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Author
Sung, James Lee

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Mechanisms of Nodal Metastasis in Prostate Cancer

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular and Medical Pharmacology

by

James Lee Sung

2012
ABSTRACT OF THESIS DISSERTATION

Mechanisms of Nodal Metastasis in Prostate Cancer

by

James Lee Sung

Doctor of Philosophy in Molecular & Medical Pharmacology

University of California, Los Angeles, 2012

Professor Lily Wu, Chair

Prostate is cancer is the most commonly diagnosed cancer in men worldwide and the second biggest killer among cancer related deaths in men. Due to advances in both detection as well as treatment, the disease can often be caught early when it is still confined within the prostate gland. The prognosis of patients when the disease is still confined within the organ is generally good. However, the prognosis in patients when the disease has spread to regional lymph nodes is very poor. Metastatic prostate cancer accounts for nearly all the mortality associated with prostate cancer. Numerous clinical studies have documented the importance of lymph node involvement in predicting patient survival. These data suggest that not only is lymph node involvement an important indicator of systemic metastases, they may be an active participant in the process. In the present report, we provide insight into mechanisms that are involved in prostate cancer
metastasis to lymph nodes. First, we examine the relationship between the lymphangiogenic mechanisms and prostate dissemination to lymph nodes. Second we, offer insight into lymph node specific homing mechanisms used by prostate cancer cells. Finally, we describe a strategy to interrogate the significance of lymph nodes as a reservoir of malignant cells that can fuel future metastatic dissemination. A better understanding of the mechanisms involved in lymph node metastasis will allow us to better treat the metastatic disease.
The Dissertation of James Sung is approved

Hong Wu

Jonathan Braun

Oliver Hankinson

Lily Wu, Committee Chair

University of California, Los Angeles

2012
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LIST of ACRONYMS

PCa Prostate Cancer
PIN Prostatic intraepithelial neoplasia
DC Dendritic Cell
PLND Pelvic lymph node dissection
PSA Prostate specific antigen
LNI Lymph node involvement
VEGF-C Vascular endothelial growth factor –C
VEGFR-3 Vascular endothelial growth factor receptor -C
LYVE-1 Lymphatic endothelial receptor-1
PDGF Platelet derived growth factor
IGF Insulin like growth factor
FGF Fibroblast growth factor
HGF Hepatocyte growth factor
SCID Severe combined immunodeficient
CMV Cytomegalovirus
RL Renilla Luciferase
GFP Green fluorescent protein
RFP Red fluorescent protein
HDAC Histone deacetylase
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VITA

Education

University of California, Los Angeles
Ph.D., Molecular & Medical Pharmacology
Expected 2012

University of California, Los Angeles
UCLA Extension
Certificate in Science with a concentration in microbiology, immunology and molecular genetics
2002-2003

University of California, Los Angeles
UCLA Extension
Certificate in General Business Studies with a concentration in finance
2001-2002

Dartmouth College
B.A. Biochemistry
Minor: Neuroscience
1995-1999

Experience

University of California, Los Angeles
Molecular and Medical Pharmacology
Graduate student researcher
2004-Present

University of California, Los Angeles
Microbiology, Immunology and Molecular Genetics
Laboratory Technician
2003

Merrill Lynch
Capital wealth management
Intern
2002

Dartmouth College
Microbiology, Immunology and Molecular Genetics
Laboratory Technician
1999-2000

Awards and Honors

Graduate Student Research Mentorship Award
2007-2008

Publications and Presentations


I. INTRODUCTION
Prostate Carcinoma (PCa) is the most commonly diagnosed cancer in men and the second leading cause of cancer deaths in the U.S. It is estimated that 1 in 6 men will be diagnosed with prostate cancer in their lifetime, and that 1 in 34 men will die from the metastatic disease.(1) The incidence and mortality rates for prostate cancer are highest in the United States and Western Europe, particular among black men in the United States.(2, 3) Though the specific factors that contribute to prostate cancer remain unknown, epidemiological studies suggests that environmental as well as life-style factors are likely contributors. In support of this theory, prostate cancer rates are lower in Asia than the rest of the world; however, these rates increase when Asians immigrate to North America.(2) In addition to environmental and life-style factors, studies have revealed that genetic abnormalities, both in inherited susceptibility genes, RNASEL, MSR1, as well as spontaneously arising somatic changes, NKX3.1, PTEN, CDKN1B, are important molecular mechanisms which drive the progression of prostate cancer.(2, 4)

Prostate cancer is heterogeneous and multifocal in nature.(2) It has a defined pattern of progression starting from early prostatic intraepithelial neoplasia (PIN) to locally invasive adenocarcinoma and ultimately to androgen insensitive metastastic disease.(2) Localized prostate cancer, when confined to the organ, is much more manageable and treatment options such as radical prostatectomy and radiation therapy are often curative(5-7). However, spread of the disease into regional lymph nodes usually indicates a very poor prognosis.(8, 9) The metastatic disease, which accounts for nearly all of the prostate cancer related deaths(10), is often treated with androgen deprivation therapy. However, the disease often progresses to a hormone refractory state that becomes resistant to therapy.(11)
Metastasis is a complicated process that involves many steps. It requires malignant transformation, invasion into and out of the vasculature, survival during periods of anoikis, and ability to grow in a foreign site. (12, 13). Spread of malignant cells can occur through both blood vessels (hematogenous) as well as lymphatic vessels (lymphogenous). Experimental studies suggest that metastasis is a very inefficient process and that the biggest barrier for malignant cells is the ability to survive and grow at the secondary site. (14, 15) Metastasis is not entirely a random process, as different types of cancers display very distinct organ specific patterns of metastasis. (14) In prostate cancer, the bones and lungs are the most common sites of metastasis, although metastasis to liver and the brain have been noted as well. (16, 17)

Metastasis to regional lymph nodes is one of the earliest events in the metastatic process and enlargement of drainage lymph nodes is a common feature of many solid tumors. (9) Lymph nodes are non-vital organs located along lymphatic vessels throughout the human body. They are comprised mainly of lymphocytes such as B, T, and Dendritic Cells and are the site of many important immunological processes. (9)

Assessing the extent of metastatic spread in regional draining lymph nodes is an important staging tool in the treatment of locally advanced prostate cancer. (18) Extent of lymph node involvement is performed by pelvic lymph node dissection (PLND) of suspect lymph nodes that are then examined for presence of metastases both visibly and histologically, often with H&E. (8) More sensitive immunohistochemical methods are also frequently used while even more sensitive PCR based methods are also being investigated. (18) Increase in lymph node involvement usually warrants more aggressive therapy more tailored to treat the systemic disease.
The extent of PLND is at the discretion of the attending physician who is often assisted by a specific set of guidelines or nomograms. Nomograms predicting lymph node involvement are based on findings in lymph node involvement from many clinical cases. These nomograms include a variety of risk factors including observance of extraprostatic extensions, gleason score of prostate biopsies, and PSA levels. Many radiologically based imaging techniques can also be used to assist in collection of occult lymph nodes.

Standard PLND usually involves removal of lymph nodes in the obturator fossa and external iliacs and can be prescribed in low risk cases. Extensive PLND is often used in high-risk cases and involves removal of more distant lymph nodes including those in the internal and common iliacs. In some cases, even more distant lymph nodes that are outside the general confines of the pelvis are removed. In total, there are more than 30 lymph nodes that can be removed during PLND and used to assist in staging. Long-term survival outlook of node positive patients with low LNI is improving due to earlier detection and better therapeutics. Furthermore, there is growing evidence that PLND has a long term therapeutic benefit suggesting that the early metastatic disease can be contained within the lymph nodes and treated with androgen deprivation therapy. In this regard, a better understanding of metastatic mechanisms will lead to better markers to stage the disease as well as better therapeutics to manage the disease.

It is commonly believed that lymph node metastasis is mediated primarily by spread through lymphatic vessels as a consequence of tumor-supported lymphangiogenesis. Tumor lymphangiogenesis is regulated, to a great degree, by the vascular endothelial growth factor C (VEGF-C). Expression of VEGF-C has been
documented in many different cancers. (22) In addition, other stromal elements within the tumor such as tumor-associated macrophages have also been shown to express VEGF-C. (23) The receptor for VEGF-C, VEGFR-3, is expressed primarily on lymphatic endothelial cells. Activation of VEGFR-3 supports the growth of new tumor associated lymphatic vessels.

In prostate cancer, expression of VEGF-C and its primary receptor VEGFR-3 has been documented in both clinical specimens as well as in experimental models, however, precise nature of the contribution of this axis to lymph node metastasis remains to be determined. In clinical studies, there is strong evidence to suggest that increased expression of VEGF-C correlates with lymph node metastasis. However, it is unclear if this increase in lymph node metastasis is a result of increase lymphatic vessel density, as correlations between lymphatic vessel density and nodal metastasis have been inconsistent. (9) In light of these observations, more extensive investigation of the mechanisms that mediate VEGF-C enhanced lymph node metastasis is needed.

Organ specific metastasis is mediated in part by chemokines and their cognate receptors. Chemokine receptors belong to a family of G-protein coupled receptors that are normally expressed on cells of the immune system. These receptors bind to a family of chemokines, ‘chemotactic’ cytokines that are important regulators of leukocyte trafficking to both inflamed tissues and lymphoid organs. Studies have shown that chemokine receptors are important mediators of many metastatic processes. In prostate cancer, expression of CXCR4 has been shown to contribute to the propensity of prostate cancer to metastasize to the bone, (24) while autocrine signaling through CXCR1 and
CXCR2 has been demonstrated to enhance numerous aspects of metastatic behavior, including lymph node metastasis.(25)

The contribution of chemokine receptors to metastasis has been studied in many different cancers. From these studies, CCR7 has emerged as an important mediator of lymph node metastasis. The ligands for CCR7, CCL19 and CCL21, are expressed highly in the lymph nodes. Experimental studies in breast and melanoma have demonstrated that CCR7 enhances metastasis to lymph nodes.(26, 27) The presence of CCR7, and its correlation to lymph node metastasis, has been documented in many cancers; however its significance in prostate cancer metastasis is still unclear.(28)

Lymph node involvement has long been known to be an important predictor of both patient survival as well as systemic metastases in cancer.(16, 29) Although the importance of assessing lymph node involvement is not disputed, there is much debate as to the contribution of lymph node metastases to distant organ metastasis. One view suggests that lymph nodes are merely indicators of already existing systemic metastases. While the opposing view argues that metastatic lymph nodes are important mediators of systemic metastasis.(30, 31) Numerous clinical studies have attempted to address these opposing viewpoints but results have been mixed, as a result, the optimum course of treatment of node positive patients remains controversial. To address these different viewpoints, a system to target and stably mark metastatic cells in the lymph node and track their spread throughout the metastatic process would be very useful and would provide more insight into a very complex process.

Metastasis to regional lymph nodes is one of the earliest metastatic events in the progression of prostate cancer. Our current understanding of lymph node metastasis
implicates expression of lymphangiogenic growth factors and chemokine receptors as two important mechanisms that can mediate this process. In the following report, we provide additional evidence to support the significance of these factors in contributing to lymph node metastases. Furthermore, we describe a strategy to assess the contribution of lymph node metastases to systemic spread.
II. VEGF-C DRIVES LYMPH NODE METASTASIS OF PROSTATE CANCER
LYMPH NODE INVOLVEMENT IS ROUTINELY ASSESSED TO STAGE LOCALLY ADVANCED PROSTATE CANCER AND USUALLY DENOTES A POOR OUTCOME. IT IS COMMONLY THOUGHT THAT METASTASIS TO REGIONAL LYMPH NODES IS MEDIATED PRIMARILY BY LYMPHATIC DISSEMINATION. IN SUPPORT OF THIS THEORY, MANY STUDIES HAVE FOUND STRONG CORRELATION BETWEEN VEGF-C EXPRESSION TO LYMPH NODE METASTASIS. HOWEVER, DESPITE THE STRONG CORRELATION, THERE IS STILL MUCH CONTROVERSY SURROUNDING THE PRECISE NATURE OF THE RELATIONSHIP BETWEEN INCREASED VEGF-C EXPRESSION AND THE LYMPH NODE METASTASIS OF PROSTATE CANCER. THE AIM OF THIS STUDY WAS TO EXPLORE THE SIGNIFICANCE OF VEGF-C EXPRESSION TO LYMPH NODE METASTASIS IN PROSTATE CANCER. MORE SPECIFICALLY, WE INVESTIGATED THE IMPACT OF MODULATION OF THE LYMPHANGIGENIC AXIS THROUGH VEGF-C ON BOTH LYMPHATIC VESSEL DENSITY AS WELL AS LYMPH NODE METASTASIS. WE OBSERVED THAT OVEREXPRESSION OF VEGF-C INCREASED BOTH THE FREQUENCY OF LYMPH NODE AS WELL AS SYSTEMIC METASTASIS. CONVERSELY, VEGF-C INHIBITION USING A LIGAND TRAP (sVEGFR-3) RESULTED IN REDUCED LYMPH NODE AND SYSTEMIC METASTASES. WE OBSERVED THAT DIFFERENCES IN LYMPH NODE AND SYSTEMIC METASTASIS BY VEGF-C MODULATION STRONGLY CORRELATED WITH CHANGES IN LYMPHATIC VESSEL DENSITY. FURTHERMORE, MANIPULATION OF THE LYMPHANGIgenic AXIS HAD NO EFFECT ON TUMOR GROWTH RATE OF TUMORS. THESE FINDINGS STRESS THE IMPORTANCE VEGF-C IN MEDIATING LYMPH NODE AS WELL AS SYSTEMIC METASTASIS AND HIGHLIGHT THE POTENTIAL OF VEGF-C DIRECTED THERAPY TO CONTROL PROGRESSION OF THE METASTATIC DISEASE.
INTRODUCTION

Metastatic dissemination to lymph nodes is thought to occur via lymphatic vessels and this is supported by many clinical studies that show a strong correlation between expression of lymphangiogenic growth factors, as well as increases in lymphatic vessel density, in primary tumor biopsies to lymph node metastases.(9) Characteristics of both the lymphatic vessels and the lymphatic system support the notion that lymphatic spread may be the preferred route of systemic dissemination.

The lymphatic system is comprised of lymphatic vessels and associated lymphoid organs such as lymph nodes and spleen. One of the primary functions of the lymphatic system is to serve as a drainage system where excess interstitial fluid, containing plasma proteins as well as leukocytes, is collected into lymphatic capillaries from peripheral tissues. This fluid passes through regional lymph nodes and eventually drains into larger lymphatic vessels and ultimately makes its way back into venous circulation via the thoracic duct and left subclavian vein.(32) The structure of lymphatic vessels is quite different from that of blood vessels. As one of their main functions is to serve as a drainage system, lymphatic vessels lack the tight inter-endothelial junctions and the smooth muscle coverage of their blood vessel counterparts. As a result, lymphatic vessels are much more permeable to both fluid and cells and under far less pressure than blood vessels.(32) Furthermore, lymphatic endothelial cells are attached to the extracellular membrane via specialized anchoring filaments that aid in vessel permeability during increases in tissue pressure during body movement, which is one way by which fluid circulates throughout the lymphatic system.(32) Because lymphatic vessels are more
permeable than blood vessels and under far less pressure, it is hypothesized that lymphatic vessels are the preferred route of metastatic dissemination.

Traditionally, research in lymphangiogenesis has been overshadowed by work in angiogenesis, however, the discovery of the first lymphangiogenic factor, VEGF-C as well as identification of lymphatic specific endothelial markers such as VEGFR-3, LYVE-1, Prox-1 and Podoplanin have ushered in a wave of intense research into lymphangiogenic mechanisms. Growth of new lymphatic vessels occurs by sprouting of as extensions from pre-existing lymphatic vessels.(33) Furthermore, studies have shown that lymphatic capillaries appear to be less prone to changes in structure than their blood vessel counterparts: they form much fewer sprouts, exhibit much less tendency to reconnect and converge, and they are much less likely to retract or change in size or form.(32)

VEGF-C belongs to a family of highly conserved glycoproteins that regulate vasculogenesis. The VEGF family consists of VEGF-A,-B, -C, -D, and –E and bind to there cognate tyrosine kinase receptors (VEGFR-1,-2, and -3). The VEGFs have overlapping functions and have been shown to have affinity for more than one receptor. VEGF-C binds to VEGFR-3 with high affinity but can be proteolytically processed after secretion. This processed form can also bind VEGFR-2 with high affinity.(9) While the expression of VEGFR-3 is has been shown to be ubiquitously expressed on endothelial cells in early embryonic vasculature, its expression becomes restricted to the lymphatic endothelium in later stages.(32) Ligand binding of VEGF-C to VEGFR-3 has been shown to increase endothelial cell proliferation, migration, sprouting, and vascular permeability. Since the discovery of vascular endothelial growth factor-C (VEGF-C), many other
growth factors have been identified with lymphangiogenic properties. These include: VEGF-D; Ang-1, -2; PDGF; IGF-1,-2; FGF; and HGF among others.(9)

The expression of VEGF-C has been correlated to tumor lymphatic density as well as metastasis to regional lymph nodes in a variety of cancers including gastric, colorectal, lung, breast, and prostate.(9, 21) Despite this correlation, the significance of increased tumor lymphatic density in promoting lymph node metastasis is controversial. In the present study, we examined the significance of the VEGF-C/VEGFR-3 axis in modulating both tumor lymphangiogenesis and lymph node metastasis.
MATERIALS AND METHODS

**Cell lines and cell culture.** The androgen-independent, androgen responsive CWR22-Rv-1 prostate cancer cell line was maintained in RPMI media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The androgen-dependant human prostate cancer cell LAPC-9 was a kind gift from Dr. Charles Sawyers (Memorial Sloan Kettering Cancer Center). LAPC-9 xenografts were maintained by passage through male SCID/Biege mice. NIH-3T3 fibroblast cell line was maintained in DMEM media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

**Lentiviral production and tumor cell transduction.** Lentivirus was produced by calcium phosphate mediated tranfection of lentiviral plasmids into 293T cells. Viral supernatant was collected at 48, 72, and 96 hours post transfection. For in vivo metastasis studies, prostate cancer cell lines were transduced with lentivirus expressing the Renilla luciferase gene under the control of the cytomegalovirus promoter (CMV-RL). For overexpression studies, cells were transduced with pCCL-CMV-VEGF-C-IRES-EGFP, pCCL-CMV-IRES-EGFP (control), or pCCL-CMV-sVEGFR-3-IRES-EGFP. Tumor cells were infected using viral supernatant at a multiplicity of infection (MOI) of 1 with polybrene for 6 h incubation.

**Immunohistochemistry.** Tumors, lymph nodes and lungs were harvested and fixed in 3% paraformaldehyde overnight at 4°C. Sections (5um) were stained with human anti-Cytokeratin (Biogenex) and developed using DAB substrate (Biogenex). For vasculature stainings, sections were stained with anti-LYVE-1 (RELIATECH) or anti-CD31 (BD Biosciences) antibodies. Images were processed and quantified using Image J software.
**Animal Work and Optical Imaging.** All animal care and procedure were done in accordance with the University of California animal Research Committee guidelines. For subcutaneous studies, CWR22Rv-1 (5 x 10^5) were implanted subcutaneously above the right shoulder of immunodeficient SCID/beige male mice (Taconic), tumor size was measured regularly using digital calipers and by noninvasive optical imaging as follows. After administration of the *Renilla* luciferase substrate, coelenterazine (1mg/kg i.v.), anaesthetized mice (i.p. injection of a mixture of ketamine and xylazine) were imaged using a cooled IVIS CCD camera (Xenogen). Images were analyzed with IGOR-PRO Living Image Software (Xenogen). LAPC-9 cells (1 x 10^6) were co-implanted with non-tumorigenic NIH-3T3 fibroblasts (1 x 10^6) above the right shoulder of immunodeficient SCID/beige male mice (Taconic). Tumors were resected when they reached 1cm in diameter (4 weeks) and mice were sacrificed 12 weeks post implantation. For orthotopic implants, transduced LAPC-9 cells (2.5 x 10^5) suspended in Matrigel were implanted in the surgically exposed prostate region of SCID/beige male mice. Cells (in 10 uL/lobe) were implanted at the base of the exposed seminal vesicles in each dorsolateral lobe. Incisions were closed with vicryl sutures (Novartis) and tumor growth was monitored optically over the course of the next 3 weeks.
RESULTS

VEGF-C overexpression increases lymph node metastasis in a subcutaneous xenograft model of prostate cancer

Numerous clinical studies in prostate cancer have correlated VEGF-C with lymph node metastasis. To further investigate the link between lymphangiogenesis and lymph node metastasis, we asked whether we could alter the metastatic spread of tumor cells to draining lymph nodes by modulating the VEGF-C/VEGFR-3 axis. We chose the aggressive CWR22Rv-1 xenograft model of prostate cancer to test this hypothesis. CWR22Rv-1 prostate cancer cells stably expressing Renilla luciferase were lentivirally transduced to express either hVEGF-C, a soluble VEGF-C trap (sVEGFR-3), or a GFP empty vector control. Transduced CWR22Rv-1 cells were implanted subcutaneously into SCID/beige mice and tumor growth was monitored by caliper measurements for 3 weeks until tumors reached the ethical limit of ~1.5cm. We observed no significant difference in growth rate between control and VEGF-C expressing tumors while sVEGFR-3 tumors exhibited a significant delay in tumor establishment which was followed by similar growth kinetics (Figure 1A). Ex vivo bioluminescent imaging of organs revealed a dramatic increase in disseminated tumor cells in the draining lymph nodes by VEGF-C overexpression (Figure 1B). VEGF-C overexpression increased metastasis to brachial and axillary nodes by 1.8 fold and 2.8 fold respectively. Conversely, lymph node metastases were reduced to <20% in sVEGFR-3 groups relative to controls (Figure 1C). These increases in lymph node metastasis also correlated strongly to increases in systemic lung metastases (Data not shown). Immunohistological analysis of lymphatic vessel density in tumors revealed a strong correlation between lymphatic vessel density and extent of
metastases (Figure 1D-E). Furthermore, despite the ability of processed VEGF-C to bind VEGFR-2, we saw no significant changes in blood vessel density, as assessed by CD31 staining. Collectively these data suggest that the VEGF-C/VEGFR-3 axis is an important facilitator of lymph node metastases.

FIGURE 1

Figure 1. Overexpression of VEGF-C increases lymph node metastasis in the CWR22Rv-1 subcutaneous model of prostate cancer. A. CWR22Rv-1 tumor cells expressing Renilla luciferase and either VEGF-C, sVEGFR-3, or GFP (Ctrl) were implanted subcutaneously in SCID/beige mice (n=4 per group). Tumor growth was monitored by digital caliper measurements until they reached ethical size limit. B. Ex vivo bioluminescent signal from metastatic lymph nodes (Ax, axillary; Br, brachial), a representative is shown for each group. C. Quantification of optical signal, average per group. D. Marginal and intratumoral vascular density was analyzed by Lyve-1 (lymphatics, red) and CD31 (blood vessels, green) staining in tumor sections, representatives are shown. Tumor boundary is marked by the dotted white line in the tumor margin, tumor is to left of line. E. Quantification of vasculature density, average per group. Histology scale bar, 20 Am. Error Bars, +/- SE. *, P<0.05, **, P< 0.01
Inhibition of VEGF-C decreases lymph node metastasis in an orthotopic model of prostate cancer

Previous work with prostate cancer metastasis models indicates that the surrounding tumor microenvironment plays an important role in influencing the dissemination of tumor cells. Observations in multiple prostate cancer cell lines suggest that, in general, subcutaneous models are much less metastatic than orthotopic models. This discrepancy is in part attributable to differences in the stromal environment of the tumor. The prostate gland is rich in both blood and lymphatic vasculature and models that are weakly metastatic subcutaneously are strongly metastatic when implanted orthotopically and readily metastasize even without any added lymphangiogenic drive. We next evaluated whether inhibition of VEGF-C could suppress lymph node metastasis in an orthotopic model of prostate cancer.

LAPC-9 prostate cancer cells stably expressing Renilla luciferase were transduced with lentivirus expressing sVEGFR-3 or control GFP vector and implanted orthotopically into the prostates of SCID/beige mice. We observed, no significant difference in tumor growth rates, which was monitored by non-invasive bioluminescent imaging (Figure 2A). Mice were sacrificed 16 days post implantation and regional lymph nodes were bioluminescently imaged *ex vivo* to determine the extent of metastasis. We observed a 7-fold and 12-fold decrease in peri-aortic and mesenteric lymph node signals respectively, in sVEFGR-3 mice relative to controls (Figure 2B). Histological analysis of lymph nodes, confirmed these results (Data not shown). Subsequent analysis of lymphatic vessel density revealed dramatic morphological changes in lymphatic vessels in the sVEGFR-3 treated group. Specifically, marginal lymphatics between the tumor and adjacent normal tissue were severely disrupted by VEGF-C inhibition. (Figure 2C). Marginal lymphatics
in treated mice displayed much more discontinuity and lacked structural integrity.

Furthermore, we frequently observed invasion of tumor cells into the marginal lymphatic vessels of control tumors, such instances were rarely seen in treated mice. Once again we saw no significant differences in blood vessels density.
Figure 2. VEGF-C inhibition reduces metastasis to regional lymph nodes in the LAPC-9 orthotopic model of prostate cancer cell. A. LAPC-9 prostate cancer cells expressing Renilla luciferase and either sVEGFR-3 or GFP (Ctrl) were implanted orthotopically into SCID/beige mice (n=10 per group). Tumor growth (Day 10, 15) was monitored by bioluminescent imaging until mice were sacrificed (Day 16). B. Regional lymph nodes (periaortic, mesenteric) were optically imaged for Renilla luciferase activity, quantification of signal (average per group) is shown. C. Lymphatic density at the interface of the prostate, seminal vesicle, and tumor xenograft was analyzed by Lyve-1 (red) staining. Lymphatic vessels in treated mice exhibited lack of vascular integrity. Arrows indicate tumor cell invasion into lymphatics. No difference was seen in intratumoral blood vessel density as indicated by CD31(green) staining. Histology scale bar, 20 Am Bars, Bars, SE. *, P < 0.05.
Overexpression of VEGF-C locally in the stromal compartment increases lymph node and systemic metastases

Accumulating evidence has revealed that stromal components within the tumor are an important aspect of tumor biology.(34) It is reported that in some prostate tumors, stromal elements composed primarily of myofibroblasts as well as fibroblasts can account for over 50% of the intra-tumoral area.(35) We speculated whether expression of VEGF-C in the stromal compartment of the tumor, could also increase the malignant spread of tumor cells.

LAPC-9 prostate cancer cells expressing Renilla luciferase were co-implanted with NIH 3T3 fibroblasts expressing VEGF-C (or control) into the right shoulders of SCID/beige mice (n=6 per group). Tumor growth was monitored by caliper measurements as well as bioluminescent signal intensity (Figure 3A-B). After 3 weeks, when tumors reached ~1cm in diameter, tumors were surgically removed and mice were given an additional 6-8 weeks to allow micrometastases to grow out. Mice were then sacrificed and tissues were analyzed for the presence of any metastases. Bioluminescent imaging of organs revealed metastases both in the lymph nodes (4/6) and lungs (4/6) of VEGF-C expressing mice (Figure 3C). In contrast, no lymph node metastases were observed in control mice and only one mouse presented with lung metastases. Furthermore, VEGF-C expressing tumors displayed a dramatic increase in lymphatic vessel density (Figure 3D). Presence of lymph node as well lung metastases was further confirmed by cytokeratin immunohistochemistry (Figure 3E-F). In many cases, metastatic lymph nodes were completely taken over by tumor cells while lung lesions were commonly micrometastatic in size. These result suggest that local overexpression of
VEGF-C in the stromal compartment, is sufficient to drive both regional lymph node as well systemic metastases.

FIGURE 3

Figure 3. Overexpression of VEGF-C in the stromal compartment increases lymph node metastasis in the LAPC-9 subcutaneous model of prostate cancer. A-B. LAPC-9 prostate cancer cells expressing Renilla luciferase were co-implanted with 3T3 fibroblasts expressing either VEGF-C or GFP (Ctrl) were implanted subcutaneously into SCID/beige mice (n=6 per group). Tumor growth was monitored by bioluminescent imaging as well as digital caliper measurements until tumors reached ~1cm, at which time tumors were surgically removed. Metastases were given 8 weeks to grow out before animals were sacrificed C. Ex vivo optical signal revealed dramatic increase in metastases (lymph nodes, lung) of VEGF-C/3T3 mice. D. Intratumoral lymphatic vessel density was assessed by Lyve-1 immunohistochemistry. E-F. Presence of tumor cells in metastatic organs was verified by cytokeratin immunohistochemistry.
DISCUSSION

The importance of lymphangiogenesis in contributing to lymph node metastasis has been demonstrated in numerous clinical as well as experimental studies in many different carcinomas. The majority of these studies has centered around the VEGF-C/VEGFR-3 axis and have shown the importance of this axis in driving lymph node metastasis. Since the discovery of VEGF-C as the first lymphangiogenic specific growth factor, there have been tremendous advances in our understanding of the process of lymphangiogenesis, however, there is still much to be elucidated, especially in regards to the relationship between lymphangiogenesis and lymph node metastasis in prostate cancer. Here, we report that modulation of the VEGF-C/VEGFR-3 axis has a significant impact on the both lymphatic density in the primary tumor as well as the extent of lymph node and systemic metastases. Furthermore, local lymphangiogenesis driven by stromal cells within the tumor was also able to drive lymph node as well as systemic metastasis.

Results from multiple studies have shown that expression of VEGF-C correlates with lymph node metastasis in prostate cancer.(36-38) However, the importance of lymphatic vessel density in mediating lymph node metastasis is still under scrutiny. Early studies using VEGFR-3 as a marker of lymphatics documented strong positive correlation with nodal metastasis.(37) However, more recent studies using more specific markers of lymphatic endothelial (LYVE-1, D2-40 (Podoplanin) cells have shown conflicting results and correlation was only seen using more specific criteria such as peri-tumoral lymphatics as well as tumor cell invasion into lymphatic vessels.(9, 38-40)

Several lines of evidence indicate that peri-tumoral lymphatics maybe a more reliable prognosticator of lymph node metastasis than intra-tumoral lymphatics. First,
ferritin particles that were injected intra-tumorally were not efficiently drained into intra-tumoral lymphatics.\(^{(41)}\) Second, specific inhibition of intra-tumoral lymphatics had no effect on lymph node metastasis in a human xenograft model of prostate cancer.\(^{(42)}\) Third, the intra-tumoral lymphatics are often collapsed and non-functional due to high interstitial pressure within tumor.\(^{(41)}\) Despite these observations, the contribution of intra-tumoral lymphatics is still under debate as the both positive and negative correlations between intra-tumoral lymphatic vessel density and lymph node metastasis have been observed in different cancers.\(^{(43-45)}\)

In the subcutaneous CWR22Rv-1 model of prostate cancer the specific contributions of intra-tumoral versus peri-tumoral lymphatics was hard to discern as we saw decreases in both with VEGF-C inhibition. Intra-tumoral lymphatics were defined as those vessels that were confined within the margin of the tumor while peri-tumoral lymphatics were defined as those vessels surrounding the perimeter of the tumor that were not in direct contact with the tumor. Using these criteria, overexpression of VEGF-C resulted a dramatic increase in intra-tumoral lymphatics while overall peri-tumoral lymphatics were decreased. However, this conclusion is somewhat misleading since the increases in intra-tumoral density by VEGF-C overexpression was extremely high in the marginal zone within tumor periphery, almost as if peri-tumoral lymphatics were actually invading into the tumor. These observations suggest that more specific criteria maybe needed to decipher the separate contributions of intra- versus peri-tumoral lymphatics to lymph node metastasis.

We extended our studies into a more clinically relevant orthotopic model and also made similar observations. Inhibition of VEGF-C decreased both intra-tumoral as well as
peri-tumoral lymphatics; however, more profound was the affect of VEGF-C inhibition on peri-tumoral lymphatics. In control mice, the rich lymphatics within the normal prostate tissue was readily visible at the tumor periphery, and in many cases, we observed invasion of tumor cells into surrounding lymphatics. In stark contrast, lymphatics extending from the adjacent normal tissue into the margin of the tumor were severely disrupted by VEGF-C inhibition. These lymphatic vessels were much more disjointed and less elaborate than control tumors which displayed elongated and interconnected lymphatics. Furthermore, rarely did we observe lymphatic vessel invasion of tumor cells in the treatment group, possibly due to a loss of vascular integrity in these vessels. These results, in a more clinically relevant model, support the significance of peri-tumoral lymphatics, as well as tumor cell invasion into lymphatics, as important mediators of lymph node metastasis.

Another interesting finding from these studies is that in both the subcutaneous CWR22Rv-1 and LAPC9/3T3 models, we noticed a strong correlation between the presence of lung metastases to the presence of lymph node metastases. Cases of lung metastases were very rare in mice without lymph node involvement. These observations allude to a sequential nature of dissemination of metastatic tumor cells and implicate metastatic colonization of regional lymph nodes as an important intermediate step in systemic metastasis to lungs. Furthermore, in the LAPC9/3T3 subcutaneous model, the lung metastases were observed 8 weeks after surgical resection of the primary tumor suggesting a stronger contribution by metastatic lymph nodes, although we cannot rule out that these metastases were originally derived from the primary tumor and were in a
period of dormancy. We are currently exploring strategies to investigate the significance of lymph node metastases in contributing to systemic metastases.

Previous studies in our lab have revealed that expression levels of VEGF-C in multiple cell lines correlates to both their lymphangiogenic and metastatic potential \textit{in vivo}. In this study, we report that modulation of VEGF-C has significant impacts on both lymphatic vessel density as well as lymph node metastasis. Inhibition of VEGF-C resulted in decreases in lymphatic vessel density that was concomitant with decreases in both lymph node and systemic metastasis. Furthermore, we did not observe significant changes in either tumor growth or blood vasculature. These findings imply that reduction of lymph node and subsequent lung metastasis in these models is most attributable to a decrease in lymphatic vessel density. These results also demonstrate the potential of VEGF-C directed therapy to reduce both lymph node and systemic metastases. This is an important finding from a therapeutic standpoint, as it demonstrates that inhibition of VEGF-C has significant potential to inhibit the metastatic process. Therapy using VEGF-C inhibition in combination with existing therapeutics such as androgen ablation may help to better manage the early metastatic disease.

In further support of this combination approach, androgen withdrawal has been shown to increase VEGF-C expression in LNCaP prostate cancer cells.(46) In part, this increase in VEGF-C expression is mediated by reduction in levels of the prostate-specific homeobox protein NKX3.1, which represses VEGF-C transcription through co-recruitment of histone deacetylase 1(HDAC1).(47) Interestingly, NKX3.1 is frequently deleted in prostate cancer and is associated with more metastatic phenotype.(48, 49) Thus, VEGF-C may also play a significant role in the metastatic progression of hormone
refractory disease. Currently, several therapeutics targeting the VEGF-C/VEGFR-3 have been developed, these drugs include antibodies targeting VEGF-C and VEGFR-3 as well as small molecule inhibitors which target multiple VEGFR family members.(50)
III. CCR7 CONTRIBUTES TO THE LYMPH NODE METASTASIS OF PROSTATE CANCER
ABSTRACT

One of the earliest events in the metastasis of prostate cancer is dissemination to regional lymph nodes. In the clinical setting, lymph node involvement is routinely used for staging in the management of locally advanced prostate cancer. Though the importance of lymph node involvement in staging in prostate cancer is well accepted, the mechanisms involved in these metastatic processes have much yet to be elucidated. A growing body of literature in the past decade has implicated the involvement of chemokine receptors in systemic as well as lymph node metastasis. Here we report elevated expression levels of the chemokine receptor CCR7 in the lymph node metastases in a xenograft model of human prostate cancer. We observed similar trends in a subset of clinical patient samples upon immunohistochemical analysis of primary tumors and associated metastases. CCR7 is a chemokine receptor normally expressed by certain subsets of immune cells. The ligands for CCR7, CCL19 and CCL21, are expressed highly in lymph nodes, where they control the recruitment of CCR7 positive cells. We hypothesize that prostate cancer cells may also use this pathway to enhance metastatic colonization of regional lymph nodes. In support of this, we found that activation of CCR7 on prostate cancer cells enhances their directional motility and invasiveness towards a CCL21 gradient as well as their adhesiveness to the extracellular matrix membrane protein vitronectin.
INTRODUCTION

There is a need for greater understanding of the mechanisms that lead to prostate cancer metastasis, as it accounts for nearly all prostate cancer associated mortality. Metastasis is not entirely a random process as different cancers have defined patterns of organ specific metastasis. In prostate cancer, malignant cells preferentially metastasize to the bones, lungs, and lymph nodes. It is commonly believed that the chemokine receptor expression profile of malignant tumor cells may contribute to the propensity of different cancers to metastasize to different organs.(51) Chemokine receptors are a diverse group of seven transmembrane G-protein coupled receptors with over 20 family members.(52) Activation of these chemokine receptors, normally expressed on leukocytes, initiates a cascade of signals that culminates in changes in cell motility, cell surface adhesion, and cell survivability.(53) The ligands for these receptors come in two general classes: Homeostatic chemokines are expressed constitutively in lymphoid organs and direct leukocyte trafficking throughout organs of the immune system. Inflammatory chemokines are expressed transiently during times of infection or injury and help direct leukocytes to sites of inflammation.(54) The chemokine/chemokine receptor system is indispensable for the function of the immune system.

CCR7 is a chemokine receptor that is normally expressed by certain subsets of immune cells such as mature dendritic cells (DCs), B cells, naïve T cells, and some memory T cells.(55, 56) The ligands for CCR7, CCL19 and CCL21, are expressed primarily by stromal cells in secondary lymphoid organs such as lymph nodes and spleen, where they guide cells into specific areas within the organ.(55) The involvement of CCR7 in the homing and positioning of cells into para-cortical T-cell areas of the lymph
node to mediate many aspects of the immune response, such as T-cell activation as well as B-cell maturation has been well established.\(^{(55, 57)}\) In addition to its importance in regulating intra-nodal traffic, CCR7 also has important functions in mediating lymphocyte extravasation through lymphatic vessels. CCL21 secretion by lymphatic endothelial cells has also been shown to be important for extravasation of CCR7 positive cells through lymphatic vessel walls.\(^{(58)}\) Furthermore, presentation of CCL21 on the surface of lymphatic vessels through binding with lymphatic endothelial specific surface proteins such as podoplanin may facilitate this process.\(^{(55)}\)

Experimental studies in both breast cancer and melanoma models have implicated a role for CCR7 in mediating the lymph node metastasis of tumor cells.\(^{(27, 54)}\) Furthermore, retrospective studies analyzing clinical specimens have correlated CCR7 expression with lymph node metastasis in a wide variety of cancers including breast, cervical, melanoma, gastric, non-small cell lung, and hepatocellular carcinoma.\(^{(28)}\) Results of these studies show that expression of CCR7 by tumor cells enhances their motility, invasiveness, as well as survivability. Despite the significance of CCR7 in lymph node metastasis, its role in the lymph node metastasis of prostate cancer is unclear.

In the present study we provide evidence implicating a role for CCR7 in prostate cancer metastasis. We observed increased expression of CCR7 in the lymph node metastases in a human xenograft model of prostate cancer as well as increased staining for CCR7 in a subset of lymph node metastases in a tissues array of prostate cancer specimens. \textit{In vitro} data also suggests that ligand stimulation of CCR7 in prostate cancer cells increases their motility, adhesiveness, and viability under serum starved conditions.
We hypothesize that CCR7 is a mechanism that is utilized by prostate cancer cells to enhance metastasis of lymph nodes.
MATERIALS AND METHODS

Cell lines and Cell culture. The androgen-dependant human prostate cancer cell LAPC-9 was a kind gift from Dr. Charles Sawyers (Memorial Sloan Kettering Cancer Center). LAPC-9 xenografts were maintained by passage through male SCID/beige mice. NIH-3T3 fibroblast cell line was maintained in DMEM media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. CWR, PC3, and LNCaP-C42 cells were maintained in RPMI media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Prior to all migration, invasion adhesion, and proliferation assays, CWR, PC3, and LNCaP-C42 cells were serum starved for 24 hrs in serum free media.

Lentiviral transduction of cells. LAPC-9 prostate cancer cells were transduced (3 MOI) with lentivirus carrying a (CMV) promoter-driven Renilla luciferase (RL) reporter gene. NIH 3T3 fibroblasts were transduced (1 MOI) with CMV-VEGF-C-IRES-EGFP (or control CMV-IRES-EGFP). CWR, PC3, and LNCaP-C42 cells were transduced at (1 MOI) with either CCR7-GFP or GFP-ctrl viral vectors. Cells were infected using viral supernatant for a 6 hour incubation in the presence of polybrene.

Animal Work and Optical Imaging. All animal care and procedure were done in accordance with the University of California animal Research Committee guidelines. LAPC-9 cells (1 x 106) transduced with Renilla luciferase were co-implanted with non-tumorigenic NIH-3T3 fibroblasts (1 x 106) expressing VEGF-C (or control) subcutaneously above the right shoulder of immunodeficient SCID/beige male mice (Charles Rivers) Tumor size was measured by noninvasive imaging as follows. After administration of the Renilla luciferase substrate, coelenterazine (1mg/kg i.v.),
anaesthetized mice (i.p. injection of a mixture of ketamine and xylazine) were imaged using a cooled IVIS CCD camera (Xenogen). Images were analyzed with IGOR-PRO Living Image Software (Xenogen). Tumors were resected when they reached 1cm in diameter (4 weeks) and mice were sacrificed 12 weeks post implantation. Portions of metastatic organs were made into single cell suspension and re-implanted subcutaneously into SCID/beige mice. Mice were sacrificed and metastases harvested once tumors reached 1cm in diameter.

**Immunohistochemistry.** Tumors, lymph nodes and lungs were harvested and fixed in 3% paraformaldehyde overnight at 4°C. Sections (5um) were stained with human anti-Cytokeratin (Biogenex) and developed using DAB substrate (Biogenex). Immunohistochemical staining of CCR7 (R&D Systems) was performed by the UCLA pathology core.

**Real-time reverse transcription-PCR analysis.** Total tissue or cellular RNA was extracted using TRIzol reagent (Invitrogen). RNA was isolated according to the TRIzol procedure. RNA was quantified and assessed for purity by UV spectrophotometry and gel electrophoresis. RNA (1ug) was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). Real time PCR was done using 1ul cDNA (~40ng), Quantace 2x master mix (Bioline) and 10ul/L each of the following primers:

**CXCR4:**
F: GCAAGGCGATCCATGTCATCTACA  
R: CAGACGCAACATAGACCACCTTT

**CCR7:**
F: TTACCTTGTCATCATCCGCACCCCT  
R: TATGAAGACCACGACCACAGCGAT
Reactions were performed using a MyiQ iCycler RT-PCR machine (Bio-Rad) under the following conditions: 40 repeats of 95°C of 15s, 60°C for 30s, and 72°C for 30 s. Data was analyzed using Bio-Rad iQ5 software.

**Migration and Invasion Assays.** Cell migration assays were performed in serum-free RPMI with 8.0-µm pore inserts on a 24-well Transwell (Corning, Falscon, USA.) Cells were serum starved for 24hrs prior to plating. Cells were detached with citric saline and 1 x 10^5 cells were seeded in the upper chamber. After incubation at 37°C for 6 hrs, non-migratory cells on the upper membrane were removed with a cotton swab; cells that migrated on the lower surface of the membrane were fixed with 3% PFA in PBS and then stained with DAPI (Invitrogen). Images of migrated cells were taken using a Nikon Eclipse fluorescent microscope (Nikon Eclipse) and then quantified by ImageJ software. Invasion assays were performed using BD biocoat invasion inserts. Cells were serum starved for 24 hrs and 5 x 10^4 cells were plated in the upper will and allowed to migrate for 24hrs. Invaded cells were fixed, imaged, and quantified as mentioned above. In both assays, cells were stimulated with either 200ng/ml CCL21 (R&D Systems) (or 0.1% BSA control) or CCL21 expressing (or control Firefly luciferase) adenovirus conditioned media. To generate conditioned media, A549 cells were plated to 70-80 % confluency before infection with adenovirus (3 PFU). Cells were infected overnight and then switched to serum free media, which was collected in 48 hrs. For antibody neutralization experiments, cells 1µg/ml of anti-human CCL21 (R&D Systems) was added to the lower chamber of the transwell.

**Vitronectin Binding Assays.** 96 well microtiter plates were coated overnight at 4°C with 1µg/ml vitronectin. (R&D Systems) Plates were washed twice with PBS and incubated
with 1% BSA in PBS for 1 hr at 37°C. Cells were serum starved for 24 hrs prior to the assay. Cells were detached with citric saline and 1x 10^5 cells per well were plated in the presence or absence of CCL21 (200ng/ml). Cells were allowed to attach for 1-2 hrs at 37°C. Non-adherent cells were removed by washing 3 times with PBS. Serum free media was replaced and remaining adherent cells were quantified using CCK-8 cell viability assay (Dojindo).

**Proliferation and Apoptosis Assays.** Cells were serum starved for 24 hrs and plated in 0.1% serum RPMI media. 1 x 10^4 cells per well were plated in 96-well microtiter plate and stimulated with either 0.1% BSA (ctrl), CCL21 (200ng/ml) or EGF (50ng/ml). Individual plates were plated for assessment of proliferation on different days. Cell proliferation was measured using CCK-8 cell viability assay. For apoptosis assays, 2 x 10^4 cells were plated in 12-well plates and simulated with either 0.1% BSA or CCL21 (200ng). After plating and addition of stimulus, plates were not manipulated and the entire contents of the well, adherent and non-adherent cells, were used in analysis. Cells were stained with Annexin V and Propidium Iodide (PI) (Propidium Iodide) (BD cell death assay kit) and then analyzed by flow cytometry.
RESULTS

CCR7 is overexpressed in the LAPC9 metastatic model of prostate cancer

Previously we described a prostate cancer xenograft model using overexpression of VEGF-C in fibroblasts to drive metastases. Using this robust model, we questioned whether there would be differences in gene signatures between the metastases and the primary tumor. Using the LAPC-9/3T3 VEGF-C model, we generated both lymph node and lung metastases. Because LAPC-9 is a xenograft model and not amenable to in vitro studies, we enriched for metastatic cells by making single cell suspensions of portions of metastatic organs and re-implanting them subcutaneously into SCID/beige mice. Due to the scarcity of metastatic tumor cells in distant organs, the overall take rate of metastatic re-growths was very poor. We were successfully able to re-grow metastases from 2 of the mice. When these metastatic re-growths were compared genetically to the primary tumor we found increase expression of both chemokine receptors CXCR4 as well as CCR7 in the lymph node metastases (Figure 1).
**FIGURE 1**

![Graphs](image)

**Figure 1. Increased expression of CCR7 in the lymph node metastases of the LAPC-9 subcutaneous prostate cancer model.** A-B. Portions of tissues from metastatic organs were re-implanted subcutaneously into SCID/beige mice and outgrowths of metastatic clones were analyzed by RT-PCR for expression of CXCR4 and CCR7 using human specific primers. Expression of genes was normalized to cytokeratin 8. *p<0.001, **p<0.05.

**CCR7 is expressed in clinical human prostate cancer specimens**

There are few reports documenting the expression of CCR7 in prostate cancer. (59, 60) To further substantiate our findings that CCR7 was expressed in prostate cancer, we extended our investigation into tissue arrays from clinical prostate cancer specimens. We examined a tissue array of 30 primary tumors with matched lymph node metastases for expression of CCR7. We observed CCR7 staining either in the primary tumor or lymph node metastasis in greater than 50% of samples. In 30% of the cases, we observed an increasing trend in CCR7 staining in the lymph node versus the primary tumor (Figure 2A) While in some cases we observed the reverse (Figure 2B). Collectively, these results validate previous reports of CCR7 expression in prostate cancer, and combined with our findings from the LAPC9 model, support the notion that CCR7 is involved in lymph node metastasis.
Figure 2. CCR7 is expressed in clinical samples of human prostate cancer and lymph node metastases. Immunohistochemical analysis of a prostate cancer tissue array consisting of primary tumor tissues and matched lymph node metastases revealed CCR7 staining in many samples. A. Increased CCR7 staining in lymph node metastases, relative to primary tumor, in a subset of patients (10/30). B Decreased CCR7 staining in lymph node metastases in a subset of patients (10/30).

Examining the effect of CCR7 overexpression on metastatic behavior

Although the significance of CCR7 in contributing to lymph node metastasis has been studied in experimental models such as breast and melanoma.(28) The role of CCR7 in contributing this process as not been explored in prostate cancer. We screened several commonly used prostate cancer lines (CWR, DU145, PC3, LNCaP-C42) for expression of CCR7 to use in our future studies. However, CCR7 expression was found to be very low (Data not shown). We cloned out the hCCR7 and inserted it into lentiviral expression vector for use in our studies. We constructed two versions of the CCR7 overexpression vector: a CCR7-fus-RFP fusion vector for fluorescence microscopy as well as a CCR7-IRES-GFP dual expression vector for use in all functional studies. Control vectors (RFP,
GFP) were also created. Stable CCR7 expressing cell lines (CWR22RV-1, PC3, LNCaP C42) were created by infection at 1 MOI. Overexpression was verified by RT-PCR as well as fluorescence microscopy, which confirmed expression of the receptors on the surface of the cell(Figure 3A-B).

FIGURE 3

Figure 3. Lentiviral CCR7 construct is efficiently expressed and targeted to the membrane.
A. PC3 and LNCaP-C42 prostate cancer cells expressing either CCR7-fus-RFP or RFP (Ctrl) were analyzed by fluorescence microscopy for cellular localization of proteins. CCR7-f-RFP was localized to the surface while RFP (Ctrl) cells displayed more cytoplasmic distribution of protein. B. Quantitative RT-PCR of CCR7-IRES-GFP and GFP (Ctrl) revealed robust induction CCR7 expression in transduced cells.

**CCR7 enhances prostate cancer cell migration and invasion**

Enhanced migratory capacity is a hallmark feature of chemokine receptor activation. We evaluated whether CCR7 could mediate chemotaxis of prostate cancer cells toward a chemokine gradient. Adenoviral vectors expressing hCCL21 were used to infect A549 cells to generate CCL21 conditioned media. CWR-CCR7 cells were serum starved for 24 hours and plated in the upper well of a transwell migration chamber. The
CCL21 conditioned media (or control conditioned media) was placed in the lower well and cells were allowed to migrate for 6 hours. Migration of CWR-CCR7 expressing cells was enhanced almost 2-fold toward CCL21 conditioned media (Figure 4).

FIGURE 4

A.

**Figure 4. Activation of CCR7 on primary tumor derived CWR22Rv-1 prostate cancer cells enhances chemotaxis toward a CCL21 concentration gradient.** CWR cells (1x10^5) expressing CCR7 (CWR-CCR7) were plated into the upper well of a transwell (24-well plate). Adenoviral hCCL21 conditioned media (or control) was used to in the bottom chamber as stimulus. Cells were allowed to migrate for 6 hours at which time migrated cells were fixed, stained and quantified by fluorescence microscopy. hCCL21 conditioned media greatly enhanced the migration of CWR-CCR7 cells.

To insure that enhanced migration toward CCL21 was not due to other factors produced by A549 cells in the conditioned media, the assay was repeated using CCL21 neutralizing antibodies. Neutralizing antibodies reduced migration to levels to control
conditioned media. In addition, CWR-GFP control cells were unable to migrate towards CCL21 conditioned media (Figure 5).
Figure 5. CCR7 mediated chemotaxis is inhibited by a CCL21 neutralizing antibody. The migration of CWR-CCR7 cells is inhibited by the addition of an anti-CCL21 neutralizing antibody. Neutralizing antibody was added to both upper and lower chambers. CWR-GFP control transduced cells were unable to migrate towards a CCL21 concentration gradient.
To further substantiate these findings, we used metastases derived PC3 and LNCaP-C42 prostate cancer cells stably expressing CCR7. Once again, we found activation of CCR7 increased tumor cell chemotaxis toward a CCL21 concentration gradient (Figure 6A,D). We repeated the experiment several times and consistently saw 60-80% increases in migration in both cell lines, a representative experiment is shown. In addition, CCL21 was also able to enhance the invasion of PC3 cells through an extracellular matrix membrane (Figure 6C). Collectively, these results suggest that CCR7 can enhance the mobility and invasiveness of prostate cancer cells in response to CCL21.
Figure 6. Activation of CCR7 increases chemotaxis, invasion, and adhesion of metastasis derived cell lines PC3 and LNCaP-C42. A, C. PC3 and LNCaP-C42 tumor cells expression CCR7-IREs-GFP were plated into the upper well of a transwell (24-well plate). Recombinant CCL21 (200ng/ml) or control (0.1% BSA in PBS) was placed in the bottom chamber as stimulus. Cells were allowed to migrate for 6 hours at which time migrated cells were fixed, stained and quantified by fluorescence microscopy. CCL21 increased the migration of cells toward CCL21 gradient. B, D. CCL21 stimulation increases the binding of cells to vitronectin. Cells (1×10^5) were plated in 96 well plates and stimulated with CCL21 (200ng/ml) for 1-2 hrs. Unattached cells were washed off and media was replaced with serum free media. Number of remaining adhered cells was quantified by CCK-8 assay. E. CCL21 stimulation enhances tumor cell invasion through a basement membrane protein layer. PC3-CCR7 cells (5×10^4) were placed in the upper well of a transwell invasion chamber. CCL21 (200ng/ml) was placed in the bottom well and cells were allowed to migrate for 24 hrs. Invaded cells were fixed, stained and quantified by fluorescence microscopy.

**CCR7 enhances prostate cancer cell binding to extracellular matrix proteins**

In leukocytes, stimulation of chemokine receptors initiates a cascade of events that not only results in increased chemotaxis, but also in increased adhesion to endothelium. In T-cells, ligand stimulation of CCR7 increases their avidity to endothelium primarily through activation of lymphocyte function associated antigen-1 (LFA-1, αLβ2 integrin). Similarly, activation of chemokine receptors in prostate
cancer cells have shown to enhances the adhesive of properties of prostate cancer cells, and this increase is has been shown to be mediated in part by αvβ3 integrins which bind to vitronectin.(61) We speculated whether CCR7 could also activate downstream pathways that lead to enhanced binding to vitronectin. Binding of prostate cancer cells to vitronectin coated plates was assayed in response to CCL21. Binding of PC3 and LNCaP-C42 cells to vitronectin was increased 10-20% by CCL21 stimulation (Figure 6 B,E). These results indicate that CCR7 activation can increase cancer cell adhesion to vitronectin. Furthermore, this data also support the notion that different chemokine receptors, in some instances, can share common downstream effector pathways.

**CCR7 activation has no significant impact on proliferation or apoptosis during serum starved conditions**

Recent literature indicates that chemokine receptors may have more function than the mechanical processes traditionally ascribed to chemotaxis (migration, invasion and adhesion). They have also been demonstrated to increase proliferation of cells, promote survival from anoikis, and contribute to chemotherapeutic resistance.(62-64) To determine whether stimulation of cells with CCL21 could serve as a growth factor to enhance proliferation of cancer cells. PC3 and C42 cells were serum starved for 24 hours then plated in serum starved conditions. Recombinant CCL21 (200ng/ml) (or control) was added to the cultures. Proliferation was evaluated over the course of one week. We observed only a minimal enhancement of proliferation in both PC3 and C42 cells over the course of one week (Figure 7A,B)
Figure 7. Activation of CCR7 has no significant effect on the cell proliferation in serum free conditions. A-B. Stimulation of CCR7 does not significantly increase the cell viability of either PC3 or LNCaP-C42 cells under serum starved conditions. Cells ($5 \times 10^5$) were plated in 96-well plate and stimulated with human CCL21 (200ng/ml), murine CCL21 (200ng/ml), or control (0.1% BSA in PBS); cell proliferation was quantified by CCK-8 assay. *p<0.001, **p<0.005.

We next evaluated whether CCL21 stimulation offered any apoptotic protection under serum-starved conditions. Cells were serum starved for 24hrs and plated in serum starved
conditions. CCL21 (or ctrl) was added to cultures and plates were not disturbed until the day of collection. On the day of analysis, entire contents of the well, both suspending as well as adherent cells were analyzed. Cells were analyzed by FACS for Annexin V and Propidium Iodide staining during the course of one week (Figure 8 A,B). We observed no significant differences in apoptosis of cells stimulated by CCL21 relative to controls. These results suggest that CCR7 activation does not significantly enhance cell viability or prevent apoptosis in serum starved conditions.
Figure 8. Activation of CCR7 does not protect against apoptosis in serum free conditions. 

A-B. Stimulation of CCR7 does not significantly decrease the apoptosis of either PC3 or LNCaP-C42 cells under serum starved conditions. Cells were plated in 12-well plates and simulated with either 0.1% BSA or CCL21 (200ng). After plating and addition of stimulus, plates were not manipulated and the entire contents of the well, adherent and non-adherent cells, were used in analysis. Cells were stained with Annexin V and Propidium Iodide (PI) (Propidium Iodide) (BD cell death assay kit) and then analyzed by flow cytometry.

Activation of growth factor pathways in conjunction with chemokine receptor pathways have been shown to increase the metastatic behavior of cancer cells. (65)
Furthermore, in breast cancer cells expressing CXCR4, a basal level of EGF was required for chemotaxis toward a CXCL12 gradient. (66) Many mitogenic growth factors such as EGF have been shown to increase the proliferation of prostate cancer cells. (67) We tested whether CCL21 stimulation in combination with EGF would further increase the viability of these cells in serum starved conditions. CWR cells were serum starved for 24 hrs and plated them in serum starved conditions for 3 days. Cells were treated with CCL21, EGF, or a combination of both. Similar to previous results, CCL21 had little affect on cell viability alone, however stimulation with CCL21 in combination with EGF had a significant additive affect on cell viability (Figure 9). These data suggest that although CCR7 activation has no significant effect on cell viability alone, it can synergize with and enhance the effects of other growth factors.
Figure 9. Activation of CCR7 in combination with EGFR has a synergistic effect on the proliferation of CWR cells. Cells (1x10^4) were plated in 96-well plate and stimulated with CCL21 (300ng/ml) with and without EGF (100ng), cell proliferation was quantified by CCK-8 assay. *p<0.01, **p<0.001, ***p<0.05.
DISCUSSION

There is growing belief that metastatic lymph nodes can contribute to systemic metastases and that chemokine receptors are important mediators of this process. To further investigate this metastatic process, we employed an un-biased lymph node metastasis model of prostate cancer, in which heightened lymphangiogenic growth signal was provided by stromal fibroblasts. We found increased expression of both CXCR4 and CCR7 in lymph node metastases. The expression of CXCR4 in prostate cancer is well established and numerous studies have reported its ability to mediate many aspects of metastatic behavior such as migration towards a chemokine gradient, invasion through a basement membrane protein layer, and adhesion to bone marrow endothelium. Though the importance of CXCR4 in prostate cancer metastasis is well accepted, the significance of CCR7 in this capacity as well as its relevance in prostate cancer biology has not yet been fully investigated.

A few isolated reports have implicated the functional contribution of CCR7 to prostate cancer lymph node metastasis. In an interesting case report, a patient presenting with generalized lymphadenopathy was found to have disseminated prostate cancer lymph node metastases, which expressed an increased level of CCR7 in the tumor epithelium. In addition, two comprehensive gene expression studies also support the involvement of CCR7 in prostate cancer progression. For instance, Wallace et al. found that CCR7 was one of several genes that were elevated greater than 1.5 fold in the prostate tumors of African American men, who traditionally have higher incidence and mortality rates of prostate cancer relative to European men, implicating a role for CCR7 in contributing to a more aggressive phenotype. A study by Taylor et al. further
corroborated these results in an extensive collection of primary tumors and metastases and found that 8% (10/131) of primary tumors as well as 21% (4/19) of metastases exhibited elevated levels of CCR7, implicating a role for CCR7 in lymph node metastasis.

To gain further impetus on the pro-metastatic role of CCR7 in clinical scenario, we performed CCR7 immunohistochemical staining on a collection of tissue microarrays from prostatectomy and pelvic lymphadenectomy specimens from men with node-positive prostate cancer.\(^{(69)}\) We observed that over 50% of the patients displayed intense CCR7 staining in either the primary tumor or lymph node metastasis. Furthermore, 30% of lymph node metastases exhibited elevated expression of CCR7 relative to the primary tumor (10/30). These data again lends support to the clinical relevance of CCR7 in the lymph node metastasis of prostate cancer.

Due to the negligible expression of CCR7 in all the common prostate cancer cell lines, we decide to create a CCR7 expression lentiviral vector to investigate the impact of CCR7 activation on metastatic behavior in prostate cancer models. The ability to sense and migrate towards chemokine gradients is an essential feature for all leukocytes and is critical for leukocyte trafficking to different organs. We first examined the ability of CCR7 stably expressing cells to migrate towards a CCL21 gradient. By using CCL21 that was delivered by a variety of methods, including recombinant protein, adenoviral expression vector, and vault nano-particle encapsulated (Data not shown), we showed that CCR7 expressing prostate cancer cells exhibited an increased ability to migrate towards CCL21. In addition, increase in chemotaxis toward CCL21 was observed in multiple CCR7 expressing prostate cancer cell lines originated from lymph node metastasis and bone metastasis. These results provide strong evidence that expression of
CCR7 on prostate cancer cells enhance their directional motility towards CCL21 gradients.

Another critical step in the metastatic process is the ability of circulating tumor cells to be able to arrest at a secondary site and then invade out of the vasculature and into the surrounding tissue. We assessed the ability of CCR7 activation to mediate these processes. We found that stimulation by CC21 enhance the ability of tumor cells to bind to vitronectin, an extracellular matrix protein that is abundantly expressed in many tissues. Furthermore, stimulation of CCL21 also significantly increased the ability of tumor cells to invade through a basement membrane protein layer.

Next, we evaluated the affects of CCL21 stimulation on cell proliferation and survivability. We found that CCL21 stimulation had no effect on the apoptosis of cells in serum starved conditions; furthermore, we observed only minimal increases in cell proliferation under these same conditions. However, stimulation in combination with growth factors synergistically increased the proliferation of cells under serum free conditions. We conclude that CCL21 stimulation in cell culture settings has no significant effect on cell proliferation or survivability, but it can potentially enhance the ability of other growth factors to induce proliferation.

Taken together, our data suggest that expression of CCR7 in prostate cancer cells confers increased ability to the tumor cells to migrate towards a chemotactic gradient of its cognate ligand, CCL21, and also enhance the ability of tumor cells to exit circulation by increasing their matrix adhesive properties. We hypothesize that CCR7 expression gives malignant tumor cells an advantage in colonizing draining lymph nodes. Thus,
therapies targeting the CCL21/CCR7 axis may be a beneficial strategy to prevent metastatic colonization in lymph nodes and dissemination to distant organs.
IV. CONSTRUCTION AND VALIDATION OF A LENTIVIRAL CRE/LOX SYSTEM TO VALIDATE THE SEQUENTIAL STEPS OF METASTASIS
ABSTRACT

The extent of lymph node involvement can predict the survival of many prostate cancer patients. Despite this strong correlation, the contribution of positive lymph nodes to distant organ metastasis still remains very controversial. Mixed findings from clinical studies concerning the survival benefits of surgical intervention (PLND) to remove occult lymph nodes have contributed to the debate. Recent findings from our lab are in support of the theory that metastatic lymph nodes are mediators of the systemic disease. However, similar to previous studies, these findings are based strongly on correlative observations. A system to track the dissemination of cells from the lymph node would greatly help our understanding about the progression of systemic disease. In pursuit of this, we have developed a strategy using a lentiviral vector based tracking construct that is activated by adenoviral mediated delivery of CRE recombinase. We have validated the functionality of the lentiviral construct in vitro and demonstrated its feasibility in vivo. Use of this tracking strategy will enable us to determine the involvement of metastatic lymph nodes in systemic dissemination.
INTRODUCTION

In the clinical setting, lymph node involvement is routinely used for staging in the management of locally advanced prostate cancer to determine the course of therapy. However, despite the importance of lymph node involvement and its correlation to distant organ metastasis, there is still much debate surrounding the contribution of metastatic lymph nodes to distant organ metastases. One theory suggests that distant organ metastasis is driven mainly by hematogenous spread and that the extent of lymph node metastasis is merely an indicator of the extent of already present systemic metastases. The second theory suggests that lymphatic spread via lymph nodes mediates systemic spread. Several lines of evidence from our studies support the latter theory:

First, the magnitude of systemic metastases strongly correlated with magnitude of lymph node metastases in many experimental studies. Second, inhibition of VEGF-C had little impact on blood vasculature, suggesting that modulation of metastases was due changes in lymphogenous spread. Third, we observed lung metastases in mice 8 weeks after surgical removal of the primary tumor in the subcutaneous LAPC9/3T3 model, suggesting that tumor cells likely originated from the metastatic lymph nodes; although a long dormancy period of metastatic tumor cells from the primary tumor, is possible.

It would be useful to have a tool to be able to track the passage of tumor cells as they pass to through the lymph node. Her we present a strategy to validate the sequential steps of metastatic dissemination through regional lymph nodes. Previously, our lab has demonstrated the ability to use adenoviral vectors to target lymph node metastases in a subcutaneous xenograft model of prostate cancer. This strategy will be used to deliver a molecular tag to malignant cells in the lymph node that will allow future
identification of cells. However, the stability of the tag throughout the metastatic process precludes use of adenoviral vectors that can only provide transient expression. To circumvent this, we have devised a strategy based upon the ability of CRE recombinase to mediate permanent site-specific genetic changes by recombination at homologous loxp sites. Adenoviral vectors expressing CRE will be used to alter the gene expression of tumor cells that harbor a molecular switch that changes the fluorescent properties of the cell. Under normal conditions, cells express only RFP, however, in the presence of CRE, RFP is floxed out and only GFP is expressed. Here we report the construction and preliminary validation of this tracking tool. Using this approach, we can track the fate of metastatic cells as they spread from the lymph node.
MATERIALS AND METHODS

Cell lines and Cell culture. CWR22Rv-1, Du145 cells were maintained in RPMI media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

Lentiviral transduction of cells. CWR22Rv-1, Du145 cells were transduced (3 MOI) with lentivirus carrying a CMV-lox-RFP-lox-GFP (RFPloxpGFP) construct. Cells were infected using viral supernatant for a 6 hour incubation in the presence of polybrene.

PCR. DNA was extracted using DNeasy Kit (Qiagen) according to manufacturer's protocol. PCR was performed using Platinum Taq (Invitrogen) under the following cycling conditions: 35 repeats of 94°C of 30s, 55°C for 30s, and 72°C for 60s.

Real-time reverse transcription-PCR analysis. Cellular RNA was extracted using TRIzol reagent (Invitrogen). RNA was isolated according to the TRIzol procedure. RNA was quantified and assessed for purity by UV spectrophotometry and gel electrophoresis. RNA (1ug) was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). Real time PCR was done using 1ul cDNA (~40ng), Quantace 2x master mix (Bioline) Reactions were performed using a MyiQ iCycler RT-PCR machine (Bio-Rad) under the following conditions: 40 repeats of 95°C of 15s, 60°C for 30s, and 72°C for 30 s. Data was analyzed using Bio-Rad iQ5 software.

Animal Work and Optical Imaging. All animal care and procedure were done in accordance with the University of California animal Research Committee guidelines. Du145 cells (1 x 10^6) transduced with RFPloxpGFP were injected into the hock’s space and carpus of Nude mice. Injection of tumor cells was followed by paw injection of
adenoviral CRE. For optical imaging, anaesthetized mice (i.p. injection of a mixture of ketamine and xylazine) were imaged using the MAESTRO fluorescence imager

**Immunohistochemistry.** Lymph nodes were harvested and fixed in 3% paraformaldehyde overnight at 4°C. Sections (5µm) were stained with human anti-Cytokeratin (Biogenex) and developed using DAB substrate (Biogenex). For lymphatic and GFP stainings, sections were stained with anti-LYVE-1 (RELIATECH) or anti-GFP (BD Biosciences) antibodies.
RESULTS

Construction of the RFPloxpGFP tracking vector

We constructed the tracking vector by modification of an existing plasmid, pTomo-HA-AKT, which was a kind gift from Dr. Hong Wu. The modification of the plasmid entailed removal of portions of the plasmid encoding the AKT gene as well as the IRES sequences in between the floxed RFP and the downstream eGFP. The plasmid was restriction digested with BamHI, Nhel and then blunt end ligated back together. The resulting lentiviral expression vector consists of a CMV promoter driving a floxed RFP followed by GFP (CMV-loxp-RFP-loxp-GFP). Under normal conditions, the CMV drives the expression of the complete construct. However translation stops and the end of the RFP sequence. Thus cells are naturally RFP positive when not activated. In the presence of CRE, the RFP with the stop codon is permanently looped out and only GFP is expressed (Figure 1).
**Figure 1. Genetic validation of RFPloxP-GFP construct**

**A.** Floxed RFP-GFP construct was created by blunt ended ligation of pHA-TomoAKT after restriction digestion with BamHI and NheI.

**B.** CWR22Rv-1 cells expressing CMV-loxP-RFP-loxP-GFP were transfected with adenovirus at MOI 1.5 and genomic DNA was purified and recombination efficiency was analyzed by PCR. Primers flanking the loxp sites showed complete removal after 48hrs.

**C.** CWR22Rv-1 cells expressing CMV-loxP-RFP-loxP-GFP were transfected with adenovirus at MOI 1.5 and genomic RNA was purified and recombination efficiency quantified by RT-PCR. RFP gene expression was completely abolished by recombination while, GFP expression increased significantly.

**In vitro validation of the RFPloxpGFP vector**

We next evaluated the efficiency of our system *in vitro*. CWR prostate cancer cells stably transduced with the RFPloxpGFP construct were infected with adenoviral CRE. Genetic analysis of both genomic DNA as well as mRNA revealed no trace of RFP 48hrs post adenoviral infection (Figure 1B,C) In addition, GFP mRNA expression was
enhanced up to 7-fold by adenoviral infection. Flow cytometry was used to confirm RFP and GFP expression at the protein level. Interestingly, although we found little trace of RFP at either the DNA or RNA level after 48hrs, RFP protein levels were quite high as determined by flow cytometry. Although GFP protein expression had turned on, RFP was still not turned off presumably due to stability of protein. RFP signal diminished when cells were analyzed 5 days later but was not completely gone (Figure 2A). We repeated the study in Du145 prostate cancer cell lines and found the residual half-life of RFP to be much shorter and virtually all cells were GFP single positive after one week (Figure 2B.)
Figure 2. Flow cytometric validation of vector. A. Flow cytometry of CWR22Rv-1 expressing CMV-loxP-RFP-loxP-GFP revealed abundant presence of RFP protein 48 hrs after adenoviral infection as well as GFP induction. Cells were passaged and analyzed again in 7 days. Results showed, decreased, but still not the disappearance of residual RFP protein. B. Repeat studies in Du145 cells much better kinetics of RFP degradation and most RFP protein was gone after 7 days.

In vivo validation of the tracking vector

Having validated the construct in vitro, we moved to validate our construct in vivo.

We chose to continue our studies using the Du145 model, which displayed better switching kinetics. We next tested whether we could mediate this switch in vivo. Nude
mice were injected in both left and right carpus and hock’s space, followed 30min later by paw injection of adenoviral CRE. MAESTRO imaging verified positive injection into mice and animals were sacrificed 2 weeks later (Figure 3A). Ex vivo florescence imaging using MAESTRO imaging confirmed presence of both RFP as well GFP cells in the drainage lymph nodes off all mice (Figure 3B,C) suggesting that adenoviral CRE was able to mediate the switch in vivo of at least a percentage of cells. To further substantiate these findings, we used immunohistochemistry to verify the presence of both cytokeratin positive and GFP positive prostate cancer cells in the lymph node (Figure 3D.) These results suggest that adenoviral delivered CRE can mediate the activation of the RFPloxpGFP construct in vivo.
Figure 3. Adenoviral delivery of CRE can mediate recombination in vivo. A. Du145-CMV-loxp-RFP-loxp-GFP cells (1x10^6) were injected into the carpus and hock’s space in nude mice followed 30 min later by paw injection of Adenoviral CRE. Mice were imaged directly after tumor cell injection by MAESTRO fluorescent imager to verify injection of cell. B. Mice were sacrificed 2 weeks post injections and lymph nodes were harvested and ex vivo imaged fluorescently for presence and color of tumor cells. GFP positive cells indicate positive recombination. C. Quantification of GFP and RFP fluorescent signal intensity using MAESTRO imaging software. D. Immunohistochemical analysis of cytokeratin and GFP confirmed the presence of GFP positive tumor cells in the lymph nodes.
DISCUSSION

Lymph node involvement is a strong predictor of the extent of systemic metastasis, despite this observation; there is much debate as to the contribution of lymph nodes as a reservoir to systemic spread. To address this controversy, we have developed a lineage tracing strategy to track the spread of malignant cells from metastatic lymph nodes. Specifically, we designed a system to genetically mark tumor cells with red fluorescent protein (RFP) and convert tumor cells that transit through draining lymph nodes by expressing green fluorescent protein (GFP). We have constructed a floxed RFP and GFP lentivirus tracking vector, designated as RFPloxpGFP, which can be converted from RFP to GFP expression by CRE recombinase. The CRE recombinase will be delivered into draining lymph node by an adenoviral vector as published studies from our group have demonstrated the lymphotropic properties of adenoviral vector.(70) Feasibility of this track system was assessed by first marking several prostate tumor cell lines with RFPloxpGFP. The transduction of marked tumor cells with CRE expressing adenoviral vector (AdCRE) resulted in very efficient elimination of RFP at both the genetic as well as protein level. The AdCRE transduced tumor cells were also efficiently converted to expressing GFP. Flow cytometric as well as PCR based analysis revealed that GFP expression was efficiently activated 48 hours after adenoviral infection. Although no evidence of RFP expression could be detected by PCR based detection methods, flow cytometry detected residual RFP expression as long as one week after inactivation by CRE.

Having validated the functionality of the construct in vitro, we next evaluated the functionality of the construct in vivo. Previously in our lab we demonstrated that paw of
Injection of both tumor cells and adenoviral particles follows predictable drainage patterns through specific lymph nodes. Using this information, tumor cells stably marked with the tracking construct were injected into the carpus and hock’s space of mice followed 30 minutes later by paw injection of adenoviral CRE. Mice were sacrificed after two weeks and tumor cells in the drainage lymph nodes were evaluated for activation of the tracking construct. Ex vivo fluorescent imaging as well as immunohistochemical analysis of draining lymph nodes confirmed the presence of GFP positive tumor cells, thus confirming efficient activation of the tracking construct by adenoviral mediated gene delivery. We also observed the presence of un-activated RFP tumor cells in the lymph nodes of mice. This would suggest that many tumor cells were not efficiently targeted by adenovirus.

Application of this tracking system will be useful in verifying the sequential steps involved in systemic metastasis and evaluating the significance of metastatic lymph nodes as important intermediate steps in metastatic progression. Furthermore, the system could be also used to mark cells at different time points throughout the metastatic process to determine kinetics of systemic spread through regional lymph node. These different approaches will allow us to begin to answer some difficult questions, such as are metastatic lymph nodes important reservoirs of malignant cells that fuel systemic dissemination? Or are they just the trail left behind by malignant cells that have already exited the lymphatic system and entered systemic circulation? Is there a lag phase between initial lymph node colonization and systemic spread? The answers to such questions also have very important therapeutic implications as a better understanding of
the process of systemic dissemination as well as the timing of events that occur during this process will assist in staging the disease in node positive patients.
V. CONCLUSION
Metastatic prostate cancer is the second leading cause of cancer death in the U.S. and it is estimated that 28,170 men will die of the disease in 2012. Although prostate cancer frequently metastasizes to the bones, lungs and liver; the first manifestations of the metastatic disease is in the draining lymph nodes within the pelvis. For this reason, assessment of lymph node involvement is an important staging tool in the treatment of prostate cancer. Extent of lymph node involvement is an important predictor of survival outcome and strongly correlates to the extent of systemic spread. Furthermore, accumulating evidence implicates metastatic lymph nodes as mediators of systemic spread. Thus, a better understanding of mechanisms involved in nodal metastasis will allow us to better predict and manage the metastatic disease.

Metastasis is a complicated process that involves many steps. Two critical steps in this process are: 1) the ability of the tumors to create new vasculature through which they can spread and 2) the ability of tumor cells to home and invade into distant organs. In the present study we explore two important facets of these critical steps: lymphangiogenesis and chemokine receptor mediated homing. In addition, we propose a strategy to help define the contribution of metastatic lymph nodes to systemic dissemination.

Metastasis of malignant cells to lymph nodes through lymphatic vessels is supported by tumor driven lymphangiogenesis. Modulation of the lymphangiogenic axis through overexpression and inhibition of VEGF-C resulted in significant changes to both lymphatic density and lymph node metastases. Overexpression of VEGF-C resulted in the increase in magnitude of both lymph node and systemic metastases. Conversely, we saw decreases in both lymph node and systemic metastases with VEGF-C inhibition. We observed that changes in the magnitude of metastases directly correlated with lymphatic
vessel density in both subcutaneous and orthotopic models suggesting that VEGF-C dependant modulation of metastases are mediated primarily by changes in lymphatic vessel density. Though we cannot conclusively delineate the separate contributions of intra- versus peri- tumoral lymphatic density to magnitude of metastases, we observed much more profound deficits to peri-tumoral lymphatic vascular integrity with VEGF-C inhibition in the orthotopic model. Furthermore, these defects were associated with decreases in tumor cell invasion into peri-tumoral lymphatics. These observations support the theory that peri-tumoral lymphatic vessel density as well as tumor cell invasion into lymphatics plays an important role in lymph node metastasis. These findings highlight the importance of the VEGF-C/VEGFR-3 axis in mediating both lymph node as well as systemic metastases and suggest that inhibition of this axis could be a useful way to treat the metastatic disease.

Lymph node metastasis is mediated in part by the expression of specific chemokine receptors on tumor cells. Of these receptors, CCR7 has been investigated in many different tumor types and has emerged as an important facilitator of lymph node metastasis. Despite its growing reputation in mediating this process, its significance in the lymph node metastasis of prostate cancer has not been explored. Using a pre-clinical model of prostate cancer, we found increased expression of CCR7 in the lymph node metastases. Analysis of clinical prostate cancer specimens also revealed increased expression of CCR7 in the lymph node metastases in a subset of patients. Activation of CCR7 resulted in enhancement in many aspects of metastatic behavior including increased migration towards a CCL21 gradient, increased invasion through a basement membrane protein layer, and increased binding affinity to the extracellular matrix protein
vitronectin. Furthermore, stimulation by CCL21 was able to synergize with other growth factors to significantly increase proliferation, however, CCL21 alone had only minimal effects on proliferation and offered no protection from apoptosis in serum starved conditions. Collectively, these data are supportive for a role for CCR7 in mediating lymph node metastasis and suggest that CCR7 activation plays a larger role in the migratory response than in the survival or proliferation of tumor cells.

Lymph node involvement is an important predictor of both patient survival as well as extent of systemic metastases. Despite, these strong correlations, the extent that metastatic lymph nodes actually contribute to systemic disease is still unclear. To better understand the relationship between lymph node and systemic metastases we have constructed and validated a lentiviral-tracking vector that can be employed to mark malignant cells in metastatic lymph nodes and track the fate of these cells throughout the metastatic process. This strategy will help us to determine the contribution of metastatic lymph nodes to systemic dissemination to distant organs. That development of systemic metastasis is a sequential process, first involving regional lymph nodes has important therapeutic implications for the treatment of node positive disease as positive nodes could also serve as source of malignant cells that can support systemic dissemination. Thus a strategy to evaluate the fate of metastatic cells in the lymph node has importance not only in our understanding of the metastatic process, but also in developing better ways to stage the disease. To this end, use of metastasis markers such as lymphangiogenic growth factors as well as chemokine receptors could also be valuable in staging the metastatic disease. Moreover, therapeutics designed to inhibit these pathways, in combination with current therapies, may help to better manage the metastatic disease.
REFERENCES


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