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Cancer is the second leading cause of death in the United States, making it a major public health issue. Due to increased efficiency in detecting and treating cancer, primary tumors account for only 10% of cancer mortalities. Today, the majority of cancer related deaths are due to metastasis and relapse after therapy, which current cancer treatments fail to prevent. Recently, cancer stem cells (CSCs) have emerged as being responsible for metastasis and relapse. CSCs are cancerous cells with stem cell characteristics including self renewal and the ability to evade chemotherapy and elimination by the immune system. A part of the innate immune system, Natural Killer (NK) cells provide the first line of defense against cancerous cells. NK cells kill cancerous cells through release of cytotoxic granules, a process regulated by activating and inhibitory receptors at the NK cell surface recognizing specific surface molecules on a tumor. Of the NK cell receptors, signaling via NKp44 is pivotal in determining the fate of tumor cells because it possesses both activating and inhibitory functions and is only expressed on activated NK cells.

In this study, expression of Proliferating Cell Nuclear Antigen (PCNA), an inhibitory ligand of NKp44, is identified on the surface of a Diffuse B Cell Lymphoma, Prostate, and Breast cancer cell lines in novel association with Human Leukocyte Antigen Class I molecules. By blocking interactions between NKp44 and the PCNA/HLA I complex, NK cell mediated cytotoxicity and IFN- γ secretion is enhanced. Finally, prostate and breast cancer cells expressing PCNA at the cell surface express several molecular signatures of cancer stem cells which increase the ability of these cells to survive the metastatic process.

EXTRACELLULAR PROLIFERATING CELL NUCLEAR ANTIGEN
AS A MARKER AND THERAPEUTIC TARGET
FOR CANCER STEM CELLS

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CHAPTER I

INTRODUCTION

The immune system is comprised of specialized effector cells, molecules, and organs that defend the body against foreign invaders and tumor cells. Protecting the body through layered defenses, the immune system is split into innate and adaptive branches. The innate immune system provides the first line of defense, including physical and chemical barriers as well as specialized cells which detect and eliminate bacteria, viruses, and tumor cells [1]. Cells of the innate immune system include neutrophils, eosinophils, basophils, monocytes, and natural killer (NK) cells [1]. Although it provides the first response against potential threats, the innate immune response is limited in that it provides a non specific response, meaning innate counter measures are independent of sensitization to specific antigens. If the innate immune system is unable to completely remove a threat, the adaptive immune system is activated.

Dependent on antigen presenting cells identifying threats, collecting antigen, and alerting other effector cells, the adaptive immune system hones its response to improve recognition, targeting, and elimination of the threat [1]. The adaptive immune system is comprised of B cells and T cells, which are called into action by dendritic cells presenting specific antigens of pathogens or tumors collected in the periphery [1]. During adaptive immune responses, the cells of the innate immune system help guide and activate the proper effector mechanisms employed by B cells and T cells [1]. One benefit of activating the adaptive

immune system is the generation of immunological memory, which allows the immune system to react much faster and stronger the next time the specific threat is encountered [1].

Natural Killer Cell Function

Developing from hematopoietic stem cells within the bone marrow, NK cells are a specialized population of lymphocytes playing a vital role in the innate immune system by recognizing foreign invaders as well as internal threats from cells that are virally infected or cancerous [2-4]. NK cells were first reported in 1975 as a lymphocyte larger than a B or T cell, containing distinct cytoplasmic granules, and naturally cytotoxic against certain tumor cells without stimulation or sensitization [5,6]. Today, we know NK cells play a vital role in control of viral and bacterial infections, rejection of transplants, tumor immunosurveillance, and reproduction [1,7]. However, knowledge of how NK cells choose targets for elimination and how this process is regulated is incomplete.

NK cells interact with other cells through surface receptors which recognize ligands on the target cell surface, either conveying an activating or an inhibiting signal to the NK cell nucleus [2,4]. The balance of signals received by the NK cell governs their activation, proliferation, and effector functions [2,4]. Traditionally, inhibitory killer cell immunoglobulin like receptors (KIRs) and killer cell lectin-like receptors (KLRs) bind cell surface Human Leukocyte Antigen Class I (HLA I) molecules expressed by healthy human cells and signal through domains known as immunoreceptor tyrosine-based inhibitory motifs (ITIMS) [2,4,8]. When HLA I interacts with inhibitory receptors, dominant inhibitory signaling transmitted by

ITIMS prevents activation and cytotoxic action by the NK cell against normal, healthy cells of the body. NK cells may also be inhibited by cytokines released by regulatory cells of the immune system, such as regulatory T cells and myeloid suppressor cells [9].

Activating receptors, including the Natural Cytotoxicity Receptors (NCRs), NKG2D, and 2B4, bind ligands induced by cellular stress, infection, or tumor transformation [2]. Activating signals are transmitted through immunoreceptor tyrosine-based activating motifs (ITAMs) located in the cytoplasmic tail of the receptor or through ITAMs in adaptor molecules which associate with activating receptors at the cell surface [2,4]. Therefore, when a target cell over expresses activating ligands or ligands for multiple activating receptors, NK cells eliminate that target by releasing preformed cytotoxic granzymes and perforin stored as granules or activate apoptosis pathways in the target cell [2,4,10].

Upon activation, NK cells also release Interferon Gamma (IFN- γ), the proinflammatory cytokine Tumor Necrosis Factor Alpha (TNF- α), and granulocyte-monocyte colony stimulating factor (GM-CSF) [7,9]. IFN- γ is a potent cytokine which can influence both the innate and adaptive immune responses. During early stages of infection, IFN- γ produced by NK cells bolsters macrophage antimicrobial functions as well as other defense mechanisms against viruses [9]. In later stages of infection, IFN- γ shapes the adaptive immune response by stimulating antigen presenting cells and CD8⁺ T cells, shaping the Th1 immune response [9,11]. IFN- γ also activates regulatory T cells; thus, as NK cells are activated, so too are the cells which can regulate effector functions of NK cells to create balance in the immune response [11]. GM-

CSF released by NK cells also alters the immune response as it stimulates development of neutrophils, monocytes, and dendritic cells [9].

In order to initiate a cytotoxic response, NK cells require activation, which is achieved in several manners [10]. First, cytokines such as Interleukin (IL)- 2, IL-12, IL-15, and IL-18 activate and stimulate proliferation, cytotoxicity, and IFN- γ release [9]. These cytokines are released by dendritic cells in response to inflammation and can increase NK cell cytotoxicity up to 200 fold [7,9,12]. Specifically, IL-2 and IL-15 up regulate expression of the anti-apoptotic protein bcl-2 which increases NK cell survival [9]. NK cells can also be activated through a phenomenon called Antibody-Dependent Cellular Cytotoxicity (ADCC) by utilizing a surface receptor, CD16, which binds the constant heavy chain portion of immunoglobulins known as the Fc fragment [9,13]. In this event, the NK cell recognizes a target cell covered in antibody and facilitates the elimination of the target. Finally, NK cells can be activated through direct interactions between ligands on target cells and activating receptors of the NK cell as described above.

NK cells disseminate throughout all tissues, including the lymph nodes, spleen, peripheral blood, liver, and bone marrow [1,7]. NK cells are defined as CD3⁻CD56⁺ lymphocytes, which are subdivided into CD56^{bright} and CD56^{dim} subsets [1,7,14]. The CD56^{dim}CD16⁺ subset are highly cytolytic when interacting with target cells and make up the majority of NK cells in the blood and spleen [1,7,14]. Mostly found in lymph nodes and tonsils, the CD56^{bright}CD16⁻ subset regulates immune responses by producing IFN- γ , TNF- α , TNF- β , GM-CSF, IL-10, and IL-13 in response to IL-1, IL-10, IL-12, IL-15, and IL-18 produced by dendritic cells and macrophages [1,7,14,15]. CD56^{dim}CD16⁺ NK cells also regulate the immune response by

editing dendritic cell populations through cytotoxic action [13]. Accounting for their presence in the lymph nodes, CD56^{bright} NK cells express the chemokine receptor CCR7; likewise, the location of CD56^{dim} NK cells in the periphery is influenced by expression of CXCR1 [15].

NK cells derive from CD34⁺ hematopoietic progenitor cells in the bone marrow, but how and where NK cells mature is still in question [11]. Developmental studies suggest NK cells develop in secondary lymphoid tissue where CD56^{dim} NK cells are derived from CD56^{bright} subsets [11]. While NK cells are especially known for innate functions, mounting evidence also shows NK cells possess adaptive immune system properties, including immune memory, clonal expansion during infection, and receptors with antigen specificity [7,16,17].

NK Cell Based Immunotherapies

Despite the protective measures employed by the immune system, malignant diseases still develop by evading immunosurveillance. In regards to tumor formation, the immune system can be subverted in several ways. First, the tumor may generate an immune-privileged site. This can occur through production of immunosuppressive cytokines, such as IL-10 and Tumor Growth Factor (TGF)- β , which stifle antitumor immune responses or skew the immune response towards a response with less ability to fight the tumor [7]. Some tumors are capable of impairing dendritic cell responses, activating regulatory T cells to suppress T cell specific tumor responses, or directly eradicating immune cells by inducing apoptosis [7]. Finally, tumors may also down regulate expression or shed tumor associated antigens to prevent detection [7]. Immune system dysfunction may also lead to tumor evasion. For example, many cancer

patients exhibit NK cell aberrations. These abnormalities include defects in expression of activating receptors or intracellular signaling molecules, over expression of inhibitory receptors, and defects in proliferation or cytokine production [7]. Cancer only becomes clinically detectable once the tumor evades the immune system. While current therapies such as radiation and chemotherapy are efficacious in killing tumors, these therapies also have toxic and immunosuppressive side effects [7]. Thus, immunotherapies designed to activate antitumor immune responses by harnessing the immune system have gained much attention. In recent years, NK cell based immunotherapies have become a powerful tool to combat cancer.

An early immunotherapy strategy was systemic administration of cytokines stimulating NK cells, such as IL-2, IL-12, or IL-15 [7]. Once stimulated by cytokines, NK cells become lymphokine-activated killer cells (LAK) and exhibit greater cytotoxicity through enhanced proliferation and cytokine production as well as increased expression of cytotoxic effector molecules [7]. Unfortunately, patients experienced severe side effects after systemic cytokine treatment and the antitumor activity of LAK cells was limited. As an alternative approach to circumvent these issues, autologous LAK cells activated and expanded *ex vivo* are adoptively transferred, which greatly improves antitumor immunity without major side effects in several cancers [7]. Since autologous LAK cells are derived from the patient, antitumor immunity can be stifled through inhibition by HLA I. This has been demonstrated in several clinical trials where no significant clinical responses were observed after reinfusion of *in vitro* activated autologous LAK cells [7]. Blocking of inhibitory receptors by anti-KIR antibodies can increase NK cell cytotoxicity and is currently being evaluated in clinical trials. Alloreactive NK cells from a

donor individual, which do not recognize HLA I molecules in the patient, have also been used to increased antitumor immunity [7]. Allogenic NK cell adoptive transfers have progressed to Phase II clinical trials for several cancers.

Alternatively, some immunotherapies utilize the ability of NK cells to recognize targets coated in antibody through ADCC. For instance, an anti-CD20 antibody, Rituxumab, is used to treat non-Hodgkin's lymphoma [7]. Antibodies used in this setting are often altered, or humanized, to increase NK cell ADCC function while minimizing antibody induced toxicity. Antibodies may also be linked to cytokines, which has been shown to increase NK cell synapse formation with antibody coated targets, enhancing antitumor responses [7]. NK cell lines, such as NK-92, are also a potential source for NK cell based immunotherapies as these cells can be easily maintained, expanded, and enhanced *in vitro* before adoptive transfer.

Identification and characterization of NK cell receptors and their ligands over the last two decades have shed light on the molecular mechanisms of NK cell activation by tumor cells. In addition to the activating and inhibitory receptors, NK cells express several members of the signaling lymphocyte activation molecule (SLAM/CD150) family that play an important role in the regulation of NK cell function. Previous work in our laboratory has identified 2B4 (CD244) and CS1 (CD319, CRACC) as two members of the SLAM family that promises targeting of NK mediated cytolytic function against tumor cells [18-21]. Soluble form of the ligand for 2B4, CD48, is elevated in patients with lymphoid leukemia. 2B4 signaling by recombinant antigen-specific chimeric receptors costimulates NK cell activation to leukemia and neuroblastoma cells [22]. This indicates that antigen-specific 2B4 expressing NK cells may be useful in adoptive

immunotherapy to treat leukemia and other cancers [22]. A monoclonal antibody against CS1 receptor (Elotuzumab, Huluc63) has shown promising results against multiple myeloma. Anti-CS1 monoclonal antibody enhances killing of myeloma cells by activating natural cytotoxicity and inducing ADCC [23]. A combinational therapy of anti-CS1 monoclonal antibody and bortezomib has showed better promise in patients with relapsed/refractory multiple myeloma [24].

Finally, NK cells can also be modified genetically to express chimeric receptors. In this approach, a single chain variable fragment receptor of an antibody specific for a tumor antigen is fused to intracellular signaling machinery [7]. When this receptor recognizes the tumor associated antigen, NK cells activate antitumor defenses. With numerous ways to employ NK cells in cancer therapy, the future use of NK cell based immunotherapies is driven by further understanding how NK cells recognize and interact with tumor cells.

Natural Cytotoxicity Receptors

Among the activating receptors is a specialized group of receptors called the Natural Cytotoxicity Receptors, which play a key role in recognition and killing of tumor and virally infected cells. Comprising the NCRs are the NKp44, NKp30, and NKp46 receptors. Binding of one or more of these receptors with a specific ligand induces strong NK cell activation and cytotoxicity [25]. However, the NCRs work best as a team to identify potential targets for optimal recognition and elimination of target cells [26]. This is evident through increased cytotoxicity when multiple NCRs are triggered versus an individual receptor, suggesting

simultaneous NCR ligand expression of target cells [25-27]. Several studies have identified and characterized NCR ligands (Figure 1.1). For example, NKp30 is known to bind B7-H6, a member of the B7 family expressed exclusively on tumor cells [28]. While many NCR ligands remain unidentified, they are believed not to be expressed by normal cells but induced under pathological conditions [29].

Heparan Sulfate Proteoglycans (HSPGs) have been identified as co-ligands involved in the recognition of tumor cells by the NCRs [30,31]. HSPGs are macromolecules found at the cell surface of mammalian cells or in the extracellular matrix. Consisting of a core protein carrying numerous glycosylations with heparan sulfate chains covalently attached, HSPGs are known to contribute to events during cell adhesion, migration, proliferation, and tumorigenesis [32]. Interestingly, each NCR recognizes distinct forms of HSPGs [31]. HSPGs are known for numerous glycosyl modifications, which allow for attachment of soluble proteins such as growth factors, chemokines, and potentially damage associated molecular pattern molecules (DAMPs) [33].

DAMPs are molecules typically sequestered intracellularly, but serve as endogenous danger signals when improperly released from tumors or damaged cells [34]. Normally residing intracellularly, DAMPs lack secretion signals but can be actively secreted by nonclassical pathways [34]. DAMPs are most often released after trauma, ischemia, or other tissue damage and initiate early inflammatory responses often in the absence of pathogens [34]. Heat shock proteins, high-mobility group box 1, hyaluronan, and heparan sulfate represent a few DAMPs known to date [35]. DAMPs may also serve as ligands to members of the NCR family. Human

Leukocyte Antigen-B associated Transcript 3 (Bat3), also known as BAG6, is considered a DAMP due to its release from tumor cells [36]. Bat3 is typically located in the nucleus where it plays an essential role in controlling the acetylation of p53 in response to cellular DNA damage [36]. However, upon nonlethal heat shock, nuclear Bat 3 relocates to the cell membrane of tumors where it serves as a ligand for NKp30 [36,37]. Interestingly, this study also found Bat 3 colocalizes with HLA I, opposing previous reports that NCRs do not associate with HLA I molecules [8,37,38].

Of the NCRs, NKp44 is unique and significant for several reasons. First, expression of the receptor is restricted to activated NK cells that can initiate an immediate cytotoxic response [39]. Thus signals transduced through the receptor act as a final gateway in governing NK cell effector function. Second, NKp44 activating function is implicated in HIV related T cell decline as expression of an activating ligand for NKp44 is induced in uninfected CD4 T cells by the gp41 envelope protein of HIV [40]. Finally, NKp44 expression is responsible for a dramatic increase in killing of many tumor cell lines and cross linking the receptor results in the release of cytotoxic granules, IFN- γ , and TNF- α [27,38,39,41-43].

NKp44

NKp44 is a transmembrane glycoprotein of the Immunoglobulin (Ig) Superfamily selectively expressed on the surface of activated NK cells, induced by IL-2 or IL-15 [38,39,42,44]. While only found on activated NK cells in circulation, NKp44 is constitutively expressed by a specialized subset of NK cells in the decidua, implicating a role for NKp44 during placentation

[39,44,45]. NKp44 is also expressed on a subset of interferon producing cells located in human tonsils and NK-22 cells in mucosal associated lymphoid tissues [46-48]. NKp44 has a molecular weight of 44 kDa and structurally consists of a 169 amino acid extracellular domain followed by 23 and 63 amino acid sequences in the transmembrane and cytoplasmic tail domains respectively [39,49]. A 55 amino acid domain connects the extracellular Ig domain to the transmembrane segment and has 13 predicted O-glycosylation sites and a single N-glycosylation site [39,49]. Crystallography of the receptor demonstrates a surface groove made by two facing β hairpin loops extending from the Ig fold core stabilized by a disulfide bridge between Cysteine 37 and Cysteine 45 (Figure 1.2) [49]. The Ig domain contains an arrangement of positively charged residues at the groove surface, suggesting NKp44 ligands are anionic [49]. Also, the groove appears wide enough to host a sialic acid or an elongated branched ligand. The cytoplasmic tail of NKp44 contains a tyrosine sequence resembling an ITIM [39,50]. This motif is functional and inhibits the release of cytotoxic agents and IFN- γ [39,42,50]. NKp44 surface expression is dependent on its association with the ITAM containing DAP 12 accessory protein linked to NKp44 through Lysine 183 in the transmembrane domain [39]. Upon recognition of activating ligands, signalling transduced through the ITAMs in Dap 12 result in release of cytotoxic agents, Tumor Necrosis Factor- α , and IFN- γ [38,50].

Originally discovered as an activating receptor, NKp44 is now known to possess dual functions conveying either activating or inhibiting signals [39,42,50]. Proliferating Cell Nuclear Antigen (PCNA) is the inhibitory ligand for NKp44 (Figure 1.1) [42]. PCNA is a nuclear protein found in all replicating cells which encircles DNA and increases processivity of DNA replication, but is also involved in DNA repair and cell cycle control [51]. NKp44 recognizes PCNA expressed

on exosomes shuttled to the surface of tumors cells when in contact with NK cells [42].

Recognition of cell surface PCNA colocalizing with HLA I on the cell surface inhibits NK cell cytotoxicity and IFN- γ release [42,52].

A truncated isoform of mixed-lineage leukemia-5 (MLL5) is an activating cellular ligand for NKp44 [53]. This MLL5 isoform contains a specific exon encoding a C-terminus which interacts with NKp44 [53]. Typically located only in the nucleus, MLL5 is a lysine methyltransferase implicated in hematopoietic differentiation and control of the cell cycle [53]. Contrary to normal MLL5, the isoform recognized by NKp44 is not found in the nucleus, but in the cytoplasm and endoplasmic reticulum, destined to be expressed at the cell surface [53]. Interestingly, the isoform does not contain a transmembrane sequence or glycosylphosphatidylinositol anchor, but does contain a glycosaminoglycan attachment site near the C-terminus, which likely accounts for its anchoring at the cells surface [53]. This correlates with recognition of heparan sulfate proteoglycans by NKp44. While MLL5 is expressed in normal tissue, the isoform recognized by NKp44 is only present on tumor and transformed cells [53]. Since PCNA and MLL5 are typically only located in the nucleus and cytoplasm, their presence on the cell surface qualifies these molecules as DAMPs, which suggests the NCRs may be pattern recognition receptors which recognize DAMPs sequestered to the cell surface by HSPGs or other molecules.

NKp44 in Tumor Recognition and Tumor Escape

NKp44 is implicated in recognition and killing of numerous types of cancer: Neuralblastoma, Choriocarcinoma, Pancreatic, Breast, Lung Adenocarcinoma, Colon, Cervix, Hepatocellular carcinoma, Burkitt lymphoma, Diffuse B Cell Lymphoma, Prostate [27,41-43,52]. While most of these ligands have not been identified, they appear to be cell cycle regulated, with down regulation of expression during mitosis [41]. Recognition of tumor cells is partially mediated through charged based binding of NKp44 with HSPGs on the surface of tumor cells [30]. Specifically, the 2-*O*-sulfation of iduronic acid and *N*-acetylation of glucosamine on HSPGs are important for interaction with NKp44 [31]. Glycans containing α 2,3-*N*-acetylneuraminic acid are also recognized on the surface of cancer cells by NKp44 [54]. Of note, recognition of HSPG only evokes IFN- γ release by NK cells, not cellular cytotoxicity [30]. Thus, HSPGs are believed to only be a coligand for NKp44 as well as the other NCRs, potentially facilitating binding with other cellular ligands, such as DAMPs.

While NK cells utilize NKp44 to recognize and kill targets, tumors may also exploit NKp44 to escape NK cell recognition. By engaging NKp44, as well as the other NCRs, tumors can induce NK cell death via up regulation of Fas Ligand in the NK cell, inducing Fas mediated apoptosis [25]. Tumors may also down regulate NKp44 surface expression by shedding soluble MHC Class I chain-related molecules or by releasing indoleamine 2,3-dioxygenase and prostaglandin E2 [55,56]. The latter two molecules are released by mesenchymal stem cells as well, inhibiting NKp44 expression in the tumor microenvironment [57]. Additionally, tumors can regulate NKp44 ligand expression to escape NK cell killing, as is the case with Acute Myeloid

Leukemia (AML) [58]. Finally, tumor cells may induce expression of exosomal PCNA when physically contacted by NKp44 expressing NK cells to inhibit NK cell effector function [42].

NKp44 in Viral Recognition

In addition to tumor surveillance, NK cells are also the first line of defense against viral infections where NKp44 plays a prominent role. NKp44 is responsible for recognition of Influenza and Sendai virus hemagglutinins via $\alpha 2,6$ -*N*-acetylneuraminic acid, requiring sialylation of NKp44 [54,59]. Binding to hemagglutinin enables lysis of viral infected cells. Specifically, NKp44 recognizes hemagglutinins from H5-type influenza virus strains [60]. Two flaviviruses, Dengue and West Nile, are also recognized by NKp44. Envelope proteins of these viruses, particularly domain III of West Nile, directly bind to NKp44, increasing lysis of infected cells and NK cell IFN- γ release [61]. Finally, NKp44 is implicated in recognizing a ligand expressed on cells infected with Vaccinia virus [62].

Similar to tumors, viruses have evolved to escape NK cell detection by down regulating expression of NKp44 ligands. In the case of Kaposi's sarcoma-associated herpes virus, extracellular ligand expression is reduced during de novo infection. Interestingly, during lytic infection, only surface levels of the NKp44 ligand are reduced as overall cellular levels are unchanged, indicating a defect in cellular trafficking [63]. Furthermore, while the NKp44 ligand is typically located outside of the nucleus, during lytic infection the ligand is found localizing to the nucleus [63]. This localization is concurrent with a burst of lytic gene expression, mainly consisting of immune related genes [63].

While NKp44 plays a role in recognizing many viruses, its role in HIV infection is most significant. A hallmark of HIV infection is the progressive depletion of CD4⁺ T cells via destruction of both uninfected CD4⁺ T cells and HIV-infected CD4⁺ T cells. In regards to NK cells, HIV modulates both the expression of NK cell receptors and their ligands. NKp44 is no exception as it is expressed at a lower surface density on in vitro activated NK cells from HIV-1 patients compared to healthy controls, resulting in decreased killing of various tumor target cells [64-66].

HIV also modulates NKp44 ligand expression. MLL5, an activating NKp44 ligand, is expressed on uninfected CD4⁺ T cells during HIV infection, correlating with the loss of CD4⁺ T cells and increase of viral load [40]. MLL5 is only expressed in high amounts on uninfected CD4⁺ T cells and is not responsible for inducing NK lysis of HIV-infected cells [67]. To avoid NK killing of HIV infected CD4⁺ T cells, the Nef protein of HIV-1 retains MLL5 intracellularly, preventing cell surface expression and interaction with NKp44 [68]. Studies by Vieillard et al. have shown a highly conserved 3S peptide motif of the HIV-1 gp41 protein is involved in the induction of MLL5 on the surface of uninfected CD4⁺ T cells. An envelope protein of the HIV virus, gp41 is vital for viral entry into target cells [40]. The 3S peptide of gp41 binds to CD4⁺ T cells via gC1qR, a receptor for the globular domain of complement component 1q [69]. Binding of the 3S motif to this receptor activates a signaling cascade involving PI3K, NADPH-oxidase, Rho-A, and TC10, leading to translocation of MLL5 from the cytoplasm to the plasma membrane [69]. MLL5 expressing CD4⁺ T cells are more susceptible to lysis by activated NK cells via NKp44 [40]. Understanding the role of MLL5 during HIV infection could help identify new therapeutic strategies to prevent the progressive loss of uninfected CD4⁺ T cells. Possible therapeutic

strategies are to inhibit the expression of MLL5 by using an anti-gp41 Ab or an anti-gC1qR Ab to block the 3S motif and gC1qR interaction [40,69]. Anti-3S immunization has also proven efficacious in preliminary studies in macaques [70].

NKp44 in Development

Decidual NK cells (dNK) make up 50-90% of lymphocytes in the uterine mucosa during pregnancy and constitutively express NKp44 [71-73]. In close contact with fetal extravillous trophoblasts cells invading the maternal decidua, dNK cells exhibit reduced cytotoxicity but crucially produce IL-8, Interferon-inducible protein 10, Vascular Endothelial Growth Factor, and Placental Growth Factor in response to NKp44 triggering [71,73]. Trophoblast cells and maternal stromal cells of the decidua both express unidentified NKp44 ligands [71,73]. This ligand may be PCNA as the protein is over expressed in trophoblast cells during the first trimester [74]. As an inhibitory ligand for NKp44, extracellular PCNA expression on trophoblast cells would help explain the diminished ability of dNK cells to lyse trophoblasts despite low levels of classical HLA I expression [73]. Invasion of trophoblasts into decidua facilitates proper placentation and NK cells help govern how far trophoblasts infiltrate (Figure 1.3) [75]. dNK cells also help reorganize the spiral arteries to facilitate appropriate blood transfer between the mother and fetus at the placenta [73,75]. Thus, alterations in dNK cells and invasion of fetal trophoblast cells are implicated in pregnancy complications, such as pre-eclampsia and tubal pregnancies [75]. Altered NKp44 expression is also proposed to play a role in miscarriage [76]. Since fetal trophoblast and maternal decidual cells express an NKp44 ligand, NKp44

constitutively expressed on dNK cell plays a crucial role in proper development of the placenta in pregnancy that requires further study.

Cancer Treatment Failure and Cancer Stem Cells

Many people today take their good health for granted. Yet, the stark reality of life is that a vast majority of the population will one day receive news that they have cancer. This diagnosis typically means a primary tumor has been found in the patient. Current therapy options for cancer treatment are fairly successful as the overall death rates for most cancers have steadily decreased over the last ten years [77]. However, the major problem with cancer arises years later, when the cancer comes back. For many patients, the return of cancer is accompanied with the cancer's spread to another part of the body. Ultimately, the primary cause of mortality in cancer is relapse and metastasis, as the primary tumor accounts for only 10% of deaths [78]. Therapy has clearly increased the survival rate after diagnosis, but often fails in the form of complete recovery. Thus, the current therapeutic approach to treating cancer has fundamental flaws. Most pharmaceuticals used in current treatment options target rapidly dividing cells within the tumor that are terminally differentiated and lack the capacity to self-renew [79]. The problem with current therapies is the failure to address the cellular complexity which makes up a tumor and drives its growth.

Cells within a tumor are actually very heterogeneous, composed of a range of different cell types including immune, mesenchymal, endothelial, and epithelial cells (Figure 1.4) [78,80]. Epithelial cells of a solid tumor vary in terms of differentiation states ranging from terminally

differentiated cells, which make up the bulk of the tumor growth and mass, but also cells which are less differentiated, and even a smaller subset of cells which retain self renewing properties. These self renewing cells generate the mass of heterogeneous cells comprising the tumor and are termed Cancer Stem Cells (CSCs) [80]. They are rare and often quiescent, thus they escape conventional therapeutics and detection in most patients [78,80]. CSCs also retain protective measures of normal stem cells against cellular damage and cytotoxicity [79]. This includes the ability to expel chemotoxic reagents, apoptotic factors, shed complement and Fas ligand, and evade immune cell detection [78,79]. Most important, like normal stem cells, CSCs also retain the ability to self renew, which often results in asymmetrical division, yielding an identical daughter cell and a more differentiated daughter cell, which maintains overall tumor growth [78,80]. CSCs are identified by their capacity to initiate new tumors through serial transplantation in mice xenograft models where tumor heterogeneity is reestablished [79,80]. CSCs are postulated to participate in metastasis and be responsible for relapse after therapy. One important distinction to make is CSCs may not be the cells which originate the primary tumor itself, but are somehow derived from the primary tumor [80].

Much debate surrounds the theories of cancer formation. The stochastic, or clonal evolution model, says any cancer cell, through accumulation of appropriate mutations, can acquire traits that promote survival, aggressiveness, and metastatic potential [78]. An opposing theory, termed the cancer stem cell model, says a small population of cancer cells drive tumor growth, akin to normal proliferative growth of bone marrow, skin, and intestinal epithelium [78,79]. There are also divergent theories as to how CSCs are formed. They may arise from normal adult stem cells of the tissue through mutation, which makes the stem cell cancerous

[78,80,81]. Longevity of stem cells may facilitate this process by making them susceptible to accumulation of genetic and epigenetic mutations. However, this may not always be the case as generation of CSCs may in fact depend on individual circumstances of the tissue and its microenvironment. There is also evidence that a differentiated cell or committed progenitor cell may reacquire self renewal capabilities and stem cell properties. Recent evidence has pointed to a fundamental process required for embryonic development which, when reactivated in differentiated cells, results in dedifferentiation and generation of a cell with stem cell like phenotype, generating a multipotent cell with invasive and mobile properties [78,80,81].

Epithelial-Mesenchymal Transition and Identifying CSCs

A central concept in biology is that all cells of the body derive from other cells, which arise from a single cell, the fertilized egg [82]. Due to the complexity of cells needed to constitute a fully functional body, the fertilized egg must replicate and differentiate into cells with a multitude of phenotypes. Thus, development of an organism requires cells to move back and forth between epithelial and mesenchymal states to successively generate cells of diverse phenotypes. The Epithelial-Mesenchymal Transition (EMT) is a fundamental process during embryonic development, wound healing, and cancer progression where differentiated epithelial cells dedifferentiate into a mesenchymal cell with a stem cell like phenotype [82,83]. EMT is recognized as a mechanism in embryonic development, but can also be activated in

terminally differentiated epithelial cells in instances of tissue damage, pathological stress, inflammation, and cancer metastasis [82].

EMT involves intense changes including the loss of cell-cell adhesion and polarity, accompanied with acquisition of migratory and invasive properties [83]. Phenotypic changes during EMT also provide markers for cells which have passed through EMT, and potentially CSCs. For example, β -catenin is typically sequestered near the plasma membrane by the cytoplasmic tail of E-cadherin. However, during EMT, expression of Snail, Slug, Zeb, and Twist transcription factors decrease expression of E-cadherin, freeing β -catenin to translocate to the nucleus providing a marker for EMT and also facilitating Wnt signaling, further perpetuating the EMT phenotype. [82,84,85]. Transcription factor activation and altered gene expression is responsible for the phenotypic changes from epithelial cells to mesenchymal cells, characterized by loss of E-cadherin and increased expression of N-cadherin, Vimentin, Fibronectin, and metalloproteases [83,84]. EMT imparts mesenchymal traits producing cells that are self renewing, motile, and invasive while resistant to apoptosis, senescence, immune responses, and chemotherapy, yielding metastatic cells with a high grade of malignancy [83,84]. Such traits help explain why both immunotherapy and cytotoxic chemotherapy sometimes fail. In this light, current standards of treatment actually select for survival of CSCs and increased malignancy [86,87].

Indeed, *in vitro* work confirms ectopic expression of these transcription factors in untransformed mammary epithelial cells and ovarian cancer cells induces EMT, characterized by a $CD44^{high}/CD24^{low}$ stem cell like phenotype [88,89]. These cells are able to grow in spheroid

structures when not allowed to adhere in typical epithelial fashion. Additionally, when CD44^{high}/CD24^{low} mammary cells are implanted *in vivo*, entire mammary ductal trees are completely regenerated, vividly displaying the stem cell like action of the cells induced through EMT and subsequent redifferentiation [88]. Analogously, CD44^{high}/CD24^{low} cells sorted from prostate cancer cell lines produced significantly more aggressive and highly vascularized tumors in mice with fewer number of seeding cells compared to bulk prostate cancer cells that were not sorted or were depleted of cells with stem cell like phenotype [90-92]. These studies have generated standardized assays to identify CSCs both *in vitro* and *in vivo*. The consensus of these studies, performed on CD44^{high} cells from breast, colon, bladder, cervix, ovary, glioma, melanoma, and prostate cancer cells, show these CSCs are far more proliferative, clonogenic, tumorigenic, and metastatic in xenograft mouse models [90,92-95]. *In vitro*, CD44^{high} cells have a higher rate of proliferation in soft agar assays, the ability to form spheroids in non-adherent culture techniques with serum free media, and invade Matrigel, a basement membrane model [88,90-94]. Microarray analysis of genes expressed by CD44^{high} cells demonstrate increased expression of genes related to maintenance of stemness, such as Oct3, BMI-1, Nanog, and CD133 [90,92].

EMT involvement in formation of CSCs relies greatly on *in vitro* data as there are a number of limitations in regards to demonstrating EMT *in vivo*. Primarily, tumors are heterogeneous, containing cells of various phenotypes. Complex interactions between tumorigenesis processes and EMT create a vast amount of heterogeneity between different types of epithelial cancers, but also within a single tumor as cells will undergo varying degrees of both tumor generating mutations and EMT. Additionally, variation within tumors is also

facilitated by a cell's proximity to the vascular network within the tumor. Locations nearest the blood vessels are more conducive for survival of highly proliferative cells and provide a niche for stem cells, while locations more distant from vessels will encounter fewer nutrients and oxygen [89]. Furthermore, it is difficult to differentiate if a tumor cell has undergone EMT or if it is a stromal cell that already has a mesenchymal phenotype by using standard EMT markers [86]. It is also difficult to ascertain if the CSC identified is a parental tumor cell or a consequence of EMT. Thus, EMT as a hallmark of metastasis has yet to be fully accepted by clinicians, highlighting a need for improved cellular markers for CSCs [86].

Mechanism of Metastasis and Subtypes of CSCs

In vivo observations, accompanied with knowledge of EMT, have led to a possible mechanism of cancer metastasis. During growth of a tumor, EMT is induced by mixed signals received from the tumor microenvironment and the neighboring stroma, activating EMT transcription factors [84]. E-cadherin expression is abated and epithelial homeostasis is lost as cellular junctions are broken, allowing cells within the tumor complex to dissociate. Subsequent reorganization of the cytoskeleton enables cellular budding and extension of podia in the direction of migration [96]. Pseudopodia cellular extensions, formed during EMT, allow cells from the primary tumor to invade the surrounding tissue and break through the basement membrane [84,92,97]. Localized to the podia are receptors for metalloproteinases to degrade the extracellular matrix, which is replaced with collagen and fibronectin, while newly expressed integrins facilitate attachment [96,98]. Metastatic cells must then enter either the lymphatics

or blood vessels, survive sheer stress during transport, and extravasate into new tissue [84].

Once metastatic cells have entered new tissue, EMT signals are no longer received and the cells colonize the tissue by reverting back to an epithelial phenotype [82,84].

The transient nature of metastatic cells as well as the vast amount of heterogeneity within the tumor, in particular subareas demonstrating robust proliferation or cell cycle arrest and differential expression of nuclear β -catenin and E-cadherin, have led to the theory of two subtypes of CSCs. Stationary CSCs remain embedded in epithelial tissue and are active in benign precursor lesions [81]. They also persist in differentiated areas in all steps of tumor progression; yet, they are unable to disseminate [81]. Mobile CSCs reside mainly at the tumor-host interface and are derived from stationary CSCs through EMT [81]. In this theory, stem cell properties are already in place while EMT conveys motility, invasiveness, and arrests cellular growth until a new niche is found [81].

Pulling together the theory of CSC subsets and in vivo evidence, a model can be generated displaying the concept of EMT and the migrating stem cell (Figure 1.5). During initiation of colon cancer, normal stem cells are located within the basal crypts of normal colon mucosa. In transition to an early adenoma, stationary CSCs become embedded in the adenoma and are present in late adenomas. During progression of late adenomas, differentiated tumor cell growth begins to encroach on the physical barrier between the mucosa and submucosa, the lamina muscularis [81]. EMT becomes activated by the mixed environmental signals received at this border as the tumor cells cross the lamina into the submucosa [81]. EMT only becomes evident during the transition from an adenoma into a carcinoma where the tumor crosses the

lamina and disseminates into the mucosa [81]. Metastasis is only found from this stage onward.

Exosomes Facilitate Microenvironment Messages

Exosomes are a class of microvesicles of endosomal origin, stored in intraluminal vesicles and range from 30-120 nm in size, and are predicted to be a main actor in niche preparation for CSCs in premetastatic organs through the transfer of proteins, genetic information, growth factors, chemokines, and proteases [99-102]. Exosomes have numerous functions including stimulation of cells by surface interaction, providing a surface for assembly of clotting factors, transferring receptors, intracellular proteins, and genetic information between cells, and enabling cells to escape the immune system, drug therapies, and apoptosis [100]. Tumors are known to release a large amount of exosomes into circulation, which has been shown to correlate with a poor patient prognosis [100]. Exosomes are also implicated in metastasis through the dissemination of exosomes containing matrix metalloproteases and pro-angiogenic factors, but also enable crosstalk between tumor cells and stromal cells, which may result in transfer of oncogenic signals [100]. Thus exosomes have a vital function in the tumor microenvironment and have been reported to coordinate different cell types within a potential tumor niche to establish an environment conducive for tumor growth.

On the other hand, exosomes, particularly those derived from stem cells, also have healing powers. During injury, damaged tissue release exosomes which reprogram the phenotype of stem cells, which then acquire tissue specific functions [101]. In turn, exosomes

from the stem cells induce cell cycle re-entry of cells surviving injury, allowing for tissue regeneration [101]. Studies demonstrate coculture of bone marrow cells with injured lung cells induces expression of lung specific genes and proteins in the bone marrow cells [101]. This phenomenon was dependent on exosomes released from the injured lung cells. Furthermore, in a rat model of hepatic injury, exosomes isolated from liver stem cells accelerate recovery of the liver after hepatectomy, dependent on the RNA and microRNA content of the exosomes [102]. Therefore, interactions between a stem cell and the local niche via exosomes have a critical role in defining not only the phenotype of the stem cell, but also the behavior of the localized tissue.

Exosome function has only recently been appreciated in tumor biology and immunity. Tumor cells are known to release exosomes, which is correlated with poor prognosis and resistance to chemotherapy [101]. Exosomes are also implicated in tumor cell escape from immune cell surveillance through shedding immune activating ligands and complement, but also by inducing apoptosis in responding immune cells [101]. While numerous studies have identified a plethora of functions for exosomes as well as associated proteins, few if any studies have addressed the function of CSC derived exosomes.

B Cell Lymphoma

Diffuse large B-cell lymphoma (DLBCL) is the most common lymphoid disease in adults, accounting for about 30% of Non-Hodgkin's Lymphomas in Western Countries [103]. DLBCL is described as a heterogeneous group of lymphoid diseases composed of large cells with

vesicular nuclei at least twice the size of a small lymphocyte [103]. Cells also exhibit basophilic cytoplasm and a high proliferation rate [103]. Patients usually present symptoms of rapidly growing lymph nodes or extra nodal masses [104]. Cancerous cells often totally replace normal lymph node cells and can infiltrate the soft tissue surrounding the lymph node [104]. Cells can be identified through typical B cell markers, including CD19, CD20, CD22, and HLA-DR [104]. DLBCL is subdivided into four classes: DLBCL not otherwise specified, DLBCL with predominant extra nodal location, large cell lymphoma of terminally differentiated B cells, and borderline cases [103]. Each class is then further divided into a complex organization based on morphology and pathology.

A hallmark of DLBCL is reciprocal chromosomal translocation between one of the immunoglobulin loci and a proto-oncogene [105]. Translocation may occur during B cell development in the bone marrow during the recombination of the B cell receptor, aberrant somatic hypermutations in memory B cells, or during immunoglobulin class switching [105]. Specifically, 30-40% of cases contain a chromosomal translocation resulting in a rearrangement of *BCL-6*, a transcriptional repressor which controls germinal center formation [103,104]. This results in unchecked proliferation of B cells in the germinal center and prevents their differentiation into a post germinal center resting B cell [105]. Thus most DLBCL are believed to originate from germinal center B cells or their descendants [105]. Translocation of *BCL-6* also inactivates p53, rendering the B cells susceptible to DNA mutations [103]. DLBCL also exhibits constitutive activation of NF- κ B [106]. Inhibiting this signaling pathway causes cell death and growth arrest in G1, suggesting the NF- κ B pathway may be a target for therapies [106].

DLBCL are very aggressive, but also curable. When detected early, 80-85% patients are cured without relapse in the first 5 years after treatment; however, at advanced stages, about half of patients will experience relapse within 5 years after treatment [103]. Around 30% of those in advanced stages of disease relapse within one year with poor prognosis [103]. Current treatment includes a combination of chemotherapy and Rituxumab treatment, a monoclonal antibody against CD20 [103]. Advances in therapy after relapse are needed to overcome resistance to chemotherapy, enabling patients to proceed to stem cell transplantation measures [103]. Novel agents to treat DLBCL are currently being investigated. These therapies include immunomodulating agents which inhibit angiogenesis, induce apoptosis, inhibit pro-survival factors, alter cytokines, and stimulate immune responses [103]. Recently, a novel monoclonal antibody with increased affinity for CD20 demonstrated antibody-dependent complement mediated cytotoxicity and strong induction of apoptosis [103]. Histone deacetylase inhibitors have also been studied as they regulate the cell cycle, survival, angiogenesis, and immune responses [103].

Prostate Cancer

Prostate cancer is the most prevalent cancer and second leading cause of cancer associated deaths in men, accounting for an estimated 30,000 deaths in 2013 [107]. Upon initial diagnosis, prostate cancer is typically treated surgically or with radiation therapy [108,109]. In the event disease recurs or is detected too late, the primary treatment is androgen deprivation, the removal of testosterone from the blood by pharmacological

intervention or surgical castration [108,109]. Eventually, patients become refractory to this treatment, developing castration resistant disease, which is incurable [109,110]. Most cases are detected by monitoring for elevated blood levels of prostate-specific antigen (PSA) followed by a biopsy [108,109]. PSA is a serine protease, believed to liquefy seminal fluid, which is released into the blood by prostate tumors [111]. For the majority of men receiving surgical treatment, 80% are successful and metastatic disease does not occur within 15 years [108]. Despite treatment, some patients develop metastatic disease with a average survival of about 16 months [108].

The glandular epithelium of the prostate is composed of a bilayer of basal and luminal cells [109]. The luminal cells secrete prostatic fluid and PSA (Figure 1.6) [110]. Sporadically entrenched in the bilayer are a few rare neuroendocrine cells [109]. A matrix of laminin, collagen, and stromal cells surround the epithelium [109]. In contrast, loss of basal cells and alterations in the matrix are found in prostate tumors (Figure 1.6) [109]. Tumor cells are often less differentiated and co-express integrins and keratins which normally segregate healthy luminal and basal cells [109]. Thus, prostate tumors are thought to originate from disruptions in differentiation pathways between the two cells types; yet, the cell of origin giving rise to the tumor has not been clearly identified [109]. Understanding the origin of prostate cancer is ultimately hindered by the lack of knowledge in regards to the process of normal prostate epithelial differentiation. As with most epithelial organs, the luminal cells of the prostate are always being shed and replaced by cells from the basal layer via differentiation [109]. However, confusion arises through a consortium of mouse studies suggesting prostate cells derive from potentially three different epithelial progenitor populations [109]. Additionally, mice, unlike

humans, are not prone to developing spontaneous prostate cancer and there are significant structural differences between the mouse and human prostate [109]. In response to these issues, current prostate cancer researchers have developed techniques which model *in vivo* differentiation of human prostate cells *in vitro*.

One of the main genetic alterations in prostate cancer involves the androgen receptor (AR) [109]. In normal prostate epithelium, AR signaling is required for secretory functions but inhibits cell proliferation [109]. However, the opposite is true in prostate tumors as they are heavily dependent, at least initially, on AR signaling for growth and survival [109,110]. Thus androgen deprivation therapy is used to promote tumor regression [108,110]. Eventually tumors develop mutations to upregulate AR signaling and become resistant to therapy [109]. Common mutations in prostate tumors also include amplification of the oncogene Myc, loss of the tumor suppressor Pten, and the fusion of a promoter sequence regulated by AR upstream of Ets genes, which are involved in differentiation, invasion, and proliferation [109].

The cause of prostate cancer is unknown, but genetic, age, and environmental factors appear to play an important role [112]. Inflammation is also believed to play a role in development of prostate cancer as well as other cancers [108]. Interestingly, CD4⁺, CD8⁺, and regulatory T cells are found in the prostate; however, CD8⁺ T cells are not functional [108]. Studies in humans and mouse models indicate T cells found in cancerous prostates become tolerant to prostate specific antigens and develop a regulatory phenotype [108]. Tolerance is partially abated through androgen deprivation, which reverses involution of the thymus that naturally occurs with age [108]. This induces an increase in naïve T cells released from the

thymus, resulting in increased infiltration of CD4⁺ T cells into the prostate which have an activated phenotype [108]. These studies suggest T cell tolerance may contribute to prostate cancer progression, but also offer potential avenues for immunotherapy in combination with androgen deprivation. Most immunotherapies targeting prostate cancer aim to activate populations of T cells which specifically recognize PSA, either through loading PSA into antigen presenting cells or by vaccination [108]. PSA is ideal since it is primarily expressed only by the prostate. Other immunotherapies under investigation include using a monoclonal antibody against prostate specific membrane antigen labeled with a radioisotope to enable localization of radiotherapy directly to the tumor mass [108].

DU145 and PC3 are both classical cell lines used to study prostate cancer. DU145 was derived from a brain metastasis while PC3 was derived from bone metastasis [113]. DU145 cells express both transcripts and androgen receptor protein, while PC3 does not express either [113]. However, both cell lines are able to grow independent of androgen [113]. Both cell lines lack expression PSA but express keratin 5, which marks basal prostate cells, and keratins 8 and 18, which mark luminal cells [113]. In terms of invasive potential, PC3 is more invasive than DU145; however, both cell lines exhibit considerably increased invasive potential over the LNCap cell line, another classical prostate cancer cell line [114].

Breast Cancer

In 2013, over 230,000 women in the United States are estimated to be diagnosed with the leading cause of cancer in women, breast cancer [107,115]. Globally, breast cancer is the leading cause of cancer associated death in women and accounts for 23% of cancer diagnoses in women [116]. While the incidence of breast cancer has increased over the last 30 years, mortality rates have decreased thanks to drastic improvements in screening and treatment [115]. Breast cancer describes a heterogeneous group of tumors with numerous differences in markers, receptor expression, genomic abnormalities, and ultimately oncogenic generating events. BRCA1, BRCA2, p53, and PTEN are a few of the most significant genes which develop mutations in breast cancer [117]. These genes play various roles in DNA repair, cell cycle control, and proliferation [117]. The advent of microarray technology to analyze gene expression has facilitated the identification of four breast cancer subtypes: luminal A, luminal B, basal like, and human epidermal growth factor receptor 2 (HER2) enriched [116,118]. Each of the groups can be further stratified based on gene expression. For example, about 80% of basal like tumors are triple negative, lacking expression of the oestrogen receptor, progesterone receptor, and HER2 [118]. With further research, more molecular subtypes will be identified, further complicating the taxonomy by which breast cancer is molecularly defined.

Complete breast removal and aggressive chemotherapy treatments have given way to hormonal and targeted therapies through improved research and characterization of tumors [115]. Estrogen and its receptor are prime regulators of breast cancer progression; thus, drugs which modulate the estrogen receptor, like tamoxifen and raloxifene, are frequently used to

hormonally treat breast cancer [119]. Therapies that target other molecules, such as HER2, are also in clinical trials [119]. Of women diagnosed with early stage breast cancer, 30% will develop metastatic lesions, which are responsible for most breast cancer associated deaths [116,120]. Breast cancer metastasis can occur early, even before diagnosis of a primary tumor, but can take up to 20 years to form a secondary tumor in another organ [120]. Long periods of dormancy in metastatic cells have recently been attributed to metastasis-specific immunosurveillance [121,122]. These studies identified CD8⁺ T cells and NK cells as mediators controlling the outgrowth of metastasis [120-122]. Deficiencies of either, especially NK cells, accelerates establishment of metastatic lesions. Other studies demonstrate impaired functionality of NK cells from peripheral blood and primary tumors of patients with breast cancer, further implicating NK cells in breast tumor surveillance [120].

The MCF7 and MDA-MB-231 breast cancer cell lines were established in the late 1970s and are widely used cell lines to study breast cancer. Both cell lines were derived from metastatic cells in pleural effusions from invasive ductal carcinomas [123]. However these cell lines differ in several ways. MCF7 cells are of a luminal epithelial phenotype, are weakly invasive, lack expression of vimentin, and express the Estrogen and Progesterone receptors, while MDA-MB-231 cells carry a mesenchymal phenotype, are highly invasive, express vimentin, and do not express Estrogen or Progesterone receptors [123,124]. Interestingly, MCF7 cells treated with doxorubicin can selectively express mesenchymal characteristics such as vimentin, suggesting doxorubicin initiates an epithelial to mesenchymal transition in the cells [123]. Expression of vimentin in MCF7 cells allows for successful invasion and increased

motility [123]. Mouse studies confirm MDA-MB-231 cells are far more aggressive and metastatic compared to MCF7 cells [123].

Significance

This study will improve scientific knowledge in the NK cell field by demonstrating a novel means by which NK cells can recognize target cells. NKp44 plays a pivotal role in NK signaling as it is only expressed on activated NK cells. Thus, signals received through NKp44 decisively polarize the balance of NK signaling in order to activate cytotoxicity, or deactivate. Thus identifying and characterizing potential NKp44 ligands on the surface of tumors is vital to understanding NK cell function and why some tumors escape killing. This knowledge will allow for the development of novel therapies harnessing NK cell receptor-ligand pair interactions.

Since numerous cancers and disease states induce NKp44 ligand over expression, identifying NKp44 ligands is paramount and will significantly impact therapeutic strategies across all medicine. In addition, this work proposes a novel method of cellular surveillance through pattern recognition. In this regard, NCRs of the NK cell recognize soluble intracellular molecules, DAMPs, which reach the cell surface due to pathologic conditions. At the cell membrane, DAMP molecules associate with docking proteins, such as HLA I and potentially HSPGs, which influence the signal transduced through the NCR. This study is also of special significance as it challenges the current paradigm of NK cell receptor interactions with HLA I as the NCRs are believed to not interact with HLA I [8,38].

Because current cancer therapies fail to provide a permanent solution through complete recovery, new approaches must be used to target those cells which are responsible for relapse and metastasis, potentially CSCs. This study demonstrates a novel yet simple model to study the core process of metastasis and generation of migrating CSCs. Such models can help develop reliable surrogate assays which are desperately needed to enhance drug development in fighting cancer. Furthermore, this study demonstrates the potential of PCNA as a novel marker for CSCs. Currently, markers for EMT and CSCs do not encompass all tumors, as antigenic markers vary between cancer types [79]. In the clinical setting, metastatic cells easily escape detection. Thus there is an urgent need to optimize markers for CSCs to increase their detection and help clinicians decide which therapies are more appropriate for the patient. Overall, identification of PCNA as a novel marker for CSCs will be a major advance in cancer biology with heavy implications in grading of tumor progression, detection of metastasis, and potential therapeutic targets of metastatic CSCs in circulation, enabling the elimination of the biggest factor in cancer associated mortalities, metastasis. Furthermore, this study also lays the ground work to establish extracellular PCNA as a novel marker of B cell lymphoma.

Hypothesis

PCNA colocalizes with HLA I on the surface of tumor cells and enables escape from NK cell effector function. PCNA is a potential marker for metastatic cancer stem cells.

This hypothesis was evaluated under three specific aims.

Specific Aim I

PCNA associates with HLA I on the surface of target cells to form a complex ligand for NKp44

Specific Aim II

NKp44 recognition of cell surface PCNA in complex with HLA I inhibits NK cell cytolytic function and secretion of IFN- γ and TNF- α

Specific Aim III

Extracellular PCNA is a marker for metastatic cancer stem cells and Diffuse B Cell Lymphoma

Figure 1.1 Natural Cytotoxicity Receptor Ligands.

NKp30 recognizes the transmembrane protein B7-H6 expressed specifically by tumor cells.

NKp30 also recognizes BAT3, also known as BAG6, released by tumor on exosomes or in soluble form, which can activate or inhibit NK cell function respectively. NKp44 recognizes PCNA resulting in inhibition of NK cell effector responses. MLL5 is an activating ligand for NKp44, released to the cell surface by uninfected CD4⁺ T cells during HIV infection. A tumor ligand for NKp46 has yet to be identified. Adapted from Koch, *Trends in Immunology* 2013 [28].

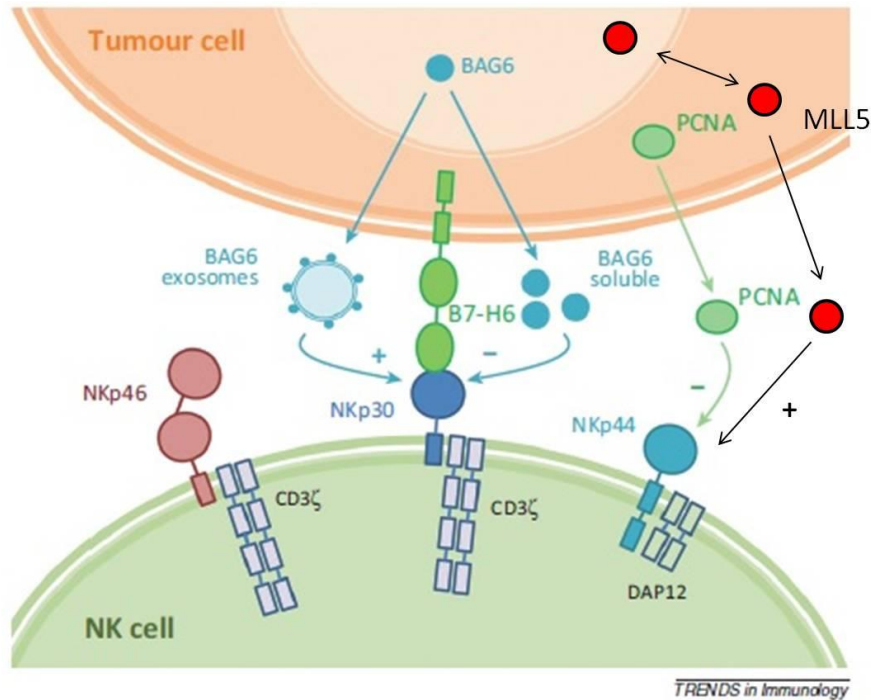


Figure 1.2 Dimeric Structure of NKp44 Binding Cleft.

The surface groove of NKp44 is made by two facing β hairpin loops extending from the Ig fold core. Patches of positively charged residues are found on opposite sides of the dimer. One protein subunit is depicted as a molecular surface while the other subunit is shown as a worm representation. Adapted from Cantoni, *Structure* 2003 [49].

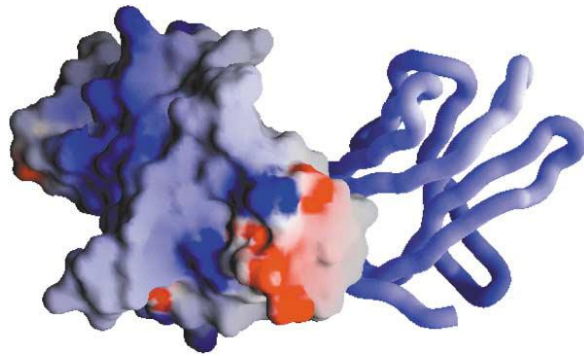


Figure 1.3 Natural Killer Cells and Development of the Placenta.

Left panel shows early stages of embryo development and implantation of blastocysts into the uterine epithelium. In the right panel, interactions between trophoblast cells, maternal cells, and decidual NK cells facilitate invasion of trophoblast cells, formation of the placenta, and remodeling of spiral arteries. Adapted from Parham, *Nature Reviews Immunology* 2013 [125] .

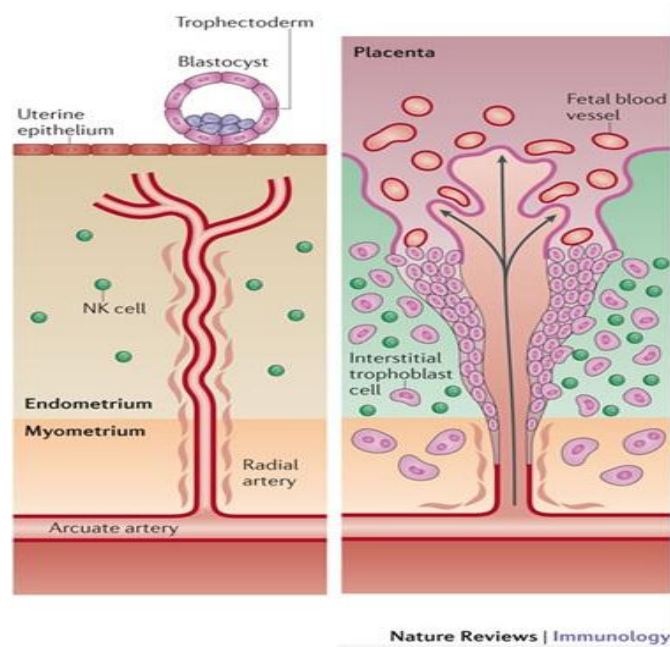


Figure 1.4 Cellular Complexity Found Within Tumors

Cells within a tumor are heterogeneous, composed of a range of different cell types including immune, fibroblast, endothelial, and epithelial cells. Epithelial cells of a solid tumor vary in terms of differentiation and rate of proliferation. Rare subsets of cells, termed cancer stem cells, retain self renewing properties. These cells drive tumor growth and contribute to metastasis and relapse after therapy.

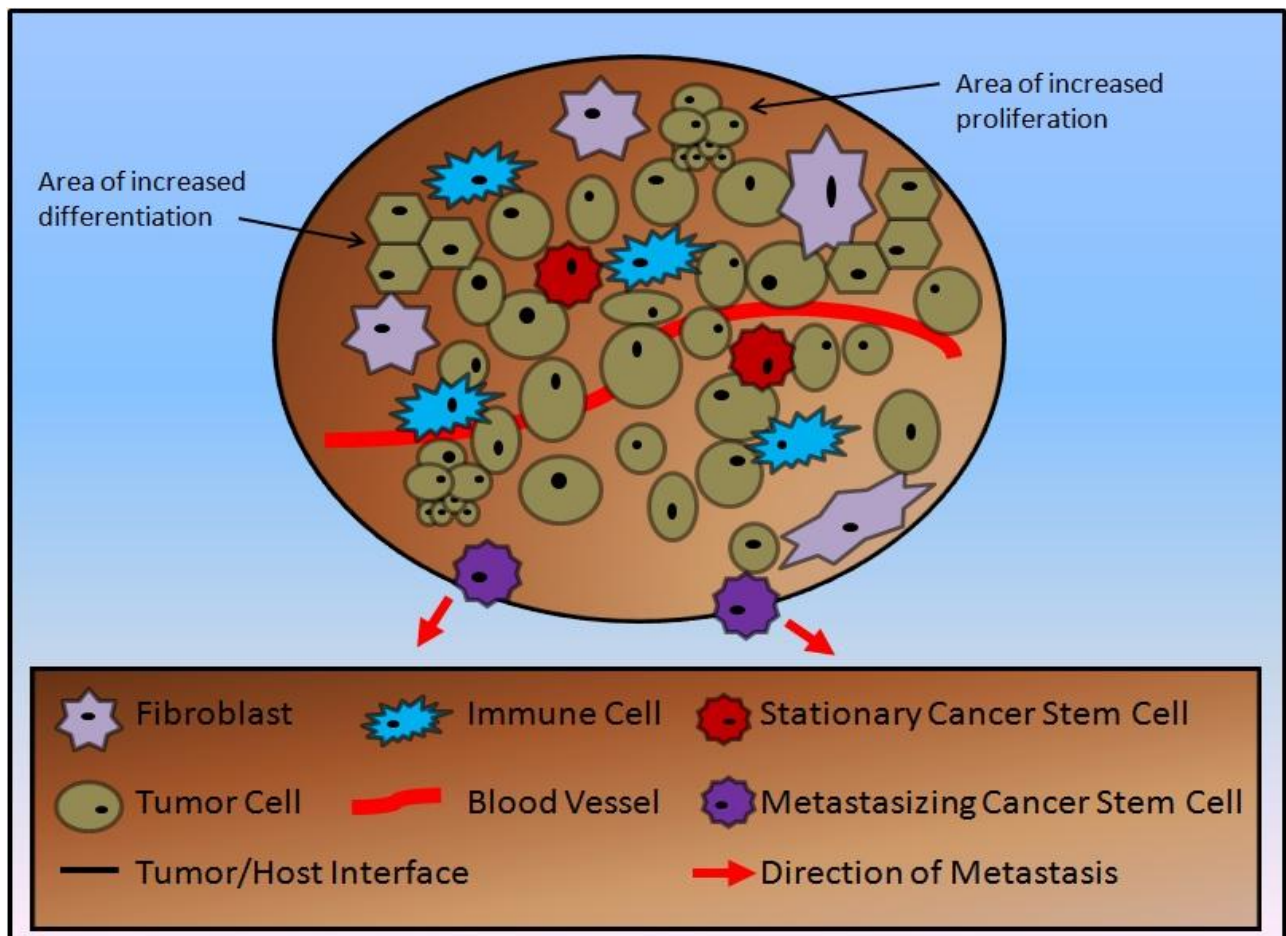


Figure 1.5 Migrating Cancer Stem Cell Model.

Normal stem cells are located in basal crypts of normal colon mucosa. Stationary CSCs become embedded in benign adenomas. In carcinomas, EMT occurs at the mucosa submucosa border, inducing migrating CSCs and metastasis from the original tumor. Adapted from Brabletz, *Nat. Rev. Cancer* 2005 [81].

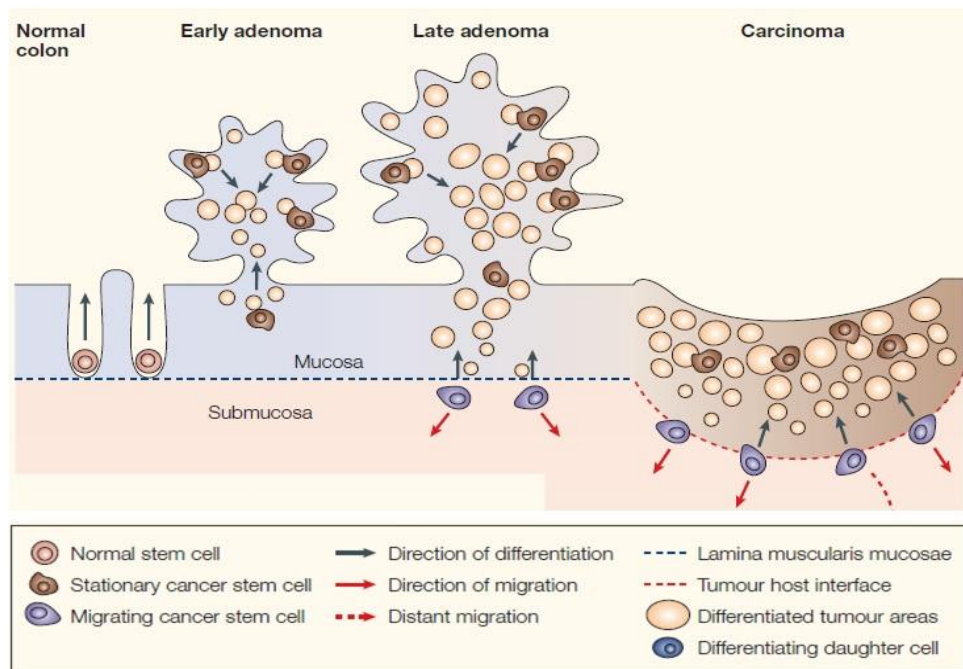
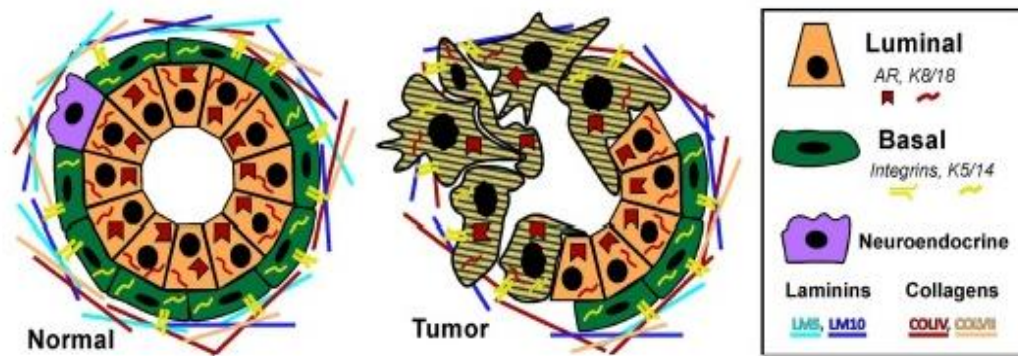


Figure 1.6 Epithelial Structure in Normal Versus Cancerous Prostate.

The normal prostate epithelium is composed of a bilayer of basal and luminal cells surrounded by laminins and collagens. In prostate tumors, the organization of the epithelium is perturbed, characterized by a loss of basal cells and alterations in the matrix. Adapted from Frank, *Frontiers in Oncology*, 2013 [109].



CHAPTER II

MATERIALS AND METHODS

Maintenance of Cell Lines

The human Diffuse B cell lymphoma cell line, DB (ATCC CRL-2289), was maintained in RPMI 1640 supplemented with 10% FetalPlex (Gemini Bio-Products), 2mM glutamine, 10mM HEPES, 10mM Sodium Pyruvate, 10mM non essential amino acids, and antibiotic-antimycotic solution containing penicillin G, streptomycin sulfate, and amphotericin B (Life Technologies). HEK-293 (ATCC CRL-1573), Jurkat (ATCC-TIB-152), MDA-MB-231 (ATCC HTB-26), PC3 (ATCC CRL-1435), DU145 (ATCC HTB-81), and MCF7 (ATCC HTB-22) cells were grown in RPMI 1640 supplemented as above, except with 10% Fetal Bovine Serum (Atlanta Biologicals). Human NK cell lines NK92 (ATCC CRL-2407) and NK92-MI (ATCC CRL-2408), constitutively expressing IL-2, was maintained in Minimum Essential Medium Alpha Medium (Life Technologies) supplemented with 12.5 % Fetal Bovine Serum, 12.5% Donor Horse Serum (Atlanta Biologicals), 0.2 mM inositol, 0.1 mM 2-mercaptoethanol, and 0.02 mM folic acid. NK92 cells were supplemented with 100 U/mL recombinant IL-2 (Gemini Bio-Products). All cells were cultured at 37°C in a humidified 5% CO₂/95% air environment.

Construction and expression of soluble NKp44-Ig fusion protein

Soluble NKp44-Ig fusion protein was previously constructed in our lab by fusing the extracellular domain of NKp44 with the Fc portion of human IgG. The extracellular domain of NKp44 was amplified by PCR (forward primer NKp44NheIFP-5' TCGCTAGCGCAATCCAAGGCTCAGGT-3' and reverse primer NKp44BamHIRP-5' CTCGGGATCCGTGTCTGCAGG GCCA-3'). The amplified product was subcloned in front of the human Fc gene at *Nhe I* and *BamH I* cloning sites in pCD5 vector, which contains the CH2 and CH3 regions of the human IgG1. Soluble NKp44-Ig fusion protein was produced by transiently transfecting the plasmid into HEK-293 cells using Fugene-6 transfection reagent (Roche Diagnostic Corporation). Cells were cultured in Optimem I (Life Technologies) reduced serum free media during transfection. Supernatants collected on days 2 and 3 after transfection were centrifuged to remove cellular debris, and then concentrated to 1 µg/ul with a 35,000 molecular weight centrifugal concentrator. Concentrated supernatants were verified for the presence of functional fusion protein by flow cytometry and western blotting. Supernatants of untransfected HEK-293 cells cultivated in Optimem I media were concentrated and used as negative controls in flow cytometry utilizing NKp44 fusion protein. A dose response curve was generated to determine optimum binding of NKp44-Ig prior to fusion protein use, which determined 70 µg of NKp44-Ig at a concentration of 1 µg/ul produces the largest shift of peak fluorescence without oversaturation.

Expression of Ligand for NKp44, HLA I, and Extracellular PCNA by Flow Cytometry

To determine NKp44 ligand expression, DB, DU145, MDA-MB-231, MCF7, and PC3 cells were incubated with 70 µg of NKp44-Ig (1 µg/ul) fusion protein for 45 minutes followed by 2.5 µg of anti-hIgG-Fc-PE (Beckman Coulter) for 30 minutes. DB cells were first incubated with Human IgG Fc fragment (Rockland) to block interactions with fusion protein and Fc receptors. Cells were incubated with 2.5 µg of W6/32 anti-HLA I antibody (Biolegend) for 30 minutes and detected with 2.5 µg anti-mIgG (H+L)-PE (Beckman Coulter) to determine HLA I expression. To block NKp44-Ig binding to DB cells, cells were first incubated with 2.5 µg of anti-HLA I. Cells were then incubated with 70 µg of NKp44-Ig fusion protein for 45 minutes followed by 2.5 µg of anti-hIgG-Fc-PE for 30 minutes. As negative controls, cells were incubated with 2.5 µg of mouse IgG2a-FITC isotype for HLA I expression or concentrated untransfected HEK293 supernatant and anti-hIgG-Fc-PE for NKp44 ligand expression. DB cells were incubated with 2.5 µg of anti-PCNA-Alexa Fluor 488 (Biolegend) versus mIgG2a isotype to establish cell surface expression of PCNA. To determine extracellular PCNA expression in DU145, PC3, MDA-MB-231, and MCF7 cell lines, cells were either gently scraped from tissue culture flasks or floating cells were harvested from the supernatant of tissue culture flasks and pelleted by centrifugation. Cells were stained with 2.5 µg of anti-PCNA-Alexa Fluor 488. All incubations were performed on ice for 30 minutes followed by analysis on a Beckman Coulter Cytomics FC 500 Flow Cytometer.

Colocalization of Extracellular PCNA and HLA I or CD63 by Live Imaging Confocal Microscopy

For HLA I and PCNA colocalization experiments, DB, DU145, PC3, MDA-MB-231, and MCF7 cells were incubated with 2.5 µg of W6/32 anti-HLA I antibody followed by 2.5 µg anti-

mouse IgG2a-Dylight 594 (Biolegend). Cells were then stained with 2.5 µg of anti-PCNA-Alexa Fluor 488 and DAPI (500 nM). For DU145, PC3, MDA-MB-231, and MCF7 cell lines, cells were seeded onto cover slips in a 24 well plate the day before analysis and cultured overnight to 95% confluency. Cells were then stained and analyzed the next day. Alternatively, on the day of analysis cells were detached from tissue culture flasks with EDTA or harvested as floating cells from the supernatant of tissue culture flasks and stained in 1.7 mL microcentrifuge tubes prior to mounting on a slide. For colocalization of PCNA and exosomes, cell were prepared in the same manner and stained with 2.5 µg anti-PCNA-Alexa 488 and .5 µg anti-CD63-Alexa 647 (Biolegend). Because exosome release is an active process, cells were gently washed and fixed with 4% paraformaldehyde immediately after staining. All cells were imaged on the Zeiss LSM 510 Confocal Laser Microscope using 40x, 1.2NA, 0.28 WD (water), C-Apochromat objective utilizing 561 nm, 405nm, 633nm, and 488 nm wavelengths.

Coimmunoprecipitation of PCNA with anti-β-2-microglobulin antibody

DB and DU145 cells were lysed utilizing a buffer consisting of 0.5% nonidet P-40, 50mM Tris-HCl pH 7.6, and 5mM MgCl₂. Immunoprecipitations were performed using Catch and Release v2.0 (Millipore) according to manufacturer's specifications. DB cells express endogenous immunoglobulins and DU145 is reported to express cancerous immunoglobulins [126]. Therefore, to prevent cross reaction and cluttering when detecting PCNA through western blotting, endogenous and cancerous immunoglobulins were removed by incubating cell lysate with affinity capture ligand prior to immunoprecipitation with anti-β-2-microglobulin. Affinity capture ligand is provided in the immunoprecipitation kit and links the Fc portion of

immunoglobulins to resin within a spin column. The pass through contains cell lysate removed of immunoglobulins derived from DB and DU145 cells. Pass through, termed precleared lysate, was analyzed by western blot to ensure removal of immunoglobulins and then used for immunoprecipitation. 500 µg of precleared cell lysate was mixed with buffer, affinity capture ligand, and 3 µg of either antibody against β -2-microglobulin (BD Biosciences) or mouse IgM isotype (BD Biosciences) and incubated in spin columns at 4°C for 1 hour. Elutions were performed with native buffer. Samples were denatured with 1% DTT, heated for 1 hour at 50°C to ensure dissociation of membrane protein aggregates and resolved on a 10% SDS-PAGE gel. After transfer to nitrocellulose, membranes were probed with anti-PCNA (1:2,500) for 1 hour and anti-mIgG2a-HRP for 45 minutes (1:2000) (Biolegend). Chemiluminescent imaging was performed with Immobilon (Millipore).

Isolation and Cultivation of Primary NK Cells

Primary NK cells were isolated from Peripheral Blood Mononuclear Cells by depletion of non-NK cells through magnetic microbead negative selection NK isolation kit (Miltenyi Biotec). Primary NK cells were cultured in RPMI media supplemented with 15% FBS and 1000 units/mL recombinant human IL-2 (eBioscience) for one week prior to use. NKp44 expression was confirmed by flow cytometry. Cells were cultured at 37°C in a humidified 5% CO₂/95% air environment.

⁵¹Cr release assay

DB cells and NK92-MI cells were incubated with Human IgG Fc fragment to block interactions with Fc receptors on both cells prior to use. DB cells were labeled with ⁵¹Cr for 1 hr at 37°C and then incubated with either 15 µg of NKp44-Ig, 1 µg anti-PCNA, 1 µg anti-HLA I, or mIgG2a isotype. Cells were then incubated with NK92-MI at ratios of 25:1, 5:1, and 1:1 for 4 hours at 37°C in a 96-well round bottom plate. NK92-MI cells were previously confirmed to express NKp44 after two weeks of culture. Percent specific lysis was compared to DB cells incubated with mIgG2a isotype antibody or with no antibody (No Blocking) as a positive control of cell lysis under unblocked conditions. Alternatively, NKp44 was blocked on NK92-MI cells with 2.5 µg of anti-NKp44 antibody (Biolegend) or 2.5 µg mIgG1 isotype control antibody prior to incubation with DB cells incubated with mIgG2a isotype control or anti-HLA I. Supernatants were collected and percent specific lysis was calculated. Experiments were performed in triplicate. ⁵¹Cr release assay was performed with primary NK cells in the same manner as with NK92-MI cells, but at ratios of 10:1, 5:1, and 1:1.

IFN-γ and TNF-α ELISA

DB cells were first incubated with Human IgG Fc fragment to block interactions with the Fc region of antibodies and fusion proteins. NK92 cells were also incubated with Human IgG Fc fragment to prevent ADCC during incubation with DB cells and antibodies. Prior to incubation with NK92 cells, DB cells were incubated with either 15 µg of NKp44-Ig, 1 µg anti-PCNA, 1 µg anti-HLA I, or 1 µg mIgG2a isotype. NKp44 was blocked on NK92 cells with 2.5 µg of anti-NKp44 antibody or 2.5 µg mIgG1 isotype control antibody prior to incubation with DB cells

incubated with mIgG2a isotype control or anti-PCNA. 1.5×10^5 DB cells and 5×10^4 NK92 cells per well were then incubated for 18 hours at 37°C in a 96-well round bottom plate. Wells were plated in triplicate. IFN- γ and TNF- α levels were measured using the Human IFN- γ and TNF- α ELISA Ready-SET-Go! (eBioscience) according to manufacturer specifications. Briefly, 96-well ELISA plates were coated with capture antibody overnight at 4°C . After washing and blocking for 1 hour, 100 μL of supernatant from DB and NK92 cell incubation was added to the ELISA plate and incubated overnight. A standard was generated with recombinant IFN- γ or TNF- α and serially diluted, with a top standard of 500 picograms/mL. After overnight incubation at 4°C , the ELISA plate was washed and incubated with detection antibody for 1 hour and detected with an avidin-HRP conjugated antibody. After addition of substrate for 15 minutes and adding phosphoric acid to stop the reaction, the plate was analyzed on a plate reader at 450 nm. Experimental wells receiving antibodies were compared to DB and NK92 incubation with no blocking and blocking with control antibody. Statistical significance was determined using a paired T Test of experimental wells receiving antibody blocking compared to positive control wells receiving no antibody.

Analysis of CD44/CD24 Expression by Flow Cytometry

DU145, PC3, MDA-MB-231, and MCF7 cells were cultured to 95% confluency in 175mL tissue culture flasks. Floating cells were first harvested by removal and centrifugation of culture supernatant. Cells attached to the tissue culture surface were then removed by gentle scraping with a sterile cell scraper in 15 mL of media. Cells were washed with PBS/1% BSA twice and 1×10^6 cells of each population were allotted to microcentrifuge tubes for antibody staining.

Cells were incubated with 2.5 µg of anti-PCNA-Alexa Fluor 488, .125 µg anti-human/mouse CD44-PE (eBioscience), and .25 µg anti-CD24-APC (eBioscience). As a control, cells were also stained with the appropriate isotype controls including, mouse IgG2a-Alexa-488, rat IgG2b-PE, and mouse IgG1-APC. Incubations were performed on ice for 30 minutes. Cells were analyzed on a Beckman Coulter Cytomics FC 500 Flow Cytometer.

Analysis of EMT/Stem cell markers by Confocal Microscopy

DU145, PC3, MDA-MB-231, and MCF7 cells were seeded to cover slips the day before analysis and cultured overnight to 95% confluency. The day of analysis, cover slips were transferred to new wells containing 150 µL of PBS/1% BSA and incubated with 2.5 µg of anti-PCNA-Alexa Fluor 488. Cover slips were gently washed with PBS/1% BSA and then stained with 1 µg anti-CD325 (N-Cadherin)-APC, 1 µg anti-CD324 (E-Cadherin)-eFluor 660, or 1 µg anti-Vimentin-eFluor 570 (eBioscience) in 150 µL of PBS/1% BSA. After washing, cells were stained for 15 minutes with 500 nM DAPI in PBS/1% BSA. Control cover slips were stained with mouse IgG2a-Alexa-488, mouse IgG1-APC, rat IgG1-eFluor 660, and mouse IgG1-eFluor 570. Floating cells were harvested on the day of analysis cells from the supernatant of tissue culture flasks and stained in 1.7 mL microcentrifuge tubes with the same antibodies. Antibody incubations were performed on ice for 30 minutes. Cover slips and stained floating cells were mounted on slides and imaged on the Zeiss LSM 510 Confocal Laser Microscope using 40x, 1.2NA, 0.28 WD (water), C-Apochromat objective utilizing 488 nm, 633 nm, 561 nm, and 405 nm wavelengths.

PCR analysis of EMT/Stem cell maintenance genes

DU145, PC3, MDA-MB-231, and MCF7 cells were cultured to 95% confluency in 175mL tissue culture flasks as before. Cells were harvested by gentle scraping with a cell scraper in culture supernatant to also collect floating cells. After centrifugation, $1-10 \times 10^8$ cells were washed and stained with 3 μ g of anti-PCNA-Alexa Fluor 488. Cells were then sorted on the Cytopia Influx Cell Sorter into positive and negative populations based on cell surface PCNA or CD44 expression. Total RNA was extracted from cells using RNA STAT-60 according to manufacturer's protocol (Tel-test Inc). cDNA was reverse transcribed from 1 μ g of RNA using Omniscript RT Kit (Qiagen) with random and oligo dT primers. cDNA was diluted after synthesis with 1X RT buffer to generate 100 ng/ μ L of cDNA template. Standard PCR was performed for the genes in table 1 with the listed primers. For further analysis of relative transcript levels, quantitative PCR was performed with the Taqman assay for PCNA, CD133, BMI-1, and Zeb1 on the StepOne Real-Time PCR System (Applied Biosystems). Expression of genes were normalized to β -actin and expressed relative to gene expression in PCNA⁻ and CD44⁻ cells.

Spheroid formation assay

DU145, PC3, MDA-MB-231, and MCF7 cells were cultured to 95% confluency in 175mL tissue culture flasks as before. Cells were harvested by gentle scraping with a cell scraper in culture supernatant to also collect floating cells. After centrifugation, $1-10 \times 10^8$ cells were washed and stained with 3 μ g of anti-PCNA-Alexa Fluor 488. Cells were then sorted on the Cytopia Influx Cell Sorter into positive and negative populations based on cell surface PCNA

expression. 1×10^6 cells of each population was serially diluted 6 times and plated on a non-adherent 24-well tissue culture plate with 1 mL of Keratinocyte Serum Free media supplemented with human recombinant Epidermal Growth Factor 1-53 and Bovine Pituitary Extract according to manufacturer's specifications (Life Technologies). Cells were incubated for 1 week at 37°C in a humidified 5% CO_2 /95% air environment. Wells were photographed under 20x and 40x magnification.

Invasion assay

DU145, PC3, MDA-MB-231, and MCF7 cells were cultured to 95% confluency in 175mL tissue culture flasks as before. Cells were harvested by gentle scraping with a cell scraper in culture supernatant to also collect floating cells. After centrifugation, $1-10 \times 10^8$ cells were washed and stained with 3 μg of anti-PCNA-Alexa Fluor 488. Cells were then sorted on the Cytopia Influx Cell Sorter into positive and negative populations based on cell surface PCNA expression. After washing in RPMI media lacking serum, 100 μL s containing 5×10^4 cells from each population was added to tissue culture inserts with .8 μm pores layered with 300 $\mu\text{g}/\text{mL}$ Matrigel (BD Biosciences) in a 24 well plate. As a control, cells were added to tissue culture inserts without matrigel. Plates were incubated overnight at 37°C in a humidified 5% CO_2 /95% air environment. The next day, tissue culture inserts were washed in PBS. Cells were stained with crystal violet and cells which migrated to the lower side of the insert were enumerated. Percent invasion was calculated as a ratio of cells migrating through matrigel to cells migrating without matrigel. In the same manner, percent invasion was determined for populations of

cells growing within the monolayer or found naturally detached, without sorting for extracellular PCNA expression.

Cell Cycle Analysis by Flow Cytometry

DU145, PC3, MDA-MB-231, and MCF7 cells were cultured to 95% confluency in 175mL tissue culture flasks as before. Cells were detached with a cell scraper in 15 mL of RPMI media and pelleted by centrifugation. After washing with PBS/1% BSA, 1×10^7 cells from each cell line was stained with 2 μ g of anti-PCNA-Alexa Fluor 488 for 30 minutes on ice. Cells were washed twice with cold PBS and then resuspended in .3 mL 50% FBS in a 15 mL tube. Cells were fixed by adding .9 mL 70% ethanol in a drop wise manner while gently mixing the cells and stored at 4°C for 2 hours. Cells were then pelleted, washed twice with cold PBS, resuspended in 1 mL propidium iodide (PI) solution (5mL PBS, 100ug/ml RNase, 10 ug/ml PI), and incubated overnight at 4°C. The next day, cells were analyzed on the Beckman Coulter Cytomics FC 500 Flow Cytometer. For Nocodazole treatment, cells were plated in 10 cm plates and grown in 10 mL of RPMI media with 10% FBS until confluency reached 95%. Cells were then halted in the G2/M phase overnight by adding 100ng/mL of Nocodazole dissolved in DMSO to culture dishes. As a control, cells in one dish received DMSO. The next day, cells were detached with a cell scraper and stained for PCNA and propidium iodide solution as before and analyzed by flow cytometry.

PCNA Expression on Peripheral Blood Mononuclear Cells

Human venous blood was withdrawn from a willing healthy volunteer. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on 15 mL Histopaque-1077 (Sigma Chemicals) in Leucosep tubes. Remaining red blood cells were lysed by adding ACK lysis buffer. Cells were washed in PBS and 1×10^6 cells were allotted into microcentrifuge tubes for staining. Cells were stained with anti-PCNA-Alexa Fluor 488 and listed groups of antibodies in separate tubes to mark specific immune cells. Anti-CD3-PE/Cy7 (BD Biosciences) was used to mark T cells. Anti-CD14-APC/Cy7 (Biolegend) and anti-CD16-APC (Biolegend) were used to identify monocytes. B cells were marked with anti-CD19-PE/Tx Red (Genway). NK cells were identified using anti-CD56-APC (Biolegend) and anti-CD3-PE/Cy7. To further characterize monocytes and T cells by 2B4 expression, cells were stained with anti-PCNA-Alexa Fluor 488, anti-2B4-PE (Biolegend), and either anti-CD3- PE/Cy7 or anti-CD14-APC/Cy7. To activate PBMCs, 1×10^6 cells were plated in a 12-well non-adherent plate to prevent attachment of monocytes. Cells were activated overnight in 1 mL of RPMI 10% FBS with 5 ng/mL phorbol 12-myristate 13-acetate (PMA) and 1 μ M ionomycin at 37°C in a humidified 5% CO₂/95% air environment. The next day, cells were removed, washed in microcentrifuge tubes with PBS/1% BSA, and stained as before and with anti-CD80-APC (Biolegend) to confirm activation. Cells were analyzed on the Beckman Coulter Cytomics FC 500 Flow Cytometer.

Table 1 PCR Primers Used to Amplify Epithelial-Mesenchymal Transition and Cancer Stem

Cell Genes

	5' Primer	3' Primer
Zeb1	TGCACTGAGTGTGGAAAAGC	TGGTGATGCTGAAAGAGACG
Zeb2	CGCTTGACATCACTGAAGGA	CTTGCCCACTCTGTGCATT
Twist1	ACCATCCTCACACCTCTGCATTCT	TGCAGGCCAGTTTGATCCCAGTAT
Twist2	AGCAAGAAGTCGAGCGAAGA	CAGCTTGAGCGTCTGGATCT
Snail1	ACCCACATCCTTCTCACTG	TACAAAAACCCACGCAGACA
Slug	CTTTTCTTGCCCTCACTGC	ACAGCAGCCAGATTCTCAT
Snail3	AAATCAATGGTGCCTGCTCT	GGCTGTCTTTGAGGGGTACA
CD133	CAGAGTACAACGCCAAACCA	AAATCACGATGAGGGTCAGC
BMI	GGAGACCAGCAAGTATTGTCCTTTG	CATTGCGCTGGGCATCGTAAG
Oct3/4	CGACCATCTGCCGCTTTGAG	CCCCCTGTCCCCATTCTTA
Nanog	CAACTGGCCGAAGAATAGCA	GCAGGAGAATTTGGCTGGAA

CHAPTER III

PCNA COLOCALIZES WITH HUMAN LEUKOCYTE ANTIGEN CLASS I MOLECULES TO FORM A COMPLEX LIGAND FOR NKp44

Elusive for ten plus years since the discovery of NKp44, ligands recognized by this NK receptor are finally coming to light. Recent evidence demonstrates NKp44 recognition of PCNA; however, numerous other reports foreshadow the discovery that NKp44 recognizes PCNA in association with another molecule which docks PCNA to the cell surface. These studies suggest PCNA may interact with HLA I on the cell surface to form a complex ligand recognized by NKp44. HLA I is an important molecule expressed by nearly all cells of the body and is vital in regulating NK cell function. When recognized by some KIR receptors of the NK cell, HLA I inhibits NK cell effector functions. Epitomizing the importance of HLA I in immune regulation is its prominent role in host versus graft disease. When tissue transplants express a different repertoire of HLA I molecules than the patient receiving the graft, the tissue is rejected by NK cells. Subsequent studies involving HLA I led to the long standing tenet that NK cell inhibitory receptors act in an HLA I restricted manner while other NK cell receptors, including the NCRs, do not interact with HLA I [38]. This study aims to challenge this paradigm, demonstrating HLA I is a part of a complex ligand recognized by NKp44.

Specific Aim I

PCNA associates with HLA I on the surface of target cells to form a complex ligand for NKp44

Rationale

In the search to identify a ligand for NKp44, several key pieces of evidence suggest HLA I plays a role in ligand formation. Betser-Cohen et al. recently found HLA I proteins coimmunoprecipitate with anti-NKp44 antibodies; reciprocally, NKp44 coimmunoprecipitates with anti- β -2-microglobulin antibodies [127]. Additionally, the Nef protein of HIV prevents surface expression of MLL5 on CD4 infected T cells, which is also consistent with the ability of Nef to retain HLA I intracellularly [68,128]. Finally, the NKp30 ligand, Bat3, colocalizes with HLA I on the extracellular membrane of tumor cells, activating NK cell effector functions [36,37]. The NCRs are known to bind HSPGs, which can sequester other proteins to the cell surface; thus, it is conceivable that HLA I may harbor protruding molecules facilitating interactions with other proteins, enabling recognition by the NCRs. Interestingly, all 50 alleles of HLA Class A, B, and C molecules harbor an Asparagine at position 86, close to the residues on the α 1 helix which determine interactions with human NK receptors [129]. This site allows for attachment of N-linked glycan structures which could enable binding of other proteins like PCNA or Bat3 [129]. Electron density mapping of the glycan structure suggests it is flexible and could serve as a ligand for other receptors or block access to HLA I molecules [129]. Additionally, HLA-A and – B are almost exclusively disialylated, resulting in these molecules having a negative charge, a characteristic of NKp44 ligands [129]. This negative charge combined with a protruding

oligosaccharide could potentially facilitate interactions with PCNA and NKp44. Taking into context with other current studies, the NCRs potentially have the ability to interact with HLA I, HSPGs, or other cell surface molecules in conjunction with soluble proteins, such as Bat 3, PCNA, MLL5 or other DAMPs on the cell surface of tumors [36]. Therefore, the NCRs may recognize DAMPs on the cell surface in association with a docking protein. These reports imply HLA I on the surface of target cells participates in NKp44 mediated recognition by NK cells.

Results

Anti-HLA I antibody blocks binding of NKp44 fusion protein

To investigate potential interactions between HLA I and NKp44, a cell line which consistently expresses a ligand for NKp44 was identified utilizing a soluble NKp44 fusion protein, NKp44-Ig (Figure 3.1). Tumor cell lines originating from various tumors models (Table 2) were analyzed for fusion protein binding by flow cytometry (Figure 3.2). Overall, cell lines analyzed express varying levels of a ligand for NKp44 while the Jurkat cell line did not express a ligand. A Human Diffuse B cell Lymphoma (DB) cell line was identified expressing elevated levels of a ligand for NKp44 and was used for further study (Figure 3.2A).

As an initial experiment to investigate potential interaction between NKp44 and HLA I, W6/32 antibody, a pan HLA I antibody which binds HLA-A, B, C, and non-classical HLA-E, was used in an attempt to block the binding of NKp44-Ig to DB cells (Figure 3.3). First, HLA I expression on DB cells was confirmed by flow cytometry (Figure 3.3B). Incubating DB cells with 2.5 µg of anti-HLA I antibody fully saturates the cells as increasing amounts do not result in any

further shift of peak fluorescence away from isotype control staining. To then test if NKp44 may be interacting with HLA I, cells were first incubated with 2.5 μ g of unconjugated W6/32 antibody. After washing, cells were then incubated with 70 μ g NKp44 fusion protein and analyzed for fusion protein binding (Figure 3.3C). Prior incubation with W6/32 antibody eliminates binding of NKp44 fusion protein to near background levels, indicating potential interaction between NKp44 and HLA I.

PCNA colocalizes with HLA I on the cell surface

Since PCNA was recently identified as a ligand for NKp44, we next sought to visualize interactions between PCNA and HLA I on the surface of live DB cells by employing confocal microscopy. We first confirmed DB cells uniformly express PCNA on the cell surface by flow cytometry in Figure 3.3D. Flow cytometry analysis indicates a single population of DB cells which express PCNA on the cell surface as cells were not fixed or permeabilized prior to staining. For confocal imaging, DB cells were first stained with 2.5 μ g of W6/32 anti-HLA I antibody followed by detection with anti-mIgG2a-Dylight 594. Cells were then stained with anti-PCNA-Alexa Fluor 488 and examined under 40x magnification. As seen in Figure 3.4, PCNA localizes only to the extracellular membrane as cells were alive when imaged and not permeabilized before staining. Additionally, PCNA is concentrated in areas which colocalize with dense pockets of HLA I on the surface of numerous cells.

To further confirm PCNA and HLA I interactions, DB cell lysate was subjected to immunoprecipitation with anti- β -2-microglobulin or control mIgM antibody of irrelevant specificity (Figure 3.5). W6/32 antibody could not be used in this experiment since it is the

same isotype (mIgG2a) as PC-10, anti-PCNA antibody, used to probe immunoprecipitation elutions. Additionally, DB cells express endogenous immunoglobulins and DU145 cells are reported to express cancerous immunoglobulins [126]. Thus, cell lysate was cleared of immunoglobulins prior to immunoprecipitation with anti- β -2-microglobulin, as detailed in materials and methods, to eliminate any potential interference. Aliquots of native elutions were reduced with 1% DTT and heated to 50°C for one hour to dissociate possible membrane protein aggregates. After resolution on a 10% SDS-PAGE gel and protein transfer, membranes were probed with anti-PCNA antibody and detected with anti-mIgG2a-HRP. PCNA is detected in elutions from DB cell immunoprecipitations performed with anti- β -2-microglobulin (Figure 3.5, lane A), and in precleared lysate used as a positive control (Figure 3.5, lanes C). However, control mIgM antibody (Figure 3.5, lane B) does not precipitate PCNA. Coimmunoprecipitation of PCNA with anti- β -2-microglobulin antibody was also confirmed in DU145 prostate cancer cells (Figure 3.5, lanes D-F).

Other cell lines found to bind fusion protein by flow cytometry were also examined by confocal microscopy for colocalization of HLA I and PCNA. When grown on cover slips, very few cells express PCNA on the cell surface, but extracellular PCNA does colocalize with HLA I (Figure 3.6). In the MCF7 cell line, PCNA is not expressed on the cellular surface when grown on a cover slip. Interestingly, DU145 cells expressing cell surface PCNA are elevated above cells which are growing in the monolayer (Figure 3.7). These results contradict earlier flow cytometry results indicating a significant proportion of cells are binding NKp44 fusion protein. Since cells must be detached from growth surfaces for flow cytometry analysis, cells either detached from monolayer growth with EDTA or naturally detached and found floating in tissue

culture supernatant were also analyzed (Figure 3.9, 3.10). Regardless of detachment mechanism, numerous cells from all cell lines which grow in a monolayer express PCNA on the cell surface, colocalizing with HLA I, when detached.

To further corroborate these results, expression of cell surface PCNA was analyzed by flow cytometry on cells which were either gently scraped off growth surfaces or those naturally detached, free floating in culture supernatant (Figure 3.11, 3.12). Similar to results obtained by confocal analysis, populations which are detached by scraping contain very few cells expressing PCNA on the cell surface while populations naturally detached are enriched for PCNA expressing cells by up to 20%.

PCNA is not exclusively located on exosomes at the cell surface

Rosenthal *et al.* originally described PCNA as a ligand for NKp44 expressed on exosomes at the surface of tumors cells when in contact with an NK cell. Therefore, cells naturally detached from monolayer growth were examined for colocalization of PCNA and CD63, a common marker found on exosomes, by confocal microscopy (Figure 3.13). While most areas of cell surface PCNA appear to colocalize with CD63 expression on exosomes, PCNA is not exclusive to exosomes and is also found in some areas which are not stained for CD63. This suggests cell surface PCNA may be found on exosomes, but PCNA is also found directly on the cell surface.

Discussion

In order to investigate interactions between PCNA and HLA I, a cell line expressing a ligand for NKp44 is needed. Chimeric fusion proteins are popular tools in molecular biology to achieve this aim. They are typically constructed by fusing the extracellular domain of a cell surface receptor to another protein which stabilizes the entire assembly and enables a method to detect binding. The chimeric NKp44-Ig fusion protein used in this study contains two extracellular domains cloned from the NKp44 receptor, enabling ligation to molecules recognized by NKp44 (Figure 3.1). Fused to the extracellular domain is the Fc region of human IgG. The Fc region can then be bound by a secondary antibody conjugated to a fluorophore, allowing detection of fusion protein binding by flow cytometry. DNA encoding the fusion protein is then cloned into a plasmid vector, enabling transfection into mammalian cells, which produce and secrete the soluble fusion protein. The supernatants of transfected cells are collected, pooled, and concentrated based on molecular weight to purify the fusion protein. Isolated fusion protein is then incubated with cells lines, enabling detection of NKp44 ligand expression.

After analyzing cell lines of various tumor models, a cell line isolated from a patient with Diffuse B Cell Lymphoma (DB) was identified as an ideal candidate for further study based on elevated expression levels of an NKp44 ligand compared to other cell lines (Figure 3.2). The cell lines examined (Table 2) display varying levels of fusion protein binding, ranging from no binding in Jurkat cells to high binding of fusion protein to MDA-MB-231 cells, similar to levels found in DB cells.

As a pilot experiment to establish potential interaction between HLA I and NKp44, a pan HLA I antibody, W6/32, was utilized. If HLA I and NKp44 interact, incubation with anti-HLA I antibody prior to NKp44-Ig should block binding of the fusion protein. First, the functionality of NKp44-Ig (Figure 3.3A) and expression of HLA I on DB cells was confirmed (Figure 3.3B). When DB cells are incubated with HLA I antibody prior to NKp44-Ig, binding of fusion protein is blocked to near background levels, suggesting NKp44 associates with HLA I (Figure 3.3C). The epitope recognized by W6/32 comprises residues from all three heavy chain domains of HLA I and β -2-microglobulin, which are crucial for proper folding of the protein and presentation of antigen [130]. Thus, NKp44 interacts with fully assembled HLA I on the cell surface and not open conformers of HLA I which do not contain β -2-microglobulin.

Next, further confirmation of HLA I forming a ligand for NKp44 was sought through confocal microscopy. Since incubation with HLA I antibody blocks binding of the fusion protein, NKp44-Ig could not be used in conjunction with HLA I antibody for colocalization. To circumvent this problem, HLA I and PCNA antibodies were utilized for confocal analysis since PCNA was recently identified as a ligand for NKp44. First, expression of PCNA on DB cells was confirmed by flow cytometry (Figure 3.3D), which indicates PCNA is expressed on the cell surface. DB cells were then stained with HLA I and PCNA antibodies and analyzed by confocal microscopy at 40x magnification (Figure 3.4). By not permeablizing cells prior to staining, PCNA is found to be located at the cell surface, colocalizing with compact areas of dense HLA I staining. Association of PCNA and HLA I was confirmed by coimmunoprecipitation in DB and DU145 cells (Figure 3.5). These data demonstrate PCNA is localized to the cell surface and associates with HLA I on the surface of DB cells.

Cell lines which express lower levels of the NKp44 ligand were also evaluated for PCNA and HLA I colocalization (Figure 3.6). In contrast to DB cells, the other cell lines binding NKp44 fusion protein originate from solid tumors born in tissue, not free floating cells of the blood like B cells. Thus, these cell lines grow in colonies as a monolayer attached to the tissue culture surface, not free floating in suspension as DB cells do. To analyze these cells in their attached state, cells were seeded on cover slips and grown over night before incubation with antibodies and confocal analysis. MCF7 breast cancer cells grown on covers lips were first analyzed for PCNA and HLA I colocalization. While MCF7 cells express HLA I as expected, they do not uniformly display extracellular PCNA, contrary to DB cells (Figure 3.6). When examining DU145 cells grown on cover slips, an interesting phenomenon came to light. Several cells expressing extracellular PCNA appear elevated about 10 μm above cells growing in the monolayer, which do not express PCNA (Figure 3.7).

A caveat to culturing immortal solid tumor cell lines on a finite surface is the inevitability of cells running out of surface area for attachment (Figure 3.8). As colonies continuously grow, eventually, surface area for new daughter cells to attach to the tissue culture surface is severely limited or nonexistent (Figure 3.8B). In this event, new daughter cells rise above the monolayer and eventually detach naturally into the supernatant (Figure 3.8C). These cells can reattach in other areas with room for growth, creating a new colony, in a process similar to metastasis.

In the DU145 cell line, it appears PCNA expression at the cell surface is preferentially found on cells raised above the monolayer due to the lack of space for attachment. Since the colony no longer has room for these cells to seat to the tissue culture surface, these cells are released into the culture supernatant and notably adopt a spherical morphology. Cells which

have naturally detached from monolayer growth can be easily harvested from tissue culture supernatant by centrifugation. When naturally detached DU145 cells are examined, numerous cells express extracellular PCNA colocalizing with HLA I, contrary to the few cells expressing PCNA at the cell surface when grown on cover slips (Figure 3.9, 3.11, 3.12). This phenomenon was exaggerated in the MCF7 cell line. While MCF7 cells grown on a cover slip express very little to no extracellular PCNA (Figure 3.6), naturally detached MCF7 cells do express extracellular PCNA which colocalizes with HLA I (Figure 3.9). This event is not unique to MCF7 and DU145 cells. Naturally detached MDA-MD-231 and PC3 cells readily express extracellular PCNA colocalizing with HLA I, contrasting the limited expression of cell surface PCNA when these cell lines are grown on cover slips. To confirm these results observed under confocal microscopy analysis, extracellular PCNA expression in populations detached from monolayer growth by gentle scraping was compared to cell surface PCNA expression in cells naturally detached (Figure 3.11, 3.12). In all the solid tumor cell lines, extracellular PCNA expression is substantially up regulated in cells which are naturally detached, confirming results obtained from confocal microscopy (Figure 3.9, 3.10).

Clearly, some mechanism exists which shuttles PCNA to the surface of cells which have detached from monolayer growth. For this to occur, natural detachment is not necessary as cells detached from monolayer growth using PBS/1mM EDTA also display this phenotype (Figure 3.10). EDTA is a calcium chelator that binds calcium and prevents adherence between cadherins, detaching cells from monolayer growth. Unfortunately, aspects of PCNA transport to the cell surface are still a mystery as the protein does not contain a secretory sequence [131]. Attempts to inhibit cell surface PCNA in DB cells using Brefeldin A were unsuccessful,

indicating PCNA is not transported via the Golgi apparatus. Unconventional protein transport to the cell surface can occur by either direct transport from the cytoplasm across the plasma membrane, lysosomal secretion, exosome derived bodies, or vesicle shedding [132].

As calcium is involved in exosome release, application of EDTA to cell cultures also induces exosome release [133,134]. Exosomes are membranous nanovesicles created by inward budding of late endosomal membranes which form intraluminal vesicles inside intracellular multivesicular bodies (MVBs) [135]. MVBs accumulate proteins destined for degradation in the lysosome; however, MVBs can be redirected to the plasma membrane enabling release of exosomes into the extracellular environment [135]. Therefore, exosomes store intracellular content including proteins, mRNA, and microRNA which can be shuttled to the cell surface instead of the lysosome [135]. Several reports have confirmed the presence of PCNA in exosomes, including Rosenthal *et al.* which identified PCNA as a ligand for NKp44 [42,135,136]. The cell lines examined in this study express PCNA on exosomes in most areas at the cell surface, as visualized through areas of colocalization of PCNA and CD63, a member of the tetraspanin family commonly used to identify exosomes (Figure 3.13). However, not all PCNA observed at the cell surface colocalizes with CD63, suggesting PCNA is also found directly on the cell surface. It is possible that PCNA is found on the membranes of exosomes and MVBs which have been incorporated into the extracellular membrane of a cell during docking and release into the extracellular environment. Alternatively, PCNA may be delivered directly to cell surface in an unknown mechanism as intracellular mechanisms of transporting PCNA is still not completely understood.

Again, DB cells differ from the other cell lines used as they grow in tissue culture as suspension cells which do not attach to tissue culture surfaces. Thus, mechanical differences between attached cells and suspension cells must be involved in the mechanism shuttling PCNA to the cell surface. Cell growth in a monolayer, attached to a surface, allows for greater cell volume compared to cells which are in suspension [137]. This is due to increased contact points between the cell and the growth surface, which allows for increased spatial growth and structural stability, enabling the cell to increase its intracellular load [137]. When a cell detaches, volume of the cell decreases, producing spatial and volumetric constraints which reduce the amount of material able to be contained intracellularly without enlargement and substantial strengthening of the extracellular membrane [137]. As the cell detaches, it must compensate for this difference by reducing intracellular load, presumably through release of exosomes. Some exosomes can be docked extracellular, attached to the extracellular membrane. If the cell reattaches to a surface, exosomes can be reabsorbed without loss of intracellular material. Therefore, the cell can quickly adapt its intracellular volume through expulsion of exosomes without significant loss of intracellular content or wasting resources synthesizing materials anew that would be lost through a simple dump of intracellular content. This mechanism likely accounts for the difference between the data presented here and that presented by Rosenthal *et al.* which describes PCNA only on the surface of tumors cells attached to a cover slip and in contact with an NK cell.

Figure 3.1 Construction of NKp44-Ig Fusion Protein

The extracellular V-like domain of NKp44 was subcloned into the pCD5 vector in front of the gene fragment encoding the second and third constant heavy region of human IgG1. The plasmid is then transfected into HEK-293 cells cultured in serum free media for 2 days. HEK-293 cells produce and secrete the fusion protein, which is collected from culture supernatant and purified by concentration with a 35,000 molecular weight centrifugal concentrator to 1 µg /ul.

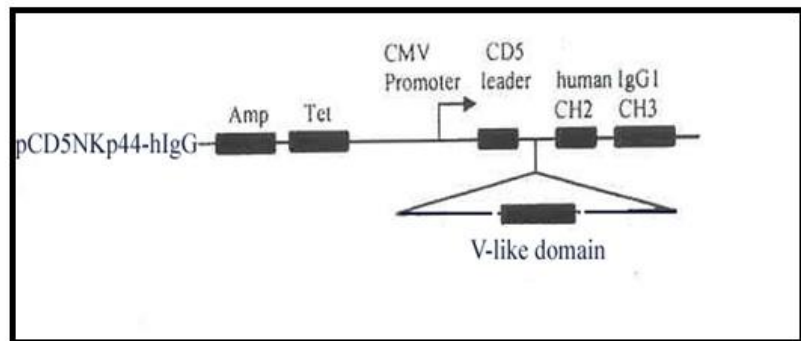
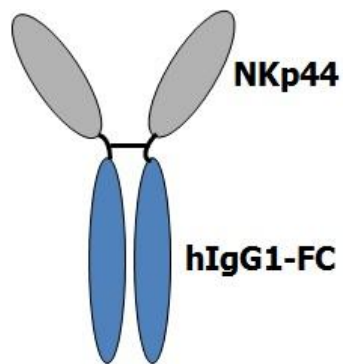


Table 2 Tissue Culture Cell Lines Evaluated for NKp44 Ligand Expression

Tissue culture cell lines modeling various cancers were evaluated for NKp44 ligand expression by flow cytometry. Cell lines binding fusion protein were utilized in subsequent experiments.

Cell Lines			
Name	Type	Growth	Fusion Protein Binding
DB	Diffuse B Cell Lymphoma	Suspension	+++
DU145	Prostate Cancer	Monolayer	++
PC-3	Prostate Cancer	Monolayer	+++
MDA-MB-231	Breast Cancer	Monolayer	+++
MCF-7	Breast Cancer	Monolayer	+
Jurkat	Acute T Cell Leukemia	Suspension	-

Figure 3.2 Identification of NKp44 Ligand Expressing Cell Lines

Various cell lines were incubated with 70 µg of NKp44-Ig fusion protein. Binding of fusion protein was detected using anti-Human IgG Fc-PE (filled histogram) and compared to negative control staining of cells with anti-Human IgG Fc-PE alone (empty histogram) by flow cytometry. Mean fluorescent intensity is indicated in the top right corner of each pane. A) Human diffuse B cell lymphoma cell line, DB. B) Human acute T cell leukemia, Jurkat. C) Human prostate cancer, DU145. D) Human prostate cancer, PC3. E) Human breast cancer, MDA-MB-231. F) Human breast cancer, MCF7.

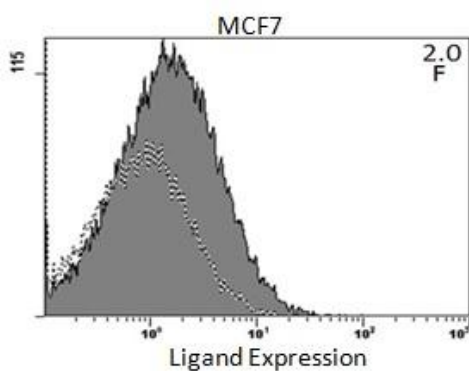
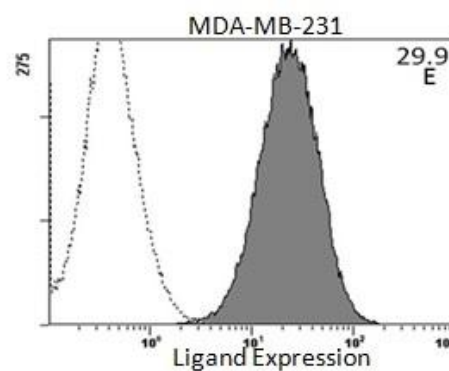
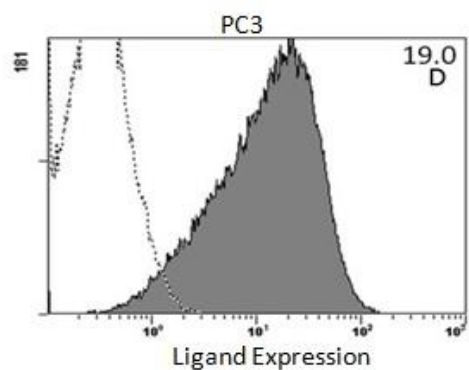
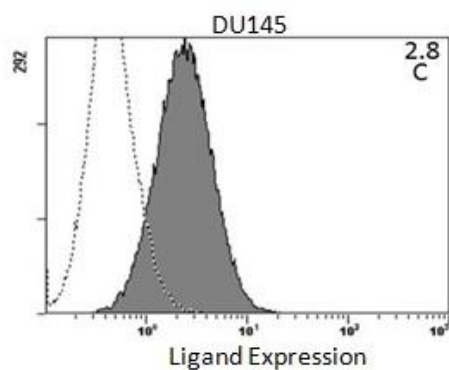
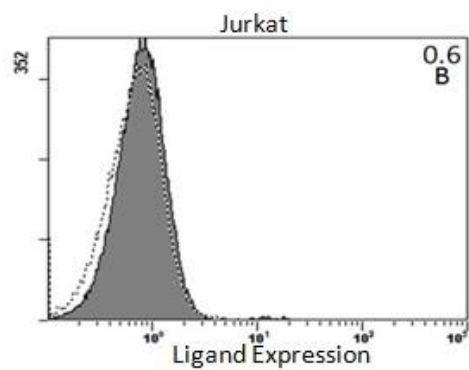
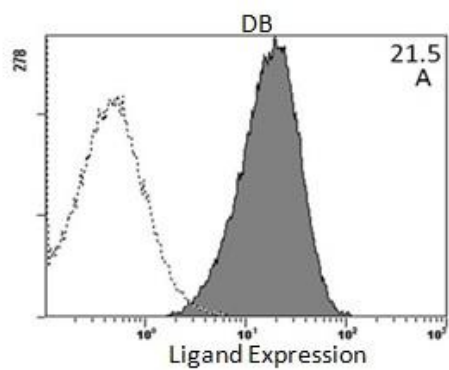


Figure 3.3 Anti-HLA I Antibody Blocks Binding of NKp44-Ig Fusion Protein on DB Cells

As an initial experiment to determine if NKp44 interacts with HLA I on the surface of DB cells, anti-HLA I antibody was used to block binding of NKp44-Ig fusion protein. Mean fluorescent intensity is indicated in the top right corner of each pane. A) Binding of fusion protein (filled histogram) was confirmed compared to negative control of anti-Human IgG FC-PE (empty histogram). B) HLA I expression (filled histogram) was confirmed on DB cells compared to isotype control (empty histogram). C) DB cells were first incubated with 2.5 µg of anti-HLA I antibody prior to incubation with NKp44-Ig fusion protein and staining with anti-human-IgG Fc-PE (filled histogram) and compared to fusion protein negative control (empty histogram). Prior incubation with anti-HLA I blocks binding of NKp44 fusion protein to near background levels. D) Cell surface PCNA expression was confirmed by staining cells with anti-PCNA-Alexa Fluor 488.

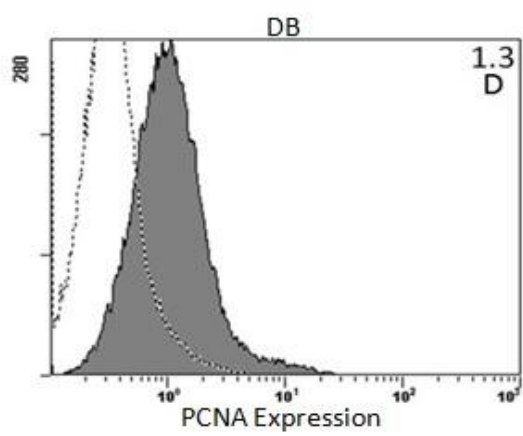
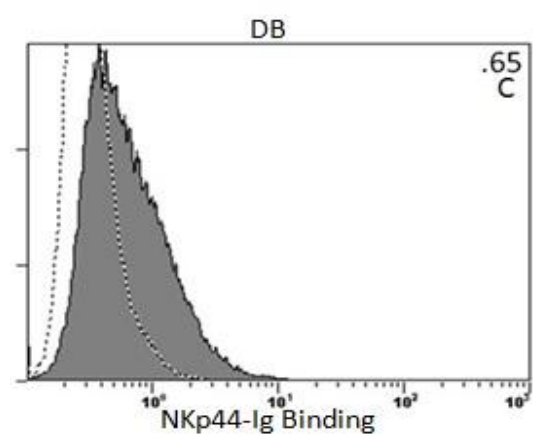
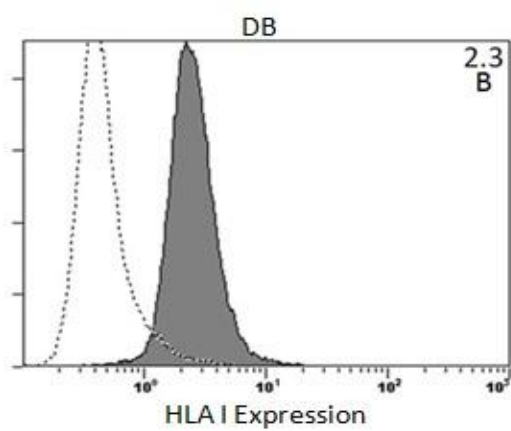
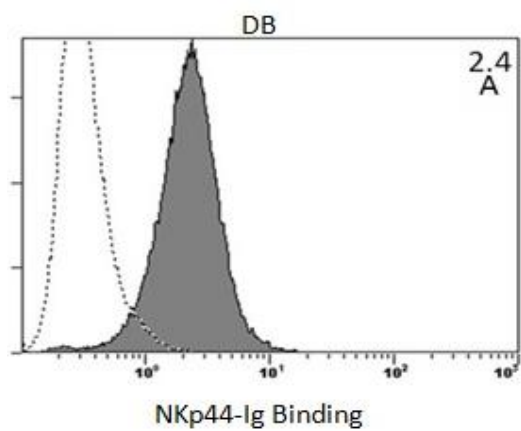


Figure 3.4 Colocalization of HLA I and PCNA on DB Cells by Confocal Microscopy

DB cells were harvested from tissue culture growth and 1×10^6 cells were first stained with anti-HLA I antibody followed by detection with anti-mIgG2a-Dylight 594. Cells were then stained with anti-PCNA-Alexa Fluor 488. To demonstrate cell surface staining, some cells were also stained with DAPI to delineate the nucleus. Since cells were not permeabilized, only cell surface PCNA was bound by antibody. Pockets of HLA I (Red) and PCNA (Green) are colocalized (Yellow) on the cell surface of DB cells and not localized in the nucleus (Violet). Cells were imaged on the Zeiss LSM 510 Confocal Laser Microscope using 40x, 1.2NA, 0.28 WD (water), C-Apochromat objective utilizing 561 nm, 405nm, and 488 nm wavelengths.

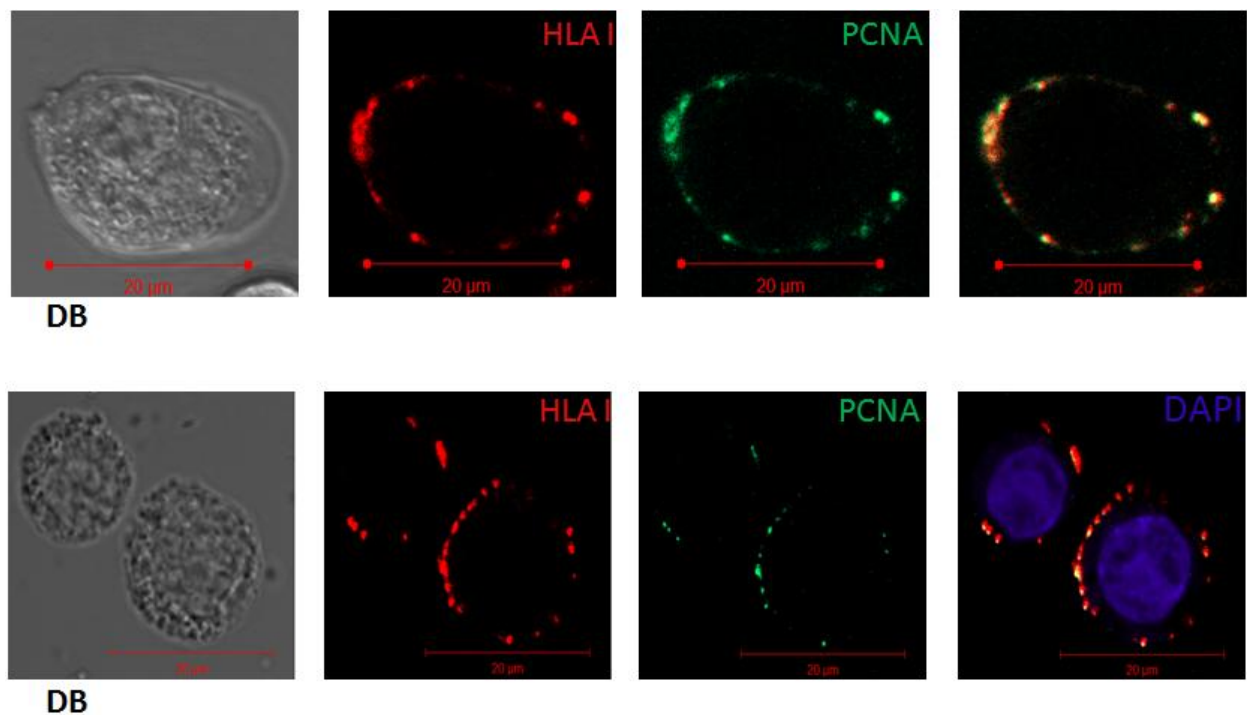


Figure 3.5 Coimmunoprecipitation of HLA I and PCNA

DB and DU145 cell lysates were first cleared of endogenous and cancerous immunoglobulins, as detailed in material and methods prior to precipitation experiments. Precleared lysates from DB (lanes A-C) and DU145 (lanes D-F) cells were subjected to immunoprecipitation with 3 μ g of anti- β -2-microglobulin antibody (lanes A, D) or mouse IgM control of irrelevant specificity (lanes B, E). Precleared cell lysate was used as a positive control (lanes C-F). Reduced samples were resolved on a 10% SDS-PAGE gel and blotted with anti-PCNA antibody.

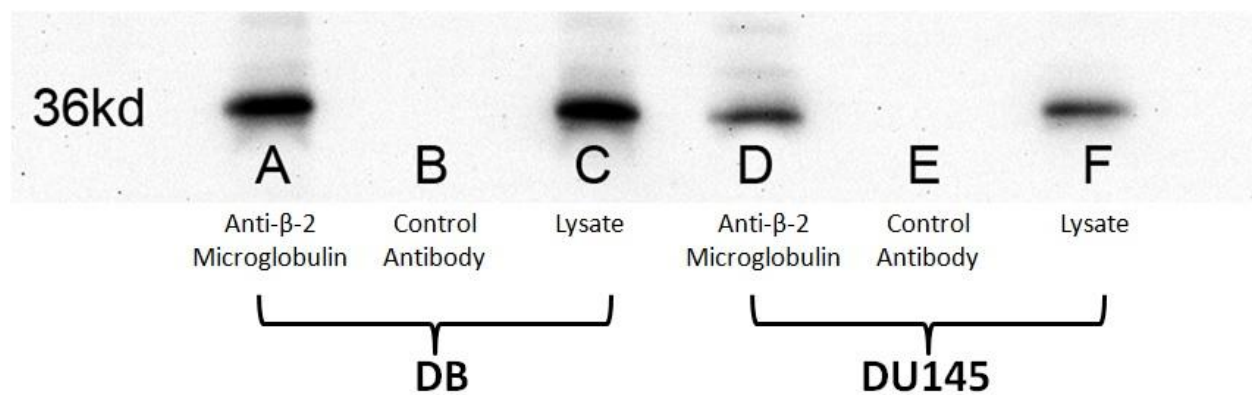
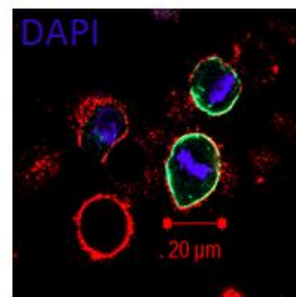
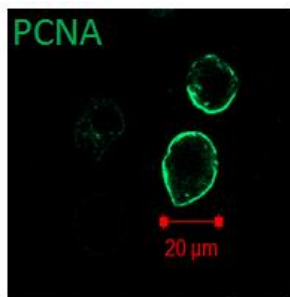
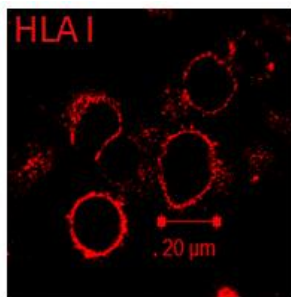
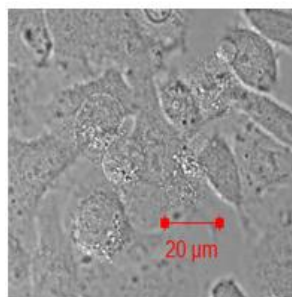
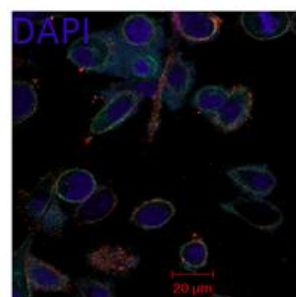
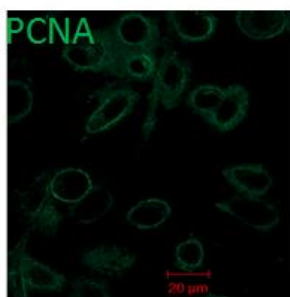
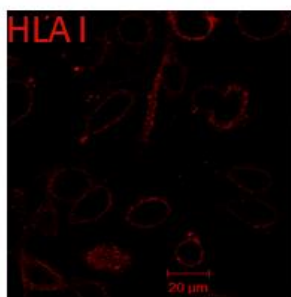
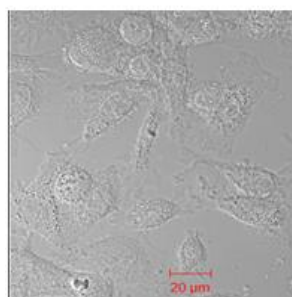


Figure 3.6 Colocalization of PCNA and HLA I on Solid Tumor Cell Lines

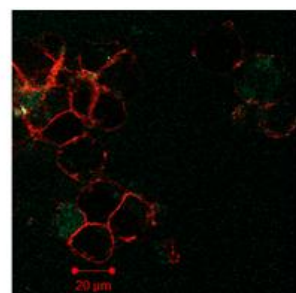
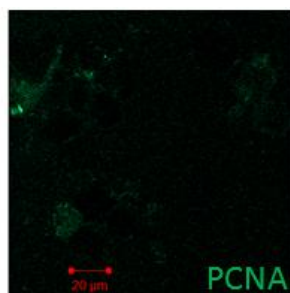
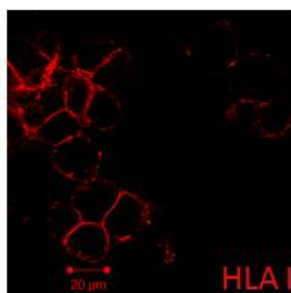
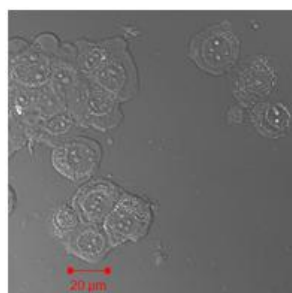
To further confirm colocalization of PCNA and HLA I, adherent cell lines were seeded onto cover slips and stained for HLA I (Red), PCNA (Green), and DAPI (Violet) as before. In DU145, PC3, and MDA-MB-231 cell lines, extracellular PCNA was sporadically found on cells and colocalized with HLA I. PC3 and MDA-MB-231 cells had more cells expressing PCNA at the cell surface than DU145 cells. MCF7 cells had very few if any cells which expressed PCNA at the cell surface, but did express HLA I. Cells were imaged on the Zeiss LSM 510 Confocal Laser Microscope using 40x, 1.2NA, 0.28 WD (water), C-Apochromat objective utilizing 561 nm, 405nm, and 488 nm wavelengths.



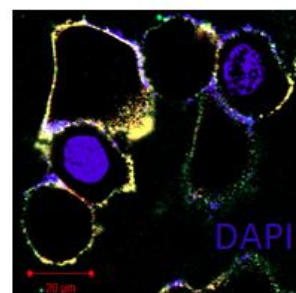
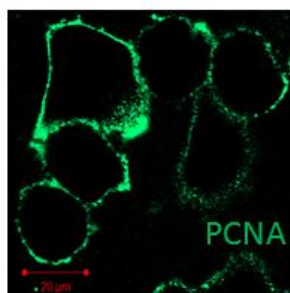
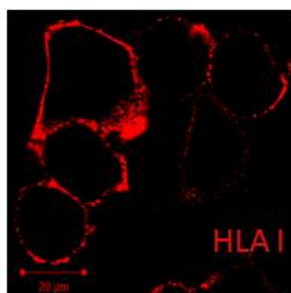
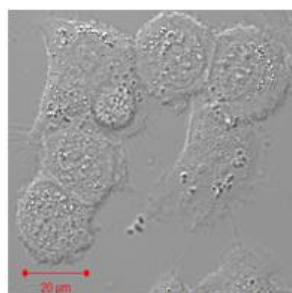
DU145



PC3



MCF7



MDA-MB-231

Figure 3.7 DU145 Prostate Cancer Cells Growing Above the Monolayer Express Cell Surface

PCNA

While analyzing cell surface PCNA expression on DU145 cells grown on cover slips, cells which were elevated above the monolayer were observed expressing PCNA (Green) at the cell surface. Utilizing Z-stack analysis, a topographical projection was created, showing cells expressing PCNA at the cell surface are elevated about 10 μm from cells within the monolayer. Due to the projection process, the topographical projection is a mirror image of the original. Arrows identify the same PCNA⁺ cells in each pane. Cells were imaged on the Zeiss LSM 510 Confocal Laser Microscope using 40x, 1.2NA, 0.28 WD (water), C-Apochromat objective utilizing 488 nm wavelengths.

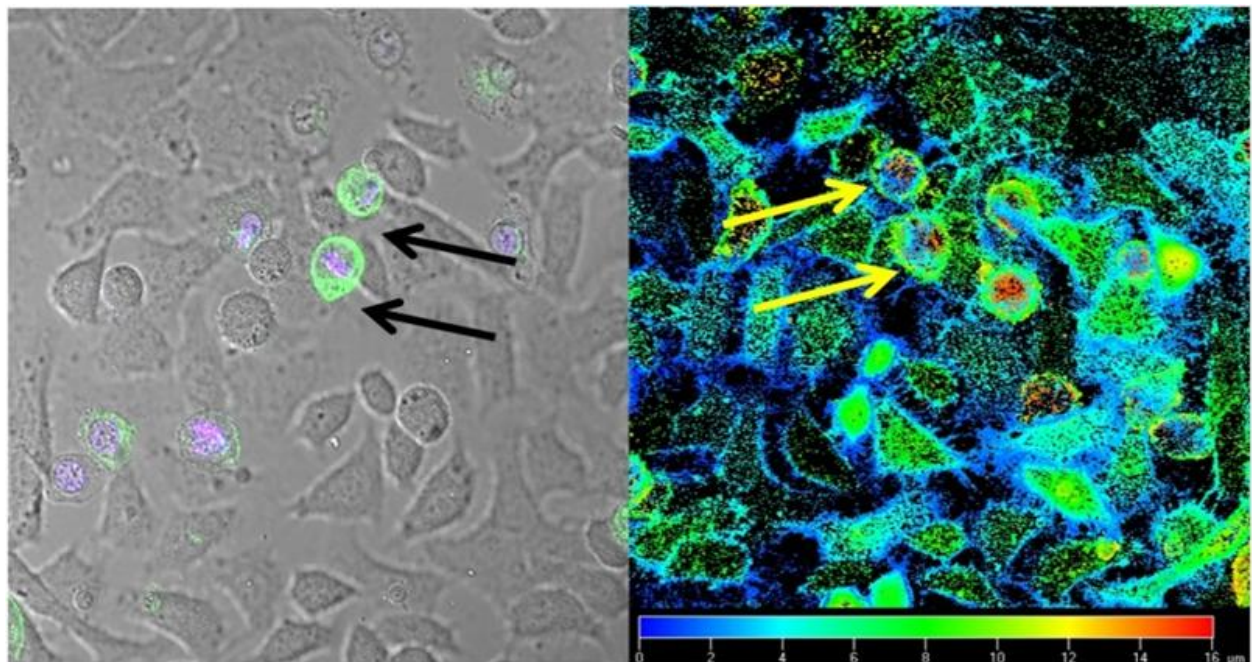
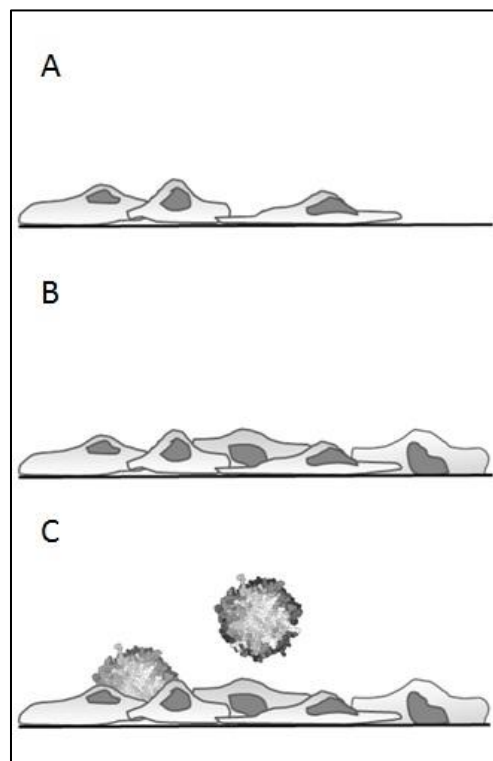


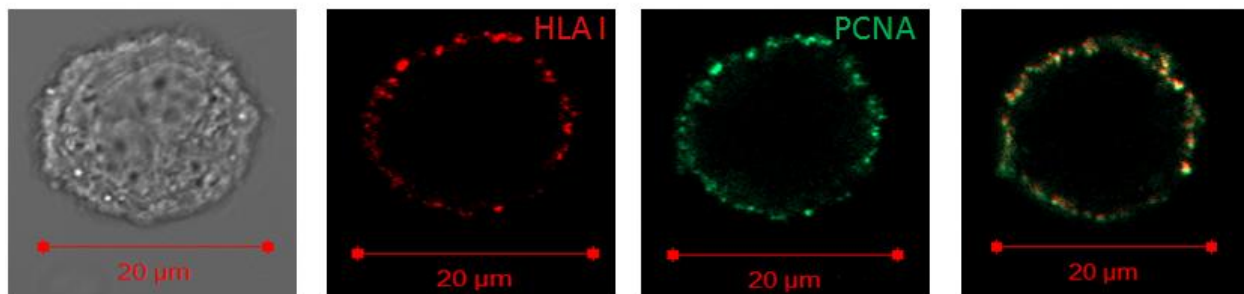
Figure 3.8 Growth Model of Solid Tumor Cell Lines

Solid tumor cell lines grow as colonies in a monolayer on tissue culture surfaces. As colonies continuously grow (A), eventually, surface area for new daughter cells to attach to the tissue culture surface is severely limited or nonexistent (B). In this event, new daughter cells rise above the monolayer and eventually detach naturally into the supernatant (C). These cells can reattach in other areas with room for growth, creating a new colony, in a metastasis like process.

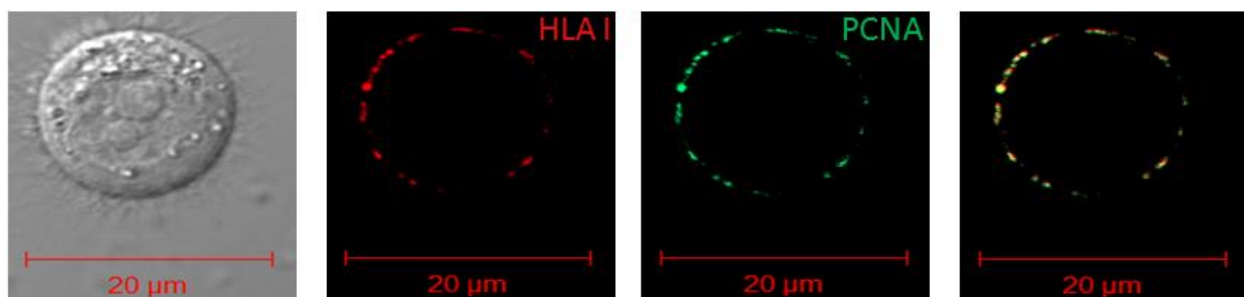


**Figure 3.9 Cells Naturally Detached from Monolayer Growth Express PCNA at the Cell Surface
Colocalizing with HLA I**

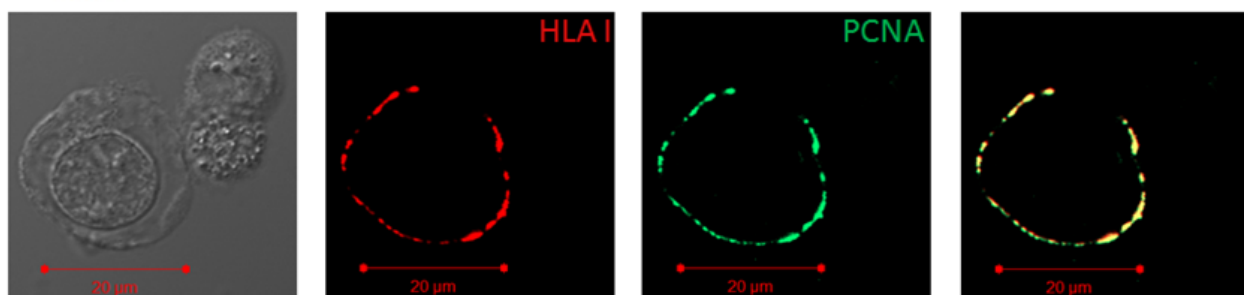
Naturally detached cells were isolated from culture supernatant of solid tumor tissue culture cell lines and stained for PCNA (Green) and HLA I (Red) as before. All cell lines expressed PCNA at the cell surface colocalizing with HLA I. There were considerably more PCNA⁺ cells in naturally detached populations compared to cells grown on cover slips. Cells were imaged on the Zeiss LSM 510 Confocal Laser Microscope using 40x, 1.2NA, 0.28 WD (water), C-Apochromat objective utilizing 561 nm and 488 nm wavelengths.



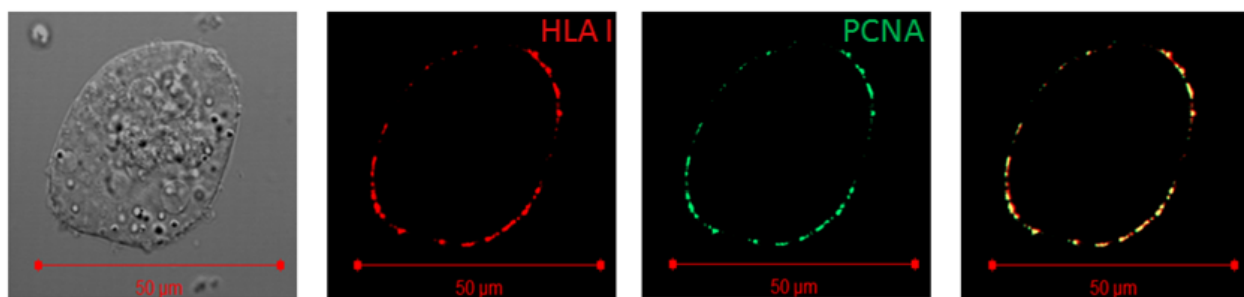
DU145



PC3



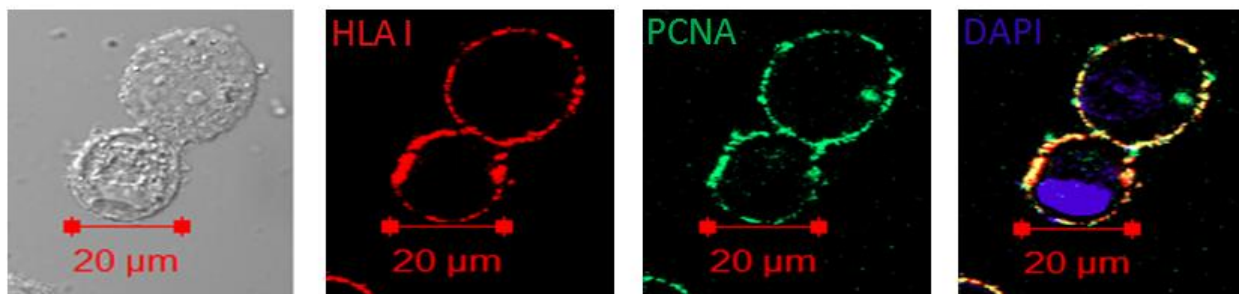
MCF7



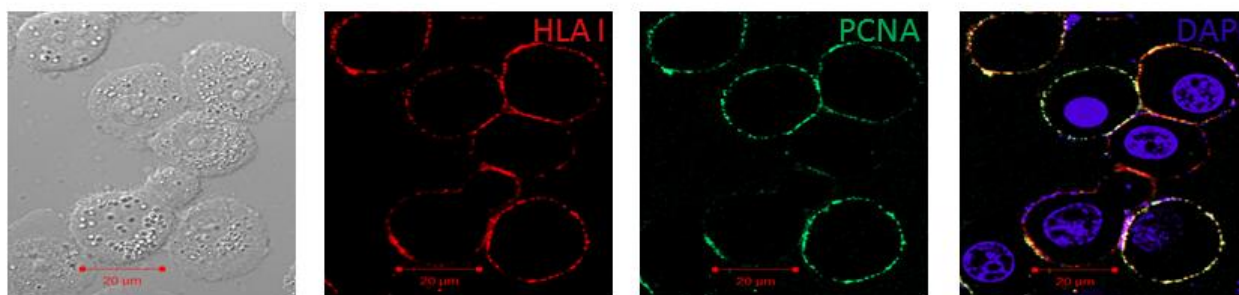
MDA-MB-231

Figure 3.10 Cells Detached from Monolayer growth with EDTA Express PCNA at the Cell Surface Colocalizing with HLA I

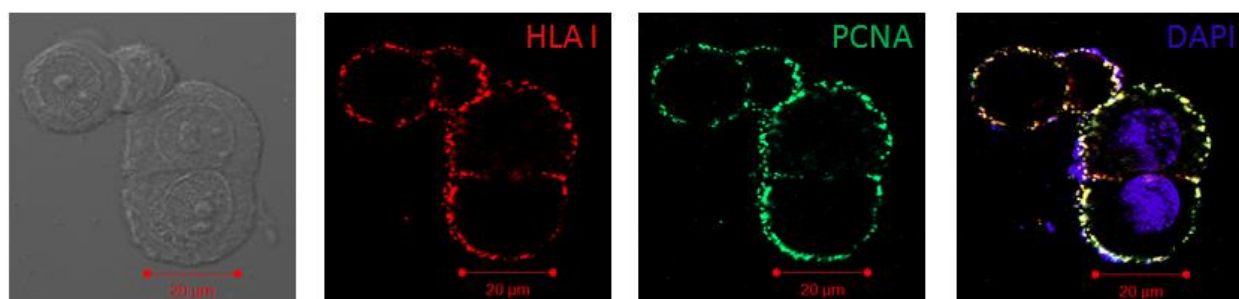
Cells were detached from monolayer growth with EDTA, a calcium chelator which prevents adherence between cadherins and facilitates removal of cells from tissue culture surfaces. Cells were again stained for PCNA, HLA I, and DAPI. In all cell lines, PCNA (Green) is seen colocalizing (Yellow) with HLA I (Red) at the cell surface. DAPI (Violet) was used to show PCNA is at the cell surface and not the nucleus as cells were not permeabilized. Cells were imaged on the Zeiss LSM 510 Confocal Laser Microscope using 40x, 1.2NA, 0.28 WD (water), C-Apochromat objective utilizing 561 nm, 405nm, and 488 nm wavelengths.



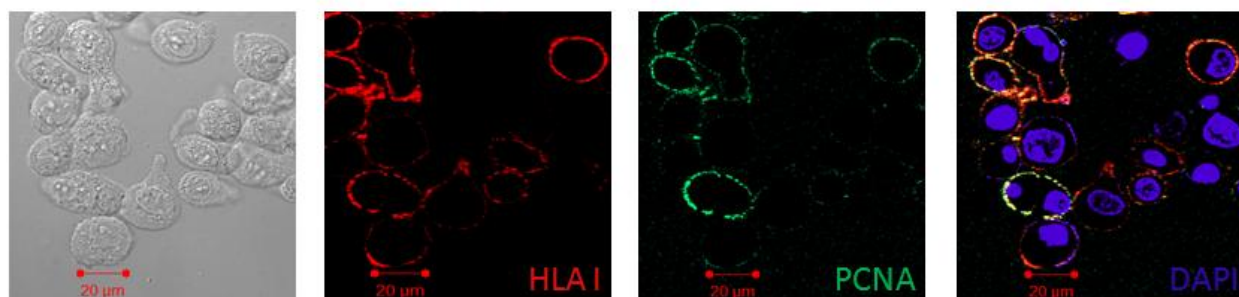
DU145



PC3



MCF7



MDA-MB-231

Figure 3.11 Analysis of Cell Surface PCNA Expression by Flow Cytometry

To further corroborate confocal microscopy results between cells grown on cover slips and those naturally detached, expression of cell surface PCNA was analyzed by flow cytometry on cells growing within the monolayer, which were gently scraped off of growth surfaces, or those naturally detached, free floating in culture supernatant. Similar to results obtained by confocal analysis, cells detached from monolayer growth by scraping contain very few cells expressing PCNA on the cell surface while populations naturally detached are enriched for extracellular PCNA expressing cells. A) DU145, 1.9% of scraped cells express extracellular PCNA, which is increased to 20.6% in cells naturally detached. B) PC3, 1.8% of scraped cells express extracellular PCNA, which was enriched to 11.2% in naturally detached cells. C) MCF7, 1.8% of scraped cells expressed cell surface PCNA, which was increased to 6.8% in naturally detached cells. D) MDA-MB-231, 2.9% of scraped cells expressed extracellular PCNA, which was increased to 20.7% in naturally detached cells.

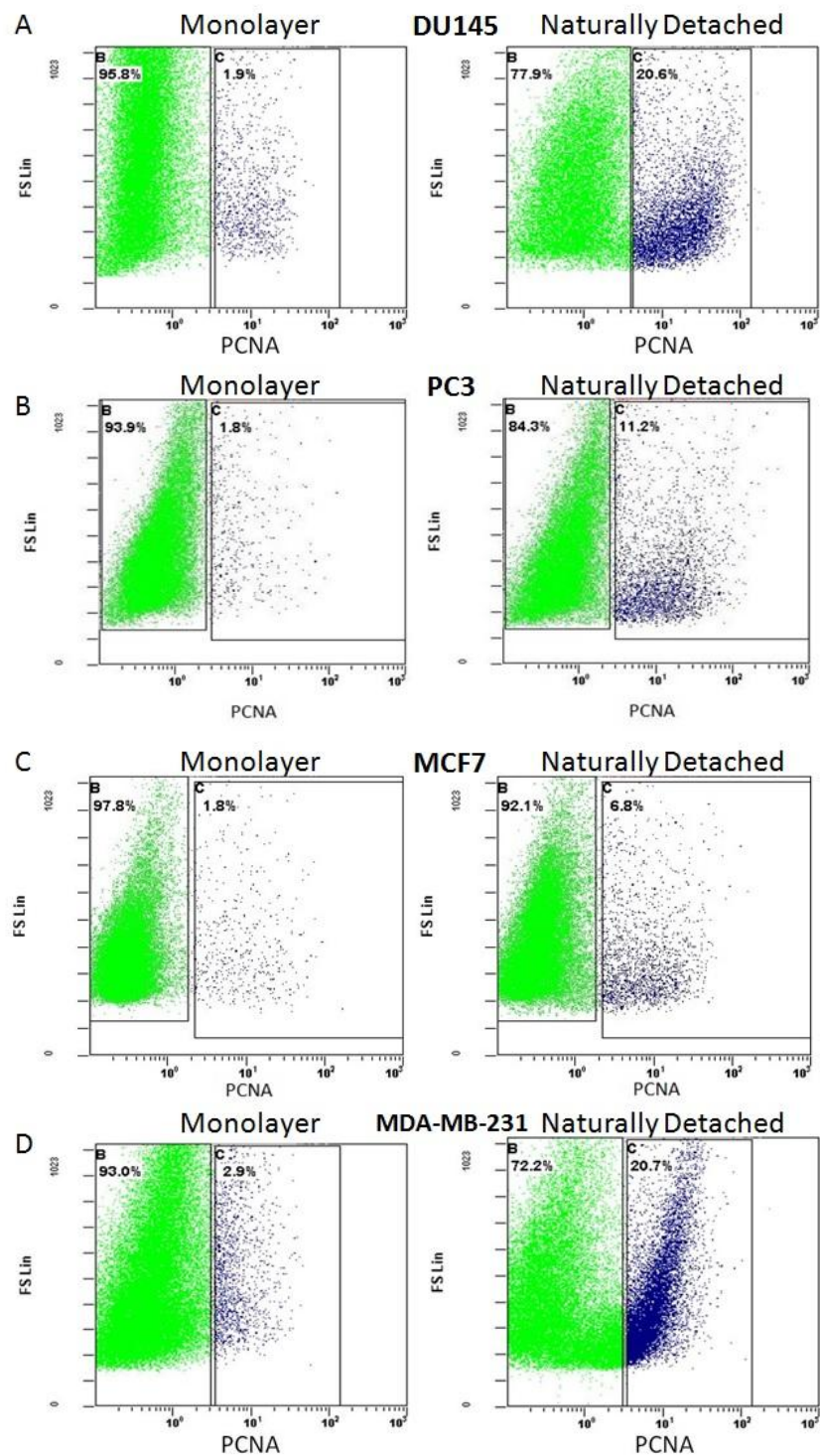


Figure 3.12 Expression of Extracellular PCNA on Monolayer and Naturally Detached

Populations

Graphical representation of percentage of cells expressing extracellular PCNA from monolayer or naturally detached populations determined by Flow cytometry from Figure 3.11. Figure is representative of two independent experiments. Bars \pm SD. * $p < .001$, Paired T-Test.

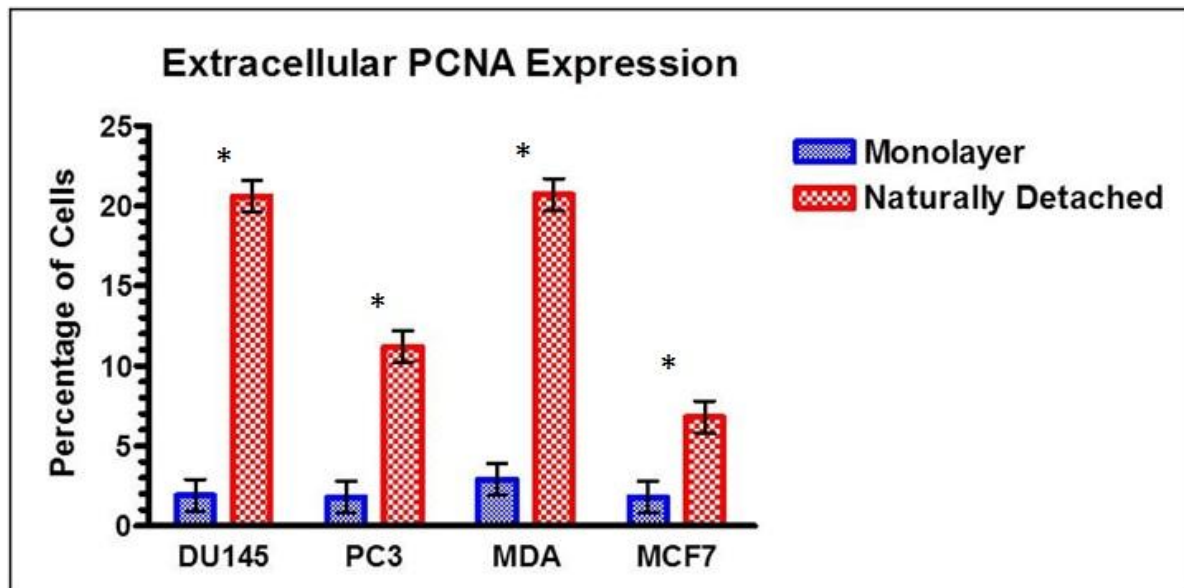
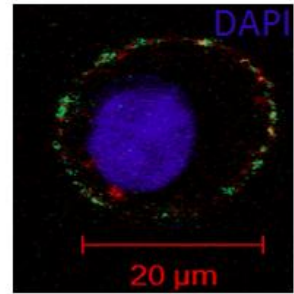
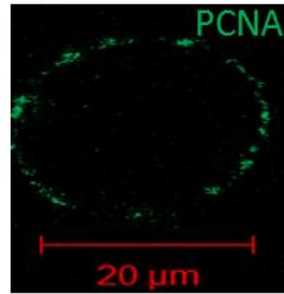
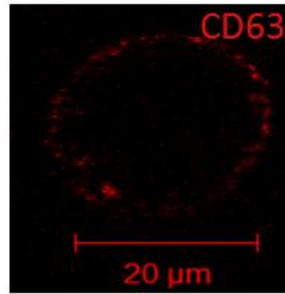
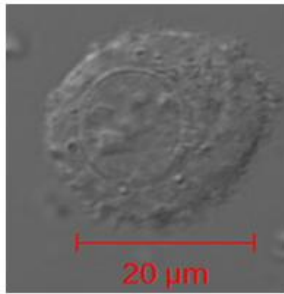
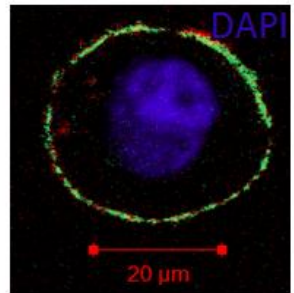
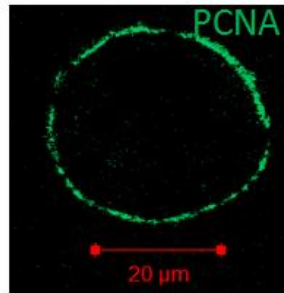
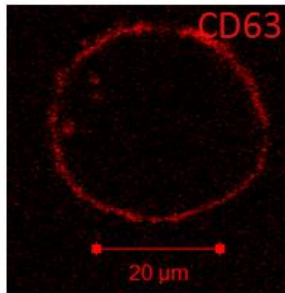
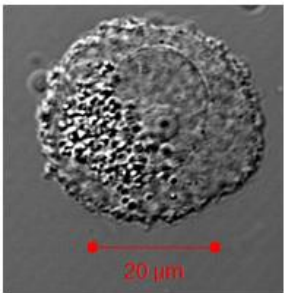


Figure 3.13 Extracellular PCNA is not Exclusively Expressed on Exosomes at the Cell Surface

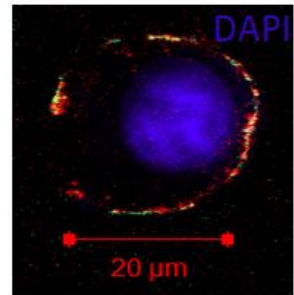
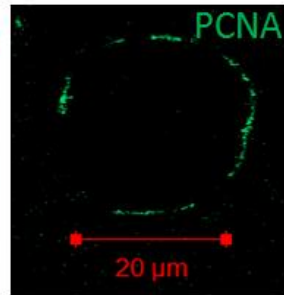
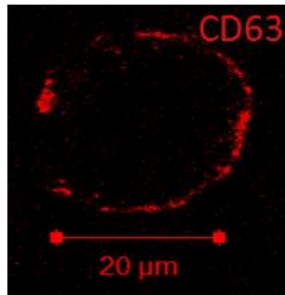
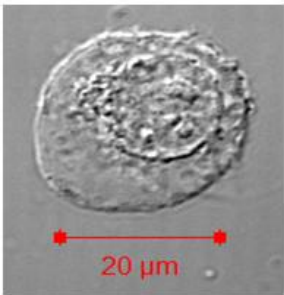
When PCNA was identified as a ligand for NKp44, PCNA was found to be on exosomes at the cell surface. Therefore we sought to colocalize cell surface PCNA in DU145 and MDA-MB-231 cells with CD63, which marks exosomes. Naturally detached cells were stained for PCNA (Green), CD63 (Red), and DAPI (Violet). While most areas of PCNA can be found colocalizing to areas marked by CD63, not all PCNA was found to be on exosomes. Cells were imaged on the Zeiss LSM 510 Confocal Laser Microscope using 40x, 1.2NA, 0.28 WD (water), C-Apochromat objective utilizing 633 nm, 405nm, and 488 nm wavelengths.



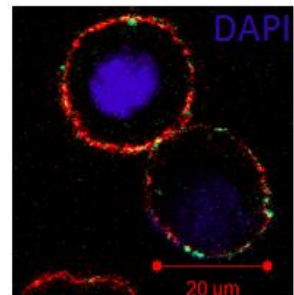
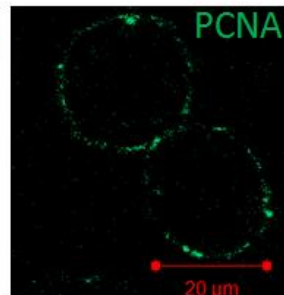
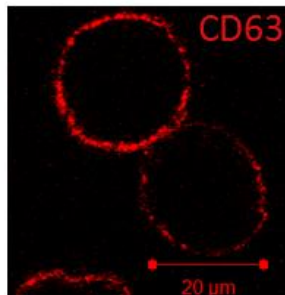
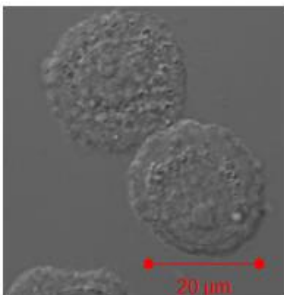
DU145



PC3



MCF7



MDA-MB-231

CHAPTER IV

PCNA INHIBITS NATURAL KILLER CELL EFFECTOR FUNCTION WHEN RECOGNIZED BY NKp44

The ultimate goal of any biomedical research is not only to acquire new understanding, but also to apply this knowledge to better the world we live in. In the realm of cancer research, this maxim holds especially true as greater understanding of why tumors form and how the immune system reacts improves therapies and saves lives. Thus, merely identifying ligands corresponding to NK cell receptors does not tell the full story. How recognition of a ligand alters NK cell functions, either inhibiting or activating effector responses must also be understood. Employment of this knowledge could one day lead to immunotherapies which alter tumor ligand expression to activate NK cell effector functions. In this specific aim, extracellular PCNA as a ligand for NKp44 is further studied to determine how this interaction affects NK cell cytolytic function and secretion of IFN- γ and TNF- α .

Specific Aim II

NKp44 recognition of cell surface PCNA in association with HLA I inhibits NK cell cytolytic function and secretion of IFN- γ and TNF- α

Rationale

NKp44 was originally described as an activating NK cell receptor in 1998 [38]. A year later, subsequent work showed NKp44 triggered NK cell activation by signaling through the physically attached DAP12 accessory molecule [39]. The same group also cloned the receptor to surprisingly reveal a tyrosine based ITIM sequence in the cytoplasmic tail of NKp44 [39]. ITIMs facilitate inhibitory signaling via phosphorylation of tyrosine within the ITIM sequence. Phosphorylation of tyrosine enables docking of inhibitory proteins containing Src homology 2 (SH2) domains, namely SH2 domain containing protein tyrosine phosphatase (SHP)-1, SHP2, and SH2 domain containing 5'-inositol phosphatase (SHIP) [50]. While the tyrosine within the ITIM of NKp44 was proven capable of being phosphorylated *in vitro*, analysis at the time could not demonstrate *in vivo* phosphorylation, recruitment of inhibitory phosphatases, or a functional role for the ITIM sequence [50]. For the next ten years, it was assumed NKp44 was purely an activating receptor with a nonfunctional ITIM sequence located in the cytoplasmic tail.

However in 2008, NKp44 was proven to inhibit NK cell effector function through recognition of cell surface PCNA [42]. By removing or mutating the ITIM sequence, Rosental *et al.* proved the ITIM sequence was indeed functional [42]. Since this is the only report detailing the inhibitory nature of NKp44, this specific aim seeks to confirm recognition of cell surface PCNA inhibits NK cell cytotoxicity and IFN- γ release, but also extend these findings to include effects on the secretion of TNF- α . Thus, the central hypothesis for this specific aim is recognition of cell surface PCNA by NKp44 inhibits NK cell effector functions, including cytotoxicity and secretion of IFN- γ and TNF- α .

Results

PCNA/HLA I complex interaction with NKp44 inhibits NK Cell Cytotoxic Function

NKp44 was originally described as an activating receptor when first discovered over ten years ago. However, recent evidence demonstrates the dual nature of NKp44 signaling with ITAMs in DAP-12 facilitating activating signaling and a putative ITIM sequence in the cytoplasmic tail of NKp44 facilitating inhibitory signaling [27,43]. Therefore, the influence PCNA colocalization with HLA I may have on cytotoxic function of NKp44 expressing NK cells was investigated utilizing the ⁵¹Cr release assay. DB cells were incubated with NKp44-Ig to block interactions with NK92-M1 cells via the PCNA/HLA I NKp44 ligand complex as a whole. DB cells were also incubated with anti-PCNA or anti-HLA I antibodies to individually block PCNA or HLA I interactions between DB cells and NK92-M1 cells. As expected, blocking interactions with the NKp44 ligand, HLA I, or PCNA increases specific lysis of DB cells (Figure 4.1A-C), indicating NKp44 is indeed acting in an inhibitory fashion when interacting with HLA I/PCNA complexes. Furthermore, the NKp44 receptor on NK92-M1 cells was blocked either alone (Figure 4.1D), or in conjunction with blocking HLA I on target cells (Figure 4.1E). Blocking NKp44 increases killing of DB cells and this killing was significantly increased with cooperative blocking of HLA I (Figure 4.1E).

In order to determine whether PCNA/HLA I complex interaction with NKp44 inhibits human primary NK cell cytotoxic function, primary NK cells were isolated from Peripheral Blood Mononuclear Cells (PBMC) of a healthy individual. The primary NK cells were cultured for one week in recombinant human IL-2 and used as effector cells after NKp44 expression was confirmed. The experiments were conducted under the same specifications used for NK92-M1

cells. As seen in Figure 4.2, blocking PCNA/HLA I complex interaction with NKp44 inhibits the cytolytic function of primary NK cells.

PCNA/HLA I complex interaction with NKp44 inhibits NK Cell IFN- γ secretion

To further investigate the inhibitory nature of PCNA recognition by NKp44, IFN- γ levels were measured in the supernatant of DB and NK92 or primary NK cell incubations by ELISA (Figure 4.3). 1.5×10^5 DB cells and 5×10^4 NK cells per well were incubated for 18 hours at 37°C in a 96-well round bottom plate. Interaction between PCNA and NKp44 was blocked in some wells by utilizing NKp44-Ig, anti-PCNA antibody, or anti-HLA I antibody on DB cells. In the same manner, anti-NKp44 antibody was used to block NKp44 on NK cells when incubated with DB cells receiving no blocking or DB cells which were blocked with anti-PCNA antibody. As a control, NK cells and DB cells were incubated without antibodies to determine a baseline of IFN- γ secretion to which wells receiving blocking antibodies were compared too.

IFN- γ levels are slightly increased, but not significantly, when recognition of the NKp44 ligand complex by NK92 cells is blocked with NKp44-Ig. However, when the ligand complex is blocked with either anti-HLA I or anti-PCNA antibody, secretion of IFN- γ by NK92 cells is significantly increased. When blocking with Anti-HLA I antibody, IFN- γ secretion is increased by almost a twofold difference. Finally, when blocking NKp44 on NK cells with or without simultaneous blocking of PCNA on DB cells, IFN- γ secretion by NK92 cells is significantly increased; however, blocking PCNA on DB cells in conjunction with blocking NKp44 on NK92 cells does not increase IFN- γ secretion over blocking NKp44 alone. Similar results were obtained when using primary NK cells as effector cells; however, compared to the NK92 cells

line, primary NK cells activated with IL-2 secrete about 4 fold more IFN- γ than NK92 cells (Figure 4.4). Signaling via NKp44 also induces TNF- α secretion; however TNF- α secretion was undetectable when incubating DB cells with NK92 or primary NK cells.

Discussion

NK cell activation and effector function is governed by the balance of signals received from activating and inhibitory receptors at the NK cell surface binding corresponding ligands on the surface of target cells. While identifying unknown ligands for NK cell receptors is significant, understanding how recognition of ligands governs NK cell functions is imperative. The development of assays to measure the level of NK cell cytotoxicity and secretion of cytokines against target cells has enabled detailed analysis of how receptor-ligand interactions affect NK cell activation and function. A hallmark assay used to measure NK cell mediated killing of a target cell is the ^{51}Cr release assay. In this analysis, target cells are loaded with radioactive Chromium, which is retained intracellularly by the target cell. Upon interaction with NK cells, target cells are killed, releasing Chromium which can then be measured to ascertain the degree of cytotoxicity employed by the NK cell. When combined with antibodies to block specific interactions between ligands and NK receptors, the role of specific ligands and receptors can be measured through variances in killing. In this manner, receptors can be deemed as activating or inhibitory, leading to a greater understanding of how NK cells function in immune surveillance.

To determine if PCNA recognition by NKp44 inhibits or activates NK cells, the ^{51}Cr release assay was employed. Radioactively labeled DB cells were incubated with NK92-M1 cells in the presence or absence of antibodies or fusion protein to block interaction between NKp44 and PCNA. Specifically, antibodies against PCNA or HLA I were used to block these molecules on DB cells prior to incubation with NK cells (Figure 4.1B, C). To block the entire PCNA/HLA I complex, NKp44-Ig was used (Figure 4.1A). When compared to killing of DB cells without any blocking or blocked with isotype control antibody, blocking PCNA or HLA I individually as well as blocking the entire complex increases specific lysis of DB cells by NK92-M1 cells. Specific lysis is also increased significantly when NKp44 is blocked on NK cells and incubated with DB cells alone or DB cells blocked with anti-HLA I antibody (Figure 4.1D, E). These results indicate NKp44 is indeed recognizing PCNA and inhibiting NK cell cytotoxicity.

The ^{51}Cr release assay was also performed with primary NK cells isolated from a healthy individual. Since NK92-M1 is a lymphoma cell line which may harbor mutations altering effector functions, utilizing primary NK cells to corroborate results in the NK92-M1 cell line strengthens the results and impact of this assay. Similar to NK92-M1 cells, primary NK cells are increasingly efficient at killing DB cells when interactions between PCNA and NKp44 are blocked (Figure 4.2). Significant differences are observed in the killing of DB cells by primary NK cells, especially when NKp44 is blocked on NK cells (Figure 4.2 D, E). These results corroborate those of Rosental *et al.* showing PCNA recognition by NKp44 does in fact inhibit NK cell killing of target cells.

As with most research experiments, there are certain limitations to the ^{51}Cr release assay, specifically with the use of antibodies to block interactions between receptors and ligands. Since recognition of HLA I is responsible for the majority of NK cell inhibition, it is no surprise that blocking of HLA I significantly increases killing of DB cells (Figure 4.1C, 4.2C). Undoubtedly, HLA I is present at the surface of DB cells both by itself and in association with PCNA. This is evident in confocal analysis of chapter 3 as not all HLA I colocalizes with PCNA.

Unfortunately, it is impossible to only block HLA I which is in association with PCNA, which would be ideal in this scenario. Even through using NKp44-Ig, which presumably recognizes PCNA and HLA I, this does not guarantee that fusion protein masks enough of HLA I to prevent its recognition by inhibitory receptors. Thus, the only true way to determine if recognition of PCNA is inhibitory is through blocking of PCNA alone, which does increase specific lysis of DB cells by NK92-M1 and primary NK cells (Figure 4.1B, 4.2B). Furthermore, while NKp44 antibody increases killing, presumably through functional blocking of NKp44 mediated inhibition, this antibody may also have stimulatory capacities by cross-linking the receptor yielding increased killing (Figure 4.1 and 4.2 D-E). However, previous reports indicate PCNA recognition results in inhibition of NK cell cytotoxicity via NKp44 and our results corroborate this outcome. Moreover, our results attempt to show the complete picture of inhibitory NKp44 recognition of target cells, underlying the role of HLA I and PCNA interaction. However, it will be interesting to know whether PCNA itself is inhibitory, or its association with HLA I is responsible for inhibition.

NK cell effector function is not limited to cytotoxic action, but also includes the secretion of IFN- γ . Therefore we further investigated the inhibitory nature of PCNA-NKp44 interaction utilizing an ELISA assay to measure the amount of IFN- γ released by NK cells when incubated with DB cells (Figure 4.3, 4.4). NK cell and DB incubations were setup in a similar manner to the ^{51}Cr release assay, utilizing antibodies against PCNA, HLA I, or NKp44 as well as NKp44-Ig. IFN- γ levels in incubation supernatants were then measured by ELISA.

To determine a baseline of IFN- γ secretion, NK92 and DB cells were incubated without blocking antibodies, which results in NK92 cells secreting about 54 pg/mL IFN- γ (Figure 4.3). When the NKp44 ligand complex is blocked with NKp44-Ig, IFN- γ secretion increases to about 62 pg/mL; however, this difference is not significant due to standard deviation. When blocking PCNA on DB cells, IFN- γ secretion increases to about 61 pg/mL. Contrary to blocking with NKp44-Ig, this difference is significant due to a small standard deviation in samples. A dramatic increase in IFN- γ secretion, up to almost 96 pg/mL, is observed when HLA I is blocked on DB cells. However this increase is not solely due to blocking of the NKp44 ligand complex. As mentioned above, anti-HLA I antibody does not specifically block HLA I molecules in complex with PCNA, but all HLA I molecules on the surface of the cell. This results in an overall decrease in inhibitory signaling received by the NK cell. The ideal scenario would be to only block those HLA I molecules which are associated with PCNA. This would allow the specific measure of whether HLA I or PCNA individually or jointly induces inhibitory signaling via NKp44. Unfortunately, this is not currently possible.

Finally, when NKp44 is blocked on NK92 cells, IFN- γ secretion is significantly increased to 103 pg/mL. When PCNA is blocked on DB cells in conjunction with blocking NKp44 on NK92 cells, no further increase of IFN- γ is observed. This is likely due to saturating effects of anti-NKp44 antibody binding all NKp44 molecules present on the surface of NK92 cells. Thus, there are no free NKp44 molecules available to recognize PCNA regardless if PCNA is blocked or not.

IFN- γ secretion by primary NK cells was also measured after incubation with DB cells utilizing the same blocking scenarios (Figure 4.4). Compared to NK92 cells, primary NK cells secrete about 4 fold more IFN- γ with a base line secretion of about 410 pg/mL. Otherwise, primary NK cell IFN- γ secretion in response to DB cells follows the exact same trend as NK92 cells. IFN- γ secretion is slightly increased when blocking with NKp44-Ig, but is increased further when blocking with either anti-HLA I or anti-PCNA antibody. Similar to NK92 cell incubations, when NKp44 is blocked on primary NK cells, IFN- γ secretion is significantly increased to about 520 pg/mL, reaching the highest detectable amount of IFN- γ by the ELISA kit.

Modulation of NK cell activity may not only depend on the DAMP molecule associated with HLA I, but also the NCR that is recognizing the motif. Due to the dual nature of NKp44 signaling, it will be of interest to determine if recognition of PCNA, HLA I, or the motif as a whole is responsible for inhibition of NK cytotoxicity. Neither NKp30 nor NKp46 has been reported to contain an ITIM sequence; however, an immunosuppressive isoform of NKp30 resulting from a single-nucleotide polymorphism in the 3' untranslated region has been reported [138]. Thus, NCRs may exhibit both inhibitory and activating functions. Whether the divergence of NKp44 function depends on the individual DAMP molecule recognized or the binding of DAMP molecules to HLA I remains to be elucidated. Interestingly, DAMP molecules

such as High-Mobility Group Protein B1 and S100A8/9 have the ability to bind heparin sulfate and heparin sulfate proteoglycans, which are known to be coligands involved in NCR dependent recognition of tumor cells resulting in secretion of IFN- γ but not cytotoxicity [30,31,34]. We postulate a DAMP molecule may be the missing link in heparin sulfate dependent recognition of tumor cells, which would then elicit full NK cell effector function.

These results also bring further attention and new function to DAMP molecules, or proteins which are located and function intracellularly, but somehow localize to the extracellular membrane, despite lacking a traditional secretory leader sequence [139]. These proteins are released by cells which have become injured in the absence of infection due to ischemia, hypoxia, transformation, chemotherapy, or other trauma [34]. Analogous to Toll-Like Receptors recognizing pathogen-associated molecular patterns, the NCRs may represent a class of NK receptors that participate in pattern recognition of DAMP molecules, whose identities may reflect the intracellular health of a cell in addition to the traditional method of HLA I presenting self peptide. In this manner, HLA I may also present DAMP molecules for identification by the NCRs, which would then regulate NK cell function potentially dependent on the DAMP molecule present and the NCR engaged. Knowledge of the identities and nature of DAMP molecules that bind to HLA I or other cell surface molecules to form ligands for the NCRs will shed light on NK cell recognition of target cells under healthy and disease conditions and offer novel therapeutic targets.

Figure 4.1 PCNA/HLA I Complex Interaction with NKp44 Inhibits NK92-MI Cell Cytotoxic

Function

The lysis of DB cells by NK92-MI cells was determined by a standard ^{51}Cr release assay. DB cells and NK92-MI cells were first blocked with Human IgG Fc fragment to prevent reverse binding of fusion protein and antibody dependent cellular cytotoxicity. DB cells were loaded with ^{51}Cr and incubated with NKp44-Ig (A) or anti-PCNA (B) or anti-HLA I (C). DB cells were then incubated with NK92-MI cells at varying target to effector cell ratios for 4 hours at 37°C . Level of killing was compared to DB cells incubated with mIgG2a isotype antibody or no antibody, which served as a positive control (No Blocking) of cell lysis under unblocked conditions.

Alternatively, NK92-MI cells were incubated with anti-NKp44 or mIgG1 isotype control antibody prior to incubation with DB cells incubated with no antibody (D) or anti-HLA I (E). When interactions between PCNA/HLA I and NKp44 are blocked, percent specific lysis is increased, indicating NKp44 recognizes the ligand complex in an inhibitory manner. Figure is representative of two independent experiments performed in triplicate. Bars \pm SD. * $p < .05$, ** $p < .01$, *** $p < .0001$, ANOVA.

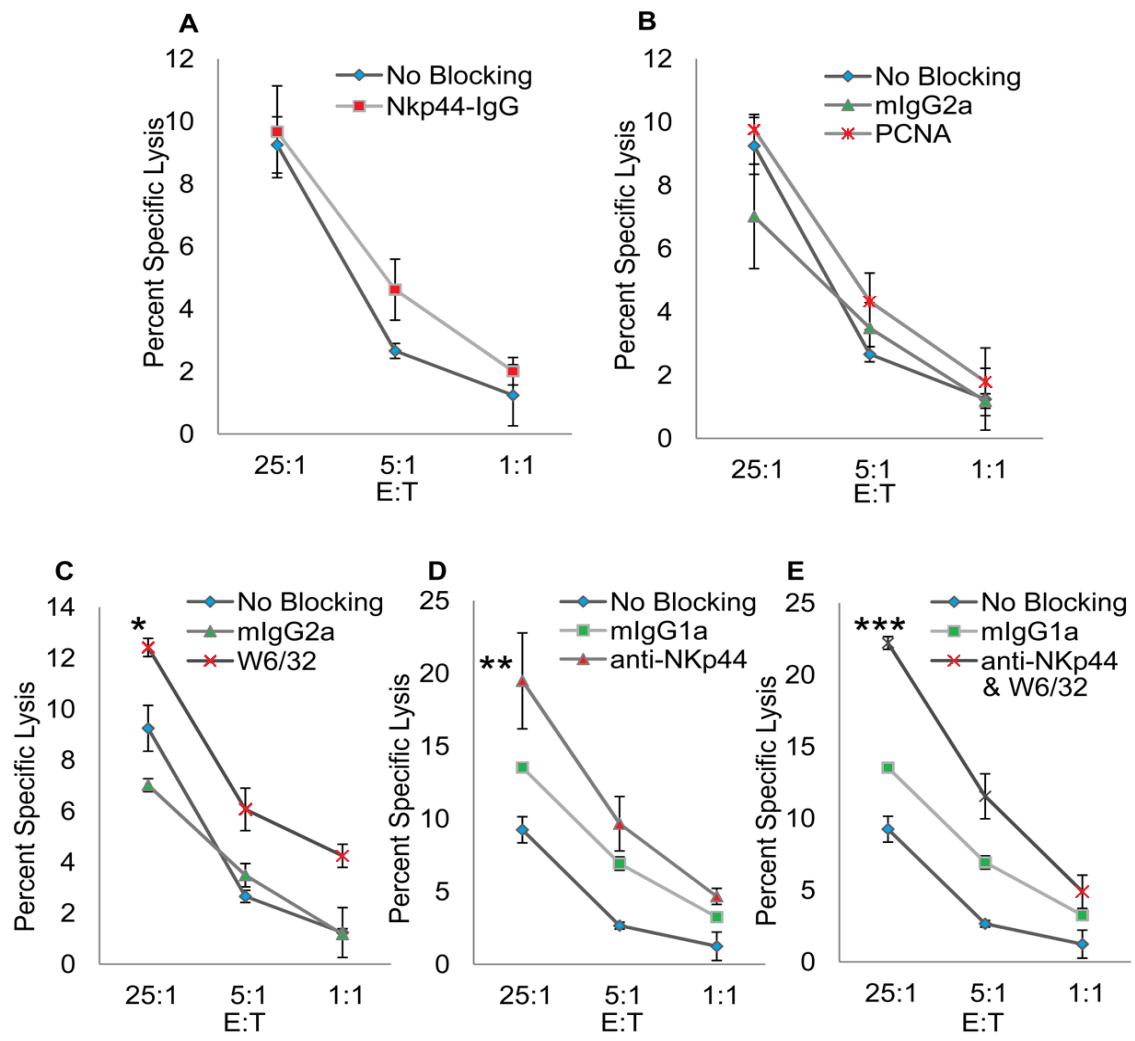


Figure 4.2 PCNA/HLA I Complex Interaction with NKp44 Inhibits Primary NK Cell Cytotoxic Function

In order to confirm the results in Figure 4.1, we performed a ^{51}Cr release assay with primary NK cells isolated from Peripheral Blood Mononuclear Cells. Primary NK cells were cultured in 1000 units/ml recombinant human IL-2 for one week, at which point NKp44 expression was confirmed prior to use. DB cells were loaded with ^{51}Cr and incubated with NKp44-Ig to block the entire NKp44 ligand complex (A) or anti-PCNA (B) or anti-HLA I (C) to individually block PCNA or HLA I interaction with NKp44. DB cells were then incubated with primary NK cells at 10:1, 5:1, and 1:1 effector to target cell ratios for 4 hours at 37°C. Level of killing was compared to DB cells incubated with mIgG2a isotype antibody or no antibody (No Blocking). In figure D, NKp44 was blocked on primary NK cells with anti-NKp44 or mIgG1 isotype control antibody prior to incubation with DB cells incubated with no antibody. NKp44 on primary NK cells was again blocked with anti-NKp44 and incubated with DB cells incubated with anti-HLA I in figure E. Again, NKp44 recognized PCNA/HLA I complex in an inhibitory manner. Bars \pm SD. * $p < .05$, ** $p < .01$, *** $p < .0005$, ANOVA.

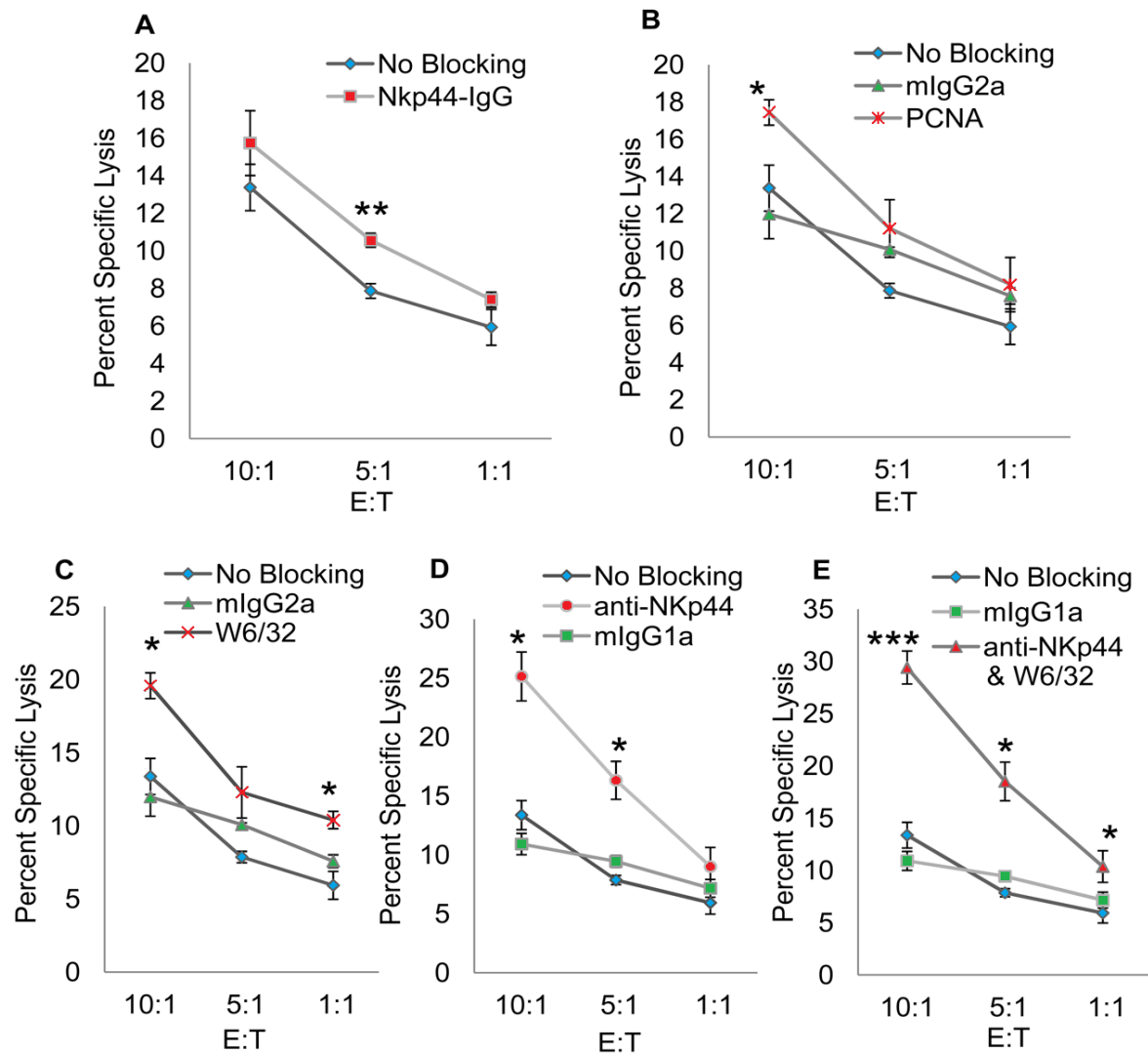


Figure 4.3 PCNA/HLA I Complex Interaction with NKp44 Inhibits NK92 IFN- γ Secretion

1.5×10^5 DB cells and 5×10^4 NK92 cells per well were incubated for 18 hours at 37°C in a 96-well round bottom plate. Interaction between PCNA and NKp44 was blocked in some wells by utilizing NKp44-Ig, anti-PCNA antibody, or anti-HLA I antibody on DB cells. In the same manner, anti-NKp44 antibody was used to block NKp44 on NK cells when incubated with DB cells receiving no blocking or DB cells which were blocked with anti-PCNA antibody. As a control, NK92 and DB cells were incubated without antibodies to determine a baseline of IFN- γ secretion to which wells receiving blocking antibodies were compared too. Figure is representative of two independent experiments. Bars \pm SD. * $p \leq .002$, ** $p < .00001$

Paired T-Test

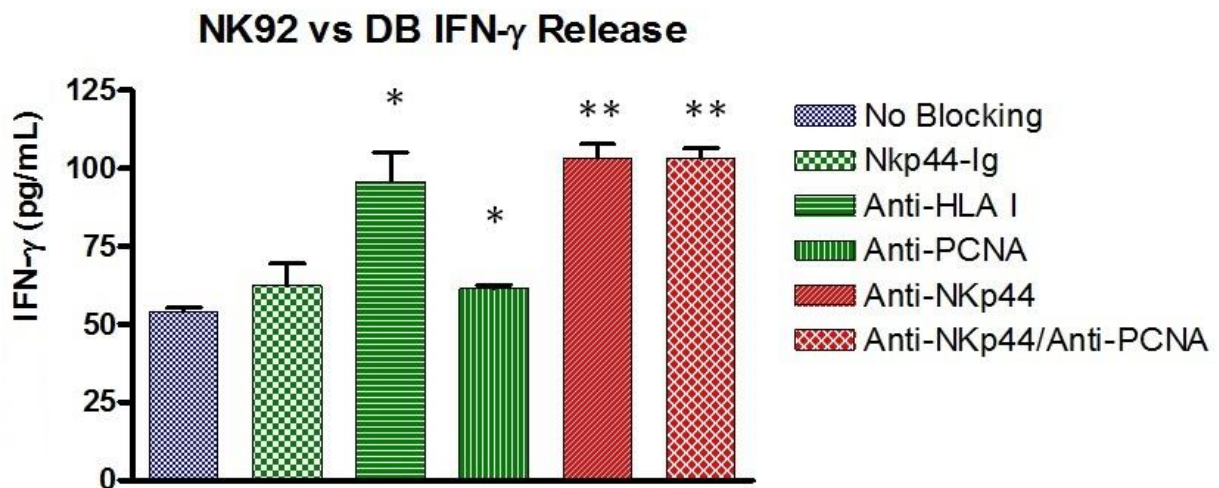
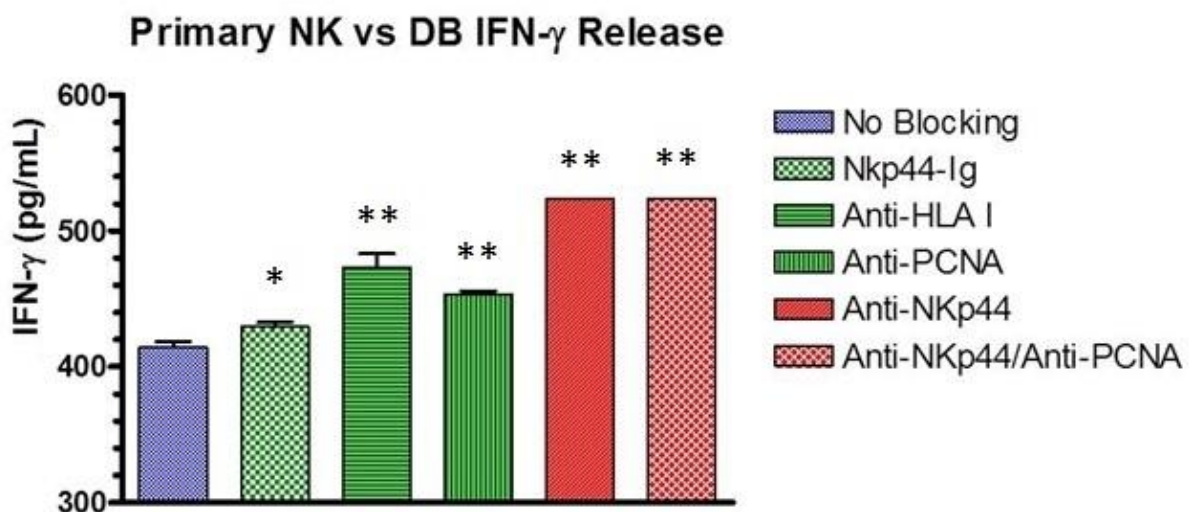


Figure 4.4 PCNA/HLA I Complex Interaction with NKp44 Inhibits Primary NK Cell IFN- γ

Secretion

IFN- γ secretion was measured in the supernatant of DB and primary NK cell incubations by ELISA. 1.5×10^5 DB cells and 5×10^4 primary NK cells per well were incubated for 18 hours at 37°C in a 96-well round bottom plate. Interaction between PCNA and NKp44 was blocked in some wells by utilizing NKp44-Ig, anti-PCNA antibody, or anti-HLA I antibody on DB cells. In the same manner, anti-NKp44 antibody was used to block NKp44 on NK cells when incubated with DB cells receiving no blocking or DB cells which were blocked with anti-PCNA antibody. As a control, primary NK cells and DB cells were incubated without antibodies to determine a baseline of IFN- γ secretion to which wells receiving blocking antibodies were compared too. When NKp44 interaction with the ligand complex is blocked, IFN- γ secretion is increased, indicating NKp44 is recognizing PCNA/HLA I in an inhibitory manner. Figure is representative of two independent experiments. Bars \pm SD. * $p \leq .008$, ** $p < .001$ Paired T-Test



CHAPTER V

CHARACTERIZATION OF TARGET CELLS EXPRESSING EXTRACELLULAR PCNA

PCNA was originally discovered as a nuclear antigen in proliferating cells through its reaction with an autoantibody in the sera of some patients with Systemic Lupus Erythematosus [140]. Since its discovery and cloning, numerous works have detailed PCNA as a highly conserved protein in eukaryotes, archaea, and bacteriophages where it plays an essential role in DNA replication [141]. PCNA forms a homotrimeric ring like structure which encircles double-stranded DNA, forming a sliding platform. This platform functions as a docking site for DNA polymerases, ligases, and other factors involved in the replication of DNA [51,141,142]. More recent studies highlight the plethora of proteins PCNA interacts with in numerous biological processes, including DNA repair, control of the cell cycle, and chromatin remodeling [51,131,141-143].

While PCNA is synthesized in all stages of the cell cycle, expression levels are increased in early S phase in order to support DNA replication and ultimately cellular replication at the entry of mitosis [142,144]. Due to its role in DNA replication and cell cycle control, PCNA also holds important value in cancer research as an indicator of differentiation and proliferation [145]. Cancer is essentially defined by uncontrolled cellular growth; thus, PCNA levels are elevated in cancer cells compared to normal cells, up to an 8 fold increase in protein levels

[142,146]. As a result, PCNA has been used as a diagnostic marker and predictor of aggressiveness in numerous cancers including prostate, colorectal, esophageal, bladder, and breast cancer [144,147-152]. Immunohistochemical analysis of PCNA expression in tumors has been used by pathologists to not only grade tumors, but assess the severity of disease as well as prognosis. For example, prostate cancer patients with lower PCNA expression levels are afforded increased survival rates over those with higher expression levels [153]. Additionally, PCNA expression in prostate biopsies after radiotherapy correlate with treatment failure [154]. Since the presence of PCNA at the cell surface has only recently come to light, the historical use of PCNA to analyze tumors focused on nuclear PCNA and its role in DNA replication and cell division, requiring permeabilization of tissue for analysis. Therefore, extracellular PCNA may serve as a novel biomarker which may indicate aspects of tumor severity, specific mutations, and metastatic potential which has yet to be appreciated. In this specific aim, tumor cells harboring extracellular PCNA are characterized in order to revolutionize the use of PCNA as a marker of dangerous tumors.

Specific Aim III

Extracellular PCNA is a marker for metastatic cancer stem cells and Diffuse B Cell Lymphoma

Rationale

Novel biomarkers are needed to detect and identify dangerous tumor cells. Since primary tumors account for only 10% of cancer related deaths, identifying markers on tumor

cells which resist treatment or potentially become metastatic is vital [78]. Observed in the first specific aim, a colony of tumor cells growing in tissue culture will eventually run out of room for daughter cells to attach to the surface in the confines of the colony. As the parental cell divides to produce the daughter cell, the new cell is released into the supernatant, lacking room in the immediate vicinity for attachment. This process is reminiscent of cells metastasizing away from a primary tumor which has grown to full capacity in its environment.

To date, several theories have been proposed outlining mechanisms of metastasis. The stochastic, or clonal evolution model, states any cancer cell, through the accumulation of mutations, can acquire traits which promote survival, aggressiveness, and metastatic potential [78]. An alternative theory is the cancer stem cell model, which proposes a small population of cells drive tumor growth [78,79]. Cancer stem cells (CSCs) may arise through mutations in normal adult stem cells due to their increased longevity, making them susceptible to accruing numerous mutations [78,80,81]. Evidence also supports the ability of a differentiated cell to reacquire self renewal capabilities via an epithelial to mesenchymal transition (EMT) [82,83]. This process involves the dedifferentiation of a cell, imparting a stem cell like phenotype with enhanced abilities to expel chemotoxic agents, invade surrounding tissue, and become mobile [78,79,82,83]. Several recent studies have identified potential CSC in tumor cell lines, including those of prostate and breast cancer [88,91]. Thus, the first part of this specific aim focuses on evaluating cells expressing extracellular PCNA for stem cell characteristics, including expression of cell surface antigens, transcription factors, spheroid formation, invasion, and cell cycle progression.

One aspect of a valuable biomarker is it specifically targets a cell of interest. Since the DB cell line uniformly expresses PCNA at the cell surface, extracellular PCNA may serve as a marker for B cell lymphoma enabling detection and specific therapeutic targeting of these malignant cells. In order to assess the feasibility of using extracellular PCNA as a marker for Diffuse B Cell Lymphoma, PCNA expression on the surface of freshly isolated and *in vitro* activated PBMCs was analyzed to evaluate the extracellular expression of PCNA in PBMCs of healthy individuals. Therefore, in the final specific aim, we hypothesize that extracellular PCNA is a marker for dangerous cancer stem cells and a unique marker of B cell lymphoma.

Results

Expression of EMT/CSC phenotypic markers in extracellular PCNA⁺ cells

Over the last 15 years, numerous groups have sought to identify and characterize CSCs. The consensus of these studies has identified a stem cell like phenotype marked by high expression of CD44 and low or no expression of CD24 [88-90,92,94,155]. Originally investigated due to heterogeneous expression in breast cancer, CD44^{high}/CD24^{low} was found to distinguish tumorigenic from nontumorigenic cells upon xenografting into immunocompromised mice [156]. Therefore, CD44 and CD24 expression was analyzed on extracellular PCNA⁺ cells either gently removed from monolayer growth by scraping or those found naturally detached, free floating in culture supernatant (Figure 5.1). Expression of CD24 and CD44 was examined by gating on populations of cells expressing PCNA at the cell surface.

In the DU145 cell line, extracellular PCNA⁺ populations do not express CD24; however, CD44 is heterogeneously expressed in four populations consisting of negative, low, intermediate, and high expression levels (Figure 5.1, 5.2A). When compared to cells removed from monolayer growth, percentages of CD44 high and CD44 intermediate populations are decreased in floating cells with a concomitant increase in percentages of CD44 negative and low populations (Figure 5.2A). The PC3 cell line exhibits the same general trend (Figure 5.1, 5.2B). Similar to the DU145 cell line, the percentage of the CD44 high population is decreased in naturally detached cells while the percentages of CD44 negative, low, and intermediate populations are increased when compared to PC3 extracellular PCNA⁺ cells removed from monolayer growth. Contrary to DU145, the PC3 cell line does contain a population of CD24 expressing cells which mainly express high and intermediate levels of CD44 (Figure 5.1).

In the MDA-MB-231 breast cancer cell line, CD24 expression is largely absent in both monolayer growth and naturally detached populations (Figure 5.1). In extracellular PCNA⁺ populations removed from monolayer growth, CD44 expression spans both high levels and intermediate levels without a clear demarcation between the two populations. Upon further comparison in the MDA-MB-231 cell line, CD44 expression is generally decreased in naturally detached cells (Figure 5.2C). This is visible through decreased density of cells above 10^2 log fluorescence intensity in the CD44 high population and extension of the CD44 intermediate population to low expression levels, and the appearance of a small CD44 negative population in naturally detached cells (Figure 5.1).

Opposed to the previous three cell lines, expression of CD24 and CD44 is drastically different in the MCF7 cell line (Figure 5.1). Cells expressing PCNA at the cell surface in this cell line can be separated into three separate populations: CD44⁻CD24⁻, CD44⁻CD24⁺, and CD44^{low}CD24⁺. Compared to the other cell lines, extracellular PCNA⁺ MCF7 cells express much lower levels of CD44, completely lacking a high expressing population, and express higher levels of CD24, which is mostly absent in some of the other cell lines. However, when comparing expression of CD44 between cells removed from monolayer growth and those found naturally detached, a pattern emerges which is similar to the other cell lines. In naturally detached extracellular PCNA⁺ MCF7 cells, percentages of cells expressing intermediate levels of CD44 decrease while CD44⁻ population percentages increase (Figure 5.2D).

It is important to note the labeling of high CD44 expression is relative to CD44 expression within populations of cells expressing PCNA at the cell surface. Prior to gating on cell surface PCNA⁺ cells, populations which do not express PCNA at the cell surface express CD44 at higher levels, up to one log higher than the denoted high populations of extracellular PCNA⁺ cells. While high expression of CD44 is a marker for CSC, lower expression of CD44 has been found to identify metastatic cancer cells [157,158]. These results suggest extracellular PCNA may mark a constituent of cells which have lost cell-cell contact in epithelial structures and are more likely to metastasize.

In addition to CD44 and CD24, other markers have been proposed which identify cancer stem cells and cells undergoing EMT. During EMT, specific transcription factors redefine how cells in an epithelial sheet interact. Most of these transcription factors repress epithelial

adhesion, mainly through decreasing expression of E-Cadherin, a hallmark of EMT [83,84]. During this process, Vimentin expression is up regulated [83,84,88]. Vimentin is a component of the cytoskeleton responsible for maintaining cell integrity [91]. Therefore, we examined cell lines for expression of vimentin by confocal microscopy on cells which were found naturally detached in culture supernatant (Figure 5.3). Vimentin expression is found only on cells expressing extracellular PCNA when naturally detached. This is the case for all cell lines except MCF7, which has previously been noted not to express vimentin [123].

Expression of EMT/CSC genes in PCNA⁺ cells

In addition to evaluating phenotypic markers of EMT and CSCs, expression of EMT transcription factors and stem cell maintenance genes were examined by standard PCR. Cells were sorted based on CD44 or extracellular PCNA expression and analyzed for expression levels of transcription factors listed in table 1 in materials and methods. Specifically for CD44, the highest and lowest 10% of cells expressing CD44 were sorted as CD44⁺ and CD44⁻ respectively. This method was used since some cell lines express very high levels of CD44, lacking a true CD44 negative population. Genetic markers were found to be differentially expressed amongst the cell lines analyzed (Table 3). Both prostate cancer cell lines, DU145 and PC3, express Zeb2, Slug, and Oct 3-4 while breast cancer cell lines do not express these EMT transcription factors. Twist2 expression is only found in DU145 cells while Snail-1 is only found in PC3 cells, but neither transcription factor is expressed in any of the breast cancer cell lines. All cell lines are

negative for expression of Twist1, Snail-3, and Nanog. All four cell lines express BMI and ZEB1, while expression of CD133 is found in all cell lines except DU145.

Because both extracellular PCNA and CD44 positive and negative populations express these genetic factors, quantitative real time PCR was used to assess transcript expression levels between these populations (Figures 5.4 and 5.5). Messenger RNA expression of BMI-1, Zeb1, CD133, and PCNA were further analyzed by quantitative PCR since these genes are expressed by almost all cell lines. In populations sorted based on expression of extracellular PCNA, only MDA-MB-231 cells exhibit a difference in PCNA expression levels (Figure 5.4A). While PCNA transcripts are equally expressed between extracellular PCNA⁺ and PCNA⁻ populations in DU145, PC3, and MCF7 cells, extracellular PCNA⁺ MDA-MB-231 populations express 5 fold more PCNA mRNA than populations of cells lacking cell surface PCNA. On the other hand, populations sorted based on expression of CD44 variably express PCNA transcripts with CD44⁺ populations generally expressing less PCNA mRNA than CD44⁻ populations (Figure 5.5A).

While expression of CD133 mRNA is not detected in DU145 cells, CD133 transcript levels are increased in PC3, MDA-MB-231, and MCF7 cells harboring extracellular PCNA (Figure 5.4B). Expression of CD133 mRNA is not significantly different in MDA-MB-231 populations sorted based on extracellular PCNA expression; however, CD133 expression in extracellular PCNA⁺ populations of PC3 and MCF7 cells are significantly increased by a 2.5 fold difference compared to populations of cells lacking cell surface PCNA. When assessing expression of CD133 mRNA based on CD44 expression, CD44⁺ populations express less CD133 transcript compared to CD44⁻ populations (Figure 5.5B).

Of all the genes analyzed, levels of BMI-1 mRNA vary the greatest between populations sorted by presence or absence of extracellular PCNA (Figure 5.4C). Populations expressing PCNA at the cell surface across all cell lines dramatically express more BMI-1 transcript than extracellular PCNA⁻ populations. DU145 PCNA⁺ populations express nearly 5 fold more BMI transcripts than negative populations while both PC3 and MCF7 extracellular PCNA⁺ populations express about 3 fold more BMI-1 transcripts. The largest increase in BMI-1 mRNA expression is seen in MDA-MB-231 extracellular PCNA⁺ populations with a near 9 fold increase. Populations sorted by CD44 expression follow the same trend as previous genes, expressing less BMI-1 mRNA in CD44⁺ populations compared to CD44⁻ populations across all the cell lines (Figure 5.5C).

Finally, expression of Zeb-1 mRNA was also measured by quantitative PCR and found to vary between populations sorted for expression of cell surface PCNA across the cell lines analyzed (Figure 5.4D). Zeb-1 mRNA levels are decreased in extracellular PCNA expressing populations of DU145 and MCF7 cell lines. In contrast, populations expressing cell surface PCNA in PC3 and MDA-MB-231 cell lines express more Zeb-1 mRNA, with the largest increase consisting of a 7 fold difference in the MDA-MB-231 PCNA⁺ population. Finally, Zeb-1 transcript expression is decreased in CD44⁺ DU145, PC3, and MCF7 populations, but equally expressed between CD44⁺ and CD44⁻ populations in MDA-MB-231 cells (Figure 5.5D).

Analysis of CSC functional characteristics in PCNA⁺ cells

Another aspect identifying CSCs is their functional abilities to self renew and invade surrounding tissue. The ability to form clonal spheres under conditions of nonadherent, serum free culture assesses the potential of CSCs to self-renew, a unique property of CSCs under such culture conditions [78]. DU145, PC3, MCF7, and MDA-MB-231 cells were sorted based on extracellular PCNA expression and purified PCNA⁺ and PCNA⁻ cells were cultured in a 24-well nonadherent plate in Keratinocyte Serum Free Media supplemented with human recombinant Epidermal Growth Factor and Bovine Pituitary Extract. Plates were examined for spheroid formation after one week. Overall, no significant difference is observed in the ability of PCNA⁺ or PCNA⁻ cells to form spheroids (Figure 5.6). Both populations appear to contain some spheroids, but also single cells and large aggregate of cells. Therefore, extracellular expression of PCNA does not appear to select for the ability to self-renew in nonadherent growth conditions.

To assess the ability of extracellular PCNA expressing cells to invade surrounding tissue, a matrigel invasion model was utilized. Matrigel is a gelatinous mixture of laminins and collagens which mimic the tumor microenvironment. By layering matrigel onto a porous transwell insert, the invasive ability of cells can be assessed. Cell lines were again sorted based on expression of extracellular PCNA. Populations of PCNA⁺ and PCNA⁻ cells were resuspended in serum free media and layered on top of matrigel in transwell inserts. Media containing 10% serum was added below the insert to serve as a chemoattractant. After overnight incubation, cells which invaded matrigel and migrated to the bottom of the transwell insert were enumerated.

Alternatively, cells removed from monolayer growth by gentle scraping and cells found naturally detached in tissue culture supernatant were also assessed for invasive potential without sorting into populations based on extracellular PCNA expression. In the DU145 cell line, cells expressing PCNA at the cells surface invade matrigel by more than 50% over cells not expressing extracellular PCNA. Unfortunately, invasion assays performed in the PC3 and MDA-MB-231 cell lines were not consistent between separate sorting events. To partially circumvent this problem, matrigel invasion was compared between cells growing within the monolayer and those which are naturally detached. DU145 and PC3 cells exhibit similar invasion kinetics between monolayer and naturally detached cells; however, naturally detached cells in the MDA-MB-231 cells line invade matrigel in higher percentages than cells found growing within the monolayer. Neither MCF7 cells sorted for PCNA expression nor MCF7 cells growing within the monolayer or naturally detached are invasive.

Cell cycle analysis in PCNA⁺ cells

Like normal adult stem cells, CSCs are believed to maintain a state of quiescence in the G0 stage of the cell cycle [87,159,160]. Therefore, DNA content of PCNA⁺ cells was analyzed with propidium iodide staining and flow cytometry analysis to determine cell cycle stage (Figure 5.8). Cells were first stained for extracellular PCNA, and then fixed and stained with PI. Overall, PCNA⁺ cells from DB, DU145, MDA-MB-231, and MCF7 cell lines are enriched for cells in the G2/M phase, when the cell is in a double diploid state after DNA replication. This enrichment is accompanied by a reduction in the number of cells in G0/G1 phase. While the percent of cells

in the G0/G1 phase decreases in PCNA⁺ cells of the PC3 cell line, percentages of cells in the G2/M phase are not increased as much as the other cell lines. Notably, in all cell lines, PCNA⁺ cells identify a subpopulation of cells which is polyploid, containing more than 2 copies of the chromosome, which drastically contrasts from PCNA⁻ cells which do not display any polyploid cells. PCNA⁺ cells also label a small population of apoptotic cells, which is minimal or absent in PCNA⁻ cells, except in MCF7 cells.

Since PCNA⁺ populations are enriched for cells in the G2/M phase, PCNA expression was analyzed in cells treated with Nocodazole, which arrest cells in the G2/M phase of the cell cycle by interfering with microtubule polymerization (Figure 5.9). MDA-MB-231 and PC3 cultures treated with Nocodazole increase expression of cell surface PCNA, while DU145 and MCF7 treated cells do not. Interestingly, cells treated with Nocodazole from all cell lines adopt a spherical morphology, contrasting elongated morphologies observed in normal cell culture or cells treated with DMSO as a control (Figure 5.10). These results indicate PCNA may also mark cells which are double diploid and about to enter mitosis as well as cells which are polyploid and may suffer mutations affecting cell cycle check point proteins detecting DNA damage.

Evaluation of cell surface PCNA as a potential marker of B cell lymphoma

Finally, potential value of extracellular PCNA as a marker for B cell lymphoma was assessed since DB cells expressed copious amounts of PCNA on the cell surface. The presence of cell surface PCNA on PBMCs from a healthy donor was first analyzed (Figure 5.11). Cells were stained with PCNA antibody in conjunction with antibodies identifying T cells, NK cells, B

cells, and monocytes. Neither B cells nor NK cells in circulation express PCNA on the cell surface. However, some PBMCs do express PCNA on the cell surface, including a small percentage of CD3⁺ T cells and monocytes. PCNA⁺ monocytes are further characterized as the CD14⁺2B4⁺CD16⁻ subset. In a subsequent experiment, PBMCs were activated overnight with PMA and ionomycin and again assessed for cell surface PCNA expression (Figure 5.11). When PBMCs are activated, CD14⁺CD16⁻ monocytes again express PCNA at the cell surface; however, in contrast to freshly isolated monocytes, cell surface PCNA also marks a small constituent of CD14⁺CD16⁺ monocytes. Likewise, a small population of T cells still expresses PCNA after activation. More importantly, in contrast to freshly isolated B cells, activated B cells appear to up regulate expression of cell surface PCNA. Finally, only a minor population of activated NK cells expresses PCNA at the cell surface. Overall, it seems PCNA may be a feasible marker for B cell lymphoma; however, further studies in B cell lymphoma patients will be warranted to fully assess PCNA as a biomarker for this disease.

Discussion

The existence of cancer stem cells is currently a hot topic of research and debate. Many studies have identified CSCs *in vivo* and *in vitro*, yielding potential markers and assays to identify these dangerous cells. Identifying CSC specific markers is vital to the development of novel therapies which specifically target CSCs instead of only targeting the highly proliferative cells which make up the bulk of the tumor [78]. However, because tumors arise in different tissues, there is not a universal marker which identifies CSCs across all types of tumors.

Furthermore, marker expression *in vitro* is dependent on the cell line used as well as the extent of passaging and isolation method [78]. Since phenotypic markers vary, the ideal identification of CSCs relies on evaluating the potential for self-renewal and tumor initiation [80]. Sphere forming assays and serial colony-forming unit assays are commonly used *in vitro* to assess self-renewal; however, these assays are not a bona fide method to identify CSC and caution must be exercised when interpreting results [78,80]. Likewise, assessing expression of genes and signaling pathways associated with stemness is also faulty as these genes are often activated in cells due to malignant transformation [78,80]. Thus, CSCs can only be defined operationally through their ability to seed new tumors in immunocompromised mice when injected in limiting numbers [80,84,88]. While this assay is viewed as the gold standard for identifying CSCs, the assay also has limitations. The primary concern is the effect of the microenvironment of the host animal on transplanted cells [78,80]. It is possible that transplantation may not only select for CSCs, but also cells which have acquired genetic or epigenetic instability imparting greater survivability in the host [80]. Ultimately, identifying CSCs by *in vitro* measures is not sufficient and must be validated with *in vivo* assays [78,80].

CSCs were recently identified in prostate and breast cancer cell lines with a CD44^{high}CD24^{low} phenotype [88,91]. These cells are not only characterized by decreased expression of E-cadherin and self-renewal ability, but also form deeply rooted tumors which invade neighboring tissue in NOD/SCID mice [88,91]. These tumors exhibit increased vascularity and up regulated expression of vimentin [91]. When compared to tumors formed by injection of CD44⁻ cells, tumors originating from CSCs are far more aggressive and require fewer cells to form tumors [91]. To begin assessing the ability of extracellular PCNA to mark

CSCs, CD44 and CD24 expression was analyzed on cells expressing PCNA at the cell surface in DU145, PC3, MDA-MB-231, and MCF7 cell lines (Figure 5.1, 5.2). Since cell surface expression of PCNA is enriched in naturally detached cells floating in culture supernatant, expression of CD44 and CD24 was compared between populations naturally detached and populations removed from monolayer growth by gently scraping.

When comparing CD44 expression across all four cell lines, a general pattern emerges. Overall, CD44 expression is decreased in extracellular PCNA⁺ populations which are naturally detached. CD44 is a cell surface glycoprotein involved in cell-cell interaction, cell adhesion, and migration. As mentioned above, high expression of CD44 is believed to mark CSC populations in solid tumors; however, since CD44 is involved in cellular attachment, lower expression of CD44 is shown *in vivo* to mark metastatic or migrating CSCs which have broken off from the main tumor [157,158]. In this manner, high expression of CD44 may specifically mark stationary CSCs while lowered expression of CD44 may mark migrating CSCs. Therefore, since extracellular PCNA⁺ populations also express lower levels of CD44 (Figure 5.2), cell surface PCNA may be a marker for CSCs which are metastasizing. This also adds weight to the observation of cells naturally detaching from monolayer growth as a rudimentary metastatic process as these cells are enriched for cell surface PCNA and decreased CD44 expression.

Like CD44, CD24 is also an adhesion molecule. Originally discovered in B-cells and neutrophils, CD24 is a mucin-like glycosylphosphatidylinositol linked cell surface glycoprotein which binds to P-selectin and facilitates rolling of leukocytes on endothelium during inflammation [161,162]. Interaction between CD24 on metastasizing breast cancer cells and P-

selectin on endothelial cells is believed to facilitate rolling and extravasation of metastatic cells into the lungs [161,163]. More recently, CD24 is found to mark basal and luminal epithelial cells of the prostate and mammary glands [162]. Specifically within the prostate, CD24 marks transit-amplifying cells which are differentiating into luminal cells [162]. In other reports which seem to contradict CD24^{-/low} expression as a marker for CSCs, CD24 expression induces rapid cell spreading, motility, and invasion [164]. In addition, CD24 expression is associated with poor prognosis in prostate and breast cancer [162]. Ultimately, the use of CD24 as a marker for CSCs is tissue specific as CD24 is a negative selection marker for prostate CSC but a positive selection marker for CSCs in ovarian, colorectal, liver, and pancreatic cancers [162].

Of the cell lines analyzed in this study, only the PC3 and MCF7 cell lines express CD24 in populations of cells expressing extracellular PCNA (Figure 5.1). In PC3 cells, this population is minor compared to the CD24⁻ population; however, in the MCF7 cell line, the majority of the extracellular PCNA⁺ populations express CD24. Since the function of CD24 in tumor cells and its use as a CSC marker are confounded in the literature, it is difficult to ascertain the meaning and function of CD24 related to this study. CD24 expression may mark cells which are undergoing differentiation, especially in the PC3 prostate cancer cell line. On the other hand, CD24 may mark potential metastatic cells with increased propensity to extravasate and colonize tissue; however, this notion is seemingly contradicted by the increased expression of CD24 in MCF7 cells, which is the least invasive cell line used in this study.

Because of enormous heterogeneity in cancer, identifying markers of more aggressive or potentially metastatic tumors is difficult. Assessing these factors is critical in deciding proper

treatment methods. Beyond CD44 and CD24 expression levels, phenotypic markers of EMT are also used to identify potential CSCs and metastatic cells. During the process of EMT, E-cadherin expression is decreased and replaced with expression of N-cadherin and Vimentin, markers of mesenchymal cells [83,84,88]. E-cadherin is an important regulator of epithelial integrity by negatively regulating cellular growth [165]. Therefore, the switch in cadherin expression is commonly found in aggressive tumor cells which have undergone EMT. In terms of adhesion strength, N-cadherin is much weaker than E-cadherin, and mediates increased motility via association with growth factor receptors [165].

Expression of vimentin, an intermediate filament of the cytoskeleton, is correlated with enhanced motility and invasion in prostate and breast cancer [166]. Vimentin networks span from the nucleus to the plasma membrane, attaching to organelles in between and maintaining the overall structure of the cell and the cytoplasm [165]. Essential to providing structural integrity to a cell changing shape, vimentin interacts with actin, microtubules, and other filaments [166,167]. Through cycles of phosphorylation and dephosphorylation, structural components of the cell are disassembled and reconstructed to enable cell motility. Of the intermediate filaments, vimentin is the most sensitive to the phosphorylation cycle [167]. Thus vimentin expression in a cell which is becoming motile is vital. While the vimentin gene is transcriptionally inactive in normal epithelial cells, increased expression is associated with tumorigenic events and increased migration and invasion [165]. Of note, vimentin is typically considered an exclusively intracellular protein, but is found on the surface of some tumor cells and secreted by activated macrophages [165]. Vimentin is also a possible ligand for NKp46; thus, vimentin is most likely involved in immune functions which remain to be discovered [165].

Naturally detached cells from solid tumor cells lines were analyzed for expression of vimentin and cell surface PCNA by confocal microscopy (Figure 5.3). Interestingly, only cells which express PCNA at the cell surface are positive for vimentin expression. DU145, PC3, and MDA-MB-237 cells express vimentin exclusive to cells expressing PCNA at the cell surface. However, MFC7 cells do not express vimentin, even on the very few expressing very low levels of cell surface PCNA. This is most likely due to the specific nature of MCF7 cells compared to MDA-MB-237, especially considering the poor invasive potential of the MCF7 cell lines [123,168,169].

Expression analysis of genes which maintain the stem cell state and those involved in implementing the transition of epithelial cells to mesenchymal cells is another way to potentially identify CSCs. Some of these genes facilitate the acquisition of CSC traits via EMT, which enable cells to metastasize and colonize. Such genes include the Snail, Slug, Zeb, and Twist transcriptions factor families [82,84,85,88,98]. These transcription factors fully induce EMT, repressing epithelial characteristics and promoting mesenchymal phenotypes [83]. More importantly, these genes also activate immunosuppressive cytokines and regulatory T cells, enabling metastatic cells to escape immunosurveillance [83]. Expressed in various combinations in numerous tumor types, these transcription factors are linked to invasion and metastasis [78].

Many of the EMT transcription factors repress the expression of E-cadherin, resulting in a loss of the epithelial phenotype. Twist and Slug transcription factors are also known for suppressing apoptosis, contributing to cell survival in adverse environments and resistance

against therapy [84]. Furthermore, Zeb1 negatively regulates the expression of the miR-200 family of micro RNAs [84]. The miR-200 family is known to suppress expression of BMI-1, which is a critical gene supporting the stem cell state [84]. Thus Zeb1 not only represses E-cadherin expression but also allows maintains expression levels of genes like BMI-1, which confers self-renewal and metastatic potential [78,170].

BMI-1 functions as an epigenetic chromatin modifier which targets many genes and is implicated in the maintenance of stem cells in various tissues as well as the ability to self-renewal [170]. BMI-1 inhibitors have recently been utilized in colorectal cancer models to irreversibly impair tumor growth by targeting self-renewal, offering an exciting new approach to treat this cancer [170]. In addition to BMI-1, other transcription factors such as Oct 3-4 and Nanog maintain the stem cell like state of embryonic stem cells and CSCs [89,90,92]. The expression of these genes and CD133 was determined in cells sorted based on expression of extracellular PCNA or CD44. CD133, or Prominin 1, is a pentaspan transmembrane glycoprotein expressed in some stem cell populations and believed to mark CSC populations in prostate and breast cancer [78]. The function of CD133 in stem cells is still unclear, but is known to localize on microvilli and membrane protrusions [78].

The presence or absence of expression of these genes was first determined by traditional PCR (Table 3). Most genes are found to be differentially expressed between prostate and breast cancer cell lines. For example, only prostate cancer cells lines express Zeb2 and Slug transcription factors as well as the Oct3-4 stem cell maintenance gene. Within the prostate cancer cell lines, DU145 cells exclusively express Twist2, while PC3 cells exclusively

express Snail1. Several genes, including BMI-1, Zeb1, and CD133 are expressed across almost all cell lines analyzed. A drawback to using traditional PCR is the inability to detect differences in expression levels of these genes. Consequently, transcript expression levels of BMI-1, Zeb1, and CD133 as well as expression levels of PCNA mRNA were determined by quantitative real time PCR. Expression of these gene transcripts were analyzed in extracellular PCNA⁺ and CD44⁺ cells relative to their expression in extracellular PCNA⁻ and CD44⁻ cells respectively, and normalized to expression of β -actin (Figures 5.4 and 5.5).

PCNA mRNA levels were first analyzed to determine if simple over expression of the PCNA gene may be responsible for extracellular localization of PCNA. When sorted based on expression of extracellular PCNA, differential expression of PCNA mRNA is only found in MDA-MB-231 (Figure 5.4A). In this cell line, populations harboring extracellular PCNA expressed about 5 fold more PCNA mRNA. DU145, PC3, and MCF7 populations expressed similar levels of PCNA mRNA regardless of whether PCNA was located at the cell surface or not. When populations of cells were separated based on cell surface CD44 expression, PCNA mRNA was generally expressed at lower levels in CD44⁺ populations; however, the maximum difference in expression was only 0.5 fold less than CD44⁻ populations (Figure 5.5). While statistically significant differences in PCNA expression based on CD44 are found in DU145 and MDA-MB-231 populations, a nominal difference of 0.5 fold is relatively small and not likely to hold much functional significance. However, a 5 fold increase in PCNA mRNA levels in MDA-MB-231 extracellular PCNA expressing populations is a significant increase likely to result in a functional difference. In this cell line, cell surface PCNA expression may be due to over expression of the gene. Up regulated expression may result from increased proliferation in populations of cells

expressing PCNA at the cell surface. On the other hand, over expression of PCNA may also reflect mutations to the p53 tumor suppressor gene.

p53 is a sequence-specific transcriptional activator which initiates multiple biological pathways including apoptosis, senescence, inhibition of cell cycle progression, differentiation, and DNA repair [171]. As a vital gene in preventing of tumorigenesis, it is activated by DNA damage, chromosomal aberrations, activation of oncogenes, hypoxia, and many more stimuli [171]. Since p53 is paramount in opposing tumorigenesis, mutations in p53 can be detrimental to normal cellular function and result in cancer formation. Indeed p53 is known as one of the most frequently mutated genes in cancer and it is estimated that nearly 50% of human tumors carries a p53 mutation [172]. Most mutations are found in the DNA binding domain of p53 or alter the overall structure of p53 to perturb this domain [172,173]. Mutant p53 has been extensively studied and is known to contribute to polyploidy, higher DNA mutation rates, and increased cell replication, migration, and invasion while elevating resistance to proapoptotic signaling [173].

Mutant p53 has also been shown to transactivate gene expression nonspecific of binding to p53 response elements within the promoter of a gene. PCNA is a prime example. Low levels of wild type p53 activate PCNA transcription, allowing for normal DNA replication in the absence of genotoxic stress [174]. In response to DNA damage, p53 and PCNA levels simultaneously increase [175]. In this manner, increasing levels of p53 promote PCNA transcription to allow participation in DNA repair. However, as p53 levels continue to increase, PCNA transcription is paradoxically decreased [176]. On the other hand, mutant p53 only

activates transcription of PCNA at high levels [174]. Transcriptional control of PCNA by mutant p53 does not rely on wild type p53 response elements in the promoter of PCNA, but is due to the high concentration of mutant p53 through its increased stability [177]. Of the cell lines analyzed here, only the MCF7 cell line harbors wild type p53 [178]. Thus, p53 mutations specific to the MDA-MB-231 cell line may induce over expression of PCNA.

CD133 is a relatively new marker used to identify potential CSC. While an antibody against CD133 is commercially available, confidence in using this antibody is questionable. When cell lines used in this study were analyzed for binding of anti-CD133 by flow cytometry, only 0.01% of cells were positive for CD133 expression. This is roughly the same percentage of cells which nonspecifically bind isotype control antibody or which autofluoresce at the same fluorescent intensity as cells binding CD133 antibody, eroding confidence in using this antibody to identify surface expression of CD133 in these cell lines. Accordingly, expression analysis of CD133 mRNA was used as a more valid approach. Using this method, CD133 transcript expression is found to be increased in PC3 and MCF7 populations expressing extracellular PCNA (Figure 5.4B). CD133 expression was not detected in DU145 populations and is equivalently expressed in MDA-MB-231 populations in both extracellular PCNA⁺ and PCNA⁻ populations. In populations of cells sorted based on CD44 expression, CD133 mRNA is decreased in CD44⁺ populations in PC3, MDA-MB-231, and MCF7 cell lines (Figure 5.5B). Again, CD133 expression is not detected in populations of DU145 cells. This data suggests extracellular PCNA may mark CSC in a similar manner to CD133; however, expression of CD133 mRNA does not guarantee expression of CD133 protein at the cell surface.

Zeb1 transcript levels were also analyzed as a means to measure the potential occurrence of EMT in sorted populations. When sorted based on expression of extracellular PCNA, only PC3 and MDA-MB-231 cell surface PCNA⁺ populations increase expression of Zeb1, while DU145 and MCF7 cells of the same population marginally decrease expression (Figure 5.4D). Zeb1 expression in PC3 cells is only marginally increased by a 2 fold difference; however, MDA-MB-231 populations expressing PCNA at the cell surface express Zeb1 nearly 7 fold higher than the PCNA⁻ population. These results indicate PC3 and MDA-MB-231 extracellular PCNA⁺ populations may be undergoing EMT events. However, since the MDA-MB-231 cell line has a mesenchymal phenotype, Zeb1 expression may be naturally elevated in this cell line and not indicative of an EMT event.

Finally, and perhaps most importantly, levels of BMI-1 mRNA were analyzed to establish the self-renewal capabilities of sorted populations. Interestingly, extracellular PCNA expressing populations across all cell lines significantly express more BMI-1 transcript levels (Figure 5.4C). PC3 and MCF7 extracellular PCNA expressing populations exhibit an appreciable 3 fold increase in BMI-1 mRNA. Even more robust increases in BMI-1 transcript levels are seen in DU145 and MDA-MB-231 extracellular PCNA⁺ populations with 5 fold and 9 fold increases respectively. When sorted based on CD44 expression, CD44⁺ populations of all cell lines express less BMI-1 mRNA compared to CD44⁻ populations (Figure 5.5C). These results suggest extracellular PCNA is a marker for cells with increased ability to self-renew, a vital component needed for the maintenance of CSCs and metastatic cells once secondary tissue sites are colonized.

It is interesting to note the differential expression of BMI-1 and Zeb1 in DU145 and MCF7 PCNA⁺ cells (Figure 5.4C and D). It may seem contradicting that BMI-1 expression is increased while Zeb1 expression is decreased in PCNA⁺ cells of these cell lines, since both genes can be used to mark CSCs. However, it is important to note that BMI-1 and Zeb1 genes identify two separate aspects of CSCs. Zeb1 is a measure of EMT, which can impart stem cell characteristic upon a cell, while BMI-1 is involved in regulation and maintenance of an already present stem cell state. Therefore, the upregulated expression of BMI-1 in PCNA⁺ DU145 and MCF7 cells indicates an already present stem cell state which is being maintained by self-renewal. On the other hand, the decreased expression of Zeb1 indicates PCNA⁺ cells in DU145 and MCF7 cell lines are not undergoing EMT or generating novel stem cells from epithelial cells. Conversely, the up regulated expression of both BMI-1 and Zeb1 in PC3 and MDA-MB-231 cell lines indicates an already present stem cell state in PCNA⁺ cells and that new CSCs are potentially being generated through expression of Zeb1. Furthermore, differential expression of the genes between the prostate and breast cancer cell lines are likely rooted in the characteristics of these cell lines, especially considering MDA-MB-231 and PC3 are more invasive and aggressive compared to MCF7 and DU145 cells [114,123,168,169].

It also must be noted that expression of genes cannot be directly compared between PCNA and CD44 expression since these values are relative to each specific negative population. Additionally, this comparison would be erroneous since CD44 low or negative populations are composed of both PCNA⁺ and PCNA⁻ cells. Additionally, results we obtained in CD44⁺ cells do not correlate with previous studies identifying increased expression of EMT and stem cell genotypic markers in these cells. The major reason our results differ from previously published

results is the source of cells analyzed. While this work utilizes cells sorted directly from cell culture, previous publications utilized cells isolated from xenografts [90,94,179]. More precisely, these groups implanted DU145 cells into an immunocompromised mouse, allowed a tumor to form, and then isolated the tumor and sorted out CD44⁺ cells [94]. Thus, direct comparison to these results cannot be made due to potential changes induced by drastically different culture environments. Another possible reason for this difference is gating strategy during cell sorting. These publications did not specifically identify their gating strategy when isolating CD44 high expressing cells. Therefore, the threshold of CD44 expression may be a major factor in distinguishing heterogeneity in a cell line. Finally, pitfalls associated with using immortalized cell lines in research have been well noted, especially alterations in phenotypes due to mutations required for immortality [180-183]. However, cell lines are still recognized as a solid basic model to initiate studies and base line evidence.

Another method to identify cancer stem cells is the sphere forming assay. This assay is used to establish the ability of tumor cells to self-renew under serum-free conditions in suspension growth, a unique ability of CSCs [78]. Self-renewal is a vital characteristic in the colonization of new tissue and formation of a macroscopic metastasis [88]. While this assay has been utilized in numerous reports, there are several factors which require consideration while interpreting results. First, some cell lines portray this property ubiquitously [78]. This suggests the ability to form spheres may not equivocally identify cancer stem cells across various cell lines; alternatively, those cell lines may retain stem like properties conveying this ability to all cells. Second, it is often difficult to discriminate between aggregates of cells and actual spheres [78]. Overall, the ability of extracellular PCNA expressing cells to form spheroids is inconclusive

as single cells, some spheroids, and aggregate of cells in both PCNA⁺ and PCNA⁻ populations are observed (Figure 5.6).

In addition to the functional ability of cells to form spheres, the ability of cells to invade matrigel was also investigated. By mimicking the microenvironment surrounding tumors, the ability of cells to invade matrigel *in vitro* is used to assess the potential ability of cells to invade tissue surrounding a tumor *in vivo*, a first step in the metastatic process. Similar to results in the sphere formation assay, experiments assessing the invasive ability of cells sorted based on expression of extracellular PCNA are inconclusive for some cell lines. Populations of cells from prostate and breast cancer cell lines were sorted multiple times; however, percentages of invasive cells were inconsistent between different sorts in the MDA-MB-231 and PC3 cell lines, sometimes varying by up to 60%. Similar difficulties were experienced while assessing the ability of these cells to form spheres. This is most likely due to stress induced by the sorting process. Results were consistent in the DU145 cell line when sorting based on extracellular PCNA expression. Cells expressing PCNA at the cell surface are found to invade matrigel by more than 50% over cells which do not express extracellular PCNA. Alternatively, the invasive potential of monolayer and naturally detached cells was assessed. Cells from monolayer growth and naturally detached populations are equally invasive in the DU145 and PC3 cells line. However, naturally detached cells in the MDA-MB-231 cell line are far more invasive than cells growing within the monolayer. MCF7 cells are not invasive in both scenarios.

Stem cells found within a solid tumor are often quiescent, residing in the G0 stage of the cell cycle and multiplying at a slow rate [159,160]. Stem cells maintain this state to ensure long

term maintenance of the stem cell compartment, only allowing a small number of stem cells to enter the cell cycle when warranted [160]. Since chemotherapy drugs target rapidly replicating cells, quiescent cells are tolerant to these drugs because they have time to repair DNA damage prior to entering mitosis [159]. Thus it is believed quiescent CSCs may be responsible for relapse after therapy [78,80,159,160,184]. DNA content of extracellular PCNA⁺ and PCNA⁻ cells was analyzed by flow cytometry using propidium iodide (PI) staining (Figure 5.8). PI intercalates with nucleic acids; therefore, when RNA is degraded, linear assessment of PI staining yields the DNA content of a cell which is used to determine cell cycle stage. In DB, DU145, MDA-MB-231, and MCF7 cell lines, populations expressing cell surface PCNA are increased for cells in the G2/M phase and decreased for cells in the G0/G1 phase when compared to PCNA⁻ populations. The PC3 cell line does not follow this trend exactly as the percentage of cells in the G2/M phase is only marginally increased compared to the other cell lines. However, percentage of cells in the G0/G1 phase is dramatically reduced in cell surface PCNA⁺ PC3 cells similar to the other cell lines. DU145 and MDA-MB-231 cell lines exhibit the largest difference in percentages of G2/M phase populations based on extracellular PCNA expression. More importantly, in all cell lines, PCNA expression at the cell surface marks a subpopulation of cells which are polyploid, containing more than two copies of the chromosome. This significantly differs from extracellular PCNA⁻ populations which have very few polyploid cells.

Since cell surface PCNA⁺ cells are generally increased for percentages of cells in the G2/M phase, the relationship between cell surface PCNA expression and G2/M phase of the cell cycle was investigated further. At this stage of the cell cycle, cells have completed duplication of DNA and are entering mitosis. Since PCNA is heavily involved in DNA replication, the

necessity for PCNA at this stage is likely diminished, aside from PCNA's role in DNA repair and cell cycle regulation mechanisms. Therefore, cell cultures were treated overnight with nocodazole, which inhibits microtubule polymerization and halts cells at the G2/M phase, and cells were again assessed for stages of the cell cycle based on extracellular PCNA expression (Figure 5.9). As expected, all cell lines are enriched for cells in the G2/M phase with drastic decreases in percentages of cells in the G0/G1 phase. Extracellular PCNA expression was then compared between populations treated with DMSO as a control and populations treated with nocodazole. MDA-MB-231 and PC3 cells increase expression of extracellular PCNA after nocodazole treatment, but MCF7 and DU145 do not. Overall, this suggests cell surface PCNA expression can mark cells in the G2/M phase. Additionally, extracellular PCNA marks cells which are polyploid and may be affected by mutations in cell cycle check points or mechanisms detecting DNA damage. Since CSCs generally reside in the G0 phase of the cell cycle, this data suggests extracellular PCNA may mark CSCs which have reentered the cell cycle.

While CSCs found within a tumor are believed to be quiescent, cell cycle analysis has yet to be performed on metastatic cells. Compared to cells within a solid tumor, characteristics of metastatic cells differ greatly, potentially including cell cycle control. By residing in the G2/M phase, metastatic cells can acquire several advantages to survive the metastatic process. During metastasis, cells are subjected to shear stress in the pressurized environment of the blood stream and lymph flow. Spherical cellular morphology provides increased structural support against this stress and also facilitates increased motility and invasion [185]. When an elongated cell is subjected to the same shear stress, it is quickly disintegrated. Due to increased

structural stability, metastatic cells are known to adopt a spherical morphology while non-metastatic cells are elongated[185].

Interestingly, after nocodazole treatment, cells are visibly more round and appear slightly detached from the tissue culture surface compared to cells treated with DMSO as a control, which look like normal elongated cells in monolayer growth (Figure 5.10). Therefore, cells residing in the G2/M phase with a spherical structure may be more resilient during the metastatic process. Metastatic cells expressing PCNA at the cell surface would be further strengthened by expression of vimentin. Additionally, metastatic cells must divide after reaching a new tissue to form a metastatic lesion. Residing in the G2/M phase would convey another advantage to metastatic cells by enabling immediate entry into mitosis. On the other hand, quiescent cells would require reentry into the cell cycle and replication of DNA, requiring more time, signals from the microenvironment, and nutrients to form a metastatic lesion.

A high quality biomarker specifically targets a cell of interest. Since DB cells express elevated levels of cell surface PCNA, the potential value of PCNA as a biomarker for B cell lymphoma was assessed. Such a marker would be of great importance in the clinical setting as it would allow for better diagnosis of this disease and also targeted therapy for malignant B cells. Extracellular PCNA expression was analyzed on PBMCs isolated from a healthy donor (Figure 5.11). B cells in circulation do not express PCNA on the cell surface, nor do NK cells. However, a small population of $2B4^{+}$ T cells and $CD14^{+}2B4^{+}CD16^{-}$ monocytes do express PCNA on the cell surface. PMA/Ionomycin activated PBMCs were also analyzed for cell surface PCNA expression. After activation, $CD14^{+}$ monocytes decrease expression of cell surface PCNA, while

CD14⁺CD16⁺ monocytes increase expression of extracellular PCNA. Percentages of T cells expressing cell surface PCNA remain relatively stable after activation. However, there is an increase in percentages of activated B cells expressing cell surface PCNA compared to freshly isolated B cells. Finally, a small population of NK cells express cell surface PCNA after activation compared to absence of extracellular PCNA in freshly isolated NK cells.

From this data, it appears PCNA may be of value to identify B cell lymphoma. Even though monocytes and some T cells express PCNA at the cell surface in circulation, B cells expressing PCNA at the cell surface could be discriminated for diagnosis by utilizing an antibody against CD19, a B cell marker. This may be of no significance since B cell lymphoma is characterized by increased numbers of B cells in circulation, which would render isolated PBMCs as mostly malignant B cells. In regards to therapeutic options, targeting cell surface PCNA may be of value, although potential therapies might target populations of monocytes and T cells. While loss of monocytes would put the patient at risk for developing infections, so does most current therapies for B cell lymphoma. Additionally, monocytes are relatively short lived. A deficiency in monocyte number is usually corrected by induction of hematopoiesis in the bone marrow. Further research is needed to assess any potential increased efficacy of targeting PCNA over current therapies. While unable to reach a conclusive answer, this study lays the ground work for obtaining IRB approval to study cell surface PCNA expression in patients with B cell lymphoma.

In conclusion, the data garnered in this specific aim suggests extracellular PCNA may be a marker for metastatic CSC. This notion is supported by the intermediate, low, or no

expression of CD44 and low expression of CD24 (Figure 5.1), as well as the expression of vimentin only on cells expressing extracellular PCNA (Figure 5.3). Extracellular PCNA⁺ cells also express some EMT transcription factors and genes related to maintenance of the stem cell state more than PCNA⁻ cells, most importantly BMI-1 (Figure 5.4). Additionally, populations of naturally detached cells, which are enriched for expression of extracellular PCNA, have the same or elevated potential to invade tissue surrounding a tumor.

On the other hand, the ability to form spheroids is inconclusive (Figure 5.6) and the majority of PCNA⁺ cells are found to be in the G2/M phase of the cell cycle, not in the G0/G1 phase as would be expected for quiescent cells (Figure 5.8). It is a possibility, however, that due to culture conditions or immortalization, CSCs could be abnormally active in terms of proliferation and not quiescent as believed to be *in vivo*. Furthermore, cells in the G2/M phase as well as naturally detached cells adopt a spherical morphology. In this manner, cells expressing PCNA at the cell surface could have several distinct advantages to survive metastasis. First, by adopting a rounded morphology, these cells can survive mechanical stresses during metastasis. Second, residing in the G2/M phase will allow these cells to immediately initiate mitosis. Finally, by expressing PCNA at the cell surface, these cells will escape NK cell mediated killing.

In order to ultimately establish extracellular PCNA expression as a marker for CSCs, PCNA⁺ cells must be xenografted into immunocompromised mice and evaluated for tumor formation at limiting numbers. Taken as a whole, these results potentially suggest an alternative hypothesis, that extracellular PCNA marks cells entering mitosis and cells which are

polyploid. Mitosis is a very active cellular process which changes numerous aspects of the cell, especially the extracellular membrane. As the membrane originally encompassed one cell, after mitosis, the membrane is divided between two cells. This event may alter expression of surface markers, like CD44. Since vimentin is heavily involved in facilitating changes in cell shape, it undoubtedly also plays a role in mitosis as the cell not only drastically changes shape, but also must reorganize and distribute organelles and cytoplasm anchored to the vimentin network. Furthermore, EMT transcription factors and stemness genes may have unknown functions in mitosis. Finally, cell surface PCNA may be a marker for B cell lymphoma which deserves further research (Figure 5.11).

Figure 5.1 Expression of CD44 and CD24 on Cells Expressing Extracellular PCNA in Solid Tumor

Cell Lines

Cells from solid tumor cell lines were harvested by either gently scraping to remove cells from monolayer growth (Monolayer) or collecting tissue culture supernatant to gather naturally detached cells (Naturally Detached). CD44 and CD24 expression was determined by flow cytometry by gating on populations of extracellular PCNA expressing cells.

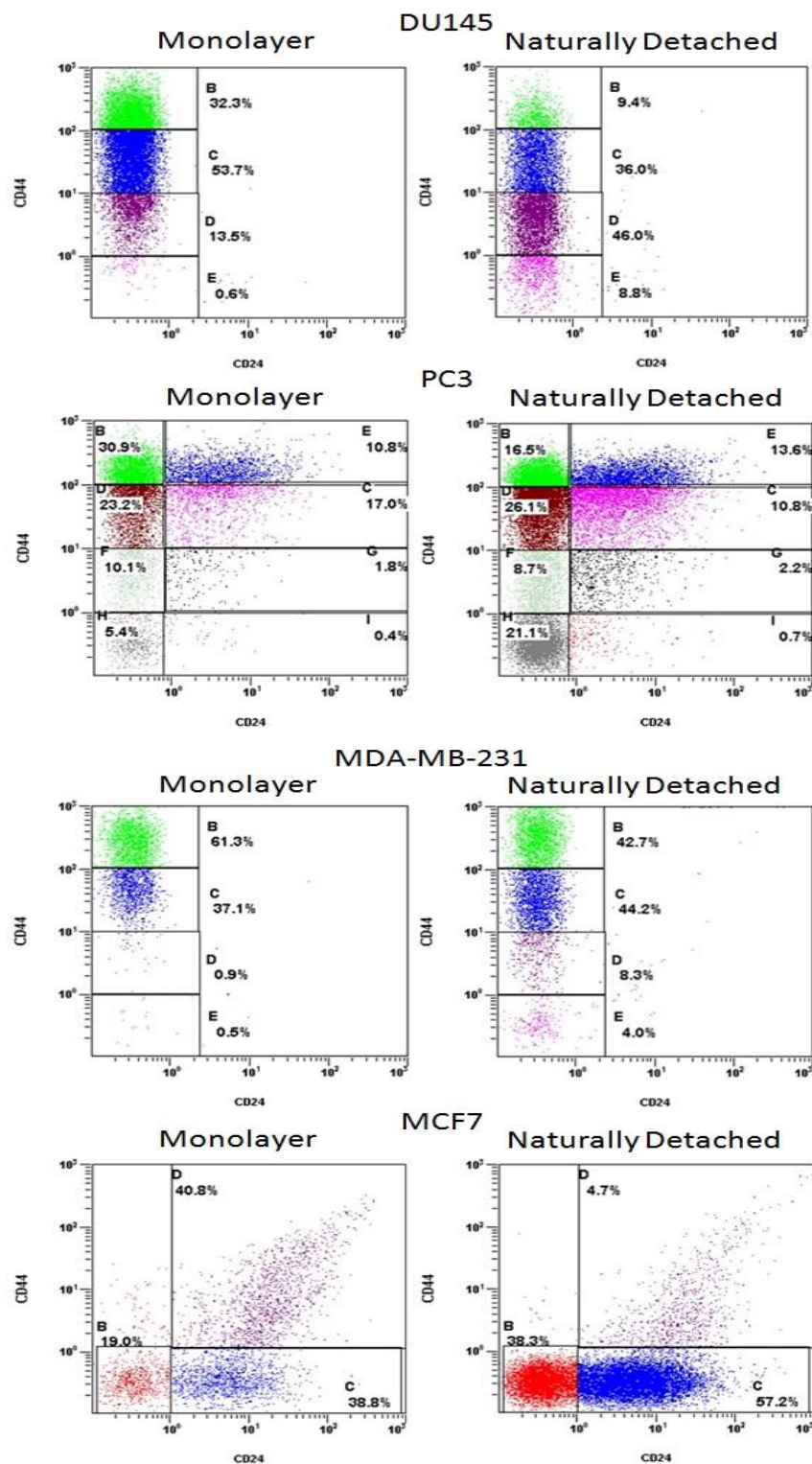


Figure 5.2 CD44 Expression on Populations of Monolayer and Naturally Detached Cells

Expressing Extracellular PCNA

Graphical representation of CD44 expression analyzed by flow cytometry in populations of cells detached from monolayer growth or found naturally detached. CD44 expression was determined by gating on populations of cells expressing extracellular PCNA. In the DU145, PC3, and MDA-MB-231 cell lines, extracellular PCNA expressing cells found within the monolayer express higher levels of CD44 compare to extracellular PCNA expressing naturally detached cells, which express lower levels of CD44. Bars \pm SD.

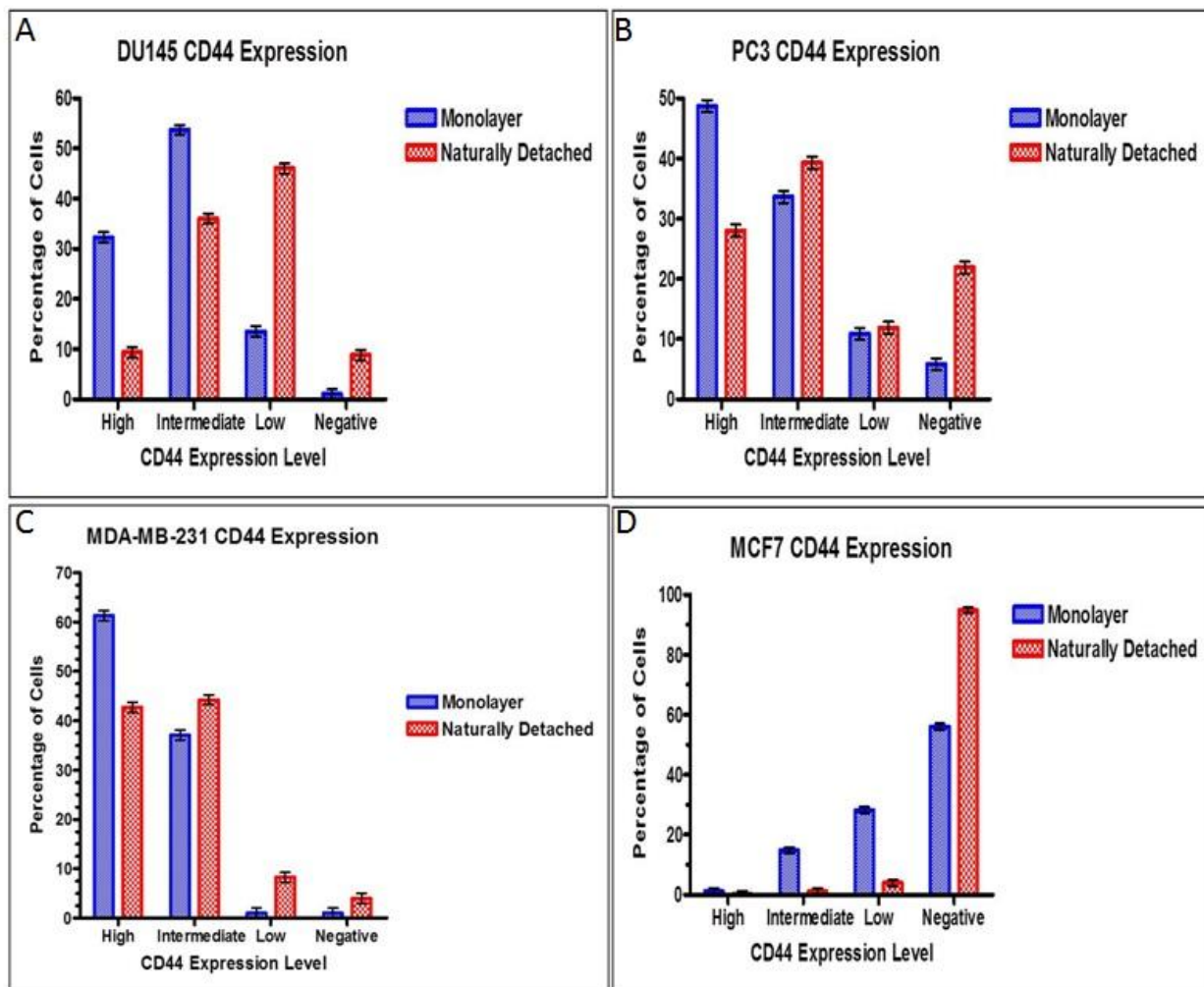
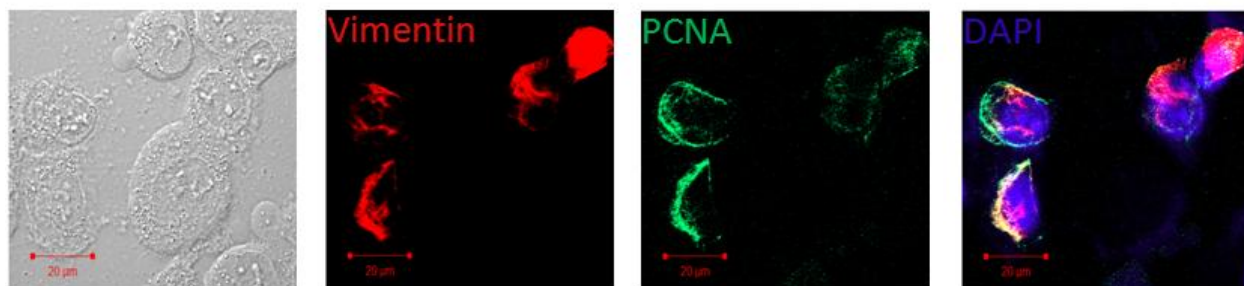
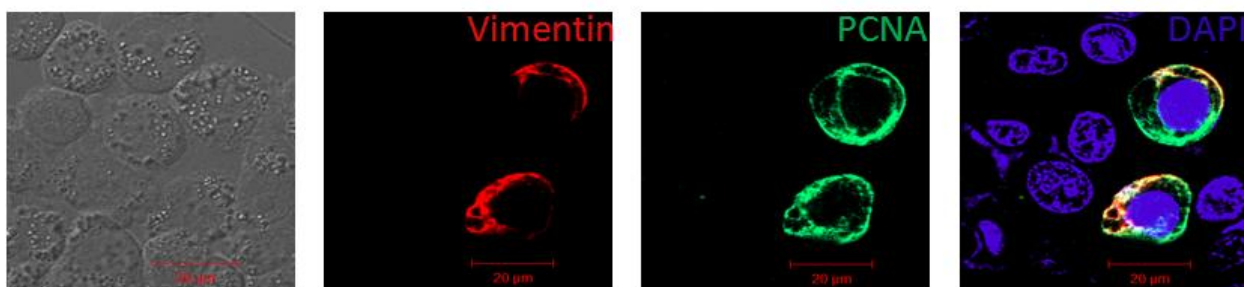


Figure 5.3 Vimentin Expression is Exclusive to Cells Expressing Extracellular PCNA

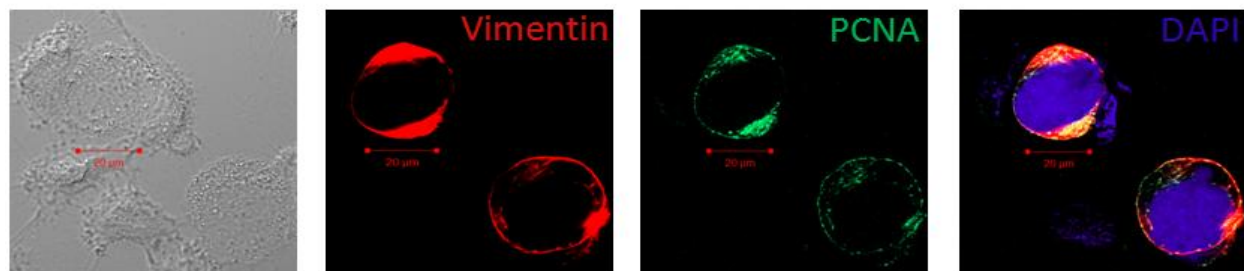
Naturally detached cells from solid tumor cell lines were stained for Vimentin (Red), PCNA (Green), and DAPI (Violet). DU145, PC3, and MDA-MB-231 cells expressed Vimentin only on cells which also expressed extracellular PCNA. MCF7 cells did not express Vimentin. Cells were imaged on the Zeiss LSM 510 Confocal Laser Microscope using 40x, 1.2NA, 0.28 WD (water), C-Apochromat objective utilizing 561 nm, 405nm, and 488 nm wavelengths.



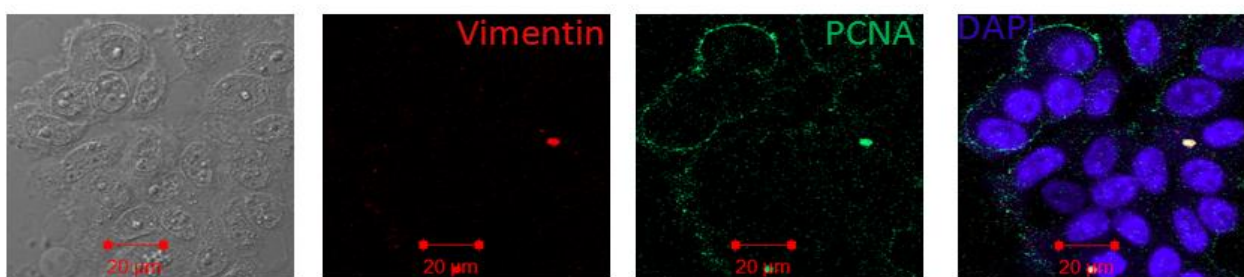
DU145



PC3



MDA-MB-231



MCF7

Table 3 Expression of Epithelial-Mesenchymal Transition and Cancer Stem Cell Genes in Select Populations of Solid Tumor Cell Lines

Solid tumor cell lines were sorted based on expression of extracellular PCNA or CD44. Total RNA was extracted from positive and negative populations from each cell line and converted to cDNA. Expression of genes related to generation of CSC through EMT or genes which maintain the stem cell state were analyzed by standard PCR for presence (+) or absence (-). Genes highlighted in red were selected for qualitative expression analysis.

	Zeb1	Zeb2	Twist1	Twist2	Snail-1	Slug	Snail-3	CD133	BMI	Oct 3/4	Nanog
DU145 PCNA -/+	+/+	+/+	-/-	+/+	-/-	+/+	-/-	-/-	+/+	+/+	-/-
DU145 CD44 -/+	+/+	+/+	-/-	+/+	-/-	+/+	-/-	-/-	+/+	+/+	-/-
PC3 PCNA -/+	+/+	+/+	-/-	-/-	+/+	+/+	-/-	+/+	+/+	+/+	-/-
PC3 CD44 -/+	+/+	+/+	-/-	-/-	+/+	+/+	-/-	+/+	+/+	+/+	-/-
MDA PCNA -/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	+/+	+/+	-/-	-/-
MDA CD44 -/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	+/+	+/+	-/-	-/-
MCF7 PCNA -/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	+/+	+/+	-/-	-/-
MCF7 CD44 -/+	+/+	-/-	-/-	-/-	-/-	-/-	-/-	+/+	+/+	-/-	-/-

Figure 5.4 Quantitative PCR Analysis of Select EMT/CSC Transcripts in Extracellular PCNA^{+/-}

Populations of Solid Tumor Cell Lines

cDNA isolated from populations of solid tumor cell lines sorted for the presence or absence of extracellular PCNA were analyzed for relative transcript expression levels of PCNA, CD133, BMI, and Zeb1 by qualitative real time PCR utilizing the Taqman assay on the StepOne Real Time PCR System. Relative expression was normalized to β -actin of each population and compared to PCNA⁻ populations. Statistical significance, indicated in each pane, was determined by paired T-test. ND, not detected. Bars \pm SD. Figures are representative of two independent experiments.

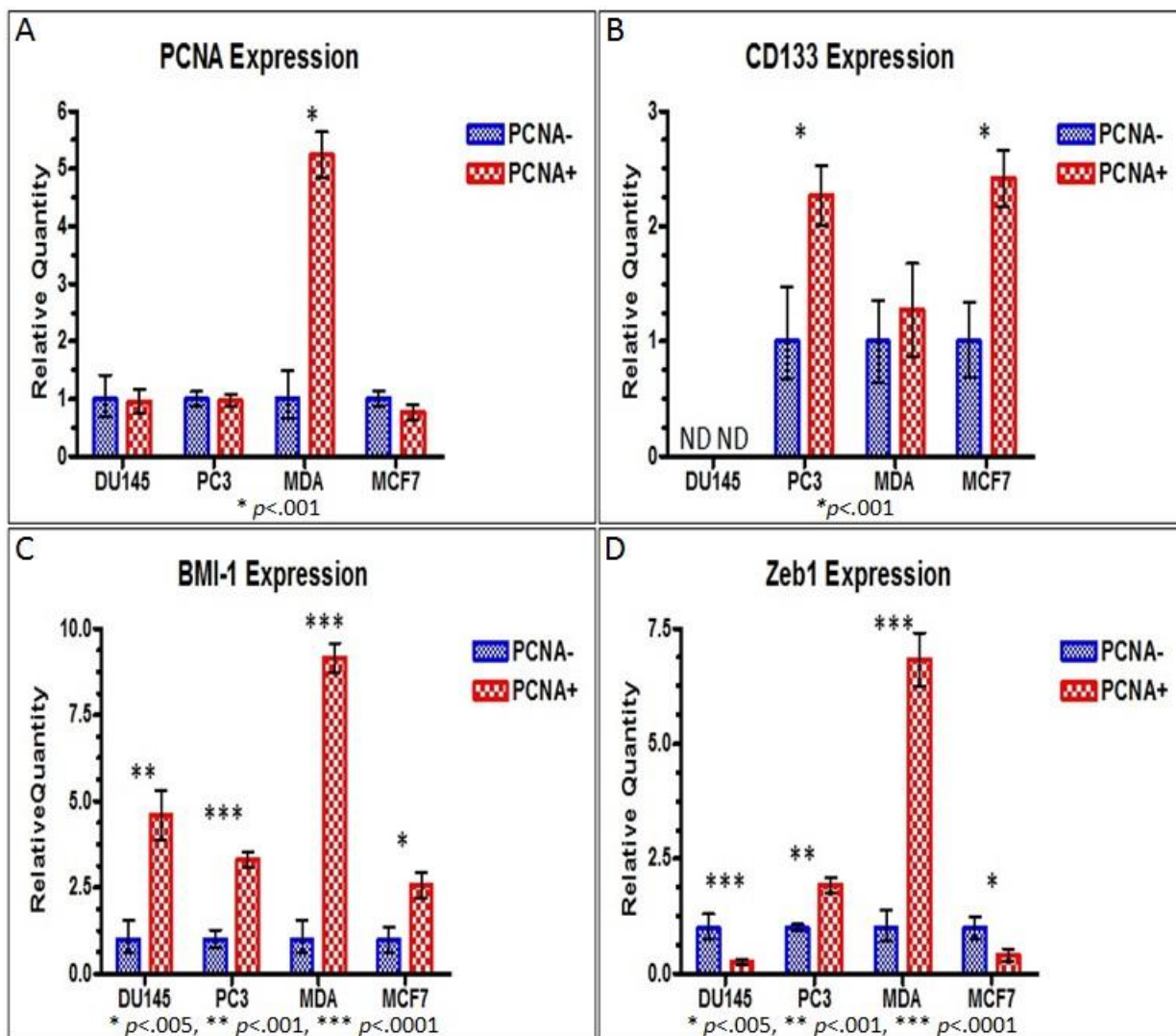


Figure 5.5 Quantitative PCR Analysis of Select EMT/CSC Transcripts in CD44^{+/+} Populations of Solid Tumor Cell Lines

cDNA isolated from populations of solid tumor cell lines sorted for high expression of CD44 or low expression of CD44 were analyzed for relative transcript expression levels of PCNA, CD133, BMI, and Zeb1 by qualitative real time PCR utilizing the Taqman assay on the StepOne Real Time PCR System. Relative expression was normalized to β -actin of each population and compared to CD44⁻ populations. Statistical significance, indicated in each pane, was determined by paired T-test. ND, not detected. Bars \pm SD. Figures are representative of two independent experiments.

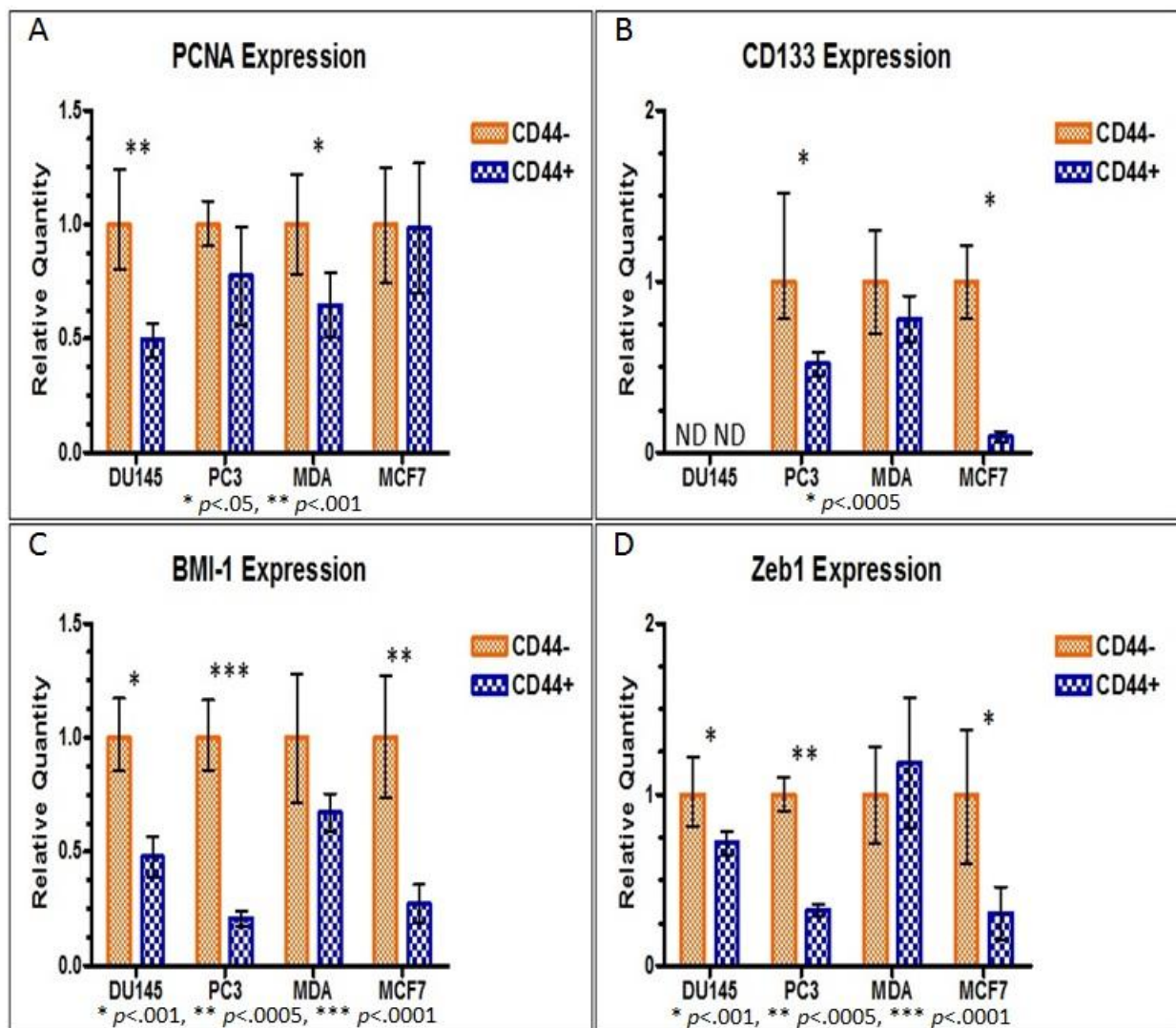


Figure 5.6 Spheroid Formation Assay

To evaluate the ability of extracellular PCNA⁺ cells to self-renew, solid tumor cell lines were sorted based on the presence or absence of extracellular PCNA. Cells were then serially diluted into 24-well nonadherent plates in Keratinocyte Serum Free Media supplemented with human recombinant Epidermal Growth Factor and Bovine Pituitary Extract, incubated for one week, and imaged under 40x magnification. Under these conditions, cells which have the ability to self-renew form spheroid structures. Both PCNA⁺ and PCNA⁻ cells appeared to be capable of forming spheroid structures; however, spheroids were difficult to distinguish from aggregates.

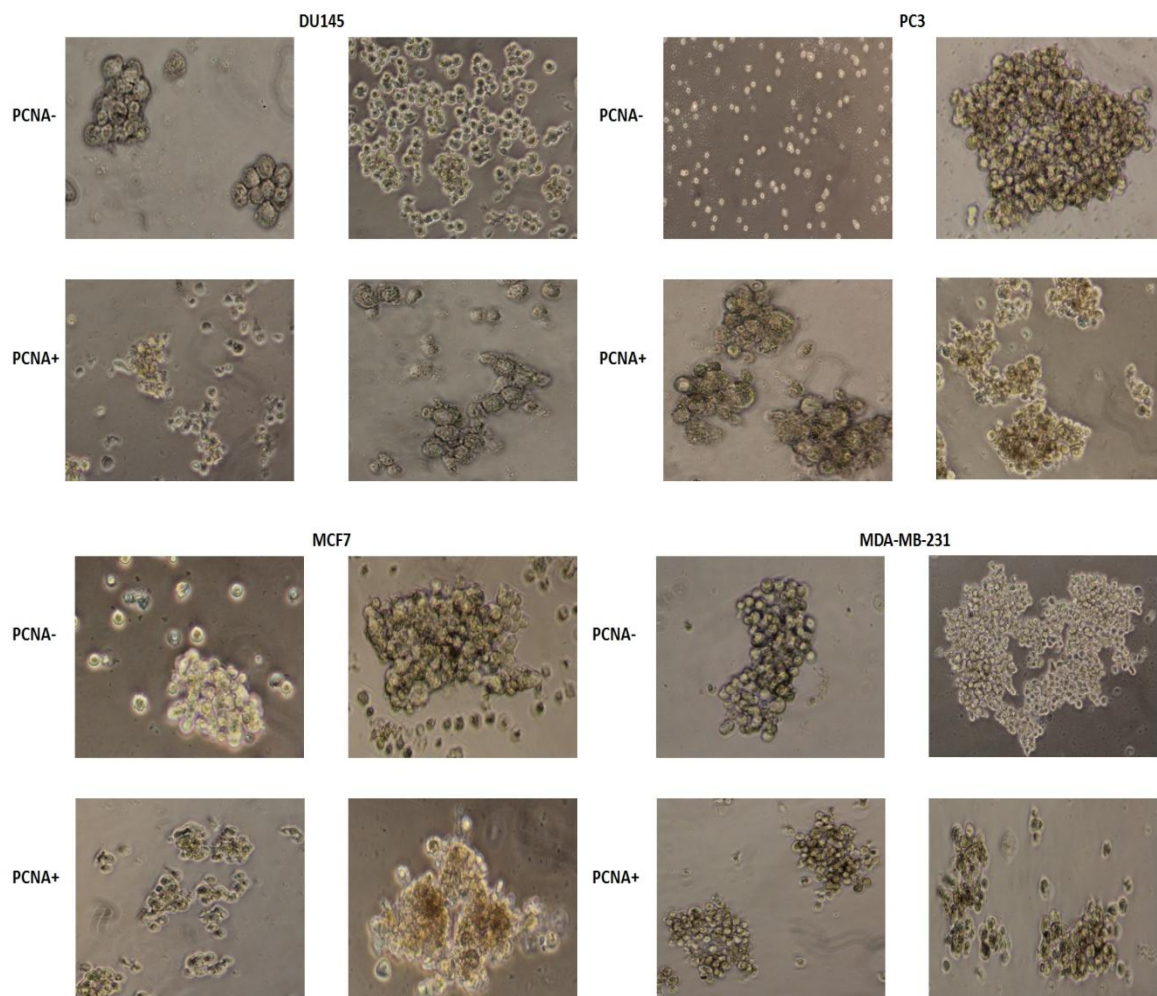


Figure 5.7 Matrigel Invasion Assay

DU145 cells were sorted based on expression of extracellular PCNA. Populations of PCNA⁺ and PCNA⁻ cells were resuspended in serum free media and layered on top of matrigel diluted to 300 µg/mL in transwell inserts. Media containing 10% serum was added below the insert to serve as a chemoattractant. After overnight incubation, cells which invaded matrigel and migrated to the bottom of the transwell insert were enumerated. Figure is an average of two independent experiments. Bars \pm SD. A) Percent invasion of PCNA⁺ and PCNA⁻ populations from the DU145 cell lines. B) Alternatively, cells removed from monolayer growth by gentle scraping and cells found naturally in tissue culture supernatant were also assessed for invasive potential without sorting into populations based on extracellular PCNA expression.

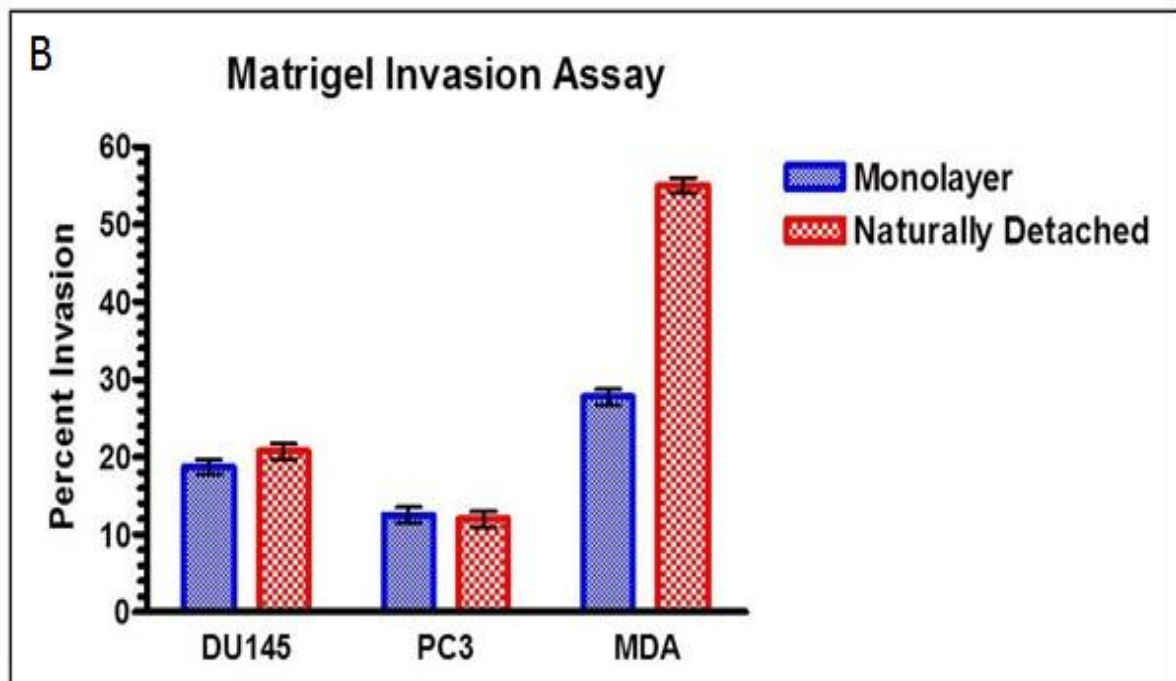
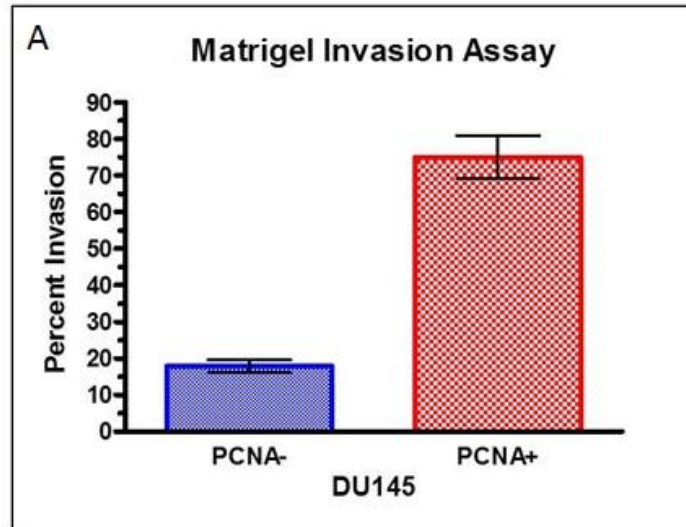
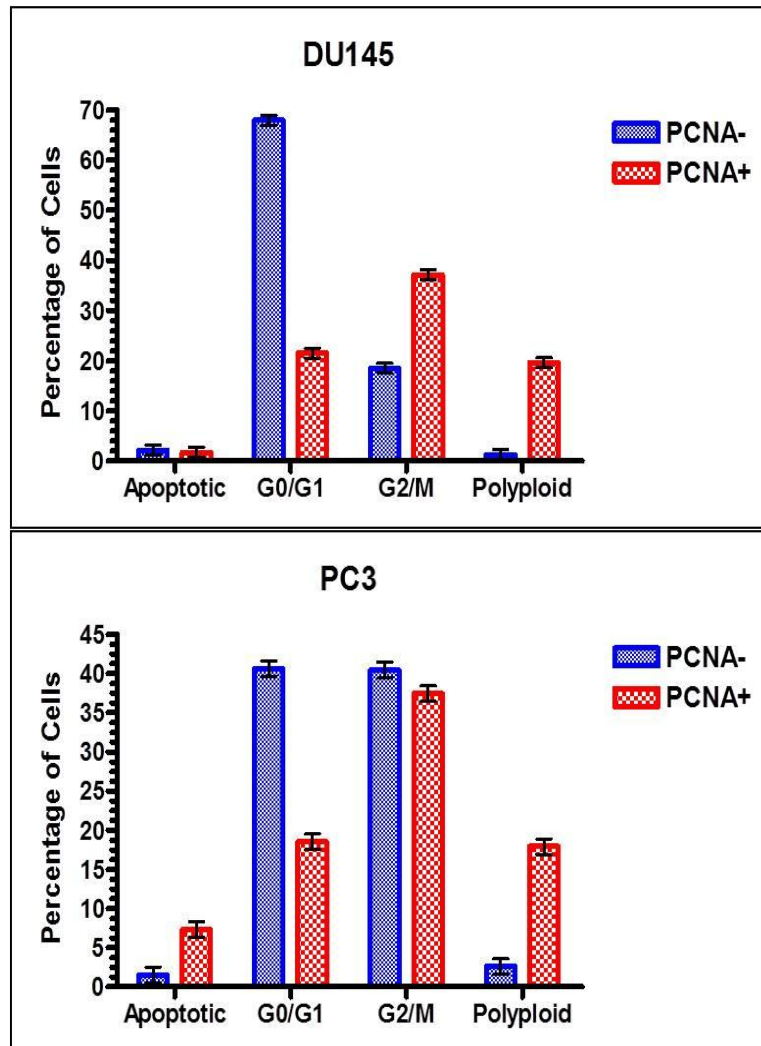


Figure 5.8 Cell Cycle Analysis of Extracellular PCNA Expressing Cells

Solid tumor cell lines and DB cells were stained for cell surface PCNA expression followed by fixation with 70% ethanol and overnight incubation in propidium iodide solution. Cells were analyzed for DNA content by flow cytometry based on presence or absence of extracellular PCNA expression. Presence of extracellular PCNA marks cells which are in the double diploid G2/M phase as well as polyploid cells in all cell lines. Figure is representative of two independent experiments. Bars \pm SD.



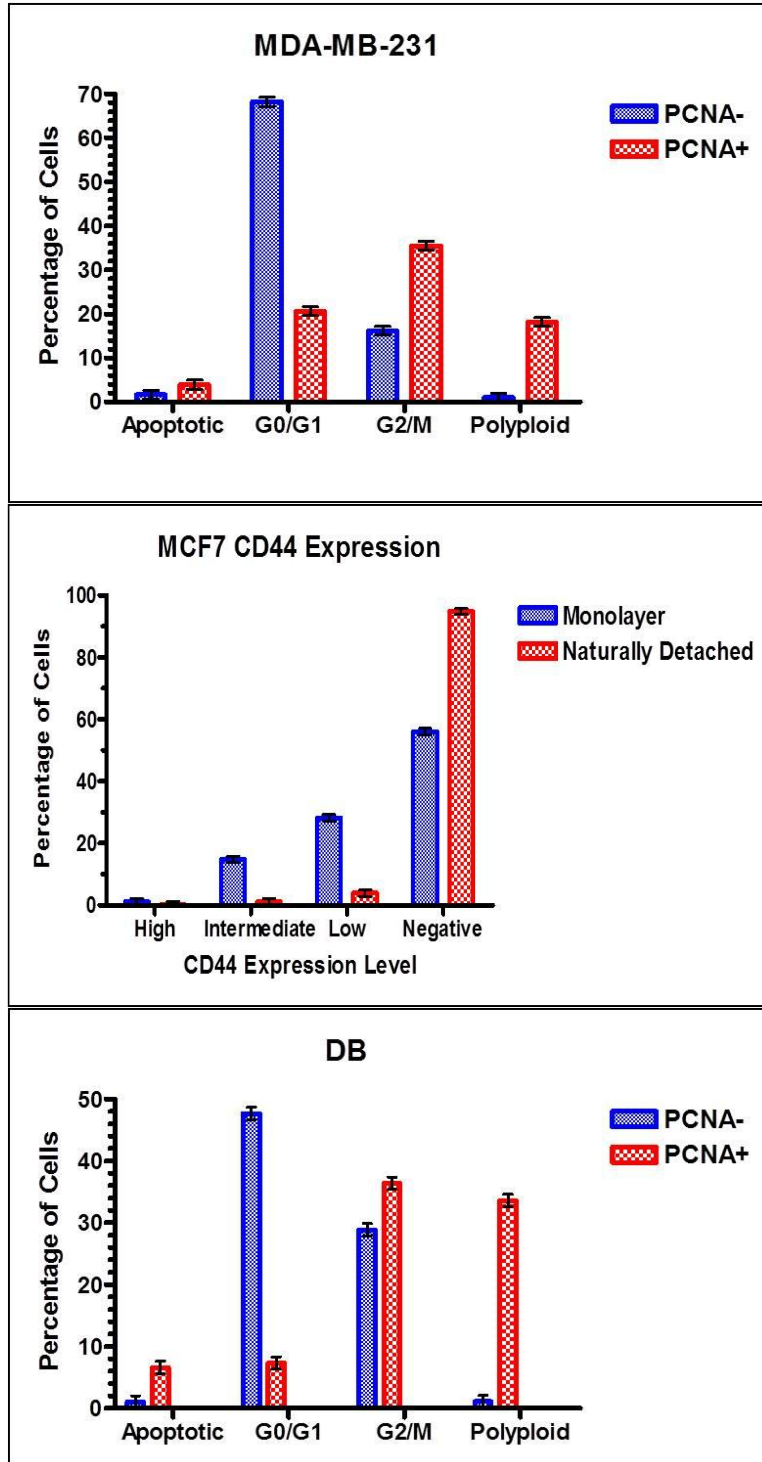
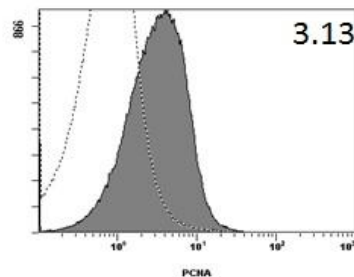
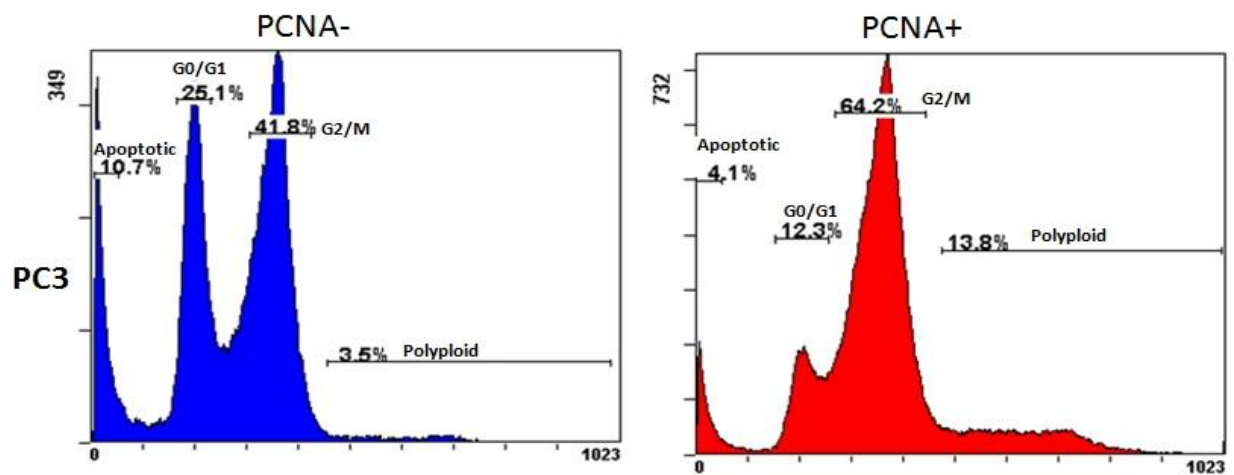
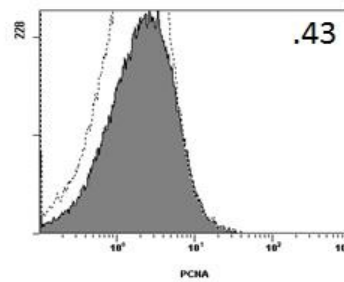
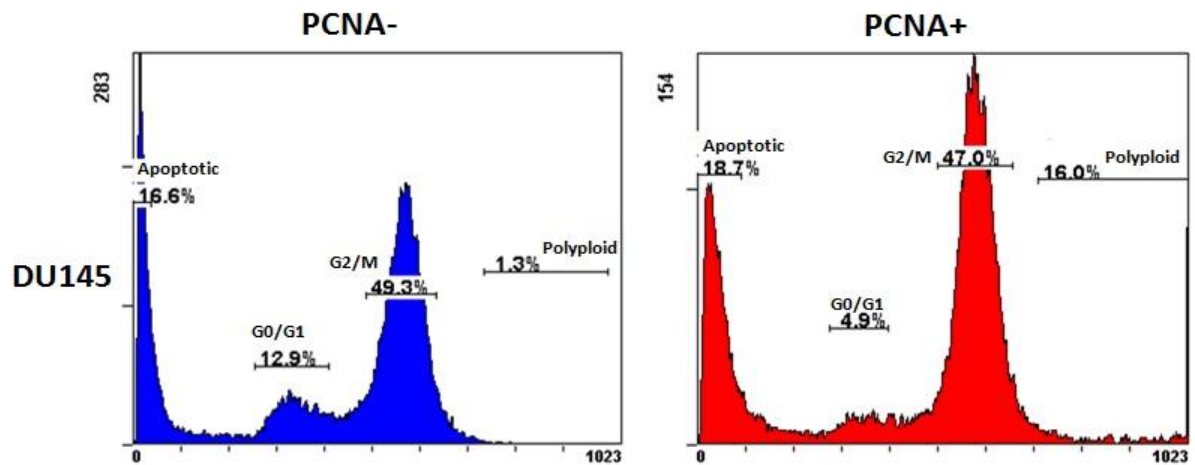


Figure 5.9 Cell Cycle Analysis and Extracellular PCNA Expression After Nocodazole Treatment

Cells were treated overnight with Nocodazole to halt cells in the G2/M phase of the cell cycle.

Cells were then stained for PCNA and with propidium iodide solution as before and analyzed for DNA content by flow cytometry. Histograms below the analysis of cell cycle stage display expression of extracellular PCNA on cells treated with nocodazole (filled histogram) compared to extracellular PCNA expression on cells treated with DMSO as a control (empty histogram).

Mean fluorescent intensity is indicated in the top right corner.



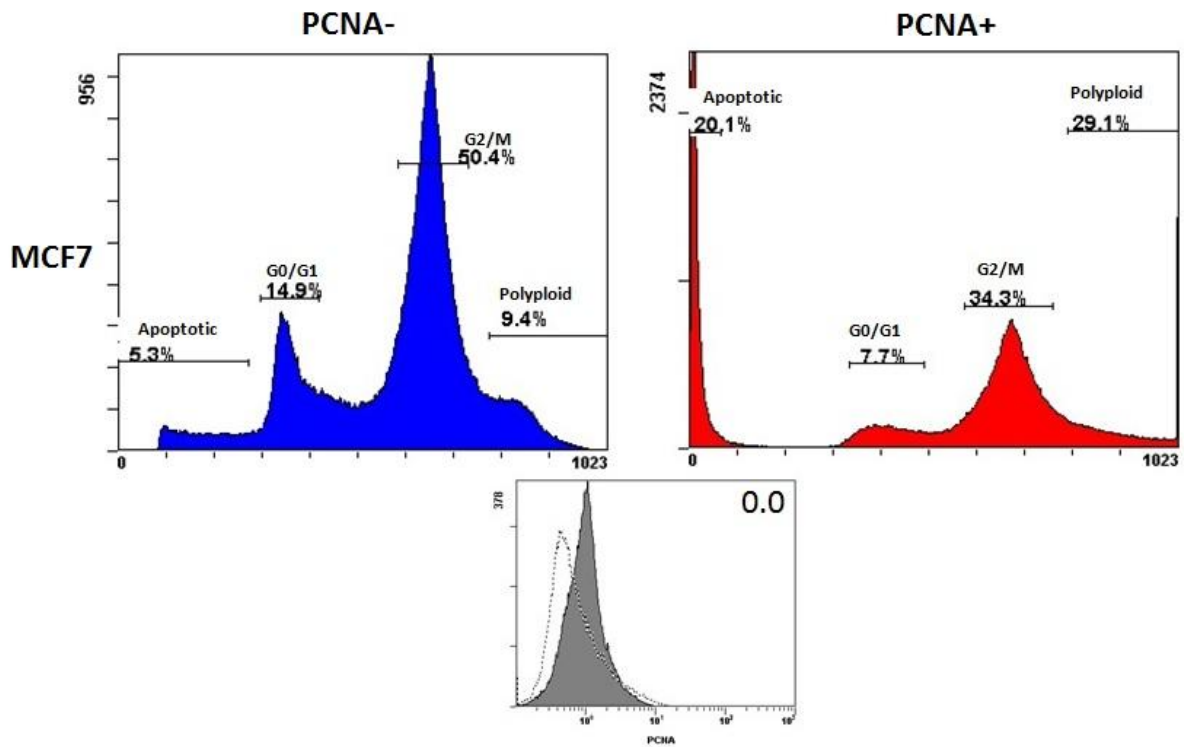
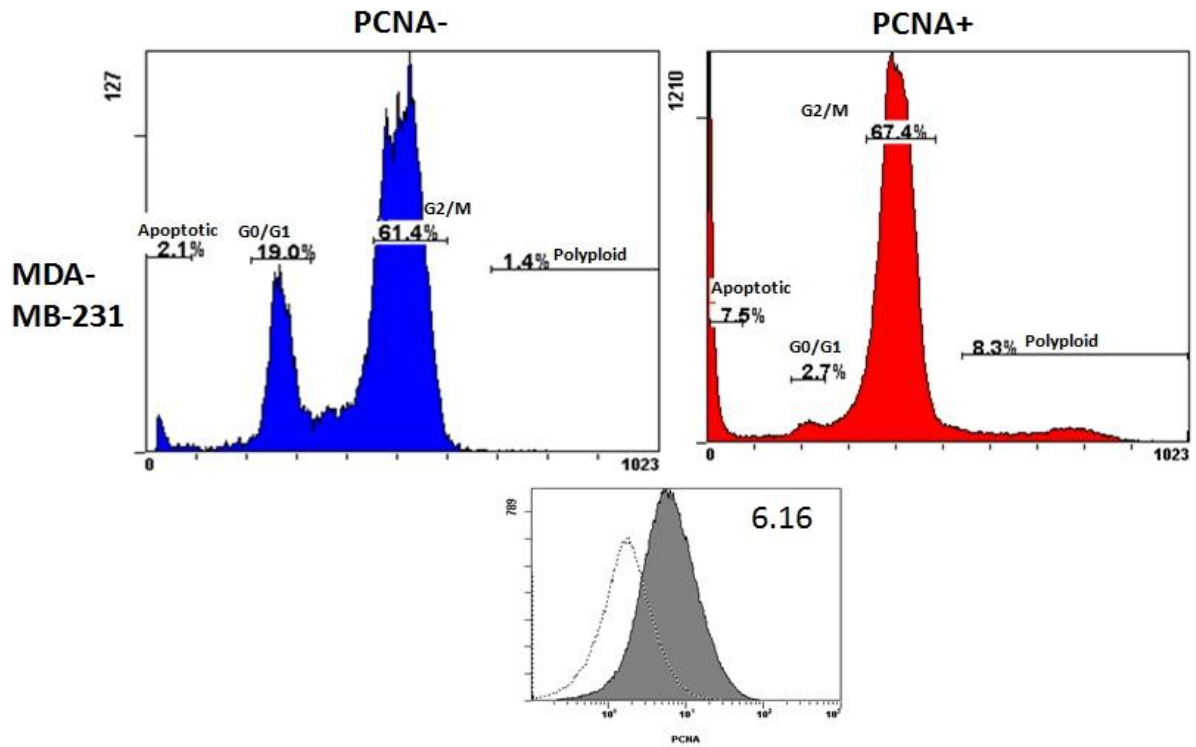
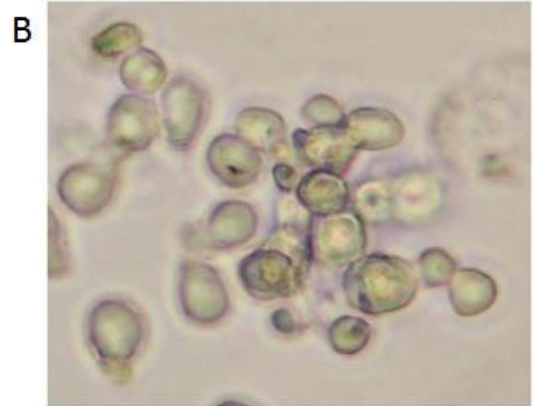
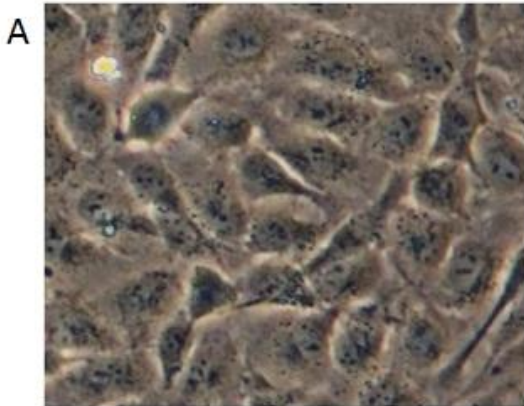


Figure 5.10 Cells in G2/M Phase of the Cell Cycle Adopt a Spherical Morphology

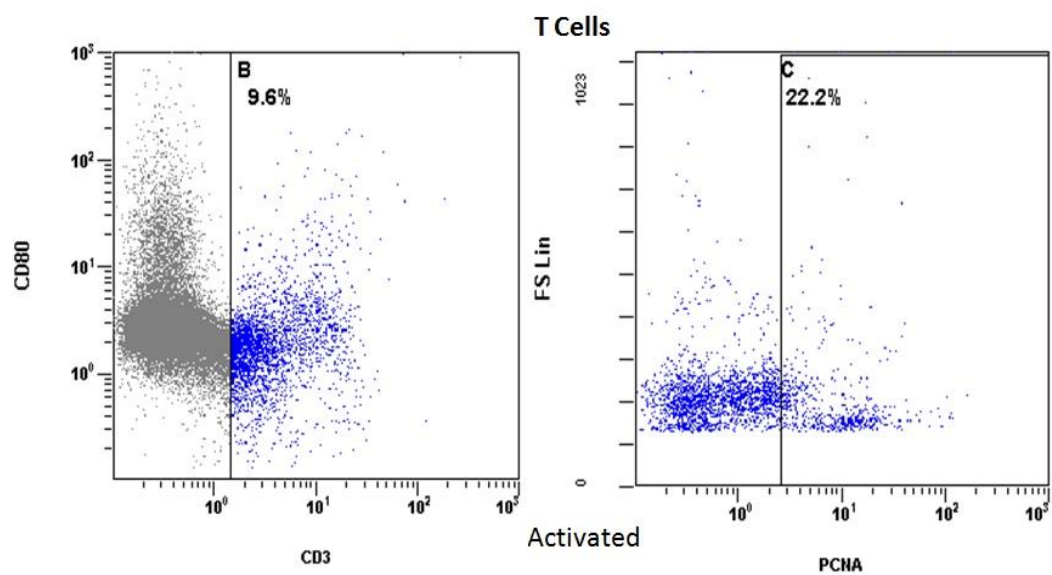
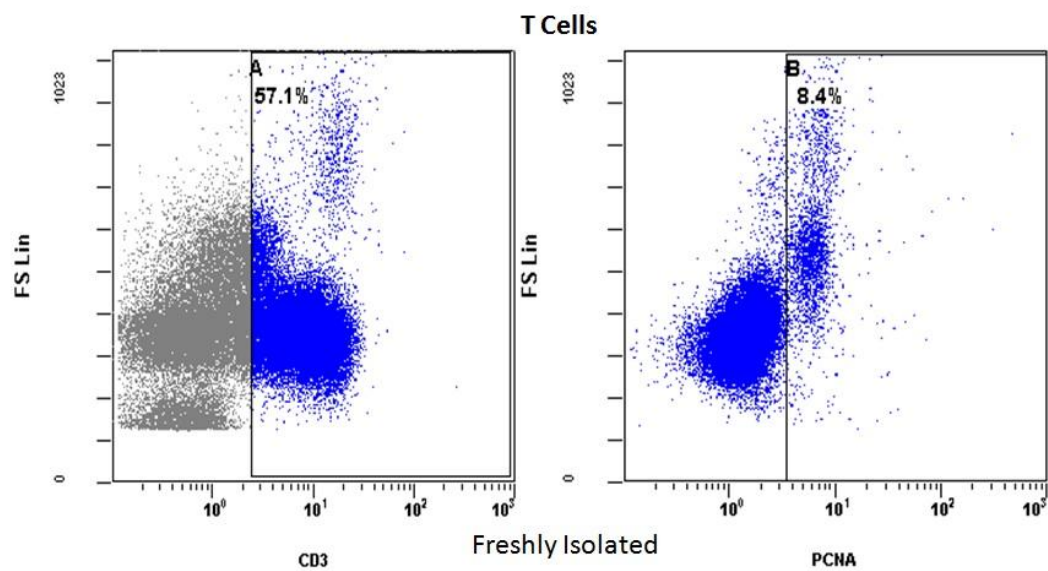
Cells were treated overnight with Nocodazole to halt cells in the G2/M phase of the cell cycle.

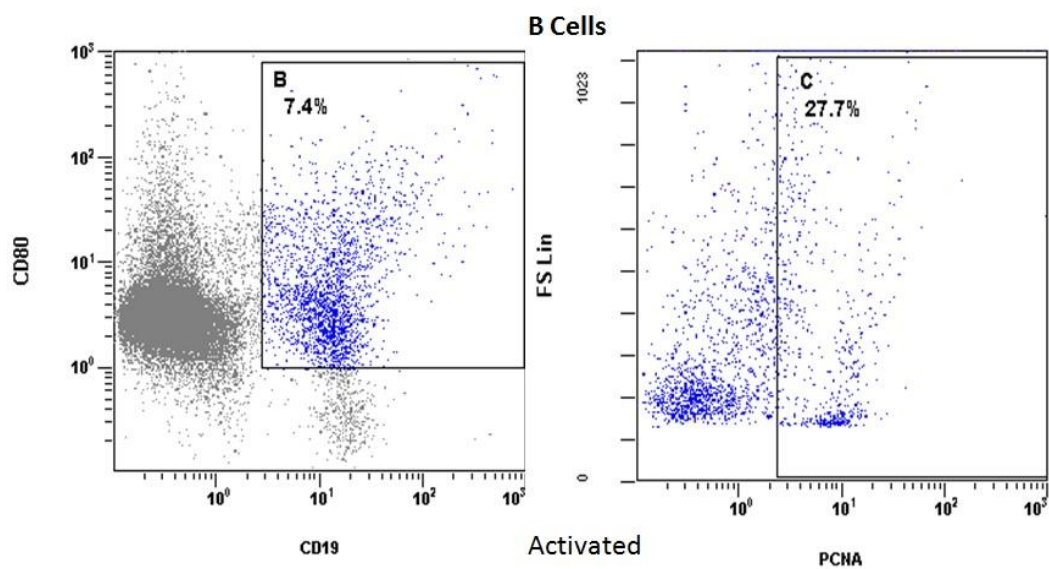
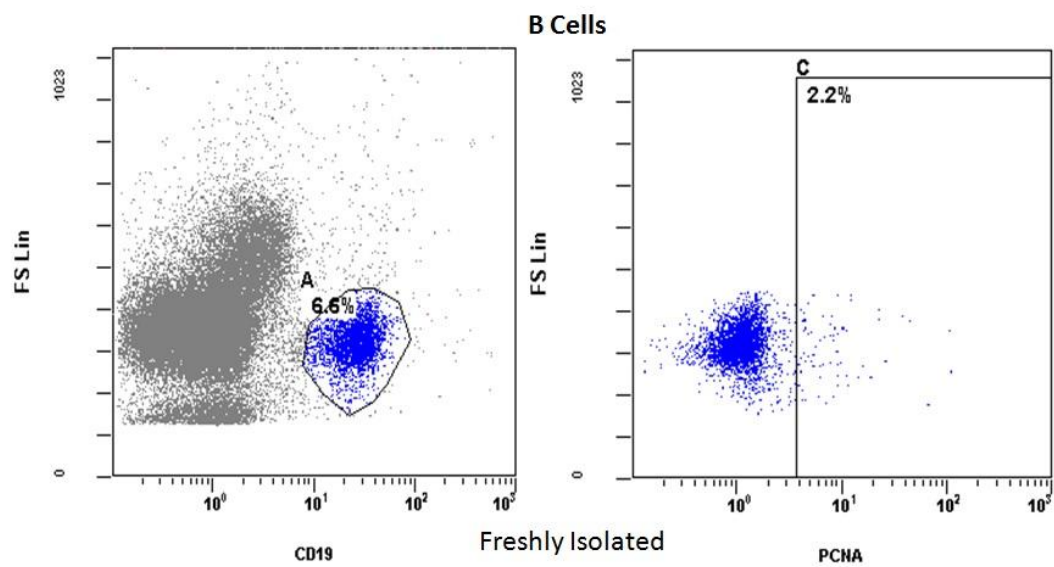
Cells residing in the G2/M phase adopt a spherical morphology compared to elongated morphology of cells treated with DMSO as a control. Spherical morphology affords metastatic cells with increased structural support to survive mechanical stress encountered during metastasis. Cells were imaged under 40x magnification. A.) Cells treated with DMSO (control). B.) Cells treated with Nocodazole

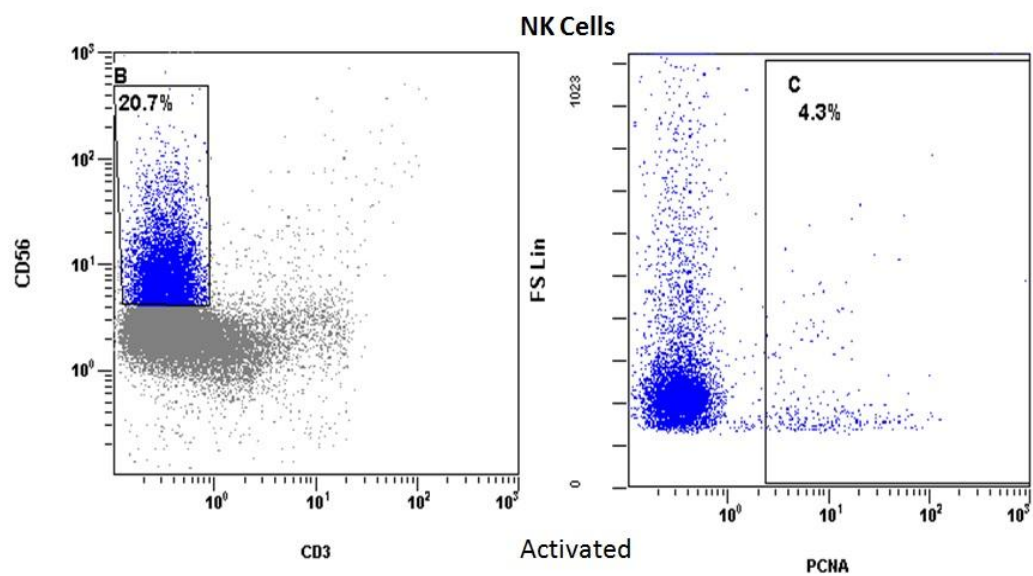
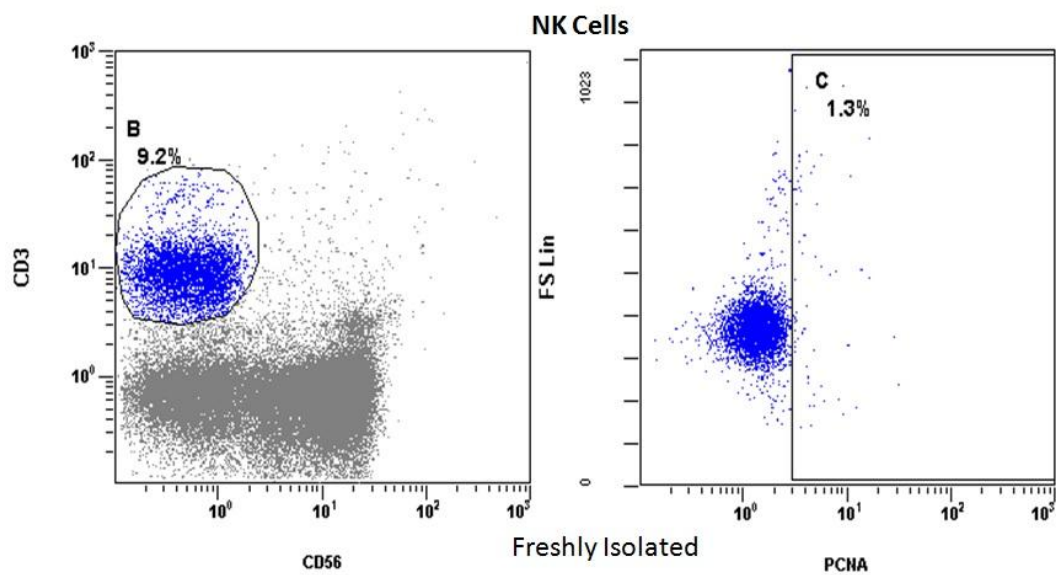


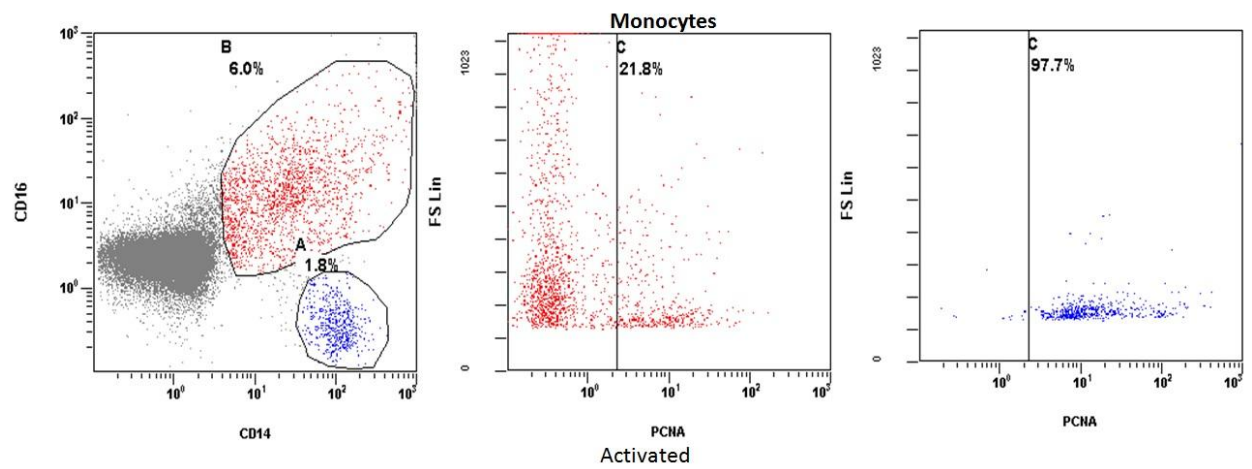
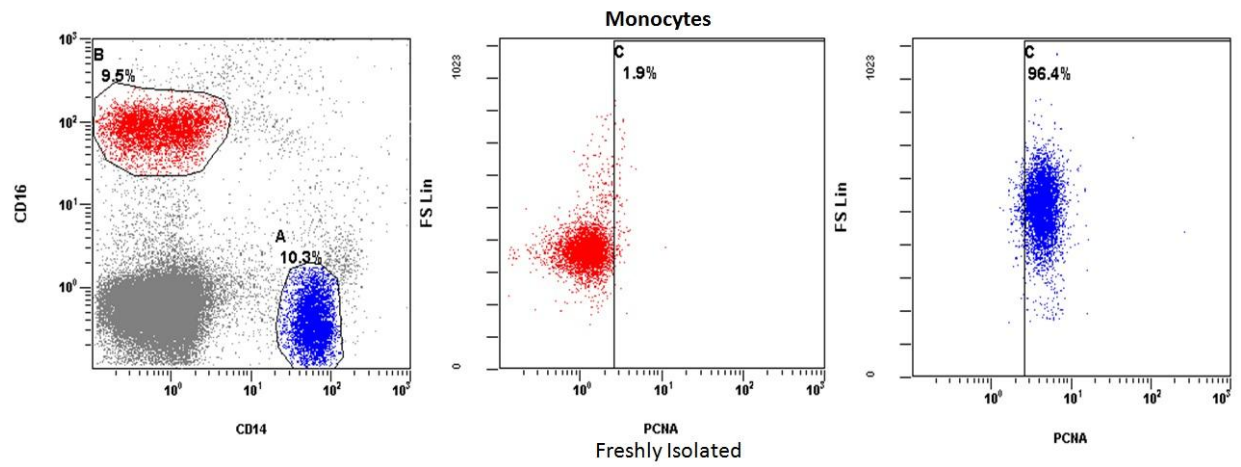
**Figure 5.11 Extracellular PCNA Expression of Freshly Isolated and In Vitro Activated
Peripheral Blood Mononuclear Cells**

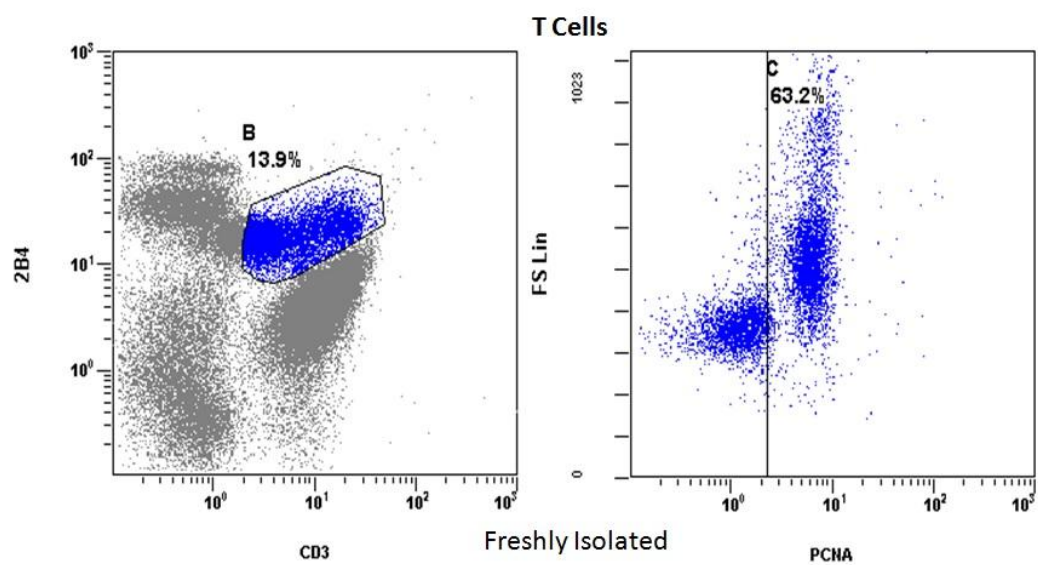
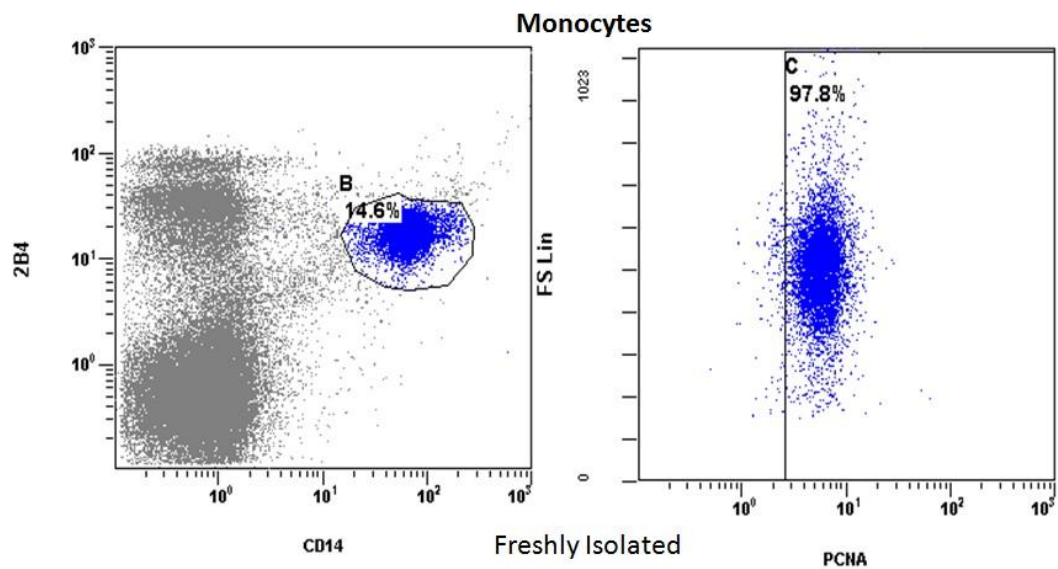
PBMCs were isolated from the blood of a healthy volunteer. Cells were stained for PCNA and markers for T cells, B cells, monocytes, or NK Cells. Additionally, 2B4 expression was evaluated on freshly isolated monocytes and T cells. For *in vitro* activation, PBMCs were cultured overnight in the presence of PMA and Ionomycin. Expression of CD80 was assessed to confirm activation. Extracellular PCNA expression was determined by gating on specific populations of PBMCs. Freshly isolated CD14⁺CD16⁻2B4⁺ monocytes and some 2B4⁺ T cells express PCNA at the cell surface. After activation, T cells still express PCNA at the cell surface while extracellular PCNA levels were slightly upregulated on NK cells. Freshly isolated CD14⁺CD16⁻ monocytes express cell surface PCNA while CD14⁺CD16⁺ monocytes increase expression of extracellular PCNA after activation.











Chapter VI

CONCLUSION

In this study I have investigated the role of interactions between the NK cell receptor NKp44 and tumor cells. First, I identified PCNA/HLA I on the surface of tumors cells as the molecular complex that interacts with NKp44. Second, by interacting with this complex, NKp44 inhibits NK cell effector function. Finally, I have shown cells which express PCNA at the cell surface display cancer stem cell characteristics and provided a basis to investigate cell surface PCNA as a marker of B cell lymphoma.

The majority of people today go about their daily business completely unaware of the battle being waged inside their body. To remain healthy, the immune system must protect the body against foreign pathogens, such as bacteria and viruses, which are encountered on a daily basis. However, an even greater threat which accounts for one in four deaths in the United States lurks on the inside, cancer [107]. An estimated 1.6 million new cancer cases and about 580,000 cancer related deaths were projected to occur in the United States in 2013 [107]. Thanks to major advances in cancer research, cancer death rates declined by 20% since their peak in 1991 to 2009 and continue to drop [107]. However, cancer is still a major public health concern, not only in the United States, but also globally. Most of the advances in cancer research have directly contributed to better detection and treatment, which has reduced the

number of cancer mortalities due to primary tumors to 10% [78]. However, metastasis and relapse after therapy are now the major killers associated with cancer. To address this issue, more research is needed to understand why metastasis and relapse occur and why therapy and the immune system fail to eliminate those cells. Additionally, novel therapies are needed to replace or supplement current therapies which fail to remove the cells responsible for metastasis and relapse.

Within the immune system, NK cells are on the front line of defense against cancer. Utilizing cell surface receptors to recognize specific proteins on the surface of other cells, NK cells discriminate between healthy and cancerous cells of the body, facilitating the removal of cancerous cells via cytotoxic granules while sparing healthy cells. Due to their sentinel like function, NK cells represent an attractive immune based therapy option which can be harnessed to combat cells responsible for cancer metastasis and relapse after traditional therapy. Of the cell surface receptors expressed by NK cells, NKp44 is a strong candidate for development of NK cell based immunotherapy since it is only expressed on activated NK cells and can either inhibit or activate NK cell effector function. The unique expression and signaling of NKp44 places this receptor at the gateway of NK cell decision making in regards to effector functions. NKp44 inhibits NK cell effector functions through recognition of cell surface PCNA and activates effector function via recognition of MLL5 [42,52,53]. As described in chapter 4, cancer cells are able to escape NKp44 mediated NK cell killing by expressing PCNA which associates with HLA I at the cell surface. Therefore, NKp44 based NK cell immunotherapy can be implemented by blocking recognition of extracellular PCNA by NKp44.

Offering further explanation as to how PCNA inhibits NK cell effector function through NKp44, data presented in chapter 3 demonstrate PCNA associates with HLA I on the surface of cancer cells. HLA I is primarily recognized by NK cell inhibitory receptors; thus, PCNA and HLA I form a complex ligand for NKp44 and the presence of HLA I may be responsible for inhibitory signaling. Interestingly, previous reports show NKp30, an NK cell receptor belonging to the NCR family along with NKp44 and NKp46, recognizes Bat3 colocalizing with HLA I on the surface of tumor cells [36,37]. Contrary to NKp44 recognition of PCNA, NKp30 can both activate NK cells when Bat3 is recognized on the cell surface, but also inhibit NK cells when Bat3 is soluble [28]. This suggests inhibitory signaling is not specifically dependent on the recognition of both a DAMP molecule and HLA I. However, there is a key difference between NKp44 and NKp30 in regards to their dual signaling nature. NKp44 inhibitory signaling is transmitted via an ITIM sequence in its cytoplasmic tail while NKp30 is only inhibitory through alternative mRNA splicing [39,42,138]. Thus, NKp44 can facilitate both activating and inhibitory signaling from the same molecule; but, NKp30 requires expression of a truncated version to facilitate inhibitory signaling. Additionally, soluble NK cell receptor ligands are known to inhibit NK cell effector functions [186,187]. In this manner, inhibitory signaling via NKp30 is probably more reliant on the soluble nature of Bat3 rather than alternate splicing of the NKp30 receptor.

More importantly, these data suggest the NCRs may be a class of DAMP receptors since MLL5, Bat3, and PCNA are all molecules usually located intracellularly but are inappropriately presented at the cell surface when cells are stressed. While Bat 3 and PCNA are proven to interact with HLA I, all of the NCRs also recognize HSPG molecules. Postulated to be coligands for the NCRs, HSPGs are macromolecules found on the cell surface carrying numerous

glycosylations which can sequester molecules like growth factors and DAMPs to the surface of cells. Future study will be required to determine if the individual DAMPs recognized by the NCRs or the docking molecule is responsible for activating or inhibitory signals. To date, only viral and bacterial ligands have been identified for NKp46, so it remains to be seen if NKp46 also recognizes DAMP molecules at the cell surface.

In regards to demonstrating HLA I and NKp44 interaction, this study contains several limitations. First, we show NKp44 interacts with HLA I through one degree of separation by demonstrating PCNA interaction with HLA I as PCNA is known to be recognized by NKp44. However, previous reports demonstrate coimmunoprecipitation of NKp44 and HLA I and figure 3.3 demonstrates the ability of anti-HLA I antibody to block binding of NKp44-Ig fusion protein to DB cells. Additionally, figures 3.4, 3.5, 3.6, 3.9, and 3.10 shows PCNA and HLA I associate utilizing colocalization by confocal microscopy and coimmunoprecipitation of PCNA with anti- β 2-microglobulin. Second, it is possible anti-NKp44 antibody activates NK cells by crosslinking the receptor in effector function assays as opposed to only blocking binding of PCNA/HLA I complex. However, blocking with NKp44-Ig fusion protein and anti-PCNA antibody increases effector functions in both NK92 and primary NK cells, demonstrating NKp44 is recognizing PCNA in an inhibitory fashion.

In addition to identifying association between PCNA and HLA I on the surface of cancer cells, this study also characterizes cells expressing extracellular PCNA. Since extracellular expression of PCNA enables cancer cells to escape NK cell effector functions, revealing the phenotypic, genotypic, and functional characteristics of these cells is of great value. As

discovered in chapter 3 and further investigated in chapter 5, extracellular PCNA may mark metastatic cancer stem cells. This hypothesis was conceived through observation of PCNA at the cell surface in the DU145 cell line. Work detailing the association of PCNA and HLA I originated using DB cells, a diffuse B cell lymphoma cell line. DB cells uniformly express PCNA at the cell surface, contrary to the initial report detailing NKp44 recognition of PCNA only on cancer cells in physical contact with NK cells [42]. The major difference between this study and Rosental *et. al* was the cell line used. DB cells are free floating cells which grow in suspension while cells used in Rosental's study are from a solid tumor cell line which grow attached to tissue culture surfaces. To rectify this difference, expression of extracellular PCNA was investigated in several solid tumor cell lines from prostate and breast cancer models. Similar to results obtained by Rosental *et. al*, very few solid tumor cells express PCNA at the cell surface when grown on cover slips and analyzed by confocal microscopy and flow cytometry (Figure 3.6 and 3.12). While suggesting expression of cell surface PCNA may be an inducible event in solid tumor cell lines, this contradicts the binding of NKp44-Ig fusion protein and anti-PCNA antibody to solid tumor cell lines examined by flow cytometry. Since cells must be detached from tissue culture surfaces to be analyzed by flow cytometry, detachment may be an event inducing expression of PCNA at the cell surface. This idea is further supported by observation of extracellular PCNA expression on DU145 cells growing above a confluent monolayer containing cells which do not express PCNA at the cell surface (Figure 3.7).

Solid tumor cell lines grow as a monolayer of colonies on tissue culture surfaces. These cells continuously grow and colonies eventually run out of room for new daughter cells to seat to the tissue culture surface after replication (Figure 3.8). Since these cells are immortalized,

they continuously grow regardless of spatial constraints preventing a new cell from attaching within the monolayer. At this juncture, newly divided cells begin growing above the monolayer. Eventually these cells lose attachment to monolayer cells and are released into culture supernatant where they may reattach in another area with ample room for growth, creating a new colony. This event is reminiscent of metastasis, albeit in a rudimentary form. Like cells which are partially detached and growing above the monolayer, cells which are naturally detached and free floating in culture supernatant express PCNA at the cell surface, remarkably, in all the solid tumor cell lines examined (Figure 3.9). This was also true for the MCF7 breast cancer cell line which express very little to no cell surface PCNA when grown on cover slips. Upon further investigation, cells do not have to be naturally detached to express PCNA at the cell surface. Cells which are detached with EDTA, used to detach cells for flow cytometry analysis, also express PCNA at the cell surface at a higher frequency than attached cells (Figure 3.10). This conclusion was confirmed by comparing PCNA expression in cells which are gently scraped from tissue culture surfaces to cells which are naturally detached and collected from culture supernatants (Figure 3.11 and 3.12). These observations prove cell detachment can induce extracellular PCNA expression and suggest cell surface PCNA may mark metastatic cancer cells. Furthermore, this also shows *in vitro* techniques should be used with caution as they can alter the phenotype of cells during analysis.

Since metastasis is responsible for a vast majority of cancer related deaths, recent cancer research has focused on understanding the process and origin of metastasis. Over the last ten years, cancer stem cells serving as the origin of metastasizing cells have emerged as a popular theory. CSCs are rare, often quiescent, self-renewing cancer cells believed to generate

the mass of heterogeneous cells comprising solid tumors. CSCs are able to resist cellular damage and cytotoxicity by expelling chemotoxic reagents, apoptotic factors, and evading the immune system. Thus, CSCs are believed to be responsible not only for metastasis but also relapse after therapy. CSCs may be generated by mutation of normal stem cells or the reverse differentiation of cancer cells by an epithelial to mesenchymal transition into cells with stem cell properties. Numerous research groups have proposed phenotypic, genotypic, and functional markers to identify CSCs, but no single marker appears to identify CSCs across all cancer types. Ultimately, transplanting low numbers of potential CSCs into immunodeficient mice resulting in generation of aggressive tumors is the gold standard for identifying these cells.

Investigated in chapter 5, extracellular PCNA expressing cells portray several characteristics of metastasizing CSCs. First, CSCs are generally characterized by a $CD44^{hi}CD24^{-/low}$ phenotype [88,91]. However, when cells are metastasizing, CD44 expression is down regulated [157,158]. In Figures 5.1 and 5.2, percentages of cells expressing extracellular PCNA which are naturally detached are increased for expression of lower levels of CD44 compared to cells removed from monolayer growth by gentle scraping. This strengthens the observation of cells naturally detaching as a rudimentary metastatic process and also indicates extracellular PCNA may mark metastasizing cancer stem cells. Furthermore, upon confocal microscopy analysis, vimentin expression was exclusively found only on cells expressing PCNA at the cell surface. Vimentin is an intermediate filament expressed by mesenchymal cells which create an intracellular network spanning from the nucleus to the plasma membrane, strengthening the overall structure of the cytoplasm and the cell as a whole. Vimentin is vital in supporting cells which are changing shape and becoming motile. Thus, vimentin expression is correlated with

enhanced motility and invasion and marks CSCs. Vimentin expression only on cell surface PCNA expressing cells is found in all the solid tumor cell lines analyzed except MCF7, which is previously reported not to express vimentin. Interestingly, vimentin was found to colocalize with extracellular PCNA in several cells, suggesting PCNA may interact with vimentin. The mechanism behind PCNA transport within the cell is still a mystery. This data not only further implies extracellular PCNA is a marker for metastatic cells, but also implies vimentin may facilitate transport of PCNA to the cell surface.

Genotypic markers are also useful in identifying potential CSCs. Many of the genes used to identify CSCs are expressed during EMT events, such as Snail, Slug, Zeb, and Twist transcription factor families. These transcription factors repress the epithelial phenotype and are linked to increased invasion and metastasis. Besides EMT related transcription factors, genes which regulate normal stem cells are also used. Such genes include Oct 3-4, BMI-1, and Nanog. BMI-1 is of particular interest due to its function as an epigenetic chromatin modifier targeting genes maintaining the stem cell state and self-renewal [170]. Recently, BMI-1 inhibitors were shown to irreversibly impair tumor growth in colorectal cancer by impairing self-renewal [170]. Figure 5.4C strikingly shows BMI-1 mRNA is up regulated in cells expressing extracellular PCNA from all the solid tumor cell lines examined. This suggests cell surface presentation of PCNA may mark cells with increased capacity to self-renew.

Expression of CD133, or Prominin 1, was also analyzed. This molecule, whose function in stem cells is unclear, is proposed to identify CSCs in breast and prostate cancer. Like BMI-1, expression of CD133 mRNA is increased in extracellular PCNA expressing cells from all the solid

tumor cell lines except DU145, which did not express CD133 (Figure 5.4B). This further suggests extracellular PCNA may identify CSCs. Transcript expression of the EMT transcription factor, Zeb1, was found to only be up regulated in cells expressing PCNA at the cell surface in the PC3 and MDA-MB-231 cell line, while decreased in DU145 and MCF7 (Figure 5.4D). This suggests EMT may be occurring only in the PC3 and MDA-MB-231 cell line. However, Zeb1 expression in MDA-MB-231 is most likely due to the mesenchymal phenotype of this cell line and not EMT. Overall, gene analysis suggests extracellular PCNA may mark CSCs which have the ability to self-renew.

Stem cells are long lived cells, only replicating when needed. Thus stem cells generally reside in the G0 stage of the cell cycle in a state of quiescence and only enter into the cell cycle when stimulated. In Figure 5.8, the cell cycle stage of cells expressing PCNA at the cell surface was analyzed. Generally, extracellular PCNA expressing cells are enriched for populations of cells in the G2/M phase, not the G0 phase. At this stage of the cell cycle, cells have duplicated their DNA and are preparing to enter mitosis where the necessity for PCNA is diminished. This was further confirmed in figure 5.9 using nocodazole to freeze cells in the G2/M phase by inhibiting microtubule polymerization. When stalled at this stage, PC3 and MDA-MB-231 cell lines increase expression of PCNA at the cell surface, but DU145 and MCF7 cells do not.

Surprisingly, extracellular PCNA is found to also mark cells which are polyploid, containing more than 2 copies of the chromosome, in all the cell lines analyzed. Populations of polyploid cells are minimal or absent in cells not expressing PCNA at the cell surface. This suggests extracellular PCNA marks cells prepared to enter mitosis as well as polyploid cells

which may be affected by mutations to p53, other cell cycle check point proteins, or mechanisms detecting DNA damage. Such cells generally succumb to apoptosis.

Unfortunately no study could be found detailing the cell cycle of metastasizing cells. While CSCs found within a solid tumor are normally quiescent, cells which are metastasizing could gain particular advantages by residing in the G2/M phase. By residing in this stage during metastasis, a cell could immediately divide once reaching a new microenvironment instead of requiring signals and nutrients to support and initiate DNA replication. More importantly, naturally detached cells expressing PCNA at the cell surface and also cells residing in the G2/M phase adopt a spherical structure. This structure is important because it increases the structural integrity of the cell as it encounters shear stress of the blood stream and lymphatic system during metastasis [185]. Non-metastatic cells are typically more elongated morphologically than metastatic cells which adopt a spherical shape [185]. This rounded shape also increases the ability of cells to move and enter vessels [185]. However, extracellular PCNA does not mark all spherical cells because cell surface PCNA is expressed only on specific subsets of PBMCs, which are all spherical. In the case of extracellular PCNA expressing cells, structural integrity is further fortified by vimentin expression. It is also possible that CSCs found within tumor cell lines are abnormally active compared to *in vivo* CSCs. Because cell lines are immortalized, cells can infinitely replicate without exhaustion or depletion of stem cells.

Assays to assess CSC phenotypic, genotypic, and functional characteristics contain several drawbacks as discussed earlier. Namely, many of these assays do not positively identify CSC without a doubt, even engraftment into an immunocompromised mouse; thus, markers,

functions, and the overall existence of CSCs is a highly debated subject in cancer research. This research is limited by the use of immortalized tumor cell lines which may behave differently compared to primary cells in the body and harbor mutations which alter phenotypes and functions. Many cell lines are generated from isolated metastases, such as DU145, which was generated by immortalization of a prostate tumor metastasis from the brain.

Finally, extracellular PCNA was evaluated as a potential biomarker for Diffuse B Cell Lymphoma by examining cell surface PCNA expression in freshly isolate and *in vitro* activated Peripheral Blood Monocytes from a healthy donor. Since DB cells, a human diffuse B cell lymphoma cell line, uniformly express PCNA at the cell surface, extracellular PCNA may be expressed on the surface of B cells in patients with B cell lymphoma. Because biomarkers must be specific to the disease state, expression of PCNA on the cell surface of PBMCs must be minimal for extracellular PCNA to be a viable marker for B cell lymphoma. As shown in Figure 5.11, B cells in circulation do not express extracellular PCNA, but expression is moderately increased after activation. CD14⁺2B4⁺CD16⁻ monocytes are the main population of PBMCs expressing PCNA at the cell surface in circulation. This would not hamper the use of extracellular PCNA as a marker for B cell lymphoma because B cells can be easily differentiated from monocytes utilizing other antibodies. Additionally, B cell lymphoma is characterized by increased number of B cells in the blood which would make monocytes a very minor population found in PBMCs from patients. Overall, this suggests extracellular PCNA may be a feasible marker for B cell lymphoma and warrant further investigation in patients.

Going forward, numerous studies and applications will spawn from this work. First and foremost, the presence and function of extracellular PCNA expressing tumor cells must be evaluated in an *in vivo* mouse model. This will determine if cell surface PCNA is indeed a marker for CSC or metastatic tumor cells. If proven true, numerous applications can be envisioned, including using cell surface PCNA as a clinical tool to identify metastatic cells in patients and improved staging of tumors upon biopsy. For years, intracellular PCNA has been used as a measure of replication to grade tumors. With this data, extracellular PCNA could become a new tool to grade tumors in terms of self-renewal and metastatic potential. Extracellular PCNA could also be used to specifically target metastatic tumor cells, improving upon current therapeutic techniques which often miss these dangerous cells. Finally, further work must be completed to determine if cell surface PCNA is a feasible marker for B cell lymphoma. This simply requires analysis of PBMCs in patients with B cell lymphoma. Again, numerous applications are possible, including better clinical diagnosis of B cell lymphoma as well as improved therapeutic options to specifically attack malignant B cells.

In conclusion, the studies presented here suggest extracellular PCNA is a marker of metastatic cancer stem cells with increased ability for self-renewal through BMI-1 expression, increased motility due to vimentin expression, increased survival during metastasis by adopting a spherical morphology, and most importantly, the ability to evade NK cell mediated anti-tumor immunity. With this knowledge, future NK cell based immunotherapies can be engineered to target these cells to decrease the chances of metastasis and relapse after therapy, which account for the vast majority of cancer related deaths.

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