes; in these

keratinocytes, melanin forms a protective cap over the nucleus to shield the DNA from the damaging effects of UV rays (11).

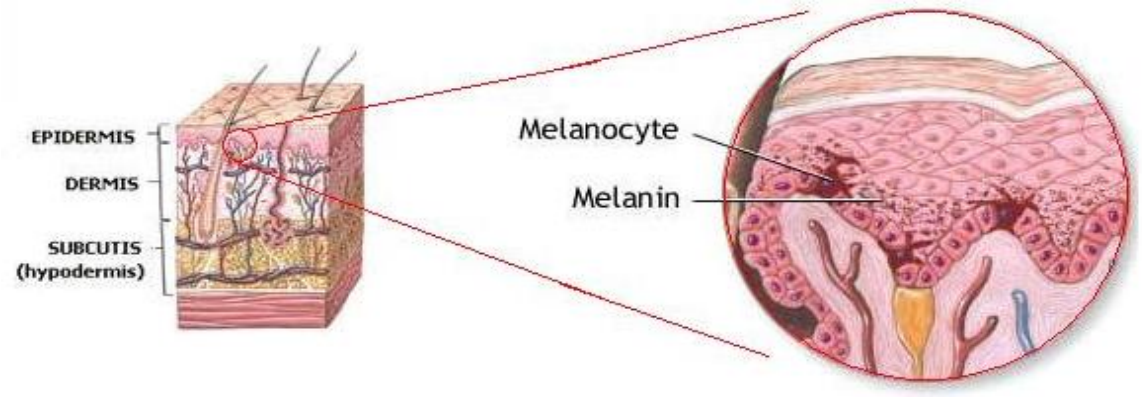

Figure 1.1 The Skin adapted from http://www.web-books.com/eLibrary/Medicine/Physiology/Skin/Skin.htm. The skin is composed of various layers and cells. Melanocytes reside in the outermost epidermis of the skin.

MELANOMA

Melanoma is cancer arising from transformed melanocytes. Even though malignant melanoma represents only 4% of all skin cancers, it is the most deadly, accounting for 75% of deaths from skin cancer (12). In 2003, approximately 54,000 new cases of cutaneous melanoma were diagnosed and 7,600 people died from this disease in the United States (13). In 2009, the numbers increased to 121,840 and 8,650, respectively (14). According to the Melanoma Research Foundation, malignant melanoma is one of the most rapidly increasing types of cancer with an annual incidence increase of ~ 4%. Melanoma is the most common cancer in young adults ages 20-30, and it is the leading cause of cancer death in females ages 25-30 (8).

MELANOMA PROGRESSION

It is thought that melanoma progression follows the Clark model of tumorigenesis where a collection of clonally inheritable events generates a cancer cell with metastatic capabilities that can enter the deeper dermis. That is, a melanocyte transitions into a benign nevus (mole) before acquiring migratory properties to become classified as malignant. For a melanoma cell to become invasive, they must acquire "vertical growth-phase" (VGP) properties rather than remaining at the "radial growth-phase" (RGP), which had previously only allowed them to grow in the epidermis (**Figure 1.2**) (15). There are a number of known molecular changes that are most commonly found in human melanomas and not in melanocytes. These include activating mutations of BRAF (16), silencing mutations of E-cadherin (17), and adverse mutations of p16/ARF (18). Thus, the pathways involved in melanoma progression are numerous and complex, and require further investigation.

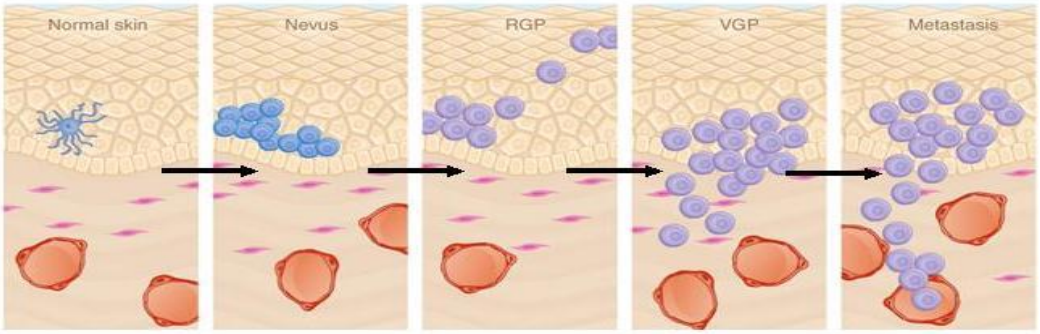

Figure 1.2 Melanoma Progression adapted from (19). Melanocytes accumulate genetic changes that allow the progression from benign nevus to metastatic melanoma.

MELANOMA TREATMENT

Surgery resulting in excision of the melanoma is the most successful method of treatment. Yet, malignant melanomas gradually obtain the ability to invade, migrate, and metastasize to the lungs, liver, bone, and brain. These highly inoperable secondary tumors are responsible for the high mortality rate of melanoma cancer patients. Alarmingly, 94% of stage IV melanoma patients with detectable metastasis die from melanoma within 5 years of diagnosis (20). Current therapeutic treatments, including standard chemotherapy treatments, high doses of interferon-α-2b, or regimens of high doses of IL-2 therapy against melanoma have largely been unfruitful and with dangerous side effects (21, 22). The average duration of benefits from these therapies is usually in the range of months. Therefore, further work is needed to better understand factors and genes that may be used as targets to prevent melanoma metastasis. Furthermore, these dire results stress the importance of not only early diagnosis, but also of melanoma prevention.

RISK FACTORS

Interestingly, a positive family history accounts for 5-12% of all melanoma cases, where having one first-degree relative with melanoma indicates a 2-fold increase in life-time risk. This finding foreshadows the importance of key gene mutations in the development of this disease. The CDKN2A gene mutation is the most common gene mutation found in families with multiple melanoma patients (23, 24). This gene encodes two proteins considered to be tumor suppressors of melanomas, INK4A/p16 and ARF,

which have been shown to inhibit improper cell cycle progression and cell proliferation (25, 26).

Though inherited genetic mutations account for a large number of melanoma cases, epidemiological studies have also unearthed several risk factors for melanoma: 1) race and skin type, 2) propensity to develop moles throughout life, and 3) excessive exposure to sunlight. Hereditary family traits such as fair skin that freckles or burns easily without tanning are considered risk factors, regardless of family history of melanoma incidence. Caucasians have the highest incidence of melanoma while people possessing darker skin, and thus, more melanin, such as Asians and African Americans are less susceptible (27).

Regarding sun exposure, studies show that intense and intermittent exposure to the sun is more detrimental and may increase melanoma incidence compared to cumulative exposure (28, 29). One theory is that consistent cumulative exposure may "prime" the DNA-damage response program in the cell to be more active, allowing for a better checking system that can fight against malignant transformation of melanocytes into cancer cells. However, other studies show that sun burns in early childhood can also increase the risk of melanoma (30, 31). Animal studies show that even though UV light cannot induce melanoma in mice by itself, it is able to promote melanoma development when combined with chemical skin carcinogens such as DMBA (32-34). Sun exposure, though there is much debate about the mechanism by which it increases melanoma risk, is clearly an established risk factor for melanoma.

OBESITY AND MELANOMAS

Over 60% of the U.S. population is considered overweight or obese (35). Obesity is an increasing concern as it can lead to numerous human disorders such as cardiovascular diseases and the metabolic syndrome; recently, obesity has also been classified as a risk factor for numerous cancers (36). Epidemiological studies show that obesity is a risk factor for melanoma: obesity increases the risk of developing subcutaneous melanoma (37) as well as the risk of developing malignant melanoma (38). The mechanism by which obesity affects melanoma, however, is not known. The objective of our study was to determine how obesity increases the invasive ability of melanomas. Obesity may provide factors that promote a more mesenchymal cell phenotype; thus, our specific objective was to determine if obesity affects the expression of genes associated with EMT.

Animal studies show that obesity promotes melanoma tumor progression and metastasis. Pandey *et al*. showed that diet-induced obesity promoted the growth of melanoma tumors (39). Mori *et al*. showed that genetically obese mice (ob/ob leptin-deficient and db/db leptin receptor deficient) developed a higher number of B16BL6 pulmonary metastases than lean control mice (40). Mori *et al*. also showed that the retention time of B16BL6 cells tagged with luciferase was increased in the lungs of obese mice compared to control lean mice. Evidence suggests that obesity promotes tumor development by inducing a pro-cancerous microenvironment by increasing systemic levels of growth and inflammatory factors (41). This inflammatory environment has been hypothesized to be responsible for increasing cancer risk in obese individuals (42,

43). In Chapter 2 of this thesis, we focused on determining the effect of systemic factors found in the serum of genetically obese ob/ob mice on their ability to modulate the mesenchymal phenotype and metastatic abilities of melanoma cells.

The cancer microenvironment can be comprised of cancer-associated fibroblasts (FACs), macrophages (TAMs), and adipocytes (44-46). It is feasible that these non-cancer cells can promote melanoma progression. In Chapter 3 of this thesis, we determined if adipocyte media affected the aggressiveness of B16BL6 melanoma cells. The adipocytes can produce factors such as the cytokine Interleukin 6 (IL-6), which can affect cancer progression (47). Thus, Chapter 3 also assessed the role of IL-6 in adipocyte media-induced invasiveness of melanomas.

EPITHELIAL-TO-MESENCHYMAL TRANSITION (EMT)

The Epithelial-to-Mesenchymal Transition (EMT) is a developmental program whereby an epithelial-type cell acquires a mesenchymal phenotype that allows it to acquire migratory and invasive abilities necessary in the various steps of cancer progression (**Figure 1.3**). The EMT program is often characterized by the aberrant activation of transcription factors including Snai1, Slug, Twist, and TGF-β1. These transcription factors collectively help inhibit cell-to-cell adhesion that keeps cells anchored to each other. They do this by down-regulating anchorage proteins such as E-cadherin and increasing the expression of mesenchymal markers such as Vimentin (48-50). This program is often activated during embryogenesis as the epithelia need to be highly plastic in processes such as organogenesis and gastrulation (Type 1 EMT); EMT

also normally occurs in adulthood most prominently during fibroblasts/mesenchymal cell recruitment in wound healing (Type 2 EMT). However, it can be activated in an abnormal manner during cancer progression, namely metastasis (Type 3 EMT) (51). We will discuss EMT in the context of cancer metastasis in this thesis.

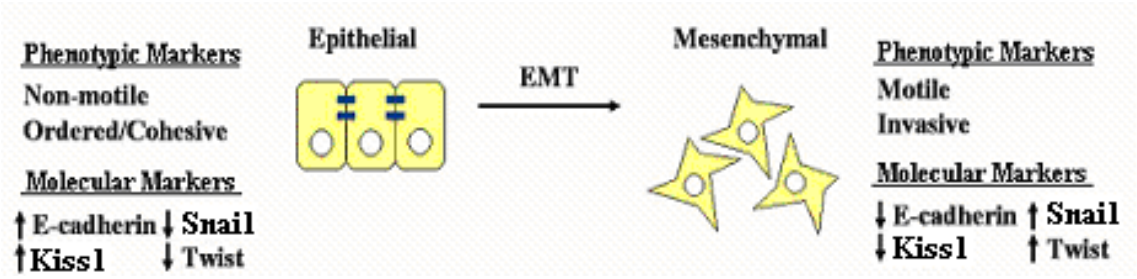

Figure 1.3 Epithelial to Mesenchymal Transition (EMT). During EMT, epithelial-type cells acquire genetic changes such as increased expression of Snai1 and Twist that lead to decreased expression of proteins such as E-cadherin and Kiss1. These changes promote a mesenchymal-type phenotype.

In cancer progression, the process of metastasis is one of the six hallmarks of cancer, coined by Weinberg *et al*. back in 2000 and again in their updated review in 2011 (52, 53). It is widely accepted that, for successful metastasis to occur, a cell must acquire EMT properties to convert itself from a benign cell to a malignant cell. Others have identified EMT as a major determinant of melanoma metastasis in cancer patients (54). Key molecular changes that are commonly studied in melanomas in association with their function in EMT include B-raf, Wnt5A, and TGF-β/SMAD (55-58). These genes may cooperatively increase cell plasticity associated with the EMT phenotype. On the other hand, these genes may down-regulate metastasis suppressor genes such as Kiss1, which has been shown to inhibit melanoma metastasis (59). In the present study, we determined

the effects of obesity on EMT and on the expression of genes associated with this phenotype, such as Snai1 and Kiss1.

SNAI1

Snai1 is a transcription factor identified to be important in EMT and the metastasis of melanoma (60, 61). Snai1 was discovered to be involved in EMT because mice and fly embryos that are homozygous recessive for Snai1 fail to gastrulate properly due to defects in neural crest closure which requires functional EMT (62, 63). Snai1 belongs to the snail superfamily of zinc-finger transcription factors that recognizes E-boxes on promoter regions using its C-terminal DNA-binding and nuclear localization domain; currently, several hundred strong binding E-box clusters and potential targets in the genome have been identified (64, 65). Its mode of action involves gene repression using the conserved SNAG N-terminal domain in vertebrates (66).

Snai1 is a key regulator of EMT (67, 68). The transcription factor Snai1 is an important regulator of metastasis because it down-regulates the expression of E-cadherin and other proteins that are involved in cell-to-cell adhesion (69, 70). Furthermore, an inverse correlation between Snai1 and E-cadherin has previously been reported in melanoma (71). Others have shown that Snai1 repression of E-cadherin requires Histone Deacetylase (HDAC) activity, specifically that of HDAC 1 and 2 which deacetylates Histones H3 and H4 at the E-cadherin promoter region in conjunction with the co-repressor mSin3A (72). High levels of Snai1 promote tumor invasion by decreasing the adhesive ability of the cancer cells; in humans, Snai1 expression is associated with distant metastasis and tumor recurrence (73, 74).

Aberrant Snai1 expression has also been linked to the up-regulation of several mesenchymal genes even though it is a transcriptional repressor. Genes up-regulated by Snai1 expression include mesenchymal genes such as vimentin, fibronectin, Wnt-5A, and delta-EF1 (75, 76) . It is likely that the up-regulation of these genes is an indirect effect of Snai1's ability to repress the expression of their inhibitors. Many of these genes promote EMT; namely, delta-EF1 is a transcriptional repressor of E-cadherin, and Wnt-5A up-regulation has been linked to the down-regulation of important melanoma metastasis suppressors such as Kiss1. Other genes up-regulated by Snai1 expression include MMP9, a protein that can degrade extracellular matrix proteins to allow cancer cells to escape into the blood or invade secondary tissues in order to establish metastases (77, 78). Thus, the diversity of Snai1 gene targets makes Snai1 a very important regulator of cancer progression by affecting EMT.

In the literature, numerous complex signaling circuits have been shown to regulate the expression of Snai1, such as the phosphatidylinositol 3-kinase (PI3-K), mitogen-activated protein kinases (MAPKs), glycogen synthase kinase 3-beta (GSK-3β) and the NF-κB pathway (79). Specifically, NF-κB can bind to the promoter region and up-regulate the expression of human Snai1; similarly, the B-raf pathway that is often mutated in melanoma cells can feed into the NF-κB pathway and increase Snai1 expression (55, 80). The well-studied TGF-β pathway has also been shown to up-regulate the synthesis of Snai1 in various cancer cells, including the highly metastatic human A375 melanoma cell line (81).

Chapters 2 and 3 of this thesis investigate the effect of systemic factors on Snai1 expression. Circulating factors associated with inflammation and obesity have previously been linked to the increased expression of Snai1. TNF-α is one such factor that has been shown to affect the expression and stability of Snai1. Wu *et al*. showed that TNF-α induces a NF-κB-mediated Snai1 stabilization through its ability to induce CSN2 [COP9 signalosome (CSN) complex component] expression. CSN2 prevents the phosphorylation of Snai1 by GSK-3β, which increases the stability of Snai1; by blocking phosphorylation of Snai1, the ubiquitin-mediated degradation of Snai1 is prevented (82). We note that phosphorylation is a key mechanism that regulates Snai1 expression because it dictates Snai1's cellular localization in the cytosol or nucleus. Others show that upon phosphorylation of a Ser-rich sequence adjacent to the protein's nuclear export sequence (NES), Snai1 undergoes a conformational change that allows better interaction with the CRM1 transporter that facilitates nuclear export of Snai1 (83). TNF-α can also activate Akt, which ultimately activate NF-κB resulting in the up-regulation of Snai1 (84). Lastly, TNF-α can also increase the stabilization of Snai1 via the Wnt/β-catenin pathway by suppressing GSK-3β activity (85).

Pertinent to our study, the cytokine Interleukin 6 (IL-6) has recently emerged as a possible contributor of Snai1 expression; thus, it is thought to be an important player in the progression of numerous cancers, including melanomas. IL-6 is a cytokine that can elicit both a pro-inflammatory or anti-inflammatory response in the body. It is produced mainly by macrophages as an immune response, but adipocytes are also able to secrete IL-6 (86). IL-6 is a ligand that binds to its receptor complex, IL-6R alpha and gp130 in

target cells. This complex goes on to activate STAT3 or SHP-2-mediated RAS signaling pathways and affects numerous cellular processes (87). Epidemiological studies show that IL-6 is elevated in the serum and adipose tissue of obese patients (88-90). In addition, IL-6 is a serological marker for malignant melanoma (91). Animal studies also show that mice inoculated with B16BL6-melanoma exhibited high systemic levels of IL-6 (92). Though these findings are correlative, they suggest that IL-6 may play an important role in the invasiveness of melanoma. It is feasible that IL-6 increases the aggressiveness of melanomas by promoting a mesenchymal phenotype via genes regulated by the NF-kB pathway, such as Snai1 (93).

Though these findings are currently correlative, cell culture studies have also assessed the effect of IL-6 on cancer cells. Interestingly, IL-6 has been implicated as both an inhibitor and an enhancer of cell phenotypes such as cell growth in cancer cells (94, 95). In some cases, others have shown that IL-6 is capable of switching from being an inhibitor to a stimulator of proliferation when the tumor itself begins to produce it in an autocrine manner (96). In accordance with this finding, others show that some human malignant melanoma cell lines endogenously produce detectable levels of IL-6 in an autocrine fashion (97). Thus, IL-6 may be an important obesity-associated cytokine that leads to exacerbated melanoma metastasis.

IL-6 has been shown to increase the expression of Snai1, and consequently EMT, in some cancers (98, 99). Even though the study by Sullivan *et al*. showed that Snai1 induction by IL-6 was not as drastic as the induction by TNF-α, we cannot disregard the finding that IL-6 may modulate EMT. We also note a relationship between TNF-α and

IL-6 because TNF-α is a strong inducer of NF-κB, which in turn can increase the expression of IL-6 in some cells (100). IL-6 may indirectly induce NF-κB activation by activating the PI3K/Akt/NF-κB pathway where the NF-κB inactivating IκB is degraded through IKK stimulation (101). Thus, IL-6 and TNF-α may be having a cooperative function in stimulating the expression of Snai1 via NF-κB. Thus, Chapters 2 and 3 of this thesis investigate the effect of obese serum and IL-6 on the expression of Snai1 in B16BL6 melanoma cells.

KISS 1

Kiss1 has been identified as a suppressor of melanoma metastasis (102). The Kiss1 gene was discovered to be localized on chromosome 1, and its expression was shown to inhibit the metastatic ability of human melanoma cells (103). The Kiss1 receptor GPR54 was also shown to inhibit the metastatic ability of B16BL6 melanoma cells (104). Thus, the Kiss1 signaling pathway seems capable of inhibiting melanoma metastasis.

Kiss1 is a secreted protein that is processed into peptides of different lengths termed kisspeptins (KPs) (105). Others show that KPs can be cleaved by Matrix Metalloproteinases (MMPs), which abolish the ability of these peptides to induce focal adhesions to prevent invasion (106). Studies also suggest that Kiss1 affects the metastases of cancer cells by decreasing the expression of MMPs, such as MMP9 (78, 107).

In terms of cancer prognosis, high levels of Kiss1 in human melanoma samples strongly correlate with a positive prognosis and low levels with a worse prognosis. This

underlines the importance of Kiss1 in melanomas because in other cancers such as breast cancer or bladder cancer, the expression of Kiss1 does not always correlate with a positive outcome (108, 109). In melanomas, others show that Kiss1 mRNA expression is reduced by 50% in primary melanomas exceeding 4mm (110). Patients with melanoma tumors of this size have a rapid drop in 5-year survival rate (111). Shirasaki *et al*. showed that both primary melanoma tumors greater than 4mm deep that have gained the ability to invade and metastatic melanomas have significantly less Kiss1 compared to primary non-invasive melanomas (110, 112). Thus, Kiss1 appears to be a good prognostic marker of melanoma progression.

SUMMARY & PERSPECTIVES

With few effective treatment options available, malignant melanoma is a formidable cancer that is currently the fifth most common cancer in men and women (6). Cancer patients with a diagnosis of metastatic melanoma survive, on average, approximately 6-9 months (113). Therefore, it is crucial that we increase our knowledge of the mechanism by which melanomas metastasize, so that better therapies for the prevention and treatment of melanoma metastasis can be developed. Obesity is prevalent in our society, and the study herein will contribute to our understanding of how obesity may affect the metastatic ability of melanomas.

Chapter 2 will determine the effect of obesity on the invasive ability of B16BL6 melanoma cells and on EMT genes.

Chapter 3 will investigate the effects of adipocyte conditioned media and IL-6 on B16BL6 cell invasion and Snai1 expression.

Chapter 4 will provide an overall discussion and future directions for this thesis project.

CHAPTER 2: OBESITY PROMOTES A MESENCHYMAL CELL PHENOTYPE IN B16BL6 MELANOMA CELLS

ABSTRACT

In 2009, malignant melanoma was responsible for approximately 9,000 deaths in the United States. These deaths are often associated with aggressive metastasis to secondary sites such as the lungs. Epidemiological and animal studies suggest that obesity is a risk factor for melanoma. Others have shown that B16BL6 melanoma cells metastasize more aggressively in obese ob/ob than in lean mice. However, the mechanism by which obesity promotes B16BL6 melanoma metastasis in ob/ob mice has not been identified. In the present study, we used serum obtained from control and ob/ob leptin-deficient obese mice to determine if obese serum increases the aggressive phenotype of melanoma cells. Results showed that ob/ob serum has higher levels of resistin, insulin, tPAI1, IL-6, TNF-α, and MCP-1 compared to control serum. We showed that ob/ob serum increases the invasive ability of B16BL6 melanomas. To understand the mechanism by which ob/ob serum increases the invasive ability of melanomas, we determined the effect of ob/ob and control serum on genes associated with the Epithelial-to-Mesenchymal Transition (EMT). Cancer cells with a mesenchymal phenotype have a higher metastatic ability. Snai1 and Twist are genes that are strongly associated with EMT and metastasis of melanomas. Our results showed that ob/ob serum increased the expression of Snai1 and Twist. Moreover, ob/ob serum increased MMP9 activity and decreased the expression of E-cadherin and the metastasis suppressor gene Kiss1. In summary, our results suggest that obesity may increase the metastatic ability of melanoma by promoting a mesenchymal cell phenotype.

INTRODUCTION

Epidemiological and animal studies suggest that obesity is a risk factor for melanoma (37-38, 40). Studies by Dennis *et al*. showed that obesity increases the risk of developing subcutaneous melanoma (37). Moreover, Samanic *et al*. showed that obesity increases the risk of developing malignant melanoma (38). In animal studies, Mori *et al*. demonstrated that obesity promotes pulmonary metastasis of B16BL6 melanoma in obese ob/ob mice (40). However, the mechanism by which obesity promotes melanoma metastasis is not thoroughly known.

Ob/ob mice were used as our model of obesity. These mice are leptin-deficient and are grossly overweight due to hyperphagia (abnormally increased appetite and food consumption); they also exhibit hyperglycemia and severe insulin resistance (114). Interestingly, ob/ob mice have been recognized to have lower incidence of cancers such as pulmonary and breast cancer (115). In regards to melanoma progression, studies have shown conflicting results in ob/ob mice. Some have observed no difference in B16BL6 melanoma growth rate when cells are injected orthotopically (40). Others show that melanoma cells grow faster when injected subcutaneously in ob/ob mice; still others, however, show that both growth rate and metastasis from subcutaneous B16BL6 cell injection are more inhibited in ob/ob mice than control lean siblings (116). This resistance to metastasis, though not fully explained, was attributed to increased immunocompetence observed as a more acute proliferative response by splenic lymphocytes from ob/ob mice towards a T-cell mitogen compared to the response by the lumphocytes from control mice. It is possible, however, that the discrepancy is due to the over-abundance of subcutaneous WAT (white adipose tissue) in these ob/ob mice, which could be a confounding factor when studying subcutaneous B16BL6 melanoma tumor growth and subsequent metastasis.

In this ob/ob mouse model, intravenous (i.v.) injection of B16BL6 melanoma cells has been used previously to study experimental metastasis (40). This approach overcomes the issue of excess subcutaneous WAT that one may encounter with typical spontaneous metastasis models that require subcutaneous tumor cell injections. The experimental metastasis model also bypasses the need to remove the primary tumor after it reaches a certain size because metastasis usually develops in these animals at a much later stage than what is humanely allowed. It is true that this method limits the study of important early steps of metastasis including intravasation, which is one of the rate-limiting steps of metastasis (117). Yet, the experimental metastasis model still allows researcher to study the later stages of metastasis that occur after the cancer cells have invaded the bloodstream. The same study by Zijlstra *et al*. shows that the establishment and growth of micrometastases at the secondary organ is also an important rate-limiting step of metastasis for some cancers (117). Others have supported the view that the early steps in metastasis may not be as important as the steps of extravasation and post-extravasation growth of the individual cancer cell in contributing to the inefficiency in metastasis (118-120). Similarly, though some have found a positive correlation between the number of circulating cancer cells and poor prognosis, others have found no connection between the two (121-124). This suggests that it is critical to investigate the steps in melanoma metastasis after extravasation using these B16BL6 melanoma cells in ob/ob mice, which can be more accurately observed in an experimental metastasis model such as in our study.

Obesity may increase the metastatic ability of melanoma cells by promoting a mesenchymal cell phenotype. Studies show that cancer cells with a mesenchymal phenotype have a higher ability to metastasize (125). The transcription factors Snai1 and Twist are genes that promote the Epithelial-to-Mesenchymal Transition (EMT) (48, 126).

Moreover, Snai1 and Twist have been shown to increase the ability of cancer cells to migrate, invade and metastasize (127). Furthermore, overexpression of Snai1 and Twist increases cell motility and leads to a significant loss of cell-to-cell adhesion (128). The mesenchymal phenotype is also associated with higher expression of Matrix Metalloproteinases (MMPs) such as MMP9 (129). MMPs play a key role in the metastatic cascade, because they allow cancer cells to degrade the extracellular matrix (ECM), and thus, allow the cancer cells to invade the basement membrane from where cancer cells can eventually metastasize to secondary sites (78).

Alternatively, obesity may promote melanoma metastasis by simultaneously leading to a decrease in the expression of genes known to inhibit metastasis. Metastasis suppressor genes encode proteins that have the ability to hinder the establishment of metastases (130). Metastasis suppressor genes, such as Kiss1, have the ability to inhibit the metastatic ability of melanomas (131). Thus, it is feasible that obesity increases the metastatic ability of melanomas by increasing the expression of pro-metastatic genes such as Snai1 and Twist and also by decreasing the expression of genes that inhibit melanoma metastasis, such as Kiss1.

In the current study, we determined the effects of control and obese serum on the invasiveness of B16BL6 melanoma cells. Results showed that ob/ob serum increased the ability of B16BL6 melanoma to invade. The higher invasive ability of melanoma was associated with increased expression of Snai1, Twist, and MMP9. Furthermore, ob/ob serum increased the expression of Snai1 in the nucleus and down-regulated E-cadherin expression at the cell membrane of B16BL6 cells. Ob/ob serum also increased MMP9 activity. Additionally, ob/ob serum decreased the expression of the metastasis suppressor gene Kiss1. Overall, our study suggests that obesity may increase the aggressiveness of

melanomas by promoting a mesenchymal cell phenotype and by down-regulating the expression of the metastasis suppressor gene Kiss1.

MATERIALS AND METHODS

Cancer cells and cell culture reagents

The highly metastatic B16BL6 melanoma cells were kindly provided by Dr. Isiah J. Fidler, University of Texas at MD Anderson, Houston TX. The non-metastatic B16F1 melanoma cells were purchased from American Type Culture Collection (ATCC, Chicago, IL). They were maintained in Dulbecco's modified minimum essential medium (Invitrogen, Carlsbad, CA), pH 7.4, containing 10% heat-inactivated fetal bovine serum (Invitrogen) and 1% antibiotic-antimycotic solution (CellGro, Manassas, VA). The cells were grown at 37°C in a humidified atmosphere of 5% CO_2. For cell culture studies, B16BL6 cells were treated with either 5% ob/ob mice serum or 5% ob/+ heterozygous control mice serum. Serums from mice for each group (control or ob/ob) were pooled together after the serum analysis was completed.

Obese ob/ob mice

All animal procedures and methods employed in our study was approved by the Animal Care and Use Committee at the University of Texas at Austin. Pathogen free male ob/ob and ob/+ heterozygous control C57BL/6J mice were purchased from The Jackson Laboratory (JAX, Bar Harbor, ME) at 6-8 weeks old. They were housed according to National Institutes of Health (NIH) guidelines (National Research Council, 1996). They were singly housed and maintained on water and a chow diet (Research Diets, New Brunswick, NJ) for 15 weeks (n=12). We measured body weight, food consumption, and liquid consumption on a weekly basis. After 15 weeks on the diet, mice were euthanized using CO_2 and blood was drawn by cardiac puncture before cervical dislocation was

performed on each mice. The blood was allowed to coagulate at room temperature for 30 mins. Serum was collected and frozen in liquid nitrogen. Liver and visceral adipose tissues were similarly collected and flashfrozen in liquid nitrogen for RNA analysis at the time of killing. Both tissue and serum were stored at -80°C until analyzed.

Body composition analyses in mice

Body composition was determined in non-anesthetized mice using an EchoMRI Composition Analyzer system (Echo Medical Systems, Houston, TX) as instructed by the manufacturer. Percent body fat was determined at 10 weeks into the study (n=12).

Blood glucose levels

To determine if ob/ob mice were hyperglycemic compared to control mice, we measured blood glucose level after fasting. At the 8th week of the study, mice were fasted for 7hrs and their blood glucose levels were determined using a Glucometer Elite (Bayer, Elkhart, IN) (n=12).

Glucose Tolerance Test (GTT)

To determine the effect of obesity on glucose clearance, we performed the GTT assay. Briefly, mice were fasted for 14 hrs and then injected with 20% glucose in PBS intraperitoneally. Then, a razor blade was used to make a small cut at the tail vein of each mouse and glucose was measured in the blood using a Glucometer Elite (Bayer, Elkhart, IN) at 0, 15, 30, 60, and 120 mins post-injection. GTT was examined at the 9th week of the study (n=12).

Insulin Tolerance Test (ITT)

The ITT assay tests how efficiently endogenous glucose is cleared from the blood as a response to insulin administration. ITT was performed after mice were fasted for 7 hrs.

They were then given an intraperitoneal injection of $0.75Ukg^{-1}$ insulin in PBS (Insulin: Sigma Aldrich, St. Louis, MO). A razor blade was used to make a small cut at the tail vein of each mouse, and blood glucose was measured using a Glucometer Elite (Bayer, Elkhart, IN) at 0, 15, 30, 60, and 120 mins post-injection. Area under the curve (AUC) was calculated to determine insulin sensitivity. ITT were examined at the 8th week of the study (n=12).

Serum analysis

To determine obesity's effect on systemic factors in the blood, we measured leptin, insulin, Interleukin 6 (IL-6), Tumor Necrosis Factor-alpha (TNF-α), resistin, tissue Plasminogen Activator Inhibitor-1 (tPAI-1), and Monocyte Chemotactic Protein-1 (MCP-1) in the serum of each mouse at the 10^{th} week of the study. We measured these factors with the Millipore's Multiplex MAP Mouse Serum Adipokine Panel kit (Millipore, Billerica, MA) as instructed by the manufacturer (n=12).

Invasion assay

We used the Boyden chamber assay to determine the effects of obese mice sera on the ability of B16BL6 melanoma cells to invade (132). Briefly, the top chamber membrane was coated with a 1:10 dilution of BD Matrigel^{TM} (BD Biosciences, Becton Drive Franklin Lakes, NJ), then, $5x10^4$ B16BL6 melanoma cells were placed in the top chamber in serum-free DMEM with 0.1% Bovine Serum Albumin (BSA). The lower chamber was filled with DMEM containing 5% ob/ob serum or 5% control serum. Cells were allowed to migrate from the top side towards the bottom side of each camber for 28 hrs. After 28 hrs, cells that remained on the top side were removed using a Q-tip. Cells that invaded to the bottom side were fixed and stained using Siemens Diff-Quick Stain Set (Siemens, Malvern, PA). Stained cells were visualized and quantified by microscopy. To determine

the average number of cells that migrated for each well, we counted 3 random fields in each well at 200x. Each treatment had 3 wells per experiment, with each experiment being independently performed three times (n=3).

Wounding assay

The wounding assay was used to determine the effects of high and low glucose on the ability of B16BL6 melanoma cells to migrate. This was done to determine if glucose levels can affect the ability of these cells to migrate. Ob/ob mice are hyperglycemic, and thus, it is possible that the high glucose levels found in ob/ob mice may affect the aggressiveness of melanoma cells to metastasize (133). In the wounding assay, cells are plated to 100% confluency and a scratch is drawn in the middle of the plate. The decrease in gap distance is measured over time and quantified; the larger the gap distance that remains, the less the cells have migrated. Briefly, B16BL6 cells were grown on a 24-well plate until they were confluent. Then, cells were grown in FBS-free DMEM overnight. The next day, wells were washed twice with PBS and a scratch was drawn on each well using a p200 pipette tip. After two more washes with PBS to get rid of cell debris, the following cell culture media was added: 1000mg/L glucose DMEM (low glucose) or 4,500mg/L glucose DMEM (high glucose). Both media was supplemented with 5% FBS. Each well was photographed at the time of treatment (0 hr) and after 9 hrs of incubation (9 hr) at 40x. The difference in gap distance was measured (9 hr time point – 0 hr time point) to quantify cell motility. The wounding assay was done in the absence of mitomycin C; however, the assay was terminated after 9 hrs, which is less time than the 16-18 hrs needed for B16BL6 cells to double (134). Thus, cell migration is most likely not due to changes in cell proliferation. Each experiment was repeated three times with each group having 3 wells per experiment (n=3).

Quantitative real time PCR (qRT-PCR)

Total RNA was collected from three replicate of cells that were serum starved overnight and then treated with 5% ob/ob serum or 5% control serum for 24 hrs. The RNA was extracted using an RNeasy Mini Kit according to the manufacturer's instructions (Qiagen). Using 1μg of RNA for each sample, reverse transcription was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Primers for 18S, IL-6, TNF-α, Snai1, Twist, MMP9, and Kiss1 were purchased from Integrated DNA Technologies (IDT, Coralville, IA) and were as follows: 18S F: GCATGGCCGTTCTTAGTTGGTGGA, B: TCTCGGGTGGCTGAACG-CCA; IL-6 F: GCTGGTGACAACCACGGCCT, B: AGCCTCCGACTTGTGAAGTGGT; TNF-α F: CTCTTCAAGGGACAAGGCTG, B: CGGACTCCGCAAAGTCTAAG; Snai1 F: CACCTCCAGA-CCCACTCAGAT, B: CCTGAGTGGGGTGGGAGCTTCC; Twist F: CCACGCTGCCCTCGGA-CAAG, B: CCAGGCCCCCTCCATCCTCC; MMP9 F: GCCCACCGTCCTTTCTTGTTGGA, B: GGGAGAGGTGGTTTAGCCGGTG; Kiss1 F: GCAAGCCTGGGTCTGCAGGG, B: CGACTGC-GGGAGGCACACAG.

Quantitative RT-PCR was performed with a SYBR GreenER qPCR kit (Invitrogen) in a Mastercycler ep Realplex Real-time PCR thermocycler (Eppendorf North America, Hauppauge, NY). The relative expression levels of target genes were normalized to the housekeeping 18S rRNA. Amplification specificity was confirmed by melting curve analysis. Each gene was measured in quadruplicate and the average ΔCt was taken from the 3 replicate wells before fold change was calculated using the ΔΔCt method. At least three independent sets of samples were performed for each gene (n=3).

Immunofluorescence Microscopy

To determine protein localization of Snai1 and E-cadherin, Immunoflurescence microscopy was used. Wax pencils were used to mark a closed circle on microscope

coverslips. B16BL6 cells were plated into the circles and allowed to attach for 24 hrs before being FBS-starved overnight. They were subsequently treated with 5% ob/ob serum or 5% control serum for 24 hrs. Cells were washed twice with PBS and fixed in 4% formalin/PBS for 10 mins. Then, cells were subsequently washed twice with PBS before being stored in PBS at 4°C overnight. The next day, cells were permeabilized with 0.1% Triton-X100/PBS and neutralized with 100uM glycine/PBS, before being treated with antibodies against Snai1 or E-cadherin (Cell Signaling, Danvars, MA and Santa Cruz Biotechnology Inc., Santa Cruz, CA) and appropriate fluorescently-conjugated secondary antibodies (Cell Signaling and Abcam, Cambridge, UK) as recommended by the manufacturer. After three washes with 0.2% Tween20 in PBS, cells were counterstained with two drops of DAPI/antifade (Millipore) according to the manufacturer's instructions for detection of cellular nuclei. After 15 mins of incubation, coverslips were placed onto microscope slides and sealed with nail polish, and images were taken with the Zeiss Axiovert 200 M fluorescent microscope at UT Austin's ICMB Core Facility. Images of control and experimental cells were acquired under identical exposure conditions for comparative analysis (n=3).

Western blot analysis

To determine the total protein expression of E-cadherin in B16BL6 cells in response to DMEM, control serum or ob/ob serum treatments, western blot analysis was carried out on whole cell lysates. For this purpose, B16BL6 cells were grown to about 70% confluency in 10 cm^3 plates. Cells were rinsed with PBS and then FBS-starved overnight. Then, cells were treated with control DMEM, 5% control serum, or 5% ob/ob serum in DMEM for 24 hrs. After the various treatments, cells were rinsed twice with PBS, trypsinized, and centrifuged at 10,000 rpm for 5 mins at 4°C. The supernatant was

discarded, and the pellets were stored in -80°C overnight. The next day, cells were washed twice with PBS before being lysed in 300uL RIPA buffer (Thermo Scientific, Waltham, MA) supplemented with a protease inhibitor cocktail (Roche, South San Francisco, CA). After a 45-min incubation on ice, cells were centrifuged at 10,000 rpm for 10 mins at 4°C. The supernatant was collected and protein concentration was measured using the Bradford Assay. 50ug of protein was loaded and run on an SDS-PAGE gel at 100-150V for 1 hr. The gel was subsequently transferred to a PMSF membrane, blocked in 5% milk, washed numerous times in TBST, and probed for E-cadherin (Santa Cruz Biotechnology Inc.) and β-actin (Cell Signaling) and appropriate HRP-conjugated secondary antibodies (Santa Cruz Biotechnology Inc.) before incubation with ECL and film exposure.

Zymogen gel assay

To determine MMP9 activity in B16BL6 cells after different treatments, we performed the zymogen gel assay. Briefly, B16BL6 cells were treated with 5% ob/ob serum or 5% control serum for 24 hrs. 1.5mL of this media was subsequently collected and concentrated using a centrifugal protein concentrator (Millipore). 20uL of protein concentrate was mixed with a 2X zymogen loading dye to a final volume of 40uL. This sample was loaded onto a zymogen gel (Bio-Rad Laboratories, Hercules, CA) to determine MMP9 activity as described previously (135). Three separate assays were performed (n=3).

Statistical analysis

All experiments were analyzed for significance using the independent Student's t-test in SPSS (PAWS version 18). P-values $\leq$ 0.05 were considered significant, and all data is represented as the mean ± SEM.

RESULTS

Ob/ob mice

Others have shown that ob/ob mice are hyperglycemic and insulin resistant (114, 136). We verified these previous findings. Final body weights for control mice was 33.73g while ob/ob mice had a final body weight of 56.65g ($p<0.05$). Results showed that ob/ob mice had significantly higher body fat levels than control mice (**Figure 2.1A**). We also showed that ob/ob mice are hyperglycemic and hyperinsulinemic compared to control mice (**Figure 2.1B, 2.2A**). Our results similarly showed that ob/ob mice were insulin resistant as determined by the GTT and ITT assay (**Figure 2.1C, D**). The GTT assay measures how quickly injected glucose is cleared from the blood. The ITT assay tests how quickly endogenous glucose is cleared from the blood in response to insulin administration. Results showed that ob/ob mice cleared blood glucose at a significant lower rate than control mice, thus, suggesting that they are indeed insulin resistant ($p<0.05$).

Adipokine and pro-inflammatory factors in ob/ob mice

As expected, systemic levels of leptin in ob/ob were undetectable (**Figure 2.2A**). However, other adipokines such as resistin and tPAI-1 were significantly higher in ob/ob mice (**Figure 2.2A**). In agreement with previous reports, we showed that ob/ob mice have higher levels of pro-inflammatory factors; systemic MCP-1, IL-6, and TNF-α were significantly higher in ob/ob mice (**Figure 2.2B, 2.2C**). Additionally, IL-6 and TNF-α were elevated in the adipose tissue of ob/ob mice, but only TNFα was elevated in the livers of these mice (**Figure 2.2D**).

Ob/ob serum increased the invasiveness of B16BL6 melanoma

To determine if the factors that increased the metastatic phenotype of B16BL6 melanoma cells in ob/ob mice were found in the blood, we determined the ability of serum from ob/ob and control mice to affect the invasive ability of melanoma cells in cell culture conditions. For this purpose, we tested the effect of ob/ob serum on the ability of B16BL6 cells to invade through a Matrigel^{TM}-coated chamber compared to control serum treatment. Results showed that B16BL6 cells exposed to ob/ob serum were more invasive than B16BL6 cells exposed to control serum (**Figure 2.3**).

High glucose increased B16BL6 melanoma cell migratory ability

Evidence suggests that high systemic glucose levels can increase the risk of cancers (133, 137). Thus, it is possible that high levels of glucose in ob/ob mice can affect the metastatic ability of melanoma cells. Using the wounding assay in cell culture conditions, we determined the effects of a high-glucose media on the ability of B16BL6 cells to migrate compared to low-glucose media. Results showed that high glucose levels increased the ability of B16BL6 cells to migrate compared to low glucose lovels (**Figure 2.4**). Thus, it is possible that once the melanoma cells reach the blood, the high levels of growth factors (e.g., insulin) and pro-inflammatory factors (e.g., IL-6) in conjunction with high glucose levels promote the metastatic ability of melanoma cells.

Ob/ob serum increases the expression of Snai1, Twist, and MMP9

To better understand the mechanism by which ob/ob serum affects the invasive phenotype of melanomas, we determined the effect of ob/ob serum on the expression of genes associated with the Epithelial-to-Mesenchymal Transition (EMT). The mesenchymal phenotype in cancer cells is associated with a strong ability of cancer cells to invade and metastasize (138). The gene expression of both Snai1 and Twist

transcription factors has been established to be strongly associated with the mesenchymal phenotype of cancers such as melanomas (56, 139). To determine if the metastatic ability of B16BL6 melanoma cells were associated with the expression levels of Snai1 and Twist, we measured their levels in highly metastatic B16BL6 and non-metastatic B16F1 melanoma cells. Our results showed that Snai1 and Twist were up-regulated while E-cadherin was down-regulated in highly metastatic B16BL6 cells compared to non-metastatic B16F1 melanoma cells (**Figure 2.5A**). To determine if the expression of Snai1 is modulatable by the presence and absence of serum, we exposed the highly metastatic B16BL6 to either no serum or 10% fetal bovine serum for 24 hrs. Results showed that Snai1 expression was increased in B16BL6 cells treated with 10% FBS compared to FBS-starved cells (**Figure 2.5B**). Thus, these results showed that expression of Snai1 can be modulated by different serum.

Next, we exposed the highly metastatic B16BL6 melanoma to ob/ob serum to determine if obese sera would increase the expression of Snai1 and Twist. Results showed that the ob/ob obese serum significantly increased the expression of both Snai1 and Twist (**Figure 2.5C**). Through Immunofluorescence microscopy, we also determined that ob/ob serum increased Snai1 expression in the nucleus of B16BL6 cells and correspondingly decreased E-cadherin expression at the cell membrane compared to control serum (**Figure 2.5D**). We confirmed a decrease in total E-cadherin protein expression in B16BL6 cells after ob/ob serum treatment (**Figure 2.5E**). Thus, we showed that ob/ob serum increases Snai1 expression and decreases E-cadherin expression, which are hallmarks of EMT (126).

In some cells, the Matrix Metalloproteinase 9 (MMP9) expression is regulated by Snai1 (77). MMP9 allows cancer cells to invade surrounding tissue by degrading the extracellular matrix (107). Since ob/ob serum not only increased the ability of melanomas

to invade but also up-regulated the expression of Snai1, we expected the obese serum to increase the expression of MMP9. Indeed, results showed that ob/ob serum increased the expression of MMP9 in B16BL6 cells (**Figure 2.5C**). Furthermore, ob/ob serum modestly increased the amount of active MMP9 compared to control serum (**Figure 2.5F**).

Effects of ob/ob serum on Kiss1 expression

Alternatively, obesity may increase the ability of melanoma cells to metastasize by promoting a mesenchymal phenotype and also by decreasing the expression of genes known to inhibit metastasis. Metastasis suppressor genes can slow down or prevent metastasis (131). Kiss1 is a metastasis suppressor known to inhibit melanoma metastasis (110). Our results showed that ob/ob serum decreased the expression of Kiss1 in B16BL6 cells (**Figure 2.5C**).

Effects of insulin, resistin, and TNF-α on Snai1, Kiss1, and MMP9 expression

To determine which obesity-related factors found in the serum of ob/ob mice affected the expression of Snai1, Kiss1, and MMP9, we treated B16BL6 melanoma cells with purified insulin, resistin, and TNF-α to measure the expression levels of these genes. We showed that resistin treatment increased Snai1 and MMP9 expression, though not significant, while it decreased Kiss1 levels significantly (**Figure 2.6A**). Insulin treatment up-regulated MMP9 but decreased Kiss 1 (**Figure 2.6B**). Lastly, we showed that Kiss1 expression decreased in a time-dependent manner after TNF-α (1ng/mL) treatment (**Figure 2.6C**). Thus, these obesity-related factors may additively contribute to the modulation of the aggressiveness of B16BL6 melanoma and the EMT phenotype.

DISCUSSION

Consistent with previous reports, we showed that leptin-deficient ob/ob mice exhibit numerous obesity-related symptoms such as excess body weight, excess body fat, insulin resistance, hyperinsulinemia, and chronic inflammation (114). Moreover, ob/ob mice had a local (e.g. adipose and liver tissue) and systemic inflammatory environment as reflected by high levels of IL-6, TNF-α, resistin, tPAI-1, and MCP-1 in the serum compared to control mice. We also showed that ob/ob mice have higher glucose levels than control mice, and that higher glucose levels increased the ability of melanomas to migrate in cell culture conditions. Hence, the higher levels of glucose in ob/ob mice may not only meet the higher nutritional requirements of melanoma cells, but also increase their ability to migrate and invade to secondary sites. Thus, the pro-cancerous environment found in the blood of ob/ob mice may allow melanoma cells to enhance their survival and to establish metastases in secondary tissue.

Mori *et al*. previously showed that B16BL6 melanoma cells established much more numerous and grossly larger metastases in the lungs of obese ob/ob mice than in control mice (40). Our results suggest that the serum of obese ob/ob mice may indeed increase the metastatic phenotype of melanoma. The invasive ability of a cancer cell is an important functional phenotype necessary for successful metastasis (140). Supporting Mori *el al*.'s results, we showed that ob/ob serum increased the invasive ability of B16BL6 melanoma cells compared to control serum. To understand the molecular mechanisms by which ob/ob serum increased cell invasion, we determined its effect on the expression of genes linked to cell invasion and metastasis. In fact, we showed that ob/ob serum increased the expression of Snai1 and Twist, genes that are associated with the mesenchymal phenotype (68). We focused our attention on these transcription factors because they have been shown to modulate EMT and the aggressive phenotype of

melanoma cells (60, 71). Melanoma cells with an epithelial phenotype are less aggressive than those with a mesenchymal phenotype (54). Cancer cells that transition towards a mesenchymal phenotype often lose the expression of cell adhesion proteins that keep the cells anchored together and prevent them from breaking away from the primary tumor (141). In some cancers, the transcription factors Snai1 and Twist decrease the expression of E-cadherin, which is a protein found in the cell membrane that plays a key role in cell adhesion: it allows cells within tissues to adhere to each other (142, 143). Our results showed that treatment of melanoma cells with ob/ob serum increased nuclear localization of Snai1 and decreased the amount of E-cadherin at the cell membrane.

Factors found in the serum of ob/ob mice such as IL-6 and TNF-α have been shown to increase the expression of Snai1 (98, 99). Thus, it is feasible that the pro-inflammatory environment found in ob/ob mice promotes a mesenchymal phenotype by increasing the expression of Snai1 and Twist to affect the metastatic ability of B16BL6. Indeed, we showed that resistin and insulin also affected the expression of Snai1 and MMP9. Others have shown that overexpression of Snai1 and Twist can increase both the mesenchymal phenotype and the metastatic ability of melanoma cells (56, 60, 128). Moreover, Snai1 also increases the expression of MMP9, a protein that can degrade extracellular matrix proteins; the degradation of the extracellular matrix proteins may allow cancer cells to escape into the blood or invade secondary tissues in order to establish metastases (77, 78). In our study, we showed that ob/ob serum increased the expression of MMP9 and its activity in B16BL6 melanoma cells, which may explain why B16BL6 melanoma cells have a higher invasive ability in the presence of ob/ob serum.

Besides affecting genes associated with EMT, obese serum may also increase the metastatic phenotype of melanoma cells by decreasing the expression of genes known to inhibit metastasis. Metastasis suppressors are genes that have been identified to stop or

slow down metastasis (144). In our study, we measured the expression levels of Kiss1, which was originally discovered for its ability to suppress the metastatic ability of two human melanoma cell lines (103). Studies suggest that one anti-metastatic function of Kiss1 may be to decrease the expression of Matrix Metalloproteinases (MMPs), such as MMP9 (107, 78). Interestingly, others show that Kiss1 is also down-regulated in cancer cells that acquire a mesenchymal phenotype (145). We found that treatment of B16BL6 melanoma cells with ob/ob serum decreased the expression of Kiss1. Furthermore, we showed that both resistin and insulin decreased Kiss1 expression and increased MMP9 expression in B16BL6 melanoma. Similarly, TNF-α treatment decreased Kiss1 expression in a time-dependent manner. Thus, systemic obesity-related factors may collectively modulate EMT and the aggressive phenotype of melanoma cells. In summary, as **Figure 2.7** depicts, our results suggest that systemic factors found in the blood of ob/ob mice may contribute to increasing the aggressiveness of melanoma cells to metastasize by promoting a mesenchymal phenotype and by decreasing the expression of the metastasis suppressor gene Kiss1.

ACKNOWLEDGEMENTS

This work was supported by the American Cancer Society grant ACS RSG CNE-113703 and by grants from the National Institutes of Health: National Cancer Society grant NCI 1K22CA127519-01A1 and National Institute of Environmental Health Sciences Center grants ES09145 and ES007784.♣

♣ This work and the following figures have been previously published in **Kushiro K**, Nunez NP. *Ob/ob serum promotes a mesenchymal cell phenotype in B16BL6 melanoma cells.* Clinical and Experimental Metastasis doi: 10.1007/s10585-011-9418-4.

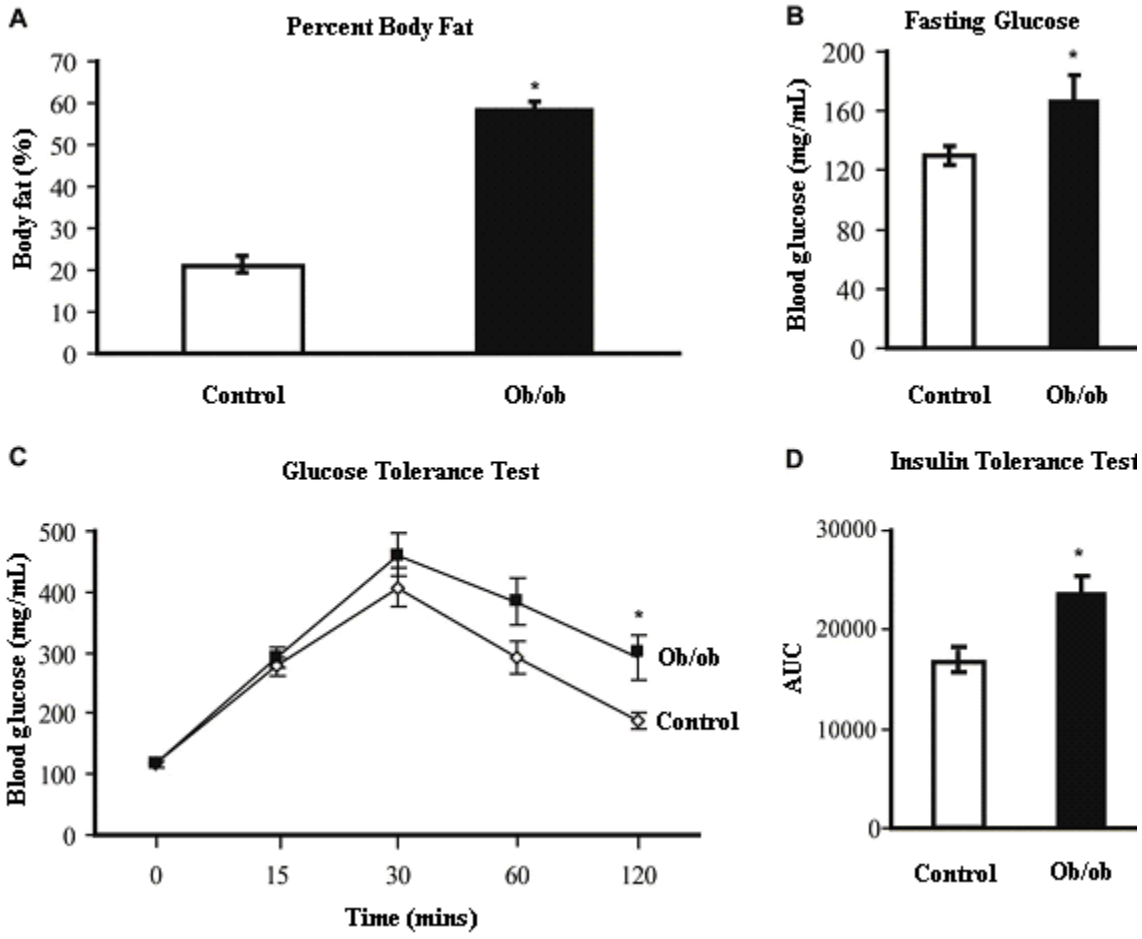

Figure 2.1 Ob/ob mice. Mice were given water and food ad lib for 15 weeks. A) Ob/ob mice had higher percent body fat and B) higher fasting glucose levels in their blood than control mice. Mice were insulin resistant as observed by the C) Glucose Tolerance Test (GTT) and D) Insulin Tolerance Test (ITT) assays results (mean ± SEM, n=12 mice/group, * is $p < 0.05$)

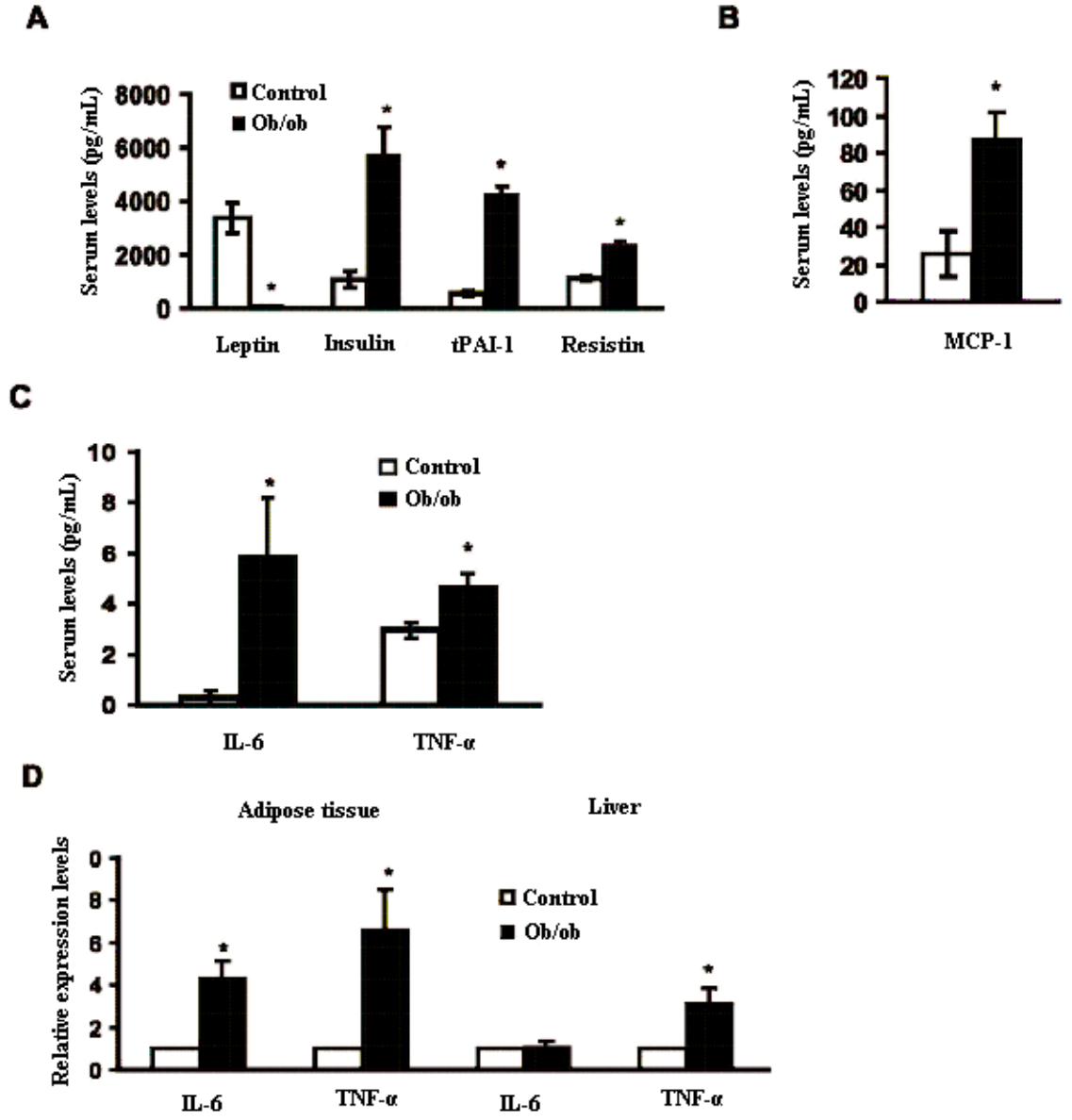

Figure 2.2 Systemic factors in ob/ob mice. Mice were given water and food ad lib for 15 weeks. At the 10th week of the study, serum was collected and analyzed for circulating factors. Ob/ob mice had A) undetectable leptin, higher levels of insulin, t-plasminogen activator inhibitor-1 (tPAI-1), resistin, B) Monocyte chemoattractant protein-1 (MCP-1), C) Interleukin 6 (IL-6), and Tumor Necrosis Factor alpha (TNF-α) compared to control mice (mean ± SEM, n=12 mice/group, * is $p < 0.05$). At the end of the study, adipose tissue and liver were collected and analyzed using qRT-PCR for inflammatory factors. D) Ob/ob mice had higher levels of IL-6 and TNF-α in adipose tissue. Ob/ob mice had higher levels of TNF-α in the liver; however, IL-6 levels in the liver were similar to those of control mice. (mean ± SEM, n=3 mice/group, * is $p < 0.05$)

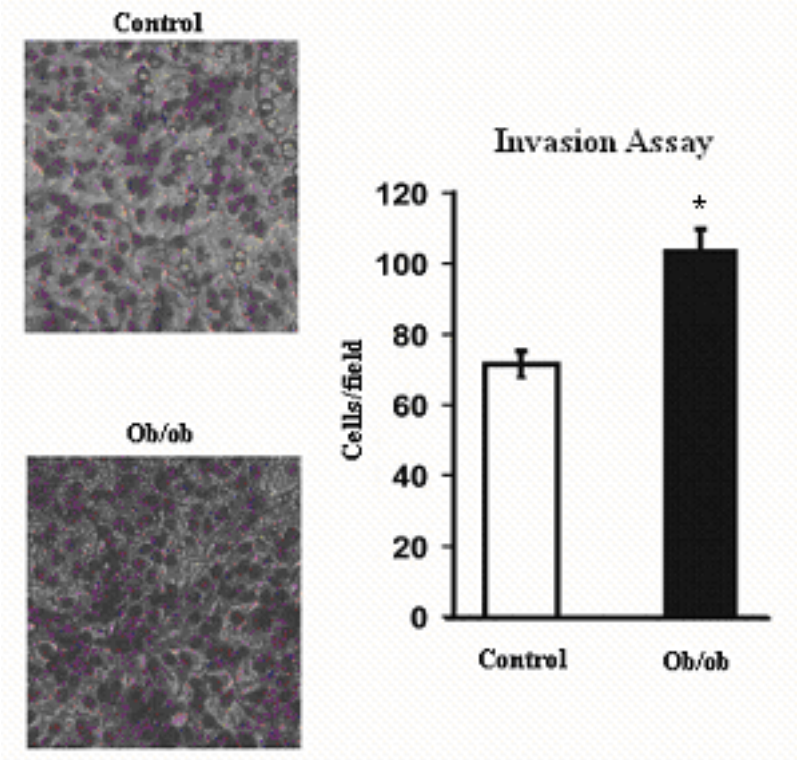

Figure 2.3 Ob/ob serum increases B16BL6 cell invasion. B16BL6 cells were exposed to 5% ob/ob serum or 5% control serum for the invasion assay. The images shown are pictures of cells that invaded during the 24 hour period. Number of B16BL6 cells that invaded in the ob/ob and the control serum over the 24 hour period. Results expressed as mean ± SEM, n=3 separate experiments with 3 replicate for each condition, * is $p < 0.05$.

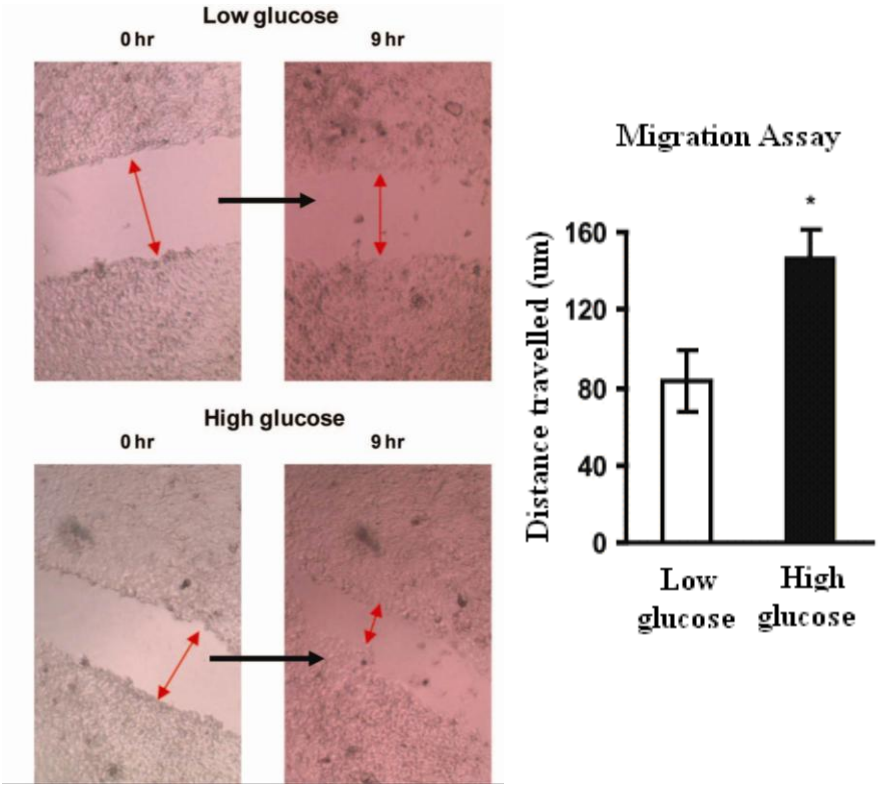

Figure 2.4 High glucose increases B16BL6 cell migration. B16BL6 cells were treated with DMEM media containing either high (4,500 mg/L) or low (1,000mg/L) glucose, and cell motility was determined using the wounding assay after 9 hrs. High glucose exposure increased the ability of B16BL6 cells to migrate. B) Representative pictures of cell motility and quantification of the assay results (mean ± SEM, n=3 separate experiments with 3 replicate for each condition, * is $p < 0.05$)

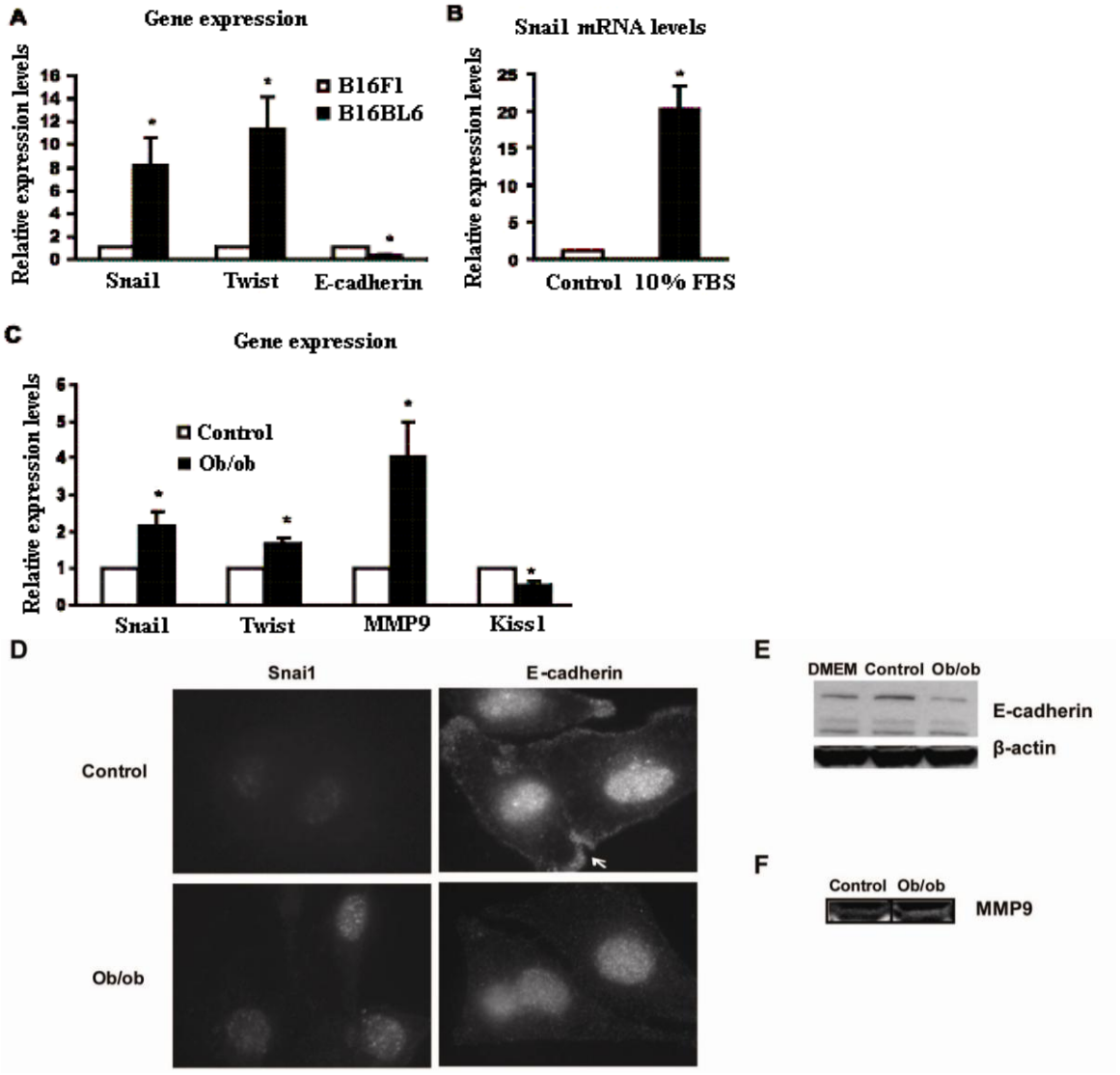

Figure 2.5 B16BL6 gene expression and MMP9 activity. B16F1 or B16BL6 cells were treated with DMEM, 10% FBS, 5% ob/ob serum or 5% control serum for 24 hrs as indicated and analyzed by qRT-PCR or Immunofluorescence microscopy. A) Metastatic B16BL6 cells have higher Snai1 and Twist mRNA levels than non-metastatic B16F1 cells. Conversely, B16BL6 cells express less E-cadherin than B16F1 cells. B) B16BL6 cells treated with 10% FBS express higher Snai1 mRNA levels than FBS-starved cells. C) Ob/ob serum increased Snai1, Twist, and Matrix Metalloproteinase 9 (MMP9) mRNA levels. Conversely, ob/ob serum decreased the expression of the metastasis suppressor Kiss1 (mean ± SEM, n=4 separate experiments with 3 replicates for each condition, * is $p< 0.05$). D) Nuclear expression of Snai1 and localization of E-cadherin in B16BL6 cells (n=3). White arrow indicates cell-cell junction. E) Total E-cadherin expression in B16BL6 cells. F) Active MMP9 secreted by B16BL6 cells (n=3).

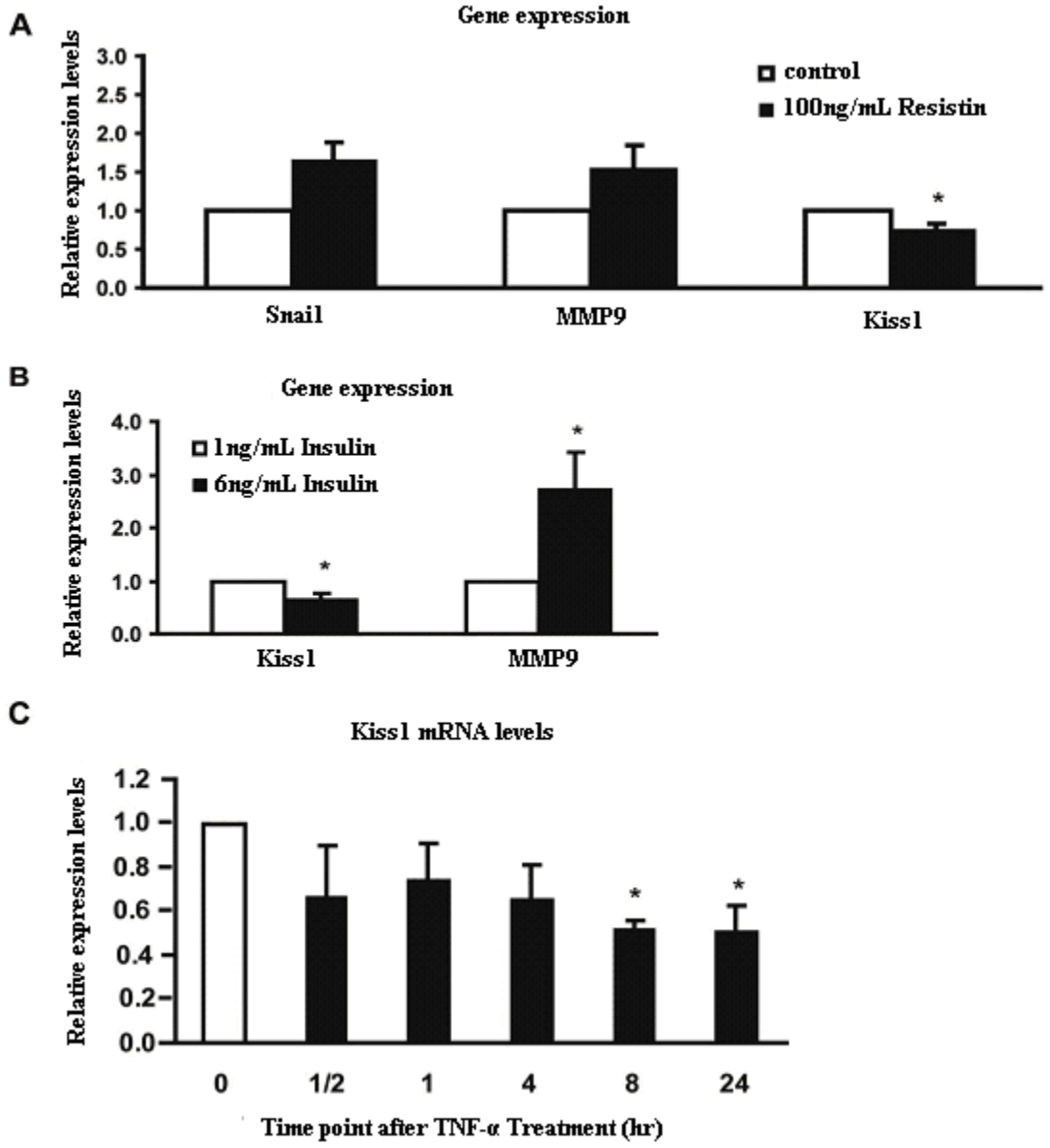

Figure 2.6 Effects of obesity factors on Snai1, Kiss1, and MMP9 expression. B16BL6 cells were treated with resistin (100ng/mL), insulin (1 and 6ng/mL) and TNF-α (1ng/mL) for 24 hrs unless indicated otherwise, to determine the expression of Snai1, Kiss1, and MMP9 through qRT-PCR. A) Effects of resistin on Snai1, MMP9 and Kiss1 mRNA expression. B) Effects of insulin on Snai1, MMP9 and Kiss1 mRNA expression. C) Effects of TNF-α (1ng/mL) on Kiss1 mRNA expression. (mean ± SEM, n=3 separate experiments with 3 replicate for each condition, * is $p < 0.05$)

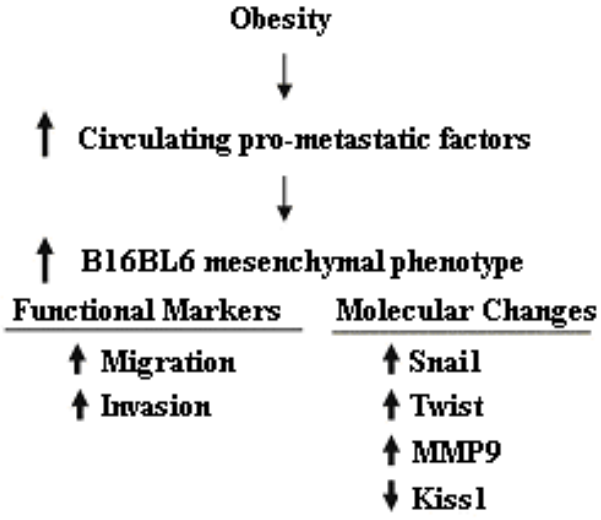

Figure 2.7 Proposed mechanism by which ob/ob serum increases the metastatic ability of B16BL6 melanoma. Obesity is characterized by high systemic levels of glucose, IL-6, and TNF-α. These factors may increase the mesenchymal phenotype by increasing the expression of Snai1, Twist, and MMP9, and thereby increase the invasive ability of B16BL6 melanoma cells. Moreover, such pro-cancerous environment may simultaneously decrease the expression of metastasis suppressor such as Kiss1, and thus further increase the metastatic phenotype of melanoma.

CHAPTER 3: ADIPOCYTES PROMOTE B16BL6 MELANOMA CELL INVASION AND THE EPITHELIAL-TO-MESENCHYMAL TRANSITION

ABSTRACT

Epidemiological and animal studies suggest that obesity increases the metastatic ability of malignant melanoma, though the mechanism is not known. In the present study, we assessed the ability of 3T3L1 adipocytes to modulate B16BL6 melanoma cell invasion and the Epithelial-to-Mesenchymal Transition (EMT). For this purpose, we induced the differentiation of 3T3L1 fibroblasts to adipocytes. Then, we collected the cell culture media from both fibroblasts and adipocytes and determined their effect on the invasive ability and EMT gene expression of B16BL6 melanoma cells. Results showed that adipocyte media increased B16BL6 cell invasiveness. The higher invasive ability of B16BL6 melanoma cells was associated with increased expression of EMT genes such as Snai1, MMP9, Twist, and Vimentin. Additionally, the expression levels of the cell-to-cell adhesion protein E-cadherin and the metastasis suppressor gene Kiss1 were down-regulated in these B16BL6 cells. Also, adipocytes had high levels of the pro-inflammatory cytokine Interleukin 6 (IL-6). Treatment of B16BL6 cells with IL-6 elicited effects similar to those observed with the adipocyte media; IL-6 was able to promote the invasive ability of B16BL6 melanoma cells, increase the expression of Snai1, and decrease Kiss1 expression compared to FBS-starved cells. IL-6 neutralization of the adipocyte media, however, did not have a visible effect on the adipocyte media-induced invasion and Snai1 staining. In summary, adipocytes may secrete factors besides IL-6 that are important in increasing the invasive ability of B16BL6 melanoma cells by promoting EMT and decreasing the expression of genes such as E-cadherin and Kiss1.

INTRODUCTION

Studies suggest that obesity increases the ability of malignant melanoma to metastasize (38, 40). Dennis *et al*. showed that obesity increased the risk of developing subcutaneous melanoma (37); furthermore, Samanic *et al*. demonstrated that obesity increased the risk of developing malignant melanoma (38). In animal studies, Mori *et al*. showed that obesity promotes B16BL6 melanoma pulmonary metastasis (40). Approximately 60% of the U.S. population is considered overweight or obese (146). In obesity, fat cells produce inflammatory factors such as the cytokine Interleukin 6 (IL-6), which can lead to the development of a low-grade inflammation environment (47). This inflammation environment has been hypothesized to increase cancer risk (147).

The microenvironment surrounding the melanoma cells may indeed play an important role in obesity's ability to promote metastasis. Obesity may cause a pro-cancerous microenvironment by providing growth factors and inflammatory factors that can increase metastasis (41). Some of the cells that comprise the cancer microenvironment include cancer-associated fibroblasts (FACs), macrophages (TAMs), and adipocytes (44-46). These cancer-associated cells are capable of secreting factors that can impact cancer metastasis (148, 149).

Our present objective was to determine if adipocyte-conditioned media promotes melanoma metastasis. For this purpose, we differentiated 3T3L1 fibroblasts into adipocytes, and then investigated the effect of 3T3L1 fibroblast and adipocyte media on B16BL6 melanoma cell invasiveness and the Epithelial-to-Mesenchymal Transition (EMT). EMT is a critical cellular program for the initiation of the metastatic cascade (150). Cancer cells with a mesenchymal phenotype have a higher propensity to metastasize than those with an epithelial phenotype (125). Increased expression of the

transcription factors Snai1 and Twist are associated with increased EMT and increased ability of cancer cells to migrate, invade, and metastasize (48, 126).

The aberrant expression of Snai1 and Twist can lead to a significant loss of cell-to-cell adhesion proteins such as E-cadherin (128). The mesenchymal phenotype is also associated with high expression of Matrix Metalloproteinases (MMPs) such as MMP9 that can degrade the extracellular matrix (ECM) and allow the cancer cells to metastasize (129). Thus, inflammatory cytokines such as IL-6 may promote EMT and increase the metastatic ability of melanoma cells. Furthermore, increased EMT has been associated with decreased expression of various metastasis suppressors (56). Metastasis suppressor genes encode proteins that have the ability to inhibit the establishment of metastases (130). Metastasis suppressor genes such as Kiss1 can inhibit the metastatic ability of melanomas (131). Thus, it is feasible that adipocytes secrete factors that promote the metastatic ability of melanomas by increasing the expression of pro-metastatic EMT genes and by decreasing the expression of metastasis suppressors such as Kiss1.

In the present study, we showed that adipocyte media increased B16BL6 cell migration and invasion. Increased motility and invasion are hallmarks of metastasis and rely on the induction of EMT (68). We further showed that adipocyte media increased the expression of EMT-associated genes such as Snai1, Twist, MMP9, and Vimentin, while it decreased the expression of E-cadherin and Kiss1 in B16BL6 cells. Lastly, we showed that the cytokine IL-6, which was increased in adipocytes, could also promote B16BL6 cell invasion, increase Snai1 expression, and decrease Kiss1 expression to levels comparable to the adipocyte media. Neutralization of IL-6 in the adipocyte media, however, did not nullify the media's promotive effects on cell invasion and Snai1 expression. Therefore, our results suggest that the adipocyte media may increase the metastatic ability of melanoma cells through other factors besides IL-6. Further studies

will need to be performed to confirm the role of IL-6 in the metastatic ability of melanomas.

METHODS AND PROCEDURES

Cancer cells and cell culture reagents

B16BL6 melanoma cells were kindly provided by Dr. Isaiah J. Fidler, University of Texas MD Anderson Cancer Center, Houston TX. They were maintained in high glucose Dulbecco's modified minimum essential medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (Invitrogen) and 1% antibiotic-antimycotic solution (CellGro, Manassas, VA), and grown at 37°C in a humidified atmosphere of 5% CO_2. For cell culture studies, B16BL6 cells were treated with 5% adipocyte media, 5% fibroblast media, or control DMEM with no phenol red (Invitrogen). Purified IL-6 was purchased and reconstituted in PBS containing 0.1% BSA to a concentration of 10ug/mL and stored in -80°C until used (R&D Systems, Minneapolis, MN). Cells were treated with 1ng/mL IL-6 in control DMEM for the IL-6 treatments at the various time points.

Differentiation of 3T3L1 fibroblasts to adipocytes

To test the effect of 3T3L1 adipocytes on B16BL6 cell invasion and EMT gene expression, 3T3L1 fibroblasts were purchased from the American Type Culture Collection (ATCC, Chicago, IL; no. CL-173). They were maintained in DMEM supplemented with 10% FBS until they were differentiated into adipocytes as described previously (151). Adipocyte differentiation was confirmed through visual observation of lipid droplets as well as Oil Red O staining as previously described (152). For collection

of fibroblast or adipocyte media, 3T3L1 cells were split into two equal plates. For fibroblast-conditioned media, cells were allowed to proliferate until the plate was 70% confluent. Cells were rinsed twice with PBS, and DMEM was subsequently added. After 24 hrs, the media was collected, centrifuged at 10,000 rpm for 10 mins at 4°C, and the supernatant was collected and stored at -80°C (Fibroblast Media). The other plate was differentiated into adipocytes accordingly, and after full differentiation (day 14), adipocytes were rinsed twice with PBS, and DMEM was subsequently added. After 24 hrs, the media was collected, centrifuged at 10,000 rpm for 10 mins at 4°C, and then the supernatant was collected and stored at -80°C (Adipocyte Media). These conditioned media were then used to determine their effect on B16BL6 melanoma migration and invasive ability as well as their ability to influence the EMT phenotype.

IL-6 neutralization of adipocyte media

Adipocyte media was thawed on ice before being incubated with 2ug/mL of IL-6 antibodies (Ambion/Applied Biosystems, Austin, TX) on a rotary shaker for 4 hrs at 4°C. 100uL of Protein A/G agarose slurry (Pierce Thermo Scientific, Waltham, MA) was added and subsequently incubated overnight at 4°C. Then, the media was centrifuged at 10,000rpm for 5 mins at 4°C to remove the beads. The supernatant was collected and stored at -80°C. This media was referred to as IL-6-neutralized adipocyte media throughout this manuscript. A decrease in IL-6 expression was confirmed with Western blot analysis before the media was used for subsequent assays.

Wounding assay

To determine the effect of adipocyte media on B16BL6 cell migration, we performed the wounding assay. Cells were plated to 100% confluency and a scratch was drawn in the middle of the plate. The decrease in gap distance was measured over time and quantified;

the larger the gap distance that remained after a given time point, the less the cells have migrated. Briefly, B16BL6 cells were grown on a 24-well plate until they were confluent. Then, cells were grown in FBS-free DMEM overnight. The next day, wells were washed twice with PBS and a scratch was drawn on each well using a p200 pipette tip. After two more washes with PBS to get rid of cell debris, the following cell culture media was added: 5% adipocyte media, 5% fibroblast media or control DMEM. Each well was photographed at the time of treatment (0 hr) and after 16 hrs of incubation (16 hr) at 40x. The difference in gap distance was measured (16 hr time point – 0 hr time point) to quantify cell motility. Each experiment was repeated three times with each group having 3 wells per experiment.

Invasion assay

The effect of adipocyte media on B16BL6 cell invasion was determined by the Boyden chamber invasion assay. In this assay, the top chamber was previously coated with BD Matrigel^{TM} that is composed of various extracellular matrix proteins such as laminin and collagen (BD Biosciences, Franklin Lakes, NJ). Cells were FBS-starved overnight, then rinsed with PBS before being trypsinized and counted by hemocytometry. 100,000 cancer cells were placed in the top chamber in serum-free DMEM with 0.1% Bovine Serum Albumin (BSA). The lower chamber was filled with DMEM (control), 5% adipocyte media, 5% IL-6 neutralized adipocyte media, 5% fibroblast media, or DMEM containing 1ng/mL IL-6. Cells were allowed to invade from the top side towards the bottom side of each chamber for 28 hrs. Cells that invaded to the bottom side were fixed and stained using Siemens Diff-Quick Stain Set (Siemens, Malvern, PA). Stained cells were visualized and quantified by microscopy. To determine the average number of cells

that migrated for each well, we counted 3 random fields in each well at 200x; each treatment had three individual wells. Three separate experiments were carried out.

Quantitative real-time PCR (qRT-PCR)

To determine the effect of adipocyte media on the expression of several genes associated with EMT, qRT-PCR was performed. Total RNA was collected from cells that were FBS-starved overnight and treated with control media, 5% fibroblast media, 5% adipocyte media, or 1ng/mL IL-6 for the stated amount of time. RNA was extracted using an RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). Reverse transcription was performed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), using 1 μg of RNA for each reaction. Primers for 18S, Snai1, MMP9, Twist, Vimentin, E-cadherin, and Kiss1 were purchased from Integrated DNA Technologies (IDT, Coralville, IA) and were as follows: 18S F: GCATGGCCGTTCTTAGTTGGTGGA, B: TCTCGGGTGGCTGAACG-CCA; Snai1 F: CACCTCCAGA-CCCACTCAGAT, B: CCTGAGTGGGGTGGGAGCTTCC; MMP9 F: GCCCACCGTCCTTTCTTGTTGGA, B: GGGAGAGGTGGTTTAGCCGGTG; Twist F: CCACGCTGCCCTCGGA-CAAG, B: CCAGGCCCCCTCCATCCTCC; Vimentin F: CCAGAGACCCCAGCGCTCCT, B: GCCGGAGCCACCGAACATCC; E-cadherin F: TTGAGGAGTTGAATGCTGAC, B: AGCTCGAACTTTCCAAGCAG; Kiss1 F: GCAAGCCTGGGTCTGCAGGG, B: CGACTGC-GGGAGGCACACAG.

qRT-PCR was performed with a SYBR GreenER qPCR kit (Invitrogen) in a Mastercycler ep Realplex Real-time PCR thermocycler (Eppendorf North America, Hauppauge, NY). The relative expression levels of target genes were normalized to the housekeeping 18S rRNA. Amplification specificity was confirmed by melting curve

analysis. Each gene was measured in quadruplicate, and the average ΔCt was taken from the 4 wells before fold change was calculated using the ΔΔCt method. At least three separate experiments were carried out.

Western blot analysis

To determine the protein expression of IL-6 in 3T3L1 fibroblasts and 3T3L1 adipocytes, Western blot analysis was carried out on whole cell lysates. 3T3L1 fibroblasts and differentiated 3T3L1 adipocytes were washed twice with PBS before being lysed in 300uL RIPA buffer (Thermo Scientific) supplemented with a protease inhibitor cocktail (Roche, South San Francisco, CA). After 45 minute incubation on ice, cells were centrifuged at 10,000 rpm for 10 mins at 4°C. The supernatant was collected and protein concentration was measured using the Bradford Assay. 100ug of protein was loaded and run on an SDS-PAGE gel at 100-150V for 1 hr. The gel was subsequently transferred to a PMSF membrane, blocked in 5% milk, washed numerous times in TBST, and blotted with appropriate antibodies and HRP-conjugated secondary antibodies before incubation with ECL and film exposure. Membranes were probed using primary antibody for IL-6 (Abcam, Cambridge, UK; no.ab6672) and β-actin (Cell Signaling, Danvars, MA, no. 4967L) and respective secondary antibodies (Cell Signaling, no. 7074; and Santa Cruz Biotechnology Inc., Santa Cruz, CA, no. 2768).

We also measured the protein expression of IL-6 Receptor α and Snai1 in B16BL6 cells as a response to the various treatments. For this purpose, B16BL6 cells were grown to about 70% confluency in 10 cm^3 plates. Cells were rinsed with PBS and then FBS-starved overnight. Then, cells were treated with control DMEM, 5% adipocyte media, 5% fibroblast media, or 1ng/mL IL-6 in DMEM. After the various time-point treatments, cells were rinsed twice with PBS, trypsinized, and centrifuged at 10,000 rpm

for 5 mins at 4°C. The supernatant was discarded, and the pellets were stored in -80°C overnight. Total protein was collected and quantified as described above. For Western blotting, 100ug of protein samples were loaded in each well, run, and transferred to a PMSF membrane before being probed for IL-6 Receptor α [Santa Cruz Biotechnology Inc., IL-6Rα (H-300), no. 13947], Snai1 (Abcam, no. 63371), and β-actin.

Flow Cytometry

B16BL6 cells were grown to 70% confluency in 6 cm^3 plates. Cells were rinsed with PBS and FBS-starved overnight. Then, cells were treated with control DMEM, 5% adipocyte media, or 1ng/mL IL-6 in DMEM. After the various time point treatments, cells were rinsed with PBS, trypsinized, and centrifuged at 10,000 rpm for 5 mins at 4°C. The supernatant was decanted and the pellet was subsequently vortexed thoroughly to make a single cell suspension. Cells were resuspended in 1mL cold PBS. Cells were subsequently added drop-wise into 2mL of cold 100% ethanol while vortexing and stored at -20°C for up to one week. Then, 2mL of this solution containing the cells were centrifuged at 1,600 rpm for 4 mins at 4°C. Cells were washed twice with PBS, then permeabilized with 0.1% triton-X100/PBS and treated with antibodies against Snai1 or Kiss1 (Santa Cruz Biotechnology Inc., no. sc-18134) and appropriate fluorescently-conjugated secondary antibodies (Cell Signaling, no. 4412; and Abcam, no. ab6949) as recommended by the manufacturer before being subjected to flow cytometry. Mean fluorescent intensity was used as a measure of protein expression.

Immunofluorescence Microscopy

Wax pencils were used to mark a closed circle on microscope coverslips. B16BL6 cells were plated into the circles and allowed to attach for 24 hrs before being FBS-starved overnight. They were subsequently treated with 5% adipocyte media, 5% IL-6

neutralized adipocyte media, 1ng/mL IL-6, or control DMEM for 24 hrs. Cells were washed twice with PBS and fixed in 4% formalin/PBS for 10 mins. Then, cells were subsequently washed twice with PBS before being stored in PBS at 4°C overnight. The next day, cells were permeabilized with 0.1% Triton-X100/PBS and neutralized with 100uM glycine/PBS before being treated with antibodies against Snai1 or Kiss1 and appropriate fluorescently-conjugated secondary antibodies (Cell Signaling and Abcam) as recommended by the manufacturer. After three washes with 0.2% Tween20 in PBS, cells were counterstained with two drops of DAPI/antifade (Millipore) according to the manufacturer's instructions for detection of cellular nuclei. After 15 mins of incubation, coverslips were placed onto microscope slides and sealed with nail polish, and images were taken with the Zeiss Axiovert 200 M fluorescent microscope at UT Austin's ICMB Core Facility. Images of control and experimental cells were acquired under identical exposure conditions for comparative analysis.

Statistical analysis

Experiments were analyzed for significance using the independent Student's t-test or One-way ANOVA in SPSS (PAWS version 18). P-values ≤ 0.05 were considered significant, and all data is represented as the mean ± SEM where appropriate.

RESULTS

Adipocyte media promotes B16BL6 cell motility and invasion

We determined the effect of adipocyte media on B16BL6 cell motility and cell invasion. Results showed that adipocyte media promoted the migration and invasion of B16BL6 cells to a higher degree than DMEM alone or fibroblast media (**Figure 3.1A-B**).

Adipocyte media affects EMT gene expression in B16BL6 cells

To better understand the mechanism by which adipocyte media affects the invasive phenotype of B16BL6 cells, we determined the effect of adipocyte media on the expression of genes associated with the Epithelial-to-Mesenchymal Transition (EMT). Cancer cells with a more mesenchymal phenotype metastasize better than those with an epithelial phenotype (138). The mesenchymal phenotype is associated with the expression of transcription factors such as Snai1 and Twist, MMPs such as MMP9, and the intermediate filament protein Vimentin (56, 139). Results showed that adipocyte media increased the expression of Snai1, Twist, MMP9, and Vimentin in B16BL6 cells (**Figure 3.2**). Adipocyte media also decreased the expression of the cell-to-cell adhesion marker E-cadherin in B16BL6 cells (**Figure 3.2**). EMT has been associated with a decreased expression of various metastasis suppressor genes, including Kiss1 (56). Our results are consistent with this finding, as we showed that the expression of Kiss1 was reduced by adipocyte media in B16BL6 cells (**Figure 3.2**).

Effects of IL-6 treatment and IL-6 neutralization on B16BL6 cell invasion

We measured the expression of the inflammatory marker IL-6 in both 3T3L1 fibroblasts and 3T3L1 adipocytes. The protein expression of IL-6 was higher in the 3T3L1 adipocyte culture than in the 3T3L1 fibroblast culture (**Figure 3.3A**). Thus, it is feasible that adipocytes promote the aggressiveness and EMT phenotype in B16BL6 melanoma cells via IL-6. In fact, we showed that adipocyte media increased the expression of the IL-6 Receptor α (IL-6Rα) in B16BL6 melanoma cells in a time-

dependent manner that was not observed in fibroblast media-treated B16BL6 melanoma cells (**Figure 3.3B**). In addition, we showed that direct treatment of B16BL6 melanoma cells with IL-6 also increased the expression of IL-6Rα (**Figure 3.3C**). We next determined the ability of IL-6 to affect the invasive ability of B16BL6 melanoma cells. Results showed that the IL-6 treatment increased cell invasion to levels comparable to those induced by adipocyte media (**Figure 3.3D-E**). To determine the role of IL-6 found in the cell culture media, we neutralized it using antibodies against IL-6, and then determined the effect of this IL-6-neutralized adipocyte media on B16BL6 invasion. Results showed that the neutralization of IL-6 did not significantly alter the ability of the adipocyte media to affect the invasive ability of B16BL6 cells (**Figure 3.3D-E**). Though further studies are necessary to completely rule out the effect of IL-6 on B16BL6 cell invasiveness, our results currently suggest that IL-6 may not be a critical factor in this process, given the similar results from adipocyte media treatment and IL-6-neutralized adipocyte media treatment.

Effects of IL-6 on the expression of Snai1 and Kiss1 in B16BL6 cells

To determine if the effects of IL-6 on the expression of IL-6Rα correlated with changes in the expression of Snai1 and Kiss1, we measured both their mRNA and protein levels in B16BL6 cells. Results showed that Snai1 mRNA and protein levels were modestly increased by the IL-6 treatment (**Figure 3.4A, 3.4C**). This effect of IL-6 on Snai1 was also observed with adipocyte media, which increased the protein expression of Snai1 in a time-dependent manner (**Figure 3.4B**). In contrast, fibroblast media did not affect the

expression of Snai1 in B16BL6 cells (data not shown). We determined that adipocyte media and IL-6 treatments affected Snai1 nuclear levels. For this purpose, we used immunofluorescence microscopy. Results showed that Snai1 was increased in the nucleus of B16BL6 cells as a result of both adipocyte media and IL-6 treatments (**Figure 3.4D**). However, IL-6-neutralized adipocyte media also increased Snai1 nuclear staining to levels that were similar to those in adipocyte media-treated B16BL6 cells when compared to control FBS-starved B16BL6 cells (**Figure 3.4D**). Thus, though IL-6 by itself may increase Snai1 expression as previously observed by others in other cancers, our results suggest that other factors in the adipocyte media may be more important for Snai1 expression in B16BL6 cells (98, 99).

As our results showed that both adipocyte media and IL-6 treatments increased the ability of B16BL6 cells to invade, we rationalized that these treatments may affect the expression of metastasis suppressor genes. We chose to determine the effect of IL-6 treatment on the expression of Kiss1 because it has been shown to be decreased during the EMT (56). Results showed that the IL-6 treatment decreased Kiss1 mRNA and protein expression in a time-dependent manner (**Figure 3.5A-B**). Immunofluorescence microscopy experiments confirmed these results and showed that both IL-6 and adipocyte media treatments decrease Kiss1 expression (**Figure 3.5C**).

DISCUSSION

Our findings showed that adipocytes increase the malignancy of B16BL6 melanoma cells. We showed that adipocyte media increased the ability of B16BL6 cells to migrate and invade. These effects of adipocyte media on the malignant phenotype of melanoma

cells correlated with changes in EMT gene expression. We showed that the expression of Snai1, Twist, MMP9, Vimentin, E-cadherin, and Kiss1 were modulated by adipocyte media. Additionally, we showed that adipocytes expressed higher levels of the cytokine IL-6. Moreover, treatment of B16BL6 with IL-6 elicited effects similar to those of adipocyte media on the ability of melanoma cells to invade. The IL-6 treatment also increased the expression of Snai1 and decreased the expression of Kiss1 in B16BL6 cells. However, neutralization of IL-6 in the adipocyte media did not decrease the effects of adipocyte media on the invasive phenotype of B16BL6 cells and on the expression of Snai1. Therefore, though IL-6 could have an observable effect on cell invasiveness and Snai1 expression when compared to FBS-starved B16BL6 cells, it may be an unnecessary component for the observed adipocyte media-induced effects. This suggests the existence of other factors in the adipocyte media that may have a much more significant impact on the expression of EMT-associated genes such as Snai1.

Evidence by others suggests that the EMT plays an important role in the metastatic ability of cancer cells (150). In particular, cancer cells that have acquired a mesenchymal phenotype metastasize more than those with an epithelial phenotype (125). The expression of Snai1 and Twist is highly correlated with the mesenchymal phenotype (68). In fact, others have shown that the mesenchymal phenotype and metastatic ability of melanomas are increased with Snai1 and Twist overexpression (56, 60, 128). This is pertinent to melanomas because those with an epithelial phenotype are less aggressive than those with a mesenchymal phenotype (54). As cancer cells transition towards a mesenchymal phenotype, they often lose the expression of cell adhesion proteins that

keep the cells anchored together such as E-cadherin (141). In some cancers, both Snai1 and Twist are thought to decrease the expression of E-cadherin (142, 143). We showed that adipocyte media increased the expression of Snai1 and Twist, and correspondingly decreased the expression of E-cadherin. Moreover, adipocyte media increased the expression of MMP9. Others have shown that Snai1 can increase the expression of MMP9, a protein that can degrade the extracellular matrix (77). The degradation of the extracellular matrix may allow cancer cells to escape into the blood or invade secondary tissues (78). Therefore, adipocytes may promote EMT by affecting the expression of Snai1 and Twist, which subsequently affect E-cadherin and MMP9 expression levels, thus allowing melanoma cells to invade and establish metastases.

Interestingly, EMT has been associated with decreased expression of the metastasis suppressor gene Kiss1 (145). Kiss1 was originally discovered for its ability to suppress the metastatic ability of two human melanoma cell lines (103). Studies suggest that one of the anti-metastatic functions of Kiss1 may be to decrease the expression of MMPs such as MMP9 (107). This would contribute to the inhibition of B16BL6 cell invasion. Others also show that Kiss1 is down-regulated in cancer cells that acquire a mesenchymal phenotype (145). We found that treatment of B16BL6 melanoma cells with adipocyte media led to decreased expression of Kiss1. Thus, adipocytes may simultaneously induce the expression of EMT genes and down-regulate the expression of Kiss1, which allows the melanoma cells to become more aggressive.

It is feasible that adipocytes affect the malignant phenotype of melanomas via multiple factors, including the Transforming Growth Factor (TGF-β). Others have shown

that TGF-β plays a crucial role in EMT (49, 153, 154). Interestingly, TGF-β is produced by 3T3L1 adipocytes as well (155, 156); moreover, it is highly expressed in the adipose tissue of obese individuals (156, 157). Thus, it is possible that the TGF-β in the adipocyte media was also partly responsible for the increase in cell invasion and Snai1 expression as others have already confirmed a positive relationship between TGF-β and Snai1 expression as well as cell invasiveness (154, 158). Therefore, the presence of TGF-β may be part of the reason why IL-6 neutralization of the adipocyte media did not have an observable effect on the media's ability to affect B16BL6 cells in our study even though IL-6 alone could elicit similar promotive effects from the B16BL6 cells. In conclusion, our results suggest that the adipocyte media may affect the malignancy of melanoma cells by promoting EMT and subsequently decreasing the expression of the metastasis suppressor Kiss1 independently of the presence of IL-6.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ACKNOWLEDGEMENT

This work was supported by the American Cancer Society grant ACS RSG CNE-113703 and by grants from the National Institutes of Health: National Cancer Institute grant NCI 1K22CA127519-01A1 and National Institute of Environmental Health Sciences Center grants ES09145 and ES007784.♣

♣ This work and the following figures have previously been published in **Kushiro K**, Chu RA, Verma A, Nunez NP. *Adipocytes promote B16BL6 Melanoma Cell Invasion the Epithelial-to-Mesenchymal Transition.* Cancer Microenvironment Special Issue Microenvironment and EMT doi: 10.1007/s12307-011-0087-2.

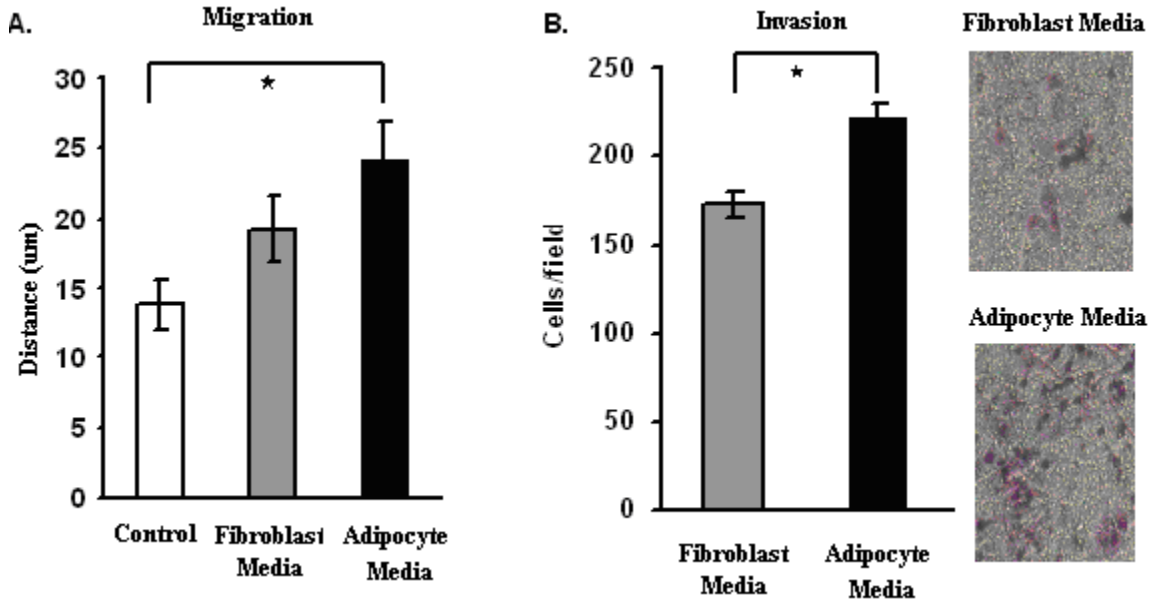

Figure 3.1 Adipocyte media increases B16BL6 cell invasion. B16BL6 cells were exposed to 5% adipocyte media, 5% fibroblast media, or control DMEM for the wounding and invasion assay. A) In the wounding assay, B16BL6 cells were exposed to their respective media for 16 hrs, and distance migrated was calculated. B) In the invasion assay, B16BL6 cells were exposed to their respective media for 28 hrs and number of cells that invaded was determined. The data are representatives of three separate experiments, presented as ± SEM (* $p<0.05$).

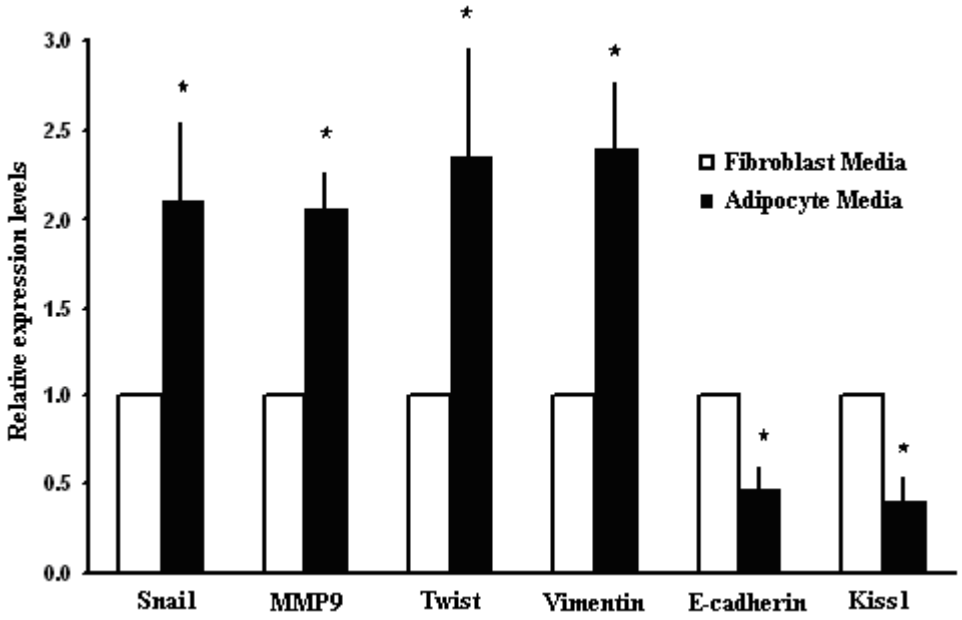

Figure 3.2 Effects of adipocyte media on EMT gene expression in B16BL6 cells. Cells were treated with 5% adipocyte media or 5% control fibroblast media for 24 hrs. Adipocyte media increased Snai1, Matrix Metalloproteinase 9 (MMP9), Twist, and Vimentin mRNA levels. Conversely, adipocyte media decreased the expression of E-cadherin and the metastasis suppressor Kiss1. Relative mRNA fold change was normalized to 18S rRNA and expressed as fold change over untreated controls. The data are representative of at least three separate experiments, presented as ± SEM (* $p<0.05$).

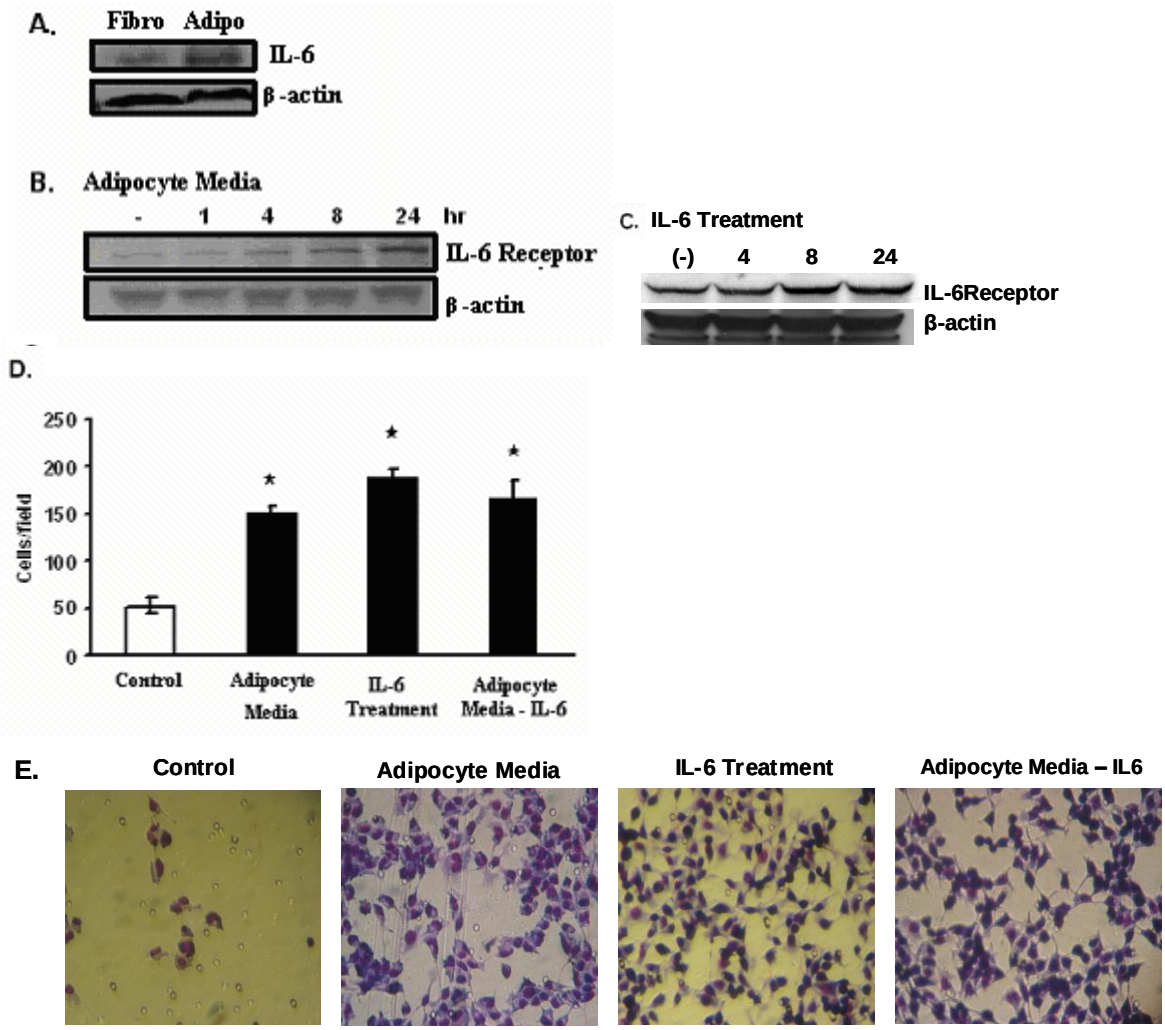

Figure 3.3 IL-6 treatment and IL-6 neutralization on B16BL6 cell invasion. A) IL-6 protein expression was increased in 3T3L1 adipocytes compared to 3T3L1 fibroblasts as observed by Western blot analysis. B) B16BL6 cells were exposed to 5% adipocyte media for the given time period, and IL-6Rα levels were measured by Western blot analysis. C) B16BL6 cells were exposed to 1ng/mL IL-6 for the given time period, and IL-6Rα levels were measured by Western blot analysis. D) B16BL6 cells were exposed to 1ng/mL IL-6, 5% adipocyte media, 5% IL-6 neutralized adipocyte media, or control DMEM for 28 hrs in the invasion assay. The average number of cells that invaded when exposed to each treatment was calculated and quantified. E) Representative pictures of fields used to quantify cell invasion. The data are representative of at least three separate experiments and presented as ± SEM where appropriate (* $p<0.05$).

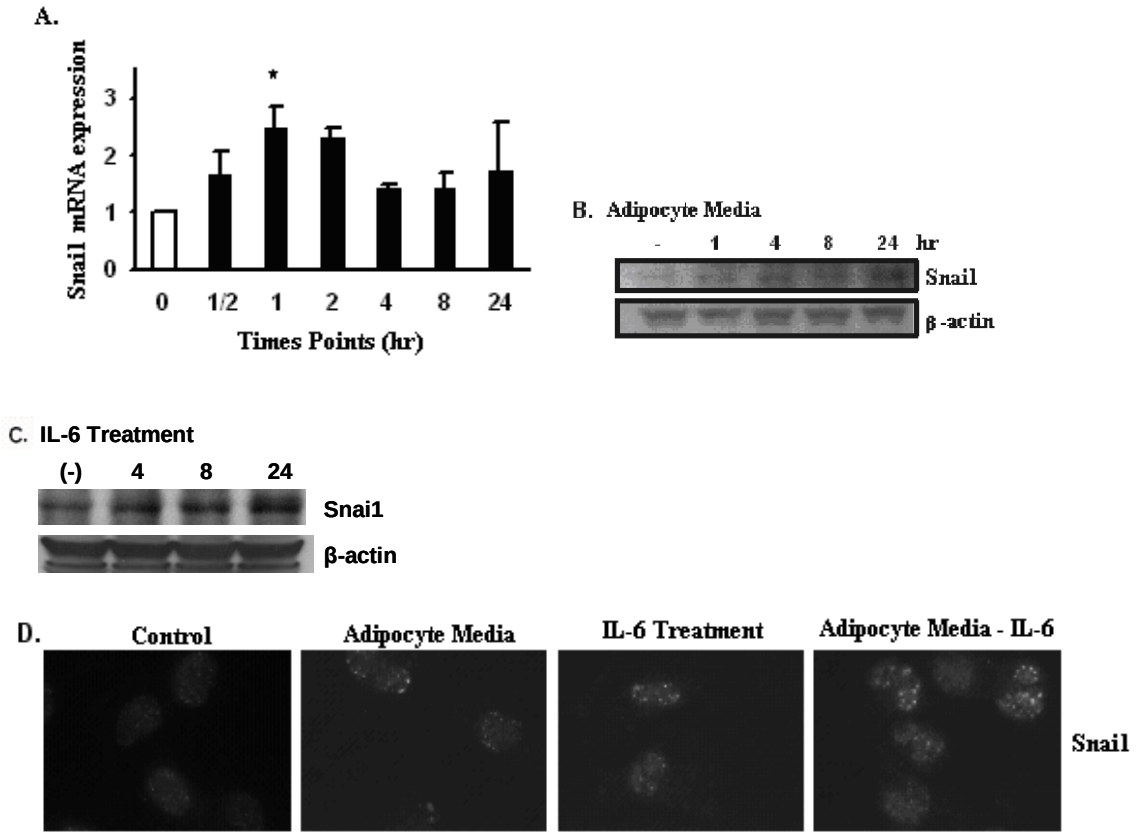

Figure 3.4 The effect of IL-6 on Snai1 expression in B16BL6 cells. B16BL6 cells were treated with 1ng/mL IL-6 for the stated time periods or as indicated. A) mRNA levels of Snai1 were increased at the time points, where the highest and significant change was observed at the 1 hour time point. B) B16BL6 cells were treated with 5% adipocyte media for 24 hrs. Snai1 protein levels were modestly increased in a time dependent manner. C) Similarly, Snai1 protein levels were increased in a time dependent manner after the IL-6 treatment. D) Nuclear staining of Snai1 was increased in B16BL6 cells treated with IL-6, 5% adipocyte media, or 5% IL-6 neutralized adipocyte media compared to control DMEM as observed by Immunofluorescence microscopy. The data is representative of at least three separate experiments and presented as ± SEM where appropriate (* $p < 0.05$).

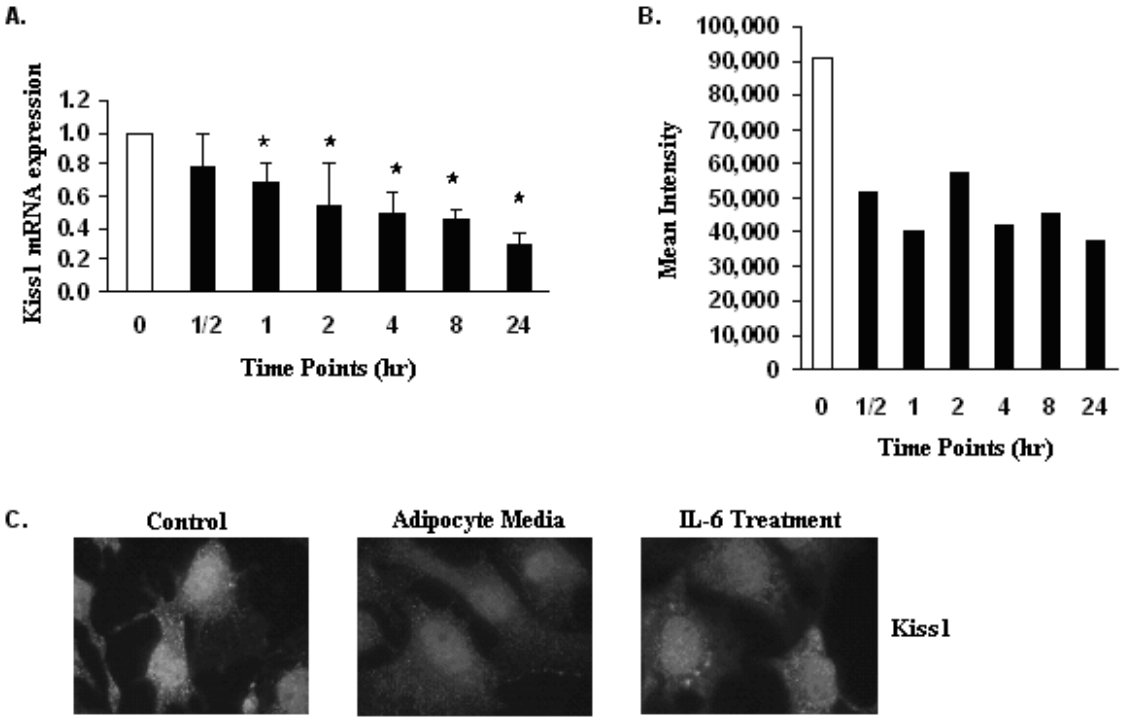

Figure 3.5 IL-6 treatment decreased Kiss1 expression in B16BL6 cells. B16BL6 cells were treated with 1ng/mL IL-6 for the stated time periods or as indicated. A) mRNA levels of Kiss1 were decreased in a time-dependent manner. B) Kiss1 protein expression was decreased after IL-6 treatment as observed by Flow Cytometry. C) Kiss1 expression was decreased in B16BL6 cells treated with 5% adipocyte media and IL-6 for 24 hrs compared to control DMEM treatment as observed by Immunofluorescence microscopy. The data are representative of at least three separate experiments and presented as ± SEM where appropriate (* $p < 0.05$).

CHAPTER 4: OVERALL DISCUSSION AND FUTURE DIRECTIONS

In this thesis, we investigated the effect of obesity on the expression of key genes associated with the Epithelial-to-Mesenchymal Transition (EMT) such as Snai1. We attempted to understand the effect of obesity on melanoma metastasis. Epidemiological studies show that obesity is a risk factor for melanoma (37, 38). Others show that in ob/ob mice, a commonly used genetic model of obesity, B16BL6 experimental metastasis is increased compared to lean control mice (40). We showed that systemic factors in ob/ob mice serum indeed increased the metastatic potential of B16BL6 cells *in vitro*; this increased invasion was accompanied by alterations in the expression profile of EMT markers – Snai1, Twist, MMP9, and metastasis suppressor Kiss1.

Obesity is most often accompanied by increased amounts of adipocytes or fat cells, and the cytokine IL-6 has been shown to be elevated in obese individuals (88-90). In an effort to understand the role of adipocytes and specific obesity-related factors that may be important in obesity-induced melanoma metastasis, we proceeded to investigate the effects of adipocyte-conditioned media and IL-6 on B16BL6 cells. IL-6 is also a serological marker for malignant melanoma (91), and some human melanoma cell lines endogenously produce IL-6 in an autocrine manner (97); these findings point to the possible importance of IL-6 in melanoma metastasis, but our study indicated that IL-6 was not the most important factor in the adipocyte media-induced effects on B16BL6 cells. We showed that both adipocyte-conditioned media and exogenous IL-6 increased B16BL6 cell invasion and altered the expression of EMT markers Snai1, E-cadherin, and Kiss1. However, neutralization of IL-6 in the adipocyte-conditioned media did not inhibit the effects of the adipocyte media on the invasiveness of the melanoma cells. Therefore, though further studies are necessary, this preliminarily suggests that IL-6 may

not be a critical component of adipocyte media-induced cell invasiveness and that other factors are more important. There are a number of factors associated with obesity, including leptin, TGF-β, IL-6, IGF-1, TNF-α, that have been linked to cancer progression and EMT (58, 82, 98, 154, 159, 160); we also showed that Insulin, Resistin, and TNF-α modestly affected the expression of EMT genes such as Snai1, MMP9, and Kiss1. Thus, it is feasible that obesity affects melanoma progression via a combination of multiple factors.

The work in this thesis has not only added further evidence towards the importance of Snai1 and EMT in the melanoma metastatic cascade, but also demonstrated that EMT can be affected by external stimuli such as obesity. It is important to continue to decipher other obesity-factors besides IL-6 that could have a more significant impact on Snai1 expression and EMT. Furthermore, Snai1 function and activity are highly modulated by post-translational modifications such as phosphorylation and ubiquitination (48). Thus, it is possible that obesity affects Snai1 by affecting its phosphorylation and ubiquitination (48). This may be a possible future directions for these studies.

Of interest, our results also showed that Kiss1 was affected by obesity. We showed that obesity decreased Kiss1 expression in B16BL6 cells. Others have shown that plasma Kisspeptin levels negatively correlates with insulin resistance and obesity (161). It would be interesting to determine if increasing systemic levels of Kiss1 may inhibit the metastatic ability of melanomas. Evidence suggests that high levels of Kiss1 in people are feasible and may not lead to detrimental side effects. For example, plasma kisspeptin levels are increased more than 7,000 times in pregnant women compared to non-pregnant women (162). This suggests that it may be physiologically possible to administer large concentrations of Kisspeptin intravenously to cancer patients. Further

investigation, however, is still necessary to determine the effectiveness of exogenous Kiss1 administration and the appropriate concentrations required to affect cancer metastasis.

ACKNOWLEDGMENT

The research in this thesis was supported by the American Cancer Society grant ACS RSG CNE-113707 and by grants from the National Institutes of Health: National Cancer Society grant NCI 1K22CA127519-01A1 and the National Institute of Environmental Health Sciences Center grants ES09145 and ES007784.

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VITA

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