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THE ROLE OF CELL ADHESION MOLECULES AND MATRIX METALLOPROTEINASES IN OSTEOSARCOMA METASTATIC POTENTIAL

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Abstract

Osteosarcoma (OS) is the most common bone cancer and the second greatest cause of cancer related death in children behind blood cancers. The American Cancer Society reports that the five year survival rate of patients with metastatic osteosarcoma is 30% even with the use of adjuvant or neo-adjuvant chemotherapy. Although, in the last 30 years there has been significant improvement in osteosarcoma of the extremities, 30-40% patients relapse within three years. Even though the use of adjuvant and neo-adjuvant chemotherapy beginning in the 1980's has increased survival, progress has since plateaued.

Work presented in this thesis specifically focuses on the role of integrin induced matrix metalloproteinases in OS metastasis. We hypothesize that the integrin-MMP expression feedback loop is a mechanism whereby metastasizing osteosarcoma cells can produce specific proteolytic enzymes required for invasion. This model predicts that integrin-mediated cell adhesion to different ECM proteins will result in the expression of different MMPs by the OS cell. To test this hypothesis, we have developed osteosarcoma lines that express high and low levels of the α2 integrin subunit. Our results demonstrate that loss of the α2 integrin subunit results in de-repression of MMP 1 and MMP 3 when grown on non-matrix substrate (plastic) and matrix substrates (fibronectin and collagen). Our data suggest a relationship between integrin ligation of specific ECM proteins and subsequent changes in MMP protein expression.

Secondly, we hypothesize that the metastatic potential of OS is influenced by the type and amount of integrin receptors present on osteosarcoma cells. To test this hypothesis, we have evaluated the integrin profile of two osteosarcoma cell lines of low

(SAOS-2) and high (LM7) metastatic potential. We found that the metastatic sub-line LM7 has lower alpha 5 integrin subunit levels when compared with the non-metastatic parental SAOS-2 cell line. This reduced alpha 5 integrin subunit expression may implicate this integrin in the metastatic process of osteosarcoma. Further, we also present data on MMP expression in the SAOS-2 parental cell line compared with the LM7 cell line. In this study, we show that the LM7 cell line expresses significantly less MMP 2 when plated on plastic and matrix substrates. Reduced MMP 2 expression in the LM7 cell line may suggest aberrant MMP 2 regulation in osteosarcoma metastasis.

Finally, due to the lack of osteosarcoma cell lines, we have isolated and partially characterized a primary osteosarcoma cell line from a patient diagnosed with Marfan Syndrome called Penn State University-Osteosarcoma-Marfan syndrome (PSU-OS-M). We have selected for tumor cells without chemical or viral induction unlike most of the OS research cell lines available. The PSU-OS-M cell line was successfully cultured *in vitro* and the cells were shown to exhibit attachment independent growth and loss of contact inhibited growth when cultured on plastic (hallmarks of transformation). We hope the establishment of this osteosarcoma cell line will better reflect human osteosarcoma. Since mutations in the fibrillin-1 gene are responsible for Marfan syndrome, we evaluated fibrillin-1 expression. Using immunoflorescent analysis, we observed reduced fibrillin deposition but normal fibronectin deposition in the PSU-OS-M cell line.

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OS	osteosarcoma
MMP	matrix metalloproteinase
ECM	extracellular matrix
FBN1	fibrillin-1 gene
CAMS	cell adhesion molecules.
MFS	Marfan syndrome
RPTPs	receptor tyrosine phosphatases
α	alpha
β	beta
TPA	12-0-tetradecanoylphorbon 13-acetate
OA	okadaic acid
ACS	American Cancer Society
CT	computed tomography
MRI	magnetic resonance imaging
Rb	retinoblastoma
q	short arm of chromosomes
p	long arm of chromosomes
p53	tumor protein 53
UMR 106-01	murine osteosarcoma cell line
K7M2	murine osteosarcoma cell line
K12	murine osteosarcoma cell line
U2-OS	human osteosarcoma cell line

HOS	human osteosarcoma cell line
SAOS-2	human osteosarcoma cell line
LM7	human osteosarcoma cell line
MG63	human osteosarcoma cell line
CS-1	hamster melanoma cell line
NCP	Negative control MG63 cells transfected with a scrambled siRNA
PSU-OS-M	Penn State University-Osteosarcoma-Marfan Cell line
REPE	Retinal epithelial pigmented cells

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Chapter 1

Background

The focus of this thesis is to evaluate the metastatic mechanisms of osteosarcoma mediated by integrins and matrix metalloproteinases (MMPs).

1.1 Extracellular matrix (ECM)

Cells secrete, assemble and remodel an insoluble network of proteins known as the extracellular matrix (ECM). The ECM consists of glycoproteins fibronectin, collagens, laminins and proteoglycans. The ECM also consists of non-matrix proteins such as growth factors. In concert, these proteins provide a flexible but durable scaffold for organization of cells into tissues. Cell adhesion to the ECM allows cell-matrix interactions and provides contact with the cells' extracellular environment. These interactions are important for cell migration, tissue organization and differentiation and play a role in remodeling and homeostasis of tissue and organ systems (Berrier and Yamada 2007). For example, bone is composed of mainly one type of ECM, collagen fibers (Vu 2001), while the lung contains fibronectin, glycosaminoglycans, laminin, type IV collagen and tensascin (Dunsmore and Rannels 1996). Furthermore, the composition of the surrounding ECM determines how a cell will react to its environment (Boudreau and Jones 1999).

Regulation of the ECM is achieved largely by the proteolytic enzymes that remodel the ECM structure and release bioactive fragments and growth factors. These enzymes include matrix metalloproteinases (MMPs), adamalysin-related membrane

proteinases, bone morphogenetic proteins and tissue serine proteinases. It is important to note that many of these proteinases not only play a role in homeostasis but also in tumor progression and metastasis. Further, many of these proteinases have been found to be expressed by responding stroma of epithelial tumors (Werb 1997). However, the role of the ECM in sarcoma tumor progression has been greatly understudied.

1.1.2 Fibrillin and Marfan Syndrome

The ECM contains several fibrillar structural elements including several non-striated fibrils with small cross sectional diameters. These smaller fibrils are classified based on average diameters. Of interest to this thesis is fibrillin-1, a 350kD connective tissue protein that is a major component of the extracellular microfibrils (Sakai et al. 1986). The fibrilin-1 gene located on chromosome 15 is approximately 200 kB genomic DNA (Biery et al. 1999) with 65 coding exons and encodes 10kB transcript (Pereira et al. 1993). Fibrillin-1 is distributed in connective tissue matrices of the skin, lung, kidney, vasculature, cartilage, tendon, muscle, cornea and cilary zonule (Sakai et al. 1986).

It is well known that mutations in the fibrillin-1 gene are responsible for Marfan Syndrome (MFS) (Dietz et al. 1991; Magenis et al. 1991; Kainulainen et al. 1994; Nijbroek et al. 1995). Marfan Syndrome affects approximately 1 in 5000 people in the US (Marfan Foundation, accessed Jan 6th, 2009). MFS is an autosomal dominantly inherited pleiotropic and highly penetrant disorder that affects patients' ocular, skeletal and cardiovascular systems (Pyeritz 2000). Ocular features include severe myopia and bilateral ectopia lentis. Skeletal abnormalities include elongated extremities, fingers and toes, above average height, scoliosis and pectus deformalities (Robinson and Booms

2001). Progressive aortic dilation associated with MFS is the main cause of morbidity in patients (Dean 2007).

Patients suffering from MFS have mutations scattered throughout the fibrillin-1 gene. Interestingly, the more severe forms of MFS have mutations clustered in exons 24-32. In addition, many of these mutations have a dominant negative effect (i.e. the mutant form of the protein impairs overall function of the microfibrils) (Robinson and Godfrey 2000).

Typically diagnosis of MFS involves evaluation of the skeletal, cardiovascular, and pulmonary systems as well as detailed family (genetic) history (De Paepe et al. 1996). However, due to extensive heterogeneity, the disease is very difficult to diagnose. Ultrasound is useful in the first two trimesters of pregnancy to determine if a fetus has MFS. If a patient has no family members with MFS or is adopted (thereby limiting detailed family history), a diagnosis is largely based on phenotype. When a family history is available, it must be confirmed that aneurysms, scoliosis, tall stature, myopia are indeed due to MFS. In general, molecular diagnosis involves evaluating mutations in the FBN1 gene of the patient. If such a mutation exists, the family members are then assessed for a mutation in the FBN1 gene. It is also important to note that sporadic cases in a family may be difficult to diagnose; particularly at an early age, especially if the individual is the first in the family to be evaluated (Pyeritz 2000).

1.2 Cell Adhesion Molecules

Cells adhere to each other and the ECM via cell surface proteins known as cell adhesion molecules (CAMs). These molecules mediate cell-cell or cell-matrix interactions. Expression of cell adhesion molecules allow cells to be organized into tissues. CAMs have three essential features; i.e. they are glycoproteins, must bind an ECM ligand and interact with the cytoskeleton. Six families of CAMs exist: integrins, cadherins, immunoglobulin-like superfamily, receptor protein tyrosine phosphatases, selectins and hyaluronate receptors.

Cadherins are calcium-dependent transmembrane adhesion molecules that typically recognize and bind to identical cadherins on the surface of other cells. Through homophillic binding, cadherins bring membranes of nearby cells together. This connection of membranes allows transmembrane or membrane-associated ligands to interact with membrane bound receptors on the opposing cell and stimulate intercellular communication via oligosaccharide, lipid or protein components of a cell membrane. Cadherins also control polarization of cells to influence cellular distribution of proteins. Cadherins may behave as ligands or receptors thus exerting signaling activity (Vleminckx and Kemler 1999).

The immunoglobulin-like superfamily consists of calcium-dependent transmembrane adhesion molecules that are similar in structure to immunoglobulins. This group is involved in antigen recognition by lymphocytes and other cells (Freemont 1998). The immunoglobin-like superfamily includes the CD3, CD4, and CD8 molecules (Lukas and Dvorak, 2004).

Receptor protein tyrosine phosphatases (RPTPs) are required in signaling, cell growth and differentiation and the cell cycle. These phosphatases possess similar catalytic mechanism arbitrated by an active cysteine, arginine and aspartic acid. It is through this component that RPTPs are capable of modulating intracellular events directly (Fauman and Saper 1996; Freemont 1998).

Selectins are a family of cell adhesion, transmembrane molecules, expressed on the surface of leukocytes and activated endothelial cells. Selectins have a lectin-like carbohydrate binding domain on the extracellular part of the molecule. There are three main groups of selectins: L-selectins (important for leukocyte homing to particular tissues on vascular endothelium), E-selectins (endothelial leukocyte adhesions molecules that mediate inflammatory reactions) and P-selectin (found in endothelial cells and α granules of platelets) (Lukas and Dvorak, 2004). Initial attachment of leukocytes, during inflammation is achieved via the selectin family which results in downstream movement of leukocytes along the endothelium through temporary, reversible, adhesive interactions called leukocyte rolling (Tedder et al. 1995).

The hyaluronate receptors are involved in a number of physiological and pathologic tissue processes as inflammation, cell growth, cell migration and tumorigenesis. Cells bind to hyaluronate, a saccharide component of the ECM, through cell surface proteins as CD44. Various isoforms of CD44 can be hyaluronate binding or non-hyaluronate binding or both which functions in lymphocyte homing, immune response and cell migration (Freemont 1998).

1.2.1 Integrins

Effects of the ECM on cells are mainly mediated by mechanotransducers known as integrins, the focus of this thesis (For reviews see: Guo and Giancotti 2004; Berrier and Yamada 2007). Integrins are cell-cell and cell-matrix adhesion molecules. These receptors are heterodimeric transmembrane glycoproteins made of alpha and beta subunits. Theoretically, any combination of α and β subunit could exist, but to date only 24 human integrins exist with 18 α subunits and eight β subunits combinations (Freemont 1998). The binding of integrins to the ECM results in the formation of focal adhesion sites. At focal adhesion sites, integrins are found on the plasma membrane and interact with the ECM and cytoskeletal proteins that allow attachment to actin filaments (Burridge et al. 1997). These focal sites permit receptor clustering, organization of the cytoskeleton and recruitment of signaling molecules to the focal adhesion. As such, these processes can allow outside-in signaling that regulate cell processes as proliferation, survival, cell migration and invasion (Larsen et al. 2006).

Integrins are capable of quickly modulating adhesive function via interactions of the short cytoplasmic integrin tails with intracellular proteins, which in turn activate conformational changes (Arnaout et al. 2005). The functions of various integrins have common characteristics: many are capable of binding the same ligand and most integrins bind several ligand molecules. In addition to cell adhesion properties, integrins are capable of interpreting signals from the outside of the cell to ultimately control cell movement, morphology, cell growth and gene expression. Furthermore, integrins can also modulate binding activities from within the cell (Ivaska and Heino 2000).

Integrins are regulated by the cells on which they are expressed and must be activated by cells in order to network with the specific ligands. Once an integrin is bound to its ligand, various signaling pathways may be activated. Typically, one or more integrins are expressed on all cell surfaces excluding mature erythrocytes (Diamond and Springer 1994). Although most alpha subunits of most integrins interact with only one beta subunit, some alpha subunits as alpha 4, 6 and V interact with multiple beta subunits (Diamond and Springer 1994).

Integrin alpha subunits usually have 950-1100 amino acid extracellular domains and can be divided into three groups. Alpha IIb, 5, 6, V and 3 integrin subunit have four divalent-cation binding sites of about 60 amino acids, and a protease cleavage site while alpha M, L, X, 1 and 2 integrin subunits are about 180-200 amino acids with three putative divalent-cation binding repeats and no protease cleavage sites. Alpha 4 integrin subunit is the only integrin in the third category and consists of three putative divalent-cation binding sites and a protease-cleavage site (Diamond and Springer 1994). The cytoplasmic domains of integrin subunits contain specific sequences that contribute to distinct intracellular functions (Juliano 1994).

Integrin beta subunits consist of approximately 675-700 amino acid extracellular domain, a hydrophobic transmembrane region and a cytoplasmic tail. Beta subunits have a cysteine-rich domain in the carboxy-terminal portion of the extracellular region and internal disulfide bonds (Diamond and Springer, 1994).

Of interest to this thesis is the α2 integrin subunit. We chose this integrin to evaluate as it is the most down-regulated integrin in cancers of epithelial origin (see section 1.2.2). The alpha 2 integrin null mice are viable, fertile and develop normally.

However, mammary gland branching morphogenesis is diminished in alpha 2 null mice (Chen et al. 2002). Additionally, α2β1 integrin is required for regulation of murine wound angiogenesis but is not necessary for re-epithelialization (Zweers et al. 2007).

1.2.2 The Role of Integrins in Metastasis

In order for a cell to be metastatic, it must be capable of moving through and invading tissues. That is, in order for cells to remove from the original tissue, metastatic cells have to release adhesion from neighboring cells and the ECM, attain migratory phenotypes, degrade and remodel the ECM. Thus, it is evident that changes in adhesion signaling are essential for tissue movement and migration (Guo and Giancotti 2004).

Variable integrin expression and its link to metastasis was first suggested in the transformation of rat1, NRK, and Nil8 cells by the Rous sarcoma virus and murine sarcoma viruses encoding the ras oncogene (Plantefaber and Hynes 1989). Subsequent studies of integrin expression in cancer cells, as detailed below, suggest that various integrin subunits can contribute positively or negatively to transformed cell phenotype. Furthermore, changes in integrin structure can be associated with a change in integrin expression (Mizejewski 1999).

Many studies have evaluated the role of integrins in carcinomas. For example, studies have shown that integrins are involved in melanoma tumor progression. Mortarini's group have shown that over-expression of fibronectin-binding integrin, α5β1 stimulates proliferation of quiescent melanoma cells (Mortarini et al. 1992). In addition, up-regulation of $\alpha_V\beta_3$ in radial growth (non-invasive) primary melanoma cells was found to support anchorage dependent and independent growth, invasive growth from the epidermis to the dermis in skin reconstructs [three-dimensional *in vitro* models consisting

of epidermal keratinocytes plated onto fibroblast-contracted collagen gels] and prevention of apoptosis in invading cells and increased tumor growth *in vivo* (Hsu et al. 1998). High expression of integrin subunits α1 and α2 has also been shown to correlate with a lower survival rate, post-initiation of chemoimmunotherapy in a study of 26 melanoma patients (Vuoristo et al. 2007).

Several groups have also demonstrated a role of integrins in the metastatic potential of breast cancer. For example α2β1, α5β1 and αvβ3 integrins have been found to be down regulated in poorly differentiated breast adenocarcinomas (Zutter et al. 1990). Glukhova's group found that tumor cells of invasive carcinomas lacked α2, α3, α6, β1 and β4 integrin expression when compared with normal mammary cells (Glukhova et al. 1995).

A few studies have also evaluated the role of integrins in the metastasis of prostate cancer. For example, one study evaluated two prostate epithelial cell lines, PC3 and LNCaP, with high and low metastatic potential, respectively. The authors demonstrate that the PC3 cell line expresses αvβ3 integrin and migrates on vitronectin (VN), a αvβ3 ligand expressed in mature bone, where prostate cancer cells preferentially metastasize. However, the LNCaP cells did not adhere or migrate on vitronectin. Nevertheless, forced expression of αvβ3 integrin in LNCaP cells generated a migratory phenotype on VN coated substrates (Zheng et al. 1999).

Although many studies have shown the role of integrins in carcinoma progression, there is limited data on the influence of integrins in neoplasms of mesenchymal origin (sarcomas), particularly osteosarcoma. However, few studies have suggested a role for integrins in osteosarcoma metastasis. Vihinen's and co-workers found that α2β1 integrin

expression was higher in virally and chemically transformed tumorigenic subclones of HOS osteosarcoma cells when compared with non-tumorigenic cell lines (Vihinen et al. 1996). However, over expression of α2 in HOS cells was not sufficient to make these cells tumorigenic. Furthermore, treatment with tumor promoters, 12-O-tetradecanoylphorbol 13-acetate (TPA) and okadaic acid (OA) led to increased transcription of the α2 integrin gene but no other integrins in cultured osteosarcoma cells (Nissinen et al. 1998). It is important to note as α2β1 integrin is normally expressed on B and T lympohocytes, platelets, fibroblasts, endothelial cells and melanoma cells. The α4β1 integrin, normally expressed in leukocytes but not in osteoblasts, was observed in primary osteosarcoma and metastatic osteosarcoma lesions of the lung and pericardium (Marco et al. 2003). These studies suggest that osteosarcoma metastasis may result from altered integrin expression.

Nevertheless, data on the role of integrins in the metastatic potential of osteosarcoma is not extensive. That is, no study has correlated the role of integrins or the genes influenced by integrins in the invasive characteristics of osteosarcoma. It is important to note that carcinomas, (of epithelial origin) by definition, are different from sarcomas (of mesenchymal origin), thus mechanisms of metastasis might be different.

1.3 Matrix Metalloproteinases (MMPs)

Matrix Metalloproteinases (MMPs) are a family of peptidases secreted into the extracellular space that degrade ECM components. MMPs can also act on non-matrix substrates including cell surface receptors and matrix bound growth regulators. Therefore, regulation of MMPs and their inhibitors (tissue inhibitors of metalloproteinases, TIMPs), is necessary for cleavage and release of growth factors and cell surface receptors during normal remodeling and homeostasis (Chang and Werb 2001).

Most MMPs are organized into basic, well conserved domains including an N-terminal signal sequence, an N-terminal propeptide, a catalytic domain and a C-terminal domain. The N terminal domain propeptide contains a conserved cysteine, which chelates the catalytic Zn ion, necessary to keep the proMMP inactive. This propeptide must be removed during activation either by other MMPs or by proteases (Ganea et al. 2007).

MMPs can be subdivided into collagenases as MMPs 1, 8, 13, 18, gelatinases as MMPs 2, 9, stromelysins as MMPs 3, 10, 11 and membrane type metalloproteinases as 1, 2, 3 and 4 (MMP 14, 15, 16 and 17 respectively). These subdivisions are based on substrate specificity, primary structure and cellular location (Clark et al. 2008). Currently, the pathways responsible for MMP activation are not well understood (Chakraborti et al. 2003). However, anecdotal reports suggest that MMPs, along with specific integrin signaling may be involved in OS metastasis (Bjornland et al. 2005).

1.3.1 The Role of MMPs in Metastasis

MMPs play an important role in normal tissue remodeling events such as embryonic development, angiogenesis, ovulation, mammary gland involution and wound healing. However, altered MMP expression is involved in a number of pathological processes such as osteoarthritis, pulmonary emphysema, tumor growth, invasion and metastasis (Duffy et al. 2000).

Many studies have demonstrated a correlation between levels (high or low) of specific MMP and increased tumor metastasis (for complete reviews see: Chang and Werb 2001; Curran and Murray 2000; Brown and Giavazzi 1995). For example, up-regulation of MMPs 1, 2, 3 (Brummer et al. 1999; Tetu et al. 2006, 9, 13 Balduyck et al. 2000) and14 (Tetu et al. 2006) has been observed in breast cancer when compared with normal cells. Up-regulation of MMP 1 has also been shown in prostate cancer (Pulukuri and Rao 2008). In oesophageal cancer, increased MMP 1 is associated with more aggressive cancers (Murray et al. 1998). Although few studies have correlated MMP expression with metastasis in sarcomas, up-regulation of MMPs 2 and 9 were evident in soft tissue sarcomas (Benassi et al. 2001). Foukas and co-workers suggested that MMP 9 could be used as a prognostic factor in osteosarcoma metastasis and survival (Foukas et al. 2002). The authors found four out of five patients with pulmonary metastasis studied, had high levels of MMP 9. Post chemotherapy, MMP 9 is expressed in surviving cancer cells.

1.4 Osteosarcoma

Bone sarcomas are rare mesenchymally-derived tumors. Approximately 2400 bone and joint cases are diagnosed per year in the US (Jemal et al. 2008). Osteosarcoma (OS), the most common type of bone tumor, can occur at any age but prevalence peaks during the second decade of life (Carrle and Bielack 2006). OS is the second most common primary bone neoplasm (ACS), the most common malignant bone tumor in children (Li et al. 2006) and is the second leading cause of cancer–related death in children behind blood cancers (ACS). Currently, the treatment of OS requires surgery of the primary tumor and chemotherapy (Ferrari and Palmerini 2007). To our knowledge, only one large scale study evaluating the prognostic factors of high grade OS has been done to date (Bielack et al. 2002).

1.4.1 Diagnosis

Today, the most widely used staging system of OS was developed by Enneking (Enneking et al. 1980). Low grade localized bone tumors are referred to as stage I and high grade as stage II. Tumors are further classified based on the anatomy of the tumor: intracompartmental (A) and extracompartmental (B). The occurrence of metastasis is classified as stage III osteosarcoma. Low grade tumors (low risk of metastasis) are usually well-differentiated and experience fewer mitotic divisions. Generally, low grade tumors are managed by conservative procedures. High grade osteosarcoma is characterized by poor differentiation, a high cell to matrix ratio, high mitotic rate, necrosis and microvascular invasion. These tumors require more aggressive treatment.

High grade OS is the most frequent subtype comprising up to 80% of patients. Males are more often affected with the disease. Bones with the fastest growth rate have

the highest occurrence of OS occurring either inside (as in the intramedullary [inside the bone] or intracortical compartment) or on the bone surface as well as in the extraosseous sites (Klein and Siegal 2006). Historically, the malignant nature of OS became obvious due to the presence of lung metastasis. Bielack's group found 1, 595 of primary osteosarcoma tumors located in an extremity and 107 in the trunk of a total of 1702 patients studied. Of the 1, 595 patients, 87% were in the leg (most commonly in the knee: distal femur, proximal tibia or proximal fibula) and the rest in the arm. About 13% of these patients presented with metastasis, with 11% having confirmed lung metastasis.

Currently, radiological finding of OS are often useful for diagnosis. An X-ray of OS typically shows an osteolytic and sclerotic appearance. OS often begins in intramedullary bone but breaks the cortex and moves into the nearby soft tissue. At the edge of the area, there is usually immature bone due to reactive bone produced by periosteum (membrane that lines the outside of bones). Irregular, cloud-like radiodensities and/or stripes of increased density perpendicular to the cortex are also evident on an X-ray. However, computed tomography (CT) and magnetic resonance imaging (MRI) scans are almost always necessary for accurate diagnosis. An MRI allows the physician to determine medullary tumor borders and epiphyseal (cartilage plate in the metaphyses of children) invasion and skip metastasis (histologically separate nodule of tumor separated from primary lesion by interval of normal tissue). A CT scan is used to confirm the presence of intra and extra-osseous extension of bone tumor. It also allows the determination of the proximity of bone tumors with the vessels prior to angiography studies (Longhi et al. 2006).

1.4.2 Etiology

Patients suffering from certain genetic conditions such as retinoblastoma, Paget's disease, enchondromatosis, hereditary multiple exostoses and fibrous dysplasia have a higher chance of developing osteosarcoma (Sandberg and Bridge 2003). Of the 1702 patients evaluated in Bielack's study, osteosarcoma resulted as a secondary malignancy in 32 cases. The propensity of certain genetic conditions to have the same etiology might suggest that these diseases have a similar genetic basis as osteosarcoma. Furthermore, genetic analysis of tumor cells from OS patients showed a homozygous loss of the Rb gene or altered Rb gene product (Hansen et al. 1985; Dryja et al. 1986). Mutations in the p53 gene have also been observed in OS patients (Longhi et al. 2006).

1.4.3 Histology

Based on the histology, osteosarcomas are sub-divided into osteoblastic, chondroblastic and fibroblastic. Osteoid production varies greatly among tumors. Osteoblastic OS has osteoid or bone as the major type of matrix. Chondroblastic osteosarcoma consists mostly of chondroid matrix, while fibroblastic osteosarcoma is mainly malignant spindle cells with little osteoid (Marina et al. 2004).

1.4.4 Cytology

Although cytogenetic studies of OS are few, examination of 111 clonally abnormal, short-term cultured tumors reveals that the majority of OS are chromosomally abnormal. These abnormalities include complex karyotypes, chromosomal breakpoints (including structural breakpoints at 1p11-13, 1q10-12), numerical abnormalities (affecting chromosomes +1, -8, -9, -10 and -18) and random abnormalities (gaps, breaks and tri-radial figures) (Bridge et al. 1997; for review see: Tang et al. 2008). Mutations in

the genes of the p53 and Rb tumor suppressor pathways have also been implicated in the pathogenesis of OS (Marina et al. 2004).

1.4.5 Treatment

Treatment of osteosarcoma requires a multidisciplinary approach. Generally, patients present with pain, swelling, localized enlargement of the extremity or fracture. Most patients present with localized disease. As mentioned, radiographs often demonstrate sclerotic and lytic lesions in the metaphyseal region of the bone. However, computed tomography and bone scanning are usually necessary to detect pulmonary and bone metastases. Death from OS most often results from pulmonary metastasis (Marina et al. 2004).

Prior to 1970, osteosarcomas were treated with amputation. However, the survival rate was minimal: 80 % of patients died from metastatic disease. Development of adjuvant chemotherapy protocols, advances in surgical techniques and improvements in radiologic staging studies has lead to increased survival (Marina et al. 2004; Wittig et al. 2002). Currently effective treatment of patients with non-metastatic OS involves resection of the primary tumor and neo-adjuvant chemotherapy (Tsai et al. 2005).

With the introduction of chemotherapeutic treatment of high grade OS, prognosis has improved the 5-year survival rate from 11% with surgical resection alone in the 1960s to 70% in the mid-1980s. Nevertheless, survival has since reached a plateau, despite advances in anticancer therapy (Chou and Gorlick 2006). In Bielack's study, 99.9% of patients received chemotherapy while 95% were operated on, 54% had tumors resected and 45% received amputations, disarticulations and rotation plasties (removal of

extremity with tumor). Typically, older patients received resections because of small tumor size. Duration of chemotherapy varied from 1-335 days.

Since OS is a relatively rare disease, the propensity towards developing new drugs is low. The most current treatments include nitrogen-containing bisphosphonates (BP) in patients with bone metastasis. Zoledronic acid, a nitrogen containing BP is currently being evaluated in clinical trials in patients with bone metastasis (Benassi et al. 2007). Additionally, interferon α has also been found to be a useful adjuvant treatment in high grade OS. The authors suggest interferon α increases chemotherapy sensitivity of OS cell lines and may be useful in conjunction with chemotherapy (Muller et al. 2005). Tomoda's group has also found that low-dose methotrexate inhibits lung metastasis and lengthens survival in rat OS (Tomoda et al. 2005) .

1.4.6 Prognosis

The prognosis of OS depends on several factors. However, the most consistent prognostic factor at diagnosis is the presence of pulmonary metastasis, which has an adverse effect (Bielack et al. 2002; Marina et al. 2004). Other factors that may determine prognosis are the location of primary site, tumor size (Bieling et al. 1996; Smeland et al. 2004) and response to chemotherapy (Marina et al. 2004).

Although prognosis and quality of life of patients with localized osteosarcoma has vastly improved in the last 30 years, there is significant recurrent disease (30-40%), usually to the lungs. The 5-year post-relapse survival rate is 28% (Fagioli et al. 2008).

1.5 The Rationale for Studying Osteosarcoma

As has been discussed before, sadly, the presence or absence of metastasis at diagnosis of OS is the most common prognostic factor. Currently, the 5-year survival of patients, with localized osteosarcoma of the extremity is 70%. However, patients with metastatic osteosarcoma do not share the same fate (Daw et al. 2005). The American Cancer Society reports that the five year survival rate of patients with metastatic cancer is approximately 30% even with the use of adjuvant or neo-adjuvant chemotherapy. Nevertheless, in the last 30 years there has been no significant improvement in osteosarcoma of the extremities: about 30-40% relapse within three years (Fagioli et al. 2008, for review see: Marulanda et al. 2008).

It is important to develop animal models for human diseases in order to establish cancer mechanisms and develop new therapeutic interventions. The ideal animal model for human osteosarcoma must have primary growth in bone (orthotopic growth) and form lung metastasis. Secondary sites of metastasis include bone and liver.

One reason for the lack of clinical progress in osteosarcoma may be the lack of an animal model that recapitulates the human disease. Currently mice genetically engineered to develop osteosarcoma, feline and canine pets that spontaneously develop OS are used as animal models. However, although genetically engineered mice models exist (discussed below), none have shown orthotopic growth in bone. Dogs with OS do have some similarity with the human disease, (histology of the primary tumor, micro-metastatic disease and progression to the lungs) but these animals typically develop the disease later in life and progression is slower than in murine models and humans (for review see: Grogan and Hansen 2003).

Murine osteosarcoma cell lines such as UMR 106-01, K7M2 and K12 have generated metastatic models of OS, providing the advantage of studying the disease in a specific and controlled background. For example, Khana's group reported the use of the K7M2 cell line and K12 cell line (Khanna et al. 2000). The K7M2 cell line, developed from the K12 cell line, was injected intra-osseously into the proximal tibia of the BALB/c mouse. Pulmonary metastases that developed were implanted into the tibial muscle flap and the cycle was repeated. The final cycle of these cells, the K7M2 cell line was more destructive when compared with the K12 cell line. The two clonal variants were characterized and several genes related to cell motility were up-regulated including integrin β4 (Khanna et al. 2001).

However, use of these murine cell lines is limited. As such, several human osteosarcoma cell lines have also been established and studied in mouse models. To date, none of these human cell lines have shown orthotopic growth in bone. Furthermore, many of these studies have been limited to four widely used human cell lines and their derivatives: U2-OS, HOS, SAOS-2 and MG63, which are briefly described below.

The U2-OS cell line was cultured in 1964 from a biopsy of osteosarcoma obtained from a 15 year old Caucasian girl. A few studies have demonstrated tumorigenicity with tail vein and subcutaneous injections into mice; however, no model has generated a truly spontaneous, metastatic mouse model (Ek et al. 2006).

The HOS cell line was established in 1970 from a 13 year old Caucasian girl. To date, there has been no successful orthotopic inoculation. However, several genetic alterations of the HOS cell line using viral and chemical agents have resulted in the

formation of cells with the ability to form pulmonary metastases in nude mice at a faster rate than the parental cell line (Ek et al. 2006).

The MG63 cell line was isolated in 1978 by Heremans and co-workers from the femur of a 14 year old boy (Heremans et al. 1978). Conflicting evidence on the tumorigenicity of this cell line exists. The cell line was described as non-tumorigenic by two groups (Kimura et al. 2002; Vihinen et al. 1996). The authors found no lung nodules or micro-metastasis 25 weeks post sub-cutaneous injection of the MG63 cell line. Additionally, Thacker's group found that nude or SCID mice injected with MG63 cells did not form tumors; however, mice that were injected with granulocyte macrophage colony stimulating factor (GM-CSF)-producing MG63 cells (with confirmed retroviral integration) were tumorigenic (Thacker et al. 1994). Also, Li et al., (2007) implanted MG63 cells into the distal femur of nude mice and found orthotopic growth two weeks later. However, this article was written in a Chinese journal and unavailable to us. As far as we could tell from the abstract, the authors did not discuss metastasis to the lungs.

SAOS-2 cells were derived in 1973 from a primary osteosarcoma in an 11 year old Caucasian girl. *In vivo* subcutaneous injections of cells into nude mice resulted in metastasis (Zhou et al. 1996). A metastatic model of the SAOS cell line was established from the lung metastases of nude mice; these were formed 6 months after lateral tail vein injection. Clones were obtained and re-injected five times resulting in the SAOS-LM6 metastatic cell line. Pulmonary metastases developed at 5-6 weeks and macroscopic lesions were observed 8 weeks after tail vein injection (Jia et al. 1999). The LM7 sub-line was developed by an additional cycling of 10^6 LM6 cells. Macroscopic lung metastases in nude mice were seen within 6 weeks following injection (Worth et al. 2002).

More recently Walkley and co-workers genetically engineered a conditional osteosarcoma mouse model (Walkley et al. 2008). This model was based on the osteoblast-restricted deletion of p53 and pRb. Many of the features of human osteosarcoma were seen: including metastatic behavior. One major limitation of this study is that a deregulated p53 or Rb gene is not ubiquitous among osteosarcoma patients. Furthermore, loss of p53 limits the clinical evaluation of drugs such as cisplatin and zoledronic acid in which a wildtype p53 gene is necessary for chemotherapeutic effects (Benassi et al. 2007).

Currently, no animal model exists that leads to orthotopic growth in bone, spontaneous metastasis, and growth in a secondary tissue. Furthermore, researchers are limited to four commonly used cell lines that may have accumulated cell culture induced artifacts during the last thirty years. As such, candidate drugs for osteosarcoma treatment cannot be tested in an animal that does not recapitulate the human disease.

1.6. Hypothesis

Our lab is interested in understanding the role of integrins and integrin-mediated MMP expression in osteosarcoma metastasis. In the long term, these mechanisms might allow the use of integrin antagonists in OS treatment similar to those used for particular epithelial cancers (for review see Jin and Varner 2004).

In epithelial cancers (carcinomas), integrins and MMPs are known to play a role in metastasis. However, little is known about the role of integrins and MMPs in sarcomas. As sarcomas receive much less experimental attention than carcinomas, there

is little understanding of the functions of these proteins in sarcomas, particularly osteosarcoma.

Integrins recognize the structure of the matrix and mediate messages to cells resulting in integrin-guided degradation of the ECM via MMPs (Ivaska and Heino 2000). As a result, many reports have suggested MMPs may facilitate tumor metastasis due to their role in ECM degradation. Therefore, it is important to establish the role between integrins and matrix metalloproteinases in the tumor invasion processes.

Some studies have linked integrin and MMP expression to carcinoma tumor progression. Activated αVβ3 integrin in human breast cancer cells and primary breast cancer cells from patients show increased migration to vitronectin and fibrinogen. Rolli and co-workers demonstrated that this migration on vitronectin was mediated by MMP-9, which in turn up-regulated αVβ3 in breast cancer cells in which this integrin was not activated (Rolli et al. 2003). Thus, these data implicate the presence of a feedback loop between integrins and MMPs. However, the data are limited on the integrin-MMP feedback loop in osteosarcoma tumor progression. Depletion of αV integrin via an intracellular antibody led to decreased expression of MMP 2 in SAOS-2 osteosarcoma cells and reduced their ability to spread on fibronectin and vitronectin (Koistinen et al. 1999). As such, a disturbance in the integrin-MMP system can be suspected in the invasive nature of human osteosarcoma. To date, there has been no study, which has evaluated the role of the integrin-MMP feedback in sarcomas, as has been done in some carcinomas.

We hypothesize that the type and/or amount of integrins present on a particular OS cell influences metastatic potential. As such, either loss or over-expression of specific

integrin receptors may alter metastatic potential. To test this hypothesis, we have developed human osteosarcoma lines that express high or low levels of alpha 2 integrin subunit. Additionally, we have compared the integrin profile of the non-metastatic SAOS-2 parental cell line and the metastatic sub-line LM7.

Secondly, we hypothesize that upon OS cell attachment to a particular class of ECM protein, MMP production is specifically altered in response: the Integrin-MMP feedback loop hypothesis. This feedback loop mechanism enables the metastasizing cells to produce the MMPs required for invasion, and this response is an element of metastatic potential. In order to evaluate this hypothesis, we have evaluated MMP production in cell lines with altered integrin levels as well as in cell lines with different metastatic potential.

Chapter 2

MG63 osteosarcoma cells demonstrate an integrin-MMP feedback loop.

2.1 Introduction

Migration of tumor cells through the extracellular matrix is an important step during metastasis. Several studies have demonstrated that integrins are capable of controlling cell invasion through the ECM (see introduction 1.2.2). We hypothesized that integrins are capable of regulating the expression of matrix metalloproteinases (MMPs), a family of peptidases secreted into the extracellular space that degrade ECM components.

We chose to down-regulate the alpha 2 integrin subunit using siRNAs in the osteosarcoma cell line, MG63. This integrin subunit was chosen because the α2β1 integrin is down-regulated in several carcinomas (see Introduction 1.2.2). The study of integrin alpha subunits is essential as they allow understanding specific functions of integrin heterodimers that share beta subunits. The alpha 2 integrin subunit dimerizes specifically with the beta 1 subunit and is expressed on platelets, endothelial cells, fibroblasts and epithelial cells and recognizes type I-VIII collagens and laminins (Chen et al. 2002). In addition to its function in adhesion, the α2β1 integrin is involved in cell migration induction, activation of matrix metalloproteinases, inflammatory reactions and collagen fibrillogenesis (Zweers et al. 2007).

Matrix Metalloproteinases (MMPs) are a family of peptidases secreted into the extracellular space that degrade ECM components. MMPs cleave and release of growth factors and cell surface receptors during normal remodeling and homeostasis (see

Introduction 1.3) Although several studies have shown that altered MMP expression is also associated with carcinoma metastases (see Introduction 1.3.1), only a few studies have evaluated the influence of integrins on MMP expression in sarcomas.

We plated the alpha 2 down-regulated cells and control cells (containing a scrambled siRNA) on the ECM matrix proteins collagen and fibronectin as well as on plastic. We show that control cells express MMP when plated on plastic, fibronectin and collagen. When plated on plastic, control cells express significantly more ($p<0.05$) MMP 1 and 3 compared with when these cells are plated on ECM substrates fibronectin and collagen. Further, control cells express similar amounts of MMP 1 on both types of ECM substrates. This shows that MMP1 is specifically down regulated on fibronectin and collagen substrates. Control cells do not vary their MMP 2 production when plated on non-matrix or matrix proteins.

We found that cells down-regulated for the alpha 2 integrin subunit expressed more MMP 1 and MMP 3 when compared with control cells on all substrates evaluated. Our results suggest that alpha 2 integrin might be necessary for the repression of MMP 1 and MMP 3 and therefore implicates the alpha 2 integrin subunit in osteosarcoma metastasis.

2.2 Experimental Methods

2.2.1 Cell lines, Cell culture and Down-regulation Construct

MG63 cells (ATCC # CRL 1427) were cultured with Dulbecco's minimal essential medium (Hyclone) supplemented with 10% fetal bovine serum (Gibco) at 37ºC with a 5% CO_2 incubator. The expression vector, pSilencer 4.1-CMV neo (Ambion, Inc.), was used to transfect and constitutively express small interfering RNAs designed to down-regulate the alpha 2 integrin subunit. Sequence information was purchased (Ambion, Inc.) and 55-mer oligonucleotides were synthesized for the sense and antisense directions (IDT, Inc.). The target gene and the siRNA design number (Ambion, Inc.) for α2 was ITGA2, ID#111111. Sequences were annealed and cloned into pSilencer and confirmed by DNA sequencing. To make integrin-reduced cell lines, plasmid DNA encoding short interfering RNA was transfected into MG63 osteosarcoma cells.

Transfection was carried out according to the Invitrogen protocol using the transfection reagent lipofectamine (Invitrogen 11668-027). Briefly, on the day before transfection, MG63 osteosarcoma cells were cultured in a 6 well plate using no antibiotics. On the day of transfection, 2μg of plasmid DNA (α2 integrin subunit down-regulated cells) was diluted into DMEM without FBS and mixed gently. In another tube 20μL of lipofectamine was mixed with DMEM. The diluted DNA and lipofectamine solutions were combined at room temperature for 45 minutes. DMEM was then added to the lipofectamine solution and mixed. This was then added to the cells drop by drop and then incubated for 5 hours at 37ºC with a 5% CO_2 incubator. After 5 hours, DMEM containing FBS was added to the cells without removing the lipofectamine solution. Stable transfectants were selected by plasmid-conferred resistance to G418 (Neomycin)

at 250 µg/ml. Clonal lines were established through dilution and colony isolation. Based upon integrin subunit down-regulation as assessed by flow cytometry, the clone with the lowest alpha 2 integrin expression was chosen for use in subsequent experiments.

2.2.2 Over-expression and Transfection of α2 Integrin

MG63 cells (ATCC # CRL 1427) were cultured with Dulbecco's minimal essential medium (Hyclone) supplemented with 10% fetal bovine serum (Gibco) at 37ºC with a 5% CO_2 incubator. The expression vector, pCDNA3.1 (+) neo (Invitrogen), was used to transfect and constitutively express over-express the α2 integrin subunit. Full length α2 cDNA was purchased (ATCC 95940). Using the restriction enzymes PsOMPI (New England Biosystems) and Not I (Invitrogen), the alpha 2 integrin was cut out of pBluescript. Sequences were cloned into pCDNA 3.1 (+) using restriction sites Not I (compatible with PsOMPI) and confirmed by DNA sequencing using oligonucleotides synthesized for the sense and antisense directions (IDT, Inc.).

To generate integrin over-expressed cell lines, plasmid DNA encoding α2 integrin subunit in pcDNA was transfected into MG63 osteosarcoma cells. Transfection was carried out according to the invitrogen protocol using the transfection reagent lipofectamine (Invitrogen 11668-027). Briefly, on the day before the transfection, MG63 osteosarcoma cells were cultured into a 6 well plate without antibiotics. On the day of transfection 2µg of plasmid DNA (α2 in pcDNA) was diluted into DMEM without FBS and mixed gently. In another tube 20µL of lipofectamine was mixed with into DMEM. The diluted DNA and lipofectamine solutions were combined at room temperature for 45 minutes. DMEM was added to the lipofectamine solution and mixed. This was added to the cells drop by drop and incubated for 5 hours in the CO2 incubator. After 5 hours,

DMEM containing FBS was added to the cells without removing the lipofectamine solution. Neomycin (250µg/mL) selection was carried out two days later. Clones were analysed for over-expression of α2 integrin via flow cytometry using the FACS Scan II.

2.2.3 Flow Cytometry and Flow Sorting

Culture media were removed, cells were washed with PBS and lifted non-enzymatically with versene at 37ºC for 3-5 minutes. Cells were pelleted in DMEM (High glucose) without serum. Pellets were re-suspended in 100 µl of primary antibody solution and incubated for 30 minutes at room temperature. The primary antibody used was mouse monoclonal anti-human integrin α2β1-FITC conjugate (1:100, Chemicon CBL477F). The samples were washed with DMEM before being re-suspended in 0.5 ml of DMEM, to be analyzed by flow cytometry using a Becton Dickson FACScan. 500,000 cells/sample were used for all flow sorting experiments and a minimum of 10,000 cells were counted. Gating was set at <1% of no antibody negative control cells. Data were evaluated with BD CellQuest Pro software.

2.2.4 Integrin-MMP Feedback Loop Analysis

Experimental cell lines (MG63 control, α2 integrin under- and over-expressed) were grown on three classes of extracellular matrix proteins: collagen (1mg/mL) and fibronectin (15µg/mL) and plastic as a control. Cells were cultured in serum free conditions by using KnockOut-SR (Invitrogen). Conditioned media was collected three days later and analyzed for MMP protein expression using fluorescent sandwich ELISA beads (R&D systems) and a Luminex 100 System (Luminex 100). The Luminex system quantitatively determines protein concentrations of human MMPs using specific antibodies pre-coated on color-coded microparticles (R&D Systems). We analyzed

MMPs 1 (LMP 901), 2 (LMP 902), 3 (LMP 513), 7 (LMP907), 9 (LMP 911) and 13 (LMP 511) secreted in cell culture media. Experiments were performed in triplicate. Plastic was used as a baseline condition for comparison to cells on fibronectin and collagen.

2.2.5 Statistical Analysis

One Way Anova was used and p values less than 0.05 were considered significant. We used the GraphPad Prism 4.

2.3 Results

2.3.1 Down-regulation of alpha 2 Integrin Subunit

In order to under-express alpha 2 integrin subunit, expression vector, pSilencer 4.1-CMV neo was used to transfect and constitutively express small interfering RNAs designed to down-regulate specific integrin subunits. The pSilencer 4.1 CMV vector, shown in Figure 2.1 was used to drive high expression of cloned siRNA templates in MG63 cells. Neomycin resistance was used to select for transfected cells that express the introduced DNA.

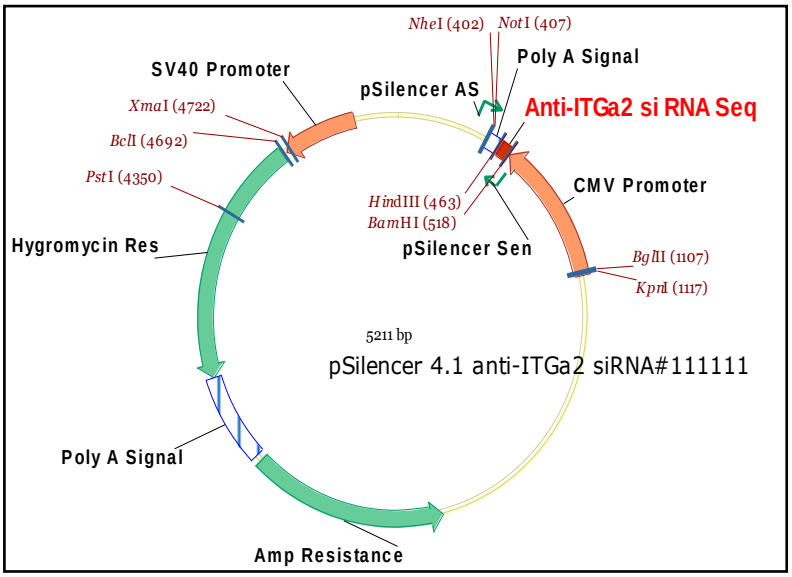

Figure 2.1: siRNA construct used to knockdown alpha 2 integrin subunit. We used pSilencer siRNA vector to knock down α2 integrin expression. The CMV promoter was used to drive α2 integrin siRNA expression. MG63 cells were transfected and selected using G418. Clonal lines were established through dilution and colony isolation.

We assessed neomycin resistant clones by flow cytometry using antibodies specific to the alpha 2 integrin subunit. Flow cytometry employ light scattering, light excitation, and emission of fluorochrome molecules to generate multi-parameter data from cells. As shown in Figure 2.2, MG63 cells were efficiently down-regulated for the alpha 2 integrin. Gating of alpha 2 integrin expression was set using MG63 cells transfected with a scrambled siRNA.

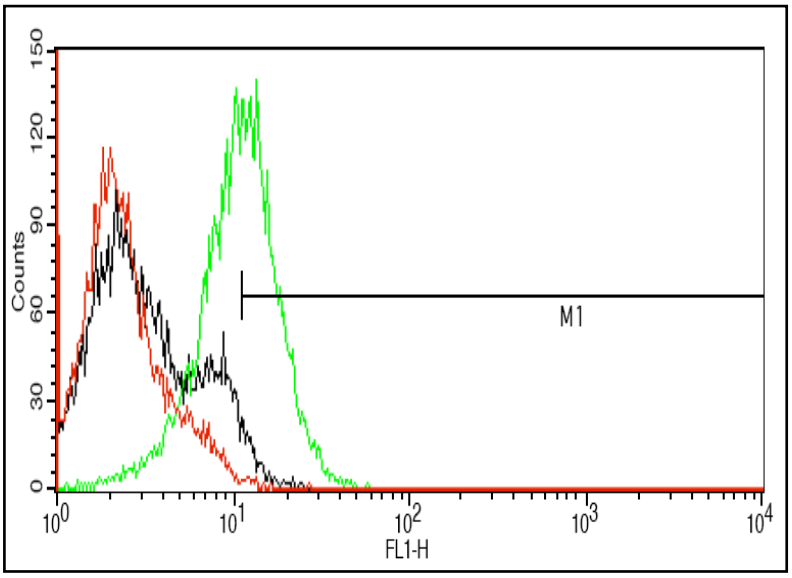

Figure 2.2: Flow cytometry showing effective down regulation of alpha 2 integrin in a MG63 daughter cell line. As described in the methods, cells were down-regulated for α2 integrin subunit in MG63 cells. The gate is set using MG63 NCP cells (red) which have been transfected with a scrambled siRNA. Effective down-regulation of alpha 2 integrin subunit for one clone is highlighted in green. Unstained MG63 NCP cells (black) were used as a no antibody control. Y-axis = cell number X-axis = fluorescence units.

2.3.2. Over-expression of alpha 2 Integrin

We then over-expressed the integrin alpha 2 subunit by cloning into over-expression vector, pcDNA 3.1 (+) shown in Figure 2.3. We transfected over-expressed α2 integrin subunit into MG63 osteosarcoma cells and selected neomycin resistant clones.

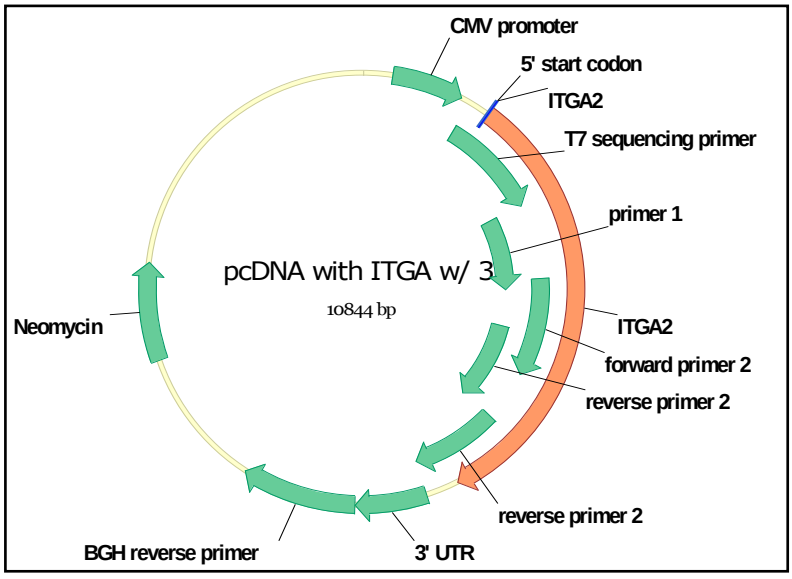

Figure 2.3: pcDNA 3.1 over–expression construct used to over-express the alpha 2 integrin subunit. Full length α2 cDNA was purchased and cloned into a pcDNA 3.1 (+) vector. The CMV promoter was used to drive α2 integrin expression. MG63 cells were transfected and selected using G418. Clonal lines were established through dilution and colony isolation.

Clones that were neomycin resistant were evaluated for up-regulation of the alpha 2 integrin via flow cytometry as shown in Figure 2.4. This figure shows the α2 integrin subunit expression was up-regulated approximately 50%. We then selected for the highest alpha 2 integrin expression via flow sorting as described in the results section.

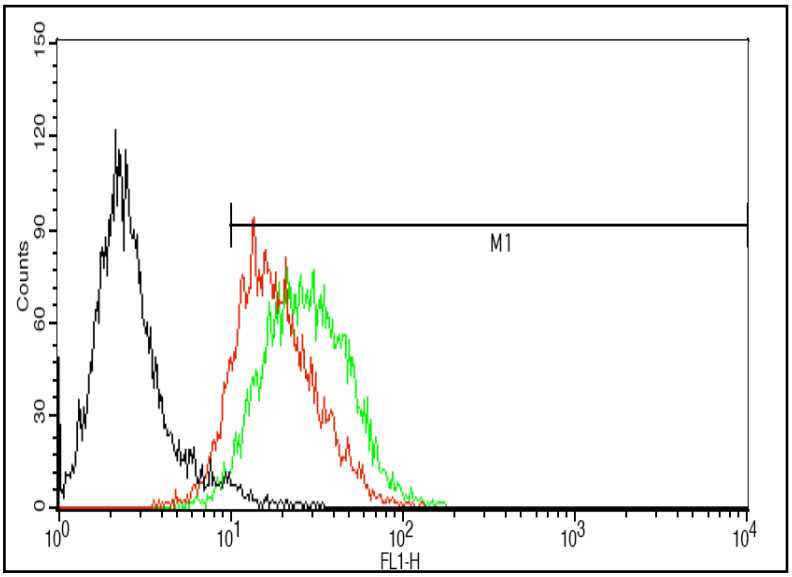

Figure 2.4: Flow cytometry showing effective over-expression of α2 integrin in an MG63 daughter cell line. As described in the methods, cells were over-expressed for the α2 integrin subunit in MG63 cells. The gate is set using MG63 NCP cells (red) which have been transfected with a scrambled siRNA. Effective over-expression of α2 integrin subunit for one clone is highlighted in green. Unstained MG63 NCP cells (black) were used as a no antibody control. Y-axis = cell number, X-axis = fluorescence units.

2.3.3 Loss of α2 Integrin Results in Increased Expression of MMPs 1 and 3

We hypothesized specific integrins mediate metastatic invasion of tissues and that loss or over expression of an integrin receptor alters metastatic potential. To experimentally address this hypothesis, we assessed MMP protein expression via the Luminex system. The Luminex system quantitatively determines protein concentrations of human MMPs using specific antibodies pre-coated on color-coded microparticles.

We evaluated the MG63 control cell line transfected with a scrambled siRNA (NCP) plated on the extracellular matrix proteins collagen and fibronectin. As seen in Figure 2.5 NCP cells express MMP when plated on plastic, fibronectin and collagen. However, when the control cells are plated on plastic they express almost 4 times more MMP 1 compared with when these cells are plated on ECM substrates fibronectin and

collagen. Further, control cells express similar amounts of MMP 1 on both types of ECM substrates. This shows that MMP 1 is specifically down regulated on fibronectin and collagen substrates. MMP 3 protein expression was lowest on matrix proteins fibronectin and collagen but was elevated on non-matrix substrate plastic. MMP 2 expression was not significantly different when plated on matrix or non-matrix substrates.

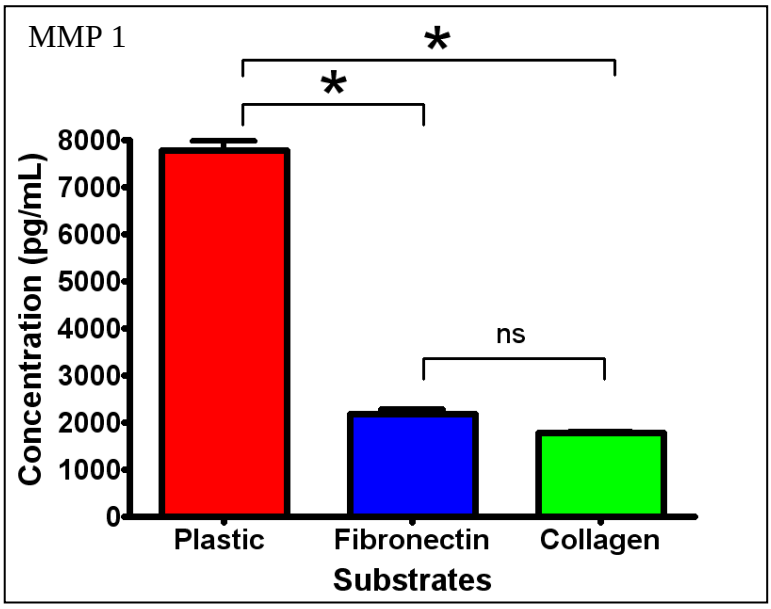

Figure 2.5: Conditioned media collected 72h after growth on plastic, type I collagen and fibronectin were evaluated from control cell line (MG63 NCP) for MMP 1 protein expression. NCP cells express significantly more ($p<0.001$) MMP 1 protein on plastic compared with matrix substrates fibronectin and collagen. NCP cells do not have significantly different (ns, $p>0.05$) MMP 1 expression on fibronectin and collagen. X-axis = substrates. Y-axis = concentration (pg/mL), n= 3.

We evaluated the genetically altered MG63 cell line that express low levels of α2 integrin and a control cell line transfected with a scrambled siRNA (NCP) on the extracellular matrix proteins collagen and fibronectin. As a baseline, we also plated our cells on plastic. As shown in Figure 2.6 down-regulation of α2 integrin results in significant ($p<0.05$) up-regulation of MMP 1 on plastic, fibronectin and collagen as compared to NCP control cells. On the plastic substrate, MMP 1 expression was increased almost 2 fold in alpha 2 down-regulated cells when compared with control cells plated on plastic. On the ECM substrates collagen and fibronectin, MMP 1 expression

increased almost 4 fold in the alpha 2 down-regulated when compared with control NCP cells as shown in Figure 2.6.

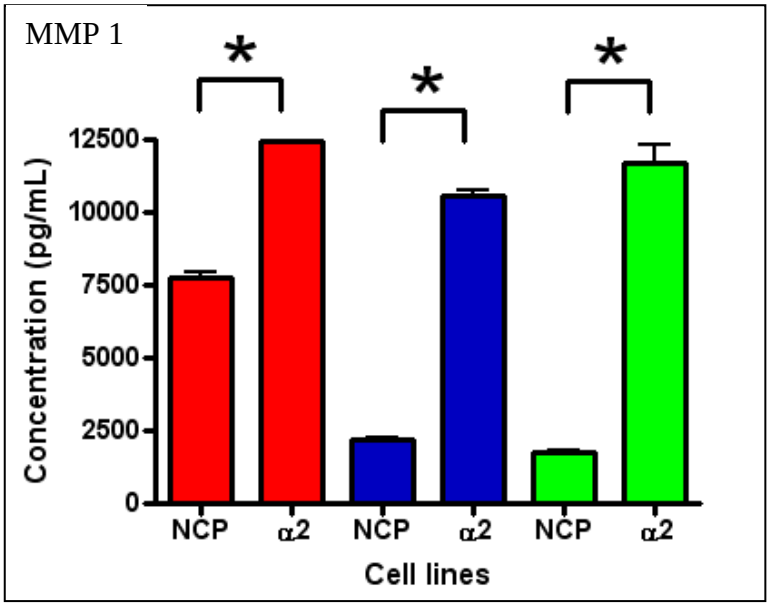

Figure 2.6: Conditioned media collected 72h after growth on plastic (red), type I collagen (green) and fibronectin (blue) were evaluated from both control cell lines (MG63 NCP) and reduced α2 integrin subunit cells (α2) for MMP 1 protein expression. α2 down-regulated cells expressed more ($p<0.01$) MMP 1 protein compared with NCP control cells plated on the same substrate. X-axis = cell lines. Y-axis = concentration (pg/mL) n= 3.

We evaluated the NCP control osteosarcoma cells and α2 integrin down-regulated cells on plastic, fibronectin and collagen as shown in Figure 2.7 for MMP 2 protein expression. There is no significant difference ($p>0.05$) in MMP 2 expression between control and down-regulated cells on non-matrix and matrix proteins. Interestingly, MMP 2 was the most highly expressed MMP we evaluated.

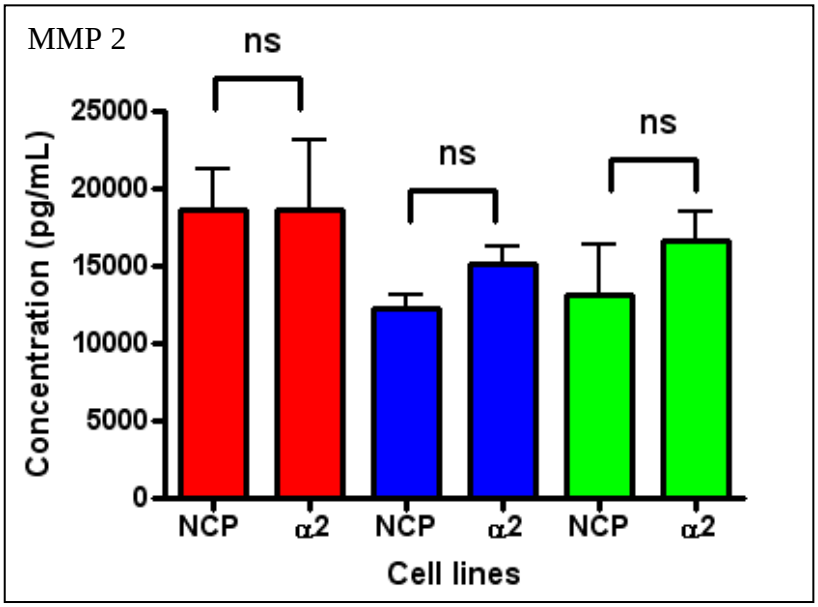

Figure 2.7: Conditioned media collected 72h after growth on plastic (red), fibronectin (blue) and collagen (green) were evaluated from both control cell lines (MG63 NCP) and reduced α2 integrin cells for MMP 2 protein expression. α2 down-regulated cells did not differ significantly ($p>0.05$) from control NCP cells in MMP 2 protein expression. X-axis = protein concentration (pg/mL), Y-axis shows cell lines, ns = not significant, n= 3.

Similar to MMP 1, MMP 3 was up-regulated in plastic and ECM substrates when compared with control cells. As shown in Figure 2.8, MMP 3 protein expression is up-regulated 1.5 fold on plastic, collagen and fibronectin. When alpha 2 integrin expression is down-regulated, MMP 3 expression is up-regulated almost 3 fold on plastic and fibronectin substrates when compared with the control cell line. On the collagen substrate there is a 1.5 fold increase in MMP 3 expression in the alpha 2 low cells.

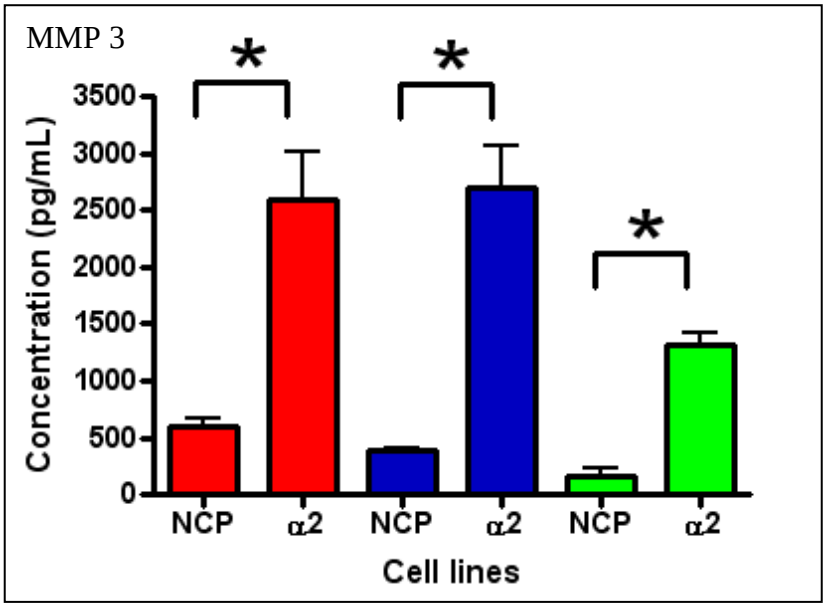

Figure 2.8: Conditioned media collected 72h after growth on plastic (red), type I collagen (green) and fibronectin (blue) were evaluated from both control cell lines (MG63 NCP) and reduced α2 integrin cells (α2) for MMP 3 protein. MMP3 protein expression significantly increases ($p<0.05$) in knocked- down cells for the α2 integrin (α2) compared with control cells (NCP). X-axis = cell lines. Y-axis = concentration (pg/mL), n= 3.

We also evaluated MMPs 7, 9 and 13 protein expression on plastic and the ECM substrates fibronectin and collagen. We found MG63 cells control or alpha 2 down-regulated MG63 osteosarcoma cells expressed negligible amounts of these MMPs (data not shown).

2.4 Discussion

It is well known that integrin expression is aberrant in carcinomas (see Introduction 1.2.2) contributing to the metastatic process. Loss or over-expression of specific integrins may contribute to an invasive phenotype and altered expression of integrins in carcinomas has long been an area of research in carcinomas. Several studies have confirmed up-regulation of $\alpha_V\beta_6$ integrin in carcinomas as lung cancer (Smythe et al. 1995), breast (Manzotti et al. 2000), thyroid carcinoma (Kitajiri et al. 2002), gastric carcinoma (Zhang et al. 2008) and oral cancers (for review see: Thomas et al. 1997). Up-regulation of the α6β4 integrin in squamous cell carcinoma was also evident when compared with normal human epidermis (Janes and Watt 2004). Additionally, lower α5β1 integrin expression is associated with development of hepatocellular carcinoma (Zhou et al. 2000). Integrin β3 over-expression suppresses tumor growth in glioblastoma (Kanamori et al. 2004). Furthermore, integrins have been used as prognostic factors in some epithelial cancers. Adachi and co-workers found reduced α3 expression associated with poorer prognosis in patients with adenocarcinoma (Adachi et al. 1998). High expression of $\alpha_V\beta_6$ is an indicator of poor prognosis in patients with colon carcinoma (Vonlaufen et al. 2001). High expression of α6 in breast cancer is correlated with a decreased survival rate in breast cancer patients (Friedrichs et al. 1995). However, studies correlating integrin expression to prognosis are limited in sarcomas, particularly osteosarcoma.

We evaluated conditioned media of MG63 cells transfected with a scrambled siRNA after attachment on plastic and extracellular matrix substrates collagen and fibronectin for MMP protein expression. We found that the MG63 control cell line

modulated their MMP protein expression depending on if they were attached to matrix proteins or plastic. MMPs 1 and 3 were lowest on matrix proteins fibronectin and collagen but were elevated on non-matrix substrate plastic. In general, MMP expression is low in tissues and is induced during ECM remodeling (Westermarck and Kahari 1999).

We chose to down regulate the collagen/laminin receptor α2β1 in an osteosarcoma cell line. Specifically, we were interested in integrin-mediated MMP proteolysis. We chose to reduce α2β1 integrin expression as this is the integrin known most commonly to be down-regulated in epithelial cancers. For example, in human malignant tumor progression, the α2β1 integrin was present in non-neoplastic fibroadenomas but were low or absent in invasive mammary cancer (Gui et al. 1995). In pancreatic cell lines, loss of alpha 2 integrin was associated with adenocarcinomas and ampullary tumors (Koukoulis et al. 1991). Loss of α2β1 integrin is also observed in carcinomas of the colon when compared to normal colon cells (Koretz et al. 1991). However, the role of this integrin in sarcoma metastasis is limited and it is important to note that epithelial cells are different form mesenchymal cells in a number of ways. While epithelial cells form closely attached layers joined by specialized structures as tight junctions, adherens and gap junctions, mesenchymal cells do not form organized layers. Mesenchymal cells are generally more motile *in vitro* than epithelial cells (for review see: Thiery and Sleeman 2006). Often, carcinogenesis involves the epithelial to mesenchymal transition (EMT) in which cells dissociate from each other and the ECM and become more motile (for review: see Savagner 2001).

Using siRNA constructs that reduce α2 integrin expression in human OS cells, we assessed integrin mediated MMP expression: the integrin-MMP expression feedback

loop. Conditioned media collected 72h after growth on plastic, type I collagen and fibronectin was evaluated for MMP protein production from both control cell lines (MG63 NCP) and reduced α2 integrin cells (α2 low). Control MG63 cells selectively down regulate MMP1 fourfold after attachment to fibronectin and collagen. In addition, down regulation is specific for MMP 1 and MMP 3 as MMP 2 remain unchanged. We conclude that signaling from the α2 integrin may repress MMP1 and MMP 3 expression, as loss of α2 results in up-regulation of MMP1 and MMP3. These data support the hypothesis that a feedback loop between integrin signaling and MMP expression is functioning in osteosarcoma cells.

Several studies in carcinomas demonstrate an integrin-MMP feedback loop. Rolli et al. (2003) found that activated integrin $\alpha_V\beta_3$ cooperates with MMP 9 to promote metastasis of the human breast cancer cell line MDA-MB 435. Furthermore, it is well established that integrin mediated alterations in cell matrix regulate MMP expression (for review see: Shapiro 1998). Baum and co-workers have shown that up-regulation of the β3 integrin in the MDA-MB-231 breast cancer cells lines increased MMP 2 production (Baum et al. 2007). Additionally, the presence of $\alpha_V\beta6$ integrin in oral squamous cell carcinomas resulted in down-regulation of MMP 13 expression (Ylipalosaari et al. 2005). This suggests that the $\alpha_V\beta6$ integrin might be essential for repression of MMP 13. In support of our data, down regulation of the α4β1 integrin during transformation led to increased MMP1 expression in invasive human breast cancer cell lines (Jia et al. 2004). Martinella-Catusse's group found that the alpha 3 integrin inhibits bronchial tumor cell line invasion via down-regulation of membrane type 1 MMP (Martinella-Catusse et al. 2001). Similar to our data, these data suggest that loss of alpha 3 integrin subunit might

result in up-regulation of the membrane type I MMP hence resulting in a more invasive phenotype. Although integrins work in concert with MMPs in normal processes, these data demonstrate that integrins may influence MMPs in pathological processes. However, few studies have evaluated the feedback loop between integrins and MMPs in metastasis and even less studies have correlated integrin mediated expression of MMPs in sarcomas.

The data presented here suggest that the alpha 2 integrin represses MMP 1 and MMP 3 expression but has no effect on MMP 2 expression in control cells and α2 knock-down osteosarcoma cells. MMP 1 is a collagenase degrades fibrillar collagen (types I, II and III), but prefers type I collagen (Duffy et al. 2000). This collagenase is expressed in the periodontal tissue, skin, bone and rheumatoid synovium (Nagase 2000). It is well understood that MMP 1 is bound by the α2β1 integrin via the I domain of the alpha 2 subunit (Dumin et al. 2001). This study supports our finding by providing a mechanism of repression of MMP 1 by the alpha 2 integrin. Increased MMP 1 expression has been demonstrated in several cancers including melanoma (Templeton et al. 1990), hereditary nonpolyposis colorectal cancers, sporadic colorectal cancers (Behrens et al. 2003) and breast (Park et al. 2008; Decock et al. 2008; Saad et al. 2000). It is interesting to note that Durko's group suppressed MMP 1 expression using an antisense fragment of MMP 1 in MIM melanoma cells (without altering the expression of MMP 2) and found reduced invasive capabilities on type IV collagen and Matrigel (type IV collagen containing basement membrane) when compared with normal or sense transfected cells (Durko et al. 1997). Furthermore, Poola et al. (2005) identified MMP 1 as prognostic marker in 73 of 103 pre-breast cancer patients. Our studies demonstrate MMP 1 expression is increased

when the alpha 2 integrin is knocked down suggesting that signaling from the alpha 2 integrin may be necessary to repress MMP 1 expression.

MMP 3 is a stromelysin of varied substrate specificity including proteoglycans, non-collagenous proteins as laminin, fibronectin and the non-helical regions of collagen IV (Duffy et al. 2000). Several studies have established that MMP 3 is up-regulated in breast cancer (Sternlicht et al. 1999; Balduyck et al. 2000; Garbett et al. 1999). Garbett et al. (1999) also showed that MMP 3 is up-regulated in colorectal cancer. However, altered MMP 3 expression has not been evaluated in osteosarcoma. Up-regulation of MMP 3 in osteosarcoma might provide a mechanism for increased degradation of non-collagenous proteins as fibronectin and laminin in osteosarcoma. Increased degradation of these ECM proteins may allow migration into the vasculature.

In combination, up-regulation of MMP 1 and 3 might result in increased proteolytic cleavage of collagen (the chief component of bone, tendons and ligaments). This up-regulation implicates these MMPs in bone cancers and metastasis. In support of this, Sasaki's group showed up-regulation of MMP 1 and MMP 3 are necessary for recruitment of osteoclastic cells for bone resorption (Sasaki et al. 2007). Therefore, loss of integrin α2 or up-regulation of MMP 1 and 3 by other genes in osteosarcoma may result in increased bone turnover. It is well established that bone turnover (reflecting bone turnover and formation) are indicators of bone metabolism disturbances (Ambroszkiewicz et al. 2006) and as such variations in normal MMP 1 and 3 expression may be used as a prognostic tool in osteosarcoma.

The data presented here also show that loss of the alpha 2 integrin does not influence MMP 2 production on plastic, fibronectin or collagen in MG63 osteosarcoma

cells. MMP 2 is a gelatinase that degrades gelatin (denatured collagen), types IV, V, VII, IX and X collagen (Nagase 2000). It is possible that MMP 2 production in osteosarcoma may be influenced by another integrin. In support of this, Silletti's group found MMP 2 associates with integrin $\alpha_v\beta_3$ forming a complex that allows vascular invasion in CS-1 hamster melanoma cells (Silletti et al. 2001). In contrast to carcinomas, it is possible that MMP 2 may not be involved in the osteosarcoma metastatic process.

2.5 Conclusions and Future Directions

Control MG63 cells transfected with a scrambled siRNA express MMPs 1, 2 and 3 when plated on plastic and matrix proteins (fibronectin and collagen). When the alpha 2 integrin is knocked down using siRNAs in MG63 osteosarcoma cells, MMPs 1 and 3 increase compared to the control MG63 cells. These data highlight the existence of an integrin-MMP feedback loop in MG63 osteosarcoma cells. Our data suggest that the alpha 2 integrin might be responsible for MMP 1 and MMP 3 repression in osteosarcoma cells. We therefore speculate that loss of alpha 2 integrin may increase metastatic potential by increasing MMP 1 and 3 production. Further investigation using additional osteosarcoma cell lines is warranted to determine if the loss of α2 integrin may be used as a prognostic factor in osteosarcoma. It is important to note that we did not down-regulate the alpha 2 integrin subunit in normal cells (for example in the hFOB osteoblast-like cell line) to determine if the alpha 2 integrin subunit is normally responsible for repressing MMP 1 and 3 expression. As such, we will also knock-down alpha 2 integrin in normal cells to evaluate the integrin-MMP feedback loop.

We are currently evaluating the effects of over-expression of the alpha 2 integrin subunit on the integrin-MMP feedback loop. We hypothesize that the up-regulation of the alpha 2 integrin may have an effect on the metastatic potential of MG63 cells. In addition, we will also evaluate alpha 3, 5 and V integrin subunits for their role in OS metastases. Further, the role of this feedback loop in determining metastatic potential in MG63 osteosarcoma cells is also currently under investigation using an *in vivo* murine model.

Chapter 3

Osteosarcoma cells of Different Metastatic Potential Differ in Integrin Expression and MMP regulation.

3.1 Introduction

Integrin expression is abberant in many carcinomas (see Introduction 1.2.2). In this chapter, we continue to investigate our overarching hypothesis that integrin profile varies between human osteosarcoma cells of low and high metastatic potential. Further, we evaluate MMP expression in these osteosarcoma cell lines of varying metastatic potential.

We evaluated SAOS-2 and LM7 osteosarcoma cell lines of low and high metastatic potential, respectively, for their integrin profiles. Jia and coworkers established metastatic sub-lines via intravenous injection of the non-metastatic SAOS-2 parental cells (Jia et al. 1999). Microscopic pulmonary metastases were repeatedly collected and cultured (following additional intravenous injections of subsequent cell lines) establishing the LM1-LM7 cell lines of increasing metastatic potential. Nude mice injected with the LM7 sub-line developed macroscopic lung metastases within 6 weeks of injection (Worth et al. 2002). We chose to use the LM7 cell line because it has the highest metastatic potential.

We found that the metastatic LM7 osteosarcoma cell line expressed lower alpha 5 integrin compared the SAOS-2 parental cell line. This cell line provides a relevant experimental model to evaluate altered integrin expression differences in osteosarcoma.

Secondly, we hypothesize that when OS cells attach to a particular class of ECM protein, MMP production is specifically altered in response. Thus, when metastasizing cells encounter an ECM protein barrier during tissue invasion, cells produce specific MMPs required for invasion, and this response is an element of metastatic potential. To address this hypothesis, we examined conditioned medium of a non-metastatic SAOS-2 parental cell line and its metastatic sub line LM7 grown on different ECM substrates and plastic. As controls we also evaluated the hFOB osteoblast-like cell line and human MG63 osteosarcoma cells.

We found that basal MMP 1, 2 and 3 expression in control hFOB cells is higher on non-matrix substrate (plastic) than matrix proteins (fibronectin and collagen). However, a similar pattern is not observed in the osteosarcoma cell lines. Greater MMP 1, 2 and 3 protein expression is observed in the MG63 cells of unknown metastatic potential when plated on plastic and matrix proteins compared with the hFOB osteoblast-like cell and the SAOS-2 and LM7 osteosarcoma cell lines. In the SAOS-2 and LM7 cell lines MMP 1 and 3 protein expression was undetectable in conditioned media obtained from these cells. However, we found that MMP 2 expression was significantly higher in the non-metastatic SAOS-2 cells when compared with the metastatic LM7 cells when plated on non-matrix or matrix proteins. These data suggest that the loss of MMP 2 expression might present a novel role for this MMP in osteosarcoma metastasis as has been observed in some carcinomas (see Introduction 1.3.1).

3.2 Experimental Methods

3.2.1 Cell lines and Cell Culture

Human MG63 cells osteosarcoma (ATCC # CRL 1427), hFOB osteoblast-like cell lines (ATCC # CRL-11372), SAOS-2 and LM7 cell lines (generously given to us by Dr. Eugenie S. Kleinerman of the University of Texas M. D. Anderson Cancer Center Departments of Cell Biology/Pediatrics) were cultured with Dulbecco's minimal essential medium (Hyclone) supplemented with 10% fetal bovine serum (Gibco) at 37ºC with a 5% CO_2 incubator.

3.2.2 Flow Cytometry and Flow Sorting

Media was removed and cells were washed with PBS and lifted non-enzymatically with versene at 37ºC for 3-5 minutes. The cells were pelleted in DMEM (High glucose) without serum. Pellets were resuspended in 100 µl of the primary antibody solution and incubated for 30 minutes at room temperature. The antibodies used were mouse monoclonal anti-integrin α2β1-FITC conjugate (1:100, Chemicon CBL477F), anti-integrin α3 (1:500 Santa Cruz, sc-6592), anti-integrin α5 (1:500 Chemicon CBL 497F) and anti-integrin αV (Chemicon: MAB1980). The samples were washed with DMEM before being resuspended in 0.5 ml of DMEM, to be analyzed by flow cytometry using a Becton Dickson FACScan. 500,000 cells/sample were used for all flow cytometry experiments and a minimum of 10,000 cells were counted. Gating was set at <1% of no antibody negative control cells. Data were evaluated with BD CellQuest Pro software.

3.2.3 MMP-Integrin Feedback Loop Analysis

Experimental cell lines (MG63, hFOB, SAO2-2 and LM7) were grown on three classes of extracellular matrix proteins: collagen (1mg/mL) and fibronectin (15µg/mL) and plastic as a control. Cells were cultured in serum free conditions by using KnockOut-Serum Replacement (Invitrogen, Cat. No.10828). Conditioned media was collected three days later and analyzed using fluorescent sandwich ELISA beads (R&D systems) and a Luminex 100 System (Luminex 100). The Luminex system quantitatively determines protein concentrations of human MMPs using specific antibodies pre-coated on color-coded microparticles (R&D Systems). We analyzed MMPs 1 (LMP 901), 2 (LMP 902), 3 (LMP 513), 7 (LMP907), 9 (LMP 911) and 13 (LMP 511) secreted in cell culture media. Experiments were performed in triplicate. Plastic was used as a baseline condition for comparison to cells on fibronectin and collagen.

3.2.4 Statistical Analysis

One Way Anova was used and P values less than 0.05 were considered significant. We used GraphPad Prism 4.

3.3 Results

3.3.1 The Metastatic LM7 cell line has Lower α5 Integrin Expression compared with Non-metastatic Parental SAOS-2 cell line

We hypothesize there will be a difference in integrin profile between cells of high and low metastatic potential. To test this hypothesis, we used the SAOS-2 and LM7 osteosarcoma cell lines to investigate integrin profiles. We chose to use these cell lines because they are of low (SAOS-2) and high (LM7) metastatic potential (Jia et al., 1996). As controls we have evaluated the BJ fibroblast cell line, the hFOB osteoblast-like cell line and the MG63 osteosarcoma cell line of unknown metastatic potential. The α5 integrin was the only integrin subunit that varied between the non-metastatic parental SAOS-2 cell line compared with the metastatic LM7 cell line, summarized in Table 3.1. The MG63 osteosarcoma cell line of unknown metastatic potential has the same integrin profile as the osteoblast-like hFOB cells.

Table 3.1: α5 integrin subunit expression is lower in metastatic LM7 cell line compared with non-metastatic parental cells

Cell lines	α2 integrin (CD49b)	α3 integrin (CD49c)	α5 Integrin (CD49e)	αV integrin (CD51)
BJ fibroblast	**High**	**Medium**	**Medium**	**Low**
hFOB	**High**	**High**	**High**	**Medium**
MG63	**High**	**High**	**High**	**Medium**
SAOS-2	**Low**	**Medium**	**Medium**	**Low**
LM7	**Low**	**Medium**	**Low**	**Low**

Table 3.1: Cell lines of different metastatic potential have varied integrin profiles. We evaluated the alpha 2, 3, 5 and V integrin subunits via flow cytometry of hFOB, BJ fibroblast, MG63, SAOS-2 and LM7 cells. The α5 integrin was the only integrin subunit that was reduced in the LM7 metastatic cell lines compared with the non-metastatic

parental SAOS-2 cell line. The MG63 osteosarcoma cell line of unknown metastatic potential has the same integrin profile as the osteoblast-like hFOB cells (see Appendix A).

3.3.2: Non-metastatic and Metastatic Osteosarcoma Cells have Different MMP Production when grown upon Various ECM Substrates

We have investigated the integrin-MMP feedback loop using SAOS-2 and LM7 cell lines, specifically because they cells are of high and low metastatic potential respectively using the Luminex system. As controls we also evaluated the hFOB osteoblast-like cell line and the MG63 osteosarcoma cell line of unknown metastatic potential for MMP protein expression. Cells grown upon three substrates varied their MMP 1 production as measured in 72h serum free conditioned medium. Osteoblast-like hFOB cells reduced MMP 1 production when grown upon collagen and fibronectin as compared to plastic. MG63 osteosarcoma cells only reduced MMP 1 production when grown upon collagen but not fibronectin. MMP 1 expression was not detectable in non-metastatic SAOS-2 and metastatic LM7 cell lines.

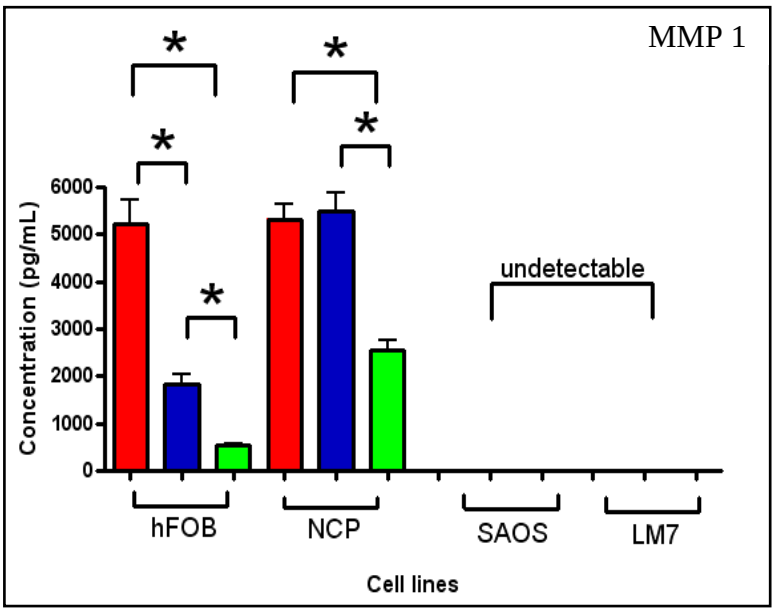

Figure 3.1: Cells grown upon three substrates varied their MMP 1 (five-fold dilution) production as measured in 72h serum free conditioned media. The hFOB cell line expressed more MMP 1 ($p<0.05$) on plastic (red) and fibronectin (blue) compared to collagen (green). The MG63 (NCP) cells expressed significantly more MMP 1 on fibronectin and plastic compared with collagen. The SAOS-2 and LM7 cell lines expressed negligible amounts of MMP 1 on all substrates. n= 3

We evaluated the hFOB, MG63, SAOS-2 and LM7 cell lines for MMP 3 protein expression. Osteoblast-like hFOB cells increased MMP 3 production when grown upon collagen and fibronectin as compared to plastic. As shown in Figure 3.2 the MG63 osteosarcoma cells expressed significantly higher MMP 3 when plated on fibronectin and plastic compared to collagen. MMP 3 expression was not detectable in the SAOS-2 and LM7 osteosarcoma cell lines.

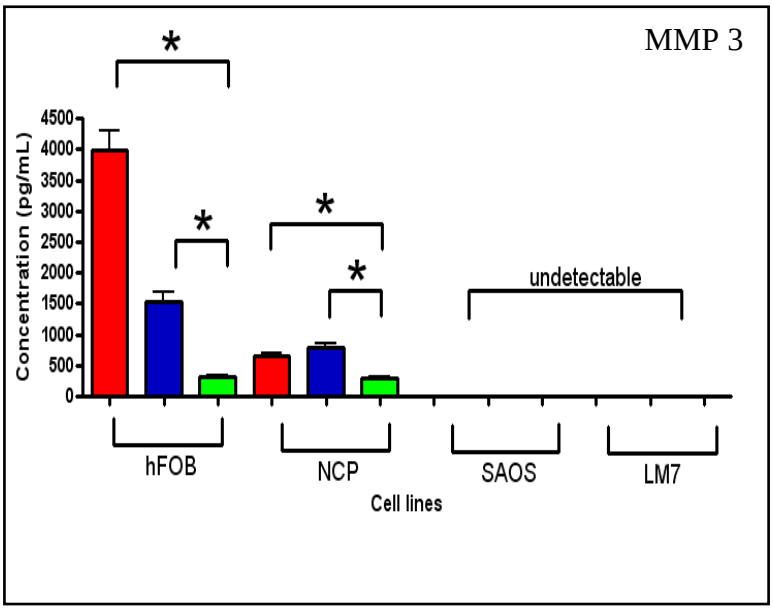

Figure 3.2: Cells grown upon three substrates varied their MMP 3 production (five-fold dilution) as measured in 72h serum free conditioned media. The hFOB cells expressed significantly more ($p<0.05$) MMP 3 on fibronectin (blue) and plastic (red) compared to collagen (green). MG63 (NCP) cells MMP 3 production was significantly higher on fibronectin and plastic compared to collagen. MMP 3 expression was undetectable in the SAOS-2 and LM7 cell lines expressed on all substrates. X-axis = cell lines, Y-axis = concentration (pg/mL), n= 3.

However, when we looked at MMP 2 expression in the cell lines evaluated, we noticed a different pattern from MMP 1 and MMP 3 protein expression in the cell lines evaluated. As shown in Figure 3.3, hFOB cells expressed MMP 2 when plated on plastic and matrix proteins. MMP 2 production of hFOB cells when plated on plastic and fibronectin were significantly higher ($p<0.05$) when compared with hFOB cells plated on collagen. The MG63 (NCP) cell line of unknown metastatic potential expressed 4 fold more MMP 2 than the other two osteosarcoma lines on the non-matrix and matrix

proteins evaluated. Further, MG63 osteosarcoma cells expressed significantly more MMP 2 on fibronectin and plastic compared with collagen. However, there was not significant difference in MMP 2 protein expression between NCP cells plated on plastic and fibronectin. Although less that the MG63 osteosarcoma cell lines, the SAOS-2 cells and LM7 osteosarcoma cell expressed MMP 2.

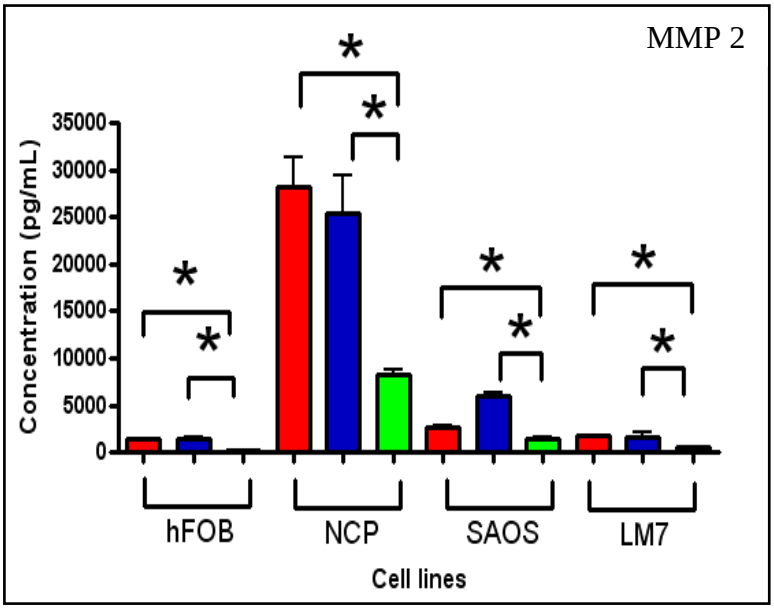

Figure 3.3: Cells grown upon three substrates varied their MMP 2 protein production (five-fold dilution) as measured in 72h serum free conditioned media. The hFOB, MG63, SAOS-2 and LM7 cells expressed MMP 2 when grown on plastic (red), fibronectin (blue) and collagen (green). hFOB. NCP, SAOS and LM7 cells expressed significantly higher ($p<0.05$) MMP 2 on fibronectin and plastic compared to collagen. X-axis = cell lines, Y-axis = concentration pg/mL, n= 3.

MMP 2 was the only MMP expressed in the SAOS-2 and LM7 osteosarcoma cells. MMP 2 expression in LM7 cell line is significantly reduced on all substrates evaluated when compared with the parental cell line plated on the same substrate. As shown in Figure 3.4, the non-metastatic SAOS-2 cell line express almost three times more MMP 2 on collagen when compared with LM7 on collagen. The SAOS-2 parental cell line also expresses approximately 6-fold more MMP 2 than the LM7 cell line when plated on fibronectin. When plated on plastic, the SAOS-2 cells express approximately 1.5 more MMP 2 protein compared with the LM7 cells.

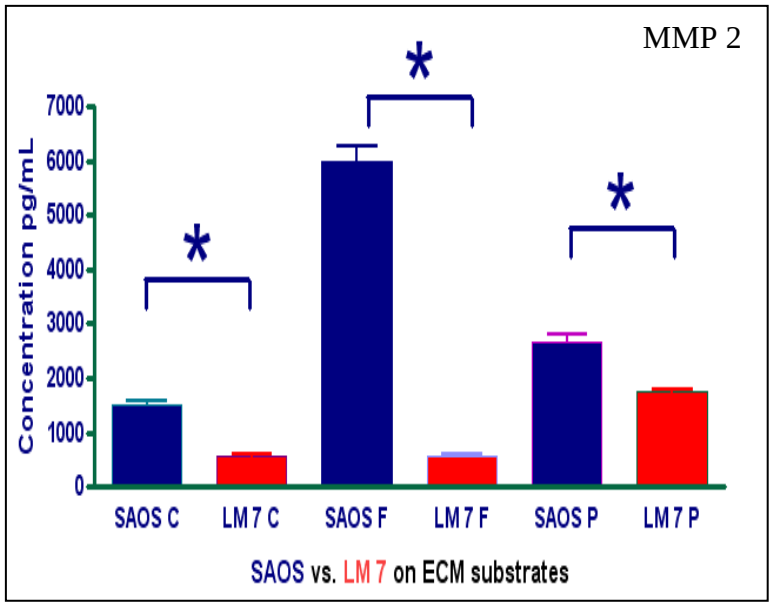

Figure 3.4: SAOS-2 and LM7 cells grown on three substrates varied their MMP 2 production (five-fold dilution) as measured in 72h serum free conditioned media. The non- metastatic SAOS-2 cell line express significantly more ($p<0.001$) MMP 2 protein than metastatic LM7 cell line on all substrates (P, Plastic, Fibronectin, C, Collagen. X-axis = cell line and substrate, Y-axis = concentration (pg/mL), n= 3.

3.4 Discussion

3.4.1: The Metastatic LM7 Osteosarcoma Cell Line Express Lower alpha 5 Integrin compared with Non-metastatic Parental SAOS-2 Cell Line

Many studies have demonstrated that integrins play a role in carcinoma metastasis (see introduction 1.3). We hypothesize that integrin profiles vary between osteosarcoma cells of low and high metastatic potential. We compared the SAOS-2 cell line of low metastatic potential with the metastatic LM7 cell line. Additionally, as controls we evaluated the BJ fibroblast, hFOB osteoblast-like and the MG63 osteosarcoma cell lines. In this study we show that the metastatic LM7 cell line express reduced alpha 5 integrin compared with non-metastatic parental cell line.

We found that the hFOB osteoblast like cell line expresses high levels of the alpha 2, 4 and 5 integrin subunit and intermediate levels of the alpha V integrin subunit relative to unstained cells. However, the BJ fibroblast cell line expressed high alpha 2 integrin levels and intermediate levels of the alpha 3 and 5 with low levels of alpha V integrin. The SAOS-2 cell line express similar levels of alpha 2, 3 and V integrin to the LM7 metastatic cell line. However, the alpha 5 integrin was higher in the SAOS-2 metastatic cell line compared with the LM7 cell line.

In these studies we observed that the metastatic LM7 osteosarcoma cell line had reduced alpha 5 integrin expression when compared with the parental SAOS-2 cell line. The α5 integrin subunit dimerizes only with the β1 integrin subunit (α5β1) and is mainly responsible for osteoblast adhesion to fibronectin (Nesti et al. 2002). Kaabeche and co-workers found that the alpha 5 integrin is essential for the initiation of apoptosis in osteoblasts (Kaabeche et al. 2005). Loss of cell attachment to the ECM triggers apoptosis

in normal, anchorage dependent cells. Our data suggest that down-regulation of the alpha 5 integrin in the LM7 osteosarcoma metastatic cells might represent a mechanism for cell metastasis in osteosarcoma cells.

Taverna's group found that mice heterozygous for the alpha 5 integrin deletion in a p53 null background developed osteosarcomas, a tumor typical of older p53 heterozygous mice but rare in null mice (Taverna et al. 1998). Further, the authors observed that these mice also developed anaplastic sarcomas often observed in p53 heterozygous mice but not nulls. Together, these data suggest that down-regulation of the alpha 5 integrin might represent a novel mechanism used by osteosarcoma cells to metastasize.

Many carcinomas also demonstrate loss of the alpha 5 integrin subunit. Seftor et al. (1993) found that lower α5β1 integrin in a melanoma cell line resulted in increased MMP 2 production and increased invasiveness, thus implicating the alpha 5 integrin in the metastatic process. Seftor and co-workers (Seftor et al. 1993) further emphasizes the existence of an integrin-MMP feedback loop in these melanoma cells. In HT29 colon carcinoma cells the α5β1 integrin is not expressed unlike in normal colon cells. Transfection of the alpha 5 integrin subunit into these cells resulted in decreased proliferation and loss of the transformed phenotype (Varner et al. 1995), further implicating this integrin in transformation. In pancreatic cancer cells, alpha 5 integrin expression was weak to moderate, and approximately 50% of the pancreatic cancer cells have complete loss of the alpha 5 integrin (Weinel et al. 1992). Although several studies have demonstrated up-regulated integrins in prostate cancer, the alpha 5 integrin subunit is specifically down-regulated in prostate cancer (For review see: Goel et al. 2008).

Several studies have highlighted possible mechanisms that implicate the α5 integrin in the metastatic process. Zhang and co-workers found that Chinese hamster ovarian cell attachment mediated by the alpha 5 beta 1 integrin, supports cell survival via the anti-apoptotic protein Bcl-2 (Zhang et al. 1995). Cells that attach with other integrins that bind fibronectin underwent apoptosis. Additionally, Cao and co-workers identified domains II and III of the integrin alpha 5 subunit as involved in cells spreading and signaling (Cao et al. 1998). Integrin alpha 5 beta 1 expression is often lost in colon cancer when compared with normal epithelial cells. Kuwada and co-workers found that alpha 5 integrin normally mediates down regulation of human epidermal growth factor 2 (HER2), exerting an anti-tumor effect (Kuwada et al. 2005). However, in many colon cancer cells HER2 is up-regulated, suggesting lost of alpha 5 integrin repression. It is possible that a similar mechanism plays a role in the osteosarcoma metastasis.

3.4.2 Integrin-MMP Feedback Loop Differs in Cell Lines of Different Metastatic Potential

We hypothesize when OS cells attach to a particular class of ECM protein, MMP production is specifically altered in response. That is, when metastasizing cells encounter an ECM protein barrier during tissue invasion, a feedback loop mechanism enables the cells to produce the MMPs required for invasion, and this response is an element of metastatic potential. To address this hypothesis, we examined conditioned medium of non-metastatic SAOS-2 parental osteosarcoma cell line and its metastatic sub-line LM7 grown on different ECM substrates for MMP protein expression. As controls we also evaluated the hFOB osteoblast-like cell line and MG63 osteosarcoma cell line for MMP protein expression. We found that osteosarcoma cells show aberrant MMP expression on

non-matrix and matrix substrates when compared with control hFOB osteoblast-like and BJ fibroblast cell lines. While, the MG63 osteosarcoma cell line express significantly higher MMPs 1, 2 and 3 than the hFOB cell, the SAOS-2 and LM7 do not express MMP 1 and 3. However, the metastatic LM7 cells line specifically down-regulates MMP 2 production on plastic and ECM substrates compared with the parental SAOS-2 cell line.

We found that in control hFOB cells, basal MMP 1, 2 and 3 expression are up-regulated on the non-matrix substrate, plastic compared with MMP protein expression on the matrix substrates fibronectin and collagen. This suggests that these osteoblast-like cells produce the least amount of MMP 1, 2 and 3 on the environment most like the bone environment *in vivo*.

However, a similar pattern to the normal hFOB osteoblast-like cell line is not observed in the osteosarcoma cell lines (MG63, SAOS-2 and LM-7) and this suggests altered MMP expression in these cells. The MG63 osteosarcoma cell line of unknown metastatic potential has higher MMP 1, 2 and 3 protein expression on plastic and matrix substrates compared with the SAOS-2 and LM7 osteosarcoma cell lines. Such findings suggest that the MG63 cell line might differ in metastatic potential when compared with the SAOS-2 and LM-7 cell lines. Many investigators have demonstrated that increased MMP regulation is correlated with increased metastatic potential in carcinomas. For example, over-expression of MMP 1 in prostate cancer cells led to increased cell invasion and migration (Pulukuri and Rao 2008). Further, the authors found increased MMP 1 expression was associated with metastases to the lung. These data suggest that MMP 1 might also play a role in osteosarcoma metastasis to the lungs, the most common site of metastases in osteosarcoma. Additionally, MMP 3 up-regulation was associated with the

epithelial to mesenchymal transition in rat mammary epithelial cells (Lochter et al. 1997). In concert with the data presented here, up-regulation of MMP 1 and MMP 3 might be a mechanism used by osteosarcoma cells to metastasize to the lungs. Our lab will investigate the metastatic potential of this cell lines in SCID mice.

Unlike the control hFOB osteoblast-like cell lines, the SAOS-2 and LM7 osteosarcoma cells of high and low metastatic potential, respectively, express negligible amounts of MMP 1 and 3 but differ in MMP 2 expression. MMP 2 expression was significantly higher in the non-metastatic SAOS-2 parental cells than the metastatic LM7 subline on every substrate tested. Although higher levels of MMP 2 are correlated with increased metastatic potential in many carcinomas, Jawad et al. (2009) found that MMP 1 silencing using shRNA resulted in increased tumor growth and vascularity of the primary tumor in a xenogenic murine model of human osteogenic sarcoma. Further, some carcinomas show an inverse relationship between metastatic potential and MMP expression. For example, high MMP 9 expression correlates with low metastasis in colon cancer (Takeha et al. 1997). Darmiento et al. (1995) found over-expression of human MMP 8 in transgenic mice reduced susceptibility to skin carcinogenesis. Additionally, MMP 8 null mice had increased propensity to skin tumors (Balbin et al. 2003). Agarwal's group compared MMP 8 protein expression in non-metastatic (MN-2C5) and metastatic (M-4A4) breast cancer cell lines. The metastatic cell line expressed a 20 fold decrease in MMP 8 expression (Agarwal et al. 2003). Loss of MMP 3 in null mice resulted in enhanced initial tumor growth rates of squamous cell carcinoma (McCawley et al. 2004). As such, further investigation will be required to determine if there is a correlation

between MMP 2 expression and metastatic potential of OS. It is important to note that sarcomas are as a group different from carcinomas.

3.5 Conclusions and Future Directions

We demonstrate that alpha 5 integrin expression is reduced in metastatic LM7 cells when compared with non-metastatic parental SAOS-2 cells. This supports our hypothesis that integrin expression may vary in osteosarcoma cells of different metastatic potential. The integrin profile data presented is the result of a single experiment and replicates are underway. Future studies in our lab will focus on the down-regulation of the alpha 5 integrin in the parental SAOS-2 osteosarcoma cells. We hypothesize down-regulation of the alpha 5 integrin may result higher metastatic potential compared with the parental SAOS-2 cell line. We are also evaluating down-regulation of the alpha 5 integrin subunit in MG63 osteosarcoma cells. Further, we will assess the integrin profile of the newly isolated PSU-OS-M cells (described in Chapter 4) to compare a recently isolated osteosarcoma cell line with osteosarcoma cell lines that have been established for decades.

In addition, we demonstrate that the hFOB osteoblast-like cell line expresses MMPs 1, 2 and 3. The MG63 osteosarcoma cells express significantly more MMPs 1, 2 and 3 compared with the hFOB, SAOS-2 and LM7 cells. Osteosarcoma cells (MG63, SAOS-2 and LM7) express different amounts of MMPs compared with the control hFOB cell line. The MG63 osteosarcoma cells expressed significantly more MMPs 1, 2 and 3 on plastic and fibronectin compared with collagen, a pattern that is different from control hFOB cells. A different pattern of MMP expression in the MG63 cells compared with the

hFOB cells may suggest distorted MMP regulation in these osteosarcoma cells compared with normal osteoblast cells.

In contrast, the osteosarcoma SAOS-2 and LM7 do not express detectable amounts of MMPs 1 and 3. The differences in MMP expression between the MG63 osteosarcoma cells compared with the SAOS-2/ LM7 osteosarcoma cells might suggest different metastatic potential among these cell lines. We also show that MMP 2 expression is significantly higher in the non-metastatic SAOS-2 cell line compared to metastatic LM7 osteosarcoma cells. Further investigation will be required to determine if there is a causative relationship between MMP 2 expression and the metastatic potential of SAOS-2 and LM7.

Our data demonstrate differences in MMP expression among the three osteosarcoma cells tested. This emphasizes the need for new primary osteosarcoma cell lines. Future studies in our lab will evaluate the MMP expression of primary osteosarcoma cells compared with osteosarcoma cells obtained from a secondary site. We anticipate that MMP expression might vary in cells obtained from the primary site compared with cells obtained from a secondary site. We will also evaluate the MMP expression of the PSU-OS-M cells we describe in Chapter 4.

In conclusion, we demonstrate that integrin profile and MMP production may vary in osteosarcoma cells of different metastatic potential.

Chapter 4

Establishment of PSU-OS-M osteosarcoma cells

4.1 Introduction

Osteosarcoma is the most common bone cancer and the second greatest cause of cancer related death in children behind blood cancers. The survival of patients with OS has plateaued in the last decade (Lewis 2007). One of the reasons that there might be a lack of clinical progress is the absence of an animal model that fully recapitulates the human disease. That is, to our knowledge, there is no animal model that allows orthotopic growth in bone, spontaneous metastasis and growth to secondary tissue. Furthermore, researchers are limited to four widely used human osteosarcoma cell lines or derivatives of these cell lines (see Introduction 1.5). As such, therapeutic drugs cannot be assessed in an animal model that recapitulates the human disease.

We believe the small number of cell lines available in research labs contributes to the lack of an animal model. In this chapter, we addressed this deficit by the isolation and characterization of a new, primary osteosarcoma cell line: PSU-OS-M. By addressing this deficit, we hope to generate an osteosarcoma mouse model. The PSU-OS-M cell line is from a patient with histopathologically confirmed osteogenic sarcoma and Marfan syndrome. We have successfully cultured these cells *in vitro*. These cells exhibit attachment independent growth and loss of contact inhibition - hallmarks of transformation.

4.2 Experimental Methods

4.2.1 Digestion of Osteosarcoma Tissue and Establishment of PSU-OS-M cell line

Tissue with histopathologically confirmed osteoblastic osteosarcoma was collected from a 40-year old female patient of surgical oncologist, Dr. Edward Fox, of the Department of Orthopaedics. The patient was diagnosed with osteosarcoma and Marfan Syndrome. She was operated on to remove the majority of the tumor at another institution. Although the tissue we obtained was small, cell culture of the samples was initiated using maceration and explantation, 37^0C digestion with 4500u/mL of bacterial type 1a collagenase (Sigma) followed by plating on cell culture plates. The cells were grown under standard culture conditions of DMEM with 10% FBS and antibiotics/ antimycotics.

4.2.2 Generation of Clonal PSU-OS-M Cells

Cloning ring was used to obtain clonal osteosarcoma cells. Briefly, the cells were washed with 1X PBS. Autoclaved rings were then placed in autoclaved Vaseline and the rings were then put on various clone populations. Approximately 150 µL of trypsin was used to fill the rings. These were then placed in the 37^0C for 5 minutes. Once cells were detached, they were spun down and re-suspended in DMEM containing FBS and plated in a 24-well plate.

4.2.3 Contact Inhibited Growth Analysis

PSU-OS-M cells were grown under standard culture conditions of DMEM with 10% FBS and antibiotics/ antimycotics for approximately two weeks.

4.2.4 Anchorage Independent Growth

Briefly, 12.5g/L soft agar stock was made using Bacto-agar (Fluka 05039) in double distilled water and autoclaved. Agar was maintained at a temperature of 44^0 and mixed with 2X DMEM media (Hyclone SH30003.03) and 20% FBS. The plate was left to harden for 10-30 minutes. Cells were trypsinized and mixed with agar/2X media and plated on agar plate. The plate was then placed in the 37^0 incubator for one week. After one week, media was added.

4.2.5 Immunofluorescence of PSU-OS-M Osteosarcoma Cells

Cells were plated at confluency on glass coverslips and grown for 7 days in standard culture conditions. Cells were washed with PBS and fixed for 30 minutes in 2.5% paraformaldehyde in PBS, pH 7.2. Cells were blocked with PBS/BSA (1 mg/ml), then incubated with the primary antibody for 60 minutes at room temperature. PSU-OS-M cells were analyzed for fibrillin (1:500) and fibronectin (1:500) rabbit polyclonal anti-fibrillin-1(Ritty et al. 1999) and goat polyclonal anti-fibronectin antibody. Cells were washed in PBS/BSA before being incubated with a chicken anti-rabbit IgG Alexa Fluor 594 conjugate and anti-goat IgG Alexa Fluor 488 (Molecular Probes) secondary antibody at a dilution of 1:800 for 30 minutes at room temperature. Hoechst 33258 dye (Sigma, St. Louis, MO) was included at a 1:2000 dilution to stain cell nuclei. Cells were washed in PBS/BSA before being mounted in 25% glycerol in sterile H_2O. A Nikon Optiphot-2 fluorescent microscope mounted with a Spot RT CCD digital camera driven by SPOT software v3.5 (Diagnostic Instruments, Inc.) was used for image capture.

4.3 Results

4.3.1 PSU-OS-M cells Demonstrate Hallmarks of Transformation

In this section we have isolated a primary OS cell line from a human patient tumor. This sample was an atypical case as this patient also had Marfan's syndrome, a hereditary defect in the fibrillin-1 gene.

We evaluated this cell for two hallmarks of cancer cells: loss of contact inhibited growth and loss of attachment dependent growth. To test for loss of contact-inhibited growth, we plated the PSU-OS-M cells on tissue culture plates. We identified nodules that appear to exhibit a loss of contact inhibition by the PSU-OS-M cells, shown in Figure 4.1.

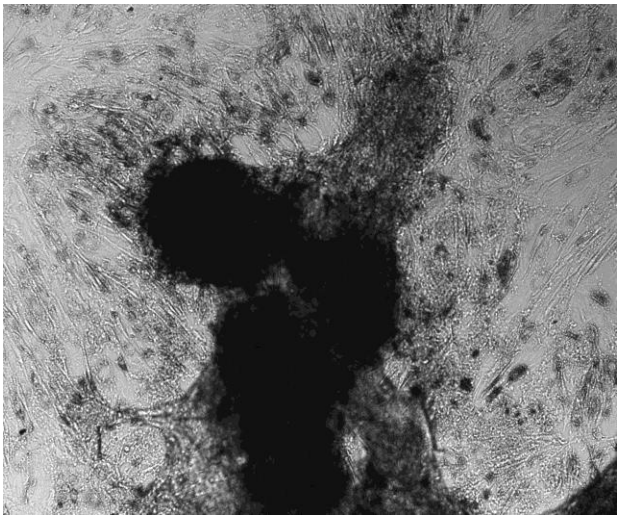

Figure 4.1: Phase contrast image of PSU-OS-M cells show loss of contact inhibited growth. These cells were obtained from a patient with osteoblastic osteosarcoma and cultured on tissue culture plates for approximately four weeks. The PSU-OS-M line also has been cultured in suspension for more than 8 consecutive weeks, showing loss of attachment dependent growth (data not shown).

We assessed the PSU-OS-M cells for another hallmark of oncogenic transformation – attachment independent growth. The PSU-OS-M cells were plated on soft agar and grew successfully for approximately 8 consecutive weeks in suspension (data not shown).

4.3.2 PSU-OS-M cells Deposit Abnormal Amounts of Fibrillin-1 Protein

We evaluated the PSU-OS-M cells for fibrillin-1 matrix assembly as patients suffering from Marfan syndrome often have a defect in fibrillin-1 assembly. Using immunofluorescence studies shown in Figure 4.2, PE and MG63 cells make and deposit fibronectin (red) and fibrillin (green). In contrast, the PSU-OS-M cells are capable of depositing normal fibronectin but deposit abnormal amounts of fibrillin matrix

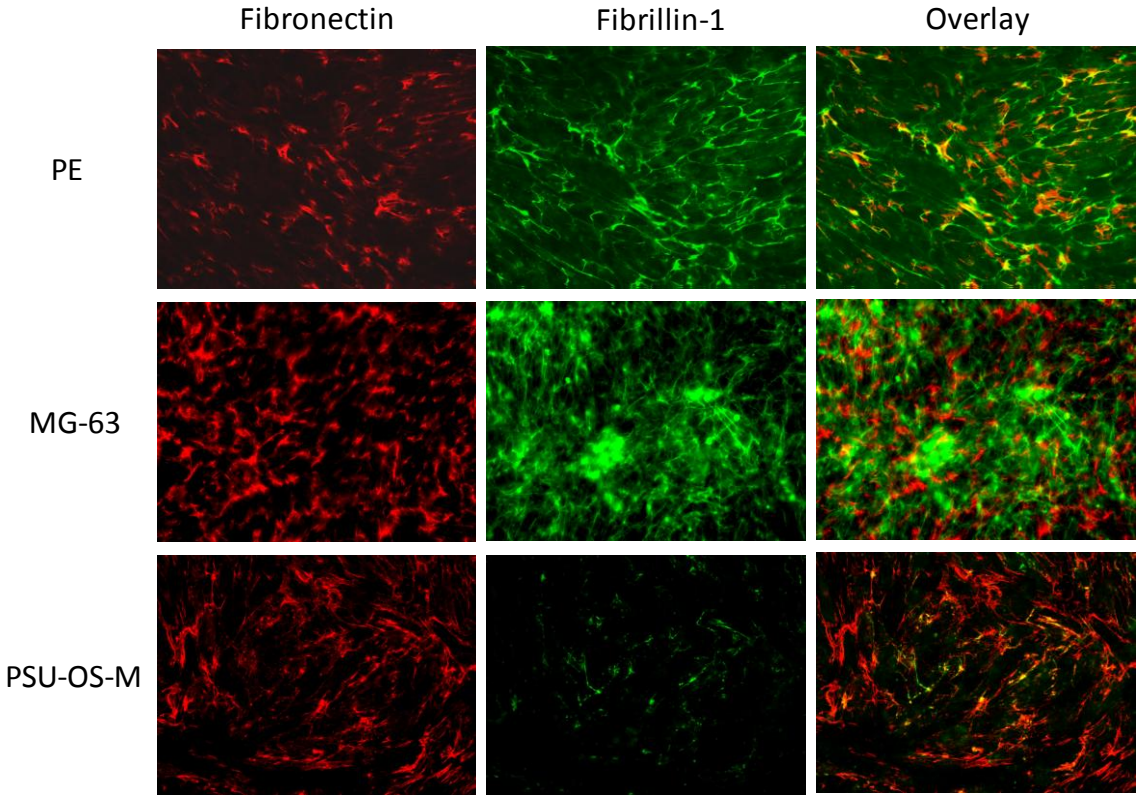

Figure 4.2: Immunocyochemistry of PE, MG63 and PSU-OS-M cells. Cells were double stained for fibronectin (red) and fibrillin (green). PE and MG63 cells deposit fibronectin and fibrillin. Despite their inability to assemble fibrillin, the PSU-OS-M cell line appears assembles fibronectin normally.

4.4 Discussion

In this chapter, we discuss the isolation and characterization of a new osteosarcoma cell line, PSU-OS-M, obtained from the primary tumor of an osteosarcoma patient. One factor that has slowed the development of OS clinical therapies is the lack of experimental cell lines and the lack of animal models that mimic the disease. Our first tissue sample was an atypical case as this patient had Marfan syndrome, a defect in the fibrillin 1 gene.

We have identified nodules when PSU-OS-M cells are grown on culture plate dishes. These nodules are indicative of loss of contact inhibited growth, typical of transformation. Contact inhibited growth is the process by which normal cells stop growing when they make contact with another cell (Abercrombie 1970). This phenomenon prevents overlapping of one cell by another. Transformed cells on the other hand are capable of evading this mechanism and overlap when cultured forming nodules.

In addition, we evaluated the PSU-OS-M cells for another hallmark of cancer, attachment independent growth on soft agar plates. We demonstrate that the PSU-OS-M cells are capable of growth in suspension for more than 8 consecutive weeks when grown on soft agar. In normal cells, attachment to the extracellular matrix is essential for survival and loss of adhesion to the ECM results in a type of apoptosis known as anoikis (Simpson et al. 2008). Normal cells are incapable of forming colonies when plated on soft agar (Freedman and Shin 1974). However, cancer cells are capable of anchorage independent growth and form colonies when plated on soft agar. This property is probably necessary for tumor cells to survive and metastasize to distant locations (for review see: Schwartz 1997).

Patients suffering from Marfan syndrome have a defect in the fibrillin-1 gene (see Introduction 1.2). Since we isolated the PSU-OS-M cell line from a patient diagnosed with Marfan syndrome, we evaluated the PSU-OS-M cell line for fibrillin and fibronectin deposition using immunoflorescence studies. As a control, we evaluated pigmented epithelial (PE) cells and the osteosarcoma cell line, MG63 for fibronectin and fibrillin matrix deposition. It is important to note that the MG63 osteosarcoma cell line is well known for depositing a fibrillin matrix *in vitro* (Ritty et al. 2003). We show the PSU-OS-M cells make fibronectin and fibrillin, but do not deposit a significant amount of fibrillin.

Another member of our lab, Robert Freed, investigated the PSU-OS-M cell line for mutations in the fibrillin-1 gene and found that the fibrillin gene contained a mutation at exon 52 encoding a premature stop codon (unpublished data). One of four mechanisms may prevent fibrillin-1 deposition into the matrix. It is possible that reduced fibrillin-1deposition may be due to a dominant negative mutation. A dominant negative mutation occurs when the mutated gene product affects the wildtype gene. Thus, the mutant fibrillin-1 protein may affect the deposition of the wildtype fibrillin-1 protein.

An alternate mechanism is that the truncated fibrillin-1 protein may be unable to be deposited into the matrix. In support of this, Ritty et al. (2003) showed that the C-terminus of the fibrillin-1 protein contains a heparin binding site necessary for matrix deposition. Heparin binding sites in matrix molecules allow induction of cytoskeletal re-organization as a substitute for integrin-mediated interactions with ECM ligands. The presence of a premature stop codon in *FBN1* of the PSU-OS-M cells, resulting in loss of the C-terminal heparin binding site which may prevent deposition of fibrillin-1 protein deposition into the ECM.

A third possible mechanism that may limit fibrillin-1 deposition into the matrix is the fibrillin mRNA may be subjected to non-sense mediated degradation due to mutation in the *FBN1* gene. Nonsense mediated decay is a cellular mRNA surveillance mechanism that allows detection of non-sense mutations (premature stop codon) to prevent the expression of truncated proteins (Frischmeyer and Dietz 1999).

A fourth potential mechanism that may prevent normal fibrillin-1 protein deposition is the abnormal protein may be targeted for degradation via quality control mechanisms of the cell (for review see: Trombetta and Parodi 2003)

4.5 Conclusions and Future Directions

The PSU-OS-M cell line demonstrated two hallmarks of transformation – loss of contact inhibited growth and attachment independent growth. We will repeat and quantitate the soft agar analysis to determine percent efficiency of nodule formation of the PSU-OS-M cell line. Additionally, we are currently evaluating the cytogenetics of PSU-OS-M cells.

The PSU-OS-M cell may be a useful cell line not only for osteosarcoma research but may also be used to study dominant negative effects of the mutated fibrillin-1 gene in Marfan syndrome. The question remains if the mutated FBN 1protein is made and deposited or degraded within the cell. Future studies in our lab will further elucidate mechanism for reduced fibrillin-1 deposition into the matrix. We will evaluate conditioned media of the PSU-OS-M cells for fibrillin-1 protein expression via a western blot.

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Appendix

Below are the results for the integrin profile of hFOB, BJ, MG63, SAOS-2 and LM7 cells. Unstained cells are considered 0%, and are subtracted from cells that are stained for specific integrins.

Alpha 2 integrin profile

Cell line	% cells stained positive
hFOB	20.00
BJ	27.78
MG63	77.77
SAOS-2	1.75
LM 7	0.58

Alpha 3 integrin profile

Cell line	% cells stained positive
hFOB	24.76
BJ	16.04
MG63	8.75
SAOS-2	23.66
LM 7	21.57

Alpha 5 integrin profile

Cell line	% cells stained positive
hFOB	4.38
BJ	26.2
MG63	7.03
SAOS-2	6.01
LM7 unstained	0

Alpha V integrin profile

Cell line	% cells stained positive
hFOB	3.52
BJ	16.48
MG63	4.26
SAOS-2 unstained	0
LM7 unstained	0