ROLE OF SURVIVIN AND ALPHA 5 INTEGRIN IN MELANOMA CELL MOTILITY AND METASTASIS

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

Survivin is an Inhibitor of Apoptosis Protein (IAP), which is highly expressed in a number of human malignancies. It is a developmentally regulated gene that is expressed during embryogenesis, but silenced in normal adult tissue. Since its discovery in 1997, survivin function has evolved to include regulation of spindle assembly in mitosis. Melanoma arises in epidermal melanocytes, and is the most lethal dermatologic cancer due to its highly metastatic nature. In melanoma, expression of survivin has been heavily correlated to advanced disease stages and poor patient prognosis. Targeting of survivin in melanoma cells leads to induction of apoptosis *in vitro* and reduction of tumor growth *in vivo*.

While the anti-apoptotic and mitotic roles of survivin have been extensively studied, the work documented in this dissertation describes a novel basis for survivin function in melanoma metastasis. Chapter 2 details a study which showed that survivin increases melanoma cell migration and invasion via Akt-dependent upregulation of α_5 integrin. I also demonstrate that overexpression of survivin increases melanoma colony formation in soft agar, and this effect could be abrogated by knockdown of α_5 integrin by RNA interference. These findings demonstrate that survivin can enhance cellular functions which are critical to the metastatic process in melanoma. To further investigate the functional role of survivin and α_5 integrin in melanoma metastasis, I used a

xenograft mouse model to determine the *in vivo* effect of survivin overexpression and subsequent upregulation of α_5 integrin on melanoma metastasis (Chapter 3). The studies show that while survivin overexpression does not result in increased tumor proliferation or decreased apoptosis, it does cause increased metastasis of melanoma cells *in vivo* and this requires upregulation of α_5 integrin. Taken together, this dissertation presents data to support an additional role for survivin function in melanoma metastasis which is α_5 integrin-dependent.

In summary (Chapter 4), I will discuss future research avenues looking mechanistically at how survivin upregulates α_5 integrin and mediates melanoma metastasis. Additionally, I will also discuss therapeutic targeting of survivin and α_5 integrin in melanoma and how the knowledge gained in this study may contribute to such efforts.

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CHAPTER 1

INTRODUCTION

Biology of human melanocytes

The skin is the largest organ in the body, and is the body's direct barrier with the physical environment. As such, the skin has a myriad of functions including protecting the body from physical, chemical or microbial insults.[1] The skin serves to regulate body temperature; shield the body from most solar ultraviolet radiation (UVR); and modulate immune responses to pathogens coming in contact with the body.[1] Skin is stratified into the epidermis, which is the external epithelial layer of skin; the dermis, a thicker and more complex layer below the epidermis; and the hypodermis, which is a layer of fatty tissue connecting the dermis to underlying skeletal components.[1] Approximately 95% of the cells found in the epidermis are keratin-containing cells known as keratinocytes (Figure 1.1).[2] The epidermis also contains two other cell types: Langerhans cells and Merkel cells, which are involved in immune response and mechanosensation respectively.[3, 4]

Melanocytes are specialized dendritic cells, which can be found in the *Stratum basale* layer of the epidermis of skin (Figure 1.1). Melanocytes produce the biochemically stable pigment melanin in lysosome-related organelles known as melanosomes,[5] which are then transferred to overlying keratinocytes through cell-to-cell contact.[6] Melanocytes and progenitor keratinocytes, found in the deeper layers of the epidermis proliferate at a slow rate. However, keratinocytes in the upper epidermis proliferate rapidly, and are eventually shed through desquamation.[6] Melanocytes also occur in other areas of the body such as: hair follicles, the inner ear, and the eye.[6] They interact with neighboring cells in these organs to mediate functions distinct from those of epidermal melanocytes.[7, 8]

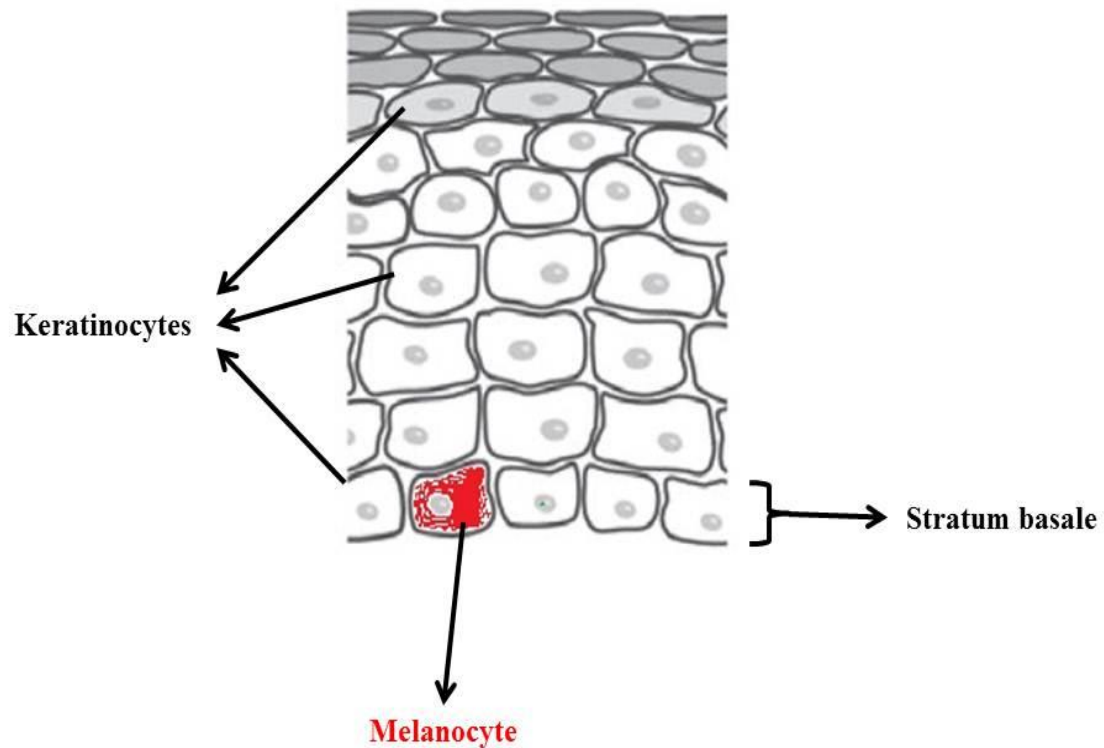

Figure 1.1 Structure of the epidermis. Melanocytes and progenitor keratinocytes can be found in the *Stratum basale* layer of the epidermis. As the major cell type in the epidermis, melanin-containing keratinocytes also occupy the upper layers of the epidermis.[1]

[1] Modified from: Raj D, Brash DE, and Grossman D. Keratinocyte Apoptosis in Epidermanl Development and Disease. *Journal of Investigative Dermatology*.2006; 126:243:257.

Melanoblasts are melanocyte precursor cells and are derived from the neural crest.[1] They develop starting in the second month of human embryogenesis and migrate through the mesenchyme as the embryo develops. By the 18th week of embryonic development, melanoblasts arrive in the epidermal layer of the skin.[1] Successful deposition, development and function of melanoblasts in the epidermis are dependent on a cohort of at least 25 genes including Kit; mutations in which lead to developmental pigmentary diseases such as piebaldism.[6, 9]

Through regulation of pigment production, melanocytes are able to carry out their photoprotective function in which melanin absorbs and refracts harmful UVR.[1] Consequently, individuals with lightly pigmented skin have a 15 - to 70 - fold increased risk of skin cancers over individuals with darkly pigmented skin, primarily due to the damaging effects of UVR.[10] There have been a number of studies which propose that melanocytes are not simply pigment producing cells. It has been shown that melanocytes produce a range of signaling molecules such as cytokines,[11] pro-opiomelanocortin (POMC) peptides,[12] catecholamines[13] and also nitric oxide in response to UVR.[14] Receptors for these signaling cues can be found on skin cells such as keratinocytes, and fibroblasts; making them potential targets for these secretory products.[15] This implies that melanocytes may act as important local regulators of a range of skin cells.

Malignant melanoma

Melanoma is a dermatologic cancer that develops in response to genetic or environmental insults in melanocytes.[16] Malignant melanoma is a significant and growing public health burden in the United States (US). In 2010, an estimated 68 000 cases were diagnosed, with roughly 9 000 deaths.[17] Melanoma incidence in the US has increased

exponentially since the 1930s.[18] It is estimated that by 2015 the lifetime risk of invasive melanoma in the US will be 1 in 50, up from 1 in 1500 in the 1930s.[18]

Fortunately, there has been steady improvement in the 5-year survival rate for localized melanoma, increasing from 82% in 1979 to 92% in 2002.[17] This is primarily because localized, nonmetastatic melanoma is curative by surgical excision. This favorable survival rate has also been aided by an increase in early detection, which in turn leads to decreased healthcare cost. It is estimated that 90% of the cost of melanoma therapy in the US is from treatment of patients with metastatic disease.[19] Patients with metastatic disease are also plagued by a poor 5-year survival rate (< 10%).[20] This again highlights the importance of early detection of melanoma.

The most common genetic risk factors for melanoma are associated with pigmentation phenotypes, such as fair skin, blond or red hair and blue eyes, as well as multiple melanocytic nevi (moles).[21] Most melanoma cases occur sporadically, with familial melanoma accounting for less than 10% of cases.[22] *Cyclin-dependent kinase inhibitor 2A* (CDKN2A), which encodes both *p16* and $p19^{ARF}$ tumor suppressor genes, has been identified as the most commonly altered gene in familial melanoma.[22] The predominant risk factor for melanoma is exposure to the DNA-damaging effects of UVR, from excessive sun exposure or from tanning beds.[18]

Developed by Drs Clark, Elder, and Guerry, the Clark Model for melanoma progression describes the histopathologic changes that may accompany the transformation from nevi (moles) to malignant and metastatic melanoma.[23] In the initial stages, melanocytic nevi become dysplastic and progress to a radial growth phase (RGP) where tumor cells are able to rapidly proliferate and spread laterally in the epidermis

(Figure 1.2). Lesions progress to an aggressive vertical growth phase (VGP), in which tumor cells invade the dermis and lead to the metastatic spread of the disease.[23] The Clarke Model is a linear progression representation of melanomagenesis. However, it must be noted that this model is not typical of how melanoma usually develops. Melanoma most often arises in melanocytes *de novo* in response to UV-induced DNA-damage.[24]

The phenotypic changes documented in the Clarke Model are representative of the underlying genetic and proteomic changes that occur to facilitate the transitions from dysplastic nevi to metastatic melanoma (Figure 1.2).[23] More than 70% of melanoma cases have been found to harbor activating mutations in key molecules like BRAF, NRAS and c-Kit.[25] These molecules are key intermediaries in signaling pathways that are initiated at the cell surface in response to growth factor or other external stimuli activation of transmembrane receptors. In response, cells are directed to proliferate, differentiate, senesce, move or die.

Common genetic alterations in signaling pathways in melanoma

In melanoma, the RAS/RAF/MEK/ERK (MAPK) and the PI3K/PTEN/AKT (AKT) networks are two major signaling pathways that are constitutively activated through genetic alterations.[26] Mutations affecting RAF, RAS and PTEN result in deregulation of apoptosis, proliferation, angiogenesis and invasion (Figure 1.3);[26] which is essential for cancer metastasis (Figure 1.4).[27] Metastasis is a complex set of processes that involves tumor cell detachment and invasion from the primary tumor, tumor cell transport and survival in the circulatory system, arrest in and extravasation from the vasculature, culminating in proliferation and outgrowth in distant sites (Figure 1.5).[28]

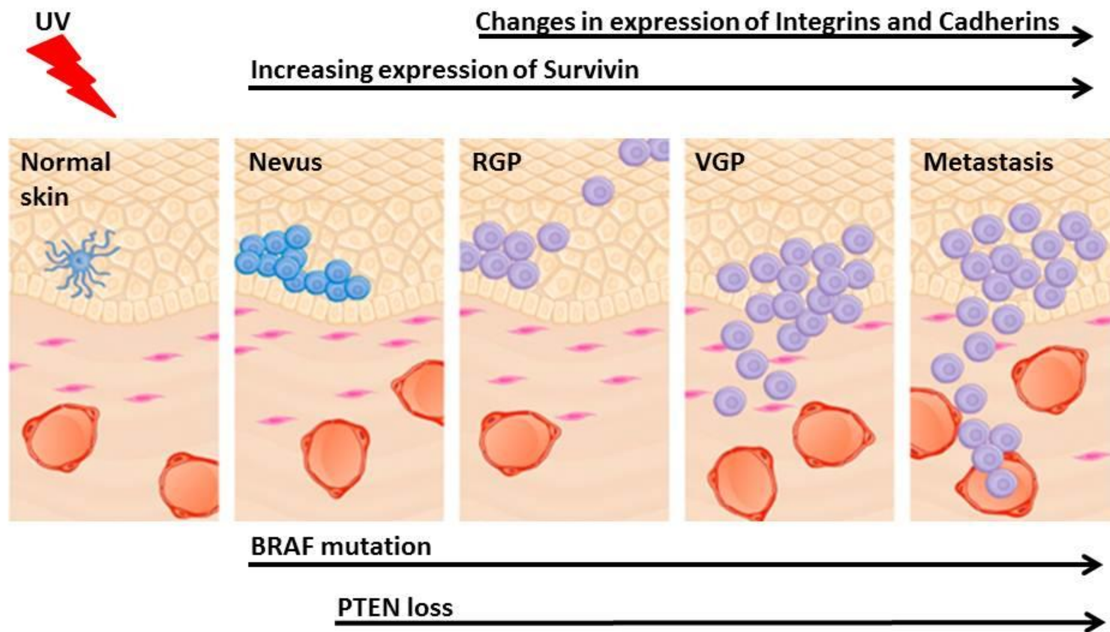

Figure 1.2 Molecular events in melanoma progression. Genetic and proteomic changes aid in the progression of melanoma from the initial stages of the disease (dysplatic nevus) to metastatic melanoma. BRAF mutations and consequent overactivation of the MAPK pathway is evident throughout disease progression. PTEN loss also helps tumor progression by increasing activity of the PI3K/AKT pathway. The vertical growth phase and metastatic melanoma are characterized by changes in cell adhesion receptor proteins like integrins and cadherins. These transmembrane proteins help the cells to create and remodel the extracellular matrix to help tumor cells progress. The expression of survivin increases as the disease progresses to aid in apoptotic resistance and metastasis.[2]

[2] Modified from: Zaidi MR, Day CP, and Merlino G. From UVs to Metastases: Modeling Melanoma Initiation and Progression in the Mouse. *Journal of Investigative Dermatology.* 2008; 128:2381-2391.

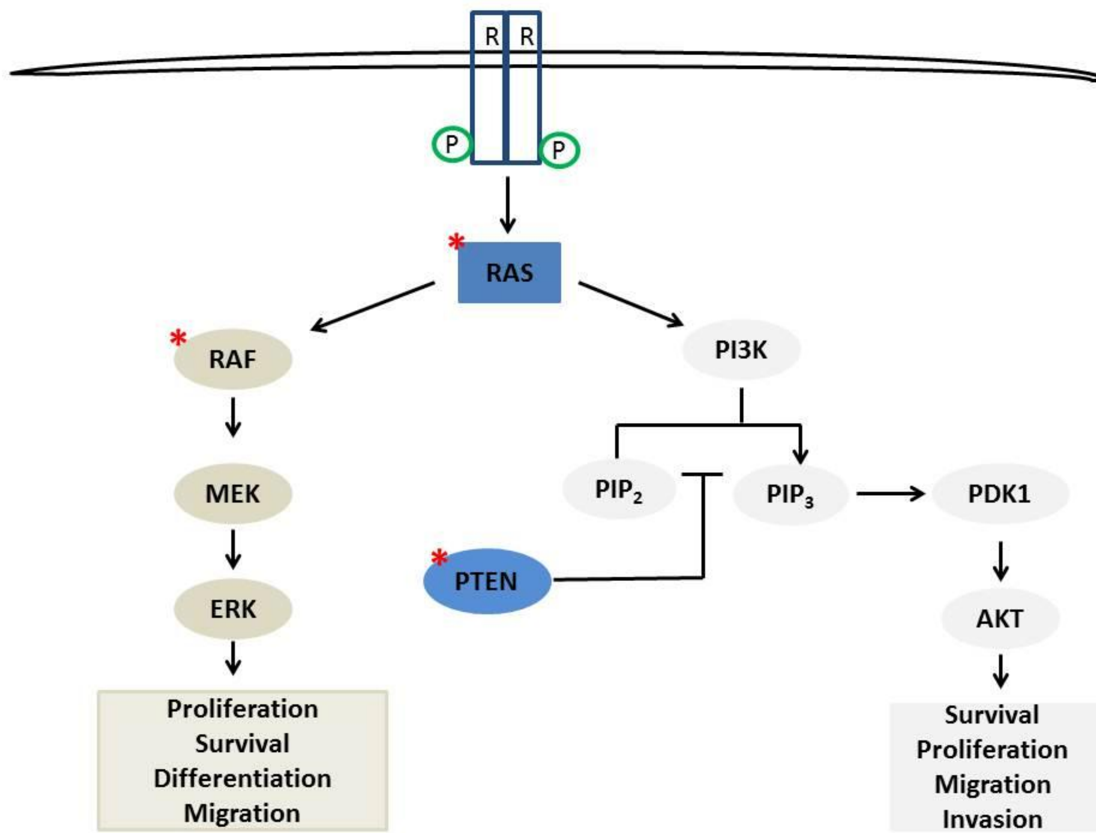

Figure 1.3 The MAPK and PI3K/AKT signaling pathways. These are two major signaling pathways that control various cellular functions. Activation of cell surface receptors (R) results in phosphorylation (P) and activation of RAS. In turn, RAS can activate the MAPK cascade through phosphorylation of RAF. RAS also activates the PI3K/AKT pathway through direct binding to PI3K. The AKT pathway may be attenuated by PTEN inhibition of the conversion of Phosphatidylinositol di-Phosphate (PIP2) to Phosphatidylinositol tri-Phosphate (PIP3). Genetic or epigenetic alterations in signaling components (*) allow for tumorigenesis and metastasis.

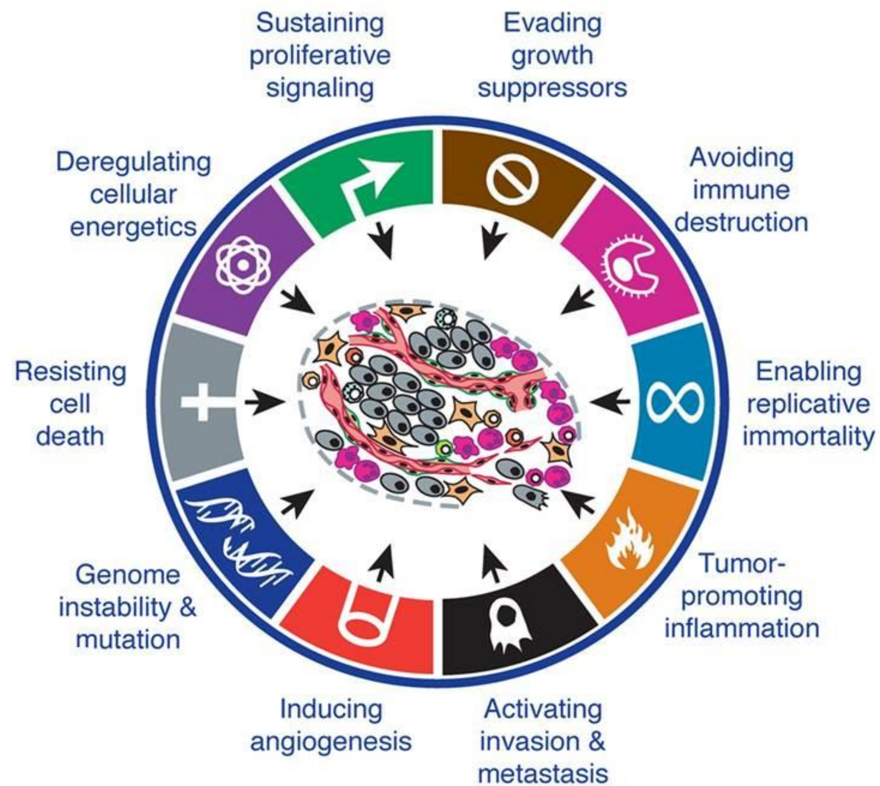

Figure 1.4 The hallmarks of cancer. Through deregulation of physiologic processes used by the cell to maintain homeostasis, tumor cells acquire functionalities that facilitate tumor progression and metastasis.[3]

[3] Modified from: Hanahan D, and Weinberg RA. Hallmarks of Cancer: The Next Generation. *Cell*. 2011; 144:646-674.

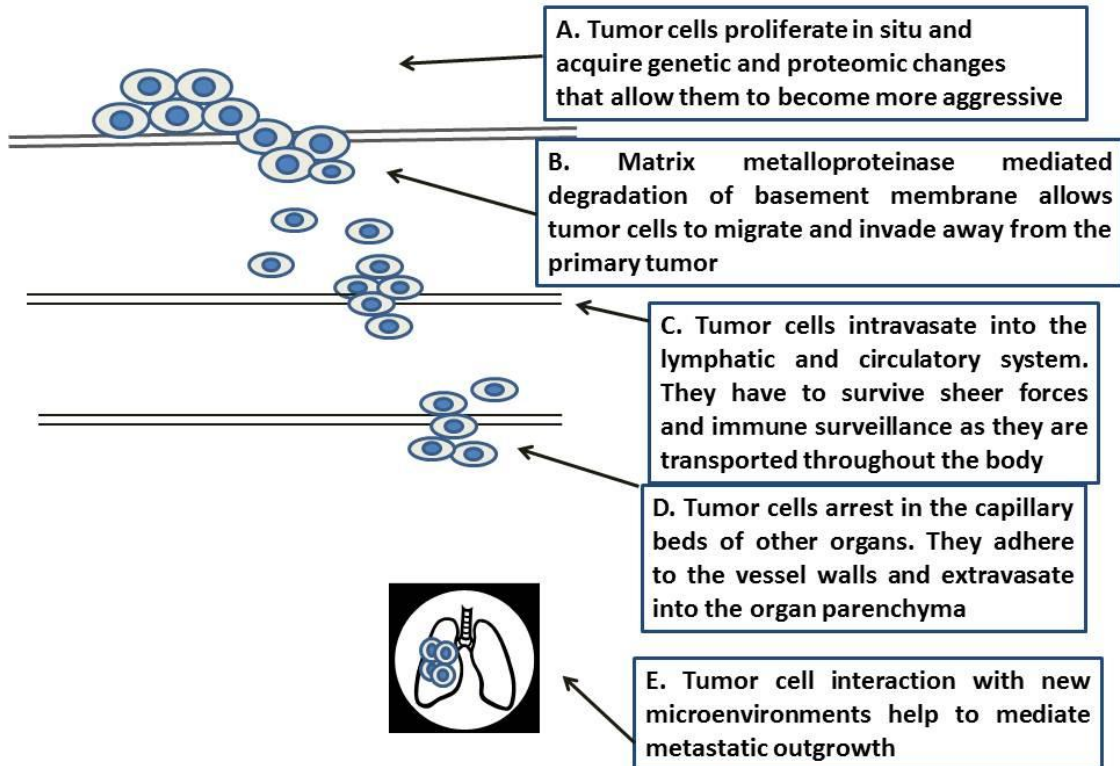

Figure 1.5 The sequential process of metastasis. Metastasis is a complex network of processes that facilitate the spread of tumor cells from the primary site. Tumor cells become detached from the primary tumor and invade through the basement membrane. Tumor cells are disseminated through the circulatory or lymphatic system. Tumor cells that survive this transport can then arrest in other organs where they undergo extravasation. Subsequently, tumor cells interact with the microenvironment to help establish new tumor colonies.

There is a significant amount of crosstalk that occurs between the MAPK and PI3K/AKT signaling pathways. RAS is a focal point in these pathways and can enhance cell proliferation through regulation of MAPK; whereas it can elicit an anti-apoptotic response through regulation of PI3K (Figure 1.3).[29] The RAS family of proto-oncogenes consists of: HRAS, KRAS, and NRAS.[30] NRAS is the most commonly mutated isoform and is present in approximately 20% of all melanoma cases.[31]

One downstream effector of RAS is RAF, which is a component of one arm of the Mitogen Activated Protein Kinase (MAPK) signaling network.[32] Through sequential phosphorylation events, MAPK signaling components are activated and regulate cell proliferation, differentiation, migration and apoptosis.[33] The RAF kinase family consists of three isoforms: ARAF, BRAF, and CRAF.[34] RAFs are serine/threonine phosphotransferases that initiate the MAPK pathway through phosphorylation of MEK. In turn, MEK phosphorylates and activates ERK (Figure 1.3).[35] ERK targets include the transcription factor Elk-1,[36] cytoskeletal proteins such as Paxillin,[37] and other kinases like Ribosomal S6 Kinase,[38] which help to execute various cellular functions. It is therefore understandable how mutations in MAPK components are advantageous to tumor cells.

BRAF mutations are found in approximately 60% of all melanoma cases and the majority of BRAF mutations in melanoma is a missense mutation that switches Valine to Glutamic Acid at codon 600 (V600E).[35] The $BRAF^{V600E}$ mutation has been detected in benign nevi, primary, and metastatic melanomas,[39] indicating that it may be a driver of early events in melanomagenesis (Figure 1.3). However, dysplastic nevi may remain dormant for extended periods of time before progressing to more advanced melanoma, suggesting that a second genetic ‘hit,’ such as loss of a tumor suppressor (ex. *p53*) is

required for BRAF mutations to facilitate tumor progression. Using a zebrafish model, studies have shown that melanocyte-specific expression of BRAF^{V600E} can induce hyperproliferation of melanocytes, akin to dysplastic nevi in humans.[40] However, combination of *p53* loss with BRAF^{V600E} was required for frank melanoma.[40]

Another commonly deregulated signaling pathway in malignant melanoma is the PI3K/PTEN/AKT pathway. Like the MAPK network, PI3K/AKT also modulates cell proliferation and survival.[41] The PI3K pathway can be activated in response to cell surface receptor (ex. Receptor Tyrosine Kinase; RTK) activation, or from direct binding of RAS to PI3K.[30] Through generation of the second messenger molecule phosphatidylinositol tri-phosphate (PIP_3), AKT is phosphorylated and hence activated (Figure 1.3).[30] Activated AKT is an important downstream effector of PI3K, which regulates the balance between cell survival and cell death.[42]

The PI3K pathway is negatively regulated by the Phosphatase and Tensin homolog (PTEN) gene.[22] PTEN dephosphorylates PIP_3, directly opposing PI3K function and thereby inhibiting phosphorylation of AKT (Figure 1.3).[30] As PI3K itself is rarely mutated in melanoma,[43] loss of PTEN is one mechanism by which PI3K/AKT signaling is constitutively activated in melanoma. PTEN loss through gene deletion or epigenetic silencing (via promoter DNA methylation) has been detected in 10% to 30% of cutaneous melanomas.[44] Mutations in RAS can also lead to ectopic activation of PI3K/AKT pathway in melanoma.[30]

Activated AKT is detected in approximately 67% of melanoma cases,[45] and its expression is inversely correlated to patient prognosis.[46] Additionally, overexpression of activated AKT in melanoma cells causes transition from the early stage RGP to the more

aggressive VGP form *in vivo*.[46] In addition to regulating apoptosis, activated AKT has also been shown to mediate melanoma cell migration and invasion.[47, 48] These signaling networks have been demonstrated to be essential for melanoma tumor growth and metastasis. The alterations in the MAPK and PI3K/AKT pathways have opened the door to the development of targeted therapies in melanoma. The next section is a discussion on therapeutic approaches that include targeting of these signaling pathways.

Therapeutic options for malignant melanoma

Until 2011, Dacarbazine (DTIC), high-dose interleukin-2 (IL-2), and hydroxyurea were the only Food and Drug Administration (FDA) approved therapies for metastatic melanoma.[25] However, none of these treatment modalities have had significant success in curtailing metastatic melanoma. DTIC is an alkylating chemotherapeutic agent which induces cell death. Low levels of efficacy and a lack of data to support a survival benefit means that DTIC has a minimal effect in patients with metastatic melanoma. High-dose IL-2 treatments are based on augmenting the numbers and activity of natural killer T-cells. However, this form of immunotherapy has only proven effective for a small percentage of patients. Pretreatment prediction of IL-2 efficacy is unknown and the high risk for multi-organ failure makes this treatment option less than ideal.

However, 2011 proved to be an historic year for the treatment of metastatic melanoma with two different drugs gaining FDA approval. Vemurafenib (Zelboraf) is a RAF kinase inhibitor which targets the $BRAF^{V600E}$ mutant prevalent in melanoma.[49] This targeted therapy has shown efficacy and was the first drug ever approved which showed significant improvement in overall survival in metastatic melanoma.[25] Vemurafenib acts by inhibiting phosphorylation of MEK and ERK, leading to G1 phase cell-cycle arrest

and apoptosis.[50, 51] While early clinical trials have demonstrated in excess of an 80% response rate among patients with V600E-positive melanomas, patient relapse is quite frequent due to acquired resistance to BRAF inhibition.[49]

Tumor cells are able to develop resistance to BRAF inhibition through a number of mechanisms which are dependent or independent of RAF.[52] RAF dependent mechanisms of resistance include: (i) upregulation of NRAS[53] or mutations in KRAS,[54] which causes reactivation of MEK/ERK signaling; or (ii) paradoxical activation of the MAPK pathway, where blocking mutated BRAF results in CRAF-mediated activation of MEK.[55] Resistance can also be acquired through bypass of RAF signaling. COT/TPL2, is a kinase which functions upstream of MEK and has been shown to activate ERK through MEK-dependent mechanisms that do not require RAF signaling.[56] Clinical trials are now underway to develop strategies to overcome resistance to RAF inhibition via combination of RAF and MEK inhibitors.[25]

The second drug approved, Ipilimumab (Yervoy), is an anti-Cytotoxic T-Lymphocyte associated Antigen 4 (α-CTLA-4) monoclonal antibody.[57] This drug elicits a therapeutic response through hyperactivation of natural killer T-cells in the immune system.[57] However, adverse immunologic side effects and limited efficacy have hampered Ipilimumab.[58] Less than 10% of patients with metastatic melanoma had complete or partial responses to this immunologic drug. However, patients that responded favorably had long-lasting disease control or remissions.[57]

The shortcomings in viable therapeutic options for metastatic melanoma highlight two key facts regarding this public health issue: (i) Early detection is critical in curtailing melanoma morbidity and mortality and (ii) A better understanding of the molecular

events regulating melanoma progression is fundamental for the development of more effective therapies for metastatic melanoma. The next section will focus on the molecular biology and function of Survivin, a protein which has been found to play a critical role in melanoma biology.

Biology of survivin: structure and subcellular localization[4]

First cloned in 1997, survivin is the smallest member of the inhibitor of apoptosis protein (IAP) family.[59] The survivin gene spans approximately 14.7 kb on chromosome 17, and its transcription is initiated from a TATA-less, GC-rich promoter. Transcription generates full-length (wild-type) survivin, along with four other splice variants.[60] Unlike other members of the IAP family such as X-linked Inhibitor of Apoptosis Protein (XIAP), and Cytosolic Inhibitor of Apoptosis Protein 1 (cIAP1), survivin has a single Baculoviral IAP Repeat (BIR) domain and also lacks the Really Interesting New Gene (RING) finger domain characteristic of other IAPs (Figure 1.6).[61] Additionally, survivin lacks a Caspase-Associated Recruiting Domain (CARD), which is critical for other IAP family members to bind and inactivate caspases, the effectors of apoptosis (Figure 1.6).[62] Survivin is also distinguished from other IAPs because of its bi-functionality as a regulator of both apoptosis and mitosis.[63]

Expression of survivin is developmentally regulated as it is ubiquitously expressed in fetal tissues, silent in most adult tissues, and then re-expressed in most cancers.[64] More recent studies using sensitive detection methods have demonstrated survivin expression in some normal adult tissues. These include vascular endothelial

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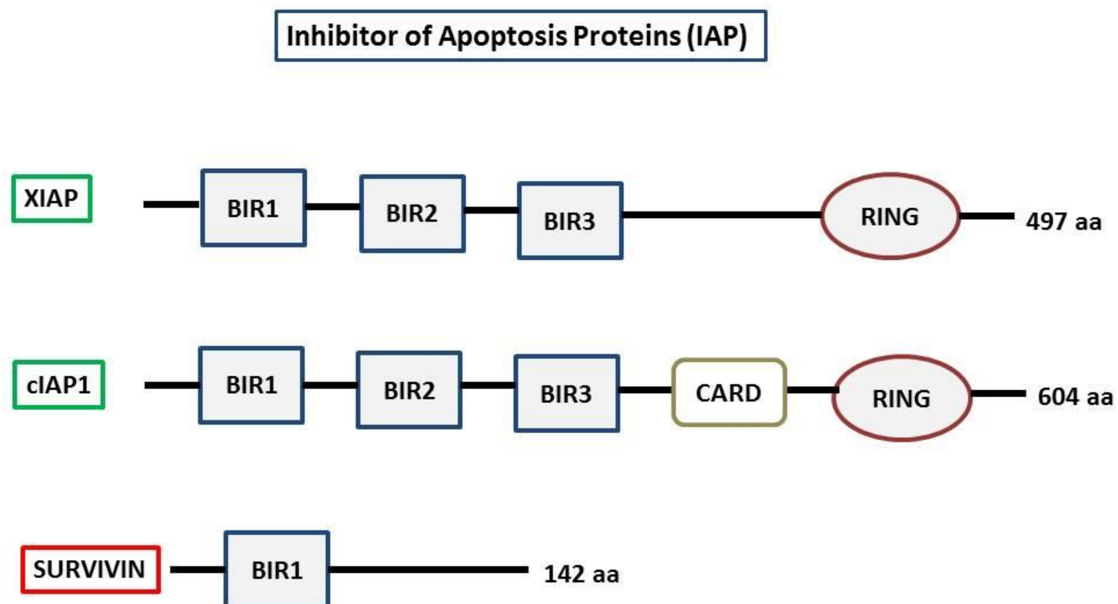

Figure 1.6 Structure of inhibitor of apoptosis proteins (IAP). Inhibitor of apoptosis proteins are classified based on the presence of the Baculoviral IAP Repeat (BIR) domain. IAPs like x-linked inhibitor of apoptosis protein (XIAP) and cytosolic inhibitor of apoptosis protein 1 (cIAP1) are much larger proteins at 497 and 604 amino acids (aa), respectively. They both contain the Really Interesting New Gene (RING) motif which is a zinc finger domain that regulates ubiquitination. cIAP1 also contains a Caspase Activating and Recruiting Domain (CARD) to mediate binding and activation of caspases. With only 142 amino acids, survivin is a smaller and unique member of the family as it contains only a single BIR domain.

cells,[65] and developing hematopoietic and immune cells.[66-68] Although there are five distinct isoforms of survivin, the predominant and most studied is the wild-type full length survivin. There has been speculation that the varied cellular functions may be attributed to different splice variants but this has been difficult to determine given the low level of expression of the minority variants.[69]

There are distinct subcellular pools of survivin located in the cytosol, mitochondria, and nucleus.[70, 71] These subcellular pools are believed to be strongly tied to its varying cellular functions. Studies have demonstrated that the nuclear pool mediates survivin function in mitosis, while the cytosolic and mitochondrial fractions are responsible for its anti-apoptotic function.[71-73] Survivin shuttles from the nucleus to the cytoplasm under the control of an evolutionarily conserved Crm1 nuclear export signal (NES). When this NES is mutated, survivin is trapped in the nucleus, proper cell division does not occur and the anti-apoptotic function of survivin is also lost.[74]

In tumor cells, survivin localizes to the inner mitochondrial membrane.[71] In response to apoptotic stimuli, survivin is trafficked from the mitochondria to the cytosol where it can inhibit apoptosis.[71] It has been shown that phosphorylation of survivin on residue Ser20 by Protein Kinase A occurs in the cytosol but not in the mitochondria, and this differential phosphorylation regulates tumor cell apoptosis by modulating the interaction of survivin with x-linked inhibitor of apoptosis protein (XIAP).[75]

Regulation of Survivin expression and posttranslational modifications[5]

Numerous studies have identified various genetic elements which exert transcriptional and translational control over survivin expression. Basal survivin gene expression is mediated through binding of SP1 to the GC rich region of the survivin promoter.[76] A role for p53 in suppressing survivin expression has been shown,[77] and our lab's more recent work has demonstrated that knockdown of either p53 or Rb protein in melanocytes is sufficient for survivin induction.[78] Although E2F2 has been previously characterized as a positive regulator of transcription, our lab identified a novel functional E2F2-binding site in the survivin promoter and found that E2F2 acts downstream of Rb to function as a negative regulator of survivin.[78] It was also found that mutation of either the p53- or E2F2-binding sites in the survivin promoter increased transcription.[78] These findings suggest that perturbations in the p53 or Rb pathways, which may occur as a result of *INK4A* loss in melanoma,[79] can result in upregulation of survivin expression.

There may also be a role for other tumor suppressors in the regulation of survivin expression in melanoma. Caveolin-1 inhibits survivin gene transcription by preventing TCF/LEF promoter binding in an E-cadherin-dependent manner, and this mechanism is operative in mouse melanoma cells leading to increased apoptosis.[80] Thus loss of E-cadherin expression, as frequently occurs in melanoma, may result in reduced activity of Caveolin-1 and consequent upregulated survivin expression. Epigenetic modifications may also be important in regulating survivin expression, as histone deacetylation can

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direct methylation and silencing of the survivin promoter,[81] although this pathway has not been investigated in melanoma.

Several posttranslational modifications have been described which regulate survivin stability and function, namely phosphorylation and ubiquitination. Cyclin-dependent Kinase 1-mediated phosphorylation of Thr34 is vital for survivin anti-apoptotic function,[82] and our lab has shown that a nonphosphorylatable Thr34Ala mutant blocks growth of human melanoma tumor xenografts.[83,84] Survivin is also phosphorylated at Ser20 by Polo-like Kinase 1[85] and at Thr117 by Aurora B Kinase;[86] while these modifications are important for mitotic regulation by survivin, their role in melanoma has not been investigated.

Survivin is highly expressed in the G2M phase of the cell cycle, and degraded via the ubiquitin-proteasome pathway during G1.[87] In various cell types, EGF and Cyclooxygenase-2 signaling inhibit its ubiquitin-mediated degradation, leading to increased apoptotic resistance.[88, 89] On the other hand, X-linked Inhibitor of Apoptosis (XIAP)-Associated Factor 1 (XAF1) is a putative tumor suppressor that can reverse the anti-apoptotic activities of survivin by targeting it for ubiquitination.[90] Finally, interaction of survivin with the chaperone protein Hsp90 has been shown to increase survivin stability and threshold for apoptotic stress in cancer cells.[87]

Survivin expression as a prognostic factor in melanoma[6]

The expression of survivin at each stage of melanomagenesis has been characterized. Survivin is absent in normal melanocytes, but our lab has shown it to be

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expressed in a broad spectrum of human melanomas (including localized and metastatic) and melanocytic nevi including dysplastic, neurotized intradermal, and Spitz nevi.[91, 92] In dysplastic nevi, survivin is primarily localized to the cytosol, although some nevi may show nuclear survivin.[93] A more recent study found positive nuclear immunoreactivity for survivin in a large subset of melanomas but much less frequently in common and dysplastic nevi.[94] In addition, nuclear survivin immunoreactivity was significantly less common in acral versus other melanoma types, in which nuclear survivin staining significantly correlated with poor survival.[94, 95]

Survivin expression is also induced upon melanocyte transformation or in response to disruption of the tumor suppressor genes *TP53* (p53) or *Retinoblastoma* (Rb).[78] Molecular profiling has identified survivin as a marker of poor prognosis and as an indicator of treatment resistance.[63] Additionally, several studies have indicated that survivin can serve as a biomarker for a number of malignancies.[96-98] With respect to melanoma, survivin expression in sentinel lymph nodes of melanoma patients is inversely correlated with progression and mortality, as 61.5% of stage III patients who exhibited survivin-positive sentinel nodes (by RT-PCR) died from their disease.[99] On the other hand, all melanoma patients with survivin-negative sentinel nodes were still alive after the 5-year observation period.[99] Survivin levels may also be predictive of treatment outcome in melanoma, as one study reported that in patients with recurrent metastatic melanoma, lower levels of survivin are associated with significantly improved survival in patients who receive postoperative adjuvant immunotherapy.[100]

Survivin function in cell division[7]

Disruption of survivin function, at least in malignant cells, results in cell cycle defects including multipolar mitotic spindles, failure of cytokinesis, and formation of multinucleated cells.[101] Expression of a dominant-negative survivin mutant in melanoma cells results in loss of G2/M DNA content and in reduced proliferation *in vitro* and *in vivo*.[83, 91] Survivin is a chromosomal passenger protein that interacts with other passenger proteins including Aurora B Kinase and Inner Centromere Protein (INCENP) to facilitate movement of the chromosomal passenger protein complex from the inner centromere during prometaphase to the midbody during cytokinesis.[102, 103] Survivin is also involved in microtubule spindle assembly and organization.[104] It is plausible that survivin may help tumor cells that have sustained DNA damage to bypass cell cycle checkpoints and proceed with cell division.

Survivin function in apoptosis[8]

Consistent with its unique protein structure compared to other IAPs, survivin exerts its anti-apoptotic function in a different manner. While conventional IAPs such as XIAP, Livin, and cIAP1/cIAP2 directly bind to pro-caspases, ubiquitinate them, or prevent their proteolytic cleavage and subsequent activation,[105] survivin does not appear to directly bind to caspases. Rather, survivin exerts anti-apoptotic control by binding to and stabilizing XIAP, which inhibits caspase-9.[106] Survivin-mediated inhibition of caspase-9 has also been shown to be dependent on binding to a co-factor Hepatitis B X-

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interacting Protein (HBXIP)[107]. The anti-apoptotic function of survivin is negatively regulated by release of SMAC/DIABLO from mitochondria, upon triggering the intrinsic cell death pathway.[108]

Our laboratory has characterized the anti-apoptotic role of survivin in normal human melanocytes and in human melanoma cells. Forced expression of survivin blocks both caspase-dependent and -independent cell death in human melanocytes.[109] In melanoma cells, our lab has demonstrated that survivin can protect against caspase-independent apoptosis.[110] Numerous studies have demonstrated the pro-apoptotic activity of dominant-negative survivin mutants in melanoma cells which increase the sensitivity to cytotoxic drugs *in vitro*,[91] and reduce melanoma tumor growth *in vivo*.[83] Using a mouse model with melanocyte-specific expression of survivin, our lab also demonstrated that survivin not only confers protection against apoptosis, but also promotes the development of UV-induced melanoma and tumor metastasis to lymph nodes.[111]

A novel role for survivin: promotion of cellular motility[9]

Cell motility encompasses both migration and invasion, which are key aspects of the metastatic process.[27] Both migration and invasion require signaling events within the cell and with the extracellular matrix for navigation of tumor cells within their microenvironment and to distant sites. Recent studies have implicated survivin in these processes, which may underlie its role in promoting cancer metastasis. Mehrotra et al., using the breast adenocarcinoma line MDA-MB-231, showed that survivin co-operatively binds XIAP and mediates both cell invasion as well as metastasis *in vivo*, independent of

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its anti-apoptotic function.[107] Survivin-mediated invasion was integrin-independent and required activation of NF-κB and cell motility kinases FAK and Src.[107]

My work has shown that survivin enhances cell migration and invasion of human melanoma cells.[48] In our system, survivin promotion of motility was found to be dependent on activation of the Akt signaling pathway and upregulation of α5 integrin (which both occur following survivin expression in melanocytes or its over-expression in melanoma cells), as blocking either of these molecules abrogated survivin-enhanced migration.[48] Knockdown of survivin by RNAi, under conditions where apoptosis was not induced, further demonstrated that survivin is required for constitutive migration and invasion of melanoma cells.[48] An additional finding was that survivin-mediated promotion of melanoma cell invasion is also dependent on activation of the MAPK pathway, as evidenced by blocking with inhibitors of Erk phosphorylation.[48] By contrast, over-expression of survivin is not consistently associated with Akt activation or α5 integrin upregulation in other (nonmelanoma) cell lines (J. McKenzie, T. Liu and D. Grossman, unpublished).

Survivin enhanced cell motility and invasion are dependent on the activation and function of cell adhesion receptors known as Integrins.[108] The following subsection will describe these cells surface receptor proteins and their impact in cancer development and metastasis.

Biology of integrins

Integrins are a family of cell adhesion proteins consisting of α and β subunits.[108] They are cell surface transmembrane receptors that recognize and bind to extracellular matrix (ECM) proteins like fibronectin, laminin, and vitronectin.[109] There are eighteen α

subunits and eight β subunits, which heterodimerize in twenty-four different combinations to enable ligand specificity and cell-type specific expression patterns.[110, 111] Integrin activation is regulated by ligand-binding affinity; the dynamics of which control cell adhesion and migration, ECM assembly and remodeling, and mechanotransduction.[112] Consequently, integrin activity contributes to embryonic development, and to diseases such as cancer.

High ligand-binding affinity denotes integrin activation, while low ligand-binding affinity results in integrin inactivation.[113] In their role as major cell surface adhesive receptors, integrins mediate bi-directional signaling across the plasma membrane to facilitate varying cellular functions (Figure 1.7). In what is termed 'inside-out' signaling, an intracellular activator (ex. Talin), binds to the cytoplasmic tail of integrins, resulting in conformational changes in the extracellular domain. Consequently, the extracellular ligand-binding affinity is increased (integrin activation).[112] Inside-out signaling controls interactions between integrins and the ECM to allow for regulation of cell migration, and ECM assembly and remodeling.[112]

'Outside-in' signaling allows for the flow of information from external stimuli to the inside of the cell. It is activated by ligand binding to the extracellular domain of integrins (Figure 1.7).[114] Ligand binding results in conformational changes in integrins that cause 'clustering,' which is the assembly of integrin heterodimers into hetero-oligomers to increase avidity.[115] Once activated and clustered, integrins mediate an array of intracellular changes. Immediate changes result in cytoskeletal rearrangements that allow cells to initiate migration and invasion.[116] More long-term effects of outside-in signaling include changes in signaling pathways and gene expression that regulate cell

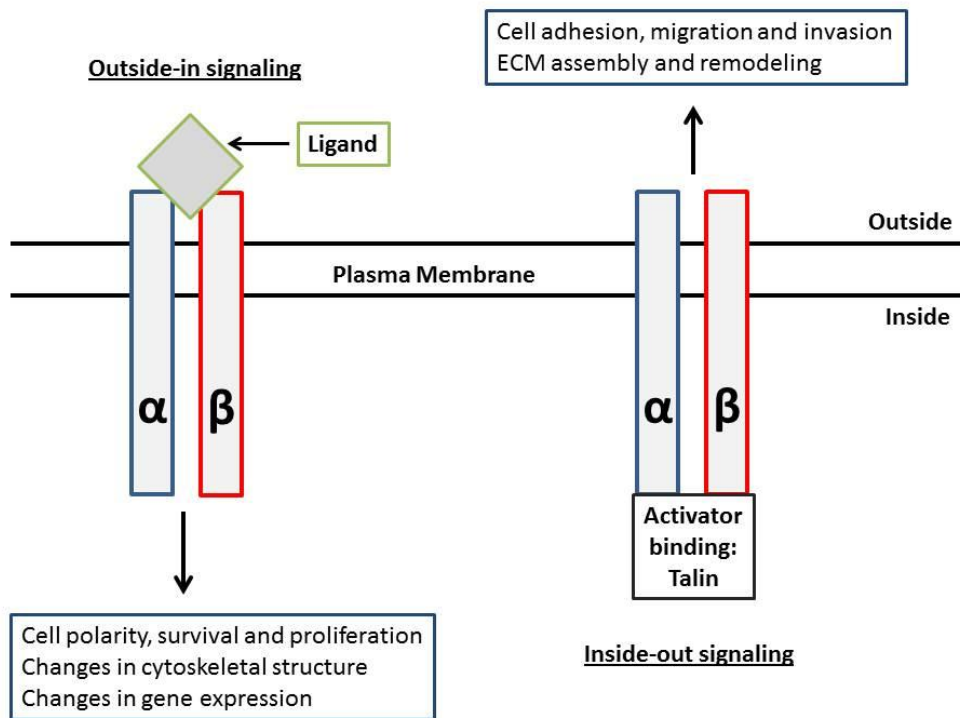

Figure 1.7 Bi-directional signaling of integrins. During inside-out signaling, activator proteins like Talin, bind to the integrin cytoplasmic tail of integrins. The subsequent conformational change results in increased extracellular ligand-binding activity of integrins. This controls processes like cell adhesion and migration. Integrins also transmit signals from the extracellular environment to the inside of the cell (outside-in). This is initiated by ECM protein ligand binding to integrins. Subsequent clustering of integrin heterodimers results in changes in cytoskeletal proteins and cellular signaling networks that control cell polarity, gene expression, proliferation and survival.

survival, growth, and proliferation.[115] Though theoretically separated, integrin bi-directional signaling events are intimately linked, and can have feedback effects on each other.[112]

Integrin function in cancer

Tumor development and metastasis is dependent on the orchestration of complex cellular processes (Figure 1.6). Considering the fundamental role played by integrins in cell adhesion, growth, motility, and survival, deregulation of integrin signaling may facilitate a number of key steps in the metastatic process. Mutations in genes encoding integrins have not been observed in cancers to date. Instead, aberrant expression of integrins increases the aforementioned cellular functions, thereby contributing to tumor development and metastasis.[117] Integrin expression has been shown to be heavily correlated with tumor development and poor patient prognosis in a number of malignancies.[118-120] During tumor progression, malignant cells change the type and levels of integrins expressed depending on the microenvironmental cues encountered.[121]

Tumor cells are able to manipulate the expression levels of integrins by using the bi-directional signaling capacity of integrins, and through interaction with components of signaling pathways like MAPK and PI3K/AKT.[117] Outside-in signaling can trigger MAPK and PI3K/AKT-mediated intracellular and gene expression changes which promote tumorigenesis.[122] This can also produce a reciprocal effect with inside-out signaling eliciting changes in integrin expression that promote metastasis through: inhibition of apoptosis, induction of proliferation, ECM remodeling, migration and angiogenesis.[123, 124]

Numerous studies have documented the multifaceted ways in which integrins contribute to tumor metastasis.[125-127] In breast cancer, β_1 integrin expression is associated with metastasis and reduced sensitivity to cytotoxic therapies.[128] Inhibition of β_1 integrin resulted in reduced tumor growth via induction of apoptosis.[129] $\alpha_v\beta_3$ integrin has been shown to increase cellular proliferation of ovarian cancer cells in an integrin-linked kinase-dependent manner.[130] Integrin-linked kinase (ILK) binds to β_3 integrin and provides a bridge between integrin signaling and growth factor signaling pathways, as its phosphorylation of AKT contributes to proliferation through activation of the PI3K/AKT pathway.[131]

In melanoma, the transition from RGP to VGP is associated with expression of $\alpha_v\beta_3$ integrin.[132] $\alpha_v\beta_3$ integrin regulates the activity of Matrix Metalloproteinase-2 (MMP-2), whose degradation of the basement membrane facilitates tumor invasion.[133, 134] Additionally, β_3 integrin promotes melanoma growth and metastasis by regulating tumor cell survival.[126]

AKT-dependent upregulation of α_5 integrin also causes increased melanoma cell motility.[48] Expression of α_5 integrin in ovarian and lung cancers has been shown to increase metastasis.[135, 136] In other settings, α_5 integrin appears to act as a tumor suppressor. In colorectal cancer, α_5 integrin downregulates expression of Her-2, and this causes decreased proliferation and tumorigenicity.[137]

The results in my dissertation will focus on the activities of Survivin and α_5 integrin and their effects on melanoma cell motility and metastasis. The research presented will show that Survivin overexpression in melanoma cells results in

upregulation of α_5 integrin. The increased levels of α_5 integrin promote cell motility and colony formation *in vitro*, as well as metastasis *in vivo*.

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CHAPTER 2

SURVIVIN ENHANCES MOTILITY OF MELANOMA CELLS BY SUPPORTING AKT ACTIVATION AND ALPHA 5 INTEGRIN UPREGULATION

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Cancer Research

Survivin Enhances Motility of Melanoma Cells by Supporting Akt Activation and α5 Integrin Upregulation

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Abstract

Survivin expression in melanoma is inversely correlated with patient survival. Transgenic mice harboring melanocyte-specific overexpression of survivin exhibit increased susceptibility to UV-induced melanoma and metastatic progression. To understand the mechanistic basis for metastatic progression, we investigated the effects of survivin on the motility of human melanocytes and melanoma cells. We found that survivin overexpression enhanced migration on fibronectin and invasion through Matrigel, whereas survivin knockdown under subapoptotic conditions blocked migration and invasion. In melanocytes, survivin overexpression activated the Akt and mitogen-activated protein kinase pathways. Akt phosphorylation was required for survivin-enhanced migration and invasion, whereas Erk phosphorylation was required only for enhanced invasion. In both melanocytes and melanoma cells, survivin overexpression was associated with upregulation of α5 integrin (fibronectin receptor component), the antibody-mediated blockade or RNA interference-mediated knockdown of which blocked survivin-enhanced migration. Knockdown of α5 integrin did not affect Akt activation, but inhibition of Akt phosphorylation prevented α5 integrin upregulation elicited by survivin overexpression. Together, our results showed that survivin enhanced the migration and invasion of melanocytic cells and suggested that survivin may promote melanoma metastasis by supporting Akt-dependent upregulation of α5 integrin. *Cancer Res; 70(20); 7927–37. ©2010 AACR.*

Introduction

Cell migration plays a central role in biological processes such as embryonic development and wound healing (1). In cancer, aberrant cell migration is involved in tumor progression and metastasis as malignant cells hijack the migratory machinery and acquire the ability to move and invade other tissues (2). The propensity to metastasize is particularly high in melanoma, a potentially fatal form of cancer arising from melanocytes in the skin (3). The probability of metastasis in melanoma directly relates to the (Breslow) depth of tumor invasion, which inversely correlates with patient survival (3). In early lesions, melanoma cells exhibit radial growth phase, which is characterized primarily by lateral migration. More advanced lesions display vertical growth phase, characterized by deeper invasion and increased risk of metastasis. Thus, deregulated cell migration is a key feature of melanoma progression, and is required for metastasis.

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Several pathways important in melanoma migration and invasion have been previously characterized. First, the mitogen-activated protein kinase (MAPK) pathway is activated in most melanomas (4), with roughly two thirds of tumors harboring activating mutations in *BRAF* (5), and constitutive MAPK activation in immortalized melanocytes confers tumorigenicity (6). Second, Akt activation is found in 70% of melanomas (7) and mediates melanoma cell migration, invasion, and metastasis in various model systems (8–10). In particular, activation of Akt has also been shown to convert a melanoma cell line from radial to vertical growth phenotype (11).

Survivin is an inhibitor of apoptosis protein that has been characterized as a regulator of both cell division and cell death (12). As a chromosomal passenger protein, it is required for proper chromosomal alignment during mitosis (13). Survivin protects cells against both caspase-dependent and caspase-independent apoptosis (14), and interference with survivin function in melanoma cells leads to spontaneous apoptosis (15) and impairs tumor growth (16). Survivin is upregulated during melanocyte transformation (17), and several studies have shown an inverse correlation between survivin expression in advanced melanoma and patient survival (18, 19). We have previously shown that transgenic expression of survivin in murine melanocytes promotes UV-induced melanoma tumor formation and metastasis (20). The metastatic phenotype in these mice was unexpected, as previous mouse tumor models based on survivin expression in uroepithelium (21) and keratinocytes (22) did not reveal metastasis.

Here we describe an alternate role for survivin in tumor progression, namely, its promotion of cellular motility. We show that expression of survivin in melanocytes and its overexpression in melanoma cells enhance cell migration and invasion. The promigratory effect in survivin-expressing cells is mediated by activation of Akt and upregulation of α5 integrin, a component of the fibronectin receptor (23).

Materials and Methods

Cell culture

Normal human melanocytes were propagated from discarded foreskins (24). Human metastatic melanoma cell lines LOX, YUSAC2 (YU2), and YUGEN8 (YU8) have been previously described (25), and HTB-66 and SK-MEL-28 (SK-28) were from the American Type Culture Collection. The human primary melanoma cell lines WM35 and WM793 were kindly provided by Meenhard Herlyn (Wistar Institute, Philadelphia, PA). The 4C7 human melanoma cells, which express wild-type human survivin in a tetracycline (tet)-regulated (tet-off) manner, have been previously described (26). All cells were grown at 37°C in 5% CO_2 humidified air.

Anoikis assay

Polyhema (Sigma Chemical Co.) was dissolved in 95% ethanol (120 mg/mL) overnight at 65°C, diluted 1:10 in 95% ethanol, then added to 6-well plates (0.5 mL/well) and allowed to dry overnight at 37°C. Melanocytes were added in growth medium and incubated for 48 hours. Apoptotic cells were detected by propidium iodide (PI; Sigma) staining and flow cytometry as previously described (14).

Migration assay

Cells were added to transwell polycarbonate membranes (Costar) after coating the lower surfaces with 0.5 mL recombinant human fibronectin (10 μg/mL; Invitrogen) in PBS overnight at 37°C. To block cellular proliferation during the assay, cells were first cultured with mitomycin C (10 μg/mL; Sigma) for 2 hours. After washing, cells were resuspended in 100 μL of serum-free medium and added to the upper surface of transwells. Each lower chamber contained 0.5 mL serum-containing media. After incubation at 37°C (optimal time determined for each cell line), nonmigratory cells on upper membrane surfaces were removed using a cotton-tip applicator. Cells that migrated through to the lower membrane surface were fixed with 4% paraformaldehyde in PBS for 15 minutes and then stained with 4′-6-diamidino-2-phenylindole (DAPI; 1 μg/mL; Sigma) in PBS for 5 minutes at room temperature. Migrating cells were quantified by manually counting 5 representative high-powered fields using a Nikon Eclipse TE300 fluorescence microscope. For some experiments, inhibitors of phosphatidylinositol-3 kinase (PI3K; wortmanin, Sigma), Akt (Akt VIII, Calbiochem), and MAPK (u0126, PD98059, Cell Signaling Technology) were also added at noncytotoxic concentrations. For antibody blocking experiments, cells were resuspended in serum-free media containing 10 μg/mL antibody against α5 integrin (IIA1, BD Biosciences), α5β1 integrin (P1D6, Covance), α6 integrin (MAB13501, R&D Systems), or control mouse IgG (Sigma), and then seeded onto the upper surface of the transwell chamber. After incubation for 3 hours at 37°C to allow cell adherence to the membrane, upper chambers were gently washed with PBS to remove antibodies and replenished with 100 μL serum-free media. For wound healing assay, a linear scratch was made using a sterile pipette tip on semiconfluent adherent cells, and indicated antibodies were added for 3 hours.

Invasion/Matrigel assay

The Matrigel matrix in BD BioCoat invasion chambers (Discovery Labware) was rehydrated with serum-free medium for 2 hours at 37°C in a humidified incubator. To block cellular proliferation during the assay, cells were first cultured with mitomycin C as above. Cells were added in 0.5 mL serum-free medium to each upper chamber, and 0.75 mL culture medium was added to each lower chamber as a chemoattractant. After incubation at 37°C (optimal time determined for each cell line), noninvading cells were removed from upper membrane surfaces and invading cells were quantified on lower membrane surfaces as described above.

Gene modulation, survivin adenovirus

Melanocytes or melanoma cells were grown to 90% confluence and then infected with a control adenovirus expressing green fluorescence protein (GFP; pAd-GFP) or a survivin-expressing GFP-tagged adenovirus (pAd-Survivin-GFP or pAd-Surv; ref. 27), kindly provided by Dario Altieri (University of Massachusetts, Worcester, MA). After 6 hours, the culture medium was changed to remove excess virus.

RNA interference (RNAi)-mediated gene knockdown

Cells were transfected at 70% to 80% confluency in 6-well plates with oligonucleotides targeting α5 integrin, Akt1/2, survivin (Santa Cruz Biotechnology) or a control scrambled sequence (17). The RNA duplexes (10 μmol/L stocks; 8 μL for α5 integrin, 6 μL for Akt or survivin) were diluted in 100 μL transfection medium (Santa Cruz Biotechnology), then combined with 100 μL transfection medium containing transfection reagent (8 μL for α5 integrin and 4 μL for Akt or survivin; Santa Cruz Biotechnology) for each well. After incubating for 30 minutes at room temperature, the transfection mixture was added dropwise to cells in 0.8 mL transfection medium per well. Plates were incubated at 37°C for 7 hours, then 1 mL of 2 times normal growth medium was added to each well.

Western blotting

Trypsinized cells were washed, lysed, and then electrophoresed and transferred to membranes as described (17). After blocking, membranes were incubated for 1 hour with primary antibodies (see Supplementary methods) dissolved in PBS containing 0.1% Tween and 5% nonfat milk. After staining with species-appropriate secondary horseradish peroxidase-conjugated antibodies (PerkinElmer Life Sciences), protein bands were visualized by enhanced chemiluminescence (PerkinElmer Life Sciences) and autoradiography.

Statistics

Analyses were performed with Prism 3.0 software (GraphPad). Data derived from multiple determinations were subjected to two-sided t tests. P values ≤0.05 were considered statistically significant.

Results

Survivin fails to protect melanocytes against anoikis

To investigate potential roles of survivin in promoting melanoma metastasis, we first examined whether its well-established capacity to confer apoptotic protection (12) could also impart resistance to detachment-induced apoptosis (anoikis). Melanocytes must acquire resistance to anoikis to survive loss of contact with keratinocytes and basement membrane which necessarily occurs as an early event in melanoma metastasis. For this purpose, melanocytes were infected with control or survivin-expressing viruses (Supplementary Fig. S1A), and then seeded onto polyhema-coated plates to prevent attachment (Supplementary Fig. S1B). As shown in Supplementary Fig. S1C and D, the apoptotic fraction was only reduced by 10% to 15% in survivin-expressing melanocytes. Although we had previously shown that survivin expression protects melanocytes against UV-induced apoptosis (14, 20), this modest protection against anoikis suggests that survivin promotion of metastasis may occur through alternate mechanisms.

Survivin enhances cellular migration and invasion

Given the importance of migration and invasion in melanoma progression and metastasis, we next investigated whether survivin expression could support either of these activities in human melanocytes or melanoma cells. Survivin was expressed in melanocytes and overexpressed in two melanoma cell lines (LOX, YU8; Fig. 1A, left), and migrating cells were visualized (Supplementary Fig. S2A). We observed that survivin expression enhanced cellular migration in melanocytes (Fig. 1A, middle) and melanoma cells (Fig. 1A, right). Similarly, by Matrigel assay, we found that survivin-expressing melanocytes showed markedly increased invasive activity compared with control cells (Fig. 1B, left). This enhanced invasive capacity was recapitulated in melanoma cell lines (LOX, WM35) overexpressing survivin (Fig. 1B, right). Thus, survivin expression in human melanocytes and melanoma cells is associated with enhanced capacity for migration and invasion.

We noted that the effect of survivin expression on melanocyte invasion was more pronounced than that on migration

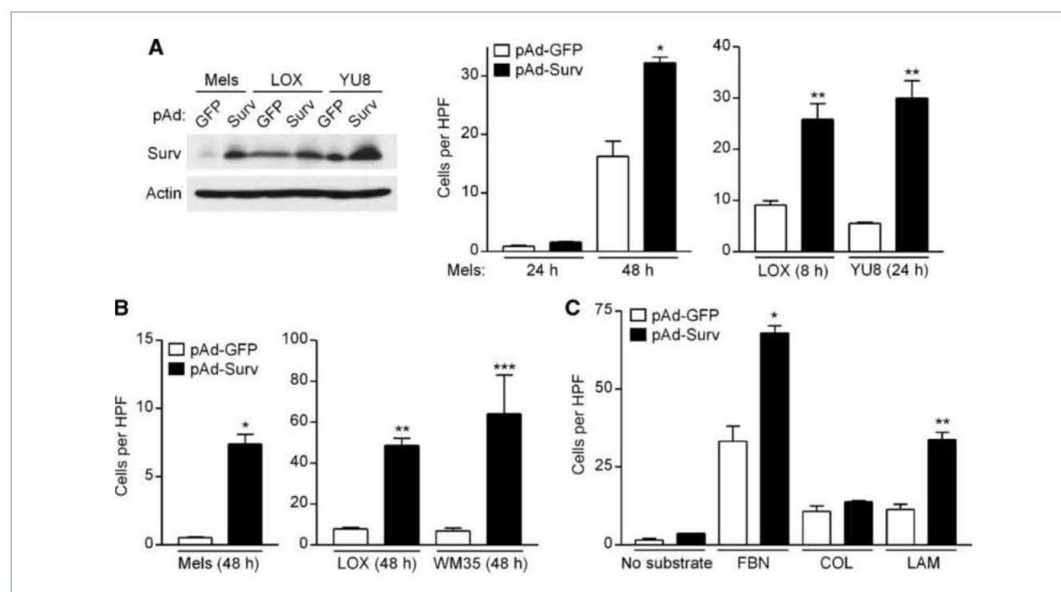

Figure 1. Survivin enhances migration and invasion in human melanocytes and melanoma cell lines. A, melanocytes (Mels) and melanoma cells (LOX, YU8) were infected with control GFP adenovirus (pAd-GFP) or pAd-expressing survivin (pAd-Surv), then 48 hours later lysates were blotted for survivin (Surv) and actin. Mels (5×10^4 per well), LOX (1×10^4 per well), or YU8 (5×10^4 per well) cells were added to transwell chambers containing fibronectin-coated inserts 24 hours after infection, and migrating cells, expressed per high-power field (HPF), were quantified 8 to 48 hours later as indicated. Error bars, SE of triplicate determinations. *, $P = 0.03$; **, $P < 0.01$. B, Mels (5×10^4 per well), LOX (5×10^4 per well), or WM35 (5×10^4 per well) cells were added to Matrigel-coated inserts 24 hours after infection, and invading cells were quantified 48 hours later. Error bars, SE of triplicate determinations. *, $P = 0.01$; **, $P < 0.001$; ***, $P = 0.02$. C, Mels (5×10^4 per well) were added to transwell chambers containing uncoated inserts (no substrate), or inserts coated with fibronectin (FBN), collagen IV (COL), or laminin I (LAM) 24 hours after infection, and migrating cells were quantified 48 hours later. Error bars, SE of triplicate determinations. *, $P = 0.003$; **, $P = 0.002$.

(Fig. 1A, middle, and B, left), and wondered whether this could reflect differential response to various extracellular matrix proteins used as substrates in the migration and invasion assays. The substrate in the migration assay was fibronectin, whereas the Matrigel (according to the manufacturer) in the invasion assay was composed of collagen IV and laminin I. Therefore, we re-examined the migratory activities of survivin-expressing melanocytes in the context of each of these substrates. Although survivin did not enhance melanocyte migration in the absence of substrate, we did observe survivin-enhanced migration on fibronectin and laminin (Fig. 1C). Melanocyte migration was increased on collagen, but no difference was seen for survivin-expressing cells on that substrate (Fig. 1C). On the other hand, although Survivin enhanced migration of YU2 melanoma cells on fibronectin (see below), there was no increased migration of survivin-overexpressing YU2 cells on laminin (not shown).

Survivin knockdown impairs melanoma cell migration and invasion

To assess the role of endogenous survivin in melanoma cell migration and invasion, survivin expression was downregulated in several melanoma cell lines using RNAi. Although RNAi-mediated depletion of survivin was found to induce apoptosis in pancreatic cancer (28) and glioma cells (29), other studies showed its partial depletion by gene targeting in melanoma cells only sensitized to drug-induced apoptosis but was not sufficient for induction of apoptosis (30). Similarly, we found in both LOX and YU8 melanoma cells that RNAi-mediated depletion of survivin (Fig. 2A and B, left) was not associated with significant apoptosis over a 72-hour period (Fig. 2A and B, middle). Importantly, this provided a window for investigating the effect of survivin depletion on migration and invasion without the potential confounding effect of cells undergoing apoptosis during the assay. Migrating

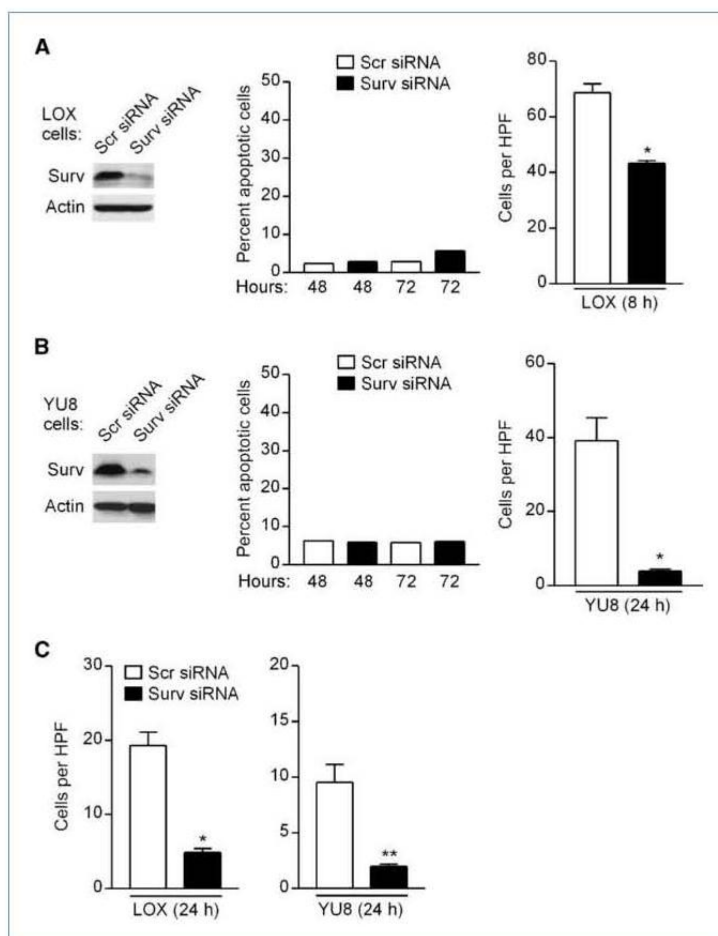

Figure 2. Knockdown of survivin impairs melanoma cell migration and invasion. A, LOX cells were transfected with scrambled (Scr) or survivin (Surv)-specific siRNA, then 72 hours later lysates were blotted for survivin and actin. Cells were PI stained and the apoptotic cell fraction was quantitated by flow cytometry 48 hours or 72 hours after transfection as indicated. Cells (1×10^4 per well) were added to fibronectin-coated inserts 48 hours after transfection, and migrating cells were quantified 8 hours later. Error bars, SE of triplicate determinations. *, $P = 0.002$. B, YU8 cells were transfected with scrambled or survivin-specific siRNA, then 72 hours later lysates were blotted for survivin and actin. Cells were PI stained and the apoptotic cell fraction was quantitated by flow cytometry 48 hours or 72 hours after transfection as indicated. Cells (5×10^4 per well) were added to fibronectin-coated inserts 48 hours after transfection, and migrating cells were quantified 24 hours later. Error bars, SE of triplicate determinations. *, $P = 0.005$. C, LOX (1×10^4 per well) and YU8 (5×10^4 per well) cells were added to Matrigel-coated inserts 48 hours after transfection with scrambled or survivin-specific siRNA, and invading cells were quantified 24 hours later. Error bars, SE of triplicate determinations. *, $P = 0.002$; **, $P = 0.009$.

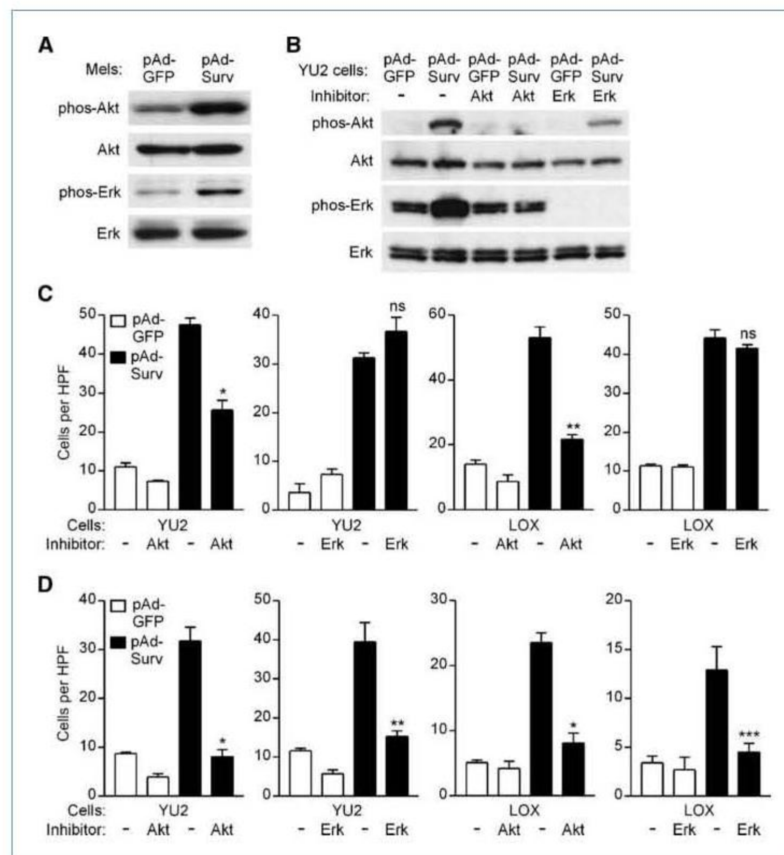

Figure 3. Role of Akt and MAPK pathways in survivin promotion of melanoma cell motility. A, melanocytes were infected with pAd-GFP or pAd-Surv for 48 hours, then cell lysates were blotted for phosphorylated (phos) Akt, total Akt, phos-Erk, and total Erk. B, YU2 cells were infected with pAd-GFP or pAd-Surv, then 24 hours later treated with diluent, 4 μmol/L Akt VIII, or 20 μmol/L Erk inhibitor u0126. After 24 hours, cells were lysed for Western blotting. C, pAd-infected cells were transferred to transwells in the presence or absence (–) of inhibitors, and migrating cells were quantitated 24 hours later. Error bars, SE of three determinations. *, $P = 0.002$; **, $P = 0.001$; ns, not significant. D, pAd-infected cells were transferred to Matrigel-coated inserts in the presence or absence (–) of inhibitors, and invading cells were quantitated 48 hours later. Error bars, SE of three determinations. *, $P = 0.002$; **, $P = 0.009$; ***, $P = 0.03$.

(Supplementary Fig. S2B) and invading cells were quantitated following transfection with control or survivin-specific RNAi. For both melanoma cell lines, we observed that reduced survivin levels significantly decreased capacity for both migration (Fig. 2A and B, right) and invasion (Fig. 2C). Taken together, these data show that endogenous survivin expression is required for melanoma cell migration and invasion.

Survivin does not affect epithelial-mesenchymal transition

We next asked whether survivin expression in melanocytes could promote an epithelial to mesenchymal transition (EMT). The EMT refers to phenotypic and genetic changes characteristic of cells that have escaped from epithelia and are primed for metastasis; conventional markers of EMT include loss of E-cadherin, increased expression of fibronectin and vimentin, and nuclear translocation of β-catenin (31). As shown in Supplementary Fig. S3, survivin-expressing melanocytes did not exhibit changes in these markers. In addition, survivin overexpression in 4C7 melanoma cells was also not associated with alteration of any of these markers. Thus, although survivin promotes melanocyte migration and invasion, its expression is not sufficient to mediate acquisition of EMT.

Role of Akt and MAPK activation

Two signaling pathways known to be important in melanoma migration include the MAPK and PI3K/Akt pathways (32). Survivin expression in melanocytes was associated with activation of both Akt and MAPK, as reflected by increased levels of phosphorylated Akt and Erk species, respectively (Fig. 3A). We used specific inhibitors of these pathways (32) to assess independently their role in survivin-enhanced migration. Because these inhibitors were found to be toxic for melanocytes (not shown), we performed these experiments only in melanoma cells. Survivin overexpression was associated with increased levels of phospho-Akt in both YU2 (Fig. 3B) and LOX cells (not shown), whereas Erk was constitutively phosphorylated. Addition of Akt VIII or u0126 efficiently blocked phosphorylation of Akt and Erk, respectively, in both YU2 (Fig. 3B) and LOX cells (not shown). In migration assays, inhibition of Akt phosphorylation blocked survivin-enhanced migration of both YU2 and LOX

cells (Fig. 3C). Similar inhibitory effects on survivin-enhanced migration were seen using the PI3K inhibitor wortmanin (Supplementary Fig. S4B). On the other hand, u0126-mediated inhibition of Erk phosphorylation did not affect survivin-enhanced migration in either melanoma line (Fig. 3C). Similarly, another Erk inhibitor, PD98059, did not block survivin-enhanced migration in either line (Supplementary Fig. S4C, left). Inhibition of either Akt or Erk, however, was sufficient to block survivin-enhanced invasion of both YU2 and LOX cells (Fig. 3D). Depletion of Akt in LOX cells by siRNA (Supplementary Fig. S5A) impaired both survivin-enhanced migration (Supplementary Fig. S5B, left) and invasion (Supplementary Fig. S5B, right). Inhibition of survivin-enhanced invasion was also seen in both cell lines treated with the Erk inhibitor PD98059 (Supplementary Fig. S4C, right). Thus, survivin promotion of melanoma cell migration requires Akt activation (but is independent of Erk activation), whereas enhanced invasion requires both Akt and Erk activation.

Survivin upregulates α5 integrin in melanocytes and melanoma cells

The substrates on which we observed survivin promotion of melanocyte migration, namely, fibronectin and laminin (Fig. 1C), are recognized by αβ heterodimeric integrin receptors. Thus, we considered the possibility that survivin may specifically promote migration and invasion through upregulation on melanocytic cells of one or more particular integrin receptor chains involved in binding to these matrix proteins. We examined in control and survivin-expressing melanocytes the protein expression of potential α and β integrin chains known to contribute to fibronectin (α5β1, αvβ3) and laminin (α1β1, α2β1, α3β1, α6β1, α6β4, αvβ3) binding (23). Survivin expression led to upregulation of the α1 and α5 integrins, with the latter being more robust, whereas expression of the other integrins was unaffected or slightly decreased by survivin expression (Fig. 4A).

The α5β1 heterodimer constitutes the predominant receptor for fibronectin (23). We observed a striking increase in α5 integrin protein levels in melanocytes at 24 hours following forced survivin expression (Fig. 4B). Melanocytes constitutively express the β1 integrin, the protein levels of which were not affected by survivin expression (Fig. 4B). Next we investigated a similar correlation between survivin and α5 integrin expression in melanoma cells. We screened a small panel of primary and metastatic melanoma cell lines for α5 integrin expression. The α5 integrin was expressed constitutively in four of six lines, with higher levels in metastatic versus localized melanoma lines (Fig. 4C, left). In five of six lines, the α5 integrin was upregulated in the context of survivin overexpression, whereas one line (SK-28) did not express the α5 integrin even after survivin overexpression (Fig. 4C, left). Thus, the connection observed between survivin and α5 integrin expression in melanocytes is largely conserved in melanoma cells. By contrast, levels of the α1 integrin were not increased by survivin overexpression in two melanoma lines (Fig. 4C, right).

The α5 integrin is required for survivin-enhanced melanocyte and melanoma cell migration

To test whether a functional correlation exists between expression of survivin and upregulation of the α5 integrin, we asked whether the α5 integrin is required for survivin-enhanced motility. First, migration of survivin-expressing melanocytes on fibronectin was examined in the presence of two different α5 integrin–specific blocking antibodies: one reactive with the α5 integrin (IIA1), the other (P1D6) blocking interaction of the α5β1 heterodimer with its ligand fibronectin. As shown in Fig. 4D (top left), the presence of either α5-blocking antibody (but not control IgG or α6-specific antibody) abrogated the effect of survivin on melanocyte migration. Although anti-α5 antibody inhibited migration of survivin-expressing melanocytes on fibronectin, it was without effect when laminin (not a ligand for the α5 integrin) was used as the substrate (Fig. 4D, top right). Similar inhibitory effects of these antibodies were observed on survivin-enhanced migration of LOX cells in a scratch (wound-healing) assay without substrate (Fig. 4D, bottom).

As a second test of the requirement for the α5 integrin in survivin-enhanced motility, we examined the effect of α5 integrin gene knockdown. These experiments were performed in melanoma cells, which are more amenable to transfection of siRNA than melanocytes. Levels of the α5 integrin were reduced in survivin-overexpressing YU2 melanoma cells following transfection of specific siRNA (Fig. 5A, left). Depletion of the α5 integrin abrogated survivin-enhanced migration of these cells (Fig. 5A, right). Similar results were seen in α5 integrin–depleted LOX cells (Fig. 5B). Despite these effects on migration, we found that knockdown of the α5 integrin in either YU2 or LOX cells did not inhibit survivin-enhanced invasion (Fig. 5C). Similarly, α5 integrin–blocking antibodies did not impair survivin-enhanced invasion of LOX cells (Supplementary Fig. S6). These data suggest that the α5 integrin is required for survivin-enhanced melanocyte and melanoma cell migration, but not invasion of melanoma cells.

Survivin-mediated upregulation of the α5 integrin is Akt dependent

Given the requirement of Akt activation and the importance of the α5 integrin for survivin promotion of migration (Figs. 3C and 4D), we examined the relationship between Akt activation and the α5 integrin in the context of survivin expression. In survivin-overexpressing cells, knockdown of the α5 integrin was not associated with significant changes in levels of phosphorylated Akt (Fig. 5A and B). Conversely, the addition of PI3K inhibitor abrogated survivin-mediated upregulation of the α5 integrin in both LOX (Fig. 5D, left) and YU2 (not shown) cells. On the other hand, addition of the Erk inhibitor u0126 did not interfere with survivin-mediated upregulation of the α5 integrin in LOX (Fig. 5D, right) or YU2 (not shown) cells. Thus, the α5 integrin seems to be downstream of PI3K/Akt, and its upregulation following survivin overexpression requires Akt but not Erk activation.

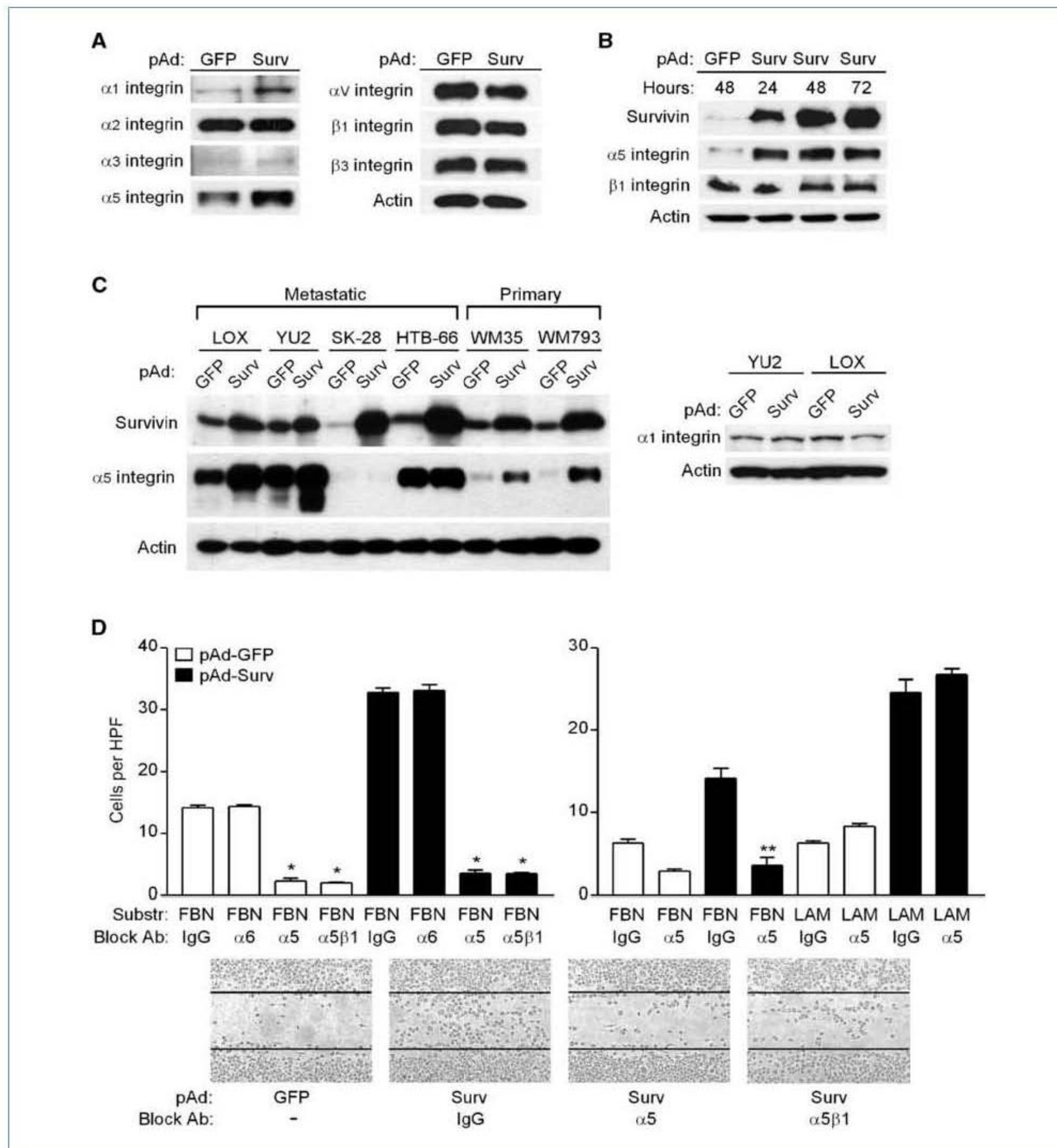

Figure 4. Survivin upregulates the α5 integrin, which mediates enhanced melanocyte migration on fibronectin. A, melanocytes 48 hours after infection with pAd-GFP or pAd-Surv were subjected to Western blotting for the indicated integrins, and actin as a loading control. Blots for α6 and β4 integrins were performed, but expression of these integrins was not detected (not shown). B, melanocytes were infected with pAd-GFP or pAd-Surv, then cell lysates were prepared at the indicated time points and analyzed by Western blotting for survivin, the α5 or β1 integrin, or actin. C, metastatic and primary melanoma cell lines were infected with pAd-GFP or pAd-Surv, then 48 hours later cell lysates were subjected to Western blotting. D, melanocytes were infected with pAd-GFP or pAd-Surv for 24 hours, then added to chambers (5×10^4 per well) with fibronectin (FBN)- or laminin (LAM)-coated inserts in the presence of 10 μg/mL of either control (IgG, α6) or α5-blocking (α5, α5β1) antibodies as indicated (Block Ab). After incubation for 48 hours, migrating cells were quantified (top left, top right). Errors bars, SE of triplicate determinations. *, $P < 0.0001$; **, $P = 0.002$. LOX cells were infected with either pAd-GFP or pAd-Surv, then after 24 hours a linear scratch was made using a pipette tip (indicated between solid lines) and indicated antibodies were added for 3 hours. Photographs were taken 24 hours later (bottom).

Discussion

Survivin is widely expressed in cancer and is thought to promote tumor development and progression through two previously characterized functions: apoptotic inhibition and mitotic chromosomal alignment (12). Indeed, multiple studies from our laboratory and others have shown that inhibition of survivin in both malignant cells *in vitro* (15, 33) and tumors *in vivo* (16, 26) precipitates spontaneous apoptosis and mitotic defects. In this study, we show a third capability of survivin: the promotion of cellular motility. We show that survivin is both necessary and sufficient to enhance

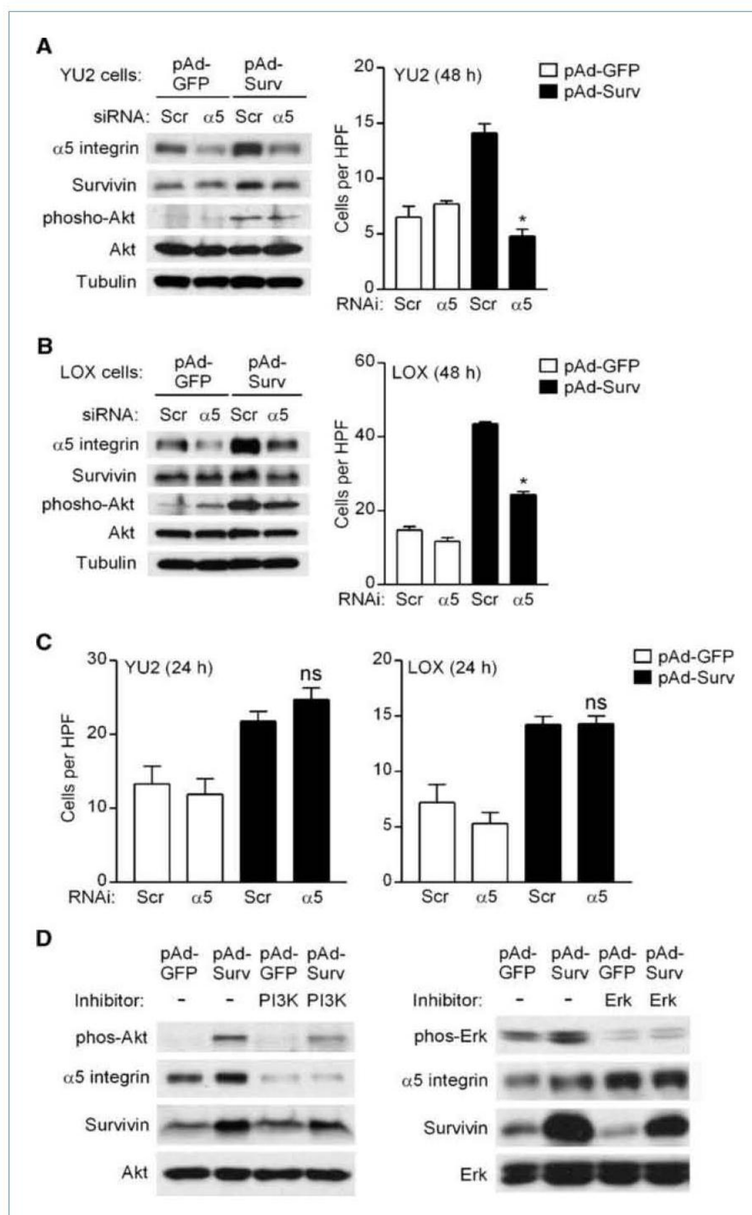

Figure 5. The α5 integrin is required for survivin-enhanced melanoma cell migration. A, YU2 cells were infected with pAd-GFP or pAd-Surv, then 24 hours later cells were transfected with scrambled or α5 integrin-specific siRNA. Cells were lysed for Western analysis after 72 hours (left), or 24 hours after transfection were added (5×10^4 per well) to chambers with fibronectin-coated inserts and migrating cells were quantitated 48 hours later (right). Errors bars, SE of triplicate determinations. *, $P < 0.001$. B, LOX cells (1×10^4 per well) were treated and analyzed as in A. *, $P < 0.001$. C, YU2 (left) and LOX (right) cells were infected with pAd-GFP or pAd-Surv, then 24 hours later cells were transfected with scrambled or α5 integrin–specific siRNA. After 24 hours, cells were added (YU2, 5×10^4 per well; LOX, 1×10^4 per well) to Matrigel-coated inserts and invading cells were quantitated 48 hours later. Errors bars, SE of triplicate determinations. ns, not significant. D, survivin-mediated upregulation of α5 integrin requires activation of Akt but not Erk. LOX cells were infected with pAd-GFP or pAd-Surv, then 24 hours later treated with diluent, 6 μmol/L PI3K inhibitor wortmanin (left), or 20 μmol/L Erk inhibitor u0126 (right). After 24 hours, cell lysates were prepared for Western blotting.

migration and invasion in normal human melanocyte and melanoma cell lines. For all the assays, cells were treated with mitomycin C to inhibit mitosis, and thus preclude any effects that may have resulted from differences in cell division associated with changes in survivin expression. The observed effects on cellular motility also seem to be independent of survivin antiapoptotic function, as both migration and invasion were significantly compromised in melanoma cells depleted of survivin under conditions not affecting apoptosis. Thus, the capacity of survivin to promote cellular migration and invasion seems to represent a novel function distinct from its other established activities.

The capacity of survivin to promote melanocyte migration on fibronectin and laminin, but not collagen, suggested the possibility that survivin expression may differentially regulate expression of various integrins known to interact with these particular substrates. We decided to focus on the functional role of the α5 integrin because we were able consistently to show dramatic upregulation of this integrin at the protein level. Given constitutive expression of the β1 integrin in melanocytes, induction of the α5 integrin should constitute a functional (α5β1) fibronectin receptor. The correlation of expression between survivin and the α5 integrin is conserved in both normal melanocytes and several melanoma cell lines. Our finding that the α5 integrin is required for survivin-enhanced melanocyte and melanoma cell migration is consistent with its described role in enhancing cell motility and cancer metastasis (34) and some studies suggesting its importance in melanoma development and metastasis. Expression of the α5 integrin has been shown in both primary and metastatic melanocytic lesions (35), and in a mouse model, inhibition of the α5 integrin effectively blocked formation of pulmonary metastases (36).

In contrast to its role in migration shown here, we found that the α5 integrin is not required for survivin-enhanced melanoma cell invasion. Although the α1 integrin is also upregulated by survivin in melanocytes, it was not similarly upregulated in melanoma cells. We have examined several melanoma cell lines by quantitative reverse transcriptase-PCR to see whether survivin upregulates other integrins known to be important for melanoma invasion such as αv and β3 (37, 38), and we have not found significant upregulation of expression of these integrin chains with survivin overexpression.[4] Expression of the αvβ3 heterodimer has been associated with transition from radial to vertical growth phase melanoma (39). An underlying mechanism may involve interaction of αvβ3 with matrix metalloproteinase-2 (40), which facilitates subsequent degradation of basement membrane (41). It is possible that other integrins are upregulated in survivin-expressing melanoma cells that allow interaction with matrix proteins other than fibronectin and underlie promotion of cellular invasion. Upregulation of other integrins may also explain our finding that survivin enhances melanocyte migration on other substrates such as laminin, which was not blocked by antibodies against the α5 integrin. However, we did not observe survivin-enhanced melanoma cell migration on laminin.

Integrins promote cell migration and invasion through interactions with extracellular matrix proteins that not only enhance adhesion, but also transmit intracellular signals (42). Signaling pathways known to promote integrin-mediated migration include activation of focal adhesion kinase and Src (43), MAPK (44), and Akt (45), which are also associated with deregulated cell migration in cancer (46, 47). We found activation of both MAPK and Akt pathways to be associated with survivin expression in melanocytes; in melanoma cells, survivin overexpression activates Akt whereas the MAPK pathway is constitutively activated. In our system, Akt activation is required for both survivin-enhanced melanoma cell migration and invasion, whereas MAPK (Erk) activation is required only for invasion. Although knockdown of the α5 integrin did not affect Akt phosphorylation, inhibition of Akt blocked the upregulation of the α5 integrin following survivin overexpression. By contrast, Erk inhibition did not interfere with survivin-induced upregulation of the α5 integrin. Our data thus suggest a pathway whereby survivin promotes melanoma cell migration via Akt-dependent expression of the α5 integrin. A role for Akt in the survivin pathway is consistent with the established role of Akt in melanoma cell migration, invasion, and metastasis (8–11). The mechanism by which survivin activates Akt is not presently clear, but Guha and colleagues (48) showed that PTEN suppresses survivin expression. Thus, it is possible that survivin may regulate Akt phosphorylation through negative feedback on PTEN. Indeed, we have seen in melanocytes and two melanoma cell lines that survivin expression results in downregulation of PTEN.[5] Akt is known to regulate integrin expression/activation by various mechanisms (49, 50). With respect to increased expression of the α5 integrin in survivin-expressing cells, in preliminary studies in melanoma cells we have found that survivin increases nuclear concentration of C/EBP and NF-1, which are the transcription factors known to regulate α5 integrin expression (51, 52).[4]

We recognize the likelihood that survivin promotes melanoma metastasis by multiple mechanisms. Clearly apoptosis inhibition is important in cancer (53), and likely plays a role in both early and later steps in tumor progression. Our finding, however, that survivin confers only modest protection against melanocyte anoikis suggests its role as a cytoprotectant may be more critical in metastatic cells where it is expressed at higher levels (25) rather than during the initial process of melanocyte escape from the epithelium. On the other hand, its role shown here in promoting migration and invasion suggests a novel basis for its critical role in early tumor development. Although conventional chemotherapy and survivin-targeted therapies (30, 54) have been aimed at inducing apoptosis in tumor cells in patients with advanced

[4] J.A. McKenzie, T. Liu, and D. Grossman, unpublished observations.

[5] J.A. McKenzie and D. Grossman, unpublished observations.

disease, blocking survivin at earlier stages may thwart melanoma cell motility and allow targeting of localized disease to prevent invasion and subsequent metastasis.

Note Added in Proof

Since the original submission of the manuscript, Mehrotra and colleagues (55) reported that survivin enhances invasion and metastasis of breast cancer cells by upregulating fibronectin and activating FAK and Src kinases. Although this provides separate validation of a role for survivin in promoting metastasis and motility, it seems that the pathway we have identified in melanoma cells seems to be distinct in several respects. First, we have not observed upregulation of fibronectin (Supplementary Fig. S3) or phosphorylation of FAK or Src in cells overexpressing survivin.[5] Second, these authors reported that survivin-enhanced motility does not require Akt activation or upregulation of integrins (55).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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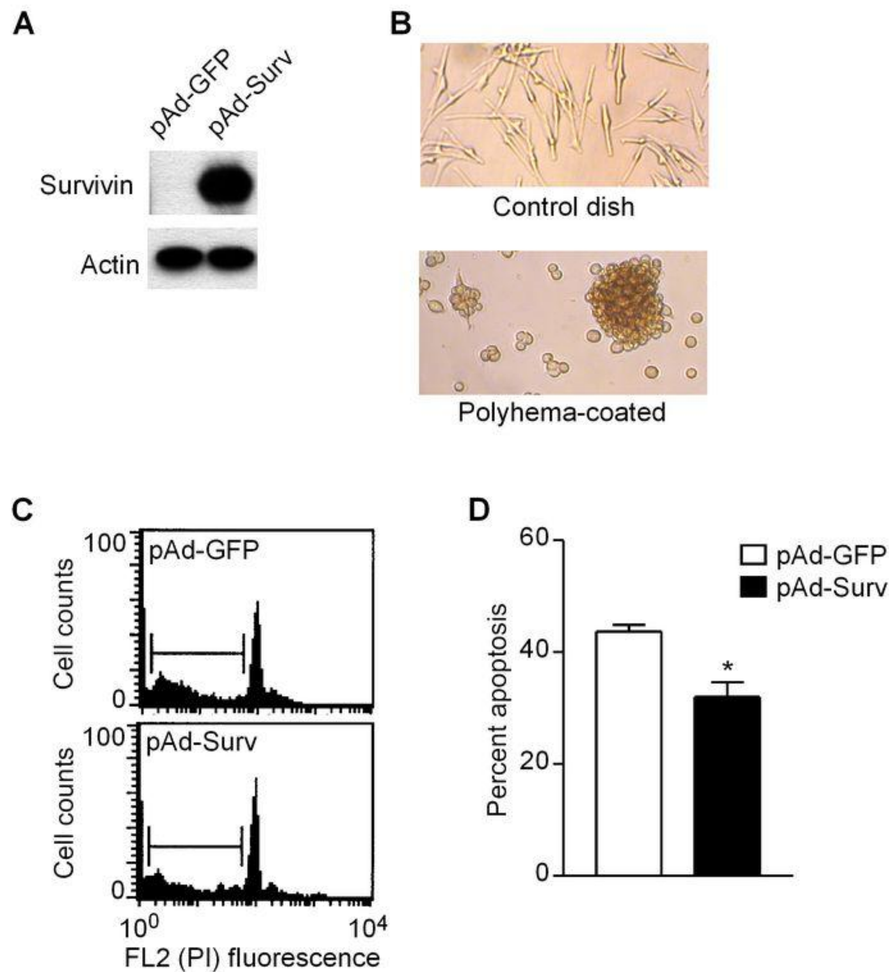
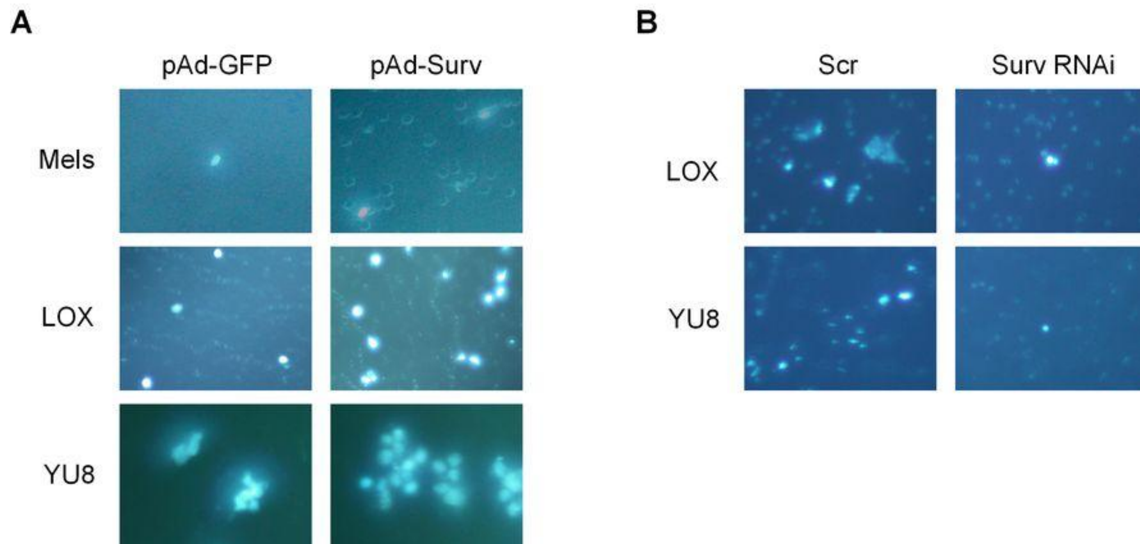
Supplemental information

Antibodies for Western blotting include Survivin (ref. 17) (1:1000), β-Actin (1:30,000; A-3853, Sigma), α-tubulin (1:1000, CP-06, Calbiochem-EMD Chemicals), α1 integrin (1:1000, sc-10728, Santa Cruz Biotechnology), α2 integrin (1:1000; #611016, BD Biosciences), α3 integrin (1:1000; AB1920, Millipore), α5 integrin (1:1000, #47051, Cell Signaling Technology), α6 integrin (1:1000; MAB1356, Chemicon), αV integrin (1:1000; #611012, BD Biosciences), β1 integrin (1:1000; #610467, BD Biosciences), β3 integrin (1:1000; #611140, BD Biosciences), β4 integrin (1:1000; #611232, BD Biosciences), E-cadherin (1:500, #610181, BD Transduction Laboratories), fibronectin (1:000, #610077, BD Transduction Laboratories), vimentin (1:500, sc32322, Santa Cruz Biotechnology), β-catenin (1:1000, #9582, Cell Signaling Technology), Akt (1:1000, #4619S, Cell Signaling Technology), phosphorylated (Ser473) Akt (1:1000, #9271L, Cell Signaling Technology), Erk1/2 (1:1000, #4675, Cell Signaling Technology), and phosphorylated Erk1/2 (1:1000, #4370S, Cell Signaling Technology).

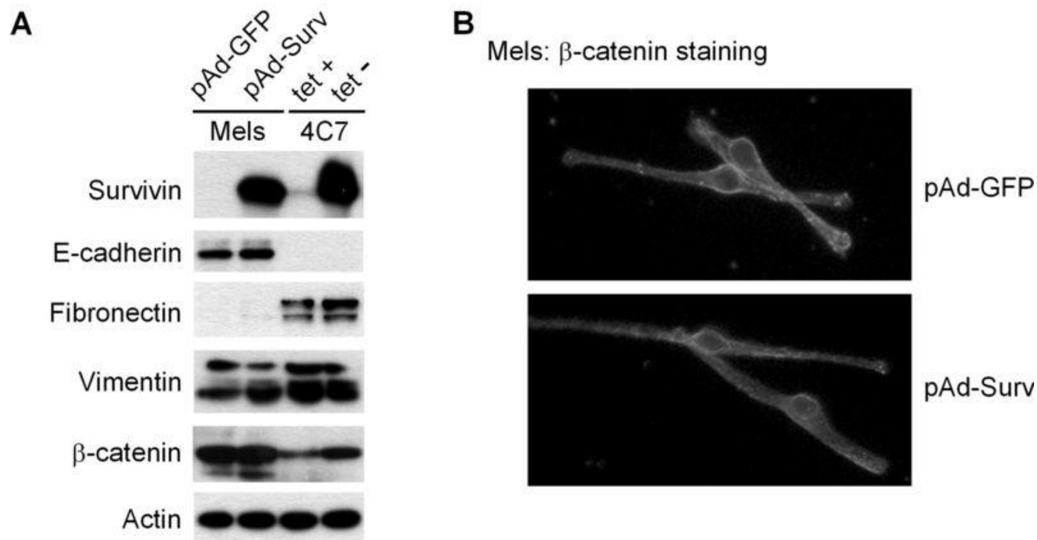
Supplemental figures



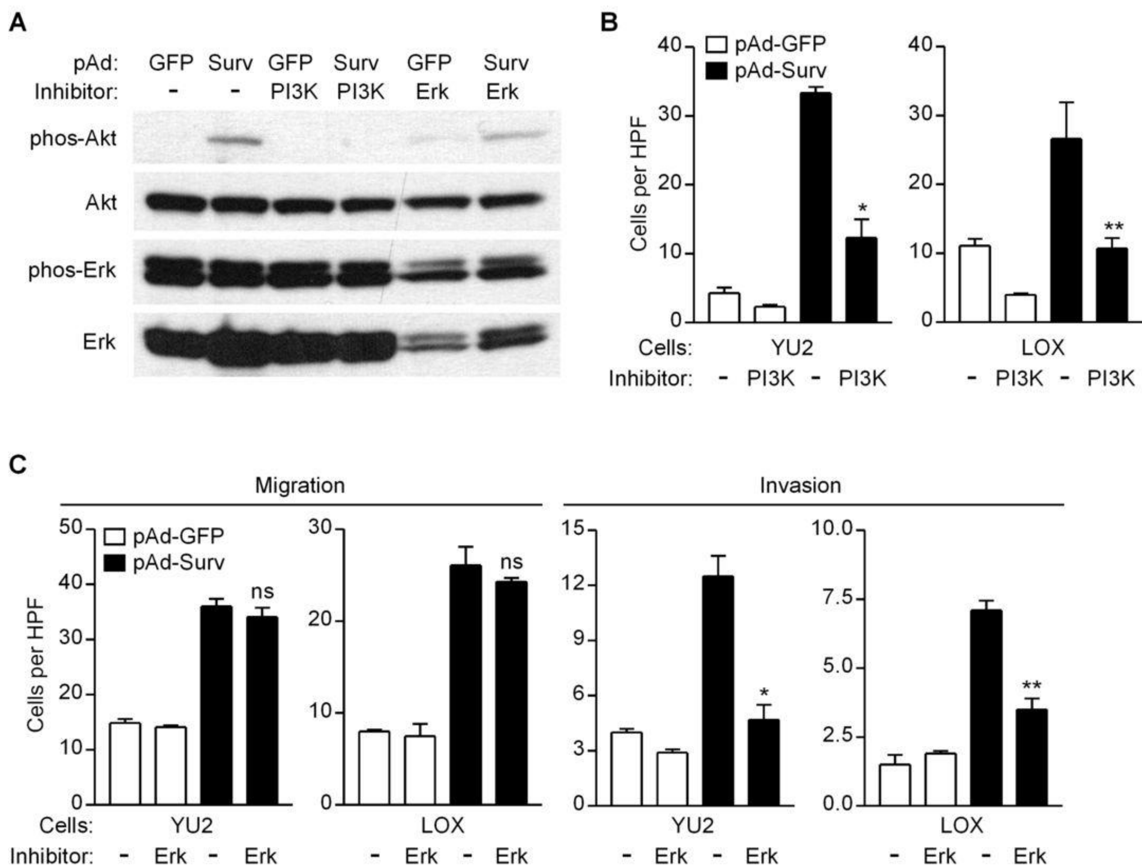
Supplemental figure S2.1 Survivin expression confers only modest protection against anoikis. Human melanocytes were infected with pAd-GFP or pAd-Surv, then 24 h later were plated into Polyhema-coated wells. *A*, Western blot demonstrating forced survivin expression in melanocytes 48 h after pAd infection. *B*, Cells (pAd-Surv) adhere to control but not to Polyhema-coated dishes. *C*, Cells were stained with propidium iodide and analyzed by flow cytometry 48 h after plating. Representative histograms with bars indicating sub-G1 apoptotic fraction are shown. *D*, Quantitation of apoptotic fractions, based on triplicate determinations. *$P<.01$.



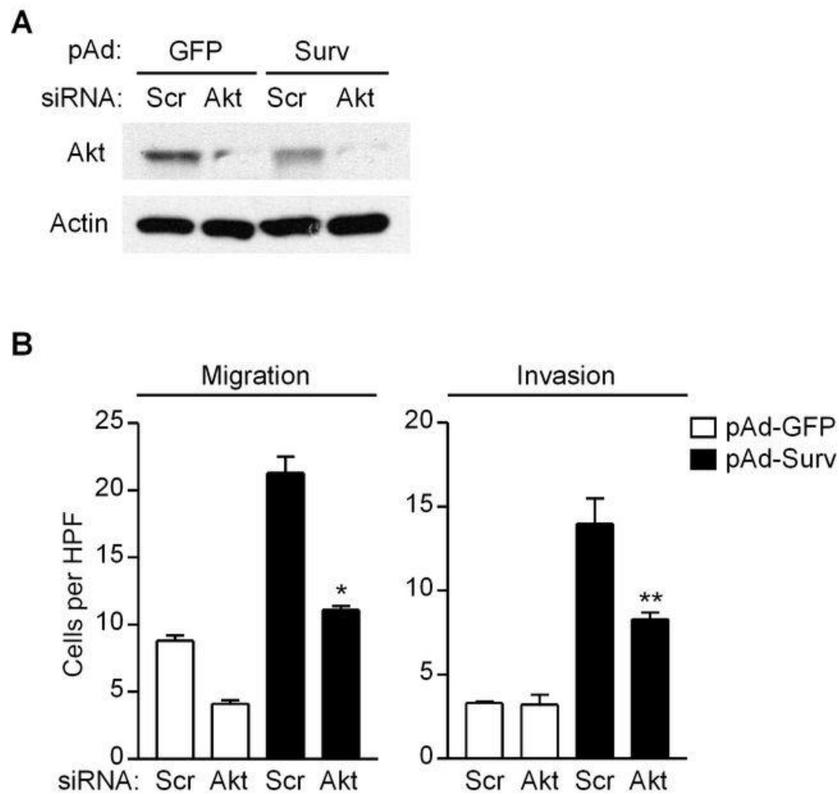
Supplemental figure S2.2 Representative images of migrating melanocytes and melanoma cells. *A,* DAPI-stained melanocytes (Mels) and melanoma cells (LOX, YU8) that were infected with pAd-GFP or pAd-Surv viruses, then allowed to migrate through transwell inserts. *B*, DAPI-stained melanoma cells (LOX, YU8) that were transfected with scrambled (Scr) or survivin-specific RNAi prior to transwell assay.



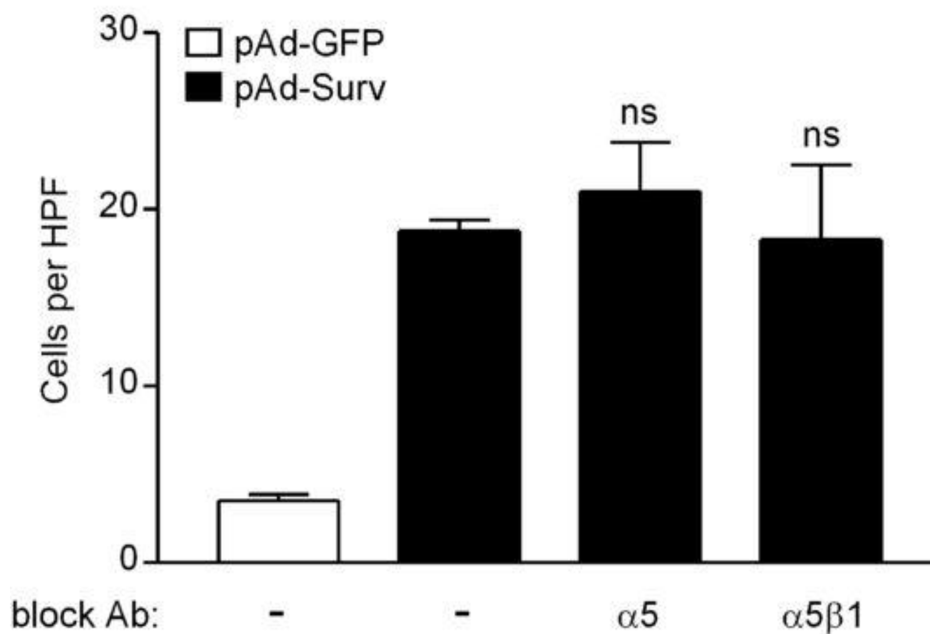
Supplemental figure S2.3 Survivin expression does not alter markers of EMT. *A*, Melanocytes were infected with pAd-GFP or pAd-Surv for 48 h. 4C7 melanoma cells were cultured for 48 h in the presence (tet+) or absence (tet-) of tet to repress or induce survivin over-expression, respectively. Cell lysates were analyzed by western blotting. *B*, Melanocytes infected with pAd-GFP or pAd-Surv for 48 h were plated onto coverslips, stained for β-catenin (1:100 dilution for 1 h) then Texas Red-conjugated goat anti-rabbit IgG (2 μg per ml for 30 min).



Supplemental figure S2.4 Effect of Akt and MAPK pathway inhibition on survivin-enhanced motility. *A*, YU2 melanoma cells were infected with pAd-GFP or pAd-Surv, then 24 h later treated with diluent, 6 μM PI3K inhibitor wortmanin, or 50 μM Erk inhibitor PD98059. After 24 h, cells were subjected to western blotting. *B*, YU2 and LOX melanoma cells were infected with pAd-GFP or pAd-Surv, then 24 h later treated with diluent or 6 μM PI3K inhibitor wortmanin. After 24 h, cells were placed in transwells and assessed for migration (24 h) in the transwell assay. Error bars represent SEM of three determinations. *P=.002, **P=.04. *C*, YU2 and LOX cells were infected with pAd-GFP or pAd-Surv, then 24 h later treated with diluent or 50 μM Erk inhibitor PD98059. After 24 h, cells were placed in chambers and migrating (*left*) or invading (*right*) cells were quantitated 24 and 48 h later, respectively. Error bars represent SEM of three determinations. *P=.004, **P=.002. ns, not significant.



Supplemental figure S2.5 Akt knockdown blocks survivin-enhanced motility. *A*, LOX cells were infected with pAd-GFP or pAd-Surv, then 24 h later transfected with either scrambled (Scr) or Akt-specific siRNA. After 48 h, cell lysates were subjected to western blotting. *B*, LOX cells were infected with pAd-GFP or pAd-Surv, then 24 h later transfected with either scrambled (Scr) or Akt specific siRNA. After 24 h, cells were placed into chambers for motility assays and migrating (24 h, left) and invading (48 h, right) were quantitated. Error bars represent SEM of three determinations. *P=.001, **P=.02.



Supplemental figure S2.6 Effect of α5 integrin blocking antibodies on survivin-enhanced invasion. LOX melanoma cells were infected with pAd-GFP or pAd-Surv viruses, then after 24 h were added to Matrigel-coated inserts in the presence of antibodies against α5 or α5β1 as indicated. Media was replaced after 3 h, then invading cells were quantitated 48 h later. Error bars represent SEM of three determinations. ns, not significant.

CHAPTER 3

SURVIVIN PROMOTION OF MELANOMA METASTASIS REQUIRES ALPHA 5 INTEGRIN

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McKenzie JA, Liu T, Jung JY, Jones BB, Welm A, and Grossman D. (2012)

Survivin promotion of melanoma metastasis requires upregulation of α_5 integrin

Abstract

Survivin is an apoptotic and mitotic regulator that is overexpressed in melanoma, and a poor prognostic marker in patients with metastatic disease. We recently showed that survivin enhances melanoma cell motility through Akt-dependent upregulation of α_5 integrin. However, the functional role of survivin in melanoma metastasis is not clearly understood. We found that overexpression of survivin in LOX and YUSAC2 human melanoma cells increased colony formation in soft agar, and this effect was abrogated by knockdown of α_5 integrin by RNA interference. We employed melanoma cell xenografts to determine the *in vivo* effect of survivin overexpression on melanoma metastasis. While survivin overexpression did not affect primary tumor growth of YUSAC2 or LOX s.c. tumors, or indices of proliferation or apoptosis; it significantly increased expression of α_5 integrin in the primary tumors and formation of metastatic colonies in the lungs. Additionally, survivin overexpression resulted in enhanced lung colony formation following i.v. injection of tumor cells. Importantly, *in vivo* inhibition of α_5 integrin via intraperitoneal injection of an $\alpha_5\beta_1$ integrin-blocking antibody significantly slowed tumor growth and reduced survivin-enhanced pulmonary metastasis. Knockdown of α_5 integrin in cells prior to i.v. injection also blocked survivin-enhanced lung colony formation. These findings support a direct role for survivin in melanoma metastasis which requires α_5 integrin, and suggest that inhibitors of α_5 integrin may be useful in combating this process.

Introduction

Melanoma arises from epidermal melanocytes, and is the deadliest type of skin cancer, due to its high propensity for metastasis.[1] Metastatic spread of tumor cells from a primary tumor to secondary organs is a highly specialized program that involves tumor cell migration, survival in the circulation, extravasation, and colonization of distant sites.[2] Despite recent therapeutic breakthroughs for metastatic melanoma with the approval of the BRAF kinase inhibitor Vemurafenib[3] and the anti-Cytotoxic T-Lymphocyte-associated Antigen 4 (CTLA4) monoclonal antibody Ipilimumab,[4] there still remains a dismal prognosis for patients with advanced disease as in the majority of patients their tumors develop mechanisms of resistance.[5, 6] Therefore, defining molecular mechanisms that govern melanoma progression may aid the development of more effective therapeutic strategies for combating melanoma metastasis.

Survivin, an inhibitor of apoptosis protein (IAP), is overexpressed in melanoma and has been identified as a biomarker of poor prognosis in metastatic disease.[7, 8] Survivin is expressed embryonically, silenced in most adult tissues, and aberrantly reactivated in malignant cells.[9] Both anti-apoptotic and pro-mitotic roles for survivin have been described in malignancies including melanoma.[10, 11] However, the role of survivin in melanoma metastasis has not been elucidated. We previously reported that survivin is required for constitutive melanoma cell motility and its overexpression promotes motility through the Akt-dependent upregulation of α_5 integrin.[12] These studies suggested a potential alternate role for survivin, independent of apoptotic or mitotic regulation, in melanoma metastasis.

Integrins are a family of α-β heterodimeric transmembrane proteins which act as receptors for cell adhesion molecules, and mediate bi-directional signaling between cells and the extracellular matrix.[13] The diverse pairings of α and β integrin subunits regulate signaling pathways that regulate proliferation, differentiation, apoptosis and motility,[14] suggesting a role for integrin-mediated signaling in tumor cell development and metastasis. Indeed, integrin expression is associated with metastatic phenotype in melanoma,[15] and signaling through $\alpha_V\beta_3$ integrin can promote melanoma metastasis in mice.[16] As noted above, we previously implicated α_5 integrin in survivin-enhanced melanoma cell motility *in vitro,*[12] suggesting a potential role for this integrin in melanoma metastasis *in vivo*.

Here we demonstrate that survivin promotes melanoma metastasis *in vivo* in an α_5 integrin-dependent manner. In this study, we investigated the effect of survivin on melanoma metastasis using of an orthotopic mouse model with melanoma cells overexpressing survivin. We found that survivin overexpression resulted in upregulation of α_5 integrin expression *in vivo*, and enhanced colony formation and metastasis to the lungs following either s.c or i.v. injection. These pro-metastatic effects of survivin were dependent on tumor cell expression of α_5 integrin.

Materials and methods

Cell culture

Human melanoma cell lines (LOX, and YUSAC2), were maintained and passaged as previously described.[12]

Survivin-expressing lentivirus

The survivin gene was PCR-amplified from human melanocyte cDNA. The PCR product was cloned into pSC-B-Amp/Kan (Stratagene, Agilent Technologies, Santa Clara, CA), confirmed by sequencing, and then subcloned into the modified pEI2 lentiviral expression vector obtained from Bryan Welm (Huntsman Cancer Institute, Salt Lake City, UT) which has been described previously.[33] Validation of the lentiviral construct and production of the virus were carried out as described.[34] For melanoma cell infection, 8 μg per mL Polybrene (AB01643-00001, Natick, MA) were added as a lipophilic reagent. Cells were maintained in culture as stable lines which consisted of >95% GFP-positive cells.

Immunoblotting

Western blotting was performed as previously described.[12]

Invasion and migration assays

Transwell migration assays were performed as previously described.[12]

Colony formation assay

The underlayer mix was created by mixing equal parts of a 1.6% SeaPlaque Genetic Technology Grade (GTG) agarose (50111, Lonza, Allendale, NJ) solution with 2X Iscove's medium (12200-036, Invitrogen Life Technologies, Grand Island, NY) containing 20% FBS and 2% penicillin-streptomycin-glutamine (PSG, Invitrogen), to give a final underlayer mix of 0.8% GTG agar with 10% FBS and 1% PSG. Underlayer mix (2 mL) was poured into sterile 6-cm plates, and allowed to solidify on a level surface at 4°C. The cell layer, consisting of 5×10^5 (LOX) or 2.5×10^5 (YUSAC2) cells in

Iscove's medium containing 0.4% GTG agarose, 5% FBS, and 0.5% PSG in 5 mL was gently poured onto the solidified underlayer in each plate. Plates were incubated at 37 ^{o}C, monitored daily, and media was replenished as needed to prevent dehydration of the agarose. After 11 d, colonies were photographed and quantitated using ImageJ software. We found that 11 d was the optimal time for growth using these cell lines. For soft agar assays incorporating RNAi, cells were seeded into agar 24 h after transfection with a scrambled or an α_5 integrin-specific siRNA. The oligonucleotides and RNAi transfection protocol are previously described.[12]

Mice

NOD.CB17/SCID mice were obtained from Jackson Laboratories (Bar Harbor, ME). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Utah.

Spontaneous metastasis assay

A total of 2 x 10^6 cells in 0.25 mL PBS were injected s.c. into the right flank. Once tumors could be palpated, the long and short dimensions (d_1, d_2 respectively) were measured using digital calipers twice weekly. Dimensions were later used to calculate tumor volume using the following equation $V = d_1 x (d_2 x d_2)/2$. Mice were sacrificed once tumors approached 2 cm^3 or the experimental endpoint was reached, and then primary tumors and lungs were harvested. For paraffin sections, tissues were fixed in 10% neutral buffered formalin and later embedded in paraffin. For cryosections, lungs were fixed in 4% paraformaldehyde and later embedded in Optimal Cutting Temperature compound (4583, Fisher Scientific, Pittsburgh, PA). For assays involving blocking of α_5

integrin, once tumor dimensions (d_1 and d_2) were approximately 0.5 cm, mice were injected i.p. twice weekly for 4 weeks with either M200 (Volociximab, 10 mg per kg) or the same dose of a control mouse IgG antibody (GWB-275D92, GenWay Biotech, San Diego, CA). M200 is an $\alpha_5\beta_1$ integrin-blocking antibody, kindly provided by Abbott Laboratories (Abbott Park, IL).

Tail vein metastasis assay

Mice were injected with 5 x 10^5 cells in 0.25 mL PBS through the tail vein. After 24 h or 14 d, mice were euthanized, their lungs resected and fixed in 4% paraformaldehyde and then embedded in Optimal Cutting Temperature compound for cryosectioning. The 24 h time point was used as an indicator of immediate extravasation, while 14 d served as a delayed time point as others have done.[35] GFP-positive tumor cells were visualized by fluorescence microscopy.

Immunohistochemistry

Sections were de-waxed by citrus clearing solvent (8301, Fisher Scientific) and rehydrated using a decreasing ethanol gradient from 100% to 70%. Antigen retrieval was performed by boiling for 20 min in sodium citrate (pH 6.0). Immunohistochemistry was performed on 5-μm tissue sections using an immunoperoxidase method (K4010, Dako, Agilent Technologies), with rabbit polyclonal antibodies against human α_5 integrin (1:300, HPA002642, Sigma, St. Louis, MO), or phosphohistone H3 (Ser10) (1:200, 9701, Cell Signaling Technology, Danvers, MA).

Tunel assay

Paraffin-embedded tumor sections (5 µm) were dewaxed and rehydrated as described above. Sections were then incubated with proteinase K (01115879001, Roche, Genetech, San Francisco, CA) at 10 µg per ml for 1 h at room temperature. Slides were then stained using the In Situ Cell Death Detection Kit TMR Red (12156792910, Roche), according to the manufacturer's protocol.

Statistics

Data are expressed as SEM of multiple replicates. All data presented are representative of at least two independent experiments. P-values were generated by two-tailed *t*-test using Prism Graphpad software (San Diego, CA).

Results

Survivin overexpression enhances melanoma cell metastatic potential

To examine the functional role of survivin in melanoma metastasis, we utilized two human metastatic melanoma cell lines LOX and YUSAC2 (YU2) to generate *in vivo* models. LOX and YU2 were stably transfected with a control lentivirus expressing GFP, or a lentivirus expressing GFP-tagged survivin. Western blotting confirmed survivin overexpression and upregulation of α_5 integrin in both lines (Figure 3.1a). We then verified their increased migratory capacity in Transwell Migration Boyden chambers. Indeed, both LOX and YU2 lines transfected with GFP-survivin demonstrated a greater than two-fold increase in the number of migratory cells compared to their GFP-transfected counterparts (Figure 3.1b).

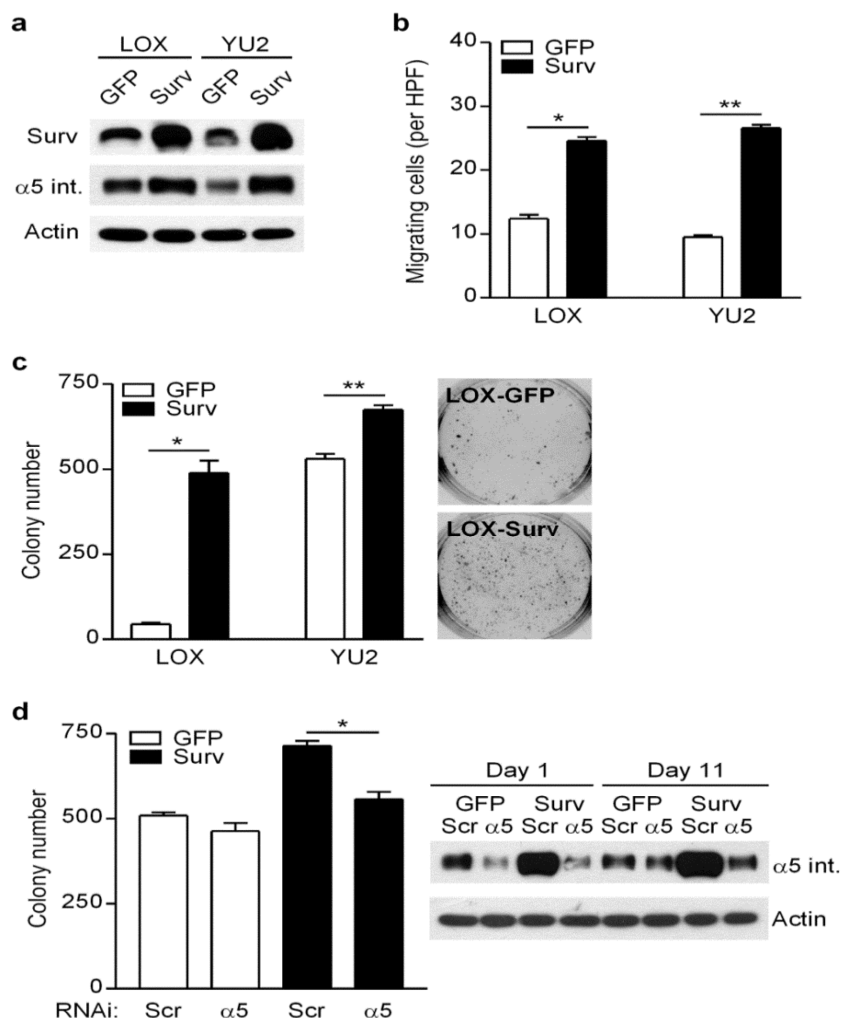

Figure 3.1 Survivin promotes melanoma cell migration and colony formation. (**a**) LOX and YUSAC2 (YU2) melanoma cells were infected with either GFP lentivirus (control) or a GFP-tagged survivin (Surv) expressing lentivirus, then 48 h later cell lysates were prepared and analyzed by western blotting for survivin, α_5 integrin, and actin. (**b**) LOX and YU2 cells stably overexpressing GFP or GFP-survivin (Surv) were added to transwell chambers (5 x 10^4 cells per well) with fibronectin-coated inserts. Migratory cells (per high-power field, hpf) were quantified 24 h later. Error bars represent SEM of triplicate determinations. *P < 0.001, **P < 0.0001. (**c**) LOX (5 x 10^5) and YU2 (2.5 x 10^5) cells overexpressing GFP or GFP-survivin (Surv) were seeded in soft agar for 11 d. Cell colonies were then photographed and quantified. Representative dishes for LOX cells shown at right. Error bars represent SEM of triplicate determinations. *P = 0.007, **P = 0.005. (**d**) YU2 cells overexpressing GFP or GFP-survivin (Surv) were transfected with scrambled (Scr) or α_5 integrin-specific siRNA. After 24 h, cells were placed in soft agar assay as in (c). Representative western blot showing knockdown of α_5 integrin shown at right. Error bars represent SEM of triplicate determinations. *P = 0.003.

Next, we examined the effect of survivin overexpression on the capacity of melanoma cells to form anchorage-independent colonies in soft agar. As shown in Figure 3.1c, LOX and YU2 cells overexpressing GFP-survivin yielded more numerous colonies than GFP-transfected control cells. To determine if α_5 integrin is required for survivin-enhanced colony formation, control and Survivin-overexpressing YU2 cells were transfected with a control siRNA or siRNA targeting α_5 integrin prior to seeding in soft agar. Knockdown of α_5 integrin effectively negated survivin-enhanced colony formation (Figure 3.1d). Thus these *in vitro* studies indicate that survivin enhances prometastatic activities in melanoma cells.

Survivin overexpression increases melanoma pulmonary metastasis in vivo

To determine whether survivin overexpression in melanoma cells could enhance metastasis *in vivo*, we performed a spontaneous metastasis assay in which control or survivin-overexpressing cells were injected s.c. into the flank of immunodeficient mice. Tumor size was monitored twice weekly for 6 weeks, then primary tumors were harvested and lungs were resected for analysis. Tumors grew rapidly, but tumor growth was not significantly affected by survivin overexpression in YU2 cells (Figure 3.2a). To further investigate the mechanics of tumor growth, we performed histological analyses to determine the proliferative and apoptotic indices of these tumors. TUNEL staining of tumor sections indicated no significant difference in apoptotic rate in survivin-overexpressing vs. control YU2 cells (Figure 3.2b). Similarly, no significant differences

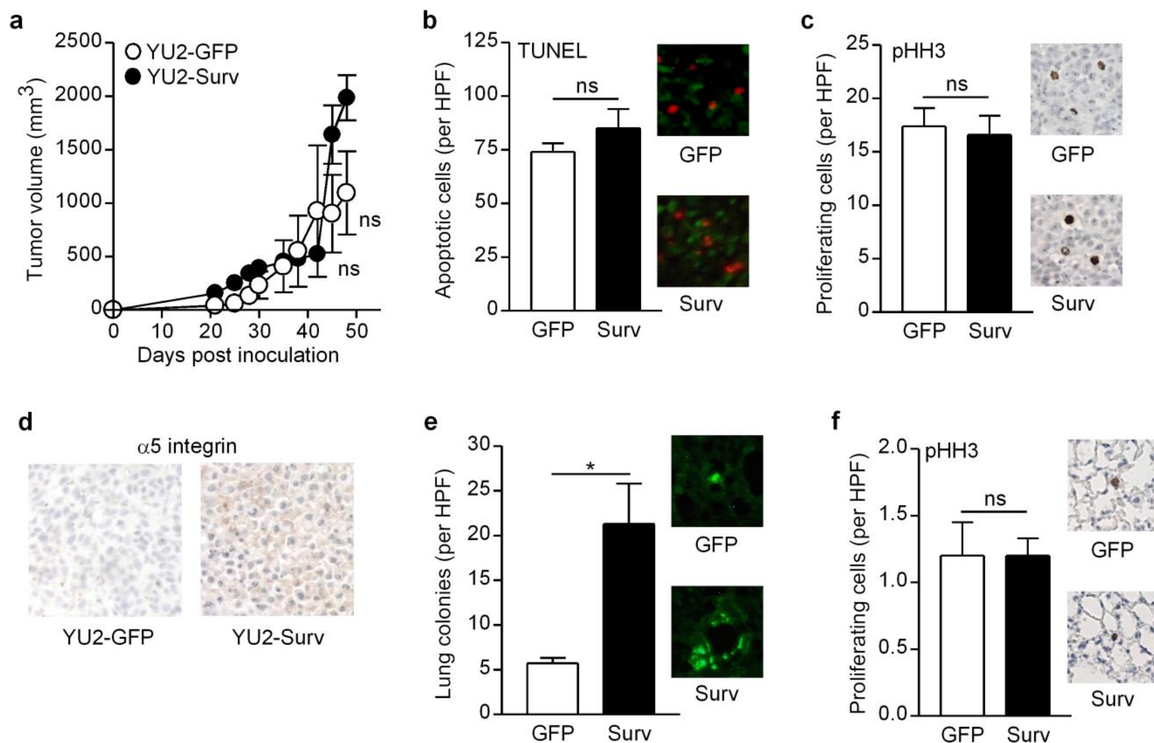

Figure 3.2 Survivin promotes metastasis of yusac2 melanoma cells *in vivo*. (**a**) YUSAC2 (YU2) cells stably overexpressing GFP or GFP-survivin (Surv) were injected s.c. into the flank of immunodeficient mice. Once tumors were palpable, they were measured twice weekly for 6 weeks. Error bars indicates SEM of 4-5 mice per group. ns, not significant. Results representative of two experiments performed. (**b**) TUNEL staining was performed, and apoptotic cells per high power field (HPF) are indicated (left panel). ns, not significant. Representative images of GFP-positive tumor cells (green) and TUNEL-positive apoptotic cells (red) are shown (right panel). (**c**) Staining tumor sections for phospho-histone H3 (pHH3) allowed quantification of proliferating cells per high power field (HPF) as indicated (left panel). ns, not significant. Representative images are shown (right panel). (**d**) Representative images of immunostaining of tumor sections for α_5 integrin. (**e**) Quantification of GFP-positive metastatic foci in lungs from mice in (a) are indicated per high power field (HPF) (left panel). Error bars indicate SEM. *P = 0.006. Representative images showing GFP-positive metastatic cells in lungs (right panel). (**f**) Phospho-histone H3 immunostaining of lung sections per high power field (HPF) as indicated (left panel). ns, not significant. Representative images are shown (right panel). All images in figure taken at x200 magnification.

were seen in proliferation as measured by phospho-histone H3 staining in survivin-overexpressing vs. control cells (Figure 3.2c). Immunohistochemistry for α_5 integrin confirmed higher levels of expression in survivin-overexpressing vs. control YU2 tumors (Figure 3.2d). Pulmonary metastases were quantified by counting GFP positive foci in serial lung sections. Notwithstanding the similarity in tumor growth, a significant increase in the number of lung metastases was seen in mice bearing survivin-overexpressing vs. control YU2 tumors (Figure 3.2e). We could not identify apoptotic tumors cells in the lungs by TUNEL staining (not shown). Importantly, there was no difference in proliferative activity of tumor cells in the lungs (Figure 3.2f), suggesting that the greater number of survivin-overexpressing cells in the lungs was a result of metastasis rather than expansion following metastasis. Similar findings were observed in companion experiments with survivin-overexpressing and control LOX tumors (Supplemental Figure S3.2). These results indicate that survivin overexpression in melanoma cells leads to increased expression of α_5 integrin and promotes lung metastasis.

Given that survivin promoted lung metastasis without affecting tumor growth; we next assessed the capacity of survivin-overexpressing cells to establish lung metastases following injection directly into the tail vein. This approach bypasses the need to navigate from the primary tumor, and measures the ability of tumor cells to survive in the bloodstream, extravasate, and colonize the lung.[17] We injected YU2-GFP or YU2-Surv cells into the tail vein, then sacrificed mice and resected the lungs to measure extravasated cells (24 h post injection) or metastatic colonies (14 d post injection). Both control and survivin-overexpressing cells showed similar number of extravasated cells in

the lungs 24 h post injection (Figure 3.3a), suggesting that survivin overexpression does not affect melanoma cell survival in the circulation or capacity to extravasate in the lungs. No difference in tumor cell proliferation in the lungs was observed either (Figure 3.3b). However, examination of the lungs 14 d following i.v. injection revealed a significant increase in lung metastases in mice injected with survivin-overexpressing cells compared to control cells (Figure 3.3c). The lack of difference in pHH3 staining (Figure 3.3d) confirmed that increased numbers of cells had become established in the lungs, rather than proliferating after reaching the lungs. Thus while survivin overexpression does not appear to affect primary tumor growth or immediate extravasation of cells into the lungs, there is enhanced capacity of these cells to establish metastatic colonies in the lung.

α_5 integrin is required for survivin-enhanced pulmonary metastasis

Our finding that overexpression of survivin in primary tumors correlated with higher expression levels of α_5 integrin (Figure 3.2d) is consistent with our previous *in vitro* finding that survivin overexpression results in upregulation of α_5 integrin in melanoma cells.[12] To determine if increased α_5 integrin expression is critical for survivin-enhanced lung metastasis, we repeated the spontaneous metastasis assay using s.c. injected cells and incorporated an $\alpha_5\beta_1$ integrin-blocking antibody (M200). We first confirmed that M200 effectively blocked survivin-enhanced melanoma cell motility *in vitro*, while a control IgG antibody did not (not shown). Mice with survivin-overexpressing tumors (YU2-Surv) were injected i.p. with M200 or the control antibody twice weekly for four weeks, and compared to mice with YU2-GFP tumors injected with

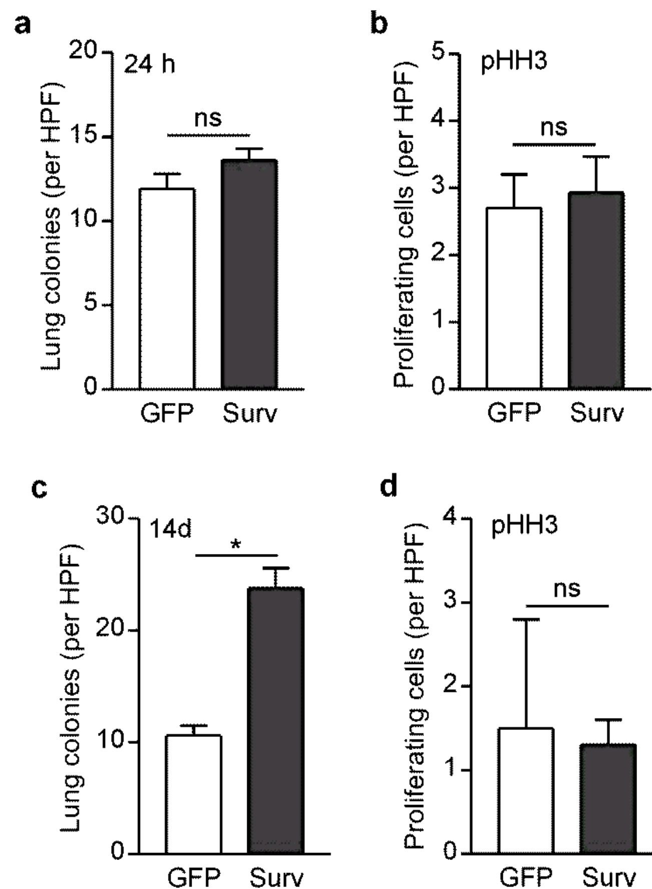

Figure 3.3 Survivin overexpression enhances melanoma cell colonization of the lungs. (**a**) YU2-GFP or YU2-Surv cells were injected into the tail vein of immunodeficient mice. Mice were sacrificed 24 h later, and the lungs resected and cryosectioned. Number of GFP-positive cells per high power field (HPF) was counted in lung sections. Error bars indicate SEM from 5 mice. ns, not significant. Representative of two experiments performed. (**b**) Proliferating tumor cells in the lungs from mice in (a) were assessed by phospho-histone H3 (pHH3) staining. Error bars indicate SEM. ns, not significant. (**c**) YU2-GFP or YU2-Surv cells were injected as in (a), then mice were sacrificed 14 d later. GFP-positive foci were quantified and expressed per high power field (HPF). Error bars indicate SEM from 5 mice. *$P < 0.001$. Representative of two experiments performed. (**d**) Phospho-histone H3 (pHH3) staining of lungs from mice in (c). Error bars indicate SEM. ns, not significant.

the control antibody. We found that tumor growth was similar between YU2-GFP and YU2-Surv tumors in mice treated with the control antibody (Figure 3.4a, Supplemental Figure S3.3). However, there was a significant reduction in the growth of YU2-Surv tumors in mice treated with M200 (Figure 3.4a, Supplemental Figure S3.3). TUNEL analysis revealed that while the level of apoptosis was similar between the control antibody-treated groups, the survivin-overexpressing tumors in mice treated with M200 contained significantly more apoptotic cells (Figure 3.4b). Similarly, M200 treatment of YU2-Surv tumors was associated with decreased tumor cell proliferation (Figure 3.4c). Consistent with our findings in Figure 2d, YU2-Surv tumors exhibited higher levels of α_5 integrin compared to control tumors in animals treated with the control antibody (Figure 3.4d). However, expression of α_5 integrin was noticeably reduced in YU2-Surv tumors from animals treated with M200 (Figure 3.4d). Consistent with our findings in Figure 3.2e, we found that among mice treated with the control antibody that those with YU2-Surv tumors had more lung metastases than mice with YU2-GFP tumors (Figure 3.4e). Importantly, examination of the lungs from mice bearing YU2-Surv tumors treated with M200 revealed fewer metastatic lung colonies (Figure 3.4e). Finally, we investigated whether the mitigating effect of α5 integrin inhibition on survivin-enhanced metastasis was related to, or independent of, decreased growth of the primary tumor. To address this question, we examined the effect of knocking down enhanced α5 integrin expression in YU2-Surv cells on their capacity to form lung mets following i.v. delivery. There was no precedent for administering M200 i.v., and we had shown that RNAi-mediated depletion of α5 integrin was effectively sustained for up to 11 d (Figure 3.1d). As shown in Figure 3.4f, RNAi-mediated knockdown of α5 integrin abrogated the survivin-

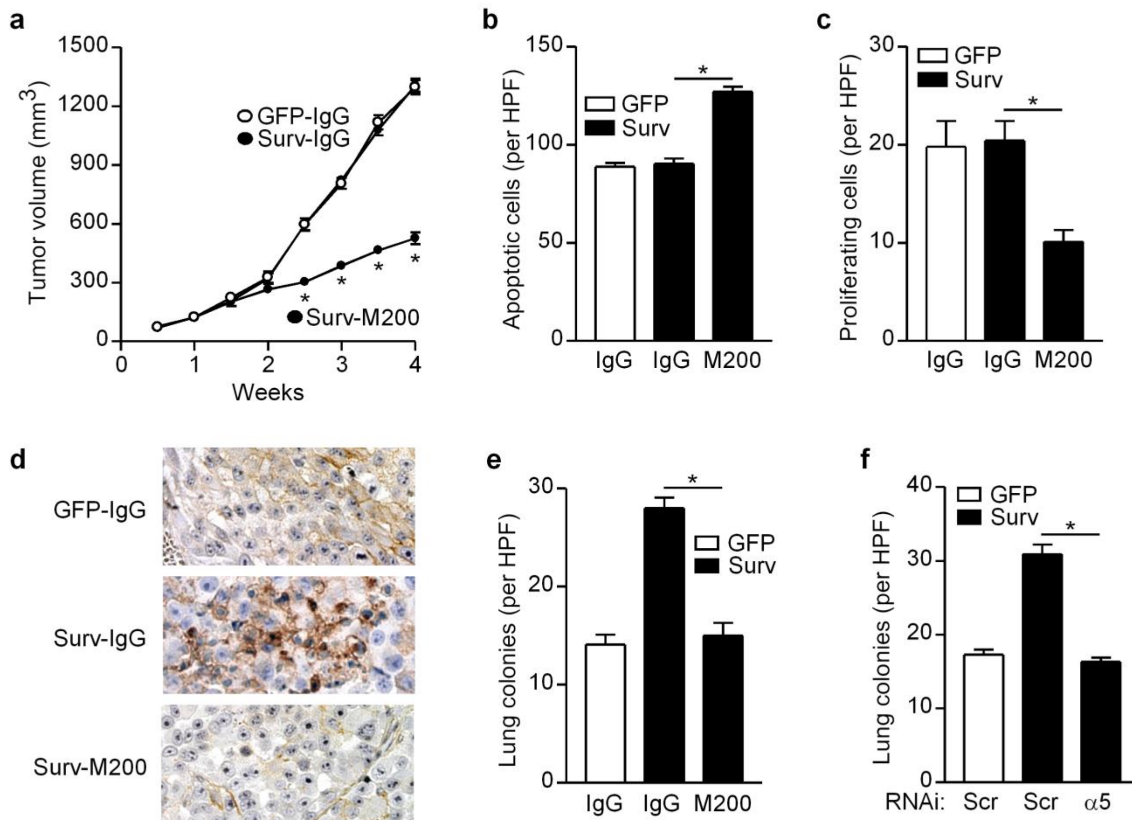

Figure 3.4 Survivin-enhanced pulmonary metastasis is dependent on α_5 integrin. (**a**) YU2-GFP or YU2-Surv cells were injected s.c. into the flank of immunodeficient mice. Once tumors were palpable, mice were injected twice weekly with M200 or a control (IgG) antibody (10 mg per kg) as indicated for 4 weeks. Error bars indicate SEM of tumor measurements in 8 mice per group. $^*P < 0.001$. Representative of two experiments performed. (**b**) TUNEL staining of tumors in (a) at experimental endpoint. Error bars indicate SEM of apoptotic cells per high power field (HPF) in 8 tumors per group. $^*P < 0.0001$. (**c**) pHH3 staining of tumors in (a) at experimental endpoint. Error bars indicate SEM of proliferating cells per high power field (HPF) in 8 tumors per group. $^*P = 0.0005$. (**d**) Representative images of α_5 integrin immunostaining of tumor sections from each group. (**e**) Lungs from mice in (a) were sectioned and number of GFP-positive foci were quantified and expressed per high power field (HPF). Error bars indicate SEM from 7-8 mice per group. Lungs could not be recovered from one mouse in the Surv-IgG group. $^*P < 0.001$. Representative of two experiments performed. (**f**) YU2-GFP or YU2-Surv cells were transfected with scrambled (Scr) or α_5 integrin-specific siRNA. After 24 h, cells were injected into the tail vein of immunodeficient mice. Mice were sacrificed 14 d later, and the lungs resected and cryosectioned. Number of GFP-positive cells per high power field (HPF) in lung sections is indicated. Error bars indicate SEM from 5 mice. $^*P < 0.0001$. Representative of two experiments performed. All images in figure taken at x200 magnification.

enhanced pulmonary metastasis. These *in vivo* results support our hypothesis that survivin promotes melanoma cell metastasis in an α_5 integrin-dependent manner.

Discussion

Survivin was originally identified as an inhibitor of apoptosis,[18] although subsequent studies revealed it to be a bifunctional protein that regulates both apoptosis and mitosis.[19] Survivin has been widely implicated in metastasis and chemoresistance in a number of malignancies including melanoma,[7, 8] breast cancer,[20] and colorectal cancer.[21] We recently reported that survivin is required for constitutive motility of melanoma cells, and that survivin overexpression increases melanoma cell motility *in vitro* via upregulation of α_5 integrin.[12] Here we demonstrate that survivin can promote melanoma cell colony formation and metastasis to the lungs in an α_5 integrin-dependent manner.

The anti-apoptotic and mitotic regulatory functions of survivin depend on cellular context and compartmentalization of survivin signaling networks.[22] In our earlier work, we showed that survivin depletion in melanoma cells reduced their motility without inducing apoptosis, and that promotion of motility in survivin-overexpressing cells was evident in the presence of a mitosis inhibitor.[12] In our experimental *in vivo* system, enhancement of melanoma metastasis by survivin also appeared to be independent of any potential apoptotic or mitotic effects. While survivin-overexpressing tumors were more metastatic, we did not observe significant differences in growth rates compared to control tumors. The lack of effect of survivin overexpression on tumor growth, consistent with our finding of no alterations in either apoptotic or mitotic indices in the tumors, suggests

that the pro-metastatic effect of survivin could not be attributed to an effect on primary tumor growth. When tumor cells were injected i.v., there was no immediate (at 24 h) effect of survivin overexpression on lung colonization, suggesting that the effect of survivin was not simply to increase cell survival in the bloodstream. Rather, our results suggest that survivin promotes extravasation and survival (but not proliferation) of tumor cells that reach the lungs, as higher numbers of lung colonies were seen in mice 14 d following i.v. injection of survivin-overexpressing vs. control cells.

We observed that the pro-metastatic effect of survivin overexpression was associated with increased tumor expression of α_5 integrin. We previously noted that α_5 integrin expression was higher in metastatic compared to primary human melanoma cell lines, and is a critical regulator of cell motility downstream of survivin.[12] Others have shown that α_5 integrin increases ovarian cancer metastasis and its expression is predictive of metastasis and poor prognosis in ovarian cancer patients.[23, 24] In melanoma, α_5 integrin promotes *in vivo* metastasis of murine B16F10 melanoma cells.[25] Integrins mediate outside-in and inside-out signaling, which may impact cell growth and death, adhesion, and motility.[26] Integrin-based interactions are also known to be involved in matrix degradation and remodeling, which can aid in tumor cell dissemination from the primary tumor.[27] We considered the possibility that increased α_5 integrin expression promoted dissemination of cells from the tumor into the bloodstream, thereby facilitating seeding of tumor cells in the lungs. To address this issue, we attempted to quantify circulating tumor cells in blood from mice with survivin-overexpressing tumors by quantitative real time polymerase chain reaction for the human melanoma marker melan-A. However, we were unable to detect such circulating cells by this method in any of the

mice over the 6-week period of tumor growth with our limit of detection being 250 melanoma cells in 0.25 mL blood (J.M., T.L. and D.G., data not shown).

Nevertheless, our results suggest that survivin-mediated upregulation of α_5 integrin in tumors promotes subsequent colonization of tumor cells in the lungs. We found that upregulation of α_5 integrin is critical for survivin-enhanced lung metastasis, as evidenced by the reduction in pulmonary metastasis seen when α_5 integrin was targeted with the antibody M200 (Volociximab). M200 treatment had the concomitant effect of reducing tumor growth, associated with higher levels of apoptosis and lower levels of proliferation in tumor cells. These findings are consistent with those of others showing that inhibitory antibodies against α_5 integrin can decrease tumor growth,[28] possibly by inducing apoptosis in tumor-associated endothelial cells.[29] We believe that this diminished tumor growth, however, is unlikely to represent the basis for the decreased metastasis resulting from α_5 integrin inhibition. To address this issue, we employed the modified metastasis assay in which tumor cells are injected i.v. rather than into the skin, which bypasses the effect of the primary tumor on the establishment of lung metastases. We found that knockdown of α_5 integrin in tumor cells prior to injection decreased survivin-enhanced lung colonization, suggesting that the reduced metastasis observed in the spontaneous metastasis assay was not solely due to the inhibitory effect of M200 on primary tumor growth.

In summary, the results of this study support a direct role for survivin and α_5 integrin in promoting melanoma metastasis, and suggest that inhibitors of α_5 integrin like M200 may be useful in combating tumor progression and melanoma metastasis. Indeed, several clinical trials have been conducted examining the efficacy of M200 in metastatic

melanoma and other cancers.[30-32] A phase II trial of M200 in patients with metastatic melanoma found that when combined with dacarbazine, about half of the participants exhibited stable disease at eight weeks, while the remaining patients suffered progressive disease.[32] It is not clear that dosing of M200 was fully optimized to block α_5 integrin in metastatic tumors, and perhaps future trials combining agents that target α_5 integrin and oncogenic kinases (such as BRAF) or immunomodulatory molecules (such as CTLA-4), may prove more fruitful.

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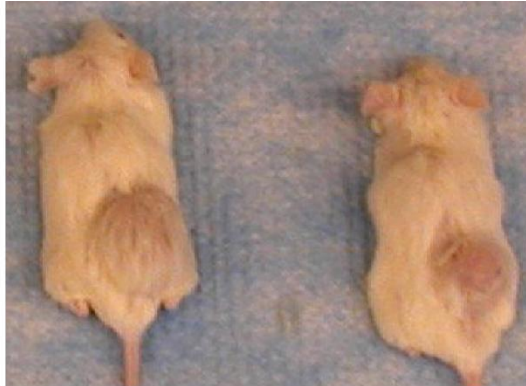
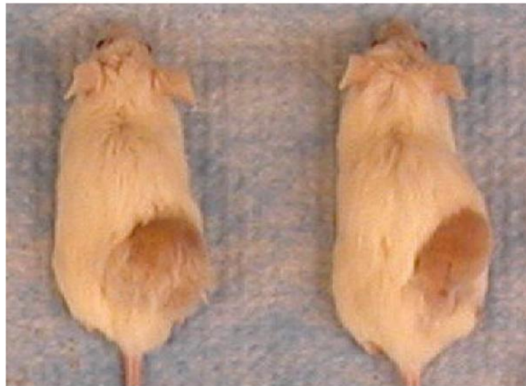
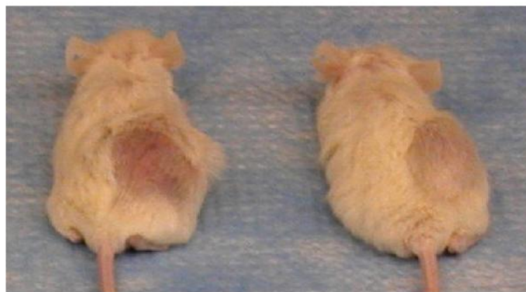
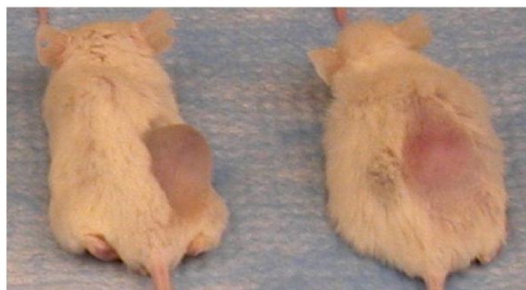
Supplemental figures

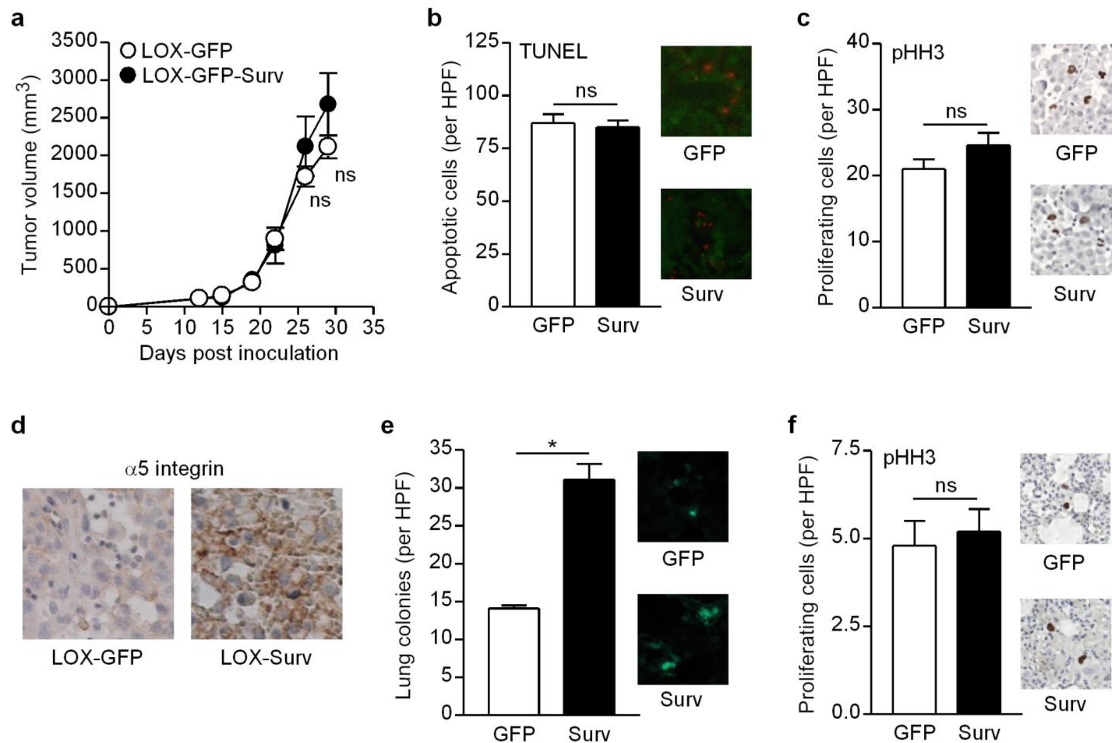




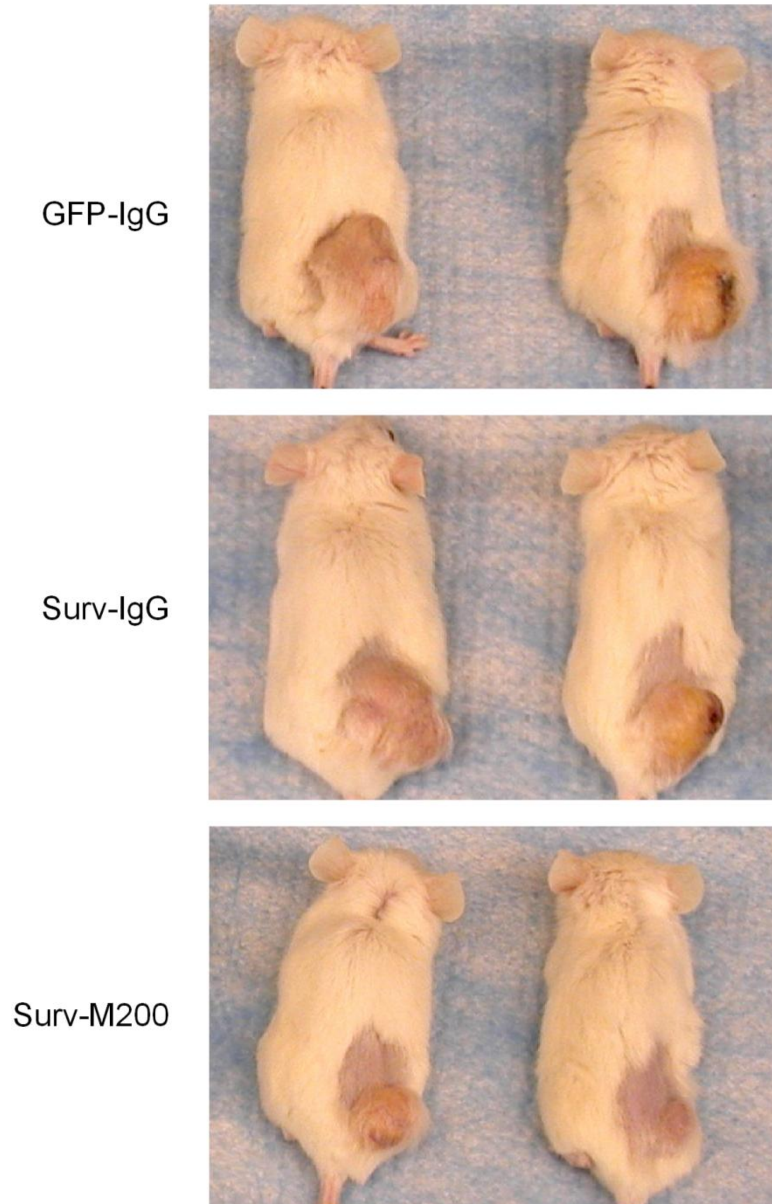




Supplemental figure 3.1 Representative images of mice following s.c. inoculation with YU2 or LOX cells overexpressing GFP or survivin. Images were taken after approximately 6 weeks once mice were sacrificed.



Supplemental figure 3.2 Survivin promotes metastasis of LOX melanoma cells *in vivo*. (**a**) LOX cells stably overexpressing GFP or GFP-survivin (Surv) were injected s.c. into the flank of immunodeficient mice. Once tumors were palpable, they were measured twice weekly for 6 weeks. Error bars indicates SEM of 5 mice per group. ns, not significant. Results representative of two experiments performed. (**b**) TUNEL staining was performed, and apoptotic cells per high power field (HPF) are indicated (left panel). ns, not significant. Representative images of GFP-positive tumor cells (green) and TUNEL-positive apoptotic cells (red) are shown (right panel). (**c**) Staining tumor sections for phospho-histone H3 (pHH3) allowed quantification of proliferating cells per high power field (HPF) as indicated (left panel). ns, not significant. Representative images are shown (right panel). (**d**) Representative images of immunostaining of tumor sections for □$_5$ integrin. (**e**) Quantification of GFP-positive metastatic foci in lungs from mice in (a) are indicated per high power field (HPF). Error bars indicate SEM. *$P < 0.0001$. Representative images showing GFP-positive metastatic cells in lungs (right panel). (**f**) Phospho-histone H3 immunostaining of lung sections per high power field (HPF) as indicated (left panel). ns, not significant. Representative images are shown (right panel). All images in figure taken at x200 magnification.



Supplemental figure 3.3 Survivin-enhanced pulmonary metastasis is dependent on α_5 integrin. Representative images of mice bearing control tumors (YU2-GFP) or survivin overexpressing tumors (YU2-Surv) that were treated with M200 or a control antibody (IgG).

CHAPTER 4

SUMMARY AND PERSPECTIVES

Dissertation summary

Survivin is a nodal protein that has been demonstrated to intersect multiple pathways required for tumor development and progression.[1] It has provided unique opportunities for basic and translational oncology, as it is a pro-oncogenic molecule that is not expressed in normal adult cells but is reactivated in malignant cells. Survivin function in the inhibition of apoptosis in tumor cells is well documented. Studies from our laboratory have demonstrated that survivin can protect melanocytes and melanoma cells from caspase independent and dependent apoptosis.[2, 3] Additionally, inhibition of survivin induced spontaneous apoptosis in melanoma cells and reduced melanoma tumor growth.[4, 5] While its importance in apoptosis goes without question, the fact that survivin is so intimately linked to a number of circuitries in the cell that work together to propagate and maintain tumors, suggests that it plays multiple roles in tumor development and metastasis.[6]

Thomas et al. showed in 2007 that melanocyte-specific expression of survivin *in vivo* decreased the susceptibility of melanocytes to UV-induced apoptosis, and surprisingly, also promoted metastasis.[7] As is highlighted in the seminal review 'The Hallmarks of Cancer,' inhibition of apoptosis is an important feature acquired by cancer cells, which is key for metastasis.[8] However, this function alone may not be sufficient to promote metastasis. Studies using *in vivo* tumor models of survivin expression in uroepithelium[9] and in keratinocytes[10] demonstrated that while survivin expression promoted tumorigenesis, no metastasis was detected. This suggests that in addition to its anti-apoptotic role in melanoma cells, survivin may promote metastasis through other mechanisms. This led us to think about the underlying functions of the cell that would

facilitate melanoma metastasis and how survivin may be involved in these processes. This chapter provides a summary of the main questions addressed in our studies of Survivin function in melanoma metastasis and the approaches used to answer said questions.

As was stated earlier in Chapter 1, metastasis requires migration and invasion of metastatic cells to allow navigation through the primary tumor and intravasation into the circulation; extravasation from vessels into target organs, and colonization of these secondary organs (Figure 1.7). To understand how survivin may be important in melanoma metastasis, we began by asking whether survivin expression in melanocytes and melanoma cells could affect cell motility. We used Transwell migration and Matrigel assays to determine the effect of survivin on migration and invasion, respectively. We found that survivin was sufficient for cell migration and invasion as its expression in melanocytes resulted in increased migration and invasion.[11] We also found that the migratory and invasive capacity of melanoma cells was greatly enhanced following survivin overexpression.[11] Given that survivin has a documented role in mitosis;[12, 13] we asked the question of whether the increased motility effect of survivin was dependent on mitosis. To do that, we conducted the migration and invasion assays under conditions where mitosis was blocked by Mitomycin C, an agent that prevents DNA synthesis and therefore blocks cell division. Under these conditions, melanocytes and melanoma cells were able to migrate and invade more in response to increased levels of survivin. This implies that these pro-metastatic activities were being mediated independent of any potential effect on mitosis. We also demonstrated requirement of survivin for migration and invasion of melanoma cells through the use of RNA interference (RNAi).[11] Under

conditions where apoptosis was not being induced by knockdown of survivin, we demonstrated that melanoma cells were less motile.[11] As such, survivin may not be promoting melanoma metastasis solely through its role as an anti-apoptotic protein.

To further determine how survivin could enhance melanoma cell motility, we carried out microarray analyses of the gene expression profile of control (GFP-expressing) and survivin-expressing human melanocytes (Appendix A.1). The profile revealed that genes involved in extracellular matrix (ECM) interactions, including α_5 integrin, were highly upregulated in survivin expressing cells (Appendix A.1). Cell adhesion molecules like integrins function to regulate cellular processes like migration and invasion.[14] Therefore, we investigated whether survivin could enhance melanoma cell motility via regulation of integrins. By examination of the protein levels of a number of α and β integrins known to be expressed in melanoma,[15] we detected that α_5 integrin levels were elevated in response to survivin overexpression in melanoma cells.[11] Inhibition of α_5 integrin by RNAi or by blocking antibody demonstrated that α_5 integrin function was required for survivin-enhanced migration.

Tumor cells are able to regulate the expression levels of adhesion molecules like integrins to transmit signals that help them to navigate away from the primary tumor.[16] Integrin control of cell movement is mediated by crosstalk with signaling pathways like MAPK, and AKT which as was discussed earlier are heavily altered in metastatic melanoma.[17, 18] Additionally, studies have shown that inhibition of components of these signaling pathways can inhibit melanoma migration and invasion.[19, 20] Our results also support this notion as we have shown that pharmacological inhibition of AKT blocks survivin mediated migration.[11]

We further examined the effect of survivin on melanoma metastasis by using an orthotopic mouse model. In this experimental system, melanoma cells stably overexpressing survivin were injected s.c. into NOD SCID mice and then tumor progression was monitored. These studies revealed that overexpression of survivin did not confer a proliferative advantage to tumors, nor did it inhibit apoptosis. However, survivin overexpression did result in increased expression of α_5 integrin in primary tumors and increased tumor cell metastasis to the lungs. Experimental lung metastasis was also increased with overexpression of survivin. The increase in metastasis was dependent on α_5 integrin, as blocking of α_5 integrin by M200 (inhibitory antibody of $\alpha_5\beta_1$ integrin), reduced the growth of primary tumors and subsequently reduced tumor metastasis to the lungs. The reduction in metastasis in response to blockade of α_5 integrin may be due to the reduced growth of the primary tumor. However, reduced metastasis may also be as a result of α_5 integrin effect at the site of metastasis. In an experimental metastasis assay, tumor cells with reduced levels of α_5 integrin were injected i.v. into the tail vein of NOD SCID mice. This allowed for examination of metastasis without the effects of a primary tumor. Our results showed that melanoma cells with less α_5 integrin were not able to form metastatic colonies in the lung as well as melanoma cells expressing higher levels α_5 integrin.

Perspectives

Survivin localizes to different subcellular compartments,[21, 22] and this allows for a variety of posttranslational modifications including phosphorylation and ubiquitination.[23-25] Consequently, the various modifications may help to distinctly regulate survivin functions.[26] The pro-metastatic roles of survivin may therefore be governed in a cell-type

specific and subcellular context specific manner. Evidence suggests that the cytoprotective function of survivin is carried out by the mitochondrial fraction.[26] Survivin is phosphorylated on Serine-20 (Ser20) by Protein Kinase A (PKA) in the cytosol. Phosphorylation at this site disrupts the binding of Survivin to its antiapoptotic cofactor x-linked inhibitor of apoptosis protein (XIAP), thereby blocking its ability to mediate cytoprotection.[26] However, PKA is unable to phosphorylate survivin in the mitochondria. Consequently, mitochondrial Survivin is able to bind to XIAP and inhibit apoptosis.[26]

The antiapoptotic function of survivin may not be required for its effect on melanoma cell motility and metastasis. As we showed using *in vitro* studies, inhibition of survivin by RNAi reduced melanoma cell migration and invasion without induction of apoptosis.[11] This is consistent with other studies that have also demonstrated that the antiapoptotic function of survivin is not required for tumor invasion and metastasis.[26, 27] In one study, a model of rat insulinoma INS-1 cells, in which stable transfection of survivin does not confer cytoprotection, was used to demonstrate increased tumor cell invasion.[27]

There are key amino acid residues within the survivin structure that are essential for its antiapoptotic and mitotic functions.[28] Mutation of these residues may help to better uncouple survivin function in apoptosis, mitosis and motility in melanoma. It is known that mutation of Threonine-34 to Alanine (T34A) in survivin disrupts dimerization of this protein and inhibits the antiapoptotic activity of survivin.[13, 29, 30] Additionally, Ser20 is known to be important for survivin function in mitosis, as survivin is phosphorylated by Polo-like Kinase-1 (Plk1) at Ser20 to help control chromosomal alignment during

mitosis.[31] These mutants could be utilized in our *in vitro* and *in vivo* models of melanoma motility and metastasis to better distinguish how survivin regulates melanoma metastasis.

α_5 integrin could be mediating survivin-enhanced metastasis on a number of levels in the metastatic cascade. In addition to facilitating tumor migration and invasion from the primary tumor, α_5 integrin may also affect the survival of tumor cells in circulation. This could be one possible point of intersection between α_5 integrin and survivin function as an anti-apoptotic protein. As an adhesive receptor, α_5 integrin could direct association of melanoma tumor cells with platelets and leukocytes in the circulation. This may allow tumor cells to form small tumor emboli that aid in keeping tumor cells in target metastatic sites.[16] Adhesion to target organ endothelium and extravasation for metastatic outgrowth could also be facilitated by the adhesive functions of α_5 integrin.

In addition to mediating cell motility, α_5 integrin could also be facilitating colonization, or outgrowth at distant sites. For successful metastatic colonization to occur, tumor cells need to be able to interact with a foreign microenvironment.[32] Microenvironment components like fibroblasts, endothelial cells, and infiltrating inflammatory cells produce cytokines, chemokines, growth factors and proteases to which tumor cells will respond. In a reciprocal manner, tumor cells will also signal to the microenvironment to remodel it to favor tumor cell colonization.[32] The bi-directional signaling capacity of integrins makes them amenable to mediating the interactions between tumor cells and the microenvironment in metastatic niches.[16]

Survivin and α_5 integrin share common functions that enable melanoma tumor cell metastasis. They are both implicated in tumor cell ability to resist apoptosis, and in

tumor cell invasion and migration. Intersection of survivin and α_5 integrin with key signaling networks like MAPK and PI3K/AKT may also promote cell survival and proliferation. Therefore, it may be difficult if not impossible to discern the individual contributions of survivin and α_5 integrin to the metastatic process in melanoma.

However, the studies in this dissertation clearly add credence to the importance of survivin and α_5 integrin in melanoma metastasis. In our *in vivo* model of melanoma metastasis, we were able to utilize M200, which blocks $\alpha_5\beta_1$ integrin function, to demonstrate the necessity of α_5 integrin for survivin-enhanced metastasis.[11] There have been a number of clinical trials conducted to determine the effect of M200 on metastatic melanoma, renal cell, and pancreatic cancers.[33-35] These studies had mixed results with some patients showing partial responses to M200 but most were unresponsive or refractory to M200 treatment.

Our studies may argue that the timing of intervention in these studies may be crucial for any real therapeutic value to be obtained with M200 treatment. In many of these studies, patients are being treated with M200 after metastases have become well established throughout the body and targeting of $\alpha_5\beta_1$ at that point is ineffective. In our *in vivo* studies, inhibition of α_5 with M200 early in tumor development, or through RNAi prior to lung colonization could stem metastatic spread and outgrowth. This suggests that the design of clinical trials based on M200 efficacy in curtailing or preventing metastasis needs to be optimized to allow for an earlier point of intervention. Perhaps, M200 could be used as an adjuvant therapeutic agent after resection of primary tumors where metastatic spread is indicated. It is possible that by blocking α_5 integrin earlier in the

disease progression, many of the advantages that allow tumor cells to propagate and metastasize may be reduced.

The combined effect of survivin and α_5 integrin on metastasis may also be context dependent. We have shown that survivin upregulation of α_5 integrin is conserved in a number of melanoma cell lines. And that α_5 integrin is required for survivin-enhanced migration.[11] In the SK-mel 28 melanoma cell line, where overexpression of survivin did not cause upregulation of α_5 integrin, cell migration was not increased (Appendix B.1); further suggesting that α_5 integrin is required to mediate survivin-enhanced motility. We investigated whether survivin upregulation of α_5 integrin was also conserved in other tumor types. This relationship was not seen in cell lines derived from other tumor types such as breast, colon and Ewing's sarcoma (Appendix C.1). Additionally, survivin expression in MCF-7 breast cancer cells resulted in increased liver metastasis *in vivo*.[27] However, the mechanism of action was alternate to α_5 integrin. It was found that survivin increased metastasis through activation of NF-κB and increased expression of activated Focal Adhesion Kinase (FAK).[27] This suggests that there is potentially a unique mechanism by which survivin is able to mediate melanoma metastasis via α_5 integrin.

In our studies, we monitored melanoma metastasis to the lungs. However, it is known that in addition to the lungs, melanoma also metastasizes to the brain, liver and bones.[36] Therefore it is possible that in our model, lung metastasis may be an incomplete picture and α_5 integrin may also mediate survivin-enhanced melanoma metastasis to other organs in the body. While we have shown a requirement for α_5 integrin in survivin-enhanced melanoma metastasis, key questions remain unanswered about the mechanisms involved. Potential future directions for this project will be discussed in the next section.

Future Directions

One important question raised by these studies is: how is upregulation of α_5 integrin in the tumor cells allowing for metastatic spread? α_5 integrin may be remodeling the ECM to allow tumor cells to move more freely out of the tumor and into the circulation to be transported to other organs. One way to address this question would be to determine the effect of α_5 integrin on matrix remodeling by assessing the activity of proteolytic enzymes like matrix metalloproteinases (MMPs). MMPs are a family of endoproteinases whose functions include degradation of ECM proteins like fibronectin, laminin and collagen.[37]

Binding of cell surface receptors like integrins to their ECM ligands can initiate cell signaling events that modulate the expression and activity of MMPs.[38] MMPs are thought to facilitate tumor invasion and metastasis through several mechanisms that include the enzymatic activity of MMPs on ECM proteins. Proteolytic processing by MMPs removes the physical barrier to tumor cell movement, as they degrade basement membranes.[37] Secondly, MMPs interact with integrins to regulate cell adhesion, which undergoes dynamic changes as tumor cells move from primary tumors to metastatic sites.[37] In cervical cancer cells, $\alpha_5\beta_1$ integrin binding to fibronectin has been shown to increase the expression and proteolytic activity of MMP-2 and MMP-9.[38] This effect was mediated by activation of AKT and NF-κB signaling.[38] Therefore, the survivin mediated upregulation of α_5 integrin in melanoma cells, may be promoting metastasis through increased MMP activity in the primary tumor.

Another potential research direction could be focused on how survivin is able to cause upregulation of α_5 integrin. We showed that survivin upregulation of α_5 integrin is

dependent on phosphorylation of AKT.[11] PTEN negatively regulates the phosphorylation of AKT and a recent study has shown that the tumor suppressor function of PTEN involves silencing of survivin gene expression.[39] This study also suggested that in a negative feedback loop, survivin expression in malignant cells can silence PTEN gene expression and function.[39] Therefore, survivin expression could relieve PTEN inhibition of AKT phosphorylation, thereby leading to upregulation of α_5 integrin (Figure 4.1). This AKT dependent upregulation of α_5 integrin may be at the transcriptional level. Transcription factors for α_5 integrin include AP-1, SP-1and NF-1,[40] some of which are known targets of the AKT pathway.[41, 42]

Other areas of research interest could include the translational and post-translational regulation of α_5 integrin. The significant increase in α_5 integrin levels in survivin-expressing melanoma cells over control cells suggests that survivin may regulate α_5 integrin at the protein level. Studies have demonstrated that protein levels of α_5 integrin are controlled through endocytic and exocytic cycling between the cytosol and the cell surface.[43] This process of integrin recycling controls whether integrins are targeted for degradation by ubiquitination, or whether they are returned to the cell surface.[43] It has been reported that Protein Kinase B (AKT) enhances cell migration through regulation of Glycogen Synthase Kinase-3β (GSK-3β) dependent α_5 integrin recycling.[44] Therefore, one area of study could examine the role of survivin in AKT mediated recycling of α_5 integrin, as this could be one way through which survivin is able to upregulate α_5 integrin expression in melanoma and thereby increase motility and metastasis.

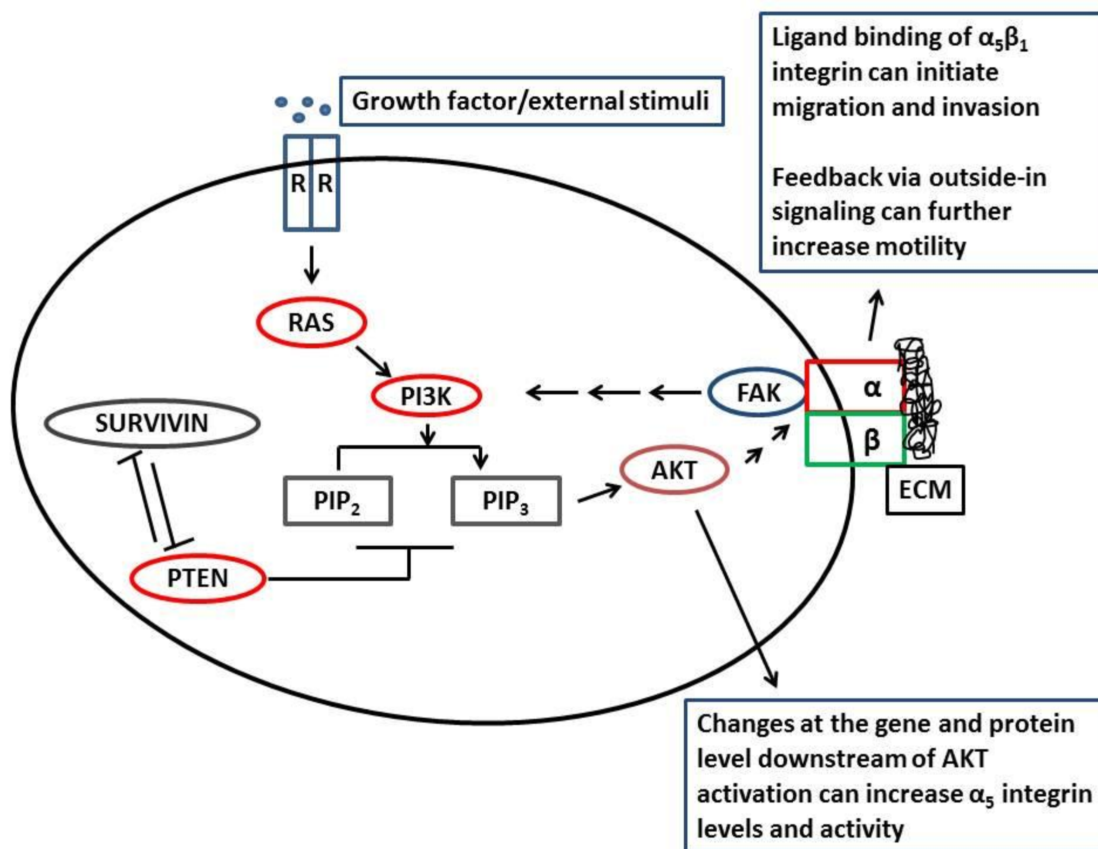


Figure 4.1 Integration of survivin and α_5 integrin function in melanoma. This is a potential model for survivin and α_5 integrin function. Increased expression of survivin in melanoma cells can lead to upregulation of α_5 integrin by relieving PTEN inhibition of AKT activation. Downstream of AKT activation, gene expression changes can lead to increased α_5 integrin to help facilitate cell motility and metastasis. In melanoma, aberrant RAS signaling can also activate the PI3K/AKT pathway. Additionally, integrin outside-in signaling can interact with the PI3K/AKT pathway via Focal Adhesion Kinases (FAK) to further stimulate this signaling pathway.

The bi-directional signaling of integrins that intersects with survivin function and with growth signaling pathways like PI3K and MAPK, can present yet another avenue for improving melanoma therapies. The $\alpha_5\beta_1$ integrin inhibitory antibody M200, which was used in the *in vivo* experiments of this study, has had mixed success in clinical trials (discussed in Chapter 3). As was addressed in the introductory chapter, resistance to RAF inhibition is a common problem associated with the use of Vemurafenib. In one mechanism of resistance, upregulation of RAS can result in activation of the AKT pathway and may lead to downstream upregulation of α_5 integrin. Therefore, targeting of RAF not only in combination with MEK, but also with α_5 integrin may help combat melanoma metastasis. The work presented in this dissertation adds another piece to the puzzle and further illustrates the intricacies involved in melanoma metastasis.

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APPENDIX A

MICROARRAY ANALYSIS OF SURVIVIN-EXPRESSING AND CONTROL MELANOCYTES

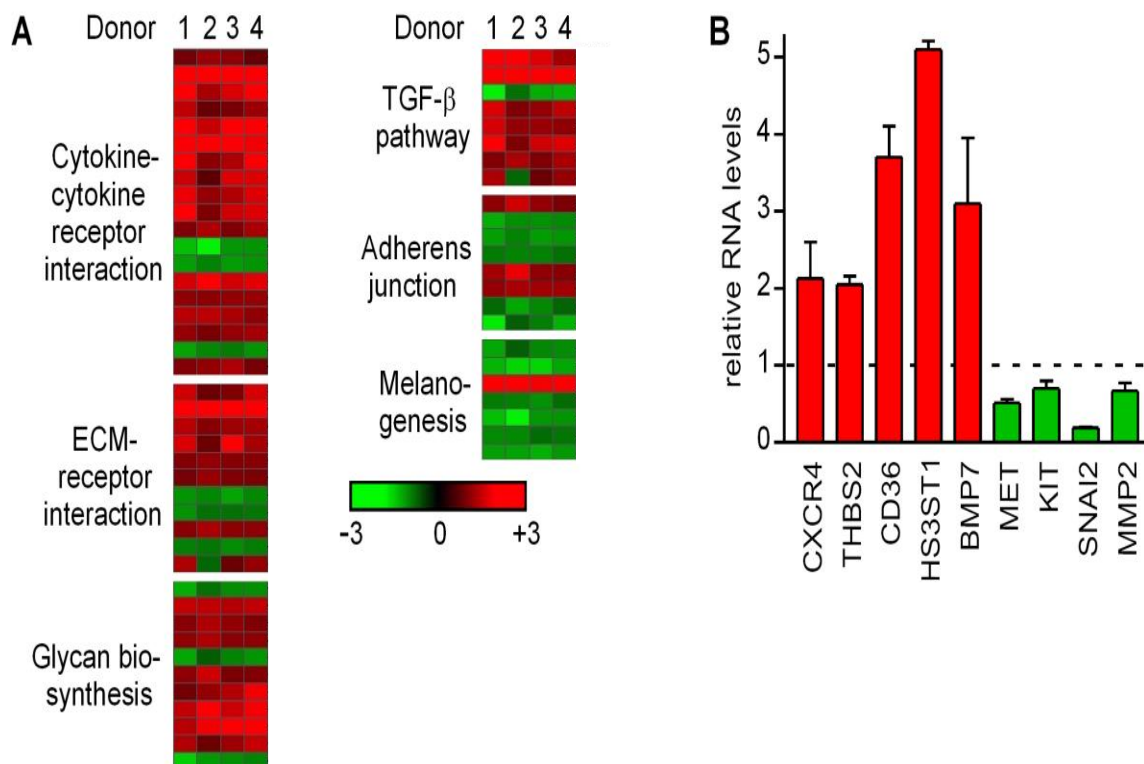

Figure A.1 Gene expression profile of survivin expressing melanocytes. Melanocytes were isolated from four different donors and infected with pAd-GFP or pAd-Surv. After 48hrs, total RNA was isolated from each group, and run in quadruplicate on Agilent Human Genome arrays. Statistical analysis of the normalized log-transformed data was performed using GeneSifter Software. Comparisons were made of paired samples to control for inter-donor variability, and pair-wise t-tests were performed with an adjusted p-value of ≤ 0.05. (A) Heatmaps of differentially affected ($p<0.05$) pathway genes. (B) Validation of selected genes by qRT-PCR. Triplicate determinations of relative RNA levels (each normalized to GAPDH = 1.0, dotted line) in pAd-Survivin - compared to pAd-GFP-infected cells. GAPDH was not affected by survivin expression. Error bars indicate SEM.

APPENDIX B

MIGRATION OF SK-MEL 28 MELANOMA CELLS OVEREXPRESSING SURVIVIN

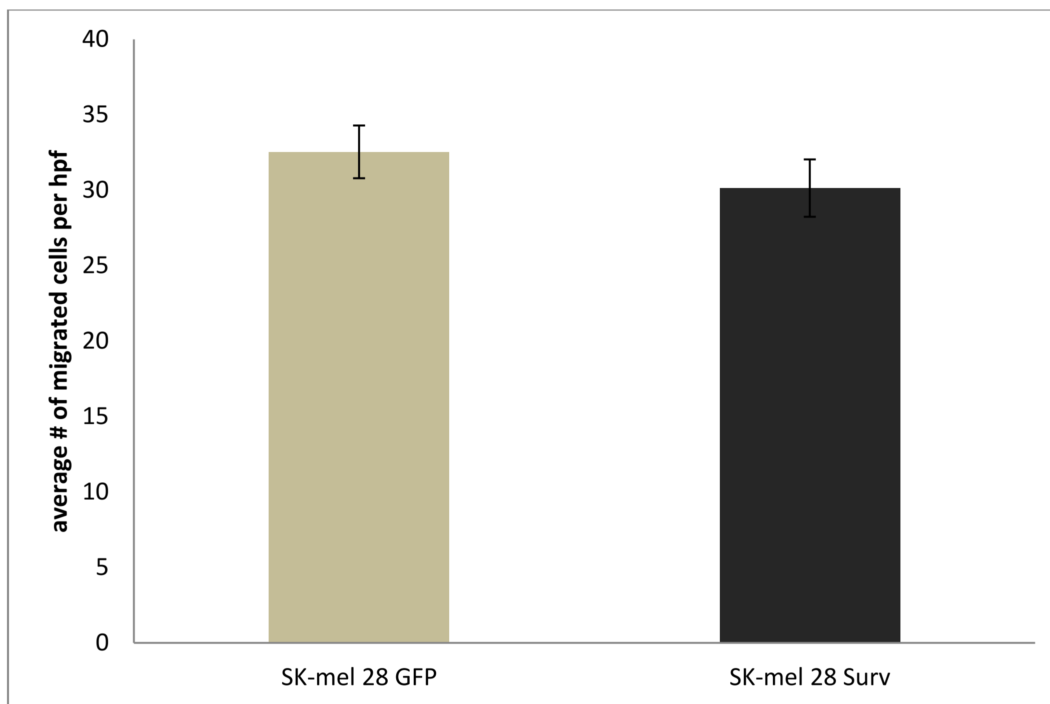

Figure B.1 Migration of survivin overexpressing sk-mel 28 melanoma cells. SK-mel 28 melanoma cells were infected with a control adenovirus expressing GFP (SK-mel 28 GFP), or an adenovirus expressing survivin (SK-mel 28 Surv). 24h later cells were treated for one hour with 10 µg/ml Mitomycin C to inhibit cell division. 15 000 cells were then transferred to each of three Transwell chambers which had been coated overnight with 10 µg/ml fibronectin. Cells were allowed to migrate for 24 hours. Migratory cells were then fixed, stained and quantitated.

APPENDIX C

SURVIVIN AND ALPHA 5 INTEGRIN EXPRESSION

IN NON-MELANOMA CELL LINES

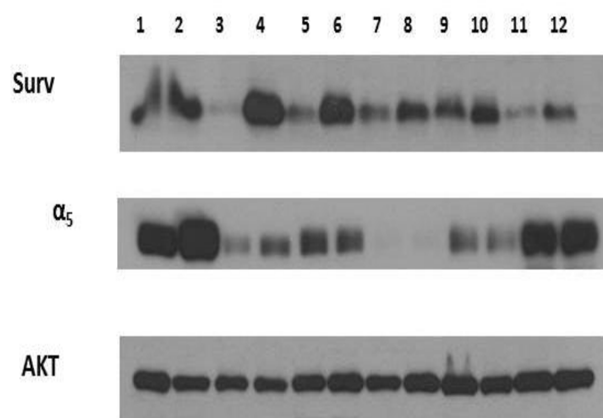

Figure C.1 Survivin and α_5 integrin expression in non-melanoma cell lines. A673 Ewing's sarcoma cells (1,2), HEK 293 transformed human embryonic kidney cells (3,4), T47D breast cancer cells (5,6), Hacat immortalized human keratinocytes (7,8), HCT 116 colon cancer cells (9,10), and MDA-MB-231 breast cancer cells (11,12) were infected with a control adenovirus expressing GFP (odd number lanes), or an adenovirus expressing survivin (even number lanes). 48h later, cell lysates were generated and subjected to western blot analysis for expression levels of survivin, and α_5 integrin. Expression of total AKT was used as a loading control.