ROLES OF THE $\alpha 2\beta 1$ INTEGRIN IN CANCER PROGRESSION AND METASTASIS

By

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Pathology

December, 2011

Nashville, Tennessee

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To my parents who have sacrificed so much for my siblings and me.

ACKNOWLEDGEMENTS

Multiple organizations and individuals have been integral in supporting this endeavor. Financial backing from the Vanderbilt Medical Scientist Training Program (NIGMS/NIH T32 GM007347), National Institute of Health Grants (RO1CA115984-01 and 2RO1CA098027-07A1), and the Vanderbilt Ingram Chair have allowed this project's realization.

I am also indebted to the people that have assisted and empowered me throughout my scientific training. Through their constant support, kindness, and friendship, I have grown personally and professionally. Firstly, I would like to thank my preceptor, Dr. Mary Zutter, for her constant mentorship and guidance in my academic growth. Secondly, I am grateful to the members of my dissertation committee. Drs. Sarki Abdulkadir, Jin Chen, and Harold Moses have enthusiastically given significant time, energy, and ideas to further my project. Dr. Andries Zijlstra and his lab members have generously provided reagents, time, and expertise throughout the years; it has been a wonderful and enlightening experience collaborating with them. Thank you to Dr. Larry Swift for chairing my thesis committee and for advice in my career development. Thirdly, I would like to express my appreciation for the Department of Pathology. The level of interaction with distinguished faculty and degree of encouragement for students has created a unique atmosphere for collaboration and academic enrichment. Fourthly, I would like to thank the members of the Zutter Lab, past and present, especially Brittney Barlow, Lynda O'Rear, Dr. Claudio Mosse, Dr. Norma Ramirez, Dr. Zhonghua (John) Zhang, Dr. Brenda Jarvis, Dr. Zhengzhi (George) Li, and Joseph Covington for their assistance on my project. Fifthly, I thank my wonderful collaborators, Sandy Olson, William Dupont, and Lisa Coussens. Finally, I would like to thank my amazing family

and friends for their faith in my perseverance, especially Fyza Shaikh and Trenis Palmer for both their constant friendship and scientific contributions to my research. I have deep gratitude for all involved because they have enriched my studies and my life.

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CHAPTER I

OVERVIEW

Cancer encompasses multiple diseases that are bound collectively by their cellular pattern of uncontrolled growth, invasion, and possible metastasis. During neoplastic progression, a complex series of genetic and epigenetic alterations occur not only within the tumor cell but also within the cells of the microenvironment. Disturbances in normal cell adhesion promote pro-migratory phenotypes that permit tumor cell invasion and metastasis. One particular cell surface adhesion molecule, the $\alpha 2\beta 1$ integrin is a key receptor for not only cell-cell and cell-matrix adhesion but is involved in bi-directional signaling for cell communication.

In this dissertation, I examined the function of this integrin on epithelial tumor cells as well as in the tumor microenvironment. Using the K14-HPV16 mouse model of inflammation-driven skin carcinogenesis on wild-type or $\alpha 2\beta 1$ integrin-null backgrounds, I elucidated the roles of this integrin during the multi-step progression towards cancer and lymphatic metastasis. I also identified an orthotopic breast cancer model system in which to study $\alpha 2\beta 1$ integrin expression specifically on the tumor or host microenvironment. The three central questions addressed were: 1) Does expression of the $\alpha 2\beta 1$ integrin by squamous cell carcinomas (SCCs) or by the inflammatory/hematopoietic cells of the microenvironment contribute to SCC development and progression? 2) Does the $\alpha 2\beta 1$ integrin alter the lymphatic vasculature, and if so, what is the mechanism for the functional changes seen with integrin loss? 3) Does the $\alpha 2\beta 1$ integrin function specifically on breast cancer cells or

other cell types to decrease intravasation and subsequent metastasis? My studies on the $\alpha 2\beta 1$ integrin in both the spontaneous K14-HPV16 tumor and in the orthotopic murine breast cancer model address these questions; I present novel discoveries on how this integrin impacts SCC progression and lymphatic metastasis, as well as how integrin expression dictates tumor cell behavior in SCC and breast cancer. These findings add to our current understanding of the $\alpha 2\beta 1$ integrin in cancer and on maintenance of the lymphatic vasculature.

My studies have implicated a role for the $\alpha 2\beta 1$ integrin in determining the growth and invasive behavior of tumor cells that is independent of the tumor microenvironment, yet influenced by the malignant cell type involved. In Chapter III, I postulated that the $\alpha 2\beta 1$ integrin would alter inflammation-driven cancer development due to its presence on several inflammatory subsets known to promote epithelial carcinogenesis in the K14-HPV16 model. However, my data demonstrated that the $\alpha 2\beta 1$ integrin had a minimal impact on chronic inflammatory populations in several tissue types. Despite this finding, I discovered a role for the $\alpha 2\beta 1$ integrin in acute mast cell responses to inflammation-driven preneoplastic progression in the skin. While the $\alpha 2\beta 1$ integrin does not significantly alter tumor formation, growth, or multiplicity, loss of integrin expression resulted in altered tumor-associated angiogenesis. Since SCCs disseminate via the lymphatic vasculature, regional lymph nodes were examined for metastasis and found to be decreased by 31.3% in $\alpha 2\beta 1$ integrin-null mice. When isolated and grown *in vitro*, $\alpha 2\beta 1$ integrin-null, primary SCC cells demonstrated decreased migration and collagen type I invasion. Additionally, orthotopic implantation of these primary tumor cells into non-K14-HPV16 transgenic, wild-type or $\alpha 2\beta 1$ integrin-null mice revealed negligible effects of the tumor microenvironment on cancer establishment and growth. These

findings indicate a role for the α2β1 integrin in modulating epithelial tumor cell behavior, which is independent of the microenvironment. Ultimately, the differences that I observed were mediated by integrin expression specifically on the tumor cells.

In Chapter IV, I performed studies on the lymphatic vasculature to examine α2β1 integrin specific effects on the lymphatic endothelium. Examination of the lymphatic system indicated that integrin loss resulted in altered SCC-associated lymphangiogenesis as well as vessel dilation in both a non-neoplastic and preneoplastic setting. Moreover, this alteration in morphology was associated with increased leakiness of integrin-null lymphatics in non-neoplastic as well as tumor tissue. The increased vessel leakiness in α2-null animals correlated with decreased expression of the tight junction marker zonula occludens-1 on the lymphatic endothelium. However, the vascular endothelial growth factors associated with lymphangiogenesis were not dysregulated in HPV/KO SCCs, compared to HVP/WT tumors, nor did VEGF-A, -C, or -D alter lymphatic endothelial cell infiltration into Matrigel plugs in wild-type or α2-null mice *in vivo.* Ultimately, the increased leakiness and defective functionality of α2-null lymphatic vessels may help account for the decreased transport of cancer cells to draining lymph nodes seen Chapter III.

Finally, in Chapter V, I elucidated the role of the α2β1 integrin on breast cancer cells. Previous work has shown that the α2β1 integrin decreased mouse mammary tumor virus-neu (MMTV-neu) tumor cell intravasation by blocking the cancer cells' invasive ability to enter the blood vasculature. Since this work was performed on global α2 integrin subunit-null mice, I have established an orthotopic model system in which I examined contributions of tumor cell-specific α2β1 integrin expression towards intravasation. In my work, I characterized α2β1 integrin expression profiles on a panel of

genetically related murine breast cancer clones, the 4T1 sister cell lines, established by Fred Miller. Orthotopic injection of parental 66c14 cells, a 4T1 sister subclone lacking $\alpha 2\beta 1$ integrin expression, into wild-type or $\alpha 2\beta 1$ integrin-null mice did not alter tumor latency, growth, or lung metastasis, demonstrating that the host microenvironment contributes minimally to breast cancer progression. While these studies need to be repeated and expanded, they have provided valuable insights into determining the $\alpha 2\beta 1$ integrin's role on tumor cells versus on cells of the tumor microenvironment. In contrast to my studies on SCC, $\alpha 2\beta 1$ integrin expression by breast tumor cells does impact the observed clinical phenotype.

A detailed background of the $\alpha 2\beta 1$ integrin in SCC, the lymphatic system, and breast cancer, with unique consideration to cancer cell dissemination and metastasis, is discussed in Chapter II. In Chapter III, I characterize the K14-HPV16 mouse on an $\alpha 2\beta 1$ integrin-null background. This section was adapted from my paper "Loss of the α2β1 Integrin Alters Human Papilloma Virus-induced Squamous Carcinoma Progression *in vivo* and *in vitro*" submitted to PLoS ONE (1). Since there were differences in lymph node metastasis with $\alpha 2\beta 1$ integrin loss, I examined the effects of this integrin on the lymphatic vasculature in Chapter IV. I demonstrate that absence of the $\alpha 2\beta 1$ integrin induced dilated lymphatic vessels, which were dysfunctional in both non-cancerous and in SCC-affected tissue. In Chapter V, I investigated the role of the $\alpha 2\beta 1$ integrin on breast cancer cells and establish a model system for studying $\alpha 2$-mediated tumor versus host microenvironment contributions in breast cancer progression. Finally, in Chapter VI, I conclude with a discussion on future research directions. This work addressed several important questions regarding the integrin's effect on the tumor cells and metastasis. I found that the $\alpha 2\beta 1$ integrin does contribute to modify the invasive nature

of SCC and breast cancer. Whether the $\alpha 2\beta 1$ integrin functions to increase or decrease cell migration and invasion, however, is dependent on the tumor cell type involved. These findings demonstrate the complexity of integrin-signaling biology. Additionally, the $\alpha 2\beta 1$ integrin plays an important role in maintenance of the lymphatic system by regulating endothelial vascular leak. My findings demonstrate an important function of the $\alpha 2\beta 1$ integrin on maintaining lymphatic vasculature integrity, thus providing novel insights into the function of this integrin in normal physiology as well as in tumor-associated lymphatics and metastasis. I believe the insights gained from these studies will help ascertain the impact of a key collagen receptor towards cancer progression, which may be highly relevant to human health.

CHAPTER II

INTRODUCTION

According to the latest Center of Disease Control statistics in 2007, cancer represented the second leading cause of death in the United States (2). After combining all types of skin cancer, these neoplasms represent the most common form of cancer in the United States. Although basal cell carcinomas are more common and least aggressive, melanomas are rare yet highly invasive and metastatic. Squamous cell carcinomas, in comparison, are the second most common skin cancer. Unlike decreasing incidence and mortality trends for breast and cervical cancer since the 1980s, skin cancer is on the rise, with over 500,000 new cases reported annually (3). Skin neoplasms, therefore, represent a significant health burden and an urgent threat for the aging population.

In addition to the traditional risk factors associated with skin cancer, such as lifetime sun exposure, chemicals, family history, male, and elderly, viral-induced skin cancer is on the rise (4). This heighten risk is related to infection with human papillomavirus (HPV). While some HPV types induce benign warts, more pathogenic strains may lead to epithelial carcinogenesis in the skin as well as at other anatomic sites. HPV-stimulated epithelial carcinoma is an extremely important health problem globally. Although attention has been predominately focused on the role of HPV in cervical cancer, HPV-induced SCCs of the head and neck are now discovered to account for 25% of mouth and 35% of throat cancers (5). Clinicopathological data suggest that HPV-associated SCC is a unique medical entity (6). Patients presenting with HPV-positive SCC have a better prognosis than patients with HPV-negative SCC,

likely as a result of different genetic alterations and gene expression profiles (7, 8). Risk factors for HPV-associated disease include an increased number of vaginal and oral sexual partners and heavy marijuana usage; the typical risk factors for head and neck cancer, tobacco and alcohol use, are irrelevant in patients with HPV-positive disease (9, 10).

Cancers arise from the accumulation of genetic mutations that alter normal regulation of cell proliferation, differentiation, organization, and motility. The genetic and epigenetic changes that endow cells with the capacity to undergo malignant transformation, progress to invasion, and proceed through the metastatic cascade have long been a major focus of cancer research. Cancer progression and metastasis, however, do not solely rely upon the genetic and epigenetic events in the tumor cell (11-13). Instead, genetic changes in the epithelial cell may promote changes in the microenvironment, including increased angiogenesis, recruitment of inflammatory cells, alterations in the extracellular matrix (ECM), and activation of fibroblasts (14). The importance of the tumor microenvironment in oncogenesis has been studied for more than a century. The hypothesis that metastasis requires crosstalk between cancer cells and the microenvironment of select organs was originally proposed by Stephen Paget in 1889. The innovation of Paget's "seed and soil" hypothesis is more acclaimed today and supported by a plethora of recent research. This hypothesis is based on the idea that neoplasms consist both of tumor and host cells (15). The "rich soil" of the tumor microenvironment consists of endothelial cells, fibroblasts, immune cells and their cell surface receptors, including integrins, growth factor/cytokines, and proteases (16-19).

Hynes *et al.* first argued that defects in cell surface adhesive receptors, in addition to altered expression of matrix components in the microenvironment, could lead to the altered cell behavior that typifies cancer progression and metastasis (20-23). As

important mediators of cell adhesive behavior, integrins play a critical role in tumor progression and metastasis. Their role in mediating adhesion to either components of the extracellular matrix or to other cells is now well established (24-29). Moreover, engagement of integrin receptors activates pro-survival signals, which has proven beneficial to cancer cell survival (30, 31).

Prominent among the integrin receptors, the β1 family of integrins has a strong foundation in cancer. Early work demonstrated that inhibition of β1 integrin ligation caused reversion of the malignant phenotype of a human breast tumor cell line to a normal, growth-arrested phenotype (25). Similarly, animals with targeted deletion of the β1 gene in mammary epithelium failed to develop cancer, suggesting that the β1 integrins are essential for cancer initiation (32). Loss of β1 integrin expression has been correlated with poor prognosis in several types of human cancer, including non-small cell lung cancer, melanoma, and oral SCC (33-35). Additionally, work using numerous *in vitro* and *in vivo* orthotopic or xenograft models indicated that the β1 integrins mediate drug resistance and stimulate metastasis in gastric, ovarian, and lung cancer (36, 37). Since the β1 integrin subunit heterodimerizes with multiple α subunits, identification of key α chains critical in cancer progression and prognosis will be vital to understanding the role of integrins in this disease process.

The α2β1 Integrin

Integrins comprise a large family of extracellular, transmembrane glycoproteins made of 18 α- and 8 β-subunit chains that combine to form at least 24 distinct, noncovalently-bound heterodimers in mammals (22, 38). Although collagens are the most abundant proteins in mammals, only 4 of the 24 integrin heterodimers have been

shown to bind collagens. The collagen receptors all consist of a β1 subunit which associates with either the α1, α2, α10, or α11 subunit (Figure 2-1) (39-41).

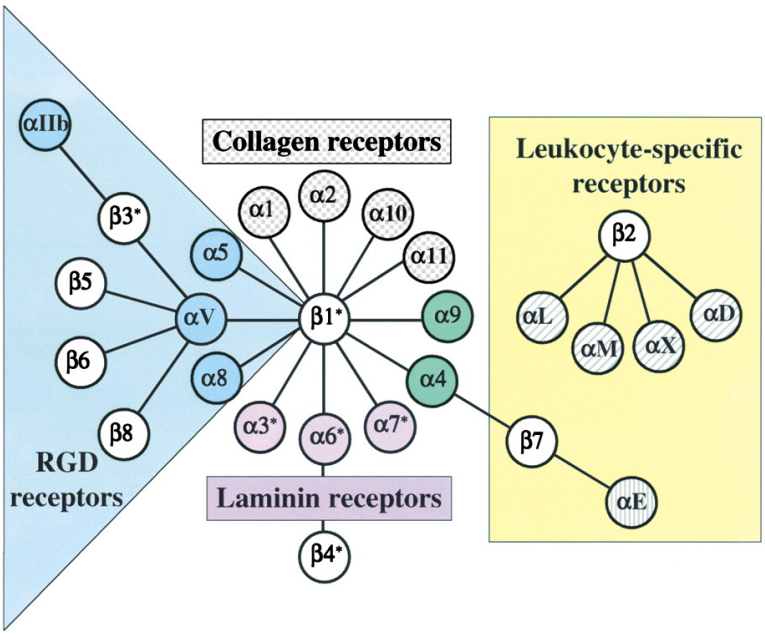


Figure 2-1. Heterodimeric associations of α- and β-subunit chains of mammalian integrins. The exact pairing of α- and β-subunits determines ligand specificity. The α2β1 integrin recognizes collagen and laminin within the extracellular matrix. *Denotes integrin subunits with alternatively spliced cytoplasmic domains. Adapted from Hynes, R, *Cell*, 2002 (22).

Integrins mediate both cell-cell and cell-ECM adhesion. Known as bi-directional signaling receptors, they transmit outside-in or inside-out signaling for communication between cells and ECM (22). Integrins have been implicated in normal development, inflammation, and innate immunity. In addition, they have roles in all stages of tumor initiation, progression, and metastasis (22, 42). Specific combinations of α- and β-subunits determine ligand specificity. The $\alpha2\beta1$ integrin (additionally known as GPIa/IIa, VLA-2, or CD49b/CD29) recognizes a number of ligands, including collagen types I, III, and IV, laminin, E-cadherin, decorin, matrix metalloproteinases, C1q, and collectins (43-46).

Although present on many cell types, the $\alpha2\beta1$ integrin is not ubiquitously expressed. High levels of the $\alpha2\beta1$ integrin can be found on epithelial, endothelial, fibroblast, platelet, and several immune system cells, such as natural killer (NK) cells, mast cells, and activated lymphocytes (47-50). Despite its existence on multiple cell types, studies creating $\alpha2$ subunit-null mice have shown the integrin is not crucial for survival. These animals display no overt abnormalities in fertility or development, thereby indicating redundancy and compensation for adhesion via the $\alpha2\beta1$ integrin (51). Although not critical for survival, the $\alpha2\beta1$ integrin functions in regulating orderly cellular proliferation, differentiation, and migration (52).

The $\alpha2\beta1$ integrin assumes a bent, inactive configuration when unstimulated. Upon activation, conformational changes occur in the protein's extracellular domain, thereby exposing the integrin's ligand binding pocket. Interestingly, all of the collagen receptor integrins contain an I-domain that is responsible for collagen recognition (53). The $\alpha2\beta1$ integrin, specifically, binds ligands via the α-subunit on its I-domain. Subtle differences in I domain structure among the collagen receptors account for their ability to

recognize distinct collagen subtypes (54). The $\alpha 2\beta 1$ integrin I-domain requires a metal cation (Mg^{2+} or Mn^{2+}) in its metal ion dependent adhesion site motif to bind its collagen ligands (55-57). Alterations in the $\alpha 2\beta 1$ integrin's conformation state depends upon receptor ligation or intracellular signaling via the unique outside-in or inside-out, bi-directional signaling (22, 58). Once activated, the $\alpha 2\beta 1$ integrin initiates signaling through a number of downstream pathways. Notably, the $\alpha 2\beta 1$ integrin influences cell motility by associations with the actin cytoskeleton. Receptor activation results in phosphorylation of focal adhesion kinase (FAK), adaptor protein recruitment, small GTPase activation, and downstream activation of additional effector molecules (59-62). These signals act in concert to regulate cell behavior, including motility, in the tissue microenvironment.

The $\alpha 2\beta 1$ Integrin in Keratinocyte Biology

Due to the protective demands imposed upon the skin, keratinocytes have evolved multiple, stratified layers of differentiated cells to prevent fluid loss and infection. The mammalian epidermis is divided into 5 layers. The basal layer, also known as the stratum germinativum or basale, consists of columnar cells that proliferate to replenish more superficial layers. These basal cells are separated from the underlying dermis by a basement membrane of collagen type IV and laminin (Figure 2-2).

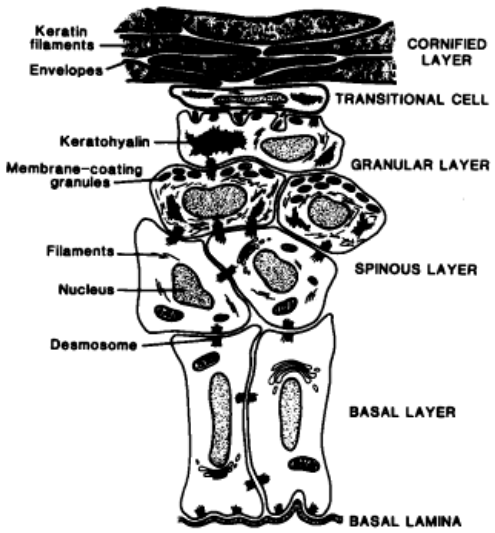


Figure 2-2. The five layers of mammalian skin. From basal to superficial, the five layers of skin are known as the stratum germinativum (basal), spinosum, granulosum, lucidum (transitional), and corneum. As skin cells proliferate and are replaced by cells from the basal layer, keratinocytes are pushed upwards, gradually differentiate, and flatten before forming the most superficial corneal layer of keratinized dead cells. In normal skin, only proliferating keratinocytes of the stratum germinativum express high levels of the $\alpha 2\beta 1$ integrin. Adapted from Eckert, R, and Rorke, E, *Environ Health Perspect.*, 1989 (63).

Of all integrins found in the stratum basale of the skin epithelium, including the $\alpha3\beta1$ and $\alpha6\beta4$ integrins, the most highly expressed is $\alpha2\beta1$. In basal cells, the $\alpha2\beta1$integrin functions to regulate proliferative re-epithelialization of the skin. In intact epidermis, however, loss of the $\alpha2\beta1$ integrin in mice caused no overt skin phenotype (64). However, when examined in human cells and skin explants, $\alpha2\beta1$ integrin-collagenase-1-type I collagen interactions were required for keratinocyte migration and wound closure (65, 66). The $\alpha2\beta1$ integrin is required for keratinocyte adhesion to collagen and for keratinocyte migration via its interaction with laminin 5 (67, 68). Furthermore, $\alpha2\beta1$ integrin expression can be found in suprabasal epidermal layers during wound healing and in benign and neoplastic diseases, indicating a function of the integrin in keratinocyte differentiation (69). These studies demonstrate a central function of the $\alpha2\beta1$ integrin in skin differentiation in response to normal physiology, healing, and neoplasia.

Role of the $\alpha2\beta1$ Integrin in Immunology

The role of the immune system in protecting the host against cancer was originally proposed in the early 1900s by Ehrlich (70, 71). Since then, the importance of both the adaptive and innate immune system in preventing tumor development is supported by both human and mouse models of immunodeficiency. Alternatively, the role of the immune system, both innate and adaptive, in stimulating cancer progression has been also been appreciated for many years. Clinical epidemiologic studies have suggested strong associations between chronic infection, inflammation, and cancer development.

As one of the major collagen receptors, the $\alpha 2\beta 1$ integrin serves an important role in the adhesion of inflammatory cells during migration towards sites of infection or neoplasm. This integrin is expressed by distinct subsets of immune cells: mast cells, neutrophils, NK cells, activated T cells, and B cells (50, 72-74). As a result, the $\alpha 2\beta 1$ integrin plays important roles in acute and chronic inflammation by modulating both innate and adaptive immune responses.

The $\alpha 2\beta 1$ integrin regulates innate immunity by impacting mast cell, NK cell, and neutrophil function. In addition to being a collagen receptor, the $\alpha 2\beta 1$ integrin binds collectins and the C1q complement protein (75). Mast cells are uniquely poised to be primary responders to injury and infection as residents of connective tissue and mucous membranes. Since mast cells play a central role in the recruitment of additional inflammatory mediators, their dysfunction impacts downstream immune responses. During wound healing of the skin, mast cell numbers are decreased significantly in $\alpha 2$ integrin-null mice compared to wild-type animals 3 days post injury (76). Ligation of the $\alpha 2\beta 1$ integrin facilitates IL-6 degranulation from mast cells to induce neutrophil recruitment in response to *Listeria monocytogenes* infection (49, 77). In addition to its role on mast cells, the $\alpha 2\beta 1$ integrin is recognized by the DX5 monoclonal antibody, which was historically used to identify pan-mouse NK cells. It was shown later that the DX5 antibody recognized the $\alpha 2\beta 1$ integrin (47). Not only has the $\alpha 2\beta 1$ integrin been used as a marker to define NK cell populations, but it serves a critical role in determining NK cell migration and effector functions in lymph nodes. NK cells expressing the $\alpha 2\beta 1$ integrin moved slowly through nodal tissue due to increased adhesion to collagen and suppression of target cell lysis *in vitro* (78). Once extravasated from the blood vasculature, neutrophils upregulate expression of the $\alpha 2\beta 1$ integrin to mediate collagen

adhesion and migration through the tissue matrix (74). In a murine model of inflammatory bowel disease, the use of function-blocking antibodies against the $\alpha 2\beta 1$ integrin reduced colitis by inhibiting neutrophil migration and activation (79). The ability of innate immune cells to bind ECM collagen has proven central to their success in responding to wounds and infection; this function is also required for adaptive immunity.

Also known as very late antigen-2 (VLA-2), α2β1 integrin expression occurs in later stages of lymphocyte activation (50). Studies using function-blocking antibodies to the α2β1 integrin reveal that inhibition decreased inflammation in animal models of delayed-type hypersensitivity, contact hypersensitivity, and arthritis by preventing T cells from adhering to collagen (80). Collagen binding to the $\alpha 2\beta 1$ integrin also reduces Fas-induced apoptosis and caspase-8 activation in T lymphocytes, thereby supporting T cell survival (81). Not only is matrix adhesion diminished, but the $\alpha 2\beta 1$ integrin also functions to alter T cell receptor signaling responses by increasing interferon-gamma production (82). Specifically, human Th17 cells are known to primarily express the α2β1 integrin as its major collagen receptor, where it plays a role in providing co-stimulatory responses to increase IL-17A, IL-17F, and interferon-gamma levels (83). Due to the $\alpha 2\beta 1$ integrin's critical role in collagen-mediated migration of inflammatory cells and effector molecule release, many have postulated upon the effects of integrin loss in chronic inflammatory processes, particularly on those seen in cancer (84). To date, little is known about how the $\alpha 2\beta 1$ integrin influences chronic inflammation associated with preneoplastic and cancerous states.

Modeling Chronic Inflammation-driven Squamous Cell Carcinoma

Squamous cell carcinomas (SCCs) constitute the second most common form of skin cancers in humans, accounting for 20% of cutaneous malignancies (85). Risk factors associated with SCC development include age greater than 50 years, male, light skin, equatorial geography, history of melanoma, UV exposure, chemical carcinogens, ionizing radiation, human papillomavirus (HPV) infection, chronic scarring conditions, and chronic immunosuppression (86-92). SCC is a malignant tumor arising from epidermal keratinocytes. It is locally invasive and capable of metastasizing to both regional lymph nodes and to distant sites, such as the lungs.

In order to model SCC disease, Drs. Arbeit, Coussens, and Hanahan developed a transgenic mouse model of epithelial carcinogenesis in which the HPV16 early region viral genes were expressed in basal keratinocytes under the control of the keratin 14 promoter (93, 94). This model of squamous epithelial carcinogenesis mimicked viral-induced tumor progression seen in human cervical carcinomas. The early region viral genes E6 and E7 are dominant oncogenes, which recognize and induce the degradation of cellular tumor suppressor proteins p53 and retinoblastoma, respectively. As a result, K14-HPV16 mice develop invasive SCCs with multiple stages of dysplasia, carcinoma *in situ*, and invasion. While the epidermis of these mice is normal at birth, animals develop epidermal hyperplasia with 100% penetrance beginning at 1-month-of-age and epithelial dysplasia with neoangiogenesis between 3- and 6-months-of-age (93). The dysplastic epidermis is characterized by dense infiltration of mast cells and neutrophils, a reactive stroma, and increased neoangiogenesis. After a year, approximately 50% of mice have developed invasive SCC, and 30% of these cancers have metastasized to local lymph nodes (94-100).

The K14-HPV16 mouse is an established model for inflammation-driven epithelial cancer development. Inflammatory responses in the K14-HPV16 mouse model have been detailed by Drs. Coussens, Berger, Hanahan, Arbeit, and their collaborators (93, 94, 101). Work with this mouse model has led to extensive characterization of the important roles of mast cells, $CD4^+$ T cells and B cells, immune complex deposition, and matrix metalloproteinase (MMP) 9 secreted from bone marrow-derived cells in the pathogenesis of SCC (96, 100, 102-106). This model clearly demonstrates the complex interplay between the malignant tumor cells and the host immune system. Notably, mast cells are required for progression of squamous dysplasia to invasive squamous cell carcinoma. Additionally, the chronic inflammatory response, keratinocyte hyperproliferation, neoangiogenesis, and incidence of SCC were markedly attenuated in mast cell deficient-K14-HPV16 mice (96). MMP9 from bone marrow-derived cells was also required for cellular transformation (107). Although MMP9-null, K14-HPV16 mice developed a robust chronic inflammatory response similar to wild-type K14-HPV16 mice, they failed to develop high-grade dysplasia or SCC. These findings suggest that mast cell stimulation of acute and chronic inflammation was necessary for neoplastic conversion, and that the phenotype is in part dependent on MMP9 secretion. The presence of $CD4^+$ T cells and B cells also decreases tumor latency and increases SCC incidence by facilitating the recruitment of neutrophils and other MMP9 secreting leukocytes (100, 103). In the absence of B lymphocytes, the incidence of invasive cancer in 12-month-old mice decreased from 47% to 6% (99). Most recently, activation of Fcγ receptors on leukocytes has been shown to help establish chronic inflammatory programs that promote *de novo* carcinogenesis in the K14-HPV16 mouse model (108).

The inflammatory cell populations shown to promote disease progression in the K14-HPV16 model are known to express the $\alpha 2\beta 1$ integrin, which functions in immune cell activation and migration. The K14-HPV16 mouse represented an important skin tumorigenesis model in which we could study the roles of the $\alpha 2\beta 1$ integrin on keratinocytes and in immunity, thereby facilitating the study of this integrin on host tumor cells and in the complex tumor microenvironment.

Impact of the $\alpha 2\beta 1$ Integrin in Squamous Cell Carcinoma

Previous studies demonstrated that the $\alpha 2\beta 1$ integrin regulates cancer progression in several tumor models, including breast, lung, bone, and prostate cancer (109-113). For example, the α2β1 integrin is highly expressed on normal breast epithelium (114). Early studies examining human breast adenocarcinoma provided initial correlative data suggesting that loss of α2β1 integrin expression may either play an important role in cancer progression or simply be a consequence of malignant progression (115). In studies using various tumor cell lines either *in vivo* or *in vitro*, the α2β1 integrin was implicated in enhancing distant metastasis (116-119).

Previous studies examining SCCs have focused on the β1 integrin subunit and integrins αvβ6, α3β1, α5β1, and α6β4 (120). The majority of studies performed on the $\alpha 2\beta 1$ integrin in keratinocytes have focused on its role in wound healing and skin differentiation, so little is known about how the $\alpha 2\beta 1$ integrin is involved in SCC disease progression. Studies examining suprabasal expression of the $\alpha 2\beta 1$ integrin have found that it does not affect malignant conversion from papillomas to SCCs (121). However, one report using two human SCC lines showed up-regulation of the $\alpha 2\beta 1$ integrin on

more invasive cells (122). These findings support a role of the $\alpha 2\beta 1$ integrin in modulating later stages of SCC disease.

Lymphatic Biology

The lymphatic system is made of single-layered endothelial cells surrounded by a discontinuous, highly permeable basement membrane (123). While normal, homeostatic functions of the lymphatic system are to maintain fluid pressure within the interstitial compartment, abnormalities in efflux and recirculation of fluid or cells within the lymphatic system can result in lymphedema, disturbed immune responsiveness, and cancer metastasis. During cancer progression the lymphatic system may serve as a conduit for metastasizing tumor cells.

Unlike the blood vascular network, which has anastomoses between the arterial and venous systems, lymphatic vessels have blind-end terminal buds (initial lymphatics), which mediate fluid uptake. Lymphatic endothelial cells (LECs) in these terminal buds form a phalanx of interdigitated endothelial cells that allow unidirectional interstitial flow into the vessel through specialized gaps between the individual cells, known as primary valves. As with the blood venous system, the lymphatic system also contains a system of valves to prevent the retrograde flow of chyle within the vessel. These structures within lymphatic collecting ducts are known as secondary valves.

Specialized junctions between the individual LECs control fluid uptake and afferent transport. On initial lymphatics, discontinuous, button-like junctions are permissive to fluid uptake. This arrangement of adhesion molecules differs from the continuous, zipper-like junctions seen on collecting lymphatics. The intercellular adhesion molecules on button and zipper-like junctions are composed of vascular endothelial cadherin and tight junction-associated proteins occludin, claudin-5, zonula

occludens-1, junctional adhesion molecule-A, and endothelial cell-selective adhesion molecule (124) (Figure 2-3).

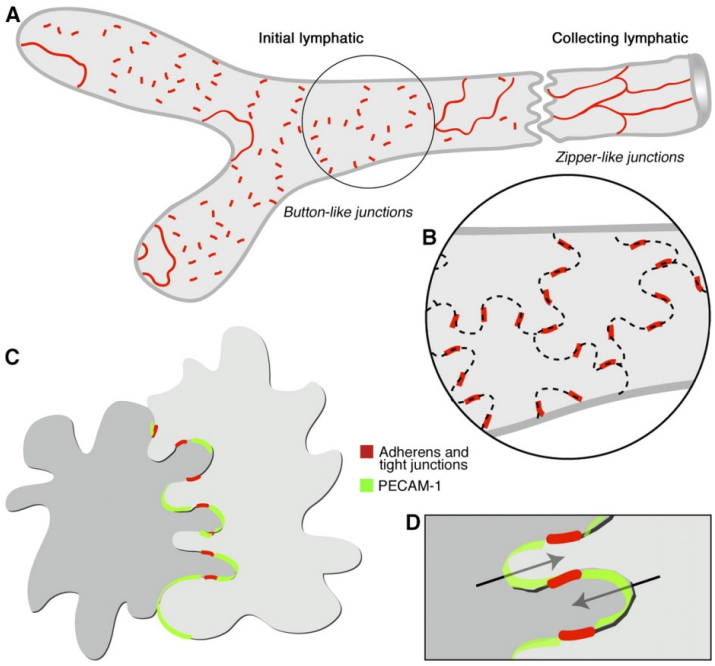


Figure 2-3. Distribution of cell-cell junctional markers on initial and collecting lymphatics that regulate sites of fluid entry. *A,* Diagram showing the discontinuous "button" junctions on the LECs of initial lymphatics versus the continuous, "zipper" distribution found on collecting lymphatics. Both types of junctions consist of adherens and tight junction proteins. *B,* Magnified view displaying the oak leaf shape of endothelial cells (dashed lines) of initial lymphatics. Button junctions (red) are localized to the sides of flaps, whereas most PECAM-1 expression is found at the tips of flaps. *C and D,* These views reveal the distribution of button junctions on the interdigitated oak leaf-shaped endothelial cells. Adherens and tight junctions at the sides of flaps direct fluid entry (arrows) to the junction-free regions at the cell tips. Adapted from Baluk, P and McDonald, D, *J Exp Med*, 2007 (124).

Fluidic movement within the lymphatic system is driven by passive, hydrostatic, and colloidal pressure gradients (123, 125, 126). Fluid enters through initial lymphatic segments via openings between button-like junctions based on differences in interstitial and intravascular pressure. Intercellular junctional distances between LECs are estimated to be 15-20 nm. With increasing pressure in the system, however, these clefts can distend to several micrometers, thereby allowing macromolecules such as colloids, cells, and cellular debris to pass into the lymphatic vessel (127). LECs are attached to the surrounding matrix by fibrillin-rich anchoring filaments. Distension of these filaments caused by pressure gradients between the interstitium and vessel interior result in fluid entry through the primary valves (128). Recent evidence has also suggested an active role of LECs in lymph transport through endothelial barrier functions that regulate ion and protein transport (129-131).

Like blood vessels, the basement membrane surrounding large lymphatic vessels is composed of more continuous layers of collagen type IV, laminin, and fibronectin. However, small lymphatic capillaries have a discontinuous basement membrane primarily composed of collagen type IV and small amounts of laminin. These differences in basement membrane compositions have a major impact on functional properties of the vessels and reflect how the discontinuous basement membrane of lymphatic vessels is essential for free fluid uptake into the lymphatic system (132).

Developmental Lymphangiogenesis

Blood endothelial and LECs share a common progenitor during embryogenesis. On embryonic day 10.5, cells from the anterior cardinal vein begin to be polarized by prospero-related homeobox gene-1 (Prox1) expression. Known as a master regulator of lymphangiogenesis, Prox1 induces the formation of lymphatic buds and sacs in the

presence of vascular endothelial growth factor (VEGF)-C. In the presence of additional signaling proteins, the lymph sacs eventually separate from their parental vein, model, and mature into a complete network of vessels separate from the blood vasculature (Figure 2-4) (133).

Studies on lineage differentiation of LECs have led to the discovery of markers for their identification, including Prox1, podoplanin (Pdpn), vascular endothelial growth factor receptor-3 (VEGFR-3), and lymphatic vessel endothelial receptor-1 (LYVE-1). Prox1 expression drives the transcription of essential genes associated with lymphatic cell identity, including the $\alpha 9\beta 1$ integrin, VEGFR-3, and fibroblast growth factor receptor (FGFR)-3 (134-137). In a similar fashion, the NFATc1 transcription factor also stimulates fibroblast growth factor receptor-3, VEGFR-3, and Pdpn expression in LECs (Figure 2-5) (138).

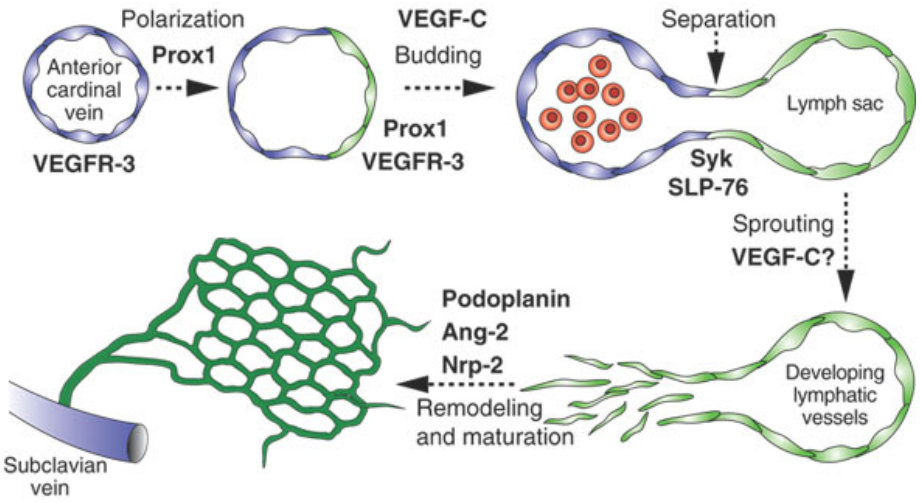


Figure 2-4. Molecular regulators of lymphatic lineage determination. The lymphatic system originates from anterior cardinal vein cells during embryogenesis on day 10.5 in mice. Polarized expression of Prox1 initiates lymphatic differentiation. In the presence of VEGF-C, lymphatic precursors bud and form lymph sacs. Complete separation of the lymph sac from its parental vein occurs through expression of two hematopoietic signaling proteins, SLP-76 and Syk. Final patterning and maturation of the lymphatic vasculature occurs from signaling through podoplanin, angiopoietin-2 (Ang-2), and neuropilin-2 (Nrp-2). Many of the regulators that dictate lymphatic lineage differentiation, such as Prox1, VEGFR-3, and podoplanin, have become useful markers for immunohistochemically identifying lymphatic vessels. Adapted from Folkman, J and Kaipainen, A, *Nat. Immunol.*, 2004 (133).

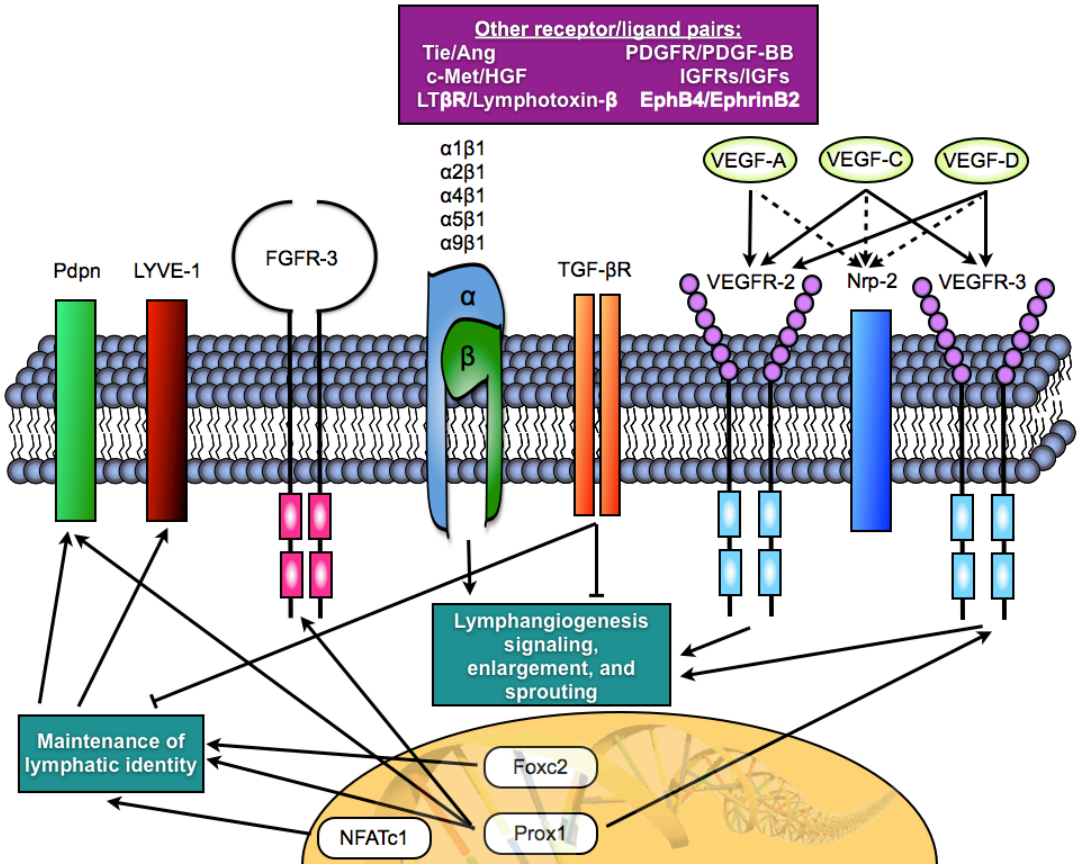


Figure 2-5. Markers for lymphatic identity and signaling pathways stimulating lymphangiogenesis. The maintenance of lymphatic identity includes surface expression of podoplanin (Pdpn), lymphatic vessel endothelial receptor-1 (LYVE-1), fibroblast growth factor receptor-3 (FGFR-3), and vascular endothelial growth factor receptor (VEGFR)-3, as well as nuclear expression of Prox1, NFATc1, and Foxc2. VEGF-A, -C, and -D stimulate lymphangiogenesis through their interactions with corresponding VEGFRs or via integrin activation. These growth factor signals ultimately lead to vessel enlargement or sprouting. Signaling through the TGFβ receptor (TGFβR), however, inhibits lymphangiogenesis (139).

Growth Factor-induced Lymphangiogenesis

Several growth factors are known to promote lymphangiogenesis, including VEGF-A, -C, and -D, as well as hepatocyte growth factor (HGF) receptor c-Met, Ephrin B2, lymphotoxin beta, and members of the fibroblast growth factor, angiopoietin, platelet-derived growth factor, and insulin-like growth factor families of secreted proteins (139, 140). C-Met is strongly expressed on LECs; its stimulation by HGF causes increased murine lymphatic endothelial proliferation, migration, and tube formation in a VEGFR-3-independent pathway. These promigratory effects are mediated by the α9 integrin subunit (141).

Multiple receptor and ligand interactions regulate normal physiologic and pathologic lymphangiogenesis. There are currently two VEGF receptors (VEGFRs) known to induce alterations in the lymphatic vasculature. VEGFR-2 activation by either VEGF-A or -C causes vessel enlargement. VEGFR-3 activation by VEGF-C or -D, on the other hand, induces sprouting lymphangiogenesis (142). Although VEGF-D is a ligand for VEGFR-3, it can induce the formation of heterodimers between VEGFR-2 and -3, thereby modifying the signaling pathway specificity induced by ligand binding (143, 144). While VEGFR-2 and -3 both can alter LEC migration and proliferation, VEGFR-2 activation modifies but is not critical for lymphangiogenesis (Figure 2-5) (142, 145). In terms of receptor affinities, VEGF-C is able to bind VEGFR-2, but it has a higher affinity for VEGFR-3 (146). VEGF-C is also less efficient at activating VEGFR-2 than VEGF-A (147). These subtle differences modulate how LECs respond to varying concentrations of growth factor stimulation.

Co-receptors to the VEGFRs are also important in regulating receptor activation. For example, neuropilin-2 (Nrp-2) binds to VEGF-C and VEGF-D. Upon VEGFR-3 stimulation, the Nrp-2 co-receptor is internalized with VEGFR-3 in endocytic vesicles,

thereby regulating VEGFR-3 activation levels (148). VEGF-C and -D activation of Nrp-2 is required for normal lymphatic development (149). The multiple ligands stimulating lymphangiogenesis, as well as the diversity of receptors to which they signal, create a complex and dynamic view of regulators for lymphatic migration, proliferation, sprouting, and tubule formation.

Lymphatic Pathology

Lymphatic vessels are essential for normal development, wound healing, and immune surveillance and activation. Mice deficient for Prox1, a master regulator of lymphatic lineage differentiation, are embryonic lethal due to arrests in anterior cardinal vein sprouting of LECs at embryonic day 11.5-12.0 (150). These animals exhibit several lymphatic system defects, including chylous ascites, due to complete absence of lymphatic endothelial differentiation from blood endothelial progenitors. Several genetic abnormalities manifest pathologically in lymphatic vessel dilation and increased leakiness, including Prox1 heterozygosity (151, 152).

Abnormalities in lymphatic function can stem from multiple causes. Normally, collecting lymphatics with zipper-like junctions efficiently transport chyle back to the thoracic duct. The continuous distribution of cellular adhesion molecules along the collecting vessel prevents fluid leak back into the interstitium (124). Loss of certain LEC-associated tight junction proteins, such as zonula occludens-1 for example, resulted in increased vessel leakiness (153, 154). Additionally, extremes in lymphatic vessel diameter lead to pathological functionality of the drainage system. Hypoplastic vessels characterize lymphedema, to which no curative treatment exists. At the other end of the spectrum, hyperplastic vessels have reduced vessel functionality due to incompetency of secondary valves within the collecting duct system (155, 156). Therefore, understanding

the relationship between these two extremes, and determining the range in which normal function can occur, will be important in defining relationships between lymphatic structure and function.

Squamous Cell Carcinoma and Lymphatic Metastasis

Metastases are responsible for approximately 90% of deaths associated with solid tumors (157). Cancer cells can metastasize to regional lymph nodes along pre-existing lymphatic vessels and by inducing the formation of new vessels (140). As with the occurrence of an angiogenic switch during tumor development, the release of pro-lymphangiogenic factors from tumor and inflammatory cells has been postulated to drive a similar "lymphangiogenic switch," which has major implications on lymph node metastasis (158). Tumor-associated lymphangiogenesis results from proliferation and migration of preexisting LECs and not from bone marrow-derived endothelial progenitor cells (159). Tumors arrive in lymph nodes via embolic tumor cell dissemination in the majority of lymphatic metastasis (160). Additionally, the intrinsic behavior and shape of tumor cells can affect their ability to enter the lymphatic vasculature. Previous studies have captured electron microscopy images of cancer cells extending protrusions through lymphatic vessel wall gaps before entering the circulation (161, 162). Prospective studies on SCC patients treated by surgical excision directly correlated larger tumor size with increased risk for local recurrence and metastasis (163). Interestingly, the most important predictor of survival in patients with head and neck SCC is cervical lymph node metastasis (164).

Multiple tumor types are known to directly stimulate lymphangiogenesis. For example, the highly metastatic prostate cancer lines PC-3 and LNCaP secrete increased levels of VEGF-A, -C, and -D, which activated VEGFR-2 on prostate LECs to increase

lymphatic vessel formation (165). Additionally, Schacht *et al.* have demonstrated increased podoplanin levels in human squamous cell carcinomas (166). Podoplanin is a mucin-type transmembrane glycoprotein and a target of Prox1, the homeobox gene known as a master regulator of lymphatic differentiation. Several investigators have demonstrated correlations between increased levels of VEGF ligands and SCC-associated lymphangiogenesis. VEGF-C in oral SCC triggers lymphangiogenesis and cervical lymph node metastasis (167). VEGF-A overexpressing skin tumors induce sentinel lymph node lymphangiogenesis, even before tumors metastasized (168, 169). Additionally, sentinel lymph node lymphangiogenesis is highly dependent upon VEGF-A producing B cells within the lymph node (170). Moreover, VEGF-A has been shown to be a potent stimulator of lymphangiogenesis, and overexpression had led to dilated lymphatic vessels with incompetent valves, decreased efficiency of flow, and delayed lymph clearance (171). Since VEGFR-2 binds all three pro-lymphangiogenic VEGF ligands, it is not surprising that, unlike VEGFR-3, VEGFR-2 is the primary receptor driving lymphangiogenesis in several cancer models, include prostate (165). These data underscore the central importance of VEGFR-2 activation in LECs. From these studies, it is evident that growth factor signaling has a dramatic impact on tumor-associated lymphangiogenesis, including in epithelial SCCs. Furthermore, the extent of lymphatic vessel development in these tumors defines clinical prognosis.

Higher lymphatic vessel density is correlated with poor survival for several cancers, including melanoma, cervical, gastric, endometrial, and head and neck SCCs (160). Recent studies indicate that the location of tumor-associated lymphatics also impacts clinical outcome. Intratumoral lymphatic development is noted to occur in breast cancer with VEGF-C overexpression and correlated with significantly enhanced lymph node and lung metastasis (172). Moreover, intratumoral lymphatics have been found in

tumor xenotransplants, in murine SCCs, and in primary human melanomas, which metastasized to regional lymph nodes (172-175). VEGF-C has been shown to induce increased tumor lymphangiogenesis, including intratumoral vessel growth, in an orthotopic murine model using MCF-7 breast carcinoma cells. Additionally, intratumoral lymphatics are found to be critical for the metastatic spread and poor prognosis in head and neck SCCs (173, 176). The growing body of evidence demonstrating the significance of intratumoral lymphatic vessel development towards cancer metastasis has called into revision the previously accepted dogma, that intratumoral lymphatics are rare and nonfunctional, due to mechanical compression from the growing tumor (177, 178). Regardless, it is generally agreed that increases in lymphatic vessel density in and around tumors increases the chance of tumor entry and formation of lymph node metastases (139).

Similar to angiogenesis, lymphangiogenesis is also induced by increasingly hypoxic tumor environments. HIF-1α expression is associated with lymphatic metastasis in various tumor types. For example, in esophageal SCC, HIF-1α may induce VEGF-C expression to increase lymphatic invasion and metastasis (179, 180). In an *in vitro* model using MDA-MB-231 breast cancer and HMVEC-dLy (a commercially available human primary lymphatic endothelial cell line from Lonza) co-cultures, hypoxia was found to increase LEC migration towards the cancer cells (181). Hypoxic conditions are also known to increase levels of TGFβ-induced protein, which increases LEC adhesion to extracellular components, such as collagen, laminin, and fibronectin via the β3 integrin subunit (182). The multiple factors that regulate lymphangiogenesis demonstrate the complexity of this process *in vivo* and further underscore how both the tumor cells and the tumor microenvironment heavily influence LEC behavior.

Integrins in Lymphangiogenesis

Several integrins have been implicated in lymphangiogenesis, including integrins $\alpha1\beta1$, $\alpha2\beta1$, $\alpha4\beta1$, $\alpha5\beta1$, and $\alpha9\beta1$ (137, 183-187). The $\alpha4\beta1$ integrin, a fibronectin receptor, is able to increase LEC proliferation in response to VEGF-A and -C stimulation, as well as increase LEC migration and invasion. Furthermore, inhibition of the $\alpha4\beta1$ integrin decreased tumor-associated lymphangiogenesis and lymph node metastasis in B16 melanoma, Lewis lung carcinoma, and panc02 pancreatic cancer mouse models (188). Additionally, the ECM elastic microfibril-associated protein EMILIN-1 is a ligand for the $\alpha4\beta1$ integrin (189). EMILIN-1 deficiency causes defects in lymphatic growth and function (Figure 2-6) (190).

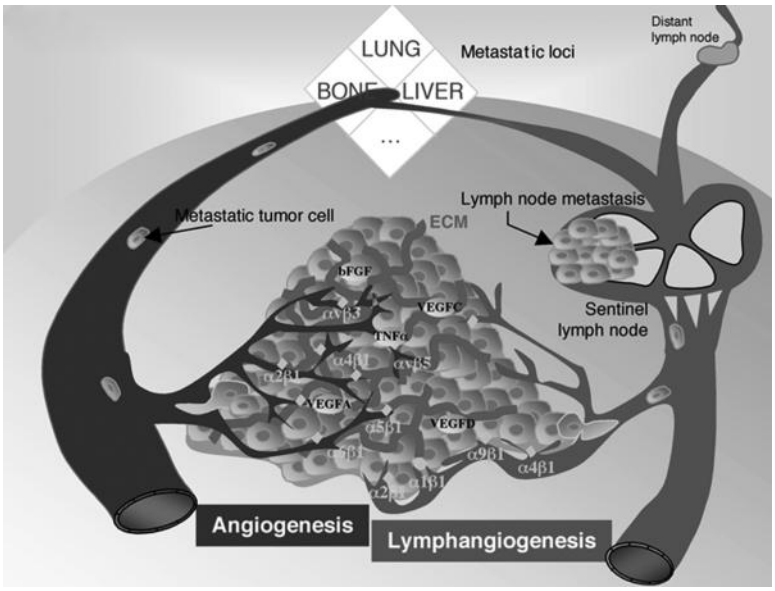


Figure 2-6. Mechanisms regulating tumor-associated lymphangiogenesis. Tumor cells secrete factors such as VEGF-A, -C, and -D, which stimulate the growth of new lymphatic vessels. These growth factors activate or upregulate expression of integrins α1β1, α2β1, α4β1, and α9β1 on lymphatic vessels to increase LEC migration and survival. Tumor-derived VEGF-A and -C also promote new lymphatic vessel growth in draining lymph nodes. New lymphatic vessels also provide routes for tumor metastasis by facilitating tumor cell transport to lymph nodes and, sometimes, to more distant tissues such as the lung. Adapted from Garmy-Susini, B and Varner, J, *Lymphat Res Biol*, 2008 (191).

Another integrin involved in lymphangiogenesis, the $\alpha5\beta1$ integrin, interacts with neuropilin-1 (Nrp-1) at adhesion sites. Trafficking and function of the $\alpha5\beta1$ integrin on endothelial cells is found to be regulated by Nrp-1/GIPC PDZ domain containing family, member 1 (GIPC-1) signaling (192). GIPC-1 binding to the SEA motif of Nrp-1 stimulates the internalization of active $\alpha5\beta1$ integrin. Blocking the $\alpha5\beta1$ integrin with a small-molecule inhibitor resulted in inhibition of inflammatory lymphangiogenesis in murine models of airway inflammation and corneal lymphangiogenesis as well as reduced *in vitro* proliferation of LECs (184, 193). These findings indicate that the $\alpha5\beta1$ integrin is involved in lymphatic sprouting due to its selective expression on new vessels. The $\alpha5\beta1$ integrin also plays a role in tumor-associated lymphatic vessel development. Endostatin inhibits LEC migration by binding to the $\alpha5\beta1$ integrin and reducing lymphangiogenesis in skin tumors (194, 195).

The only integrin with a critical role in embryonic lymphatic vessel development is the $\alpha9\beta1$ integrin. Prox1, the master regulator of lymphatic lineage differentiation, induces expression of integrin $\alpha9$ and VEGFR-3, enabling differentiating LECs to migrate towards a VEGF-C gradient (135, 137). Mice deficient for the $\alpha9$ integrin subunit die 7-10 days post-partum from chylous pleural effusions as a result of defective lymphatic valve morphogenesis (185, 196). Furthermore, lymphangiogenic growth factors VEGF-C and -D are direct ligands for the $\alpha9\beta1$ integrin (185, 197).

Through the process of angiogenesis, VEGF-A was discovered to increase $\alpha1\beta1$ and $\alpha2\beta1$ integrin expression and enable endothelial cell migration on collagen type I. Blocking antibodies to the $\alpha1$ and $\alpha2$ integrin subunit resulted in decreased blood vessel cross-sectional area and new vascular area (198). VEGF-A promotes wound healing-associated lymphatic vasculature formation via activation of VEGFR-2 and by inducing

expression of the collagen binding integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ (186). Additionally, VEGF-A is also known to induce tumor and sentinel lymph node lymphangiogenesis and promote lymphatic metastasis (168). While expression of the $\alpha 1\beta 1$ integrin was found in human lymphangiomas, there is no data on the role of the $\alpha 2\beta 1$ integrin in similar processes (199). Overall, there is little known about the role of the collagen-binding integrins in tumor-associated lymphangiogenesis and metastasis (Table 2-1).

Expression and stimulation of VEGF receptors can also be influenced by integrins. VEGFR-2, which is a central receptor in pro-lymphangiogenic signaling, is negatively regulated by the $\beta 3$ integrin (200). Additionally, VEGFR-3 can interact with the $\beta 1$ integrin subunit, such that $\beta 1$ integrin binding to collagen and fibronectin induces VEGFR-3 phosphorylation (201, 202). These findings demonstrate the complex nature of integrin-VEGFR interactions.

Table 2-1. The role of integrins in lymphangiogenesis. Multiple integrins are expressed on LECs, where they regulate various processes, including lymphangiogenesis during development, in response to inflammation, during tumor growth, and cancer metastasis. Only the $\alpha 9\beta 1$ integrin is critical in forming the lymphatic vasculature. Other integrins, such as $\alpha 1\beta 1$ and $\alpha 2\beta 1$ are upregulated in inflammation-induced lymphangiogenesis. However, little is known about the $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins on tumor-associated LECs and in mediating lymph node metastasis. The $\alpha 4\beta 1$ and the $\alpha 5\beta 1$ integrins have functional roles in tumor-associated lymphatic vessel development, with a known role for the $\alpha 4\beta 1$ integrin in mediating lymphatic metastasis. "+" indicates presence of a functional role, "-" indicates no function found, and "unknown" indicates that the functionality of the integrin has not yet been investigated in that biological process.

Integrin	Primary Matrix Ligands	Development	Inflammation	Tumor	Metastasis
$\alpha 1\beta 1$	Collagen, Laminin	-	+	Unknown	Unknown
$\alpha 2\beta 1$	Collagen, Laminin	-	+	Unknown	Unknown
$\alpha 4\beta 1$	Fibronectin	-	Unknown	+	+
$\alpha 5\beta 1$	Fibronectin	-	+	+	Unknown
$\alpha 9\beta 1$	Fibronectin, Tenascin	+	-	Unknown	Unknown

The α2β1 Integrin in Breast Cancer

Unlike SCCs, the α2β1 integrin's role in breast cancer and hematogenous dissemination has been more extensively studied. Aside from non-melanoma skin cancer, breast cancer is the most common cancer among American women (3). Human breast cancer represents a disease of multiple origins. The most common form is ductal carcinoma, followed by lobular and medullary carcinoma. Extensive work has been performed on the α2β1 integrin's role in breast cancer. As in skin epithelium, high levels of the α2β1 integrin are also found in breast tissue, specifically on normal ductal epithelium (115). During malignancy, however, expression of the α2β1 integrin is decreased in breast adenocarcinoma; studies on other types of adenocarcinoma have yielded similar results (112, 115).

Expression of the α2β1 integrin has been shown to enhance hematogenous metastasis (116-119). Studies by Ramirez *et. al.* have demonstrated that in the spontaneous MMTV-neu model of breast cancer progression and metastasis, loss of the α2β1 integrin increases cancer cell intravasation into the blood vasculature. Additionally, expression of the α2β1 integrin is a prognostic indicator of decreased metastasis and better patient outcomes (203). Examination of polymorphisms linked to altered expression levels of the α2β1 integrin in human patient breast cancers have revealed that reduction of integrin expression is correlated with poor prognosis (204). Loss of the α2β1 integrin caused reduced collagen-dependent adhesion, motility, and morphogenesis in mammary carcinoma cells *in vitro* (205). Finally, re-expression of the α2β1 integrin in integrin-null murine mammary tumor virus-induced Mm5MT cells was able to revert the malignant breast cancer phenotype to a differentiated epithelium, thus

indicating that the α2β1 integrin plays a central role in mammary gland differentiation (112, 206).

Summary

Over the past three decades, it is increasingly evident that the α2β1 integrin functions in multiple cancer types to affect cancer progression and metastasis. This dissertation will address how the α2β1 integrin alters SCC pathogenesis by examining the role of the integrin on epithelial cancer cells as well as in inflammatory, angiogenic, and lymphangiogenic processes driving cancer progression and metastasis that occur within the tumor microenvironment. Particular emphasis will be placed upon the malignant SCC cell phenotype due to presence or absence of the α2β1 integrin. Additionally, the lymphatic system, which mediates lymph node metastasis in the SCC model, will be evaluated in detail. Finally, orthotopic breast cancer models will be examined to evaluate the role of the α2β1 integrin on neoplastic cells in hematogenous metastasis.

The goal of this research was to elucidate integrin-specific contributions by the tumor cells versus the tumor microenvironment. My hypothesis was that the α2β1 integrin functions in cancer initiation or progression by modulating interactions between the squamous epithelium and/or tumor microenvironment. Furthermore, the differences in metastasis with loss of α2β1 integrin expression could result from abnormalities in the tumor-associated lymphatic vasculature. These hypotheses were evaluated through use of the K14-HPV16 mouse, a well-established model of inflammation-driven SCC. Effects of the α2β1 integrin on multi-stage epithelial carcinogenesis was assessed by crossing the K14-HPV16 mouse with either wild-type (HPV/WT) or α2 integrin subunit-null

(HPV/KO), congenic animals. Primary tumor cell lines were established *in vitro,* in which expression levels of the $\alpha 2\beta 1$ integrin could be easily manipulated. Host microenvironment contributions of the $\alpha 2\beta 1$ integrin were also assessed in an orthotopic murine breast cancer model. From these studies, we were able to define the role of the $\alpha 2\beta 1$ integrin in two separate cancer models, a spontaneous SCC model and an orthotopic breast cancer model. Additionally, we were able to investigate the role of this integrin on lymphatic endothelial cells.

This dissertation identifies a role for the α2β1 integrin in tumor cell behavior, both in the SCC and the breast cancer model, although there are disparate effects of the $\alpha 2\beta 1$ integrin depending on the tumor cell type involved. From recent studies, strong evidence suggests that the cancer cell type and the metastatic route determine how the $\alpha 2\beta 1$ integrin influences alterations in cancer formation and metastasis. In the SCC model, we examined how expression of the α2β1 integrin altered preneoplastic progression in the spontaneous K14-HPV16 tumor model. Since our initial SCC studies utilized global α2β1 integrin-null mice, we developed primary tumor cell lines to evaluate the integrin-specific impacts on the malignant epithelium versus the tumor microenvironment. We found that expression of the $\alpha 2\beta 1$ integrin directly increased migration and invasion through type I collagen *in vitro*. Additionally, when HPV/WT and HPV/KO SCC cell lines were orthotopically injected into either WT or KO hosts, α2β1 integrin-specific expression on tumor cells decreased tumor latency and increased tumor growth, regardless of host integrin status. During our initial tumor studies, we found a 31.1% reduction in lymph node metastasis in HPV/KO, compared to HPV/WT, animals. Examination of the lymphatic vasculature in α2β1 integrin-null mice revealed pathologic vessel morphology marked by increased vessel diameter. These dilated vessels

demonstrated increased colloidal carbon leakiness and diminished tumor-associated lymphatic functionality. The decreased metastasis seen in HPV/KO mice may be explained by a decreased functionality of the lymphatic vessels and/or by a decreased migratory and invasive phenotype of HPV/KO tumor cells. Finally, examination of α2β1 integrin expression by the host tumor microenvironment revealed a negligible role of the microenvironment towards altering breast cancer cell metastasis.

This dissertation concludes with a discussion on the varying contributions of the α2β1 integrin, which has proven to be tumor cell type dependent. There will also be discussion on future directions and implications these studies will have on our clinical approach to cancer and lymphatic biology. Although the α2β1 integrin plays no obvious role during normal development, my studies indicate that exposure to physiological stressors, such as cancer, unmasks functions of the α2β1 integrin to reveal its importance in tissue biology.

CHAPTER III

LOSS OF THE α2β1 INTEGRIN ALTERS HUMAN PAPILLOMA VIRUS-INDUCED SQUAMOUS CARCINOMA PROGRESSION *IN VIVO* AND *IN VITRO**

*This chapter is modified from the publication Tran et al., *PLoS ONE*, 2011 (1).

Expression of the α2β1 integrin, a receptor for collagens and laminin, is altered during tumor progression. Recent studies have linked polymorphisms in the α2 integrin gene with oral, squamous cell carcinoma (SCC). To determine the α2β1 integrin's role in SCC progression, we crossed α2-null mice with K14-HPV16 transgenic animals. Pathological progression to invasive carcinoma was evaluated in HPV-positive, α2-null (HPV/KO) and HPV-positive, wild-type (HPV/WT) animals. α2β1 integrin expression stimulated progression from hyperplasia and papillomatosis to dysplasia with concomitant dermal mast cell infiltration. Moreover, lymph node metastasis was decreased by 31.3% in HPV/KO, compared to HPV/WT, animals. To evaluate the integrin-specific impact on the malignant epithelium versus the microenvironment, we developed primary tumor cell lines. Although transition from dysplasia to carcinoma was unaltered during spontaneous tumor development, isolated primary HPV/KO SCC cell lines demonstrated decreased migration and invasion, compared to HPV/WT cells. When HPV/WT and HPV/KO SCC cells were orthotopically injected into WT or KO hosts, tumor α2β1 integrin expression resulted in decreased tumor latency, regardless of host integrin status. HPV/WT SCC lines failed to demonstrate a proliferative advantage *in vitro*, however, the HPV/WT tumors demonstrated increased growth compared to HPV/KO SCC lines *in vivo.* Although contributions of the integrin to the

microenvironment cannot be excluded, our studies indicate that α2β1 integrin expression by HPV-transformed keratinocytes modulates SCC growth and progression.

Introduction

Cancers arise from the accumulation of genetic mutations that alter cell proliferation, differentiation, and tissue organization. Infection with Human Papilloma Virus (HPV) causes 100% of cervical cancer, 90% of anal cancer, 40% of vulvar and vaginal cancer, 15%-35% of oropharyngeal cancers, and approximately 3% of oral cancers (9, 10, 207). Approximately 6.2 million new HPV infections occur each year globally, with 20 million women currently infected. Cervical cancer is the 7th most common cause of death in women worldwide. Arbeit, Coussens, and Hanahan developed a transgenic mouse model of epithelial carcinogenesis in which the HPV 16 early region genes were expressed in basal keratinocytes under the control of the keratin 14 promoter (47-49, 93, 94, 96, 100-106). This model of squamous epithelial carcinogenesis mimics viral-induced tumor progression in humans.

Cancer progression and metastasis do not solely rely upon the genetic and epigenetic events within the tumor cell, but also on changes in the microenvironment (11-13). Integrins mediate both cell-extracellular matrix (ECM) and cell-cell adhesion (22, 24-29, 38). As mediators of cell adhesive behavior, integrins play a critical role in tumor progression and metastasis (22, 42). The $\alpha 2\beta 1$ integrin, primarily a collagen and laminin receptor, is highly expressed on basal keratinocytes where it is involved in adhesion to basement membrane collagens and migration on laminin 5. The integrin is also expressed on many epithelial cells, activated endothelial cells, and some inflammatory cells (47-49, 52, 67). Previous studies suggest that $\alpha 2\beta 1$ integrin

expression is altered *in vivo* during progression of breast, lung, and prostate cancers (109-113). Recent studies have linked polymorphisms in the α2β1 integrin with oral, squamous cell carcinoma (SCC) (208). Dyce *et al*. demonstrated that human SCC cell lines that expressed high levels of the $\alpha 2\beta 1$ integrin were more invasive than cells with low integrin expression (122).

To determine the role of α2β1 integrin expression in squamous epithelial carcinogenesis, we chose the K14-HPV16 model for several reasons: 1. Expression of the α2β1 integrin on squamous epithelial cells is regulated in a differentiation-dependent manner. 2. The K14-HPV16 model investigates the complex interplay between the malignant cells and the host immune system, including a requirement for mast cells in the progression towards invasive SCC. 3. Prior data from our laboratory demonstrated α2β1 integrin expression on connective tissue mast cells and showed that the integrin participated in mast cell activation in response to specific pathogens. 4. HPV-stimulated carcinoma is highly relevant to human disease and represents a significant public health burden.

We now show that α2β1 integrin expression promotes early preneoplastic dysplasia from hyperplasia and papillomatosis to dysplasia. Decreased dysplasia in the HPV/KO mice was associated with decreased recruitment of mast cells at early time points. Although, loss of α2β1 integrin expression did not affect tumor latency, prevalence, tumor growth, or histologic grade, metastasis to the regional lymph nodes was decreased by 31.3%. Since these studies were conducted in animals in which the α2β1 integrin was globally deleted, primary tumor cell lines were developed. Isolated, primary HPV/WT, but not HPV/KO SCC cell lines, migrated rapidly and invaded through a matrix of type I collagen. Following orthotopic injection of HPV/WT and HPV/KO cells into either WT or KO mice, HPV/WT SCC cells formed tumors with a short latency and

rapid growth. In contrast, HPV/KO SCC cells either failed to form tumors or grew significantly slower than the HPV/WT tumor cells. Integrin status of the host animal did not influence tumor development. Therefore, our data from both the spontaneous *in vivo* and orthotopic primary tumor cell transplantation *in vivo* models support a role for α2β1 integrin expression by the HPV oncogene-transformed SCCs in malignant progression. The differences between the spontaneous model and the orthotopic injection model suggest that many factors play a role in tumor progression *in vivo*.

Materials and Methods

Ethics Statement—Mice were housed in pathogen-free conditions at Vanderbilt University Medical Center in strict compliance with national and institutional animal welfare regulations; all animal experiments were approved by Vanderbilt's IACUC protocol M/10/002.

Animals and Tumor Measurement—The α2β1 integrin-deficient mice on the FVB/N background were crossed with congenic K14-HPV16 transgenic mice, a generous gift from Lisa Coussens, to generate K14-HPV16, α2-null (HPV/KO) or wild-type (HPV/WT) mice (51). Non-K14-HPV16-transgenic littermates were used as controls. Mice were monitored weekly for tumor development; tumor volume was calculated as $V = 0.52 \times A \times B^2$, where V is the volume, A is the largest diameter, and B is the shortest diameter. Once tumors reached a maximum diameter of at least 10 mm, affected animals were sacrificed, and tumors, blood, ears, and superficial lymph nodes were harvested.

Histology—Tumor and ear morphology was evaluated on formalin-fixed, paraffin-embedded, and hematoxylin and eosin stained sections. Tumors were classified according to the WHO system of differentiation (1+ = well, 2+ = moderate, 3+ = poorly,

and 4+ = anaplastic/spindle cell) (94). Ear skin was analyzed for the presence or absence of hyperplasia, papillomatosis, or dysplasia. The number of toluidine blue-stained mast cells was quantitated using the Metamorph imaging system (Molecular Devices, Sunnyvale, CA) (209).

Immunohistochemistry and Immunofluorescence—Immunohistochemical identification of wide-spectrum cytokeratin expressing metastatic tumor cells was performed on lymph node sections using anti-bovine WSCK antibody, EnVision+ polyclonal labeled polymer, and DAB substrate (all from Dako, Carpinteria, CA). Immunofluorescence analysis of primary tumor cells was conducted on cells plated on coverslips with anti-LYVE-1 (Abcam, Cambridge, MA), anti-WSCK (Dako), and DAPI (Invitrogen). Secondary antibodies included Alexa Fluors 568 and 488 (Invitrogen). Imaging was performed using a Nikon Eclipse 80i microscope and quantitated using ImageJ (NIH, Bethesda, MD).

Flow Cytometry–Flow cytometric analyses of peripheral blood and infiltrates into ear skin and tumors were performed on a 3-laser, BD LSR II Flow Cytometer in the Vanderbilt Medical Center Flow Cytometry Shared Resource, and data analysis was performed using FlowJo (Tree Star, Inc., Ashland, OR). Tumor-bearing animals were sacrificed and tumor, ear, and peripheral blood leukocytes were collected and processed, as described previously (105). Cell suspensions were evaluated with either pre-conjugated BD Pharmingen antibodies against mouse Gr-1 or NK1.1, eBioscience antibodies (San Diego, CA) against mouse c-kit, CD11b, F4/80, CD3e, B220, CD4, CD25, or Foxp3, or appropriate isotype controls. Expression of the α2 integrin subunit on primary SCC cells was determined by flow cytometric analysis using anti-CD49b (BD Pharmingen) or IgG control (BD Pharmingen).

Cell Culture

Isolation of Primary Tumor Cells: Primary tumors were dissected under sterile conditions, finely minced, and cultured in primary tumor media containing DMEM-F12 (Mediatech, Inc., Manassas, VA) supplemented with 5% FBS (Atlanta Biologicals, Lawrenceville, GA), 0.01 μg/mL human EGF (Pepro Tech, Rocky Hill, NJ), 1 μg/mL hydrocortisone (Sigma-Aldrich, St. Louis, MO), 0.1 μg/mL cholera toxin (Calbiochem, La Jolla, CA), 0.3 units of insulin (Novo Nordisk, Princeton, NJ), and 100 units of penicillin/streptomycin (Mediatech, Inc.) at 37°C in 5% CO_2.

Proliferation Assay: HPV/WT or HPV/KO tumor cells (2×10^3) were plated on either collagen type I (BD Biosciences Discovery Labware, Bedford, MA) (100 μg/mL), fibronectin (BD Biosciences Discovery Labware) (100 μg/mL), or plastic and incubated in primary tumor media. Cell proliferation was evaluated every 24 hours using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega Corporation, Madison, WI), according to the manufacturer's protocol. Plates were read at 490 nm on a Molecular Devices Emax Precision Microplate Reader, and data was analyzed using Softmax (Molecular Devices).

Transfection with the α2 Integrin Subunit: The mouse full-length α2 (mα2) integrin subunit (NCBI GenBank Z29987), a gift from Jeffrey Bergelson, was inserted into the mammalian pSRα expression vector. Clones were selected and verified by sequencing. HPV/KO-2 cells were transfected with 20 μg of either pSRα-mα2 or empty pSRα vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer's protocol.

Adhesion Assay: The ability of primary tumor cells (1.7×10^4 cells/well) to adhere at 37°C for 1 hour to either collagen type I (20 μg/mL) or fibronectin (20 μg/mL)

was determined using established protocols in the presence of 2 mM $MgCl_2$ (Sigma-Aldrich) or 2 mM EDTA (Sigma-Aldrich) (210). The optical density of the plate was read at 405 nm on a Molecular Devices Emax Precision Microplate Reader, and data was analyzed using Softmax (Molecular Devices).

Migration and Invasion Assay: Cell migration and invasion assays were performed using a modification of the protocol described previously (211). HPV/WT or HPV/KO primary tumor cells (2×10^4) in primary tumor media containing 1.5% FBS were placed on the upper membrane of 0.8 μm pore transwell filters (Corning Incorporated Life Sciences, Lowell, MA), which was either uncoated or coated with collagen type I (100 μg/mL). Primary tumor media containing 5% FBS was placed in the lower chamber. The number of cells migrating to the lower chamber at 37°C after 18 hours was determined by counting the number of cells in 3 random high power (10X magnification) fields.

Orthotopic Injection of Primary Tumor Cells—Tumor cells (1×10^6) derived from two separate HPV/WT or HPV/KO lines were injected subcutaneously into the interscapular region of either WT or KO, 4-5-week-old FVB/N hosts. Tumors were measured twice a week; volumes were calculated as previously described.

Statistical Analyses—Statistics were performed using student's t-tests, unless otherwise noted. Contingency table analyses with tests for trend were used to analyze all distributions of tumor grades and tumor multiplicity. Tumor volume growth curves were analyzed by spaghetti plots with curve regression analysis to determine average slopes. Chi-squared tests were performed to determine significance of preneoplastic ear histology. Mann-Whitney U tests were performed for analyses of mast cell ear histology, and for analyzing specific groups within inflammatory populations in all flow cytometry

data. P-values of ≤ 0.05 were considered significant. Analyses were performed using Stata (StataCorp LP, College Station, TX) and GraphPad Prism (La Jolla, CA).

Results

The α2β1 Integrin Promotes HPV-induced Squamous Epithelial Dysplasia

HPV-induced squamous carcinogenesis involves the step-wise progression from hyperplasia to papillomatosis, to dysplasia, to carcinoma in situ (CIS) and finally to invasive and metastatic cancer (95-100, 103). To define the role of the α2β1 integrin in a multi-step, inflammation-driven epithelial carcinogenesis model, we crossed the α2β1 integrin-deficient mouse on an *FVB/n* background with congenic K14-HPV16 mice to generate K14-HPV16/wild-type (HPV/WT) and K14-HPV16/α2-null (HPV/KO) mice. Preneoplastic progression, including hyperplasia, papillomatosis, or dysplasia, was defined in the ear skin of HPV/WT and HPV/KO mice at 3-, 6-, or 9-months-of-age, or at the time of sacrifice due to the development of invasive, squamous carcinoma at another location. By 6-months-of-age, there were significant differences in dysplasia and papillomatosis between the two genotypes: approximately 15% of HPV/WT animals (n = 20), but none of the HPV/KO animals (n = 25), developed dysplasia. In contrast, the incidence of papillomatosis was almost double in the HPV/KO animals (p = 0.0384). Differences in papillomatosis and dysplasia between HPV/KO and HPV/WT ears were also present at later time points (9-months p = 0.0637; time of sacrifice p = 0.00169) (Figure 3-1*A*).

Inflammation has been shown to be responsible for driving neoplastic progression in K14-HPV16 transgenic animals (106). Therefore, the recruitment of inflammatory cells to the skin of HPV/WT and HPV/KO animals at early time points was investigated. There was no significant difference in the total number of CD45-positive

cells recruited to the dermis of HPV/WT and HPV/KO mice at either 3- or 6-months-of-age ($p = 0.29$ and 0.90, respectively; data not shown). At 3-months-of-age, there was also no difference in the number of dermal mast cells in HPV/WT and HPV/KO mouse ears ($p = 0.58$). In contrast, by 6-months-of-age, there were significantly fewer resident mast cells in HPV/KO than in HPV/WT ears ($p = 0.019$). Mast cell numbers decreased in ear tissue over time but were similar at 9-months-of-age and at the time of sacrifice between HPV/WT and HPV/KO ears ($n = 0.32$ and 0.23, respectively) (Figure 3-1*B and C*). While the quantity of acute mast cells was altered in the preneoplastic ears of K14-HPV16 transgenic mice, detailed studies examining inflammatory populations at the time of animal sacrifice revealed that chronic inflammation is not substantially altered in blood, non-tumorigenic ear, or tumor tissue with integrin loss. In this inflammation-driven tumor model, immune cell differences were dependent on presence of the K14-HPV16 transgene, but ultimately, the α2β1 integrin contributes minimally to long-term, chronic inflammation (Figure 3-2 and Table 3-1).

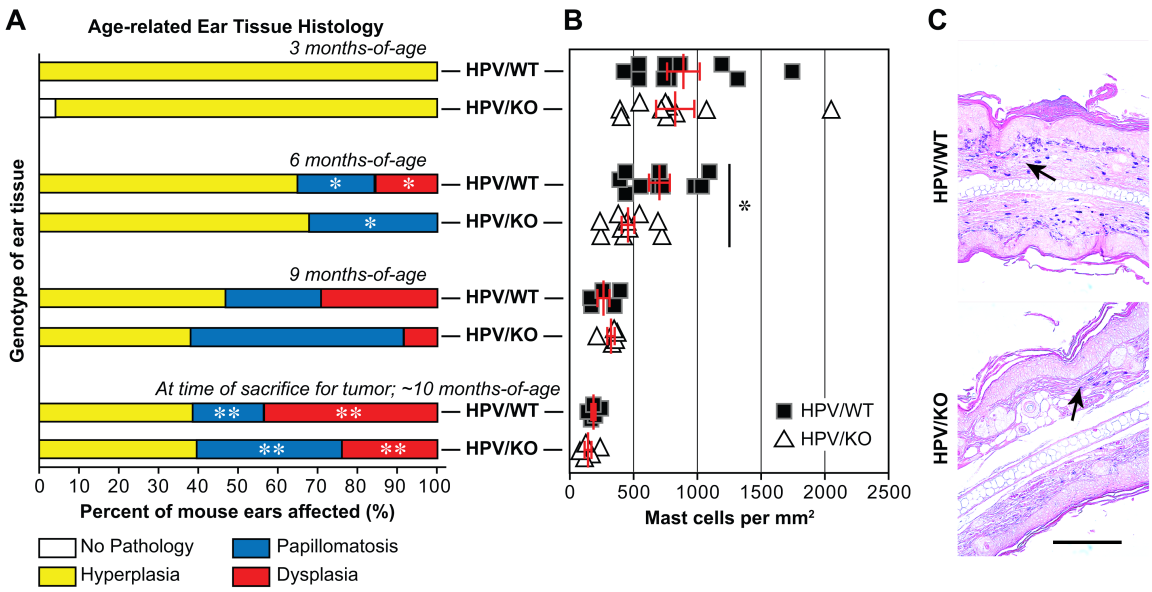


Figure 3-1. Loss of the α2β1 integrin enhanced HPV-induced papillomatosis but limited dysplasia and preneoplastic mast cell infiltration. *A*, The percentage of HPV/WT and HPV/KO animals with hyperplasia, papillomatosis, or dysplasia at 3-, 6-, and 9-months-of-age, and at sacrifice was determined by morphological examination of ear tissue. The incidence of papillomatosis was significantly increased, while dysplasia was significantly decreased in HPV/KO animals, compared to age-matched, HPV/WT littermates, at 6-months-of-age and at sacrifice (3-months $p = 0.304$, HPV/WT $n = 28$, HPV/KO $n = 27$; 6-months $p = 0.0384$, HPV/WT $n = 20$, HPV/KO $n = 25$; 9-months $p = 0.0637$, HPV/WT $n = 17$, HPV/KO $n = 13$; time-of-sacrifice $p = 0.00169$, HPV/WT $n = 95$, HPV/KO $n = 68$). *B*, Mast cell infiltration into the ear dermis of HPV/WT and HPV/KO animals was quantitated at 3-, 6-, and 9-months-of-age, and at sacrifice. Ear skin of HPV/WT and HPV/KO animals at 3-months-of-age have similar numbers of mast cells ($p = 0.58$, $n = 10$ for both groups). At 6-months, HPV/KO ears had decreased numbers of mast cells compared to age-matched HPV/WT littermates ($p = 0.019$, $n = 10$ for both groups). Over time, dermal mast cell infiltration decreased. The number of mast cells in the ear skin of HPV/WT and HPV/KO animals was similar at 9 months and at sacrifice (9 months $p = 0.32$, $n = 5$ for both groups; time of sacrifice $p = 0.23$, $n = 5$ for both groups). Bars represent mean ± SEM of 3 random images per tissue sample. *C*, A representative toluidine blue-stained section of HPV/WT and HPV/KO premalignant ear tissue at 6 months. Arrows indicate toluidine blue positive cells. Scale bar = 200 μm.

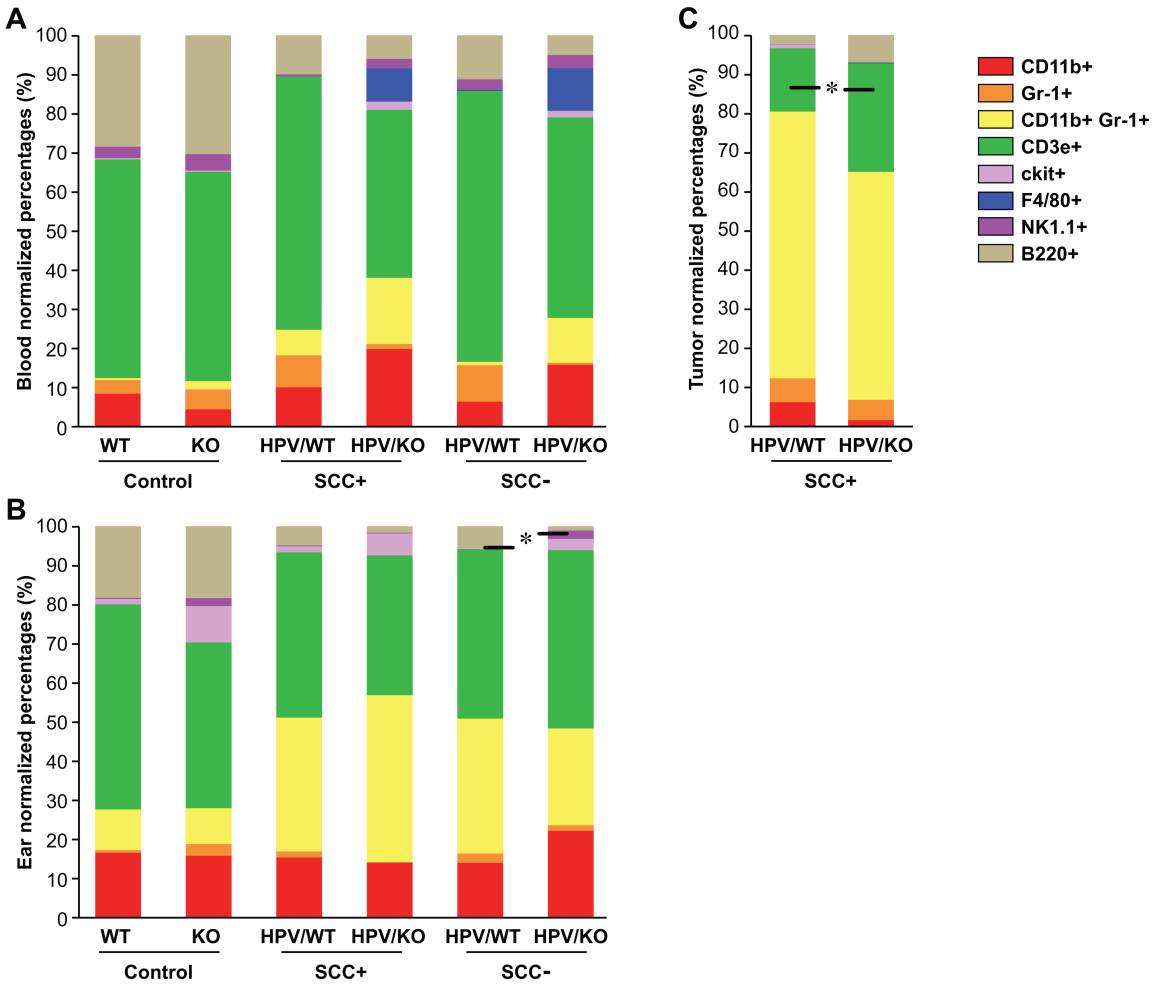


Figure 3-2. The K14-HPV16 transgene, not the α2β1 integrin, mediates a robust inflammatory response. *A-C*, Flow cytometric analysis of inflammatory cells was performed on the blood and preneoplastic ears of non-K14HPV16 transgenic wild-type (WT Ctrl) or α2-null (KO Ctrl) mice and HPV/WT and HPV/KO animals, either with (SCC^+) or without tumors (SCC^-). Similar analysis was also performed on the tumor tissue of HPV/WT and HPV/KO mice. The percentage of inflammatory cell subsets in HPV/WT and HPV/KO animals was compared to non-transgenic controls. Inflammation was highly dependent upon the presence of the K14-HPV16 transgene. Loss of the $\alpha 2\beta 1$ integrin in HPV/KO ears increased the percentage of $NK1.1^+$ cells relative to HPV/WT ears in non-tumor bearing animals ($p = 0.014$). Additionally, there was a significant increase in $CD3\varepsilon^+$ T cells in HPV/KO tumor infiltrates, when compared to HPV/WT SCCs ($p = 0.033$). (Number of samples analyzed in blood and ear tissue: WT Ctrl n = 9; KO Ctrl n = 9; HPV/WT, SCC^+ n = 12; HPV/WT, SCC^- n = 5; HPV/KO, SCC^+ n = 14, HPV/KO, SCC^- n = 4. Number of samples analyzed in tumor tissue: HPV/WT, SCC^+ n = 10 and HPV/KO, SCC^+ n = 12.)

Table 3-1. Detailed Analysis of Inflammatory Cell Populations in Blood, Preneoplastic Ears, and Tumors. WT Ctrl and KO Ctrl animals were used to verify and establish baseline inflammatory populations independent of the K14-HPV16 transgene. Chi^2 probability with ties analysis was performed on all 6 groups for each specific tissue; those found to be significant or close to $p < 0.05$ were analyzed further through inter-comparison of the 6 groups by Mann-Whitney tests. The groups in which significance was found are denoted as Genotype 1 vs. Genotype 2. Differences in inflammatory cells were found between non-K14-HPV16 transgenic, control animals and those expressing the K14-HPV16 transgene. Integrin-dependent differences were identified in the NK1.1-positive and CD3ε-positive cell populations. Non-neoplastic ear tissue in HPV/KO, SCC^- mice had increased NK1.1-positive cells than HPV/WT, SCC^- ears ($p = 0.014$). HPV/KO SCCs contained more CD3ε-positive cells than HPV/WT tumors ($p= 0.033$). T regulatory cells were defined as CD4, CD25, and Foxp3 triple-positive cells as a percentage of CD4-positive cells. (Blood and ear samples analyzed: WT Ctrl n = 9; KO Ctrl n = 9; HPV/WT, SCC^+ n = 12, HPV/WT, SCC^- n = 5; HPV/KO, SCC^+ n = 14, HPV/KO, SCC^- n = 4. Tumor tissue analyzed: HPV/WT, SCC^+ n = 10 and HPV/KO, SCC^+ n = 12).

* represents $p < 0.05$
** represents $p < 0.001$
*** represents $p < 0.0001$

Table 3-1, continued

Tissue	Marker	Chi^2 Probability with Ties	Genotype 1	vs.	Genotype 2	Mann-Whitney Test
Blood	CD11b	0.56				
Blood	Gr-1	0.010*	KO Ctrl	>	HPV/KO, SCC-	0.0055**
Blood	Gr-1/CD11b	0.17				
Blood	CD3ε	0.58				
Blood	ckit	0.79				
Blood	F4/80	0.057	WT Ctrl	<	HPV/WT, SCC-	0.02*
Blood			KO Ctrl	<	HPV/KO, SCC-	0.031*
Blood	NK1.1	0.0030*	KO Ctrl	>	HPV/KO, SCC+	0.0019**
Blood	B220	0.0001*	WT Ctrl	>	HPV/WT, SCC+	0.0056**
Blood			WT Ctrl	>	HPV/WT, SCC-	0.014*
Blood			KO Ctrl	>	HPV/KO, SCC+	0.0003***
Blood			KO Ctrl	>	HPV/KO, SCC-	0.0055**
Blood	CD4	0.19				
Blood	% Tregs of CD4+	0.023*	WT Ctrl	<	HPV/WT, SCC+	0.055
Blood			KO Ctrl	<	HPV/KO, SCC+	0.025*
Ear	CD11b	.17				
Ear	Gr-1	.77				
Ear	Gr-1/CD11b	.0011**	WT Ctrl	<	HPV/WT, SCC+	0.011*
Ear			WT Ctrl	<	HPV/WT, SCC-	0.014*
Ear			KO Ctrl	<	HPV/KO, SCC+	0.0033**
Ear			KO Ctrl	<	HPV/KO, SCC-	0.031*
Ear	CD3ε	.17				
Ear	ckit	.83				
Ear	F4/80	.077				
Ear	NK1.1	.03*	KO Ctrl	>	HPV/KO, SCC+	0.037*
Ear			HPV/WT, SCC-	<	HPV/KO, SCC-	0.014*
Ear	B220	.012*	WT Ctrl	>	HPV/WT, SCC+	0.028*
Ear			KO Ctrl	>	HPV/KO, SCC-	0.0087**
Ear	CD4	.15				
Ear	% Tregs of CD4+	.0058**	KO Ctrl	<	HPV/KO, SCC+	0.049*
Ear			KO Ctrl	>	HPV/KO, SCC-	0.021*
Tumor	CD11b		HPV/WT, SCC+	≈	HPV/KO, SCC+	.80
Tumor	Gr-1		HPV/WT, SCC+	≈	HPV/KO, SCC+	.87
Tumor	Gr-1/CD11b		HPV/WT, SCC+	≈	HPV/KO, SCC+	1.0
Tumor	CD3ε		HPV/WT, SCC+	<	HPV/KO, SCC+	.033*
Tumor	ckit		HPV/WT, SCC+	≈	HPV/KO, SCC+	.66
Tumor	F4/80		HPV/WT, SCC+	≈	HPV/KO, SCC+	.66
Tumor	NK1.1		HPV/WT, SCC+	≈	HPV/KO, SCC+	.84
Tumor	B220		HPV/WT, SCC+	≈	HPV/KO, SCC+	.27
Tumor	CD4		HPV/WT, SCC+	≈	HPV/KO, SCC+	.32
Tumor	% Tregs of CD4+		HPV/WT, SCC+	≈	HPV/KO, SCC+	.11

The α2β1 Integrin Regulates Development of Sebaceous Adenocarcinoma But Not Invasive Squamous Cell Carcinoma

To determine the impact of α2β1 integrin expression on progression from dysplasia to invasive carcinoma, tumor latency and prevalence in HPV/KO and HPV/WT animals were determined. Tumor latency was similar in HPV/KO and HPV/WT animals ($p = 0.11$) (Figure 3-3*A*). No differences exist in SCC development between HPV/WT (49.4%) and HPV/KO (58.9%) mice by 10-months-of-age ($n = 170$ and 107, respectively; $p = 0.12$). The tumor growth rate, number of tumors per animal, and anatomic location of the SCCs were indistinguishable in HPV/KO animals, as compared to HPV/WT mice (Figure 3-4*A, B*, and data not shown). Therefore, although α2β1 integrin expression promotes epithelial dysplasia, expression does not stimulate tumor progression from dysplasia to invasive carcinoma in the HPV-stimulated model of squamous cancer.

Previous studies demonstrated that α2β1 integrin expression may be associated with normal, regulated, epithelial differentiation and that altered expression of the integrin may be seen in different subtypes of cancer. To determine whether α2β1 integrin expression or lack thereof affected tumor cell differentiation, invasive HPV/WT and HPV/KO tumors were analyzed histologically. SCC differentiation was graded based on the Broder's four-tier system (94). There was no difference in tumor grade between HPV/WT and HPV/KO animals ($p = 0.57$), suggesting that the α2β1 integrin did not ultimately impact squamous differentiation in the K14-HPV16 background (Figure 3-4*C*). Even though the majority of tumors arising in the wild-type K14-HPV16 background were SCCs, occasionally, these animals developed sebaceous adenocarcinomas, either alone or in areas with concomitant SCC growth (212). In HPV/KO mice, compared to HPV/WT mice, pure sebaceous adenocarcinomas represented 13.75% versus 4.00% of the tumors, respectively ($p = 0.028$) **(**Figure 3-3*B*).

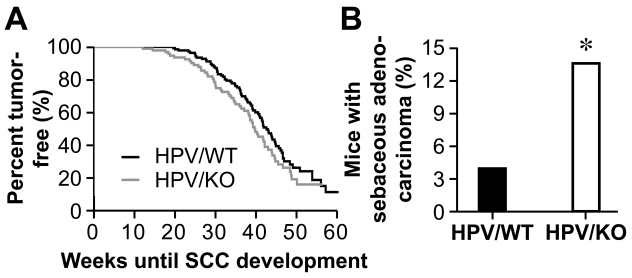


Figure 3-3. Expression of the α2β1 integrin modulates the incidence of sebaceous adenocarcinoma formation, but not SCC. *A*, Kaplan-Meier plots of tumor-free HPV/WT and HPV/KO mice. Tumor development was recorded when a visible tumor nodule formed. Latency (time to tumor development) was similar in HPV/WT (n = 146) and HPV/KO (n = 94) mice (p = 0.11). *B,* The percentage of HPV/WT and HPV/KO animals that developed either SCC or sebaceous adenocarcinoma was determined morphologically. Development of sebaceous adenocarcinoma was significantly increased in HPV/KO animals (n = 80) compared to HPV/WT mice (n = 100) (p = 0.028).

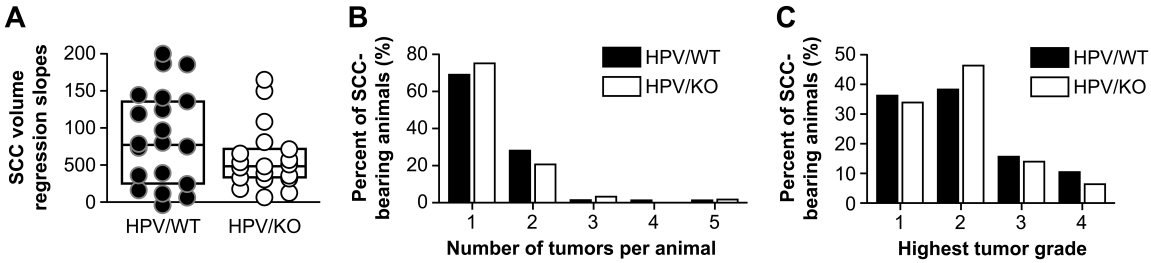


Figure 3-4. Loss of the α2β1 integrin does not alter SCC growth, multiplicity, or grade. *A*, Tumor volumes were measured weekly. The rate of tumor growth over time was calculated from tumor volume regression slopes and plotted as a function of time. No significant differences existed in the rates of SCC growth between HPV/WT (n = 22) and HPV/KO (n = 22) mice (p = 0.37). *B,* Total tumor burden for each HPV/WT (n = 97) and HPV/KO mouse (n = 73) was quantitated at the time of sacrifice. No significant differences were found for the multiplicity of tumor development (p = 0.45). *C,* Since multiple tumors may form on an animal, the highest grade scored was considered for analysis of differentiation loss. No significant differences were observed when considering the highest grade of SCC that developed in HPV/WT (n = 97) or HPV/KO (n = 73) mice (p = 0.57).

Loss of the α2β1 Integrin by HPV-Induced SCC Decreases Lymph Node Metastasis

Previous studies have shown that approximately 30% of SCCs in the K14-HPV16 mouse metastasize to regional lymph nodes (212). Consistent with the literature, in our study, 34.8% of HPV/WT tumors metastasized. In contrast, only 23.9% of HPV/KO SCCs metastasized to the lymph nodes (Figure 3-5*A*). The presence of lymph node metastasis was verified by immunohistochemical staining for cytokeratin (Figure 3-5*B*). Therefore, although there was no difference in tumor growth or tumor latency, expression of the α2β1 integrin promoted tumor metastasis to regional lymph nodes. The difference in metastasis between HPV/WT and HPV/KO animals was not statistically significant ($p = 0.14$) due to limitations of study size. However, the incidence of lymph node metastasis in HPV/KO mice was decreased by 31.3%, compared to metastasis in the HPV/WT animals. The odds ratio for developing lymph node metastasis in the HPV/WT animals relative to HPV/KO mice was 1.7 (HVP/KO mice 95% confidence interval is 18.3-54.3%; HPV/WT 95% confidence interval is 34.7-81.9%).

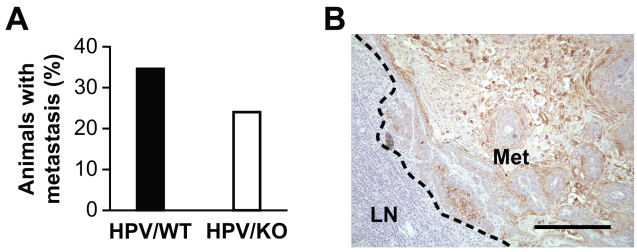


Figure 3-5. α2β1 integrin expression promoted lymph node metastasis. *A*, The percentage of HPV/WT and HPV/KO animals with SCC that developed regional lymph node metastasis was determined by morphologic and immunohistochemical analysis of superficial lymph nodes. The HPV/KO animals with tumors (n = 71) developed metastasis in 23.9% of cases; HPV/WT animals with tumors (n = 92) developed metastasis in 34.8% of cases (p = 0.14; odds ratio 1.7). *B*, Representative section of a regional lymph node from an HPV/WT animal evaluated by immunohistochemistry for detection of WSCK. The metastatic tumor cells express WSCK (Met) and are surrounded by normal lymph node parenchyma (LN). Scale bar = 200 μm.

α2β1 Integrin Expression by Squamous Carcinoma Drives Migration and Invasion

To begin dissecting integrin-dependent changes in the tumor cells versus by cells of the host microenvironment, we focused on the contribution of α2β1 integrin expression by the malignant epithelial cells in tumor progression. Primary tumor cells from HPV/WT and HPV/KO tumors were harvested and two HPV/WT (HPV/WT-1 and HPV/WT-2) and two HPV/KO (HPV/KO-1 and HPV/KO-2) squamous carcinoma cell lines were developed. The epithelial origin of the tumor cells was confirmed by cytokeratin staining (Figure 3-6*A*). The HPV/WT, but not the HPV/KO primary tumor cell lines expressed the α2β1 integrin, as determined by flow cytometric analysis (Figure 3-6*B*). Both HPV/WT cells, but not the HPV/KO cells, adhered to type I collagen in a Mg^{2+} dependent and $EDTA^{2+}$-inhibitable manner, as did a positive control, NMuMG-X2C2 (derived from the NMuMG3 line stably transfected with full length human α2 integrin subunit) (Figure 3-6*C*) (210). All cells adhered to fibronectin (data not shown). Both HPV/WT and HPV/KO cells proliferated at a similar rate on collagen, fibronectin, or plastic ($p = 0.35$, $p = 0.33$, and $p = 0.42$, respectively) (Figure 3-7). Therefore, integrin expression did not alter tumor cell proliferation of HPV-driven squamous tumor cells. Although presence of the α2β1 integrin did not alter cell proliferation, expression of the integrin stimulated cell migration and cell invasion *in vitro.* HPV/WT, but not HPV/KO, cells robustly migrated *in vitro* in a three-dimensional transwell migration assay ($p < 0.0001$) and invaded through a barrier of type I collagen ($p < 0.0001$) (Figure 3-6*D*).

To determine if α2β1 integrin expression alone could mediate the migratory ability of HPV/KO cell lines, expression of the α2β1 integrin in the HPV/KO-2 cell line was rescued by transfection with a murine α2-integrin subunit expression vector (HPV/KO-2-mα2^{+}) or control vector (HPV/KO-2-VC). As determined by flow cytometric analysis, HPV/KO-2-mα2^{+} cells expressed high levels of the murine α2β1 integrin

(Figure 3-6*E*). Re-expression of the α2 integrin subunit restored the ability of the HPV/KO-2-mα2^{+} cells to adhere to type I collagen in a Mg^{2+} dependent and $EDTA^{2+}$-inhibitable manner, when compared to HPV/KO-2-VC cells ($p = 0.015$) (Figure 3-6*F*). Restoration of murine α2-integrin expression by HPV/KO-2 SCCs also rescued the migratory and invasive ability of the tumor cells through type I collagen, when compared to the control transfectants ($p = 0.0002$ and $p < 0.0001$, respectively) (Figure 3-6*G*).

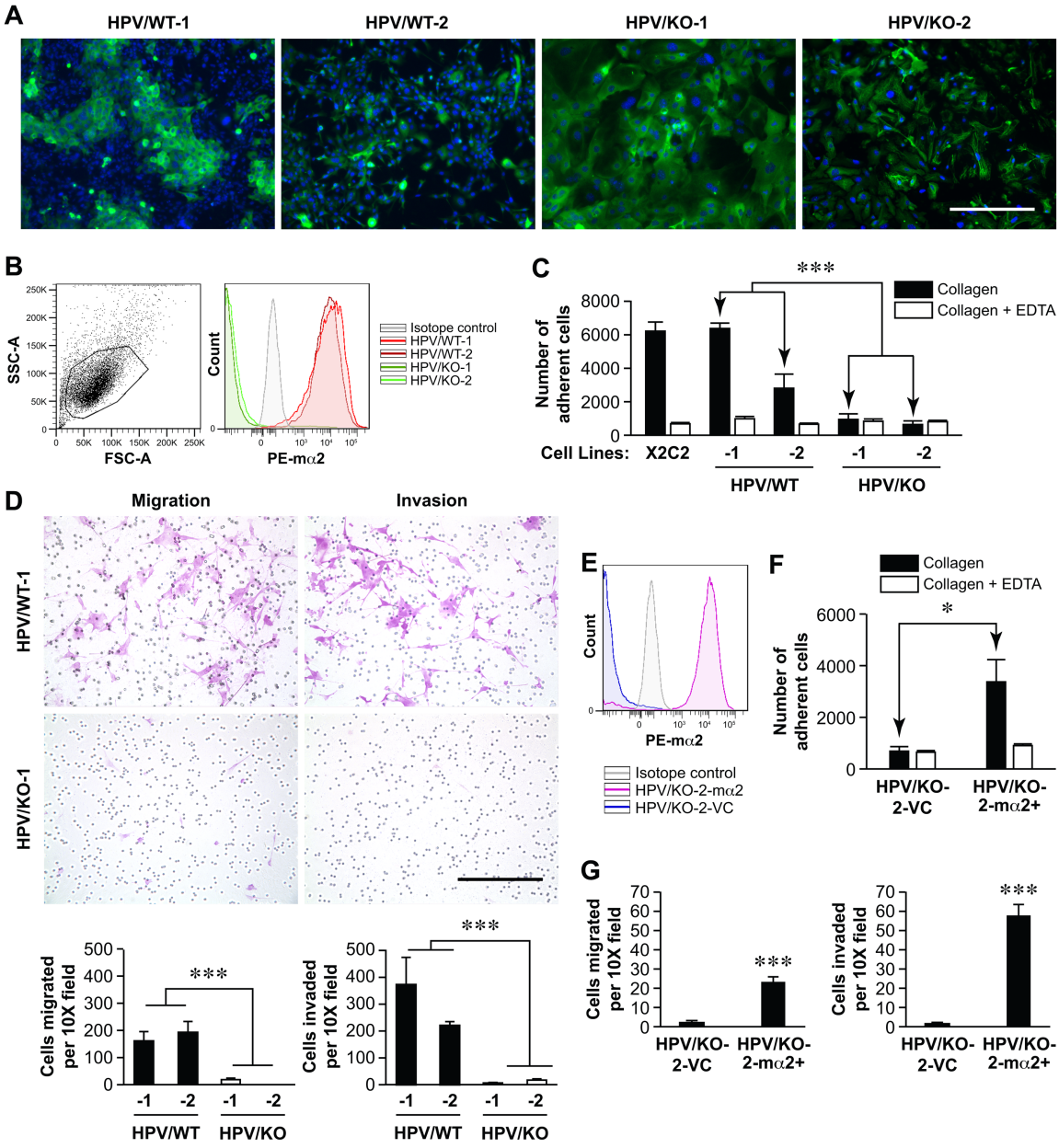

A
HPV/WT-1
HPV/WT-2
HPV/KO-1
HPV/KO-2
B
SSC-A
FSC-A
Count
PE-mα2
Isotope control
HPV/WT-1
HPV/WT-2
HPV/KO-1
HPV/KO-2
C
***
Collagen
Collagen + EDTA
Number of adherent cells
Cell Lines: X2C2 -1 -2 -1 -2
HPV/WT
HPV/KO
D
Migration
Invasion
HPV/WT-1
HPV/KO-1
Cells migrated per 10X field
Cells invaded per 10X field
***
HPV/WT
HPV/KO
E
Count
PE-mα2
Isotope control
HPV/KO-2-mα2
HPV/KO-2-VC
F
Collagen
Collagen + EDTA
*
Number of adherent cells
HPV/KO-2-VC
HPV/KO-2-mα2+
G
Cells migrated per 10X field
Cells invaded per 10X field
***
HPV/KO-2-VC
HPV/KO-2-mα2+

Figure 3-6. Expression of the α2β1 integrin stimulates SCC migration and invasion *in vitro*. *A*, Primary HPV/WT and HPV/KO tumor cell lines were stained with anti-WSCK to demonstrate the epithelial origin of the cells. *B,* Flow cytometric analysis using an α2 subunit antibody verified integrin expression on wild-type SCC lines, HPV/WT-1 and -2, and absence of integrin expression on α2-null lines HPV/KO-1 and -2. *C,* HPV/WT-1 and -2 SCC lines adhered to type I collagen in a Mg^{2+}-dependent and EDTA-inhibited manner. The X2C2 control cells that express human full-length α2 cDNA served as a positive control. The HPV/KO-1 and -2 cells failed to adhere to type I collagen ($p < 0.0001$). Bars represent mean ± SEM of 2 experiments, performed in duplicate. *D*, HPV/WT-1 and -2 cells exhibited significantly enhanced migration and invasion compared to HPV/KO-1 and -2 cells, cells ($p < 0.0001$ and $p < 0.0001$, respectively). Bars represent mean ± SEM of 3 random photos of transwell experiments, performed in duplicate. *E*, Transfection of the HPV/KO-2 line with pSRα vector containing the wild-type mouse α2 integrin subunit (HPV/KO-2-mα2) restored integrin levels to that found in wild-type SCC cells, as determined by flow cytometric analysis. *F*, Expression of the transfected mα2 subunit in HPV/KO-2-mα2 cells rescued their ability to adhere to collagen, compared to empty vector control transfectants (HPV/KO-2-VC) ($p = 0.015$). Bars represent mean ± SEM of 2 experiments, performed in duplicate. *G,* The ability of the HPV/KO-2-mα2 transfectants to migrate and invade was restored, compared to HPV/KO-2-VC cells ($p = 0.0002$ and $p < 0.0001$, respectively). Bars represent mean ± SEM of 3 random photos of transwell experiments, performed in duplicate.

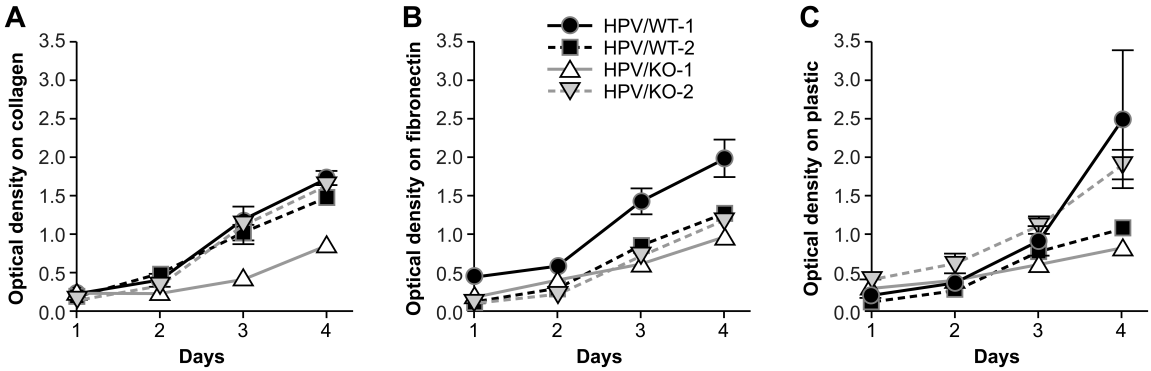


Figure 3-7. *In vitro* proliferation of primary SCC cells was unaffected by loss of the $\alpha 2\beta 1$ integrin. Proliferation of the HPV/WT-1 and -2 and HPV/KO-1 and -2 SCC lines when adherent to collagen, fibronectin, or tissue culture plastic was determined *in vitro*. Proliferation *in vitro* of HPV/WT and HPV/KO lines was similar irrespective of the matrix ($p = 0.35$, $p = 0.33$, $p = 0.42$, respectively).

α2β1 Integrin Expression by Squamous Epithelium Promotes Tumor Growth *In Vivo*

To determine the impact of α2β1 integrin expression by the tumor cells on tumor growth and latency, the primary tumor cell lines derived from HPV/WT and HPV/KO animals (HPV/WT-1 and -2 and HPV/KO-1 and -2) were orthotopically injected into nontransgenic, wild-type or α2-null mice. The HPV/WT tumor cells grew rapidly when placed in either wild-type or α2-null mice. In contrast, the HPV/KO tumor cells demonstrated increased latency ($p = 0.0003$) and markedly decreased tumor growth rates ($p = 0.034$) when compared to mice injected with HPV/WT SCC cells, regardless of recipient mouse integrin status (Figure 3-8*A and B*). The short time span of orthotopic tumor growth was not permissive for the development of spontaneous metastasis. These results demonstrate that the α2β1 integrin expression promotes tumor growth and progression of SCC in a manner independent of the host microenvironment.

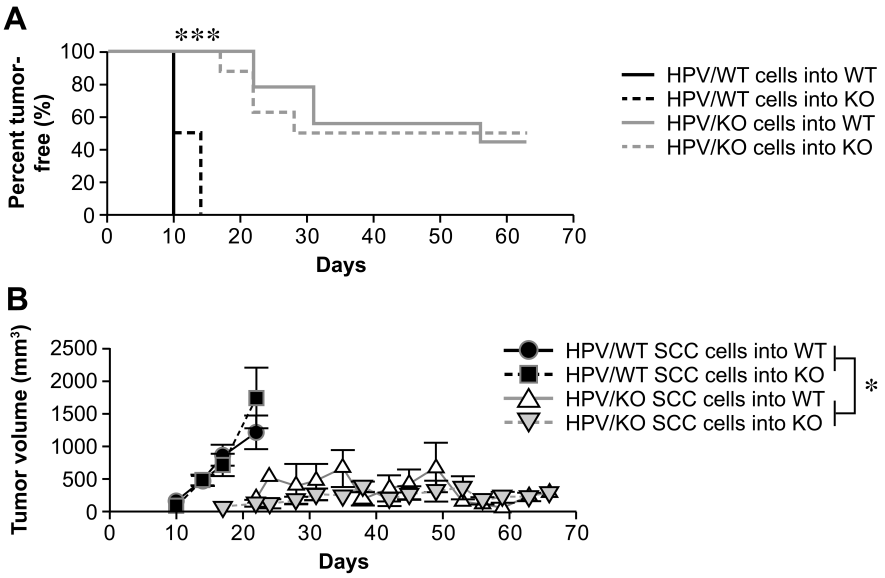


Figure 3-8. Tumor-specific expression of the α2β1 integrin caused rapid tumor formation and increased tumor growth *in vivo*, independent of host microenvironment. *A*, Orthotopic injections of SCC lines HPV/KO-1 and -2 into either non-K14-HPV16 transgenic, α2 WT or α2-null (KO) hosts resulted in increased tumor latency by approximately 5-10 days, as compared to SCC lines HPV/WT-1 and -2 ($p = 0.0003$). Host integrin status had no impact on tumor formation. Tumor latency was dependent on presence of the α2β1 integrin by the tumor cells (n = 4 WT or 4 KO hosts each for HPV/WT-1, HPV/WT-2, and HPV/KO-1 injections; n = 4 WT and 5 KO hosts for HPV/KO-2 SCC cell injections). *B*, Orthotopically injected HPV/KO tumor cells demonstrated decreased growth rate regardless of host integrin status, compared to HPV/WT SCC lines ($p = 0.034$). Bars represent mean ± SEM.

Discussion

Using the K14-HPV16 cancer model, we demonstrate that lack of $\alpha 2\beta 1$ integrin expression results in decreased progression from epithelial papillomatosis to dysplasia, increased formation of sebaceous adenocarcinomas instead of SCCs, and modestly decreased lymph node metastasis. Although global loss of the α2β1 integrin in all HPV/KO mouse cells did not affect tumor latency, growth, or multiplicity *in vivo,* primary tumor cells derived from HPV/KO animals demonstrated diminished cell migration and invasion *in vitro* and decreased tumor formation and growth when implanted orthotopically into non-K14-HPV16 transgenic wild-type or α2-null animals. Additionally, the host's integrin status did not impact tumor formation or growth, thereby suggesting that α2β1 integrin expression by the tumor microenvironment is not responsible for tumor progression in this model.

Diminished epithelial dysplasia and enhanced papillomatosis in HPV/KO mice suggest that the α2β1 integrin plays a role in regulating epithelial differentiation and promoting the initial steps of neoplasia. The mast cell reduction in 6-month-old HPV/KO mice may promote papillomatosis. Firstly, the reduction in mast cells may limit the further progression of papillomas to carcinoma. Secondly, mast cell deficient animals have been shown to be more susceptible to papilloma formation than their wild-type counterparts in other models (213). Therefore, while these inflammatory cells help drive the hyperplasia and dysplasia associated with squamous carcinogenesis, they may be affecting rates of papillomatosis differently (96). At the stage of invasive carcinoma, neither tumor latency, growth, or differentiation, i.e. grade, was different in HPV/WT and HPV/KO mice. In concordance with *in vivo* murine studies, demonstrating that dysregulated expression of the α2β1 integrin did not alter malignant conversion in SCC,

α2β1 integrin expression in the K14-HPV16 model did not affect later aspects of tumor progression (121).

Although no difference in SCC progression was noted *in vivo* using global α2-null mice*,* orthotopic injections of primary squamous carcinoma cells isolated from HPV/WT or HPV/KO mice into either non-K14-HPV16 transgenic, wild-type or α2-null animals revealed that HPV/WT tumor cells, but not HPV/KO tumor cells, engrafted and grew rapidly.. The HPV/WT tumor cells were significantly more migratory and invasive *in vitro*. Integrin loss on SCC cells resulted in reduced migration but even more striking deficiencies in invasion through collagen type I. Our data suggest that α2β1 integrin-mediated interaction of squamous carcinoma cells with type I collagen, which is abundant in the dermis of mice and humans, may function to promote cancer cell migration and invasion, as seen in other models (118, 214). HPV/KO tumor cells transfected with the wild-type mouse α2-integrin subunit failed to maintain integrin expression *in vivo*, thus preventing the analysis of integrin rescue in a SCC model of *in vivo* tumor formation and growth.

In patients with SCC, the development of lymph node metastasis is a predictor of poor outcome (163, 215). Loss of the α2β1 integrin in the K14-HPV16 model resulted in decreased lymph node metastasis to regional lymph nodes by 31.3%. This correlates with an odds ratio of 1.7 for developing lymph node metastasis in the HPV/WT animals relative to HPV/KO mice. These data were quite surprising in light of our own recently published data that the α2β1 integrin acts as a tumor metastasis suppressor in breast and prostate cancer. In the mouse mammary tumor virus-Neu (MMTV-Neu) transgenic mouse model of breast cancer, lack of $\alpha 2\beta 1$ integrin expression resulted in modestly decreased mammary tumor latency and markedly increased cancer metastasis (203).

The discordant contributions of the α2β1 integrin to metastasis in the HPV-stimulated model of SCC versus the neu-driven model of breast cancer raise interesting questions. First, the two models deal with distinctly different subtypes of carcinoma, SCC and adenocarcinoma, which arise from two disparate cells types. Perhaps the α2β1 integrin's role in regulating the multistep process of tumorigenesis is different depending on the cell of origin. Second, the metastatic route and mechanisms of dissemination are different between the two disease models. In the K14-HPV16 model, SCCs primarily metastasize via the lymphatics to regional lymph nodes. In the MMTV-neu model, cancer metastasizes primarily via the hematogenous route to the lungs. Third, the two tumor models are driven by different oncogenes that function distinctly. K14-HPV16 oncogenesis is triggered by expression of early region HPV16 oncogenes, E6 and E7, which inactivate two important tumor suppressor genes p53 and retinoblastoma, respectively. In contrast, the MMTV-Neu model of mammary cancer is driven by overexpression of the Neu tyrosine kinase that stimulates activation of the ras/map kinase cascade. Fourth, progression to carcinoma in the K14-HPV16 model requires the protumorigenic activity of inflammatory cells. Neither tumorigenesis nor metastasis in the MMTV-Neu model is dependent on recruitment of inflammatory cells. Fifth, the interaction of cancer cells with the specific collagen content of their microenvironment may be different. SCCs expressing the $\alpha 2\beta 1$ integrin may have increased migration and invasion along collagen type I fibers in the skin, whereas receptor ligation with collagen type I may not be as important in breast cancer cell motility.

In summary, the $\alpha 2\beta 1$ integrin plays a complex role in tumor progression through its contributions to both the malignant epithelial cell and within the tumor

microenvironment. Our data are the first to suggest that integrin dependent regulation of tumor progression may be specific to the tissue type and to the mechanism of oncogenesis. In c-neu/HER2-positive breast cancer, the α2β1 integrin is a metastasis suppressor. In contrast, the α2β1 integrin promotes tumor metastasis in HPV-induced squamous cancer, likely by increasing the migratory and invasive ability of cells along collagen type I.

CHAPTER IV

THE α2β1 INTEGRIN ALTERS LYMPHATIC MORPHOLOGY AND FUNCTIONALITY IN BOTH NON-NEOPLASTIC AND TUMOR-ASSOCIATED VESSELS

Lymph node staging for cancer cell dissemination is widely used as a prognostic indicator of cancer progression, yet far less is known about lymphatic versus hematogenous dissemination routes. Multiple factors influence lymphatic metastasis, including tumor cell invasiveness, tumor-associated lymphangiogenesis, and the lymph node microenvironment. As surface heterodimers involved in cell signaling, matrix adhesion, proliferation, and differentiation, integrins are emerging as key players in developmental, inflammatory, and neoplastic lymphangiogenesis. During wound healing, the α2β1 integrin is upregulated on lymphatic endothelial cells (LECs) in response to increased vascular endothelial growth factor (VEGF)-A. In Chapter III, we showed that integrin loss resulted in decreased lymph node metastasis in the K14-HPV16 murine model of squamous cell carcinoma (SCC). We hypothesized that the α2β1 integrin functions in maintenance of lymphatic integrity and in altering tumor-associated lymphatic functionality. In this Chapter, we demonstrate that loss of the α2β1 integrin resulted in altered tumor-associated lymphangiogenesis, lymphatic vessel dilation, increased leakiness from the vasculature, reduced dye transport from tumors to draining lymph nodes, and decreased expression the LEC tight junction protein ZO-1 *in vivo*. Additionally, when primary LECs were plated on Matrigel, α2-null cells demonstrated an overall increase in area covered by growing cells after one week in culture. Our findings emphasize novel roles of the α2β1 integrin in maintenance and function of the lymphatic system. The data presented here suggest a novel paradigm in

tumor dissemination via the lymphatic vasculature: destabilization of the tumor lymphatic vessels causing intravascular leak will decrease the efficiency of afferent cancer cell transport to draining lymph nodes.

Introduction

Metastasis in cancer is a key predictor of poor survival by accounting for approximately 90% of deaths associated with solid tumors (157). Often the initial site of metastasis for multiple cancers, lymph node status has been useful in staging and defining clinical prognosis (216). For example, 75% of prostate cancer patients with positive lymph nodes at the time of diagnosis eventually develop bone metastasis within 5 years, regardless of treatment (217). Notably, the most important predictor of survival in patients with head and neck SCC is cervical lymph node metastasis (164).

Multiple factors contribute to metastatic dissemination and distant colonization. Cancers and their associated inflammatory cells are well known to secrete factors that increase and/or functionally alter the blood and lymphatic vasculature (171, 218, 219). More specifically, tumors are capable of promoting their own dissemination, not only by increasing their innate invasive ability, but also by secreting pro-angiogenic and lymphangiogenic factors that increase vascular density and optimize metastatic opportunity (160, 165). These alterations have a significant impact on cancer prognosis and clinical outcome (220). Furthermore, mechanisms of tumor cell entry into lymphatic vessels can either be active, via invasion through the lymphatic endothelial phalanx, or passive, by interstitial-intravessel pressure gradients (127, 162). Similar to the hematogenous route of cancer dissemination, metastasizing tumor cells must be able to invade locally, enter the lymphatic vasculature, survive and avoid immune cell-mediated death, colonize draining lymph nodes, and grow (221).

The integrin family of cell surface, heterodimeric receptors, specifically $\alpha1\beta1$, $\alpha2\beta1$, $\alpha4\beta1$, $\alpha5\beta1$, and $\alpha9\beta1$, have recently been shown to play novel roles in developmental, inflammatory, and neoplastic-associated lymphangiogenesis (137, 183-187). The $\alpha2\beta1$ integrin, responsible for binding collagens, laminins, and other matrix and non-matrix proteins, is expressed on LECs. Although its presence is not critical for survival, the $\alpha2\beta1$ integrin functions in regulating orderly cellular proliferation, differentiation, and migration (52). While $\alpha2\beta1$ integrin expression is increased during inflammatory lymphangiogenesis, little is known about the $\alpha2\beta1$ integrin's role in normal or in tumor-associated lymphatics (186). In Chapter III, I have shown that loss of the $\alpha2\beta1$ integrin resulted in modestly decreased SCC lymph node metastasis. We hypothesized, therefore, that the $\alpha2\beta1$ integrin plays a role in lymphatic functionality and in tumor-associated lymphangiogenesis to alter metastasis.

A central aim of my studies was to examine the effect of $\alpha2\beta1$ integrin loss on the lymphatic vasculature in a non-tumorigenic setting as well as in the presence of cancer. By first understanding perturbations in lymphatic function caused by integrin loss, we can determine how cancers may co-opt these functional differences to either facilitate or impede metastasis. In order to study the lymphatic vasculature in these two settings, we used wild-type (WT) and $\alpha2$-null FVB/N mice in addition to the previously described K14-HPV16 mouse model of SCC crossed onto a wild-type (HPV/WT) or $\alpha2$-null (HPV/KO) congenic animal background.

My studies revealed that $\alpha2\beta1$ integrin loss resulted in altered tumor-associated lymphangiogenesis, marked vessel dilation even in non-neoplastic-associated lymphatics, and increased leakiness of injected macromolecules. This altered vessel morphology was not associated with developmental patterning defects of the lymphatic

vasculature, as analyzed from branching morphogenesis of colloidal carbon injected ears. Additionally, lymphatic vessels had decreased tracer dye uptake in the draining lymph nodes of HPV/KO SCCs, compared to HPV/WT animal tumors. This new data could support findings detailed previously in Chapter III, whereby a reduction in HPV/KO lymphatic functionality contributed to the decreased lymph node metastasis seen in HPV/KO, compared to HPV/WT, mice. This defect in lymphatic transport suggests that tumor-associated lymphatic vessels merely manifest the decreased lymphatic functionality seen with $\alpha 2\beta 1$ integrin loss, which existed in the absence or presence of tumor. Additionally, the increased leakiness of $\alpha 2$-null vessels may result from the observed loss of zonula occludens-1 expression by normal and tumor-associated LECs. Further examination is required, however, to determine the cause of $\alpha 2$-null vessel dilation. Initial studies revealed an increased primary LEC coverage on Matrigel after 7 days of *in vitro* culture, which may result from either increased proliferation, sprouting, or tubulogenesis.

Ultimately, my studies present a novel paradigm in the study of lymphatic-associated integrins. Until now, studies on integrins in the lymphatic system have mainly focused on developmental, inflammatory, and wounding processes. Here, I show that in an altered lymphatic hemodynamic setting, such as in states of fluid overload or neoplasia, expression of the α2β1 integrin prevents vascular leak of macromolecules, thereby increasing efficiency of fluid and potentially tumor cell transport to the draining lymph node. Finally, further studies will be performed to examine the lymphatic transport of multiple cancer cell lines in real time.

Materials and Methods

Transgenic Animal Model—Animals were housed in pathogen-free conditions at Vanderbilt University Medical Center in compliance with IACUC regulations. Previously described C57BL/6 or FVB/N WT or α2-integrin subunit null mice were used to determine lymphatic functionality in a non-tumorigenic setting (51). K14-HPV16 transgenic mice, a generous gift from Lisa Coussens, were crossed with α2-null mice on the FVB/N background to produce α2-null (HPV/KO) or wild-type (HPV/WT) mice, as described previously in Chapter III.

Colloidal Carbon Ear Injections—Mice ears were injected with tracer as previously described (218). Briefly, animals were anesthetized using a mixture of 2% isoflurane/98% oxygen and cradled in a custom-made, Crystal Clear 200 plastic mold (Smooth-on, Easton, PA). Using pre-pulled, beveled borosilicate micropipettes (Sutter Instrument, Novato, CA), a 1:1 mixture of colloidal carbon (Sanford, Oak Brook, IL):PBS was injected into the terminal lymphatics of WT and KO mouse ears; images were taken at 1- and 20-minutes post injection using a Nikon Coolpix 5700 camera adapted on an Olympus SZH10 dissecting microscope. Color images were split into red, green, and blue channels using ImageJ (NIH, Bethesda, MD). Only red channels were retained for analysis. Photos from 1 minute post injection were analyzed for the quantity of branch points between the injection site and the main collecting vessel of the ear. Images were quantitated on two lymphatic vessels per mouse ear.

Colloidal Carbon:Isosulfan Blue Tumor Injections—Mice tumor volumes were calculated as $V = 0.52 \times A \times B^2$, where V is the volume, A is the largest diameter, and B is the shortest diameter. Once tumors reached a maximum diameter of at least 10 mm, affected animals were used for tracer studies. Mice were anesthetized using a mixture of 2% isoflurane/98% oxygen and injected with 60 μL of a 1:1 mixture containing

colloidal carbon (Sanford) and 1% isosulfan blue (Tyco Healthcare Group, Norwalk, CT) either into the interior of the tumor or in the tumor margins. Animals were allowed to recover from anesthesia for 20 minutes before being sacrificed. The dye-injected tumor and all superficial draining lymph nodes were harvested. Tumor tissue was fixed in 10% PBS-buffered formalin (Fisher Scientific, Fair Lawn, NJ). Fixed tissue was paraffin-embedded, sectioned 5 μm thick, and stained with hematoxylin and eosin. The anatomical site of injection was verified histologically; the number of dye-positive lymph nodes was quantitated from each animal.

Immunofluorescence for LYVE-1, CD49b, & TO-PRO-3—Frozen tumor tissue was sectioned 8 μm thick, blocked in 3% goat and 2% horse serum (Vector Laboratories, Burlingame, CA), and stained with anti-LYVE-1 (1:800, Abcam, Cambridge, MA), FITC-conjugated anti-CD49b (1:200, BD Pharmingen, San Diego, CA), and TO-PRO-3 633 (1:400, Invitrogen, Carlsbad, CA). Goat anti-rabbit Alexa Fluor 568 (1:800, Invitrogen) was used as a secondary antibody. Tissue was mounted using Fluorogel with TES buffer (Electron Microscopy Sciences, Hatfield, PA). Imaging was performed using a LSM 510 Zeiss inverted confocal microscope in the Vanderbilt Cell Imaging Shared Resource.

Immunofluorescence of Whole Mount Ears—Mice ears were prepared as previously described (218, 222). Briefly, animals were anesthetized with a 2% isoflurane/98% oxygen mixture and injected with 100 μL of FITC-conjugated tomato lectin (Vector Labs, Burlingame, CA) retro-orbitally. After 3 minutes, mice were sacrificed and cardiac perfused with 10% PBS-buffered formalin (Fisher Scientific). The cartilage was stripped from the ventral and dorsal aspect of the ears, and tissue was fixed and stained with anti-LYVE-1 (1:500, Abcam) and goat anti-rabbit Alexa Fluor 568 (1:500, Invitrogen). Z-stack images were taken using a LSM 510 Zeiss inverted confocal

microscope in the Vanderbilt Cell Imaging Shared Resource. All Z-stacks were compressed via intensity summation and analyzed in ImageJ (NIH). Tight junctional staining of whole mount ears was performed using anti-LYVE-1 (1:500, Abcam) in addition to either anti-Claudin-5 (1:50, Abcam), anti-ESAM (1:100, Abcam), anti-JAM-1 (1:100, Abcam), anti-Occludin (1:100, Abcam), or anti-ZO-1 (1:100, Abcam). Staining with primary antibodies was followed by incubation with Alexa Fluor 568 (1:500, Invitrogen), Alexa Fluor 488 (1:500, Invitrogen), and TO-PRO-3 (1:400, Invitrogen). Tissue was mounted using Fluorogel with TES buffer (Electron Microscopy Sciences). Z-stack images were captured with a LSM 510 Zeiss inverted confocal microscope in the Vanderbilt Cell Imaging Shared Resource. Intensity of junctional adhesion protein staining on lymphatic vessels was quantitated using Metamorph using individual sections from Z-stack images (Molecular Devices, Sunnyvale, CA). Z-stacks were then merged for presentation purposes only in Figure 4-3*A*. The tight junction mean fluorescence intensity from 6 random LYVE-1$^+$ areas was used to determine relative protein expression on each tissue sample.

Immunofluorescence of Squamous Cell Carcinomas—OCT-embedded frozen tumor sections from HPV/WT and HPV/KO mice, collected previously, were sectioned 8 μm thick and acetone-fixed. Sections were stained with anti-LYVE-1 (1:800, Abcam) in addition to anti-Claudin-5 (1:50, Abcam), anti-ESAM (1:100, Abcam), anti-JAM-1 (1:100, Abcam), anti-Occludin (1:100, Abcam), or anti-ZO-1 (1:100, Abcam). Staining with primary antibodies was followed by incubation with Alexa Fluor 568 (1:800, Invitrogen), Alexa Fluor 488 (1:800, Invitrogen), and TO-PRO-3 (1:500, Invitrogen). Tissue was mounted using Fluorogel with TES buffer (Electron Microscopy Sciences, Hatfield, PA). Photos of LYVE-1$^+$ vessels were taken using a LSM 510 Zeiss inverted confocal microscope in the Vanderbilt Cell Imaging Shared Resource. Three random photos were

taken per sample. The masking function in Metamorph was used to calculate mean fluorescence intensities of the tight junction stain in all LYVE-1$^+$ areas.

Isolation of Murine Primary LECs—Primary lymphatic endothelial cells were harvested from incomplete Freund's adjuvant (IFA) injected mice according to established protocol (223). Briefly, 200 µL of a 1:1 mixture containing IFA and PBS were injected into the peritoneum of WT or α2-null C57BL/6 mice on days 1 and 15. Lymphangiomas were harvested on day 30, manually disrupted, digested by incubation for 30 minutes at 37°C in 0.5 mg/mL collagenase H (Roche Applied Science, Basel, Switzerland), and plated onto dishes containing 1% gelatin (Sigma-Aldrich, St. Louis, MO). Cells were grown in RPMI 1640 media (Gibco Invitrogen, Carlsbad, CA) containing 20% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 100 units of penicillin/streptomycin (Mediatech, Inc., Manassas, VA), 1 mM sodium pyruvate (Sigma), 2 mg/mL glutamine (Gibco Invitrogen), 100 µg/mL heparin (Sigma), and 30 µg/mL endothelial cell growth supplement (Sigma). Cells were expanded *in vitro* for at least 10 days before flow sorting on a BD FACS Aria (BD Biosciences, San Jose, CA) using anti-podoplanin and anti-LYVE-1 antibodies (eBioscience, San Diego, CA).

Plating of Primary LECs on Matrigel—Flow-sorted LECs from either 5 WT or 5 α2-null mice were combined for each experiment, which was repeated twice, with all cells plated and quantitated in quadruplicate in a 96-well plate. 2 x 10^3 primary LECs were plated onto 100 µL of growth factor-reduced Matrigel (BD Biosciences Discovery Labware, Bedford, MA) in complete lymphatic media. Cells were grown at 37°C in 5% CO_2, and photographed at 1, 2, 5, 7, and 11 days after plating.

Flow Cytometry of HMVEC-dLyAd Cells—Levels of α2β1 integrin expression on HMVEC-dLyAd cells (Lonza, Walkersville, MD) were determined by flow cytometric analysis using anti-CD49b (BD Pharmingen) or IgG control antibodies (BD Pharmingen).

Matrigel Plug Assay—For each mouse, 400 ng of recombinant VEGF-A (R&D Systems, Minneapolis, MN), VEGF-C (Cell Sciences, Canton, MA), or VEGF-D (R&D Systems) were mixed with 400 µL of growth factor-reduced Matrigel (BD Biosciences Discovery Labware). Animals were anesthetized with a mixture of 2% isoflurane/98% oxygen. 400 µL of VEGF-loaded Matrigel was injected subcutaneously, inferiorly and laterally to the right scapula. As a control, 400 µL of PBS-loaded Matrigel was injected in a similar location, inferiorly and laterally to the left scapula. After 21 days, animals were sacrificed, and Matrigel plugs were removed and fixed using a solution of 4% paraformaldehyde/10% sucrose in PBS overnight at 4°C. Tissues were then blotted, embedded into OCT, and sectioned 5 µm thick. Sections were blocked in 3% goat and 2% horse serum (Vector Laboratories, Burlingame, CA), and stained with anti-LYVE-1 (1:800, Abcam), anti-CD31 (1:800, BD Pharmingen), and TO-PRO-3 633 (1:400, Invitrogen). Goat anti-rabbit Alexa Fluor 568 (1:800, Invitrogen) and goat anti-rat Alexa Fluor 488 (1:800, Invitrogen) were used as secondary antibodies. Tissue was mounted using Fluorogel with TES buffer (Electron Microscopy Sciences). Imaging was performed using a LSM 510 Zeiss inverted confocal microscope in the Vanderbilt Cell Imaging Shared Resource. Three images were taken per sample.

Statistical Analyses—All statistical analyses were done using GraphPad Prism (La Jolla, CA). T-tests were performed on fluorescent angiographies of blood and lymphatic vasculature. Mann-Whitney tests were performed on the distribution of tumor-associated lymphatic vessels, on tight junctional analysis of whole mount ears and tumor-associated vessels, and on the analysis of Matrigel plugs. Primary LEC growth on

Matrigel was analyzed using 2-way ANOVA. P values of ≤ 0.05 were considered statistically significant.

Results

Loss of the α2β1 Integrin Results in Altered Tumor-associated Lymphatic Vessels, Where it is Normally Expressed in Both Mice and Humans

Little is known about α2β1 integrin expression on cells of the lymphatic vasculature. Besides its involvement in wound healing lymphangiogenesis, wherein the α2β1 integrin aids LEC migration, no data exist on tumor-associated lymphatic vessels (186). In Chapter III, I demonstrated that HPV/KO mice demonstrated a modest decrease in SCC lymph node metastasis by 31.3%. The reduction in lymphatic metastasis prompted the examination of lymphatic vessels in K14-HPV16 transgenic and non-transgenic animals. In order to understand these metastatic differences, I examined the distribution of SCC-associated lymphatic vessels in the K14-HPV16 mouse model. There was a significant decrease in the overall number of lymphatics in HPV/KO tumors, compared to HPV/WT tumors ($p = 0.037$). Due to the ongoing debate concerning the importance and functionality of intratumoral versus peritumoral lymphatics, vessels in both areas were examined separately (177, 178). As was noted in human SCCs, there was a correlation between the quantity of intratumoral lymphatics and metastasis (173, 176). The decrease in lymphatics was prominent intratumorally, where HPV/KO SCCs had significantly fewer vessels than HPV/WT tumors ($p = 0.0049$), but this effect was not seen peritumorally ($p = 0.66$) (Figure 4-1*A and B*).

In order to determine whether our murine LECs expressed the α2β1 integrin, non-neoplastic HPV/WT ear tissue was immunofluorescently labeled with antibodies against the LYVE-1 lymphatic specific marker and CD49b, the α2 subunit. Results

showed that the α2β1 integrin is present on LECs in our murine model (Figure 4-1*C*). Likewise, when primary human HMVEC-dLyAd cells were analyzed for surface expression of the α2 integrin subunit, high levels of the integrin were detected by flow cytometry (Figure 4-1*D*). The α2β1 integrin, therefore, is found on LECs across multiple species.

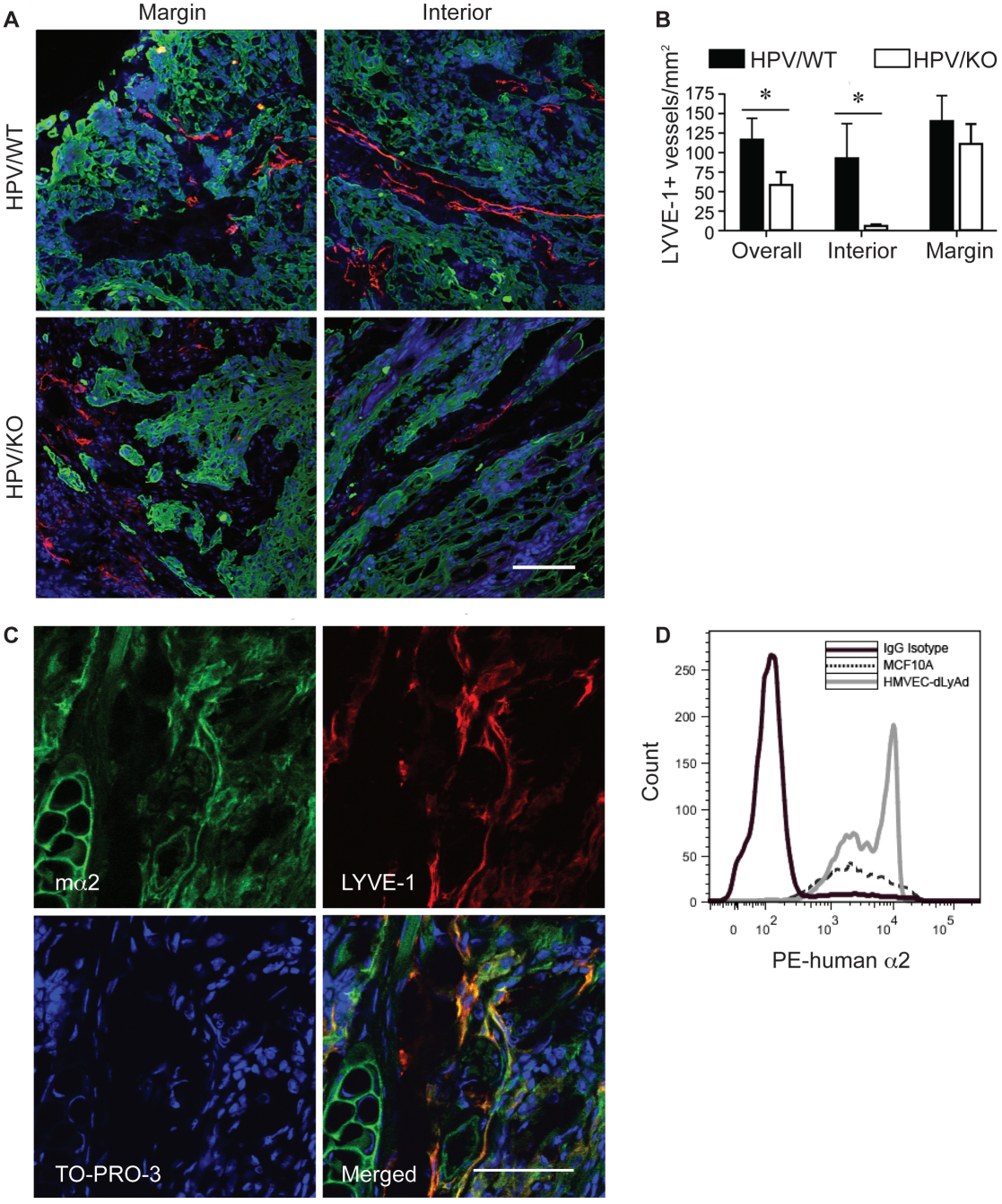

A
Margin
Interior
HPV/WT
HPV/KO
B
HPV/WT
HPV/KO
LYVE-1+ vessels/mm2
175
150
125
100
75
50
25
0
*
*
Overall
Interior
Margin
C
mα2
LYVE-1
TO-PRO-3
Merged
D
IgG Isotype
MCF10A
HMVEC-dLyAd
Count
250
200
150
100
50
0
0
10^2
10^3
10^4
10^5
PE-human α2

Figure 4-1. α2β1 integrin expression alters the distribution of tumor-associated lymphatic vessels, where it is normally found on lymphatic endothelial cells in mice and humans. *A*, Immunofluorescence analysis of tumor lymphatics in HPV/WT and HPV/KO animals is shown: wide spectrum cytokeratin positive SCC cells (green), LYVE-1^+ lymphatics (red), and TO-PRO-3^+ nuclei (blue). Scale bar = 100 μm. *B*, The number of LYVE-1^+ vessels overall, within the tumor itself, or at the tumor margin, was quantitated on 2 random peritumoral and 2 intratumoral images per sample using Image J. HPV/KO tumors (n = 7) have fewer overall LYVE-1^+ lymphatic vessels than HPV/WT tumors (n = 8; p = 0.037), but this effect was predominantly due to a decrease in the vessel number intratumorally, p = 0.0049). In contrast, the number of HPV/WT and HPV/KO lymphatic vessels was similar at the tumor margins (p = 0.66). Bars represent mean ± SEM. *C,* Immunofluorescence performed on non-neoplastic HPV/WT ear tissue revealed expression of the mouse $\alpha 2\beta 1$ integrin (mα2) (green) on LYVE-1^+ lymphatic endothelial cells (red). Cell nuclei were stained with TO-PRO-3 (blue). Scale bar = 50 μm. *D,* Primary human lymphatic HMVEC-dLyAd cells express high levels of the $\alpha 2\beta 1$ integrin, as demonstrated by flow cytometry. MCF10A cells, known to express abundant levels of the integrin were used as a positive control for staining.

Loss of the α2β1 Integrin Causes Dilation of Lymphatics, Increased Vessel Leakiness, and Decreased Drainage of Macromolecules from Primary Tumors to Lymph Nodes

The alterations in tumor-associated lymphangiogenesis prompted a further examination of the lymphatics in both non-tumor-bearing and tumor-bearing animals. Immunofluorescence on whole mount ear tissue revealed dilated lymphatic vessels in α2-null mice, compared to control WT animals ($p < 0.0001$). Similarly, HPV/KO ears also had dilated lymphatic vessels, when compared to HPV/WT mice ($p < 0.0001$). This vessel enlargement in α2-null mice is, therefore, independent of the K14-HPV16 transgene and instead directly dependent upon α2β1 integrin expression.

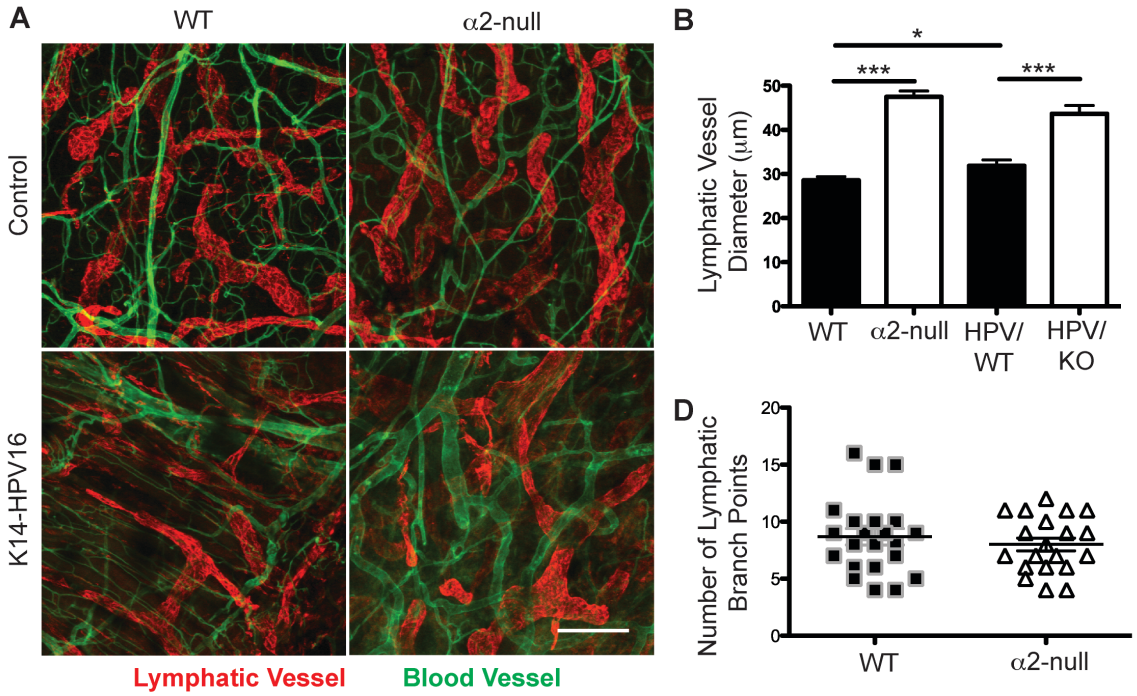
Previously, Eichten *et al.* demonstrated that K14-HPV16 mice had increased LEC proliferation in premalignant and carcinoma tissue (218). Likewise, HPV/WT lymphatic vessels were more dilated than WT, non-K14-HPV16 transgenic animals ($p = 0.032$; n = 6 and 7, respectively); however, the increase was not as dramatic in HPV/KO, compared to α2-null mice ($p = 0.096$; α2-null n = 5 and HPV/KO n = 5) (Figure 4-2*A and B*). While K14-HPV16 transgene expression does not alone account for lymphatic vessel dilation, it does increase vessel size from baseline in animals with the α2β1 integrin. Several etiologies exist for lymphatic vessel dilation, such as abnormalities in lymphangiogenesis-associated genes like Prox1, which lead to hyperplasia of the vasculature (151, 152). Vessel dilation has been associated with increased leakiness caused by reduced functionality from secondary valve incompetency (155, 156). These studies demonstrate that alterations in lymphatic vessel structure may compromise functionality.

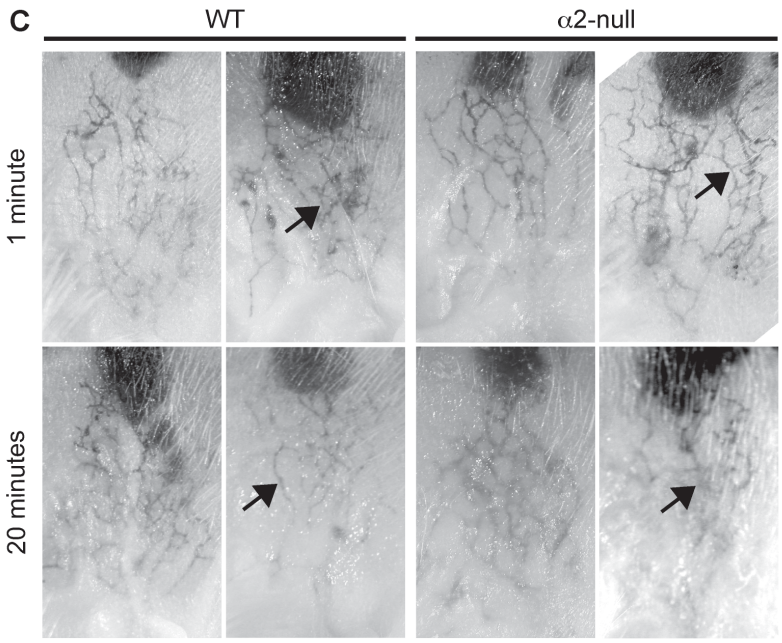
In order to determine whether the dilated vessels seen in our α2-null mice were associated with decreased functionality and leakiness of lymphatic vessels, ears from

WT and α2-null mice were distally injected with colloidal carbon, and animals were either sacrificed at 1 or 20 minutes post injection. Photos of the ears were taken immediately before sacrifice. At 1 minute post injection, there were no differences in diffusion of colloidal carbon from the lymphatic vessels. However, at 20 minutes post injection, the lymphatic vessels of α2-null mice demonstrated increased leakiness, as evident by loss of well-demarcated vessel boundaries (Figure 4-2*C*). To examine the complexity of lymphatic branching and determine differences in developmental patterning of the vasculature, the points of vessel branching were quantitated along the length of collecting lymphatics. No differences were found in the number of lymphatic branch points between WT and α2-null mice injected with colloidal carbon ($p = 0.46$) (Figure 4-2*D*).

Since α2-null lymphatics from non-neoplastic tissue demonstrated decreased functionality, it was important to test if the function of tumor-associated lymphatic vessels from HPV/WT and HPV/KO mice was also affected. SCCs were injected peritumorally into the subcutaneous tissue beneath the tumor or intratumorally within the mass of the neoplasm with a mixture of colloidal carbon and isosulfan blue, and animals were sacrificed after 20 minutes. The presence of dye in superficial lymph nodes was used as an indicator of tumor-associated lymphatic functionality. Previous studies have shown that peritumoral lymphatics are more efficient than intratumoral vessels as being conduits for metastasis (139, 224). As expected, dye injected into the peritumoral space of HPV/WT ($n = 7$) or HPV/KO ($n = 4$) mice developed more tracer-positive lymph nodes than mice with intratumoral dye injections (HPV/WT $n = 12$ and HPV/KO $n = 5$) (71.4% versus 12.5% in HPV/WT, odds ratio = 3.50; 25.0% versus 0.0% in HPV/KO mice, odds ratio = 4.71, respectively). As demonstrated in the non-neoplastic ears, tumor-associated lymphatic vessels in HPV/WT mice were more efficient at tracer transport

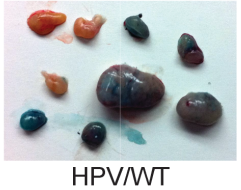
than α2-null animals. HPV/KO mice had significantly fewer dye positive lymph nodes than HPV/WT mice, regardless of the injection site. When injected into the tumor periphery, 46.4% less HPV/KO mice had dye positive nodes, compared to HPV/WT animals (odds ratio = 7.50). Upon intratumoral injection, 12.5% less HPV/KO mice had tracer positive lymph nodes (odds ratio = 8.07) (Figure 4-2*E*).





E

	Peripheral			Intratumoral		
	Positive	Negative	Percent	Positive	Negative	Percent
HPV/WT	5	2	71.4%	5	7	12.5%
HPV/KO	1	3	25.0%	0	5	0.0%



HPV/WT

Figure 4-2. Loss of the $\alpha 2\beta 1$ integrin caused lymphatic vessel dilation. Altered vessel morphology led to aberrant lymphatic function, manifested by increased leakiness in non-neoplastic ears and decreased tracer delivery from tumor-associated lymphatic vessels. *A,* Mice were injected intravascularly with FITC-conjugated dextran to highlight blood vessels (green). Whole mount ear sections were subsequently stained with LYVE-1, a lymphatic-specific marker (red). Scale bar = 200 μm. *B*, Mice null for the $\alpha 2$ integrin subunit demonstrated increased lymphatic vessel dilation, as demonstrated by a larger vessel diameter than WT mice ($p < 0.0001$). HPV/KO animals also exhibited increased lymphatic vessel dilation, compared to HPV/WT mice ($p < 0.0001$). Vessel enlargement, therefore, is dependent on absence of the $\alpha 2\beta 1$ integrin and not on the K14-HPV16 transgene. However, the K14-HPV16 transgene did increase lymphatic dilation in HPV/WT mice, when compared to non-K14-HPV16 WT animals ($p = 0.032$). This increase was not evident when comparing HPV/KO and $\alpha 2$-null mice ($p = 0.096$). WT n = 7, KO n = 5, HPV/WT n = 6, and HPV/KO n = 5. Bars represent mean ± SEM. *C*, Colloidal carbon was injected into the periphery of WT and $\alpha 2$-null ears, and photos were taken 1 or 20 minutes post injection to assess lymphatic functionality. No differences in vessel leakiness were observed at 1 minute post injection. However, 20 minutes after injection, $\alpha 2$-null lymphatic vessels demonstrated increased colloidal carbon diffusion into the surrounding tissue, seen by loss of crisp vessel delineation, when compared to WT controls. Arrows indicate representative vessels with either sharp or diffuse boundaries. *D*, Images from colloidal carbon injected ears were analyzed at 1 minute by quantitation of the number of lymphatic branches emanating from a single collecting duct. No differences existed in the number of lymphatic branch points between WT (n = 11) and $\alpha 2$-null mice (n = 10), indicating that gross developmental lymphatic patterning is unaffected by integrin loss ($p = 0.46$). *E,* HPV/KO mice had decreased functionality of tracer transport, as manifested by a decreased number of mice with dye-positive lymph nodes, compared to HPV/WT mice, from injections into the periphery of the tumor (odds ratio = 7.50) or the intratumoral space (odds ratio = 8.07). Peripheral SCC injections only resulted in 25.0% of HPV/KO mice manifesting lymphatic tracer dye in lymph nodes, whereas, 71.4% of HPV/WT mice had dye-positive nodes. Intratumoral injection of dye resulted in no HPV/KO animals having positive lymph nodes, while 12.5% of HPV/WT mice had at least one positive node. Injections into the peritumoral space demonstrated that these lymphatic vessels were more efficient at tracer transport, than intratumoral lymphatics, regardless of integrin status (HPV/WT peripheral versus intratumoral odds ratio = 3.50; HPV/KO peripheral versus intratumoral odds ratio = 4.71). HPV/WT peripheral n = 7 and intratumoral n = 12. HPV/KO peripheral n = 4 and intratumoral n = 5. Representative photo shows 6 out of 9 dye-positive lymph nodes from an HPV/WT mouse.

ZO-1 Expression is Decreased on α2-null Lymphatic Endothelial Cells

Previous studies have shown that both button- and zipper-like junctions of lymphatic vessels are composed of tight junction-associated proteins occludin, claudin-5, zonula occludens-1 (ZO-1), junctional adhesion molecule-A (JAM-A), and endothelial cell-selective adhesion molecule (ESAM) (124). In order to understand the reason for increased lymphatic vessel leakiness seen with loss of the α2β1 integrin, tight junction-associated protein levels were quantitated on immunofluorescently stained sections of non-neoplastic WT and α2-null ears. Integrin loss resulted in decreased ZO-1 levels on the lymphatic vasculature ($p = 0.016$), but no differences were detected in levels of claudin-5 ($p = 0.15$), ESAM ($p = 0.30$), JAM-A ($p = 0.83$), or occludin ($p = 0.72$) (Claudin-5, ESAM, JAM-A, and ZO-1, each, WT n = 5 and KO n = 5. Occludin WT and KO n = 7, each) (Figure 4-3*A and B*).

In order to determine whether the expression of tight junction markers was also altered during tumorigenesis, SCC sections from HPV/WT and HPV/KO mice were examined. SCC-associated lymphatics in the K14-HPV16 model revealed increased ESAM and decreased ZO-1 levels in HPV/KO mice, compared to HPV/WT animals (ESAM $p = 0.0060$, HPV/WT n = 6, and HPV/KO n = 5; ZO-1 $p = 0.018$, HPV/WT n = 4, and HPV/KO = 3). However, no differences were found in the expression levels of claudin-5 ($p = 0.20$, HPV/WT n = 8 and HPV/KO n = 8), JAM-A ($p = 0.41$, HPV/WT n = 6 and HPV/KO n = 10), or occludin ($p = 0.63$, HPV/WT n = 8 and HPV/KO n = 7) (Figure 4-3*C and D*).

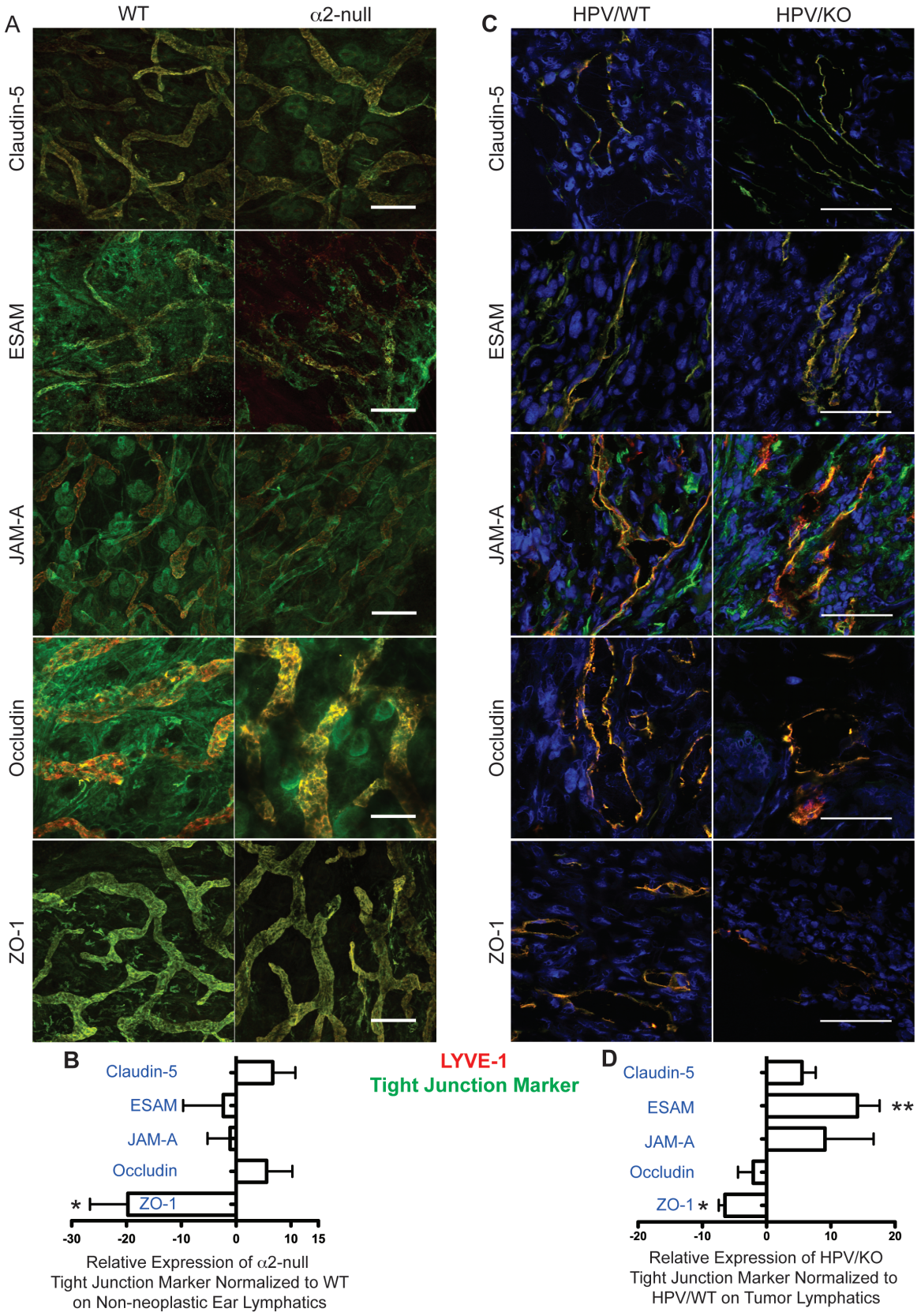

A
WT
α2-null
Claudin-5
ESAM
JAM-A
Occludin
ZO-1
C
HPV/WT
HPV/KO
Claudin-5
ESAM
JAM-A
Occludin
ZO-1
LYVE-1
Tight Junction Marker
B
Claudin-5
ESAM
JAM-A
Occludin
ZO-1
*
-30
-20
-10
0
10
15
Relative Expression of α2-null
Tight Junction Marker Normalized to WT
on Non-neoplastic Ear Lymphatics
D
Claudin-5
ESAM
JAM-A
Occludin
ZO-1
**
*
-20
-10
0
10
20
Relative Expression of HPV/KO
Tight Junction Marker Normalized to
HPV/WT on Tumor Lymphatics

Figure 4-3. Non-neoplastic α2-null mouse ear and HPV/KO SCC-associated lymphatics have decreased expression of ZO-1. *A*, Whole mount ears were harvested and stained with LYVE-1 (red) and either claudin-5, ESAM, JAM-A, occludin, or ZO-1 (green). Scale bar = 100 μm. *B*, The intensity of tight junctional marker staining in α2-null animals was normalized to the average values obtained from respective WT controls. No differences were found in the expression levels of claudin-5 ($p = 0.15$), ESAM ($p = 0.30$), JAM-A ($p = 0.83$), or occludin ($p = 0.72$). ZO-1 expression, however, was significantly diminished on α2-null lymphatic vessels, compared to WT ($p = 0.016$). Claudin-5, ESAM, JAM-A, and ZO-1, each, WT n = 5 and KO n = 5. Occludin WT and KO n = 7, each. Bars represent mean ± SEM. *C*, SCC sections from HPV/WT and HPV/KO mice were stained with LYVE-1 (red) to identify lymphatic endothelial cells, TO-PRO-3 (blue) to detect cell nuclei, and either claudin-5, ESAM, JAM-A, occludin, or ZO-1 (green). Scale bar = 50 μm. *D*, The intensity of tight junctional marker staining in HPV/KO animals was normalized to the average values obtained from respective HPV/WT controls. No differences were found in the expression levels of claudin-5 ($p = 0.20$), JAM-A ($p = 0.41$), or occludin ($p = 0.63$). ESAM expression was significantly elevated in HPV/KO lymphatic vessels ($p = 0.0060$). ZO-1 expression, however, was significantly diminished on HPV/KO lymphatic vessels, compared to HPV/WT vessels ($p = 0.018$). Claudin-5 HPV/WT n = 8 and HPV/KO n = 8. ESAM HPV/WT n = 6 and HPV/KO n = 5. JAM-A HPV/WT n = 6 and HPV/KO n = 10. Occludin HPV/WT n = 8 and HPV/KO n = 7. ZO-1 HPV/WT n = 4 and HPV/KO n = 3. Bars represent mean ± SEM.

Loss of the α2β1 Integrin Increased Lymphatic Endothelial Cell Coverage on Matrigel *In Vitro*

To assess LEC-intrinsic differences, primary cell behavior was analyzed. Benign lymphangiomas were harvested and cultured *in vitro* before being flow sorted to isolate a pure population of LECs. Primary LECs were plated onto Matrigel and allowed to sprout, proliferate, and form tubules over 11 days (Figure 4-4*A*). Loss of the α2β1 integrin (n = 10) resulted in increased LEC coverage area on Matrigel, compared to WT LEC (n = 10; $p < 0.0001$) (Figure 4-4*B*). This increased growth of α2-null LECs may result from either heightened proliferation, escape from senescence and apoptosis, increased sprouting, or increased tubule formation. Further studies are underway to determine the cause for this growth advantage.

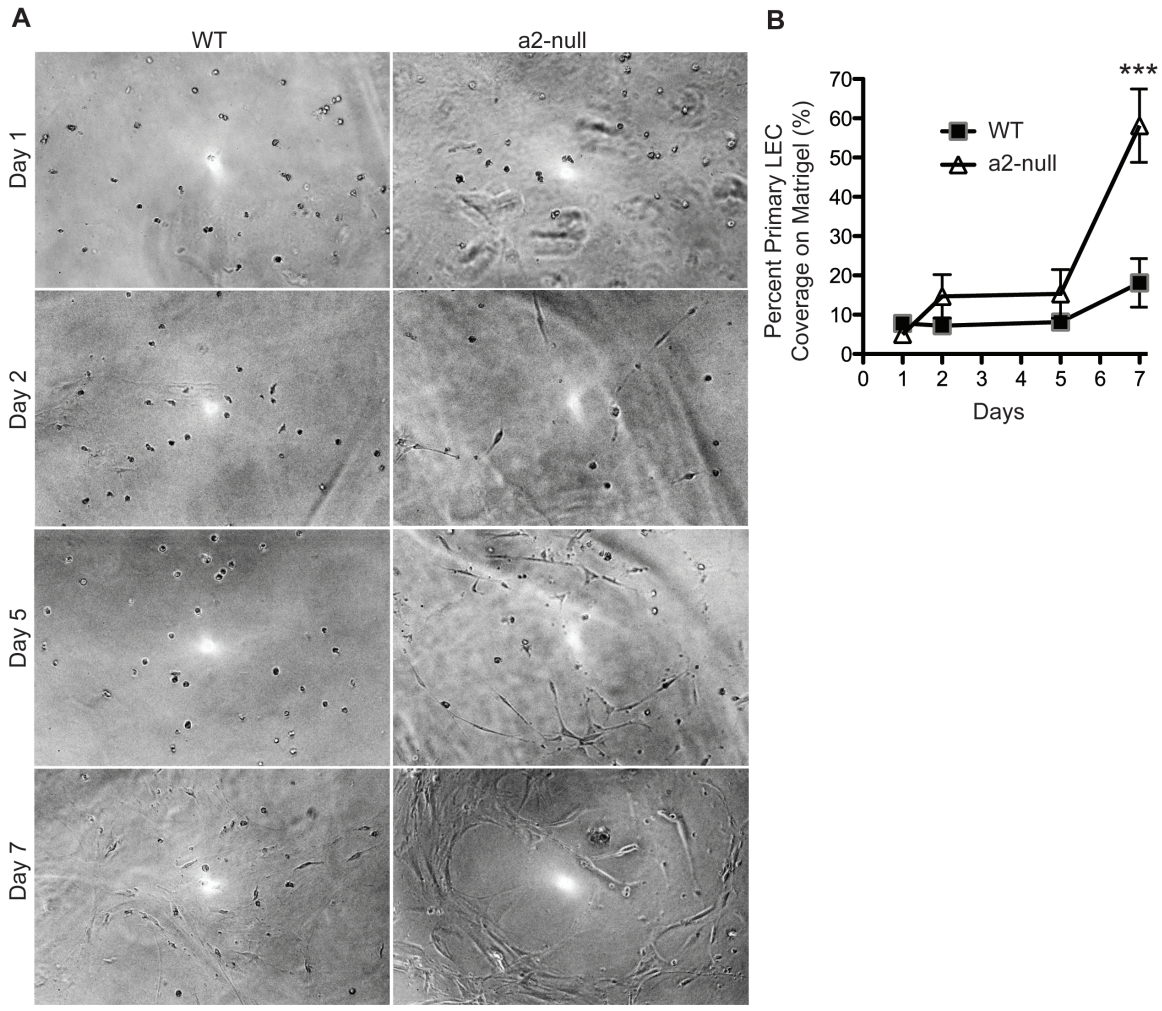


Figure 4-4. α2-null lymphatic endothelial cells demonstrate increased growth and coverage on Matrigel. *A,* Primary LECs, harvested from flow sorted WT or α2-null mice lymphangiomas, were plated onto Matrigel, and photos were taken at specific time intervals. Photos taken at 10X magnification. *B*, The percentage of area covered by LECs was determined for each photo. α2-null LECs (n = 10) demonstrated significantly increased area coverage of matrigel *in vitro*, when compared to WT LECs (n = 10) ($p < 0.0001$). Experiment performed in quadruplicate. Bars represent mean ± SEM.

Vascular Endothelial Growth Factors Do Not Contribute to Alter the Migration or Proliferation of α2-null Lymphatic Endothelial Cells

Members of the VEGF family of ligands have a well-established role in lymphangiogenesis. VEGF-A, an endothelial cell mitogen, also regulates vascular permeability (225, 226). Due to VEGF-A's role in promoting endothelial cell proliferation, leakiness, and migration, it was necessary to evaluate the effect of α2β1 integrin loss on VEGF-stimulated lymphangiogenesis (186). Matrigel loaded with VEGF-A, -C, or -D was subcutaneously injected into the lateral subscapular area of WT and α2-null mice alongside control, PBS-loaded Matrigel. No differences between WT and α2-null animals were observed in the area of Matrigel infiltrated with LYVE-1^+ LECs in response to any of the VEGFs tested (n = 3 WT and 3 α2-null animals per treatment group; VEGF-A $p = 0.30$; VEGF-C $p = 0.59$; VEGF-D $p = 0.68$) (Figure 4-5). Therefore, provided the same levels of stimulation, no differences existed in the response to any of the VEGF ligands tested. Focusing specifically on SCCs, it was important to determine whether VEGF levels were indeed similar between HPV/WT and HPV/KO mice. Using quantitative real-time PCR, no differences were detected in the endogenous transcript levels of VEGF-A or -C in HPV/WT and HPV/KO tumors; furthermore VEGF-D levels were undetectable (data not shown) (227, 228).

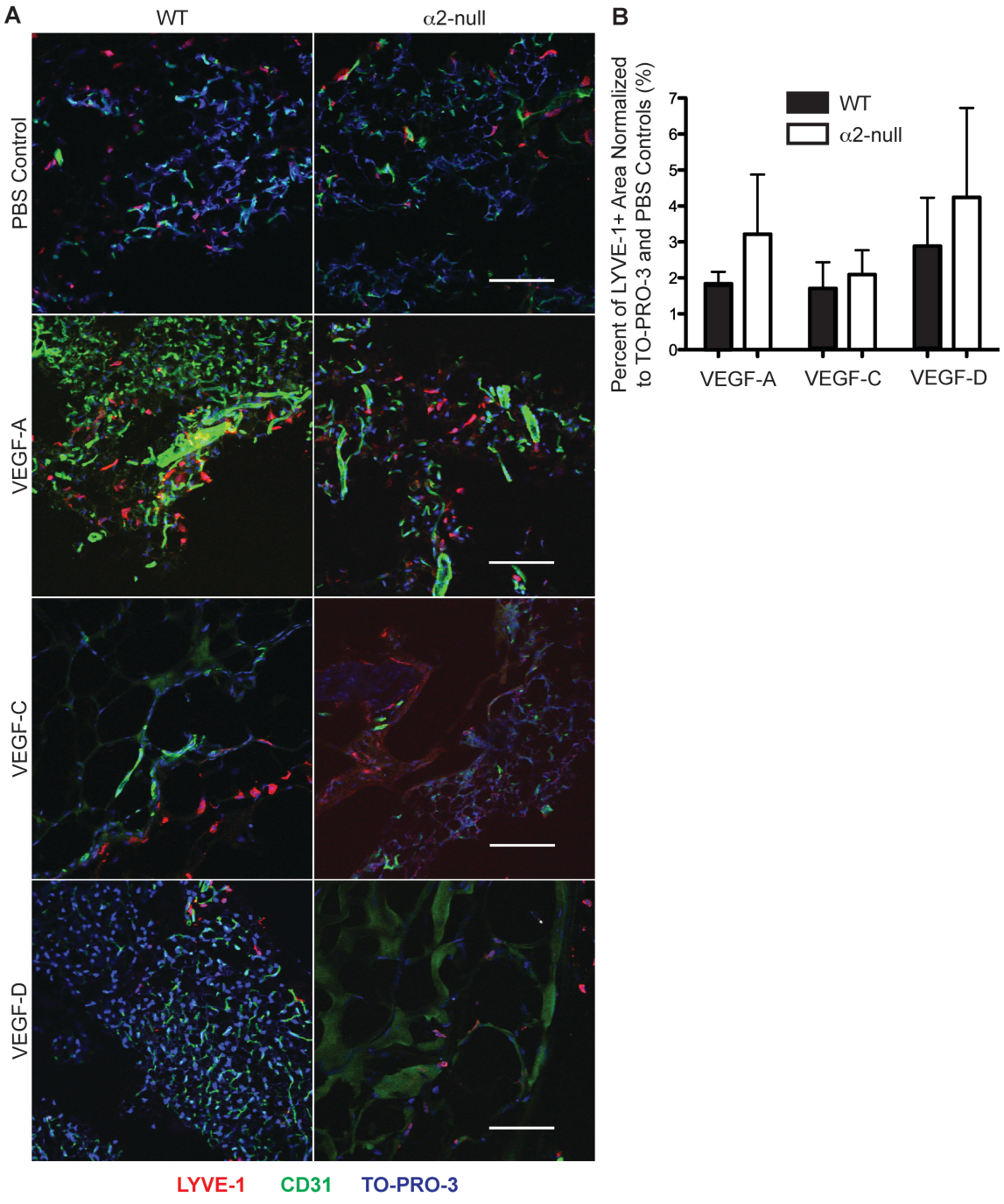

A
WT
α2-null
PBS Control
VEGF-A
VEGF-C
VEGF-D
LYVE-1
CD31
TO-PRO-3
B
Percent of LYVE-1+ Area Normalized to TO-PRO-3 and PBS Controls (%)
WT
α2-null
0
1
2
3
4
5
6
7
VEGF-A
VEGF-C
VEGF-D

Figure 4-5. The α2β1 integrin does not affect vascular endothelial growth factor stimulation of lymphangiogenesis. *A*, VEGF-A, -C, and -D, which are known to induce lymphangiogenesis, were injected in a Matrigel plug assay, along with PBS control Matrigel, and cells were allowed to infiltrate. Immunofluorescence was performed on resected Matrigel plugs using the lymphatic marker LYVE-1 (red), the blood vessel marker CD31 (green), and the TO-PRO-3 nuclear stain (blue). Scale bar = 100 μm. *B*, Quantitation was performed on the tissue sections and revealed no differences in the quantity of LYVE-1$^+$ cell coverage in the presence of VEGF-A ($p = 0.30$), VEGF-C ($p = 0.59$), or VEGF-D-loaded Matrigels ($p = 0.68$) injected into WT or α2-null mice after normalization to PBS control samples and TO-PRO-3$^+$ areas (n= 3 WT or α2-null animals in each treatment group). Bars represent mean ± SEM.

Discussion

There is strong evidence for the role of β1 integrins in lymphangiogenesis. Integrins α1β1, α2β1, α4β1, α5β1, and α9β1 have been implicated in various aspects of developmental, inflammatory, and tumor-associated lymphatic vessel formation (137, 183-187). Although the α9β1 integrin is the only one critical for survival, due to its role in embryonic lymphatic vessel development, integrins α4β1 and α5β1 have established roles in promoting tumor-associated lymphangiogenesis. Little is known, however, concerning the role of collagen binding integrins α1β1 and α2β1 in tumor lymphangiogenesis. To date, studies examining the α1β1 and α2β1 integrins have focused on their role in inflammation-induced lymphatic development (186). In human lymphangiomas, α1β1 integrin expression was elevated, but no data exists on the role of the α2β1 integrin in similar processes (199). Since all the LEC-associated integrins contain the β1 subunit, it was essential to pinpoint specific contributions of the α2 subunit in lymphangiogenesis. By employing the α2-null mouse in both a non- and a tumorigenic setting, we were able to define baseline differences in the lymphatic vasculature induced by integrin loss as well as identify perturbations in tumor-associated lymphatic function. Use of the K14-HPV16 model in an α2-null or WT background allowed for the study and manipulation of superficial, cutaneous SCCs and the identification of tumor-associated lymphatic vessel defects.

Our results revealed that the α2β1 integrin's presence on the lymphatic vasculature helped maintain vessel integrity and minimize leakiness. Loss of the α2β1 integrin led to decreased functionality of lymphatic vessels. These morphological and functional defects seen with loss of the α2β1 integrin in a non-tumorigenic setting also translated to decreased tumor-associated lymphatic vessel transport of tracer-dye to

draining lymph nodes, thus possibly explaining the reduction in nodal metastasis in HPV/KO mice, compared to HPV/WT animals, as discussed in Chapter III. It is still unclear, however, whether the reduction of tight junctional protein zonula occludens-1 on non-neoplastic and cancer-associated lymphatic vessels was a definitive cause of increased leakiness or simply a manifestation of α2-null LEC immaturity.

It is still debatable how extensively peritumoral versus intratumoral lymphatic vessels contribute as functional conduits for cancer cell metastasis. It was believed that peritumoral lymphatics act as primary mediators of metastasis, since they are patent and relatively unaffected by the growing tumor's compressive forces (229). In this chapter, I demonstrated that peritumoral vessels are more functional at transport of tracer-dye to the draining lymph node, than intratumoral lymphatics, regardless of $\alpha 2\beta 1$ integrin status. Previously, intratumoral vessels were considered nonfunctional due to vessel collapse (230, 231). However, recent studies determined that intratumoral lymphatics are still somewhat functional in facilitating cancer dissemination. When examining human SCCs, correlations have consistently been found between intratumoral lymphatics and metastasis, although no known biological explanations can be given (173, 176). In my model, HPV/KO mice have decreased intratumoral lymphatic vessel coverage and decreased metastasis, when compared to HPV/WT mice. The decrease in lymph node metastasis can now also be explained by a reduction in intratumoral lymphatic transport efficiency of SCC cells in HPV/KO mice. This is the first report of the α2β1 integrin's role in facilitating SCC lymph node metastasis.

Using primary, flow-sorted cells harvested from benign lymphangiomas, α2-null LECs demonstrated increased growth when plated onto Matrigel, compared to control WT cells. Whether this difference was the result of heightened proliferation, escape from senescence and apoptosis, increased sprouting, or increased tubule formation is yet to

be determined. Further investigation is needed to examine whether α2β1 integrin loss results in vessel hyperplasia, since several studies have detailed associations between pathological lymphatic vessel dilation and hyperplasia with reduced functionality, either by increased leakiness or incompetency of collecting duct secondary valves (151, 152, 155, 156). Regardless, the alterations in vessel morphology due to loss of the α2 integrin subunit were accompanied by reduced lymphatic functionality in the absence and presence of cancer.

LECs have unique intercellular interactions that dictate lymphatic vessel behavior. Baluk *et al.* have characterized the adherens and tight junction compositions forming punctate, “button”-like, unidirectional primary valves between LECs on initial, terminal bud lymphatics, which mediate fluid uptake. Larger collecting lymphatic vessels are composed of the same cell-cell junctions, but their distribution occurs in a continuous “zipper”-like formation along the LEC membrane to seal off the vessel and prevent intravascular leak of fluid and cells (124). Tight junction loss on endothelial cells has been associated with increased vascular permeability (232). The increased leakiness of α2-null lymphatics was associated with ZO-1 loss in non- and tumor-associated lymphatic vessels. ZO-1 has previously been shown to stabilize and tighten the endothelial barrier, thus preventing intravascular leak of chyle and other macromolecules (153, 154). While increased ESAM expression on HPV/KO LECs may also contribute functionally to alter the cohesiveness of the endothelial barrier, this effect may be specific to tumor-associated LECs, as no differences in ESAM levels were observed in non-K14-HPV16, α2-null or WT mice. ZO-1 loss, on the other hand, was consistently decreased on α2-null LECs, regardless of tumor status. Furthermore, in a murine model examining Netrin-4 overexpression, increased lymphatic vessel proliferation led to ZO-1 loss and increased lymphatic leak (233). It is still unclear whether loss of ZO-1

expression occurred as a byproduct of increased proliferation in α2-null LECs or as the primary, causative event leading to defective functionality of the lymphatic vessel. Regardless, the $\alpha 2\beta 1$ integrin is important in regulating the intercellular interactions of LECs and in determining vessel permeability.

Although many factors lead to altered tumor-associated lymphangiogenesis, such as pro-inflammatory cytokines and growth factor signaling, it was unlikely that inflammatory differences were responsible for the changes seen in $\alpha 2$-null mice. Furthermore, morphological differences in the lymphatic vasculature were observed in the absence of tumor and its associated inflammatory component. In Chapter III, no remarkable alterations were observed in chronic inflammatory populations between WT and $\alpha 2$-null mice or between HPV/WT and HPV/KO mice to suggest altered immune contributions towards lymphangiogenesis. Additionally the VEGF ligands known to alter lymphangiogenesis did not have a role in mediating the lymphatic differences seen in my studies. Therefore, differences observed in the lymphatics and in clinical metastasis are likely the result of intrinsic differences in the LECs. It is also possible that the increased invasive ability of HPV/WT SCCs contributed towards the elevated levels of metastasis seen in regional lymph nodes, compared to HPV/KO SCCs. However, the tracer-dye studies provide compelling evidence that $\alpha 2$-null lymphatic vessels are less effective at transport of macromolecules, and possibly cells, to the collecting nodes.

An important question raised from my studies into lymphatic vascular leak asks why the pathology seen in α2-null mice is not readily evident since birth. Instead, α2-null animals exhibit no overt phenotypes in fertility or development, and animals have no gross lymphatic dysfunction typically observed with loss of important genes regulating lymphatic development (234). As with other studies performed using the α2-null mouse,

perturbations in the normal physiology of the animal are required to reveal important functions of the integrin. Challenges, including wound healing, infection, or cancer, expose critical roles of the integrin in maintenance of homeostasis (64). While the $\alpha 2\beta 1$ integrin functions in regulating lymphatic vessel integrity, loss of the integrin does not significantly impede normal function. However, we have shown that overloading the drainage system by excess fluid or cancer, for example, results in a pathological reduction in drainage to the lymph nodes.

CHAPTER V

USE OF THE ORTHOTOPIC 66C14 MAMMARY TUMOR MODEL TO DETERMINE TUMOR VERSUS HOST MICROENVIRONMENT CONTRIBUTIONS OF THE α2β1 INTEGRIN IN BREAST CANCER PROGRESSION AND METASTASIS

Cancer cell dissemination from the primary tumor is a critical step in disease progression that dictates clinical prognosis and aggressiveness of the treatment regimen. As surface adhesion receptors, integrins play critical roles in the cell-cell and cell-matrix interactions that modulate cancer progression and metastasis. Using public breast and prostate cancer microarray datasets, our lab has previously shown that loss of the α2β1 integrin predicted for metastatic disease and poor clinical outcome. Furthermore, the α2β1 integrin suppresses metastasis in a spontaneous murine model of breast cancer by decreasing hematogenous intravasation of tumor cells. Since these studies were performed using global α2 integrin-null mice, I established an orthotopic model system in which to investigate the effects of α2β1 integrin expression on either the tumor cells or the cells of the tumor microenvironment towards mediating the metastatic phenotype. I demonstrated that in breast cancer, integrin expression on cells of the tumor microenvironment contribute minimally to disease progression and metastasis. Conversely, these findings suggest that tumor-cell specific expression of the α2β1 integrin regulates breast cancer disease progression. The insight gained into the biologic functions of the α2β1 integrin will help to elucidate the complex roles this collagen receptor plays in regulating tumor cell behavior with its microenvironment. Future studies will examine how the α2β1 integrin regulates a critical step in the metastatic cascade—namely intravasation of cells from the primary tumor into the systemic blood vasculature. To this end, we plan to re-express the α2β1 integrin on the

highly metastatic 66c14 mammary tumor line and test orthotopic cancer growth and hematogenous intravasation.

Introduction

A distinct feature of cancer progression and poor outcome is metastasis to distant organs, as this is the causal factor in approximately 90% of deaths associated with solid tumors (157). The metastatic cascade is thought to involve the following integral steps: local invasion, intravasation into systemic circulation, survival in the vasculature, extravasation, and colonization (221). Studies into metastasis are complicated by the elaborate interactions of the tumor cells with their microenvironment, thereby increasing the difficulty of identifying key signaling events required during this process. As such, little is known regarding how metastatic breast cancer cells intravasate the blood vasculature of the mammary gland. In breast cancer patients, hematogenous dissemination occurs early on in cancer progression, so understanding the cell-cell and cell-matrix interactions involved will significantly aid in identifying and possibly preventing progression towards a metastatic phenotype (235).

As in skin epithelium, high levels of the $\alpha 2\beta 1$ integrin are found on normal breast ductal epithelium (115). During malignancy, however, expression of the $\alpha 2\beta 1$ integrin is decreased in breast adenocarcinoma; studies on other types of adenocarcinoma have yielded similar results (112, 115). Loss of $\alpha 2\beta 1$ integrin expression caused reduced collagen-dependent adhesion, motility, and morphogenesis in mammary carcinoma cells *in vitro* (205). Re-expression of the $\alpha 2\beta 1$ integrin in integrin-null Mm5MT cells (tumorigenic cell line derived from a mouse mammary tumor virus [MMTV] breast cancer) was able to revert the malignant breast cancer phenotype to a differentiated

epithelium, thus indicating that the $\alpha 2\beta 1$ integrin plays a central role in mammary gland differentiation (112).

Expression of the $\alpha 2\beta 1$ integrin has been shown to enhance hematogenous metastasis in several tumorigenesis models (116-119). However, previous work by Ramirez *et al*. has shown that the α2β1 integrin acts as a suppressor of breast and prostate cancer metastasis. Deletion of the α2β1 integrin increased breast tumor cell intravasation *in vitro* and *in vivo* without altering tumor growth or cell proliferation. Although α2-null/MMTV-neu tumor cells demonstrated enhanced anchorage-independent growth *in vitro*, compared to WT/MMTV-neu cells, the rates of *in vivo* lung colonization were similar when these cells were injected intravenously. These data indicate that the α2β1 integrin does not regulate later stages of metastasis. Moreover, mining of human public breast and prostate cancer datasets has shown a correlation between loss of α2β1 integrin expression and increased metastasis and poor clinical prognosis (203). These findings support prior studies on human breast cancer patients, in which examination of polymorphisms linked with decreased α2β1 integrin expression was correlated with poor prognosis (204).

The MMTV-neu model of spontaneous breast cancer suggested that the increase in metastasis in α2-null animals was primarily dependent on α2 expression or lack thereof on the tumor cells and not on the tumor microenvironment. However, effects of integrin expression by cells of the tumor microenvironment cannot be completely excluded with use of the current global α2-null animal model. We hypothesized that the α2β1 integrin's role in limiting tumor cell intravasation was dependent on tumor-specific integrin expression, with minimal contributions from the microenvironment.

In order to further dissect the role of the α2β1 integrin on the tumor cells versus on cells of the tumor microenvironment in breast cancer progression and metastasis, it was imperative to identify a highly metastatic murine model system in which integrin levels could be manipulated in the host animals as well as on the tumor cells. To this end, we profiled α2β1 integrin expression on a series of sister clones that were originally isolated and characterized by Fred Miller from a single, primary murine breast carcinoma. Generated through a series of subcloning experiments examining chemoresistance, five sister subpopulations were obtained that model varying degrees of local tumor invasion and metastasis. These cell lines, which include 4T1, 66c14, 4T07, 67NR, and 168FARN cells, have proven a useful tool in studying tumor cell heterogeneity by providing a model to examine the genetics associated with metastasis. The advantage of using cells in the 4T1 series is their ability to recapitulate human disease progression in a murine model system: cancer cells may be implanted into the breast tissue, tumors begin metastasizing from the primary site spontaneously, and distant metastatic sites are similar with dissemination to the lungs and lymph nodes (236).

We demonstrate here that when 66c14 cells, which lack the α2β1 integrin, were injected into wild-type (WT) or α2-null mice, there were no differences observed in latency of tumor development, tumor growth, or metastasis to the lungs. As a result, α2β1 integrin expression by cells of the tumor microenvironment appears to play a minimal role in breast cancer progression in this orthotopic model. We are also in the process of stably re-introducing the α2 integrin subunit back into the 66c14 cells so that the effects of tumor-specific integrin expression can be evaluated. While the intravasation of these 66c14 tumor cells is also yet to be determined, tumor cell-specific expression of the α2β1 integrin is likely mediating the decreased intravasation observed

previously in our lab using the spontaneous, global α2 wild-type or null animals (203). These findings stress the importance of integrin expression in dictating cell behavior, and ultimately, cancer metastasis and prognosis.

Materials and Methods

Transgenic Animal Model—Animals were housed in pathogen-free conditions at Vanderbilt University Medical Center in compliance with the Association for Assessment and Accreditation of Laboratory Animal Care and Institutional Animal Care and Use Committee regulations. Wild-type BALB/c mice were ordered from Jackson Labs (Bar Harbor, Maine). α2-null BALB/c animals have been previously described and were the generous gift of Ambra Pozzi (234).

Tissue Culture—4T1, 66c14, 4T07, 67NR, and 168FARN cells were donated by Jin Chen and used with permission from Fred Miller (236-238). Cells were grown in DMEM (Cellgro Mediatech, Inc., Manassas, VA) containing 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 100 μM non-essential amino acid (Sigma, St. Louis, MO), 2 mM L-glutamine (Gibco, Grand Island, NY), 100 units/mL penicillin (Mediatech, Inc., Manassas, VA), and 100 μg/mL streptomycin (Mediatech, Inc., Manassas, VA) at 37°C and under 5% CO_2 conditions.

Flow Cytometry—Cells were trypsinized and assessed for expression of the α2β1 integrin using PE-conjugated anti-α2 integrin (BD Pharmingen, San Diego, CA) or PE-conjugated IgG1 isotype control (BD Pharmingen). Flow cytometry was performed on a 3-laser, BD LSR II Flow Cytometer in the Vanderbilt Medical Center Flow Cytometry Shared Resource, and data analysis was performed using FlowJo (Tree Star, Inc., Ashland, OR).

Orthotopic injection—Orthotopic injections were performed on 4-5 week old female BALB/c mice anesthetized using a mixture of 2% isoflurane (Rx Elite, Meridian, ID)/98% oxygen. 1 x 10^5 66c14 cells suspended in 50 μL of PBS were injected into the right 4th mammary fat pad, lateral to the inguinal lymph node. All surgical incisions were closed using 9 mm wound clips (BD Primary Care Diagnostics, Sparks, MD) and removed 2 weeks post surgery. Pain was managed using Buprenex (Bedford Labs, Bedford, OH) as needed in accordance with animal welfare guidelines. Mice were examined twice a week for tumor development. All tumors were measured and volumes were calculated as $V = 0.52 \times A \times B^2$, where V is the volume, A is the largest diameter, and B is the shortest diameter. Once tumors reached a maximum volume of 1200 mm^3 or abscessed, affected animals were sacrificed. All studies examining metastasis only evaluated tumors greater than 1000 mm^3. Lungs were perfused with 10% PBS-buffered formalin (Fisher Scientific, Fair Lawn, NJ) before being harvested. Lung and tumor tissues were evaluated histologically on formalin-fixed, paraffin-embedded, and hematoxylin and eosin (H&E)-stained sections. The area occupied by lung metastases was quantitated from 4X photos covering the entire tissue using a Nikon Eclipse 80i microscope. Images were stitched together using the MosaicJ plug-in and analyzed in ImageJ (NIH, Bethesda, MD) (239).

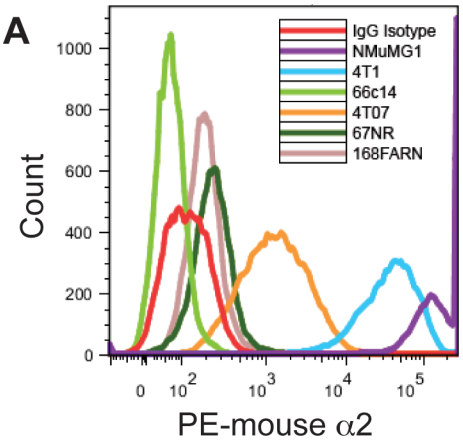
Statistical Analyses—All statistical analyses were done using GraphPad Prism (La Jolla, CA). Latency for tumor formation was examined using log-rank Mantel-Cox tests. Tumor growth rates were analyzed by imposing best-fit, exponential curve regression analysis on raw volume measurements to determine growth slopes. Contingency table chi-square tests were used to evaluate lung metastases. All other comparisons, including the regression analysis of tumor growth rates, were calculated using unpaired t-tests. P-values of < 0.05 were considered statistically significant.

Results

Expression Profiling of the 4T1 Sister Clones Revealed Absence of α2β1 Integrin Expression on 66c14 Cells

The search for an ideal, *in vivo* model system to examine effects of the α2β1 integrin on tumor versus host microenvironment cells was central to defining the role of the integrin in metastasis. In order to establish an orthotopic model for breast cancer, wherein the α2β1 integrin could be deleted on tumor cells and/or in the host animal, several criteria were required. 1) The tumor cells must have high viability *in vitro* and *in vivo*. 2) α2β1 integrin expression must be easily manipulated on the tumor cells with near complete integrin expression or loss. 3) The tumor cells should be highly aggressive, with elevated metastatic capacity. 4) The tumor cells should be immunologically compatible with recipient, host animals. In order to find an ideal breast cancer cell line for use in these studies, we evaluated the 4T1 panel of sister clones that were originally established and characterized by Fred Miller (236). Flow cytometric analysis was performed on 4T1, 66c14, 4T07, 67NR, and 168FARN cells to detect surface expression of the α2β1 integrin. Using immortalized, normal murine mammary gland (NMuMG1) cells as a positive control for α2β1 integrin expression, we compared relative integrin expression levels on the 4T1 sister clones (210). Expression of the α2β1 integrin from highest to lowest was as follows: 4T1 > 4T07 > 67NR > 168FARN > 66c14 (Figure 5-1*A and B*). Because 66c14 cells are aggressive in terms of invasion and metastasis (second only to 4T1 cells) and completely lacked the α2β1 integrin, these cells were chosen for use in our following studies.

A



B

Specimen	Geometric Mean of Fluorescence for PE-mouse alpha2
IgG Isotype	111
NMuMG1	124000
4T1	32100
66c14	60
4T07	1188
67NR	227
168FARN	184

Figure 5-1. α2β1 integrin expression on the 4T1 series of breast cancer lines revealed complete absence of integrin expression on 66c14 cells. *A*, Cultured 4T1, 66c14, 4T07, 67NR, and 168FARN cells were stained with PE-conjugated antibodies against the mouse α2 integrin subunit or IgG1 isotypes and analyzed by flow cytometry. NMuMG1 cells were used as a positive control for α2 integrin staining. *B*, Quantitative geometric means of fluorescence for the individual cell lines and isotype controls are listed. Expression of the α2β1 integrin from highest to lowest was as follows: 4T1 > 4T07 > 67NR > 168FARN > 66c14.

α2β1 Integrin Expression in the Tumor Microenvironment Contributes Minimally to Breast Cancer Progression

In order to evaluate the contribution of host microenvironment towards tumor metastasis, 66c14 cells were orthotopically injected into the mammary fat pad of WT or α2-null BALB/c mice. No differences were observed in latency of tumor formation when cells were injected into WT or α2-null backgrounds ($p = 0.73$; WT n = 25 and α2-null n = 20) (Figure 5-2*A*). Additionally, 66c14 tumors grew at similar rates in WT and α2-null mice ($p = 0.64$; WT n = 17 and α2-null n = 13) (Figure 5-2*B*).

Because 66c14 cells are known to hematogenously colonize the lung, the presence of lung metastases was analyzed. There was no difference in the percent of animals with metastases between α2-null and WT animals ($p = 0.32$; WT n = 16 and α2-null n = 12) (Figure 5-2*C*). Even when the number of lung colonies was evaluated in WT and α2-null animals, there was no difference in the quantity of nodules present ($p = 0.12$; WT n = 16 and α2-null n = 12) (Figure 5-2*D*). Because the same 66c14 cells were injected into host animals of different backgrounds, we were able to evaluate integrin-specific contributions of the tumor microenvironment towards cancer progression. As was anticipated from previous work using the MMTV-neu breast cancer model, the host microenvironment contributed minimally to cancer metastasis to the lungs (203).

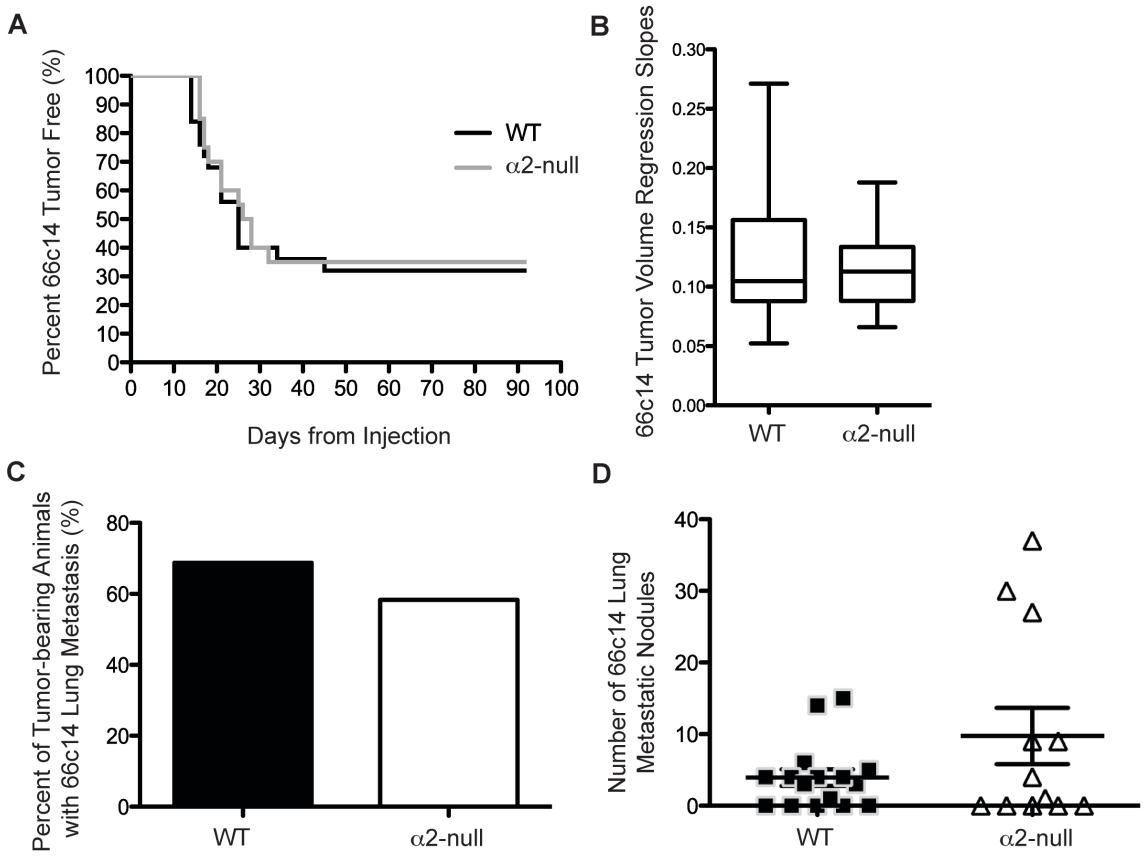


Figure 5-2. The α2β1 integrin on cells of the tumor microenvironment contributed minimally to alter breast cancer disease progression. *A*, No differences in latency of tumor development were observed when 66c14 cells were orthotopically implanted into the mammary fat pad of α2-null or WT mice (p = 0.73; WT n = 25 and α2-null n = 20). *B*, 66c14 tumors grew at similar rates in WT and α2-null mice (p = 0.64; WT n = 17 and α2-null n = 13). *C*, No differences were observed in the percentage of mice with lung metastatic colonies between α2-null and WT animals (p = 0.32; WT n = 16 and α2-null n = 12). *D*, Lungs were examined for the number of metastatic colonies, but no differences were detected between α2-null and WT animals (p = 0.12; WT n = 16 and α2-null n = 12).

Discussion

The 4T1 series of murine mammary carcinoma cells demonstrate a range of metastatic capabilities. From our data profiling α2β1 integrin expression, we were able to determine relative levels of the integrin on all 5 sister cell lines. We found that levels of the α2β1 integrin, from highest to lowest, were as follows: 4T1 > 4T07 > 67NR > 168FARN > 66c14. Based on extensive studies examining the metastatic ability of these cells, loss of α2β1 integrin expression did not correlate with metastasis. 4T1 cells, which are locally invasive and efficiently metastasize hematogenously, exhibited high levels of the α2β1 integrin. 4T07 cells, which have moderate expression of the α2β1 integrin, intravasate into the blood but fail to colonize other sites. Unlike 4T1s, 66c14, 168FARN, and 67NR cells had no integrin expression. 66c14 cells are considered the second most aggressive, after 4T1 cells, and are still efficient at dissemination via the blood and lymphatics. 168FARN cells metastasize via the lymphatics to arrest in draining lymph nodes, yet their spread does not continue beyond this secondary site. These cells also exhibit a defect in hematogenous intravasation, as no tumor cells were detected in blood cultures. 67NR cells cannot leave the primary tumor site; thus no intravasation occurs (236, 238).

There are several reasons why the α2β1 integrin does not suppress metastasis in this panel of tumor cells. 1) Gene expression was experimentally manipulated through subcloning and transfection to induce antibiotic resistance. Artificially culturing cells likely alter their gene expression profiles, selecting for cells that survive the foreign *in vitro* environment, which is drastically different from *in vivo* growth conditions (240-242). As a result, regulation of α2β1 integrin expression could be altered along with other pro-migratory adhesion molecules that may compensate for loss of integrin expression. 2) Different oncogenes may act independently or in concert in the various breast cancer

subclones to abrogate the effects of any single molecule, such as the α2β1 integrin, in metastasis (243). 3) The α2β1 integrin, in combination with other yet unidentified proteins, may be required to suppress metastasis in the breast model. 4) The interaction of tumor cells with their microenvironment may impact the degree by which tumor cells actively invade into or passively enter the vasculature (244, 245). 5) Stromal cells in the tumor microenvironment, such as fibroblasts and macrophages, may interact differently with the various 4T1 sister clones to contribute growth factors, matrix metalloproteinases, and chemotactic migratory factors to influence invasion and intravasation (245, 246). These factors all underscore the complexity of tumor metastasis and highlight the need for further studies examining the genetic profile of the cancer cells and their interaction with the tumor microenvironment. Because a range of genetic alterations likely explain the differences in cell morphology and behavior of the 4T1 sister clones, we focused on one specific line, 66c14 cells. Future experiments to lentivirally infect these cells with the α2 integrin subunit will help determine the precise contribution of this receptor in tumor cell-mediated cancer metastasis.

Because 66c14 cells intravasate into the blood vasculature, they can be quantitated by cytokeratin 19 detection. Future experiments include harvesting blood from mice implanted with α2 expressing 66c14 cells at the time of sacrifice to isolate total RNA. Keratin 19 quantitation would be subsequently performed to detect circulating tumor cells. These studies will help to determine whether integrin expression by tumor cells promotes differences in intravasation or survival in circulation. In order to study intravasation, we plan to test the ability of α2 expressing 66c14 cells to migrate through matrix and a layer of endothelial cells *in vitro* using a three-dimensional transwell assay. In rat mammary adenocarcinomas, intravasation was found to be the rate-limiting step in metastasis (247). Additionally, 20-30% of patients with absence of lymph node

metastasis still develop disease at distant sites (248). Therefore, direct routes of tumor cell intravasation into systemic circulation may be a better prognostic indicator than lymph node status alone. Studies have reported significant correlations between the presence of circulating tumor cells and poor clinical outcome in breast cancer patients (237, 249). However, the prognostic effectiveness of using circulating tumor cells in the blood has been of debate, with technical limitations on low sensitivity and high rates of false positives (250, 251). These future studies using integrin-specific expression by the 66c14 tumor cells will elucidate the differences caused by the α2β1 integrin during critical steps of the metastatic cascade. Findings will help identify specific mechanisms by which breast cancer cell dissemination occurs, in hopes of blocking disease progression in human patients.

In the MMTV-neu model of oncogenesis, the α2β1 integrin acts as a suppressor of hematogenous adenocarcinoma metastasis (203). However, α2β1 integrin expression on epithelial keratinocytes promotes lymphatic metastasis in the K14-HPV16 model of carcinoma. As was detailed in Chapter IV, the differences in metastasis likely resulted from the primary pathways utilized for cancer cell dissemination. In the murine model, breast adenocarcinomas preferentially intravasate the blood vasculature, whereas skin squamous cell carcinomas disseminate via the lymphatics. Little is known regarding how tumor cells preferentially metastasize either via the blood or the lymphatic system. Recent data suggest that certain expression profiles on the cancer cells dictate the route of dissemination (252). For example, tumor cells expressing vascular endothelial growth factor-C and the C-C chemokine receptor 7 heighten the invasive nature of cells towards lymphatics (253).

The most common forms of breast cancer are classified as ductal or lobular adenocarcinomas, since they arise from glandular tissue. Work using breast and skin

cancer murine models indicate that the α2β1 integrin impacts cancer progression differently based on the cell type of cancer origin. As was shown in Chapter III, loss of integrin expression was shown to increase development of skin adenocarcinomas in the K14-HPV16 model but did not impact squamous cell carcinoma formation. These data suggest a function for loss of the α2β1 integrin in adenocarcinoma development. When considering cancers of various origins, however, there is no definitive role of the α2β1 integrin in disease initiation, migration, or invasion. Regardless of the tumor cell type involved, however, the α2β1 integrin appears to function as a marker of disease progression, since poorly differentiated squamous cell carcinomas and breast adenocarcinomas express reduced levels of the α2β1 integrin, compared to well-differentiated tumors (115, 206, 254). As was done with poorly differentiated murine breast cancer cells, it would be interesting to re-introduce the α2 integrin subunit into HPV/KO sebaceous adenocarcinoma cells to see if a reversion to a more-differentiated phenotype occurs (206).

Many factors are known to regulate expression of the α2β1 integrin. In MDA-MB-231 breast cancer cells, loss of autocrine axon repulsion factor 3A signaling leads to decreased levels of the α2β1 integrin and increased metastasis (255). In prostate cancer, androgen receptor expression is known to increase expression of the α2β1 integrin and tumor cell binding to collage type I (256). It is likely that regulation of integrin expression is subject to the type of cancer involved. Further studies, therefore, will have to be performed to determine how the combination of α2 gene polymorphisms and transcription regulators dictate expression levels and as a result, adhesive behavior of cancer cells. Through continued studies on the α2β1 integrin, we will gain critical insights into the multiple key roles this receptor plays in the complex and multi-step process of cancer progression and metastasis.

CHAPTER VI

DISCUSSION AND FUTURE DIRECTIONS

Cancer and its associated metastases have been prominent subjects of current cancer research. In the last few decades, integrins have been discovered to play central roles in cancer progression and metastasis (257, 258). Specifically, increased β1 integrins serve as prognostic indicators of poor outcome and survival in breast cancer and SCC (259, 260). Inhibiting ligation of the β1 integrin has reversed the malignant phenotype of a human breast cancer cell line to a normal, growth-arrested phenotype (25). In addition to suppressing the malignant phenotype, the β1 integrin has been implicated in tumor initiation and progression (32, 36, 37). Due to the heterogeneity of the integrin family, which consists of at least 24 distinct αβ-heterodimers, detailed studies are essential to understanding the contribution of any one integrin in cancer. By focusing on the α2β1 integrin, I have been able to identify unique roles of this receptor in both cell-cell and cell-matrix interactions during cancer progression. Recent data from the Zutter lab indicated that the α2β1 integrin acts as a metastasis suppressor (203). However, emerging evidence suggests that the α2β1 integrin's ability to inhibit tumor metastasis may be dependent on both the subtype of tumor cell involved and the route of dissemination. The studies presented in this dissertation examined the complex contributions of the α2β1 integrin towards several key process involved in cancer progression, including preneoplasia, inflammation, and metastasis, in multiple animal models.

Studies into the α2β1 integrin have revealed significant roles of this integrin in multiple cancer types. Correlations between levels of integrin expression and cancer

aggressiveness, however, revealed no clear association. In this dissertation, I have focused on two subtypes of cancer—squamous cell carcinoma of the skin and adenocarcinoma of the breast. Not only are these two distinctly unique cancer subtypes, affecting different organs and responding differently to α2β1 integrin expression, but in the murine model, they robustly metastasize via alternative routes (SCC via the lymphatics to seed regional lymph nodes, and breast adenocarcinoma via the blood vasculature to colonize the lungs). Studying these two types of cancers has allowed me to discern specific roles of the α2β1 integrin in progression and metastatic dissemination.

In Chapter III, I showed that the α2β1 integrin alters early steps towards skin cancer formation but ultimately contributes minimally to chronic inflammation and disease progression. However, loss of the α2β1 integrin in SCC produced a reduced migratory and invasive phenotype of cancer cells *in vitro* that was also found to be independent of the tumor microenvironment when studied orthotopically *in vivo*. In Chapter IV, I examined how the α2β1 integrin maintains lymphatic endothelial barrier functions *in vivo* to prevent vessel leak into the interstitium and found that integrin loss resulted in increased LEC growth on Matrigel as well as dilated lymphatic vessels that exhibited ZO-1 loss. In Chapter V, I demonstrated that integrin expression by cells of the tumor microenvironment contribute minimally to producing the aggressive, metastatic phenotype seen with global loss of the α2β1 integrin. These results indicate that the α2β1 integrin plays complex and divergent roles dependent upon the cell type of cancer origin and upon the route of metastasis. Overall, the discoveries presented in this thesis underscore the importance of studying α2β1 integrin interactions in multiple cancer types, as the emerging data indicate multiple and varied roles of the integrin in predicting disease progression and prognosis dependent upon cancer origin. The ultimate goals of

these studies focus on impeding and inhibiting cancer development and metastasis. In this chapter, I will summarize some of the key findings that have broadened our understanding of the α2β1 integrin in cancer as well as propose future research directions.

Contributions of the α2β1 Integrin in Epithelial Squamous Carcinogenesis

Our lab had an initial interest in studying the α2β1 integrin's role in skin cancer due to its high expression levels on basal keratinocytes. In the skin, these cells are responsible for proliferation and replenishment of superficial epidermal layers. It was unknown how α2β1 integrin loss would impact the proliferative and invasive properties of epithelial cells in an oncogenic setting. Use of the K14-HPV16 mouse model of step-wise, epidermal carcinogenesis has allowed us to study the progression from preneoplasia to frank cancer in WT and α2-null mice. In Chapter III, I showed that the α2β1 integrin affected only very specific steps in squamous carcinogenesis, and its presence on cells of the tumor microenvironment, including chronic inflammatory populations, did little to alter cancer development. Instead, tumor metastasis was modestly decreased in HPV/KO mice, compared to HPV/WT animals, and HPV/KO tumor cells demonstrated decreased migration and invasion *in vitro*.

Previous studies in our lab have shown that the integrin is important in innate immunity through its ligation of C1q and the collectin family of proteins (46). Additional studies have found a role for the integrin in initiating acute mast cell responses to *Listeria monocytogenes* by recognizing C1q in circulating immune complexes (77). Not only is the α2β1 integrin important in mast cell degranulation and recruitment of neutrophils, but neutrophils themselves utilize the integrin for motility through extravascular tissue (74, 261, 262). Since discovering the α2β1 integrin's upregulation on activated adaptive

immune cells, where it has gained the name very late activation antigen-2 (VLA-2), it has been shown to promote T cell proliferation, cytokine secretion, and inhibit fas ligand-induced death (46, 263, 264). The α2β1 integrin has been examined in various murine models of delayed-type hypersensitivity, contact hypersensitivity, and arthritis using anti-α2β1 integrin antibodies (80).

In recent years, there has been increased speculation on how α2β1 integrin loss would impact the chronic inflammation associated with cancer development (84, 265). Evidence from my studies suggested a role for the α2β1 integrin only in acute but not chronic inflammatory responses. Mast cells were decreased in the HPV/KO preneoplastic ear tissue only at 6-months-of-age concomitant with increased papillomatosis and decreased dysplasia. However, mast cell counts at later time points were unaltered between HPV/WT and HPV/KO animals, indicating that this cell type contributed less towards dictating the pathologic progression to neoplasia. It is likely that the developing chronic inflammatory infiltrate commandeers the behavior of the microenvironment and that long-term inflammation, as seen in this mouse model of cancer, has functionally compensated for loss of the α2β1 integrin. The cells comprising chronic inflammatory responses in the blood, non-neoplastic ear, and tumor tissue revealed minimal differences between HPV/WT and HPV/KO animals. This finding demonstrates that the α2β1 integrin does not contribute significantly to the chronic inflammation associated with epithelial cancer progression.

In prostate cancer, the α2β1 integrin is aberrantly expressed. Levels of the integrin are increasingly downregulated in advanced stages of the primary tumor but upregulated in lymph node metastases (266, 267). Additionally, in breast cancer, progressive loss of differentiation is associated with loss of α2β1 integrin expression (112, 203). In concordance with these findings, I found expression of the α2β1 integrin

was also decreased in more highly advanced stages of SCC. These data suggest that the α2β1 integrin may be a useful indicator in determining the stage of cancer, in so far as integrin expression is decreased in higher stage, less differentiated, and more aggressive tumors. However, since lack of the α2β1 integrin did not alter SCC latency, tumor cell growth, or distribution of tumor grades, this integrin likely does not regulate progression towards any particular stage of disease in the K14-HPV16 model.

Similar studies focusing specifically on the β1 integrin subunit have revealed an important requirement for the β1 integrin in the cell adhesion, spreading, and invasion of SCC (260). It is interesting that our studies on the α2β1 integrin may narrow and corroborate these previously reported findings, implicating the significance of this specific heterodimer in the adhesive, migratory, and invasive properties of SCCs, particularly on collagen type I. It is still unclear, however, whether the decreased migratory and invasive phenotype of HPV/KO SCC cells was sufficient to produce the observed decreased rates of lymph node metastasis, or whether other microenvironmental factors contributed to this difference, such as the decreased efficiency of lymphatic transport detailed in Chapter IV. Unfortunately, the limited time span of our orthotopic SCC injection studies did not permit any statistically significant evaluation of spontaneous metastasis formation. Further studies examining *in vitro* intravasation of HPV/WT and HPV/KO SCC cells through either a WT or α2-null lymphatic endothelial barrier will have to be performed to address this question.

Previous studies from our lab have found the α2β1 integrin to be a suppressor of breast cancer metastasis (203). Why then, does the α2β1 integrin not serve as a universal inhibitor of metastasis? In Chapter III, I found that lymph node metastasis was modestly decreased in HPV/KO mice, compared to HPV/WT animals. There are several key differences between the MMTV-neu and the K14-HPV16 mouse model of cancer,

however, that may explain these altered metastatic responses. First, the two models represent disparate subtypes of carcinoma—adenocarcinoma, which arises from glandular epithelium, and squamous cell carcinoma, which arises from squamous epithelial tissue. Second, the preferred metastatic routes of the two tumors are different. MMTV-neu tumors disseminate hematogenously, while SCCs metastasize via the lymphatics. Third, the two cancer models are driven by different genes that function distinctly to drive cancer development. The viral oncoproteins E6 and E7 inactivate p53 and retinoblastoma to induce cancer in the K14-HPV16 model, while overexpression of the neu tyrosine kinase stimulates proliferation by activating the ras/map kinase cascade. It is widely accepted that the signaling pathways that ultimately lead to tumor initiation largely dictate disease progression (268). Fourth, the role of protumorigenic inflammation, while critical in driving squamous carcinogenesis, is not necessary in MMTV-neu tumorigenesis or metastasis. Fifth, the interaction and response of SCC cells towards collagen type I during migration and invasion may be more relevant towards facilitating metastasis than in breast adenocarcinoma. We postulate that these multiple factors function independent or in concert to alter metastasis.

Over the last few decades, an increasingly complex understanding of the α2β1 integrin in cancer has emerged, demonstrating that the integrin's prognostic significance depends on the type of cancer. In some neoplasms, high levels of the α2β1 integrin are associated with poor prognosis, such as in melanoma, thyroid, and ovarian malignancies. However, high integrin levels indicate better prognosis in breast and prostate cancer (203, 269-272). As detailed in Chapter III, loss of the α2β1 integrin did not alter SCC development but increased sebaceous adenocarcinoma formation. These disparate findings concerning the α2β1 integrin's role in cancer likely result from the multiple and varied roles of the integrin in various cell types and tumor

microenvironments. The findings in this dissertation, therefore, only highlight the need for continued research regarding the α2β1 integrin in cancer.

Function of the α2β1 Integrin in Regulating Lymphatic Leak and Transport

In the last several years, new interest has emerged concerning the role of integrins in lymphangiogenesis. While only the α9β1 integrin is critical in developmental lymphangiogenesis, integrins α4β1 and α5β1 have known involvements in tumor-associated lymphatics (135, 194, 195). To date, the collagen binding integrins α1β1 and α2β1 have only been studied in inflammatory and wound healing lymphangiogenesis (186). In light of the decreased lymph node metastasis observed with α2β1 integrin loss in K14-HPV16 mice, I wanted to determine if the integrin affected tumor-associated lymphatic vessel development and function.

Most studies examining α2β1 integrin-related, tumor-associated vessel development have focused on angiogenesis. The α2β1 integrin has a prominent role in promoting tumor growth by supporting formation of the blood vasculature feeding the neoplastic cells. Endorepellin, an angiostatic protein, is a ligand for the α2β1 integrin and VEGFR-2, such that systemic delivery of endorepellin was able to inhibit tumor growth and angiogenesis in mice expressing the α2β1 integrin but not in α2-null mice implanted with Lewis lung carcinoma cells (273, 274). In another study examining integrin membrane association with key signaling molecules, the α2β1 integrin was found to associate with the CD9 tetraspanin to induce growth arrest and quiescence in endothelial cells (275). Using α2 morpholino knockdown zebrafish, blood vessel sprouting was largely inhibited and existing vessels were nonfunctional (276). Interestingly in Chapter IV, α2-null lymphatic vessels also demonstrated decreased

functionality. Since angiogenesis and lymphangiogenesis are sister processes, it is not surprising that the α2β1 integrin may also play a role in the lymphatic vasculature.

The studies presented in Chapter IV demonstrated that loss of the α2β1 integrin is associated with lymphatic vessel dilation and reduced functionality, irrelevant of tumor status. Alterations in vessel leakiness were evident simply in the presence of fluid overload, a fact that also may decrease the efficacy of tumor cell transport to draining lymph nodes. Increased leakiness of tracer dye was noted in both a non-tumorigenic and tumorigenic setting, demonstrating that the integrity of the LECs was unaffected by cancer-related factors. Further experiments are underway to confirm the decreased functionality of α2-null lymphatic vessels using a modified *in vitro* intravasation experiment. Using this setup, we will be able to recapitulate our *in vivo* data by testing the specific contributions of the integrin on SCC cells versus on the LECs. Thus, we will be able to determine whether the decreased lymph node metastasis seen in Chapter III was the result of reduced HPV/KO tumor cell invasion or reduced LEC barrier functionality.

In Chapter IV, I showed that primary α2-null LECs demonstrated increased growth on Matrigel, compared to WT LECs. It has been reported that pathological lymphatic vessel dilation and hyperplasia is associated with reduced functionality, either by increased leakiness or incompetency of collecting duct secondary valves (151, 152, 155, 156). Future studies will be required to examine the contribution of secondary valve incompetency in causing decreased lymphatic functionality. In order to explain the increased leakiness of colloidal carbon from α2-null lymphatics, several tight junction proteins were examined. The vessel dilation in α2-null lymphatics was associated with a reduction in ZO-1 expression on non-tumor-associated as well as in tumor-associated HPV/KO vessels. ZO-1 has previously been shown to stabilize and tighten the

endothelial barrier, thus preventing the vascular leak of chyle and other macromolecules (153, 154). In a murine model examining Netrin-4 overexpression, increased lymphatic vessel proliferation led to ZO-1 loss and increased lymphatic leak (233). Further experiments are required, however, to determine whether the ZO-1 loss associated with α2-null lymphatics is itself the causative determinant in vessel leakiness or simply a by-product of the increased proliferation and hyperplasia seen with immature LECs.

Possible mechanisms resulting in increased α2-null vessel dilation are currently being investigated. In Chapter IV, we examined the effects of growth factors known to induce lymphangiogenesis *in vivo*, and particularly, those growth factors known to mediate endothelial cell migration and proliferation in cooperation with the α2β1 integrin (277). No differences were observed in the quantity of WT or α2-null lymphatics after VEGF-A, -C, or -D stimulation. These growth factors are, therefore, unlikely to be mediating the proliferative differences *in vitro*. We have future plans to study endorepellin, a ligand known to bind the α2β1 integrin's I domain, which triggers actin disassembly and blocks angiogenesis (273, 278). Loss of endorepellin signaling through the α2β1 integrin may cause increased LEC proliferation and the observed vessel dilation. This pathway, however, has not been studied specifically on LECs, but we postulate that endorepellin signaling may be common to all types of endothelial cells.

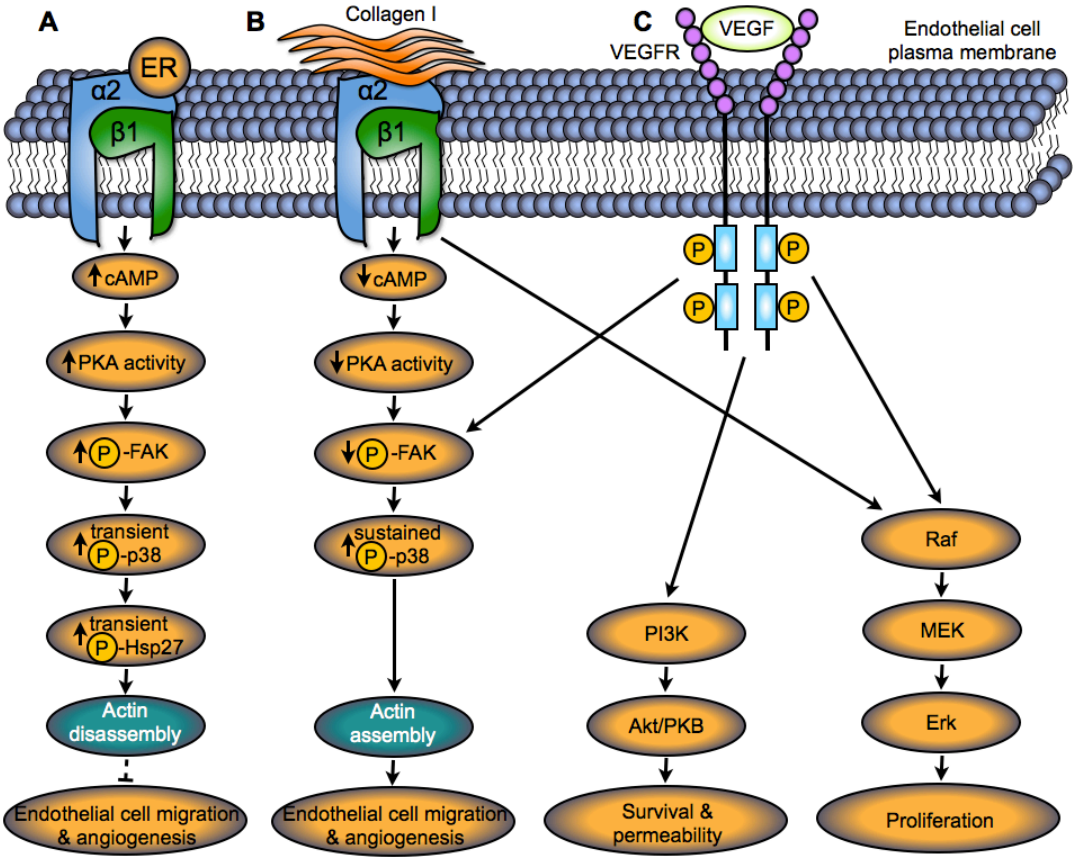


Figure 6-1. Possible mechanisms mediating the lymphatic vessel dilation and leakiness in α2-null vessels. Based on mechanisms known to influence blood endothelial cells, future studies will focus on identifying the pathway relevant to explaining our LEC phenotype. *A*, Endorepellin (ER) binds the α2β1 integrin I domain to activate cyclic AMP (cAMP), protein kinase A (PKA), focal adhesion kinase phosphorylation [(P)-FAK], and transiently induces the activation (and phosphorylation) of p38 mitogen-activated protein kinase [(p38)-MAPK] and the small chaperone and actin-capping heat shock protein 27 (Hsp27). The subsequent actin disassembly inhibits the alignment of endothelial cells and angiogenesis. *B*, Collagen I ligation of the α2β1 integrin activates many of the same signaling pathways associated with ER binding but with an opposite response. Ultimately, collagen I induces endothelial cell alignment, and increased formation of actin stress fibers and angiogenesis. *C*, Stimulation of the vascular endothelial growth factor receptors (VEGFR) associated with lymphangiogenesis, including VEGFR-2, and -3 by VEGF-A, -C, and -D ligands, can also lead to the alignment of endothelial cells, as well as increase survival, permeability, and proliferation. Based on what is known about blood endothelial cells, receptor ligation leads to increased cell survival and permeability through activation of phosphoinositide 3-kinase (PI3K) and the protein kinase B (Akt/PKB) pathways. Increased proliferation occurs through activation of Raf, MEK, and Erk (279, 280). In Chapter IV, I showed that LEC infiltration into VEGF-A, -C, or -D loaded Matrigels was similar between WT and α2-null mice. Activation of the VEGFR pathways, therefore, is likely not responsible for the differences in our animal model.

Contributions of the α2β1 Integrin in Breast Adenocarcinoma

While relatively little is known about the α2β1 integrin in skin cancer, the effects of integrin expression in breast cancer has been more extensively studied. Based on previous work in our laboratory, the α2β1 integrin decreased hematogenous metastasis to the lungs by inhibiting tumor cell intravasation (203). Since these studies were performed in global α2-null animals, the contributions of the tumor microenvironment towards intravasation and metastasis could not be ruled out. Furthermore, elaborate studies using primary tumor cells were hindered by difficulties with their *in vitro* manipulation.

In Chapter V, I examined the α2β1 integrin's role in the tumor microenvironment versus on breast cancer cells. I first demonstrated that the murine 66c14 cells, derived from BALB/c mice, were an ideal breast cancer cell line to use due to their highly aggressive nature for invasion and metastasis as well as their ease of *in vitro* manipulation. Through orthotopic implantation of these cells, I demonstrated that growth of the α2-null primary tumor and lung metastasis were unaffected by integrin status of the host microenvironment. These experiments will have to be repeated along with experiments examining tumor-specific effects of α2β1 integrin expression on breast cancer progression and metastasis. While these experiments are preliminary, they indicate that expression of the α2β1 integrin on cells of the tumor microenvironment do not significantly impact breast cancer. Conversely, integrin expression by tumor cells is likely the cause for the heightened intravasation of cancer cells seen in our original studies using the global α2-null mouse.

Further studies are underway to stably transduce the α2 integrin subunit into 66c14 cells using lentivirus. After orthotopic implantation of these cells, hematogenous intravasation of transduced α2 expressing or vector control cells will be examined by

qRT-PCR. If more 66c14-vector control cells than 66c14-α2 cells are found in the peripheral blood of WT BALB/c mice, it would indicate that tumor-specific integrin expression is the cause for differential intravasation. Ultimately, studies into the biochemical mechanism of α2β1 integrin down-regulation in advanced stages of breast cancer could allow the development of novel therapeutics in disease prevention, diagnosis, and treatment.

Summary

The studies presented in this dissertation investigated the α2β1 integrin's impact on SCC disease progression as well as its role in the lymphatic and hematogenous metastasis of SCC and breast cancer. By examining the multiple compartments known to drive cancer, it has been possible to ascertain the relevant contributions of the tumor cells, the inflammatory cells, and the additional cells of the tumor microenvironment towards mediating preneoplastic and neoplastic changes.

Previous work in our lab has focused on the role of the α2β1 integrin as a metastasis suppressor in murine and human models of breast cancer. Integrin null breast tumor cells promoted anchorage-independent growth, exhibited increased *in vitro* intravasation, and were more abundantly found in circulation than WT controls (203). SCCs, however disseminate via the lymphatic system, and loss of the α2β1 integrin resulted in modestly decreased metastasis to regional lymph nodes. Whether a tumor cell metastasizes via the blood or the lymphatic vasculature depends on several factors, including intrinsic behavior of the cancer cells and their interaction with either blood or lymphatic endothelial cells (252). Patterns of metastasis are typically unique to the tumor type (281). In this dissertation, I have examined the effect of α2β1 integrin expression by tumor cells and by cells of the tumor microenvironment, namely the

inflammatory component and the lymphatic vascular system, in neoplastic progression and metastasis. My studies have revealed a role for the α2β1 integrin in promoting the migratory and invasive ability of SCC cells on collagen type I. Additionally, I have found that integrin loss produced profound defects in lymphatic function, by inducing vessel dilation and increased leakiness. These factors could, alone or in concert, contribute to the overall reduction in HPV/KO lymph node metastasis.

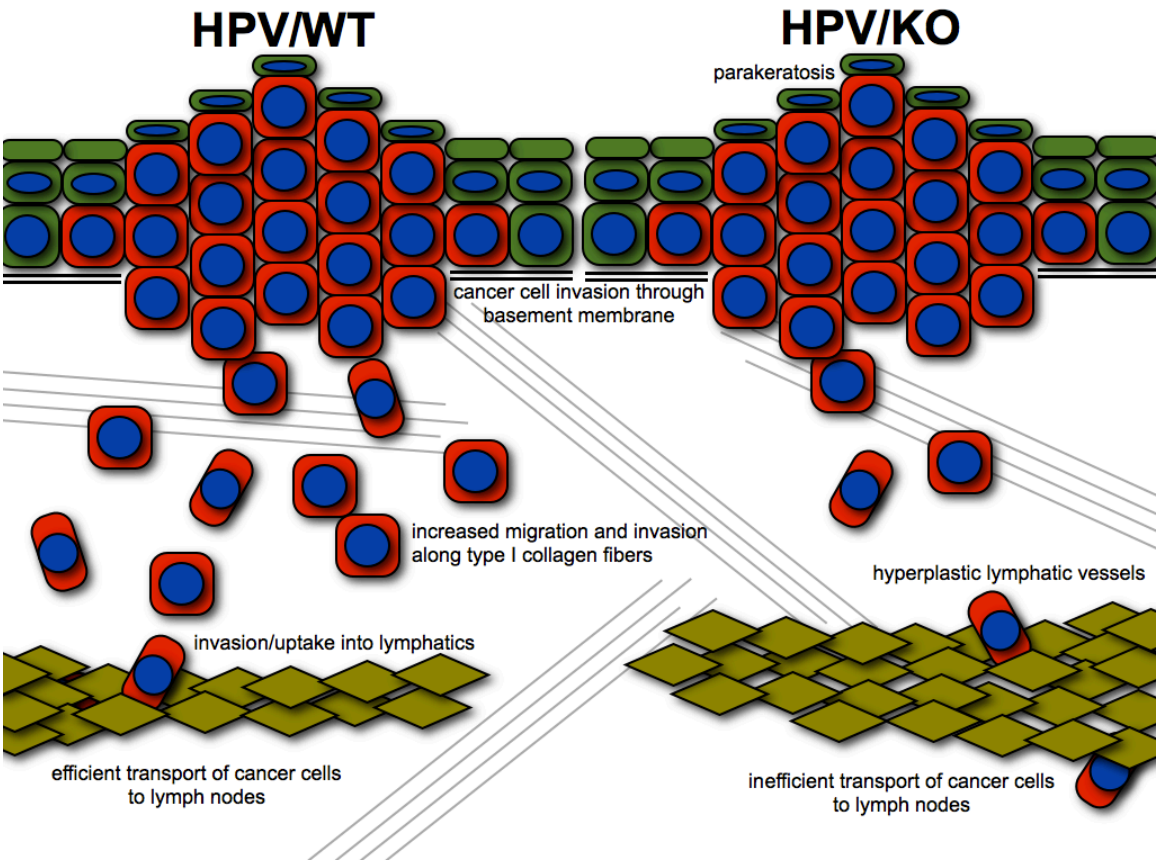


Figure 6-2. Model of defective lymphatic metastatic capacity in HPV/KO animals. Presence of the α2β1 integrin does not impact cancer development in K14-HPV16 mice, indicating that the rates of cancer cell invasion through the basement membrane are similar. However, HPV/KO tumor cells have reduced migration and invasion along type I collagen fibers *in vitro*, which are the main collagen component of dermal tissue. Additionally, the decreased rate of lymph node metastasis seen in HPV/KO animals may also be due to defective lymphatic transport of cancer cells, secondary to lymphatic vessel dilation seen with α2β1 integrin loss.

Based on previous studies using the K14-HPV16 murine model of multi-stage epithelial carcinogenesis, we determined that the α2β1 integrin differentially regulates SCC versus sebaceous adenocarcinoma (SA) formation. In this model, loss of the α2β1 integrin did not alter SCC formation but increased SA prevalence. Since the majority of breast cancers arise from glandular/ductal tissue, we have found that mammary adenocarcinomas behave similar to the cutaneous, sebaceous adenocarcinomas seen in K14-HPV16 mice. Loss of α2β1 integrin expression by both types of adenocarcinomas predict for more aggressive and advanced disease. As a result, the α2β1 integrin may be useful in predicting disease progression and poor prognosis for adenocarcinomas of multiple origins. Additional studies using the K14deltaNLef1 mouse, for example, may provide more insight into the α2β1 integrin's role in cutaneous sebaceous adenoma. K14deltaNLef1 mice have a deletion in the N-terminus of the lymphoid enhancer-binding factor 1 gene, which causes constitutive transcriptional gene activity, under the direction of the keratin 14 promoter. These animals have become a model for the development of sebaceous tumors (282, 283). A review of the literature further provides a complex and dynamic view of the α2β1 integrin's role in dictating clinical prognosis. Unfortunately, there is no clear consensus about how the integrin is associated with long-term outcomes. Instead, the α2β1 integrin's role appears specific towards the cancer subtype.

Table 6-1. Expression of the α2β1 integrin in varied cancer subtypes. Based on the cancer type in question, the α2β1 integrin predicts for divergent disease outcomes. For example, in melanoma, lung squamous cell carcinoma, chondrosarcoma, thyroid cancer, gastric cancer, ovarian carcinoma, and pancreatic cancer, increased expression of the α2β1 integrin resulted in worsen prognosis which may involve a greater association with advanced malignancy or increased metastasis. Conversely, increased expression of the α2β1 integrin on prostate, breast and colorectal adenocarcinoma is associated with a more differentiated cell phenotype and better prognosis in human and mouse studies.

Tumor type	Associated phenotypes	Organism/Model system	Reference
Melanoma	Expression associated with advanced disease and poor survival	Human tissue samples and *in vitro* cell lines	(269, 284)
Lung squamous cell carcinoma	Expression correlated with increased metastasis	Human tissue samples	(113)
Chondrosarcoma	Expression increased tumor cell migration	Human tissue samples	(285)
Thyroid	Increased expression in anaplastic carcinomas	Human tissue samples	(270)
Gastric	Increased expression in metastases	Human tissue samples	(286, 287)
Ovarian	Increased expression associated with invasion	Human cell lines *in vitro*	(271, 288)
Pancreas	Increased expression associated with malignant phenotype	Human cell lines *in vitro* & tissue sections	(289, 290)
Prostate	Decreased expression in more advanced stages of cancer	Human tissue samples	(203, 266, 267, 272, 291)
Breast	Re-expression results in differentiation of cancer cells and better prognosis	Human tissue samples & mouse	(112, 115, 203, 206)
Colorectal	Re-expression results in differentiation of cancer cells; decreased expression in advanced stages of cancer	Human cell lines *in vitro*	(292, 293)

The use of integrin inhibitors in cancer treatment began in the mid-90s and has primarily focused on blocking tumor-associated angiogenesis, and as a result, reducing the tumor's ability to grow. Studies using functionally neutralizing antibodies against the major collagen binding integrin α-subunits, α1 and α2, called Ha 31/8 and Ha 1/29, have been shown to be effective at inhibiting endothelial cells in a haptotaxis assay on immobilized collagen type I by <40% in single therapy but when used in combination, the antibodies synergized to product <90% inhibition (294). *In vivo* studies using these antibodies in A431 tumor-bearing nude mice revealed a reduction in angiogenesis and tumor growth (277).

In addition to these functionally neutralizing antibodies, endogenous inhibitors have been discovered that block the α2β1 integrin's pro-angiogenic effects. Angiocidin, originally isolated from lung carcinoma, is known to bind collagen type I as well as the α1β1 and α2β1 integrins. A 20 amino acid N-terminal peptide of angiocidin was found to disrupt *in vitro* α2β1-depdendent cell adhesion of the erythoroleukemic cell line K562, human umbilical vein cell tube formation, and *in vivo* Lewis lung carcinoma growth and angiogenesis (295). Another endogenous peptide, derived from the C-terminal cleavage of collagen type XVIII and termed endostatin, was found to bind the α2β1 integrin and inhibit chondrosarcoma growth and angiogenesis *in vivo* without affecting proliferation and migration of the cancer cells *in vitro* (296). Finally, endorepellin, which is derived from the C terminus of perlecan, recognizes the α2β1 integrin and has been shown to inhibit Lewis lung carcinoma growth *in vivo* by inhibiting angiogenesis (273, 278).

To date, the only drug specifically targeting the α2β1 in clinical trials is the small molecule inhibitor E7820. This drug has been shown to inhibit tumor growth in multiple *in vitro* tumor cell lines and two xenograft models through blocking α2β1 integrin ligation on blood endothelial cells (297-299). Currently, E7820 is in phase II trials (300). Based

on the efficacy of these compounds in blocking tumor growth and angiogenesis, it would be interesting to know how tumor-associated lymphangiogenesis will be altered with these same drugs. Not only can inhibition of angiogenesis reduce the rates of hematogenous metastasis, but based on my findings, the effect of these inhibitors on lymphangiogenesis could additionally reduce lymphatic metastasis. This additive effect resulting from blocking the α2β1 integrin should be investigated and could justify the incorporation of α2β1 integrin inhibitors in more treatment regimens against cancer.

Table 6-2. Pharmacological inhibitors of the α2β1 integrin. The antibody inhibitor Ha1/29 and endogenous inhibitors angiocidin and endorepellin have not been studied in humans. The aromatic sulfonamide derivative, small molecule inhibitor E7820 has reached Phase II studies in multiple cancer types. Endostatin, the only endogenous inhibitor of the α2β1 integrin to reach clinical trials, is not specific but instead targets multiple integrins involved in angiogenesis. All these pharmacologic inhibitors of the α2β1 integrin have proven to reduce tumor growth and angiogenesis in *in vivo* murine models of cancer.

Inhibitor	Integrin target specificity	Highest phase reached	Company	References
Ha1/29	α2β1			(277)
Angiocidin	α1β1 & α2β1			(295)
Endostatin	α5 subunit, αv subunit, & α2β1	Phase III	Medgenn Co. Ltd.	(301, 302)
Endorepellin	α2β1			(273)
E7820	α2 subunit	Phase II	Eisai Medical Research	(297, 298, 300)

The studies in this dissertation have examined the multiple and varied roles of the α2β1 integrin on both the tumor cells and in the tumor microenvironment using two separate cancer models. While no definitive rule can be applied to predict the behavior of the tumor cells or the responses of the microenvironment, the presented findings indicate that biology of the α2β1 integrin in cancer is complex and dependent on the type of cell expressing it. Additionally, the concept that tumor-associated lymphatics can be targeted to decrease their functionality and inhibit afferent transport of metastatic tumor cells to lymph nodes is promising. This finding suggests that pharmacologic α2β1 integrin inhibition could potentially block three aspects of tumor progression—primary tumor growth, angiogenesis, and lymphatic metastasis.

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