CSPG4 AS A POTENTIAL TARGET OF ANTIBODY-BASED IMMUNOTHERAPY FOR MALIGNANT MESOTHELIOMA

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ABSTRACT

Malignant Mesothelioma (MM) is an aggressive tumor associated with asbestos exposure [1]. Chemotherapy is the mainstay of MM treatment; however it only extends survival by 11 weeks. The high mortality rate and the distinctive chemo-resistance of MM underscore the need for novel targeted therapies for this deadly disease. Chondroitin Sulfate Proteoglycan 4 (CSPG4) is a cell surface proteoglycan that is highly expressed and involved in cell proliferation and migration of many tumor cell types [2]. Through binding this antigen with extracellular matrix components (ECM) components (such as Collagens and Fibronectin) and integrins, it modulates cellular activities such as polarization, adhesion and spreading via activation of signaling cascades leading to FAK, Src and ERK1/ERK2 activation [3]. Highly over-expressed in most melanoma cells, CSPG4 is associated with expression and activation of membrane type matrix metalloproteinases (MT-MMPs) at the site of contact between cells and the underlying ECM, indicating a role in cell invasion [4]. To date, no study has examined the role, if any, of CSPG4 in the progression of MM. The lack of effective therapies for MM prompts the search to identify targets to implement antibody-based immunotherapy. We report here the expression of the membrane bound Chondroitin Sulphate Proteoglycan 4 (CSPG4) in 6 out of 8 MM cell lines and in 24 out of 40 MM biopsies, with minimal expression in surrounding normal cells. CSPG4 is involved in the onset and progression of several tumors, as well as in angiogenesis. CSPG4 expression in MM cells was induced upon engagement of ECM, especially fibronectin, and increased percentage of viable
MM cells. CSPG4-specific monoclonal antibody (mAb) TP41.2 inhibited MM cell attachment to the ECM, the resulting intracellular signaling and induced apoptosis. Moreover, in vitro, CSPG4-specific mAb TP41.2 significantly reduced MM cell motility, invasiveness, wound healing and inhibited MM growth in soft agar. In vivo, mAb TP41.2 prevented human MM tumor establishment in SCID mice and significantly inhibited the growth of established MM xenografts. These results suggest that CSPG4 mAb-based immunotherapy may represent a possible novel valid approach for the treatment of MM.
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>MT-MMPs</td>
<td>Membrane-type matrix metalloproteinases</td>
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<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
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<td>Platelet-derived growth factor beta</td>
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<td>Basic fibroblast growth factor</td>
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CHAPTER 1

Malignant Mesothelioma

1.1 Introduction

Malignant mesotheliomas (MM) are highly aggressive tumors that arise from the serous membranes of the pleura, peritoneum and occasionally in the pericardial and tunica vaginalis cavities [5]. It originates from the naïve primary human mesothelial cells (HM) locally, but spreads rapidly to occupy most of the visceral and parietal surfaces. Epidemiological data and experimental studies have established the association of asbestos fibers and MM [6]. Recently, MM pathogenesis has also been linked to Simian Virus 40 (SV40) infection [7]; exposure to erionite [8]; genetic susceptibility [9]; and radiation exposure [10]. About 3,000 cases of mesothelioma are diagnosed each year in the US alone, with a median survival of 1 year from diagnosis [1].

1.2 Etiology and Pathogenesis of MM

1.2.1 Fibers: Asbestos and Erionite

Asbestos, primarily the amphiboles crocidolite and amosite, still remains to be the predominant cause of MM [10]. Generally, the latency period between the asbestos exposure and the onset of the disease in patients occurs between 30 to 40 years [11]. The mechanisms of asbestos carcinogenesis have been the topic of intensive investigation for decades and have been elucidated just recently [12]. The long latency period suggests that numerous mounting of events occur during the malignant transformation of mesothelial cells.
Recent studies have revealed that asbestos carcinogenesis is linked to the activation of the AP-1 pathway [13], which brings about cell division, and to the release of TNF-alpha (TNF-α). In addition, mutagenic oxygen radicals released mainly by lung macrophages may contribute to asbestos carcinogenesis [14; 15].

Erionite is another type of fiber, which has been demonstrated to cause MM in almost 100% of animals compared to 20% when injected with crocidolite or other types of asbestos [16]. Based on these findings, erionite is considered the most potent type of mineral fiber in inducing MM. Many regions of the world consist of erionite and recent analyses show the chemical composition of the Turkish erionite is no different to the erionite found in USA [17]. However, with the exception of Turkey, its contribution to the incidence of MM has not been evaluated.

The findings were further evaluated linking TNF-α and NF-kB to asbestos pathogenesis and MM [18]. These results concluded the critical role of TNF-α and NK-kB signaling in bringing about responses of HM cells and macrophages to asbestos exposure, and demonstrating that NF-kB activity protected HM cells from asbestos-induced cell death, allowing DNA-damaged HM cells to survive asbestos exposure and possibly grow into MM (Figure 1-1). Recently, the mechanistic model for asbestos carcinogenesis was expanded identifying the link was through the effects of the chronic inflammatory reaction by the HMGB1 release in MM [19].
1.2.2 Risk cofactors: SV40

SV40 infection has also been linked to MM pathogenesis. The relationship between this virus and mesothelioma is still not well understood as humans are not the natural hosts for SV40 [20; 21]. The unknown introduction of SV40 to the population recently has caused a huge curiosity of its pathologic effects in mesothelioma tumors. A causative association between SV40 and MM was proposed fairly recently and has been the subject of both profound consideration and some controversy [22; 23; 24]. Variations in SV40 detection contained in MM, through failed epidemiologic evidence linking polio-vaccinated cohorts with higher incidence of tumors and also the fact that SV40 was not found in MM in certain countries [10] may account to some of these controversies. However, to
address these debates, facts still remain that, millions of people worldwide were injected with the inactivated (Salk) and early live attenuated (Sabin) forms of polio vaccines that were contaminated with SV40 [25]. Inoculations of SV40 virus have produced mesotheliomas in 100% hamsters after intrapleural injection [26], demonstrating its oncogenicity [10].

Also, on the strength of current advances and emerging biological investigations and clinical observations, SV40 appears to be involved in the pathogenesis of at least a proportion of the patients with MM, where it is specifically found in the tumor cells and not in the surrounding tissues [27]. Within these cells, SV40 binds and inactivates essential tumor suppressor genes p53 [28] and pRb [29], stimulates met, Notch-1 and telomerase activity [30]. Moreover, the expression of Tag induces a high degree of aneuploidy that per se may be sufficient to promote malignant cell growth. Furthermore, SV40 causes promoter methylation, therefore, inactivating the tumor suppressor RASSF1A [31], as a result causing a more antagonistic tumor phenotype [32]. Finally, SV40-positive malignant mesotheliomas have higher AKT activity that promotes cell proliferation and cell survival [13]. The tag also plays a role by complementing the role of Tag in the cytoplasm. It functions by binding to and inhibiting the PP2A, which indirectly reinforces mitogenic extracellular stimuli [33; 34]. Together, the mechanisms of these two oncogenes promote mitogen-activated protein kinase (MAPK) and AP-1 activity, contributing to cell proliferation [35].
In addition, recent results also demonstrated the interaction between SV40 and crocidolite asbestos to be cocarcinogens and that, in the presence of SV40, significantly low amount of asbestos is adequate to induce MM [36]. These findings indicate that the risk of MM among exposed individuals because of their genetic background or because of exposure to other carcinogens. Altogether, considerable evidence supports a role of this oncogenic virus in mesothelioma pathogenesis.

1.2.3 Genetic Predisposition

Very recent results indicate that mineral fiber carcinogenesis can be influenced by genetic predisposition and to the development of MM. The latter phenomenon was demonstrated in some Turkish families causing an epidemic in Karain, Sarihidir and Tuzkoy villages of Cappadocia [17; 37; 38; 39]. About 50% or more deaths are caused by MM linking this epidemic to mineral fiber erionite, a fibrous zeolite commonly found in the stones of the built homes of Cappadocia [40]. In these families, it appears that exposure to minimal amounts of, erionite, is sufficient to cause MM [41; 42]. Moreover, MM was more frequent in certain families compared to others revealing the unusual susceptibility to erionite carcinogenesis in these families [40]. Furthermore, pedigree analysis of families who lived in homes where mesotheliomas occurred showed that these mesotheliomas seemed to be inherited in an autosomal dominant pattern. Approximately 50% of descendants of affected parents developed mesotheliomas. When members of unaffected families married into affected families, 50% of their descendants also developed mesotheliomas [17; 43]. It still
remains unknown whether genetics alone or in conjunction with erionite is responsible for these mesotheliomas, however genetics is still a key factor, because mesotheliomas do not develop in non-affected families, regardless of environmental exposure. Familial mesothelioma has also been identified in other parts of the world, specifically in the USA to asbestos susceptibility [44]. However, the size of the families is usually too small to prove genetic spread.

Also, the mesothelioma in these families might also have been linked to asbestos exposure and/or SV40 infection [12; 45]. These studies proposed that there is a putative MM susceptibility gene that is genetically altered in these families and could be the target of asbestos in sporadic MM in other parts of the world.

1.2.4 Radiation

Radiation exposure has also been linked to MM though these cases are rare [10]. Patients who received radiation treatments, specifically in the thorax or abdomen, have shown increased risks in developing MM [46]. The average time between radiation exposure and the development of MM is about 21 years [47; 48]. Moreover, studies in rats demonstrate radiation as a causative factor of MM.

The overall features shown by advanced mesotheliomas suggest complex and diverse patterns of changes in growth regulation during carcinogenesis, which involve both inactivation of tumor suppressor genes (TSGs) and activation of proto-oncogenes. This process may be facilitated by SV40 large T-antigen, which inhibits p53 leading to impaired DNA repair and apoptosis in erionite or asbestos-damaged cells (Figure 1-2).
1.3 Classification and Morphology of MM

Pleural mesothelioma is the most common type, that accounts for about 70% of all MM cases [49]. MM is subtyped into three forms according to the histological morphology: epithelial, sarcomatoid, and biphasic. The prognosis of MM is poor, and the median survival time for these three types is 18, 8, and 11 months, respectively [50; 51].
Figure 1-3: Histologic Subtypes of MM.
(A) Epithelioid, (B) Sarcomatoid, and (C) Biphasic phenotypes

Epithelioid MM is an epithelial cell type appearing relatively uniform, and is reported as having a tubular papillary structure. Each individual cell is cube-shaped having an easily identifiable nucleus. It is the most common form of the disease, occurring at about 50% to 70% of all MM patients diagnosed. However, it is considered the least aggressive and for the most part considered to respond the best to treatment, offering the best prognosis of the cell types.

Sarcomatoid MM is a spindle cell type with elongated nuclei that is not as obvious as in the epithelial type. This cell type represents approximately 7% to 20% of MM cases diagnosed. It is considered to be the most aggressive subtype and typically does not respond well to treatment, consequently, presenting poor prognosis. It is very challenging and can be very ambiguous to differentiate sarcomatoid MM from other types of sarcomas than in the epithelial form.

Biphasic MM is a combination of mixed cell types composed of both the epithelial and sarcomatoid subtypes in the same tumor, or found in specific clusters throughout the tumor. The biphasic MM makes up 20% to 35% of mesotheliomas, with prognosis being normally in-between the two cell types.
1.4 Epidemiology of MM

About 3000 cases are diagnosed annually in the United States. MM remains to be a serious problem worldwide as the incidence continues to increase worldwide (Figure 1-4) [52].

![Figure 1-4: Projected number of MM cases](image)

Surveillance, Epidemiology, and End Results Program data (SEER), 1973–2005

Risk is highest at about age 80 to 84 (Figure 1-5). The median age in reported clinical trials is frequently a decade or more younger than the median age of patients in the SEER database.

![Figure 1-5: Incidence of MM by age and year of diagnosis in men.](image)

Surveillance, Epidemiology, and End Results (SEER), 9 areas.
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<td>8</td>
<td>200,000 deaths in 10yrs Death toll to peak in 2015.</td>
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<td>340</td>
<td>10</td>
<td>Expected death toll to rise: Due to asbestos mine continuation.</td>
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<td>Sweden</td>
<td>149</td>
<td>20</td>
<td>World’s 1st country to show declining incidence due to asbestos ban.</td>
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<td>United Kingdom</td>
<td>1,862</td>
<td>39</td>
<td>To peak 2,500 deaths annually from 2011</td>
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<td>Australia</td>
<td>600</td>
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<td>13,000 deaths by 2020 World’s most threatened due to age group starting 20 and above.</td>
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<td>Japan</td>
<td>900</td>
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<td>100,000 deaths by 2040 Smoking is prevalent</td>
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<td>India</td>
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1.5 **Diagnosis of MM**

The incidence of MM will continue to rise to daunting proportions worldwide (Table 1). Despite the efforts that have progressed towards prognosis, for various reasons MM still remains to be difficult to diagnose.

1.6 **Treatment of MM**

There are no standards of care for the management of MM due to the aggressiveness of the tumor resisting conventional therapy. The treatment options for this deadly disease depend on several criteria, including performance status, pulmonary function, stage, and age of the patient [53]. Various options are included in the maintenance of the disease (see Chapter 2 section 2.3).

Due to the lack of effective conventional therapies for MM caused by the late diagnosis and the aggressiveness of this deadly disease, it is important to develop new therapeutic targets to decrease its burden that can increase the survival rate of the patients. In this research we intend to use CSPG4 as a possible novel target for a more definitive diagnosis, as well as candidate for antibody-based immunotherapy for MM. Since CSPG4 is a cell surface proteoglycan, we will explore the interaction of CSPG4 on MM cells with components of the ECM, that possibly potentiate cell survival, proliferation and migration in MM tumor progression. Inhibiting these interactions with an antibody specific for CSPG4 may lead to tumor regression. We propose to elucidate the role of CSPG4 in MM onset and progression and validate this proteoglycan as a possible novel therapeutic target of MM.
1.7 Hypothesis & Objectives

We hypothesize that the interaction of CSPG4 on MM cells with components of the ECM potentiates cell survival, growth and migration. Inhibiting these interaction with an antibody specific for CSPG4 may lead to tumor regression. To elucidate the role of CSPG4 in MM onset and progression and to validate this proteoglycan as a possible novel therapeutic target of MM, we propose to explore the following aims:

**Aim 1.** To determine the expression and diagnostic value of CSPG4 in MM.

**Aim 2.** To elucidate the mechanism of CSPG4-induced cell survival, growth and motility of MM cells. (1A) Investigate the interaction of CSPG4 with components of the ECM. (1B) Investigate the signaling cascades activated by the interaction of the components of the ECM with CSPG4 that lead to tumor cell adhesion.

To determine whether inhibiting the interaction of CSPG4 with components of the ECM prevents the establishment of MM and induces regression of established MM.

**Aim 3.** Functional analysis of CSPG4 role in MM xenografts formation/maintenance *in vivo*:

a. Preventive study – administration of MM tumor cells together with CSPG4-specific mAb on day 0.

b. Therapeutic study - administration of CSPG4-specific mAb upon established tumor growth.

The overall goal of this research is to find an effective target that can be used for antibody-based immunotherapy for MM.
We chose CSPG4 as a possible target because it has been shown to be an effective therapeutic target for melanoma.

In this research, we clarify the role of the cell surface CSPG4 in the biology of MM cells *in vitro*, to provide an increased understanding that may eventually lead to the therapeutic use of monoclonal antibodies against CSPG4 for MM.

1.8 Justification and Significance

MM is a tumor that arises from the mesothelial cells that line the pleural, pericardial and peritoneal cavities. It has been associated with exposure to asbestos and radiation and with human genetics. MM is an extremely aggressive and fatal malignancy that is all too often diagnosed too late for a cure [1]. In most patients, the tumor is unresectable and treatment is strictly palliative through chemotherapy. Several combination chemotherapy regimens have shown improved survival when compared with single agent treatment and have become the standard of care [54]. However, none result in a cure. The gold standard for therapy, which is pemetrexed/cisplatin, only increases average life expectancy by 11 weeks. Thus, there is a significant need to better understand the underlying biology of MM that can be combined with advances in drug design, immune-based to specifically target tumor-associated antigens and combine these targeted regimens with standard chemotherapeutic regimens. To be effective, in addition to specifically targeting tumor cells therapeutic interventions must simultaneously target cells of the tumor microenvironment that are essential for
neo-angiogenesis and therefore required for tumor growth [55]. Achieving this may be possible with antibody-based immunotherapy if target antigen is expressed in both the tumor cells and the neo-vasculature. While such antigens have not been identified for MM, a cell surface proteoglycan called Chondroitin Sulfate Proteoglycan 4 (CSPG4) is expressed by tumors such as melanomas, gliomas and breast cancers, and by connective tissue cells of capillaries and small blood vessels called pericytes. CSPG4 has been extensively investigated as a target for monoclonal antibody based immunotherapy in melanoma, but whether CSPG4 is expressed in MM has not been previously determined.

1.9 Innovation and Impact

Thus far, no study has evaluated CSPG4 expression or function in MM disease progression. Therefore, all the data obtained through this proposal will be novel. Through the elucidation of the mechanism by which CSPG4 induces cell survival, proliferation and migration, novel molecular targets for therapeutic intervention may be revealed. Most importantly, the results obtained through the proposed studies will provide the pre-clinical data necessary to translate an antibody-based therapeutic regimen to clinical trials for MM. With the lack of efficacious therapies available to treat patients with MM, the development of such regimens would have the potential to bring hope to patients who are diagnosed at the later stages of disease and currently have no recourse for treatment.
2.1 Introduction

Malignant mesotheliomas are deadly cancers of the serous membranes (pleura, peritoneum, pericardium, vagina and ovary) that arise from the mesothelial cells [1]. This dreadful cancer is usually caused by asbestos exposure [15], considered to be the single-biggest occupational disaster of modern times. However, more recently, MM has been linked to erionite, SV40, familial susceptibility and radiation [56]. To date the molecular mechanisms that bring about the carcinogenesis of this fatal cancer are still puzzling, which constitute major challenges in the diagnosis and treatment of the disease.

2.2 Diagnosis of MM

Currently, there pose to be differential diagnostic challenges in MM, especially with other metastatic tumors. Accurate diagnosis of MM is very important for diagnosis and for the treatment of the disease. A large number of patients present with symptoms that are associated with pleural effusion. These include, coughing, chest pains, difficulties in breathing, fatigue, weight loss and fever.

Therefore, there are no approved early detection procedures or definitive diagnostic markers for MM. Each subtype poses a difficulty for interpretation due to the similarities of morphology with other adenocarcinomas or connective tissue type tumors.
2.2.1 Imaging

This is the primary standard procedure carried out to initiate the diagnosis, when a mesothelioma patient presents with signs of pleural effusion, palpable chest or abdominal masses. Conventional evaluation tests include:

2.2.1.1 Chest X-ray

A chest x-ray usually reveals a fluid build-up between the lung and chest wall (pleural effusion), pleural plaques and thickening, however, the extent of the shape and tumor burden is then followed by using a more advanced imaging test [57].

2.3.8 Computed Tomography (CT)

Typically MM tumor masses cannot be detected using the chest x-ray alone, however, a CT scan uses a combination of x-ray and interpreted in the computer to determine the extent of the tumor [58]. Though CT scans can be used as a guide for fine needle biopsy, this technique cannot differentiate between an adenocarcinoma versus mesothelioma.

2.2.1.3 Magnetic Resonance Imaging (MRI)

The extent of the tumor and chest wall invasion is often assessed by MRI scans. This technique takes images in multiple planes using a combination of radio waves and a strong magnetic field. It is accurate and informative to assess the extent and invasion of the tumor to other local organs that is useful in the determination of the surgery and future therapy [59; 60].
2.2.1.4  **Positron Emission Tomography (PET)**

PET is widely used now as an adjunct to diagnosis despite its expensive costs and lack of coverage in most health insurances. This technique involves the administration of glucose solution by intravenous injection. The cancer cells metabolize sugars faster than normal cells thereby images show cancer cell deposits. It is useful in determining the staging of the disease especially with lymph node involvement [61].

2.2.2  **Cytopathology**

Pleural or ascitic fluid is normally drained and further assessed cytologically for its cause [62]. However, only about 35% of cases can be accurately diagnosed with this technique due to the inability to distinguish between mesothelioma cancer cells and reactive mesothelial cells. A combination of cytology and histopathology is normally advocated in order to increase the accuracy in the diagnosis of MM [63].

2.2.3  **Histopathology**

Researchers have actively sought MM biomarkers for more than 20 years. Biomarkers would be helpful in managing three clinical aspects of MM: early diagnosis, prognosis, and treatment outcome prediction. The most predominantly used technique for distinguishing MM from adenocarcinomas or other types of cancers is immunohistochemistry, “a histopathological method of analyzing and identifying cell types based on the binding of antibodies to specific components of the cell from a biopsy sample of tumor tissue” [64]. However, microscopically, it is a challenge to differentiate the MM from adenocarcinomas when stained with
haematoxylin and eosin (H&E). The development of a panel of immunohistochemical markers to facilitate the differential diagnosis of MM is very much needed.

Previously, the “markers” that were used differentiate MM from adenocarcinoma were “negative markers”, those that are expressed in adenocarcinomas, but not in MM. Among them are carcinoembryonic antigen (CEA), CD 15 (LeuM1), epithelial glycoprotein (Bg8), tumor glycoprotein (BerEp4) and tumor glycoprotein (MOC-31)[65]. This made the diagnosis confirmation more complex, because pathologists were dealing with the absence of, rather than the presence of certain markers.

However, more recently, “positive markers” for MM that are negative for adenocarcinomas have emerged. Including these markers are calretinin, cytokeratin 5, epithelial membrane antigen (EMA), HBME-1, mesothelin, N-cadherin, thrombomodulin, Wilm’s tumor gene product (WT-1), thyroid transcription factor-1 (TTF-1) and vimentin [53; 66]. Osteopontin, soluble mesothelin, and megakaryocyte potentiating factor (MPF) appear to be the most promising of the recent biomarkers, but are still subject to some limitations. Osteopontin lacks specificity for mesothelioma, while both soluble mesothelin and MPF lack sensitivity for detecting non-epithelial subtypes. It is imperative to acknowledge that while the above markers are routinely used to help diagnose the epithelial sub-type of MM, that they may also be expressed in other types of cancer, and may not necessarily apply to the biphasic or sarcomatoid sub-types of MM, which continue to present diagnostic challenges.
The distinction between MM, metastatic tumors and benign proliferative of fibrotic conditions of the serous membranes is essential in diagnostic histopathology and for later therapy. Currently, panels consisting of a small set of biomarkers do not improve the diagnostic yield and results from molecular profiling are too preliminary to be brought into daily clinical practice. While a large number of biomarkers have been assessed in biological fluids and tumor tissue for their prognostic value, none have had a widespread impact on clinical practice. In contrast, data concerning predictive biomarkers are very limited, even though they are most interesting from the perspective of clinicians. Additional prospective studies, in large and independent samples of patients, with rigorous statistical methodology and standardized laboratory techniques are now warranted to validate and define the precise value of diagnostic and prognostic MM biomarkers.

2.3 Treatment of MM

MM is a very difficult cancer to treat, with a median survival of 12 months from diagnosis regardless of the disease stage. Treatment has mostly been based upon individual status of the disease. The result is predominantly due to MM being a heterogeneous cancer with a variable clinical course and partly a matter of most patients being diagnosed at a later stage of the disease causing serious impediment to the development of a standard, stage-related, and overall therapeutic approaches. To a certain extent, the stumbling block in making progress in this disease has been that only a few randomized controlled studies
have been conducted because of the small number of patients, dubious response measurements, variable staging of the disease between the trials (surgical staging versus radiographic staging), and trial enrolled patients diverse histology.

The rationale of the management depends on several criteria such as the location of the tumor, performance status of the patient, stage of the disease, and the patient’s age.

The accepted treatment options encompass surgery, chemotherapy and radiation therapy. However, not one of the aforementioned single treatments has shown significant impact on survival. In spite of that, there is a growing number of advances in the understanding molecular dynamics of this fatal disease that have developed new potential therapeutic targets that could potentially change the clinical course of the disease [67].

2.3.1 Surgery

In most cases (80%), patients are diagnosed in stages III or IV of MM making them not suitable candidates for surgical removal of the. However, there are three types of surgical techniques used for the treatment of MM. These include video thorascopy, palliative pleurectomy/decortication (P/D) and extrapleural pneumonectomy (EPP).

Video thorascopy is a minimally invasive procedure for the treatment of MM with fewer recurrences [68].

Surgical removal of the lung (extrapleural pneumonectomy), has led to median survival times of more than 2 years, however, requiring very invasive
removal of the gross tumor. Some form of adjuvant therapy aiming at eliminating residual microscopic disease typically follows this procedure. However, only few patients are suitable for this line of surgery [69].

2.3.2 Chemotherapy

Despite the efforts on chemotherapy, MM remains a fatal disease of increasing incidence worldwide. There has been no curative chemotherapy in MM so far. However two regimens have been shown to be valuable. Pemetrexed plus cisplatin is a combination of multitargeted antifolate and platinum compound. A phase III study consisting of 448 patients showed improvement in overall survival of about 3 months and 41% response rate, with the combination compared with cisplatin alone [70].

Other combinations have included, Gemcitabine plus cisplatin has also been shown to have similar responses, offering symptomatic improvement, quality of life and a response rate of about 48% in a total of 74 patients [71]. Also, MM trials on imatinib mesylate and gefitinib to block the platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) pathways have been tested, however, no palliative response has been seen [72].

There are more combinations being tested, however, no signs of achieved response rates have been demonstrated compared to the ones mentioned above [73]. The current studies focus on the use of chemotherapy and biological agents as part of a more complex treatment schedule to have multiple therapeutic effects. As of current, the contemplated standard of care for first line palliative chemotherapy is pemetrexed and cisplatin combination, in patients with good
performance status [70]. Prophylactic dexamethasone plus supplemental folic acid and vitamin B12 are also prescribed. The most routinely used second line chemotherapy drugs are gemcitabine plus cisplatin.

2.3.3 **Radiotherapy**

Besides local postsurgical radiotherapy, the use of radiation, in MM have been very disappointing over the last 3 decades [74]. The aggressiveness and diffuse nature of the tumor on pleural surfaces makes it difficult to treat by radical radiotherapy without causing pneumonitis. Most recently and has been successful to control disease locally, is the use of intensity modulated radiotherapy after extrapleural pneumonectomy, where accurate placement of markers have been used. However, most patients die because of metastatic disease.

2.3.4 **Gene Therapy**

Gene therapy approaches have been attempted for the treatment of MM, though are still far from development [75]. Suicide gene therapy involves the transfer of a DNA molecule encoding an enzyme into tumor cells, which has no effect on its own but after administering ganciclovir, it is capable of converting that drug into toxic metabolites that destroy the tumor cells. Another application is the immunomodulatory gene therapy which involves the local delivery of a vector (i.e. vaccinia virus) producing a cytokine (i.e. IL-2 within the tumor over a prolonged periods of time mimicking immune responses that occur in organs undergoing autoimmune destruction [76]. Gene therapy continues to offer promise as a form of cancer therapy but so far, it has no recognized position in
standard MM therapy.

2.3.5 Immunotherapy

Substantial tumor regression has been supported by animal studies in tumor immunotherapies [77] yet, the results are not predictably enough to authorize standard application in clinical setting. Patients with MM usually mount an anti-MM immune response, however, one that is weak and not able to destroy the tumor [78]. Therefore, the goal of immunotherapy is to boost this weak response and induce tumor regression.

Previous studies with BCG vaccine have demonstrated a little boost with improved survival rate in 30 MM patients [79]. Moreover, cytokine therapy with interleukin-2 (IL-2) or interferon gamma (IL-γ) into the pleural cavity, promoted responses in patients with early disease but were ineffective with advanced disease [80; 81]. Furthermore, patients given recombinant interferon alpha (IL-α) or recombinant cytokine granulocyte-macrophage colony stimulating factor (GM-CSF) as a single drug, induced partial response rates of about 10-15% [82; 83]. Also, based on the preclinical models of tumor antigen mesothelin coupled to Pseudomonas exotoxin Phase I trials [84] dendritic cell vaccine immunotherapy were also initiated [85]. Although substantial tumor regression can be induced, immunotherapy is still not predictably enough to warrant standard use in clinical practice.

2.3.6 Multimodality therapy

Almost certainly, promotion of acquired immune protective response can be achieved in combination with other therapeutic forms such as chemotherapy
Therefore only recently, it was reported that a prescribed course of treatment consisting of a complicated combination including intrapleural preoperative IL-2, pleurectomy decortication, intrapleural postoperative epidoxorubicin, IL-2, adjuvant radiotherapy (30 Gy), systemic chemotherapy (cisplatin and gemcitabine), and long-term subcutaneous IL-2 provided a median survival of 26 months [87; 88].

The success of targeted therapies is still to be achieved for MM, which remains a difficult and growing problem. However, emerging evidence suggests promising data and novel targeted agents may offer disease control in the future.

2.3.7 **Antiangiogenic agents**

Agents that inhibit angiogenesis pathways are currently under investigation, considering the fact that MM tumors often tend to have high microvessel density, which has been linked with poor outcome [89; 90], however they have shown only limited activity until now. Phase II studies were conducted using monoclonal antibody against VEGF, Bevacizumab [91], or small molecule inhibitors of the VEGF receptor kinases, Sorafenib and Vatalanib [92]. Less than 5% effect has been seen, which did not meet the criteria for further investigation.

2.3.8 **Other agents**

Novel anti-cancer agents such as histone deacetylase inhibitors (HDAC) that are said to function as an inducer of apoptosis and blocking angiogenic VEGF signaling are being utilized for MM phase I trials [73].
2.4 Limitations of MM Treatments

Currently, MM is notoriously refractory to most treatments, highly limiting the management of this deadly disease. Sadly, the overall survival rate is approximately less than 2 years. Surgery is of limited applicability and is reserved for special cases, however, for curative surgery to be effective, it is particularly important that MM be diagnosed early. Unfortunately, MM is not usually diagnosed until it reaches Stage III or IV, when surgery is not an option.

Radiation therapy is often used alone or in combination with surgery. Regardless of how, it is rare for radiation therapy to provide more than short-term symptomatic relief. Chemotherapy may be used to achieve different goals, depending on the stage of the cancer at the time of diagnosis, the age and health of the patient, however it is not considered "curative". Gene therapy has produced some promising results for MM patients. It has particularly been useful in combination with chemotherapy, but it is still only available through clinical trials.

Furthermore, assessment of the efficacy and side effects of combined treatments for advanced stages is very complex, due to the lack of controls, and the variety of dosages and schemes. To be able to target these limitations, all patients must be enrolled in protocols and administer less toxic adjuvants.

In view of the apparent lack of sensitivity of MM to current immunotherapy, further approaches are still needed for it to enter standard practice for the treatment of MM. CSPG4 has been extensively investigated as a target for monoclonal antibody based immunotherapy in melanoma, but whether CSPG4 is
expressed in MM has not been previously determined.

Since no study has examined the expression of CSPG4 in MM, our preliminary studies are the first to show that several MM cell lines and biopsies from primary human MM tissue express higher levels of CSPG4 as compared to HM. Moreover, treating MM cells with an antibody specific to CSPG4 significantly reduces their viability and induces apoptosis. These data support the theory that CSPG4 proteoglycan may represent a useful target for antibody-based immunotherapy of MM.

2.5 Proteoglycans

Proteoglycans (PGs) are a heterogeneous set of macromolecules which consist of a core protein covalently attached to one or more glycosaminoglycan chains (GAGs) of either the chondroitin sulfate (CS), heparan sulfate (HS), dermatan sulfate (DS), hyaluronan (HA) or keratan sulfate (KS). GAGs are long unbranched polysaccharides consisting of disaccharide repeats. The two sugars in the disaccharide repeats consist of either a hexosamine, N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GalNAc) and the other sugar is a hexuronic acid (HexUA), either G-glucuronic acid or L-iduronic acid. Next to collagens, PGs are ubiquitously present in the body and form important components of the ECM. Based on its direct involvement with ECM, it also binds and interacts with a number of different ligands including adhesion molecules, cytokines, chemokines, and growth factors [93]. GAGs perform a variety of functions on the cell surface and in the cytoplasm, such as, cell adhesion, motility and invasion.
(Burg, 1998 #97; Fang, 1999 #98). One such proteoglycan is the CSPG4 that is also known as the High Molecular Weight-Melanoma Associated Antigen (HMW-MAA) or Melanoma Chondroitin Sulfate Proteoglycan (MCSP).

2.6 Chondroitin Sulfate Proteoglycan 4

CSPG4 is the human counterpart of the rat NG2 and mouse AN2 that was first identified in the 1970s as an integral membrane proteoglycan [94]. The genes for NG2 and AN2 share over 80% amino acid sequence identity with CSPG4 and over 90% amino acid sequence with each other (Figure 2-1).

![Figure 2-1: Comparison of CSPG4, NG2 and AN2 core protein structures. Campoli MR, et al. Critical Reviews in Immunology, 24 (4): 267-296 (2004).](image-url)
CSPG4 is an N-linked glycoprotein of 250 kDa and a proteoglycan component >450 kDa (Figure 2-2). The 250-kDa molecule is the core glycoprotein of the proteoglycan, that is composed of a chondroitin sulfate proteoglycan by its content of both 2-acetamido-2-deoxy-3-O-(β-D-gluco-4- enepyranosyluronic acid)-β-4-O-sulfo-D-galactose (ΔDi-4S) and 2-acetamido-2-deoxy-3-O-(β-D-gluco-4- enepyranosyluronic acid)- β- 6-O-sulfo-D-galactose (ΔDi-6S) chondroitin sulfate disaccharides [94; 95; 96; 97].

![Figure 2-1: Schematic representation of CSPG4 structure.](image)


CSPG4 is widely dispersed in a number of normal and transformed cells. The expression has been found in basal cells of the epidermis, chondrocytes, smooth muscle cells, melanocytes, placental villi and pericytes [98; 99]. Pericytes are connective tissue cells that are associated with vascular capillaries that make up small blood vessels. A number of clinical and experimental observations suggest that pericytes contribute to the regulation of microvascular growth and
function [100]. The expression of CSPG4 in pericytes suggests that it may be involved in neo-angiogenesis [101].

Studies have also documented the high expression of CSPG4 in pathologic conditions such as genetic and inflammatory disorders [102], tumors of the melanocytic origin [2]; and tumors of the non-melanocytic origin such as In addition to its wide expression on melanomas [103; 104]. CSPG4 is also found on glioblastomas [105; 106]; chondrosarcomas [107] and lymphoid leukemias [108].

The existence of CSPG4 on the cell surface binds ECM, where it potentiates interactions that are critical for cell function.

2.6.1 CSPG4 interactions with ECM

Predominantly, CSPG4 is found in the extracellular matrix (ECM) or associated with the cell surface of most eucaryotic cells where they bind to other matrix- and cell-associated components. Therefore, CSPG4 serve as a diverse set of adhesion receptors that mediate cell-cell and cell-ECM interactions fulfilling a variety of functions on the cell surface and in the cytoplasm.

Previous studies have shown that proteoglycans acted as co-receptors of tissue-cultured Balb/c 3T3 cells to plasma fibronectin [109]. However, a direct role of CSPG4 in liaising melanoma cell adhesion to fibronectin was first demonstrated by the identification of a cationic synthetic peptide, that was named FN-C/H-III [110]. The peptide is found to be within an A-chain derived, 33-kD carboxyl-terminal heparin binding fragment of human plasma fibronectin, and demonstrates direct binding to CSPG4. The peptide binding was also shown to
inhibit CSPG4 from binding to the FN fragment and also demonstrated to promote melanoma cell adhesion by a CSPG4-dependent $\alpha_4\beta_1$ integrin independent mechanism. These results suggested that CSPG4 interacted with the components of the ECM. This possibility was also supported by three lines of evidence obtained by the rat homologue NG2, where interaction was seen with laminin, tenasin, collagen types II, V, and VI [111]; secondly it engages with the substrata triggering cytoskeletal rearrangement and lastly the formation of lung metastases by NG2-transfected mouse cells B16F1 and B16F10 in vivo [112].

2.6.2 CSPG4 functions

The expression pattern of CSPG4 on immature progenitor cells suggested that the CSPG4 might contribute to biological processes such as cell proliferation and motility. In fact CSPG4 is often re-expressed by tumour cells, which are usually characterized by increased proliferation and migration. Moreover, the specific interactions with many biologically active proteins such as collagen I (CI), collagen IV (CIV), fibronectin (FN) and various other components of the ECM, it contributes to critical processes such as cell adhesion, proliferation, motility and survival [113].

2.6.2.1 Role of CSPG4 in Cell Migration

Recently, studies have shown that the interactions of rat homologue NG2 with ECM enhance migratory potential of cells [114; 115; 116] that could lead to activation of associated intracellular signaling cascades involved in cell attachment by the activation of Rho-Family GTPases, FAK and ERK [116]. FAK activation triggers its downstream proteins, which are all involved in mediating
cytoskeletal reorganization and ultimately migration that could potentially be inhibited by monoclonal antibodies [114; 117; 118]. These findings suggest a rationale that CSPG4 could potentially interact with the cytoskeleton directly and/or with components of the cytoskeletal-signaling complex.

Subsequently, it was shown that human MV3 melanoma cells expressing endogenous CSPG4 expression had 3-fold higher migratory ability in matrigel than human M14 melanoma cells, which do not express CSPG4 [119]. Moreover, by downregulating the expression of CSPG4 in MV3 cells, reduced cell mobility was observed. Recently, Iida’s group reported that CSPG4 triggers the activation of focal adhesion kinase (FAK), a protein tyrosine kinase downstream of p130cas, which activates various cytoskeletal components including paxillin, talin and vinculin, leading to cell migration [120]. Also, endogenous expression of CSPG4 protein in melanoma cells, verified its engagement with various ECM components including CI, CIV, vitronectin, and Matrigel, resulting in FAK phosphorylation and activation of its downstream target Src [2].

The mechanisms underlying the function of CSPG4 are still not fully evaluated. However, the available information about NG2-mediated signaling in rat cells has provided a useful background to define the series of events triggered by CSPG4 interactions with ECM components to initiate cell migration. In rat cells, similar activation lead to the extension of filopodia and lamellipodia at the cell front and an extension of retraction fibers at the cell rear end [117]. Whether these changes also occur in CSPG4 positive human tumor cells remains to be studied.
FAK activation often results from integrin-triggered signaling [121]. Interestingly, CSPG4 has been demonstrated to associate with α4β1 integrin and has been suggested to amplify integrin-mediated signal transduction in melanoma cells [117]. Altogether these findings suggest that CSPG4 may work together with integrins in the modulation of cell motility and/or migration. It still remains to be determined whether (1) CSPG4 can independently trigger Rho family GTPases-p130cas-FAK signaling events or (2) if FAK activation by CSPG4 is dependent on its interactions with integrin. On the other hand, it is also unclear whether integrin-triggered signaling events are dependent on interactions with CSPG4.

2.6.2.2 Role of CSPG4 in Survival

In addition to its effects on cell migration, NG2, has previously been linked to β1 integrin ligands for the reason that several tumor cell types demonstrate the co-expression of NG2 and α3β1 forming a complex on the cell surface [122]. For this reason, NG2-dependent activation of α3β1 integrin also has effects on cell survival due to increased signaling through the PI3K/AKT pathway [123]. Studies have shown that U251 glioma cells that are transfected with NG2 are resistant to TNFα treatment and various other chemotherapeutic drugs thought to elicit apoptosis in parental U251 cells. Moreover, silencing NG2 expression effectively restores apoptosis sensitivity in these NG2-expressing U251 cells, further confirming the association between NG2 expression and apoptosis resistance. Furthermore, this phenomenon has also been confirmed in glioma lines expressing NG2 endogenously, such as, U87 and A172, as well as in the A375
melanoma line. These results suggested that NG2-dependent apoptosis resistance is a widespread phenomenon in several tumor types with a direct correlation between NG2 expression level and both $\beta 1$ integrin activation and the level of AKT phosphorylation. Additionally, NG2-silenced U87 cells have also produced significantly smaller, slower growing tumors in NOD-SCID mice compared to NG2-expressing U87 cells and these were further inhibited by administration of TNF$\alpha$ [124].

The exact relationship between signaling networks triggered by CSPG4 and integrin await further investigations. Therefore, understanding the molecular mechanisms of CSPG4 signaling in tumor cell biology may have significant impact on the ability to regulate the means of CSPG4-targetted therapies on tumor cell growth and metastases.

2.6.2.3 Role of CSPG4 in Cell Proliferation

Previous studies have shown that NG2, the rat homologue of CSPG4, which often is re-expressed by tumor cells, contributes to increased proliferation in vitro [111]. Moreover, it has been shown that an increase in NG2 expression enhances tumorigenic and metastatic properties of mouse melanoma cells [112]. NG2 has also been reported to bind with high affinity to the human basic fibroblast growth factor (bFGF) and platelet-derived growth factor-AA (PDGFAA) [125]. Both growth factors are involved in mitogenic and survival signaling pathways in cells [126; 127]. Whether CSPG4 also binds these two growth factors remains to be investigated.
However, more recently, a study showed increased tumor cell growth rates *in vivo* when melanoma CSPG4 positive cells were inoculated into SCID mice [128]. These data were corroborated by CSPG4 transfection of melanoma cells M14 that do not express endogenous CSPG4 significantly increased their growth rates both *in vitro* and *in vivo* [129]. These indicate that functional properties of CSPG4 are similar to those displayed by NG2.

### 2.6.2.4 Role of CSPG4 in Angiogenesis

CSPG4 is highly expressed in pericytes and neovasculature both *in vitro* and *in vivo* suggesting its involvement in regulating and promoting angiogenesis, which is crucial for tumor cell survival and proliferation [98]. Increased rates of neovascularization and vasculature in tumors have been associated with increased CSPG4 expression [130]. CSPG4 expression on pericytes has only recently been appreciated. Pericytes are fibroblastic/smooth muscle-like mesenchymal derived cells that wrap around small blood vessels and capillaries [131]. They stabilize tumor blood vessels by cell-to-cell contact, as well as by providing vascular endothelial cells with survival signals. Supporting evidence demonstrates that CSPG4 expression is higher on activated pericytes than on resting pericytes that may represent a useful target for anti-angiogenic-based therapies.

### 2.6.2.5 Role of CSPG4 in Cell Invasion

CSPG4 has been reported to be important in regulating membrane-type 3-matrix metalloproteinase (MT3-MMP) and its role in the activation of pro-MMP-2 in melanoma cells [4; 132]. Other members of the MMP protease family have
been documented to bind CSPG4 as well [133]. These data suggest the facilitation of invasion and metastases through the recruitment of MMPs to the cell membrane by CSPG4.

2.6.3 **CSPG4 as a target for therapeutic intervention**

CSPG4 exhibits several features to make it an attractive target for the application of immunotherapy. Immunotherapy involves the use and manipulation of a patient's own immune system to help them fight diseases such as cancer to which the immune system would not normally respond. There are two types: (1) Active immunotherapy is cell mediated and involves highly specific treatments made with cells from the patient's own body. (2) Passive immunotherapy involves the use of components that are created outside the body. One example is antibody monoclonal therapy. These types of treatments differ from active immunotherapy in that passive treatments do not attempt to force the immune system to actively destroy cancer cells.

2.6.3.1 **Cell mediated**

The usage of CSPG4 as a target for immunotherapy has prompted researchers to question whether it is a tumor rejection antigen, since this class of tumor antigens presents as the preferred targets for immunotherapy. This possibility was examined in AN2, the mouse homologue of CSPG4 because: (1) the unavailability of CSPG4-transgenic mice to test this, and (2) due to very limited information about the generation of T-cell immunity against CSPG4. Maciag et al, established a CSPG4-transfected B16F10 melanoma tumor model to investigate the ability of this antigen to act as a tumor rejection antigen [134].
B16F10 melanoma cells were transfected with CSPG4 cDNA to induce the expression of this antigen. Transfected cells were grafted into immunocompetent syngeneic mice, which three days later were immunized with a fragment of CSPG4 expressed in *Listeria monocytogenes*. Remarkably, over 50% of the treated mice were tumor free 8 weeks post tumor challenge. Both CD4+ and CD8+ T cells played a role in the anti-tumor effect of the immunity elicited by the CSPG4 fragment, since immuno-depletion of CD4+ or CD8+ T cells abrogated the ability of the immunized mice to control tumor growth. Furthermore, adoptive transfer of CD8+ T cells isolated from mice that were immunized with the CSPG4 fragment inhibited the growth of tumors in at least 50% of the mice inoculated with B16F10 cells expressing CSPG4. The anti-tumor effect of the CSPG4-specific cellular immunity was mediated by CSPG4, because parental B16F10 cells were able to grow in the immunized mice, although, at a slow rate. The reduced tumor growth rate of the parental cells is likely due to the antiangiogenic effects of the targeting the cross-reacting mouse AN2 antigen expressed on the activated mouse pericytes. Speculation for the possibility of the infiltration of CD8+ T cells into the CSPG4 neg tumors and around blood vessels, as well as the decreased in the number of pericytes in tumors from mice immunized with the CSPG4 fragment. It is widely know that pericytes are activated in tumor stroma [135], having functional roles in vascular development, stabilization, maturation and remodeling. Activated pericytes express higher levels of CSPG4 compared to quiescent pericytes.
Previously, evaluating the potential of CSPG4 as a target of antibody-based immunotherapy, studies found that approximately 60% of the patients with advanced melanoma immunized with CSPG4 mimics developed CSPG4-specific antibodies [136;137]. The mimic used was a characterized mouse anti-idiotypic mAb [138]. The development of the CSPG4-specific antibodies in these patients correlated with significant enhanced survival rates. Moreover, some of the patients who had developed CSPG4-specific antibodies experienced regression of their metastases [139].

Early studies also showed that CSPG4-specific monoclonal antibodies could inhibit melanoma cell attachment and spreading on various ECM components, such as collagen, or collagen-fibronectin complexes [95; 140]. Moreover, migration of MV3 cells in Matrigel was inhibited by 50% when the cells were treated with CSPG4 antibody, mAb 763.74 determinant. Also, administration of CSPG4 antibody into melanoma treated SCID xenografts, markedly reduced the clonal expansion in vivo [128].

As a follow up to these findings, data from our co-investigator, Dr. S. Ferrone, show that targeting CSPG4 with a monoclonal antibody increases survival of melanoma xenografts by inhibiting CSPG4-induced signaling. Systemic administration of CSPG4-specific mAb to SCID mice implanted with pieces of xenografts derived from a human melanoma cell line (Figure 2-3). The therapy caused a significant reduction of tumor size and a significant increase in survival.
Figure 2-3: Inhibition by CSPG4-specific mAb 763.74 of human melanoma cell growth in immunodeficient mice.

MV3 cells ($1.5 \times 10^6$/mouse) were injected subcutaneously into 10 SCID mice. Tumor-bearing mice (5/group) were divided into two groups. One group was injected intravenously with CSPG4-specific mAb 763.74 (100 µg/injection) on days 14, 16, 18 and 20. The other group was injected with an isotype matched control mAb. Tumor volumes were estimated by determining the maximum length (L) and perpendicular width (W) of each tumor and applying the formula volume $=\pi/6 \times L \times W^2$. Wang X, et al. Current Molecular Medicine, 10 (4) (2010).

Consequently, systemic administration of CSPG4-specific mAb inhibited experimental and spontaneous metastases in SCID mice indicating that CSPG4-specific mAb have anti-tumor activity *in vivo*. The activity elicited by CSPG4-specific mAb, does not seem to be mediated by a complement-dependent or a cell-dependent cytotoxicity mechanism, since there was no induction of cytotoxic activity *in vitro*. Additionally, the anti-tumor activity of the CSPG4-specific mAb was not affected by depletion of NK cells in the SCID mice used. However, the inactivation of signaling pathways associated with melanoma progression could
be one suspected mechanism. Recently, scFv-FcC21 was constructed by linking the CSPG4-specific scFv C21 to a human Fc and scFv-Fc119 was constructed as the isotype control. To determine whether treatment with this anti-CSPG4 antibody blocked signaling that leads to cell migration and survival, CSPG4+ MV3 cells were treated with antibody and cell lysates were analyzed by western blotting for phosphorylated (p)-FAK, (p)-ERK1/2 and (p)-PKC, molecules previously shown to be activated by CSPG4. A significant downregulation in phosphorylation of FAK, ERK1/2 and PKC upon anti-CSPG4 antibody treatment was observed (Figure 2-4).
To confirm these results *in vivo*, MV3 cells were injected into SCID mice, animals were treated twice weekly for 4 weeks. Tumor lysates were analyzed for total and activated FAK, ERK1/2 and PKC proteins. Figure 2-5 shows similar downregulation of activated FAK, ERK1/2 and PKC proteins [141].

**Figure 2-4: Effect of CSPG4-specific scFv-FcC21 on expression and activation of FAK, ERK1/2 and PKC α signaling pathways in human melanoma cells MV3.** scFv-FcC21 was constructed by linking the CSPG4-specific scFv C21 to a human Fc. MV3 cells (2.0 × 10^4/ per well) were serum starved at 37°C for 72 hours and then incubated with either the CSPG4-specific scFv-FcC21, the control scFv-FC119, or PBS for 48 hours at 37°C. Cell lysate were tested in western blot with anti-phosphorylated (p)-FAK (Tyr397), FAK, p-ERK1/2 (Thr202/Tyr204), ERK1/2 and PKCα mAbs. Then bound antibodies were detected using ECL Plus Western Blotting Detection System, and bands were visualized using the FOTO/Analyst Investigator Eclipse System. HLA class I heavy chain (HLA class I) was used as the loading control. The experiment was performed 3 independent times. Wang X, et al. Current Molecular Medicine, 10 (4) (2010).

To confirm these results *in vivo*, MV3 cells were injected into SCID mice, animals were treated twice weekly for 4 weeks. Tumor lysates were analyzed for total and activated FAK, ERK1/2 and PKC proteins. Figure 2-5 shows similar downregulation of activated FAK, ERK1/2 and PKC proteins [141].
The overwhelming advantage of monoclonal antibodies compared with traditional pharmaceuticals is their specificity - they can be engineered to bind particular cell surface antigens. The binding of antibodies to the antigen on target cells can trigger cell death with little or no toxicity to nearby healthy cells. While antibodies are thought to kill tumor cells through three mechanisms: complement-mediated lysis, antibody-dependent cellular cytotoxicity, and activation of apoptosis, it is unknown to what degree each mechanism, or other unrecognized mechanisms, contributes to the overall tumor cell destruction.

Figure 2-5: Effect of CSPG4-specific scFv-FcC21 on FAK, ERK1/2, AKT, β-Catenin and PKCα signaling pathways in vivo. scFv-FcC21 was constructed by linking the CSPG4-specific scFv C21 to a human Fc. MV3 cells (1.5 × 10^6/mouse) were injected subcutaneously to each of 12 SCID mice on day 0. On day 7, mice were divided into 2 groups using a stratified randomization strategy. Starting on day 7, one group (6 mice) was injected intravenously with scFv-FcC21 twice weekly for a total of 6 injections and the other group (6 mice) with the control scFv-Fc119. On day 28, all tumors were removed. Lysates were prepared and analyzed by immunoblotting for the level of total and activated FAK, ERK1/2, and PKCα proteins. HLA class I heavy chain (HLA class I) was used as the loading control. Wang X, et al. Current Molecular Medicine, 10 (4) (2010).

2.7 Advantages of monoclonal antibody-based immunotherapy

The overwhelming advantage of monoclonal antibodies compared with traditional pharmaceuticals is their specificity - they can be engineered to bind particular cell surface antigens. The binding of antibodies to the antigen on target cells can trigger cell death with little or no toxicity to nearby healthy cells. While antibodies are thought to kill tumor cells through three mechanisms: complement-mediated lysis, antibody-dependent cellular cytotoxicity, and activation of apoptosis, it is unknown to what degree each mechanism, or other unrecognized mechanisms, contributes to the overall tumor cell destruction.
Other advantages of this type of therapy is the relatively limited side effects and the variety of modifications available to enhance treatment efficacy such as attaching low level radiation, toxins or chemotherapeutic drugs. For these reasons, significant efforts have focused on developing antibody-based regimens for cancer therapy, as a result of which the U.S. Food and Drug Administration (FDA) has approved numerous antibodies for use in humans.

The data from Ferrone’s group and others [128; 134; 141; 142] provide evidence for *in vivo* anti-tumor efficacy of CSPG4-specific antibodies in a different tumor model, together with the data showing over-expression of CSPG4 is restricted to tumor cells in MM and the information supporting the role of NG2 [111], provides the rationale to utilize CSPG4 as therapeutic target in MM. In this research, we investigate whether, antibody-based therapy against CSPG4 leads to regression of MM tumor cell survival.
CHAPTER 3

Expression and diagnostic value of CSPG4 in MM

3.1 Introduction

The correct diagnosis of MM often raises a challenge for pathologists. Importantly, the distinction between MM versus lung cancers in the histopathology is often misdiagnosed due to the similarity in growth patterns. For example, the epithelial subtype of MM may present with an adenomatous morphology, thus misinterpreting it to an adenocarcinoma of the lung or other organs. On the other hand, the sarcomatous subtype of MM represents a fibrous pattern resembling that of pleural fibrosis as well as other connective tissue tumors with very much like morphology. Collectively, each histological subtype represents its own diagnostic problem; therefore more markers are required to differentiate or be used in adjunct to current immunohistochemical (IHC) markers to overcome these problems. Proteoglycans, overexpressed in many tumors are potential targets for cancer. CSPG4 is highly expressed in melanoma and already represents a very useful marker for the diagnosis of melanoma [2].

Recent findings in our laboratory (Testa et al, manuscript under review) uncovered a similar molecule in a subset of melanoma and mesothelioma patients. Thus, we investigated whether CSPG4, a protein that is over-expressed in melanoma, was also expressed in MM. The cells that give rise to both these solid tumors (melanocytes for melanoma and HM for MM) come from the same origin, the mesoderm. Other similarities include mutations in p16 gene (a tumor suppressor protein) commonly found in both the tumors. Currently, no studies
have indicated the association between CSPG4 and MM therefore, we selected this antigen as a potential target for diagnosis for MM.

In this chapter, we describe the expression levels and also determine the diagnostic value of CSPG4 in MM. In collaboration with Dr Soldano Ferrone (Hillman Cancer Center, Pittsburgh) we wanted to detect the expression of CSPG4 in MM using CSPG4-specific monoclonal antibodies generated from his laboratory.

3.2 Materials and Methods

3.2.1 Generation of CSPG4-specific monoclonal antibodies

CSPG4 antibodies were not commercially available and for the generation of this thesis we collaborated with Dr. Soldano Ferrone (University of Pittsburgh, PA) who is an internationally recognized expert in immunotherapy and has published a wide literature on CSPG4. Dr. Ferrone provided CSPG4-specific mAb preparations (225.228, 763.74, TP32, TP41.2 and TP61.5), from the hybridomas he prepared and selected in his previous work [138]. The data on these mAbs have been published before the initiation of this research [2] and were presented in groups recognizing distinct and spatially distant epitopes of CSPG4.

**Experimental design:** The murine CSPG4-specific mAbs 763.74, TP32, TP41.2 and TP61.5 were all IgG1 except mAb 225.28 which was IgG2a, was generated by immunizing female BALB/c mice at 10-day intervals with three injections of 5 x 10^6 cultured human melanoma cells (Colo 38) which had been incubated with
recombinant 7-interferon (250 units/ml) for 72 h. Splenocytes from immunized mice were then hybridized with murine myeloma cells, P3-X63-Ag8.653. Hybridization, subcloning, and growth of hybridomas in tissue culture and in the peritoneal cavity of BALB/c mice were performed according to standard procedures [104]. Ascitic fluids were clarified by centrifugation, diluted, and precipitated with 50% saturated ammonium sulfate. The IgG precipitates were dialyzed against 20mM Tris buffer, pH 7.5 and chromatographed on DEAE-Sephacel (Pharmacia) in the same buffers using gradients of 0-100 mM NaCl. Purity of fractions was determined by SDS-PAGE in a 7.5% gel under non-reducing conditions. The isotype of mAb was determined by testing with the mouse monoclonal sub-isotyping kit 55015-K (Hyclone Laboratories. Logan. UT). Specificity of mAb was determined by serological binding assays and also calculated by measuring the protein concentration of the antibody solution with the Lowry method. The CSPG4-specific mAbs 225.28, 763.74, TP41.2, TP32 and TP61.5 recognize distinct and spatially distant epitopes of CSPG4 and do not cross-inhibit each other’s binding to CSPG4-positive cells [2; 94]. The mAb D2.8.5-C4B8 recognizes an epitope of CSPG4 in formalin-fixed paraffin-embedded (FFPE) tissue sections; this epitope is spatially distant from the epitopes recognized by the other four CSPG4-specific mAbs. The isotype IgG control is an anti-idiotypic (IgG1) corresponding to mAb TP41.2 recognizing idiotopes in the antigen-combining site of the immunizing anti-CSPG4 mAb. Studies to map the antigenic determinants recognized by this large panel of CSPG4-specific mAbs were conducted in order to correlate the structure with the
function of the domains present in CSPG4 [141; 143]. The antibodies produced against CSPG4 were analyzed of their spatial relationship between the different epitopes. The epitopes were mapped by a panel of mouse CSPG4 monoclonal antibodies through cross-blocking experiments and classified into about 13 groups that identified distinct and spatially distant antigenic determinants. Atleast three mAbs were classified per group (Table 2). The binding and reactivity of each mouse CSPG4 mAb appeared to be independent of the presence or absence of chondroitin sulfate.

<table>
<thead>
<tr>
<th>Group 1(^a)</th>
<th>Group 2(^a)</th>
<th>Group 3(^a)</th>
<th>Group 4(^a)</th>
<th>Group 5(^a)</th>
<th>Group 6-13(^a,d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>149.53(^b)</td>
<td>225.28(^b)</td>
<td>763.74(^c)</td>
<td>VF1-TP34(^b)</td>
<td>VF1-TP41.2(^b)</td>
<td>VF20-VT5.1(^c)</td>
</tr>
<tr>
<td>VT68.2(^b)</td>
<td>VF4-TP109(^b)</td>
<td>VT80.12(^c)</td>
<td>VF4-TP108(^b)</td>
<td>VF1-TP43(^b)</td>
<td>VF18.176(^b)</td>
</tr>
<tr>
<td>653.25(^c)</td>
<td>TP32(^b)</td>
<td>VF20-VT1.7(^b)</td>
<td>543(^b)</td>
<td>116(^b)</td>
<td>TP61.5(^c)</td>
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<td>VT75.1.1(^c)</td>
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<td></td>
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<td></td>
<td></td>
<td>724(^c)</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td>9.2.27(^ND)</td>
</tr>
</tbody>
</table>

\(^a\) Grouping of Ab is based on the ability of mAb to cross-inhibit the binding of each other to CSPG4-bearing human melanoma cells.

\(^b\) mAb do not demonstrate reactivity in Western blotting.

\(^c\) mAb demonstrate reactivity in Western blotting.

\(^d\) mAb do not cross-inhibit the binding of any CSPG4-specific mAb to CSPG4-bearing human melanoma cells.

\(^ND\) not tested in Western blotting.

**Table 2: CSPG4 Antibody grouping.**

### 3.2.2 Cell culture

All cell lines were a gift. Eight MM cell lines (PPM-Mill, Hmeso, REN, Gard, Gor, Rob, Con, Phi) were generously provided; by Drs. Harvey Pass (New York University) and Steven Albelda (University of Pensylvannia) and Melanoma cell line (Colo38) and Burkitt Lymphoma (Raji) were kindly provided by Dr.
Soldano Ferrone (Hillmann Cancer Center, Pittsburgh). Briefly, these cell lines were established from different patients with pleural malignant mesothelioma tumors. All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO₂, grown as monolayer cultures in complete Dulbecco’s modification of Eagle’s medium (DMEM) (Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY), 4.5 g/L glucose, L-glutamine and sodium pyruvate. The cells were propagated twice per week. For propagation, the medium was removed and the cells were washed one time with phosphate buffered saline (PBS) at a pH 7.4. The cells were released from the substrate by incubation with sterile 1mM EDTA (pH 8.0) in PBS for 2 minutes at 37°C. The released cells were re-suspended in culture medium, centrifuged at 1000 rpm for 5 min, and aliquoted into maintenance flasks (T75), and to cell culture petri dishes or multiwall plates for experiments. Seven cultures of HM were derived from seven separate patients with congestive heart failure who accumulated pleural fluid and underwent pleural fluid drainage (IRB #: CH14406, Pathogenesis of mesothelioma (PO1CA114047-01A). HM were cultured and maintained in DMEM supplemented with 20% FBS supplemented with 1% Penicillin Streptomycin (Gibco, Grand Island, NY) and characterized as described in [144]. Briefly, the HM cells were always observed with an inverted phase-contrast microscope (Olympus CKX41) with typical cell morphology of “fried egg”. Before starting an experiment, these cells were stained for positivity of cytokeratin, HBME-1, and calretinin, and negative staining for LeuM1, BerEp4, B72.3, and carcinoembryonic antigen [144]. Typically, HM cells take two weeks
to propagate with 100% of cells stained positive for calretinin, and at this point, they are expanded and used for experiments that are described in this thesis. All HM cells were used at passages 1-5, no further passages were used as HM became senescent between passages 6 onwards. All cell lines and HM cells were controlled and tested for *Mycoplasma* ssp infection by MycoAlert substrate (Cambrex Bio Science Rockland, ME). There were no *Mycoplasma* infections identified in all the cell lines used during the work with this thesis.

### 3.2.3 Flow cytometry analysis (FACS)

Flow cytometry was used as a method to examine the expression of CSPG4 in MM cell lines and HM cells, and to characterize the consistency in the cell surface expression using different CSPG4 monoclonal antibodies against different epitopes. This technique is widely used for cells growing in suspension, and the process of detaching adherent cells from their substrate causes extensive changes in cell morphology that can disrupt the cell surface molecules. It is important to use the right chelating agent that is not harsh to the cells and that can maintain the organization of surface molecules. MM cells were cultured to sub-confluence, then detached from plates by treatment with 1mM EDTA in PBS. Cells (1X10^6 cells/sample/50µl) were washed in PBS, and blocked with 2% BSA in PBS. Cells were incubated with CSPG4 mAbs (1µg/sample/50µl) for 1 hour at 4°C, and thereafter labeled with 100µl of secondary antibody (polyclonal goat anti-mouse immunoglobulins/RPE, goat F(ab’)2) (DakoCytomation, Denmark) at a volume ratio of 1:20 diluted in 2% BSA/PBS, and incubated at 4°C for 30 min. The antibody-treated cells were washed and re-suspended in
500µl PBS. Fifty thousand cells were counted and examined for cell surface CSPG4 expression using a flow cytometer (Becton Dickinson, CA). The cell surface expression levels were analyzed using CELLQuest Pro software (Becton, Dickinson and Company, CA).

3.2.4 Western blot assay (WB)

Cell extracts were prepared by detaching the cells with 1mM EDTA and washing with PBS and lysed (1X10^6 cells/sample/500µl) with lysis buffer [M-PER containing 25mM bicine buffer pH 7.6, 1M NaF, 0.1M PMSF and 0.2 M Na₃VO₄, protease inhibitor cocktail tablet (Roche) as per manufacturer’s instructions, for 30 min at 4°C. The supernatants were aliquoted and stored at -80 °C until Western Blot analysis for CSPG4 antigen was conducted. Total protein content was determined with a bicinchonicic acid protein assay reagent (Pierce Biotechnology, Thermo Scientific, Rockford, IL) as per manufacturer’s instructions. Fifty micrograms of prepared cell lysates were heated at 70°C for 10 min before separation with Nu-PAGE 4-12% non-reducing SDS-PAGE on Bis-Tris (Invitrogen, Carlsbad, CA) and transblotted to nitrocellulose membranes.

After blocking with 5% BSA/TBST [5% bovine serum albumin (BSA) in Tris Buffered Saline containing 0.1% Tween (TBST)], the membranes were incubated with 1µg of the appropriate primary antibodies in 5% BSA/TBST overnight at 4°C and probed with HRP-conjugated anti-rabbit or anti-mouse IgG (1µl of antibody in 2ml of 5% BSA/TBST) for 1 h at room temperature. Protein bands were detected with enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Rockford, IL) and visualized on X-ray film (GE, Healthcare). The anti-
GAPDH antibody was used as loading control.

### 3.2.5 Immunohistochemistry (IHC)

IHC is a technique to detect antigens, that is, proteins from tissues sections by exploiting the use of antibodies binding specifically to antigens in biological tissues. Paraffin embedded sections of surgically removed tumors from patients with pleural and peritoneum malignant mesotheliomas were used. Briefly, 5-µm paraffin embedded sections were incubated overnight at 56°C and deparaffinized in xylene three times and dehydrated in 100%, 95% and 70% ethanol for a duration of 5 min each. Tissue sections were washed in water and immersed in 1 mmol/L EDTA (pH 8.0) submerged in boiling water for 20 mins. CSPG4 mAbs were used in combination with the CSA II System (CSA II, Biotin-Free Catalyzed Amplification System; DakoCytomation) following manufacturer’s protocol. Tissue sections were then incubated overnight at 4°C with antibodies at 15 µg/mL. Normal mouse IgG (Santa Cruz Biotechnology) was used as negative control under the same experimental conditions. After three 5 min washings in TBST, tissue sections were incubated for 15 min at room temperature with horseradish peroxide–conjugated anti-mouse immunoglobulin antibodies. After a 15-min incubation at room temperature with fluorescyl-tyramide (an amplification reagent that is catalyzed by oxidation with the bound peroxidase), anti-horseradish peroxidase was applied and incubation was continued for an additional 15 min at room temperature. After development with substrate (VIP Substrate kit; Vector Labs), tissue sections were counterstained with Gill’s hematoxylin 1X (Fisher Scientific Company) for 1 min at room temperature,
dehydrated with 70%, 95%, 100% of ethanol and three xylene changes, followed by mounting the uncovered tissues with non-aqueous medium and covering with coverslips.
3.3 Results

3.3.1 Characterization of cell surface staining by CSPG4-specific mAbs

CSPG4 on melanoma is highly immunogenic in Balb/c mice [94]. The large collections of the CSPG4-specific mAbs have identified different epitopes (Table 2) [Desai, 1998 #394]. Ferrone’s group has also demonstrated that the mAbs within each of the 6 groups had different cross-reactivity patterns with human tissues. A number of human tumors (melanoma, glioma, head and neck cancer, basal cell breast carcinoma, pancreatic carcinoma, chondrosarcoma) have been shown to express high levels of CSPG4 using CSPG4-specific mAb 225.28 or 763.74. These 2 mAbs failed to react with melanocytes from normal skin as well as benign skin lesions, but reacted with chronic skin lesions [141].

Here we determine the expression of MM tumor cells (PPM-Mill, Hmeso, REN, Gard, Gor, Rob, Con, Phi) by testing the percentage of MM cells expressing CSPG4 with mAb TP41.2. Flow cytometric analysis of 8 MM cell lines stained with CSPG4 specific mAbs detected the expression of this antigen on Gard, Gor, Hmeso, Phi, PPM-Mill, Rob cell lines; the percentage of stained cells ranged from 52% (Hmeso) to 88% (PPM-Mill). The mean fluorescence intensity ranged from 0 to 10,00. Melanoma (Colo38) and Burkitt Lymphoma (Raji) were used as references, positive and negative controls, respectively.

Since the expression of epitopes recognized by CSPG4 specific mAbs has been found to be heterogenous both in melanoma and basal breast carcinoma cell lines [141], we characterized the specificity of the CSPG4 mAbs in MM, and compared the staining of the PPM-Mill, Hmeso and REN cell lines by mAbs.
225.28, 763.74, TP32, TP41.2, TP61.5, which recognize distinct and spatially distinct epitopes of CSPG4. The REN was CSPG4-negative. All of the mAbs produced essentially identical results (Figure 3-1).

**Figure 3-1: Characterization of CSPG4-specific mAbs in MM.**

MM PPM-Mill, Hmeso and REN cells (1X10^6 cells/sample/50µl) were incubated with 1 µg of CSPG4-specific mAbs 225.28, 763.74, TP32, TP41.2 and TP61.5. Cells were then analyzed with the FACScan flow cytometer. The CSPG4-positive Colo38 melanoma cells and the CSPG4-negative Burkitt’s lymphoma cells Raji were used as positive and negative controls, respectively.
3.3.2 Expression of CSPG4 in Malignant Mesothelioma

We verified the results obtained by flow cytometry using Western blotting. We analyzed the expression of CSPG4 in mesothelioma tumor cell lines obtained from different MM patients. Western blot analysis shows that 6 (Ren, Con, Phi, Rob, Gard, Hmeso, Gor and PPM-Mill) out of 8 MM cell lines expressed CSPG4 protein when stained with anti-CSPG4 antibody (mAb 763.74 which recognizes an epitope of CSPG4 in Western blotting, see Table 2). Expression of two components of CSPG4 (450 kDa) and small (280 kDa) were detected in the same 6 MM cell lines found by flow cytometry and no expression was detected in HM. Therefore, the molecular profile of CSPG4 synthesized by MM cell lines is similar to that of the antigen synthesized by Colo38 melanoma cell line, used as a reference. It is noteworthy that CSPG4 was not detected in lysates of primary HM isolated from pleural exudates (Figure 3-2).

**Figure 3-2: Expression of CSPG4 in Malignant Mesothelioma.**
Lysates (50 µg protein/lane) from MM cells and from HM cells were resolved by electrophoresis on a Nu-PAGE 4-12% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. Membranes were probed with CSPG4-specific mAb 763.74 or with GAPDH-specific mAb, followed by horseradish peroxidase-labelled goat anti-mouse immunoglobulins. The signal was detected by electrochemiluminescence (ECL). The CSPG4-positive Colo38 melanoma cells and the CSPG4-negative Burkitt’s lymphoma cells Raji were used as positive and negative controls, respectively.
Data also revealed that even MM cells that did not show any expression by Western Blotting were at least 1% positivity by flow cytometry analysis (Figure 3-2). However, to verify if the lack of expression observed was a generalized phenotype shared by all HM cells, we tested additional HM cells from 7 different individuals treated for heart congestive failure. CSPG4 expression in these cells was neither detected or only very low levels of CSPG4 protein was observed (Figure 3-3).

Figure 3-3: Human Mesothelial cells do not express CSPG4. Lysates (50 µg protein) from HM cells, obtained from pleural effusions of different individuals were resolved by electrophoresis on a Nu-PAGE 4-12% SDS-polyacrylamide gel and transferred to nitrocellulose membrane. Membranes were probed with CSPG4-specific mAb 763.74 or with GAPDH-specific mAb, followed by horseradish peroxidase-labelled goat anti-mouse immunoglobulins. The signal was detected by electrochemiluminescence (ECL). The CSPG4-positive Colo38 melanoma cells and the CSPG4-negative Burkitt’s lymphoma cells Raji were used as positive and negative controls, respectively.

Furthermore, the expression of CSPG4 in MM was modulated by the density of the cell culture. CSPG4 expression levels on cells from cultures plated at 80% confluence was significantly higher than that on cells harvested from cultures plated at 30%, 60% and full confluence. Therefore, cultures used as sources of the cells utilized in the experiments described in this research, were all seeded at 80% confluence (Figure 3-4).
Figure 3-4: Modulation by cell density of CSPG4 expression on MM cell lines. A. MM PPM-Mill, Phi and Hmeso cells were seeded at 30, 60 and 80% density and at full confluence and cultured for 24 hours at 37°C. Cells were then harvested, stained with CSPG4-specific mAb TP41.2 and analyzed with the FACScan flow cytometer. Results are expressed as percentage of cells expressing CSPG4 with P4 and P5 representing negative and positive cells, respectively. The CSPG4-positive Colo38 melanoma cells and the CSPG4-negative Burkitt’s lymphoma cells Raji were used as positive and negative controls, respectively. B. Mean ± SEM of CSPG4 expression at different cell densities in PPM-Mill (62.6 ± 10.4, n=4), Phi (12 ± 2.19, n=4) and Hmeso (20.5 ± 1.33, n=4) based on three experiments.

To determine whether CSPG4 is expressed in MM lesions, 30 epithelioid, 5 biphasic and 5 sarcomatoid paraffin embedded (PEAT) tissues were stained by immunohistochemistry with CSPG4 specific mAb D2.8.5 (CSPG4-specific mAb
demonstrating reactivity in Immunohistochemistry). Twenty-four out of the forty MM lesions were found positive, with minimal expression in the surrounding non-tumor tissue. CSPG4 was expressed in 100% of sarcomatoid (5/5), biphasic (5/5) and epithelioid (14/30) tested. In addition, the staining intensity was strong and homogenous in these 5 sarcomatoid lesions (Figure 3-5).

Figure 3-5: CSPG4 is highly expressed in all three MM tumor cell types despite the phenotype. Sections of a formalin fixed, paraffin embedded tissues. A CSPG4-positive melanoma lesion (a) was used as a positive control, normal pleura (b) sarcomatoid (d), biphasic (e) and epithelioid were sequentially incubated with CSPG4-specific mAb D2.8.5 overnight at 4°C and with peroxidase-conjugated goat anti-mouse IgG antibodies for 1 hour at room temperature. (Magnification 400X).

Together, these data confirm that CSPG4 is expressed in most MM cell lines (6/8) and primary MM tissues (24/40).
3.3.3 Investigation of CSPG4 expression in MM versus Lung tumors

To test whether CSPG4 can be used as a biomarker, histological distinction between MM and Lung Tumors with an optimized staining of lesions was performed using mAb D-2.8.5. Together with 30 MM lesions, a pool of 120 paraffin embedded tissues (PEAT) of lung tumor specimens from different patients containing 30 adenocarcinomas, 30 small cell lung tumors, 30 squamous cell lung carcinomas and 30 bronchioalveolar lesions were stained mAb D-2.8.5. Melanoma lesions stained with mAb D-2.8.5 or IgG control were used as positive and negative controls respectively. The results show that 90 out of 120 lung tumors express CSPG4 despite the histotypes (Figure 3-6).

Figure 3-6: Expression analysis of CSPG4 expression in Lung Tumors. Immunohistochemical staining of paraffin embedded lung tumors shows positive staining compared to normal lung.
A. Normal lung 1
B. Normal lung II
C. Adenocarcinoma
D. Small cell lung carcinoma
E. Bronchioalveolar and
F. Squamous cell lung carcinoma. Magnification 200X.
Table 3 shows the results obtained with the 120 lung tumor biopsies. The intensity of staining was scored as strong (3+), intermediate (2+), weak (1+), or negative (-). The staining of each PEAT section was scored as the average percentage of stained cells based on assessments by three independent observers (ZR and pathologists M.C. and P.B.G.)

**Table 3: CSPG4 analysis of Lung metastases.**

<table>
<thead>
<tr>
<th>Tumor cell type</th>
<th>Tumor grade</th>
<th>Score</th>
</tr>
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<tbody>
<tr>
<td>Small cell lung carcinoma</td>
<td>Undifferentiated</td>
<td>8/30 = 2+</td>
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<tr>
<td></td>
<td></td>
<td>17/30 = 3+</td>
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<tr>
<td>Squamous cell lung carcinoma</td>
<td>Poorly differentiated</td>
<td>7/30 = 2+</td>
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<tr>
<td></td>
<td></td>
<td>5/30 = 3+</td>
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<tr>
<td></td>
<td>Moderately differentiated</td>
<td>5/30 = 2+</td>
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<tr>
<td></td>
<td></td>
<td>7/30 = 3+</td>
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<tr>
<td>Bronchioalveolar carcinoma</td>
<td>Poorly differentiated</td>
<td>1/30 = 3+</td>
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<tr>
<td></td>
<td>Moderately differentiated</td>
<td>1/30 = 1+</td>
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<td>Well differentiated</td>
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<td>7/30 = 3+</td>
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<tr>
<td>Adenocarcinoma</td>
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<td>Well differentiated</td>
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<td></td>
<td>High grade</td>
<td>1/30 = 2+</td>
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</table>
3.4 Conclusions

Altogether, the data suggest that, like melanoma tumor samples CSPG4 is expressed on the surface of some, but not all MM tumor cell lines. Such expression has previously been detected in many progenitor cell types such as oligodendrocytes, astrocytes, keratinocytes, and pericytes; and tumor cells such as glioblastomas, chondrosarcomas and lymphoid leukemias [2].

We have also tested by immunohistochemistry if we can use CSPG4 as diagnostic marker for MM. We characterized a panel of five CSPG4-specific mAbs (225.28, 763.74, TP32, TP61.5 and TP41.2) and the flow cytometry results demonstrated that all antibodies gave a similar pattern of expression, confirming their specificity. We further evaluated the expression of CSPG4 in MM and HM cells. The results show CSPG4 protein is expressed in MM cell lines but not in HM cells. HM cells sometimes show signs of differential growth patterns and inconsistent morphology of a typical “egg shape” which can mimic the morphology of malignant cells. Currently, the use of IHC markers such as calretinin or cytokeratin does not differentiate between MM and HM cells but that they are of mesothelial origin. Therefore, the distinction in the expression using CSPG4-specific mAbs provides useful information to identify malignant or reactive cells versus benign cells.

Moreover, CSPG4 was expressed in MM biopsies. Expression was observed in all three phenotypes, epithelioid (14/30), biphasic (5/5) and sarcomatoid (5/5). The different tumor tissues from epithelioid and biphasic showed differential expression, however, all lesions from the sarcomatous cell
type showed high levels of expression. It would be ideal to correlate this result with aggressiveness and prognosis; however, the numbers were too small to make this correlation.

Additionally, histological distinction between epithelioid MM and adenocarcinomas of the lung or other organs poses a challenge and often requires a panel of IHC markers for a definitive conclusion. This also applies to sarcomatous MM with pleural fibrosis or other connective tissue tumors. Typically, the use of CEA, BerEp4, and CD15 favors the diagnosis of an adenocarcinoma, but are negative for MM. On the other hand calretinin, cytokeratin, HMBE-1, membranous EMA, TTF-1 and WT-1 suggest a mesothelioma. In this study, we tested the diagnostic value of CSPG4 between MM and lung tumors by concerning the differential patterns. The results showed that CSPG4 was expressed in both types of tumors, regardless of the different histotypes. The data concludes that CSPG4 cannot be used as a marker to differentiate MM from lung tumors; however, the data provides additional information and therefore can be used as an adjunct to cytology as an additional marker.

Some but not all MM cell lines were positive and the antigen CSPG4 is modulated by cell overcrowding. This is an important phenomenon to understand, the nature of CSPG4 expression and its association with other factors to maintain its function. At 30% confluence, CSPG4 expression is low perhaps due to less number of cells containing CSPG4 and stress because of lack of contact. With 80% confluence, CSPG4 expression is at its peak. Number
of cells is higher therefore more CSPG4 containing cells and CSPG4 acts as a co-receptor with other factors providing good contact between cells and exchange of signals to promote growth. Consequently, at 100% confluence, CSPG4 is expressed but at lower levels, perhaps due the overcrowding making the cells unable to interact well. These data provided us with useful information to further elucidate the function of CSPG4.
CHAPTER 4

Functional role of CSPG4 in MM cell biology

4.1 Introduction

The cell surface localization of CSPG4, as determined by flow cytometry, and despite its variable expression among the phenotypes, indicates a possible function as cell surface receptor. Also based on previous reports, one possible function is to cooperate in the interaction between tumor cells and protein components of the ECM), such as fibronectin, laminin, and collagens [93], contributing to cell adhesion and to critical processes such as cell motility and survival.

To elucidate the mechanisms of CSPG4-induced cell survival, proliferation and motility of MM cells, we investigated the interaction of CSPG4 with components of the ECM to determine its function in MM cells in vitro.

4.2 Materials and Methods

4.2.1 Antibodies & Reagents

All antibodies besides CSPG4 mAbs were purchased commercially: anti-rabbit phosphor-AKT, anti-rabbit AKT, anti-rabbit phosphor-FAK, anti-rabbit FAK (Cell Signaling Technology, Beverly, MA); anti-mouse p21 antibody, anti-rabbit cyclin D1, anti-mouse VEGF, anti-mouse MMP-2, goat anti-mouse IgG, goat anti-rabbit IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA); anti-mouse GAPDH monoclonal antibody (Chemicon International Inc., Temecula, CA); PE-labeled goat anti-mouse IgG, FITC-labeled goat anti-mouse IgG (Dako North America,
Inc. Carpinteria, CA). CSPG4 Stealth RNAi siRNA (Invitrogen, Carlsbad, CA). Fibronectin, Collagen I, Collagen IV, Laminin, Osteopontin (BD Biosciences, San Jose, CA). MTS assay (Promega, Madison, WI). FITC Annexin V Apoptosis Detection Kit I (BD Pharmigen, CA), HEMA3 kit (Fisher Diagnostics PROTOCOL, USA)

4.2.2 siRNA transfection

siRNA against CSPG4 were commercially synthesized by Invitrogen (Stealth RNAi™ siRNA Card for CSPG4, Invitrogen). Cells were transfected with siRNA using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Stealth RNAi siRNA with high GC content was used as negative control.

4.2.3 Cell viability assay (MTS)

After a pre-treatment with 10 µg/ml (2µg of antibody/ 5000 cells) TP41.2 mAb or isotype IgG control in suspension for 1 hour, MM cells (5X10³/sample/100µl) were seeded in a 96 well plate and incubated at 37°C in a 5% CO2 atmosphere for up to 72 h. Cell viability was measured using Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to manufacturer’s instructions.

4.2.4 Cell adhesion assay

Microtiter 96 well plates were pre-coated with FN, CI, CIV in PBS at a concentration of 10µg/ml (2µg of antibody/ 5000 cells) and incubated at 4°C overnight. On the next day, the wells were blocked with 2% heat inactivated BSA at 37°C for 2 h and the plate was washed with serum free DMEM media. Equal
numbers of cells (5×10^4 cells/well), suspended in 100µl medium pretreated with TP41.2 or isotype IgG control, were added to each coated well and incubated for 1 h at 37°C. The wells were washed three times with low shear to remove unbound cells, followed by fixation of adherent cells with 2% glutaraldehyde in PBS for 10 minutes. The fixative was then removed and the plate was allowed to dry for 5 minutes. Cells were then stained with 0.5% crystal violet for 45 min and washed three times with PBS. The crystal violet dye retained on the wells was solubilized in 100µl 10% (v/v) acetic acid, and the absorbance of each well at 560nm was measured using a multiwell spectrophotometer (UV-VIS).

4.2.5 Migration and invasion assays

A transwell system (8µm pore size, Corning Inc., Corning, NY) was used to evaluate cell migration and invasion. First, 100µl cell suspension (1×10^4 MM cells/sample/200µl) in serum free medium was added in the upper compartment (for migration is uncoated) or coated with matrigel (for invasion) and 600µl DMEM medium containing 10% FBS was placed in the lower compartment. Cells were pre-treated with mAbs TP41.2 or isotype IgG control (2µg of antibody/ 5000 cells) prior to seeding on the upper chambers. The chambers were further incubated for 24 and 48 h at 37°C. The filters were fixed and stained using HEMA 3 staining kit (Protocol, Fisher Scientific) as per manufacturer's instructions. After gently rinsing with water, cells on the upper surfaces of filters were removed by wiping with a cotton swab. Cells remaining on the lower surface of the filters were photographed at 40X using an inverted microscope. To quantify the migratory ability, the crystal violet dye retained on the filters was
dissolved in 10% acetic acid and absorbance was measured at 595 nm.

4.2.6 Wound healing assay

MM cells transiently transfected with CSPG4 siRNA or pre-treated with mAbs TP41.2 or isotype IgG control (2µg of antibody/ 5000 cells) were added to MM cells and cultured for 24 h. A clear area was then created with a micropipette tip. Migration of cells into the cleared area was evaluated with an inverted microscope and photographed at 24 and 48 h later using a magnification of 40X. The number of cells migrated into the blank area of the plate was quantitated using Image J software analysis. Each treatment was done in triplicates.

4.2.7 Apoptosis assay

MM cells were pre-treated with TP41.2 mAb or isotype IgG, then cultured for 24 h at 37°C before detaching with 1mM EDTA (pH 8.0). After washing the cells three times, apoptosis was analyzed using a BD Pharmingen kit according to the manufacturer’s instructions. Annexin was added first and propidium iodide was added 30 min later. Data were evaluated on a flow cytometer (Becton Dickinson, CA). The level of apoptosis was analyzed using CELLQuest Pro software (Becton, Dickinson and Company, CA).

4.2.8 Soft Agar assay

MM cells were detached with 1mM EDTA (pH 8.0) and treated with TP41.2 mAb or IgG control for 1 h in suspension. Addition of 0.3% agarose in resuspended cells were overlaid onto a bottom layer of solidified 0.6% agarose in DMEM media containing 10% FBS, at cell concentrations of 2X10^4 cells per per well of a six-well plate, and incubated for 2 weeks, respectively. Colonies were
photographed, and quantified using Image J software analysis.

### 4.2.9 Human angiogenesis PCR Array

Total RNA was isolated by chloroform and isopropanol extraction. RNA concentrations were measured on NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA quality was measured by microcapillary (Lab-on-a-Chip) electrophoresis using a Agilent 2100 Bioanalyzer using Total Eukaryotic RNA Nano chip (Agilent technologies, Wilmington, DE). Intact RNA produced two major bands (consisting of 28S and 18S ribosomal RNAs) with a high (8-10) RIN (RNA integrity number). cDNA was reverse transcribed from 4ug of RNA by random priming using ABI High Capacity cDNA RT Kit (Applied Biosystems, Foster City, CA). Real-Time quantitative PCR (qPCR) using TaqMan Gene Signature 384-well Human Angiogenesis Arrays were used to measure the expression level of 94 angiogenesis related and two control housekeeping genes (GAPDH and 18S) in duplicates. 4 Samples in duplicates were analyzed per array using 4 ug of cDNA (based on RNA amount) per sample. The arrays were run on ABI 7900HT Fast Real-Time PCR System using the SDS 2.3 software (ABI) to create a SDS file of the run. The SDS file of the qPCR run was analyzed using RQ Manager software (ABI).

### 4.2.10 Statistical Analysis

Data were collected from two to four independent experiments. The mean and SD were calculated for each data point. Differences between treated groups were analyzed by Student t-test (unpaired), one-way or two-way Analysis of Variance (ANOVA). Differences were considered to be significant of p < 0.05.
4.3 Results

4.3.1 CSPG4 mediates MM cell survival through its interactions with ECM

To examine the interplay between ECM components and CSPG4, we performed experiments in the absence of serum, by seeding MM cells on selected ECM components CI, CIV and FN, pre-coated on tissue culture dishes. The MM cell lines PPM-Mill, Hmeso and REN were selected for this chapter to compare because of their variability in the expression of CSPG4 protein as demonstrated in chapter 2. MM cells were seeded on plates pre-coated with CI, CIV and FN, under serum-free conditions and incubated for 24-48 h. MM cells adhered to CI and CIV and intact FN in a dose-dependent manner, adhesion being maximal at 10 µg/ml coating concentration. Cell attachment was greater to FN and CIV than to CI. In contrast, a very limited number of adherent cells were observed in uncoated dishes and morphological signs of cell death were evident (Figure 4-1A).

We further examined the relationship between ECM components and CSPG4 by evaluating the expression of CSPG4 after lysis and protein extraction, by immunoblotting. The expression of CSPG4 was clearly enhanced by the adhesion on ECM components in CSPG4-positive MM cells, PPM-Mill and Hmeso. In contrast, there was no effect in CSPG4-negative MM cells, REN (Figure 4-1B). This suggests that, in CSPG4-positive MM cells, CSPG4 is required and promotes the process of cell attachment by interacting with the ECM components.
Figure 4-1: The interaction between CSPG4 and the extracellular matrix (ECM) promotes MM cell adhesion.

MM cells were plated in serum-free medium on (A) uncoated tissue culture dishes, or pre-coated with different ECM components such as (B) CI, (C) CIV and (D) FN (at a concentration of 10µg/ml of ECM in PBS) for 48 hours. (A) ECM components prevent, at different extents, cell detachment in serum-free condition. Microphotographs show monolayer culture morphology for the different treatments (magnification, 100X). (B) ECM components enhance CSPG4 expression. The cellular proteins were extracted and analyzed by immunoblotting for CSPG4 expression; GAPDH was used as loading control.
4.3.2 Role of CSPG4 in MM cell growth

The mAbs 225.28, 763.74 and TP41.2, recognizing different epitopes of CSPG4 were tested for their ability to inhibit MM cell growth. Two-fold dilutions of the mAb preparation at concentrations ranging from 2.5 to 50 µg/ml were incubated for 1 hour in suspensions of PPM-Mill and Hmeso cultures (5000 cells/sample/100µl), expressing high and medium levels of CSPG4, respectively. The cultures were incubated at 37°C for 72 hours and mAbs were added every 24 hours to each culture. At the end of incubation, cell growth (MTS assay); all antibodies delayed cell growth, with a maximum effect when added at 10µg/ml. The extent of inhibition was not increased by higher doses of mAb. Only mAb TP41.2 inhibited proliferation of both CSPG4-positive MM cell lines to a similar extent. On the other hand, mAbs 225.28 and 763.74 only inhibited PPM-Mill cell growth (Figure 4-2). Therefore mAb TP41.2 was used in all other experiments.

**Figure 4-2: Effect of CSPG4-specific mAbs on MM cell lines in vitro.**
PPM-Mill and Hmeso were pre-incubated with CSPG4-specific mAbs 225.28, 763.74 and TP41.2 or isotype IgG control at the concentrations of 2.5, 5.0, 10.0, 20.0 and 50.0 µg and then cultured for 72 hours in the presence of each mAb. Cell growth was then measured by MTS assay (mean ± SEM of triplicate cultures).
The functional significance of CSPG4 and the validity of the results obtained with mAb TP41.2 (Figure 4-2) was corroborated by the results obtained by analyzing the effect of CSPG4 gene silencing on MM cell proliferation. Human sequence-specific siRNA was used to reduce the expression of this proteoglycan in MM cells. Transient transfection of PPM-Mill and Hmeso MM cells with human CSPG4 sequence-specific siRNAs down-regulated by 40-60% CSPG4 levels in MM cells compared to cells transfected with scrambled control siRNA (Fig. 4-3).

![CSPG4 and GAPDH Western Blot](image)

**Figure 4-3: Down-regulation of CSPG4 protein expression by siRNA.** PPM-Mill and Hmeso cells were transfected with CSPG4-specific siRNAs or with scramble siRNA. The down-regulation of CSPG4 was monitored by testing the cell lysates with CSPG4-specific mAbs 763.74 in Western blotting. GAPDH was used as a loading control. The density of the bands corresponding to the CSPG4 components and to GAPDH was measured using densitometry (mean ± SEM), PPM-Mill (1.039 ± 0.02742 n=3) and Hmeso (0.06872 ± 0.008025 n=3). The results are expressed as the ratio between CSPG4 and GAPDH band intensities. ***P<0.0005

Analysis by MTS assay of CSPG4 silenced PPM-Mill and Hmeso cells, cultured for 48 hours, revealed a moderate, although significant (p<0.05), reduction in cell viability, compared to the same cells transfected with scramble siRNA. Transfection with CSPG4-specific siRNA of CSPG4-negative MM cells, REN did not influence their growth, confirming the specificity of the results (Figure 4-4).
The reduction in cell viability in CSPG4 knockdown cells was significantly reversed, leveling up to the control cells when CSPG4 expression was restored (data not shown). These results indicate that CSPG4 positive cells may be dependent also on CSPG4-mediated survival signals.

Figure 4-4: Silencing of CSPG4 decreases cell viability of MM cells. CSPG4-positive MM cells, PPM-Mill and Hmeso, and CSPG4-negative MM cells, REN transfected with CSPG4-specific siRNA or with scramble siRNA and were cultured for 48 hours. Cell growth was determined by MTS assay (mean ± SEM), PPM-Mill (82.62 ± 1.359, n=6), Hmeso (84.12 ± 1.055 n=6) and REN (97.95 ± 0.7352 n=6). Asterisks indicate P<0.05 significance.

4.3.3 Role of CSPG4 in MM cell adhesion to ECM components

Another important role envisaged for CSPG4 is that of a modulator of cell-cell and cell-ECM interactions. In a previous study, CSPG4 was shown to promote cell adhesion by an alpha 4 beta 1 integrin-dependent mechanism on fibronectin [110].

To examine the interplay between ECM components and CSPG4 in MM, we grew CSPG4-positive PPM-Mill and Hmeso MM cells, as well as CSPG4-
negative REN MM cells on plates pre-coated for 24-48 hours with two-fold dilutions of CI, CIV and FN (concentrations ranging from 2.5 to 20 µg/ml) under serum-free conditions. All MM cells adhered to the ECM components; the extent of adhesion increased with the increase of each ECM component concentration (data not shown). In contrast, a very limited number of adherent cells was observed in uncoated dishes after 48 hours, where clear morphological signs of cell death were evident (data not shown).

Thus, we tested the ability of mAb TP41.2 to interfere with MM cell adhesion to the ECM. To this end, PPM-Mill, Hmeso and REN MM cells were pre-treated with mAb TP41.2 or with an isotype IgG control for 1 hour before plating onto ECM pre-coated dishes. Cells were allowed to attach for 1 hour to the various ECM components CI, CIV, and FN. The number of adherent CSPG4-positive cells treated with isotype IgG control was significantly (p<0.05) higher than that of cells pre-incubated with mAb TP41.2. In PPM-Mill (CI= 1.724 ± 0.08313, n=3, P=0.0365; CIV=2.082 ± 0.03361, n=3, P=0.0120; FN=2.595 ± 0.2526, n=3, P=0.0380) and Hmeso (CI=0.6657 ± 0.01828, n=3, P=0.0302; CIV=0.5417 ± 0.01267, n=3, P=0.0035; FN=0.7353 ± 0.01440, n=3, P=0.0262) On the contrary in CSPG4-negative REN cells no differences were observed (CI=0.8858 ± 0.01294, n=4, P=0.9063; CIV=0.8420 ± 0.009028, n=4, P=0.8042; FN= 0.9235 ± 0.01543, n=4, P=0.4866) (Figure 4-5). These results demonstrated that CSPG4 protein plays a role in cell adhesion of the MM cells.
Figure 4-5: Role of CSPG4 in MM cell adhesion to ECM components. CSPG4-positive MM cells were plated in serum-free condition on dishes pre-coated with collagen CI, CIV and FN and cultured for 30 minutes. Then cells were stained with crystal violet. Acetic acid was then added to solubilize the cells and the absorbance was measured at 560nm to determine the extent of cell adhesion. Values represent the mean ± SEM of triplicates from three independent experiments. Asterisks indicate P<0.05.

To determine whether mAbs inhibited the activation of signaling pathways associated with cell adhesion to ECM components, we analyzed the phosphorylation of AKT and FAK which are the signaling pathways activated by CSPG4 [3; 141], as well the expression of cyclin D1. PPM-Mill cells were incubated with CSPG4-specific mAb TP41.2 and cultured for 15 min, 30 min, 1 hour or 2 hours. Cells were washed after which lysates were collected and evaluated by immunoblotting. mAb TP41.2 treatment showed a decrease in the level of phosphorylated FAK (Tyr397) and AKT (Ser473) as well as down-regulated Cyclin D1 expression in cells plated on FN in the first 15 minutes (Figure 4-6). However, this decrease was transient and was not maintained throughout the 2 hours.
Figure 4-6: Role of CSPG4 in fibronectin activation of signaling in MM. PPM-Mill MM cells were grown on fibronectin-coated dishes for up to 2 hours. Cell lysates were tested in Western blotting with phosphoFAK-, FAK-, phosphoAKT-, AKT- and Cyclin D1- specific antibodies. GAPDH was used as a loading control.

4.3.4 Role of CSPG4 in MM cell survival

CSPG4 is additionally involved in phosphatidylinositol 3-kinase/AKT mediated cell survival as demonstrated in human glioma and triple-negative breast cancer cells [124; 145]. We next analyzed the effect of CSPG4-specific mAb TP41.2 in the survival of MM cells. mAb TP41.2 was added to cultures of PPM-Mill, Hmeso and REN MM cells plated on CI and FN under serum-free condition. Following a 72 hour incubation at 37°C, cell viability was measured by MTS assay. mAb TP41.2 treatment reduced the survival of PMM-Mill cells plated on CI and FN by approximately 25%. The change in cell morphology induced by mAb TP41.2 was striking and similar to that observed in cells plated on uncoated dishes: the cells rounded up and detached from the substrate, suggesting loss of
viability. Accordingly, cell viability (assessed by MTS assay) was significantly (P≤0.0001) lower compared to isotype matched control treated cells. In contrast mAb TP41.2 affected neither morphology nor viability of REN cells, lacking CSPG4 expression (Figure 4-7).

**Figure 4-7: Role of CSPG4 in MM cell survival.**
CSPG4-positive PPM-Mill and Hmeso, and CSPG4-negative REN MM cells were seeded on CI- or FN-coated dishes and cultured under serum-free conditions for 72 hours. (A) Cell morphology was observed under the contrast-phase microscope (magnification at: 100X). (B) Cell viability was determined by MTS assay. Asterisks indicate p<0.005 significance. All data represent the mean ± SD of 3 independent experiments, performed in triplicate. (Mill***P≤0.0001; Hmeso **P≤0.005; REN P=0.746).
We examined whether the reduction in numbers due to the effects elicited by mAb TP41.2 on MM cells were truly associated with antibody-induced programmed cell death which may represent a potential therapeutic candidate for MM therapy, by effectively suppressing the tumor growth through induction of apoptosis. To this end PPM-Mill and REN cells were pre-treated with mAb TP41.2 or isotype IgG control for 1 hour in suspension, and then cultured for 24 hours in the presence of 10% Fetal Bovine Serum (FBS).

The extent of apoptosis was determined by flow cytometry using Annexin V and 7-Amino-Actinomycin (7-AAD) double staining. The cytograms shown in Figure 4-8 indicate that in the cells treated with mAb TP41.2, the percentage of apoptotic cells is significantly (P<0.0001) higher than in the isotype IgG control treated group. Specifically the percentage of the apoptotic cells was at least 35.17% in the mAb TP41.2 treated cells, but was about 17.17% in the isotype IgG control.

It is noteworthy that percentage found in the isotype IgG control group is within the range of apoptotic cells of most MM cell lines in tissue culture. The pro-apoptotic effect of mAb TP41.2 appears to be mediated by its interaction with CSPG4, since no induction of apoptosis was detected in CSPG4 negative REN cells incubated with mAb TP41.2 Programmed cell necrosis was not induced by mAb TP41.2, as evident in the cytograms (Figure 4-8).
Figure 4-8: Analysis of apoptotic index of mAb TP41.2 treated cells compared with control cells.
CSPG4-positive PPM-Mill and CSPG4-negative REN MM cells were pre-incubated with mAb TP41.2 or isotype matched control for 1 hour and then cultured for 24 hours on uncoated dishes. Then the extent of apoptosis was evaluated by flow cytometry using Annexin V and 7-AAD. The percentages of live cells, of early and late apoptotic cells and of necrotic cells are indicated in the cytograms. The total percentage of apoptosis is reported in the bar graph. Asterisks indicate p<0.0001.

4.3.5 mAb TP41.2 diminishes the ECM effects on cyclin D1 stability exerted through CSPG4

We observed that CSPG4 mAbs led to suppression of growth and survival of MM cells. The balance between cell proliferation and apoptosis determines the overall rate of tumor cell progression; therefore we tested if mAb TP41.2 influences the cell cycle of CSPG4-expressing MM cells.

The progression of MM cells along the cell cycle is tightly controlled by expression and activation of a variety of cell cycle regulatory proteins. In
particular, the activation and induction of cyclin D1 plays a critical role in the G1-S phase transition. These events are, in turn, controlled by a complex array of signal transduction mediators. MM cells were seeded on ECM pre-coated dishes after pre-treatment with mAb TP41.2 and the expression of a number of cell cycle regulatory proteins, like cyclin D1 and p21 was examined. The protein p21 a tumor suppressor gene that acts as a downstream effector of p53 function mediating G1 cell cycle arrest by inhibiting cyclin-dependent kinases, which promote cell growth.

Immunoblotting analysis indicated that the treatment with isotype IgG control, in the presence of FN, had no effects on cyclin D1 expression, compared to cells cultured in the absence of ECM. Pre-treatment of mAb TP41.2 dramatically decreased the levels of cyclin D1. MM cell lysates were also examined for changes in p21 expression and a significant up-regulation in the expression of p21 was observed in TP41.2 pre-treated cells (Figure 4-9).
Figure 4-9: mAb TP41.2 down-regulates cyclin D1, and blocks GI-S phase progression in MM cells.
MM cells were pretreated with 10 ug/ml of IgG Isotype control or with mAb TP41.2 for 1 hour in suspension, and then plated on uncoated dishes or ECM pre-coated tissue culture dishes for 24 hours. (a) Cell lysates were immunoblotted for cyclin D1 and for the cyclin-dependent kinase inhibitors, p21.

4.3.6 Role of CSPG4 in MM cell motility and invasiveness

The aggressiveness of a cancerous cell is determined by its potential to invade the ECM and metastasize to distant sites. Several studies have supported the idea that the invasive and metastatic potential of cancer cells is intimately related to their motility [146].

To evaluate the effect of mAb TP41.2 on motility and invasiveness of MM cells, CSPG4-positive MMP-Mill and Hmeso and CSPG4-negative REN MM cells were preincubated with mAb TP41.2 or with isotype IgG control and then plated
on the top chamber of Transwell membranes. Cells were allowed to migrate for 48 hours toward the bottom chamber in the presence of 10% serum. mAb TP41.2 significantly reduced by two-fold cell motility, compared to that of cells treated with isotype IgG control (P<0.0004 for PPM-Mill and P<0.0009 for Hmeso). There was no difference in the rate of motility with CSPG4-negative cells, REN between the isotype and mAb TP41.2 treatments (Figure 4-10).

![Figure 4-10: Role of CSPG4 in MM cell motility.](image)

PPM-Mill, Hmeso and REN MM cells were pre-incubated with mAb TP41.2 or with isotype matched control for 1 hour. Cells were then plated on the membrane of the upper chamber of a Transwell plate and grown for 48 hours in the presence of 10% FBS. Cells grown in serum-free conditions were used as a negative control. The cells migrated to the lower surface of the membrane were stained with Giemsa staining for microscopical observation. Then cells were solubilized and the absorbance was measured at 560nm to determine the extent of cell migration. The graph indicates the mean ± S.D. from three separate experiments. The statistical differences represent comparisons versus untreated cells using Students's t test. P ≤ 0.05.

Similar results were obtained when the expression of CSPG4 was down modulated in PPM-Mill cells by transfecting CSPG4 specific siRNAs. CSPG4
silencing led to at least 40% reduction of cell migration, compared to control cells. Conversely, REN cells that were transfected with CSPG4-siRNA showed no significant differences in migratory effect (Figure 4-11).

Figure 4-11: Down-regulation of CSPG4 inhibits MM cells motility. PPM-Mill and REN MM cells were transfected with CSPG4-specific siRNAs or with scramble siRNA. Cells were then plated on the membrane of the upper chamber of a Transwell® plate and grown for 24 hours in the presence of 10% FBS. The cells migrated to the lower surface of the membrane were stained with Giemsa staining for microscopical observation. Then cells were solubilized and the absorbance was measured at 560nm to determine the extent of cell migration. Asterisk indicates P=0.0002 significance.

Matrigel invasion assay was performed on CSPG4-positive PPM-Mill and Phi, and CSPG4-negative MM cells by plating cells on the upper chamber of Matrigel-coated Transwell. Note, Hmeso did not invade, therefore, another CSPG4-positive cell line Phi was selected (Figure 3-4). A significant reduction of invasiveness was observed for both PPM-Mill and Phi cells preincubated with mAb TP41.2, as compared to cells preincubated with isotype matched control (p<0.0001 for PPM-Mill; p<0.0032 for Phi). On the other hand, pre-treatment of
REN cells with isotype control or mAb TP41.2 displayed no differences in the level of invasion (Figure 4-12).

**Figure 4-12: Effect of mAb TP41.2 on MM cell invasion.**
PPM-Mill and Hmeso MM cells were pre-incubated with mAb TP41.2 or with isotype IgG control for 1 hour. Cells were then plated on the membrane of the upper chamber of a Matrigel®-coated Transwell® plate and grown for 48 hours in the presence of 10% FBS. Cells grown in serum-free conditions were used as a negative control. The cells migrated to the lower surface of the membrane were stained with Giemsa staining for microscopical observation. The graph indicates the mean ± S.D. from three separate experiments. The statistical differences represent comparisons versus untreated cells using Student's t test. $P \leq 0.05$.

### 4.3.7 Role of CSPG4 in MM wound healing

Moreover, as the Transwell® assay measures mainly directional, chemotaxis migration towards a gradient of chemoattractant, we also performed a wound-healing assay to compare the ability of non-directional ability.

PPM-Mill and Hmeso cells were cultured to confluence, a section of the cell layer was removed with a plastic 100μl tip (“wound”) and the cells remaining on the plate were treated with mAb TP41.2 or isotype IgG control. The cleared space was inspected following a 24 and 48 hours incubation to assess the ability
of cells to fill the “wounded” area. mAb TP41.2 markedly inhibited wound closure as compared to the isotype IgG control. The effect was mediated by the interaction of mAb TP41.2 with CSPG4, since the wound healing of the CSPG4-negative REN cells was not affected by incubation with mAb TP41.2 (Figure 4-13A).

These findings were corroborated by the inhibition of wound closure of MM cells with CSPG4 down-regulation caused by siRNA mediated gene silencing The CSPG4-negative cells, REN, had similar growth rates (Figure 4-13B).
Figure 4-13: Role of CSPG4 in MM wound healing.
(A) Wound closure of PPM-Mill, Hmeso and REN MM cells treated with mAb TP41.2 or with isotype IgG control, was assessed 24 hours and 48 hours later. The wound closure extent (Image J counting) is shown in the bar graph on the right. Asterisk indicates P<0.05. (B) Wound closure of PPM-Mill and Hmeso, transfected with CSPG4-specific siRNA (siRNA) or with scramble control siRNA (scramble), was assessed 24 hours and 48 hours later. The wound closure extent (Image J counting) is shown in the bar graph on the right. Asterisk indicates P<0.05.)
4.3.8 Role of CSPG4 in anchorage-independent growth

To investigate if the inhibition of the survival suppresses anchorage independent growth, we analyzed the formation of colonies in soft agar. MM cells were pre-treated with TP41.2 or with isotype IgG control and cells were cultured in agar.

mAb TP41.2 inhibited MM cell growth in soft-agar. The number and size of colonies were markedly reduced when PPM-Mill cells were pre-incubated with mAb TP41.2, and cultured for 17 days in agar supplemented with antibodies every 48 hours. The number and size of colonies were markedly higher when the cells were pre-incubated and cultured in presence of the isotype IgG control.

There were no significant differences in the number of colonies nor the size between the REN cells treated with isotyped IgG control and mAb TP41.2 (Figure 4-13).
Figure 4-14: Role of CSPG4 in anchorage-independent growth. (A) CSPG4-positive PPM-Mill and (B) CSPG4-negative REN MM cells were pre-incubated with isotype IgG control or mAb TP41.2 then plated in soft agar. Thereafter, mAb TP41.2 or isotype IgG control were added to the cultured cells every 48 hours. The rate of cells growth was evaluated by phase-contrast microscopy 17 days later. The average number of colonies was quantified by colony counter using Quantity One software analysis and is reported in the bar graph. The size of the colonies was determined in 5 random optical fields from each plate.
Preliminary Analysis:

4.3.9 Role of CSPG4 in angiogenesis.

The above-mentioned data have elucidated the multiple roles of CSPG4 in MM progression, specifically; the interactions between the tumor cells and ECM induced activity in MM.

It is widely acknowledged that tumors require effective vascularization for their growth and expansion [147]. The role of CSPG4 in blood vessel development is very important to MM progression. Pericytes are very critical in the microvascular development. The expression of CSPG4 in pericytes has already been documented [98; 99]. These findings suggest that CSPG4 may be involved in angiogenesis.

To further examine if CSPG4 is expressed in blood vessels, we first performed an immunohistochemistry to stain MM lesions using CSPG4-specific mAb with particular focus on the blood vessels surrounding the tumors. The staining confirmed that CSPG4 is expressed on the cell surface of the pericytes of MM blood vessels (Figure 4-15A). Several molecules have been recognized as angiogenic factors [147; 148].

To determine if CSPG4 regulates the expression of these factors, we treated MM cells with mAb TP41.2 prior to seeding onto tissue culture dishes. RNA was extracted using Trizol reagent, and RT-PCR array was performed (Figure 4-15B). CSPG4-specific mAb TP41.2 down-regulated the CSPG4 expression and angiogenic –associated proteins suggesting that CSPG4 may be involved in the tumor microenvironment. We further confirmed some of the down-
regulated genes, MMP-2 and VEGF by Western Blotting (Figure 4-15C). The results show that mAb TP41.2 decreases the expression of CSPG4, MMP-2 and VEGF.

![Image](image.png)

**Figure 4-15: Regulation of angiogenesis by mAb TP41.2.**
(a) Immunohistochemical analysis of CSPG4 expression in blood vessels from MM lesions (b) Heatmap generated from PCR Array data analysis showing the regulation of gene expression in MM cells treated with mAb TP41.2. (c) PCR Array data of some genes was validated by Western blotting.
4.4 Conclusions

Previous studies have shown that an increase in CSPG4 expression in human melanoma cells or NG2 expression in rat glioma or mouse melanoma cells, is associated with enhanced growth, adhesion and migration [3; 112; 115].

Our study elucidated the function of CSPG4 in MM. Taken together; our data demonstrated that CSPG4 interacts with ECM to promote cell adhesion in CSPG4-positive MM cells. Additionally, each cell cultured on ECM when compared on plastic produces more CSPG4. This effect was more dramatic on FN. mAb TP41.2 blocked MM cell binding to CI, CIV and FN. Due to minimal reduction in binding was observed in cell adhesion, we didn’t expect much reduction in cell-associated adhesion signals within the first 15 minutes. This was a transient effect perhaps due to the presence of serum (0.2% FBS). There would be a more dramatic effect if the cells were starved and allowed to adhere in serum free conditions as to not influence other factors involved in upregulation of signaling when cells adhere. This also means that other factors are involved in the process of MM cell adhesion in conjunction with CSPG4, providing a rationale that it may act as a co-receptor. Moreover, the data could explain why there were no differences seen between mAb TP41.2 treated cells versus isotype IgG control after 15 minutes of cell attachment.

Treatment with mAb TP41.2 blocked CSPG4 mediated functions in MM and, exerted an anti-growth effect, through inhibition of the ECM mediated MM cell survival.
mAb TP41.2 elicited apoptotic cell death of MM cells, by blocking the function of CSPG4. These data also indicated that CSPG4 expressing MM cells are protected by apoptosis and that CSPG4 is responsible for maintaining their survival suggesting that CSPG4 may be a useful therapeutic target for MM intervention. Moreover, the binding of CSPG4 to ECM influenced intracellular cyclin D1 and p21 expression levels. The increase in p21 indicates MM tumor cell growth can be inhibited by CSPG4-specific mAb TP41.2 in vitro, with significant numbers of tumor cells reverting from S to G0/G1. These results correlated with the slower growth and increased cell death when MM cells were treated with mAb TP41.2.

This research also provided further evidence that CSPG4 possibly acts as a matrix co-receptor and as a modulator of MM cell motility and invasiveness, which are the key elements of the metastatic process and mAb TP41.2 was able to block these important roles in vitro. In addition, the results revealed a significant reduction in colony size and a clear delay in the process of colony formation in MM cells treated with mAb TP41.2, compared to isotype treated cells.

The data also demonstrated that CSPG4 is highly expressed on the pericytes of MM blood vessels. Treatment of MM cells with mAb TP41.2 regulated the gene expression involved in promoting angiogenesis.

These results suggest that CSPG4-specific mAb TP41.2 reduces cell number by at least 50% either killing them or inhibiting growth and reducing colony number and size possibly due to lack of migration.
Altogether, these data suggest that CSPG4 is involved in MM biological activities. CSPG4-specific mAbs blocked the function of CSPG4 abrogating its interactions with ECM components rendering MM tumor progression. These results provide the rationale to assess the ability of CSPG4-specific mAbs to block tumorigenic process *in vivo*. 
CHAPTER 5

CSPG4 sustains the growth of MM xenografts in SCID mice

5.1 Introduction

In vitro, we have shown that in MM cells, CSPG4 interacts with ECM and that this interaction influences cell growth, survival and the ability to migrate and invade (Chapter 4). Therefore, by blocking this interaction, we might be able to prevent or delay tumor growth. A delay in human melanoma and triple-negative breast cancer growth in vivo by CSPG4-specific mAbs has been demonstrated, thereby supporting our hypothesis [128; 134; 142; 145]. In this study, we tested whether blocking the interaction of CSPG4 with ECM would help control the tumor growth when mice were treated with CSPG4-specific antibodies the day after inoculation of MM tumor cells in SCID mice, we evaluated delay in tumor onset and/or reduction in tumor size, compared to controls.

5.2 Materials and Methods

5.2.1 PMM-Mill/luc cells

MM cell line PMM-Mill was characterized and cultured as previously described (section 3.2.2). PMM-Mill/luc luminescent cells was derived from the PPM-Mill cell line that was transduced with the lentiviral vector encoding the bioluminescent genetic marker (luciferase gene) driven by the ubiquitin promoter (pRRL.sin.PPT.CMV).
5.2.2 Determination of the minimum number of cells to induce a tumor

CSPG4-positive MM Cells (PPM-Mill/luc) were grown to confluence in T150 flasks supplemented with 10% FBS in DMEM medium. Cells were washed and harvested using 1mM EDTA, and centrifuged 1000 rpm for 5 mins. Cells were counted and resuspended in PBS at 500ul/mouse (Table 4). These were injected intraperitoneum (i.p.) using 1cc syringes and 23G needles. All procedures were done under sterile conditions. There were three groups of mice (5 mice/group) that received the following treatments:

Table 4: Determination of the minimal number of cells to induce a tumor

<table>
<thead>
<tr>
<th>Group number</th>
<th>Number of mice</th>
<th>Number cells/mouse/500ul</th>
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<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>500,000</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
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<td>3</td>
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All mice were monitored their tumor growths and survival. Tumor growth was measured using the In Vivo Imaging System (IVIS, Xenogen Corp) every seven days. With this machine, we used the in vivo optical imaging technology that allows us to evaluate and monitor the antibody treatment effects on tumor progression in living SCID mice. To assess tumor dimensions and localization of luminescent cells, we anesthetized the animals and i.p injections of 0.3 ML of 15 mg/ml D-luciferin was administered to each animal, bioluminescence signals of PPM-Mill/luc-inoculated mice was monitored using the IVIS system. We used
isofluorane to anaesthetize the animals while we monitoring the animals under the IVIS system. All mice were sacrificed at week 8.

5.2.3 **SCID human xenografts**

SCID mice were injected into peritoneum with $1 \times 10^6$ PPM-Mill cells engineered to express the luciferase reporter gene (PPM-Mill/luc), as previously described (Bertino, 2008 #395) suspended in 500 µL of PBS. Xenografts were visualized by luminescence, after luciferin injection, using the In Vivo Imaging System (IVIS™, Xenogen Corp., Alameda, CA), with regions of interest quantified as total photon counts, processed by Living Image software (Xenogen Corp., Alameda, CA).

The “preventive” experiments: We tested whether injecting CSPG4-specific mAb TP41.2 along with CSPG4-positive PPM-Mill MM cells prevented tumor formation. In this study there were two groups of SCID mice (5 mice/group). Mice were injected intraperitoneally (i.p.) into the lower left flank on day 0 with PPM-Mill/luc ($1 \times 10^6$ cells/ mouse) that were pre-treated with mAb TP41.2 or isotype IgG control. Tumor growth was measured using the In Vivo Imaging System (IVIS, Xenogen Corp) every seven days. With this machine, we used the *in vivo* optical imaging technology that allows us to evaluate and monitor the antibody treatment effects on tumor progression in living SCID mice. To assess tumor dimensions and localization of luminescent cells, we anesthetized the animals and i.p injections of 0.3 ML of 15 mg/ml D-luciferin was administered to each animal, bioluminescence signals of tumor/luc-inoculated mice was monitored using the IVIS system. We used isofluorane to anaesthetize
the animals while we monitoring the animals under the IVIS system. Mice were treated with mAbs twice a week (200 µg/ml/mouse/injection) for four weeks. Survival of the animals was monitored and complete necropsies were performed upon signs of death. All remaining live animals were sacrificed at week 21 (150 days) after the initial treatment (all organs were inspected). Histological evaluation of any suspicious mass and of the following organs was also performed: heart, lung, diaphragm, liver, spleen, kidneys, large and small intestine. Sections of tumor nodules and sections from each of the organs listed above were collected for analysis of apoptotic markers and signaling proteins for future research. Tumor-derived cell cultures will be analyzed as described in aim 2. Results will be compared with nonparametric Mann Whitney U test for statistical difference.

Expected Results: We expected to see that mAb TP41.2 would delay the tumor onset in vivo and to see a significant tumor reduction in treated mice compared to controls.

The “therapeutic” experiments: We tested whether injecting CSPG4-specific mAb TP41.2 in pre-formed MM xenografts induces regression. As in the “preventive” study, there were groups of SCID mice (5 mice/group). PPM-Mill/luc was established using a lentivirus encoding the luciferase gene driven by the ubiquitin promoter. Mice were injected i.p. into the lower left flank on day 0 with PPM-Mill/luc (1X 10e6 cells/ mouse) On day 5, when tumors have a mean diameter of ~ 3mm, mice will received treatments of mAb TP41.2 or isotype IgG control twice weekly. Tumor growth was measured using the In Vivo Imaging
System (IVIS, Xenogen Corp) every seven days. With this machine, we used the
*in vivo* optical imaging technology that allows us to evaluate and monitor the
antibody treatment effects on tumor progression in living SCID mice. To assess
tumor dimensions and localization of luminescent cells, we anesthetized the
animals and i.p injections of 0.3 ml of 15 mg/ml D-luciferin was administered to
each animal, bioluminescence signals of tumor/luc-inoculated mice was
monitored using the IVIS system. We used isofluorane to anaesthetize the
animals while we monitoring the animals under the IVIS system. Mice were
treated with mAbs twice a week (200 µg/ml/mouse/injection) for four weeks.
Survival of the animals was monitored and complete necropsies were performed
upon signs of death. All remaining live animals were sacrificed at week 21 (150
days) after the initial treatment (all organs were inspected). Histological
evaluation of any suspicious mass and of the following organs was also
performed: heart, lung, diaphragm, liver, spleen, kidneys, large and small
intestine. Sections of tumor nodules and sections from each of the organs listed
above were collected for analysis of apoptotic markers and signaling proteins for
future research. Tumor-derived cell cultures will be analyzed as described in aim
2. Results will be compared with nonparametric Mann Whitney U test for
statistical difference.

**Expected Results:** Based on the soft agar assay in Figure 4-14, we expected to
see that mAb TP41.2 will show slower progression of established tumors *in vivo.*
Therefore expected to see a significant tumor reduction in treated mice
compared to controls.
5.3 Results

5.3.1 Pilot study

CSPG4-positive MM cells, PPM-Mill and Hmeso, were transduced with the lentiviral vector pRRL.sin.PPT.CMV, expressing the bioluminescent reporter luciferase and identified as PPM-Mill/luc and Hmeso/luc, respectively. After several days of culture, luciferase expression was monitored by bioluminescence using the IVIS imaging system. The results showed that these established cell lines expressed luciferase (Figure 5-1).

Figure 5-1: Expression of luciferase in MM cells.
CSPG4-positive PPM-Mill/luc (A) and Hmeso-luc cells (B) were established using a lentivirus encoding the luciferase gene driven by the CMV promoter. Expression of luciferase was evaluated periodically by IVIS imaging system treating cells with 20 µl of 15 mg/ml D-luciferin. Bioluminescence signals was determined and photographs were taken.

To determine the optimal number of cells to be injected into mice for future immunotherapy studies, we conducted a pilot study using three groups of 5 mice each injected with serial cell densities (group #1= 5x10^5 cells, group #2= 1x10^6 cells, and group #3= 2x10^6 cells) and PPM-Mill/luc cells were chosen because of
the higher levels of CSPG4 expression. The results revealed that tumors arose 1 week after injection and grew at a steady rate with time (Figure 5-2).

![Figure 5-2: MM growth in SCID mice.](image)

PPM-Mill/luc cells were i.p. inoculated into SCID mice on day 0. Mice were analyzed weekly by IVIS imaging to assess tumor growth. A progressive tumor growth was observed from week 1 for all the cell densities tested.

The results demonstrated a correlation between growth and initial number of cells. When 5x10^5 cells were inoculated a slow growth was observed. In contrast, in the group with an initial amount of 2x10^6 cells, the tumor growth was fast but irregular. A stable standard growth curve was obtained with 1x10^6 cells injected, leading to a steady and stable growth throughout the duration of the experiment (Figure 5-3).
Figure 5-3: Tumor growth progression at the indicated initial cell amounts injected. Bioluminescence was expressed as total photon flux/sec

5.3.2 CSPG4-specific mAb TP41.2 inhibits the growth of MM xenografts in SCID mice

The *in vivo* anti-tumor activity of mAb TP41.2 was investigated in a preventative and a therapeutic model. SCID mice were injected intra peritoneal (i.p.) with PPM-Mill cells transduced with the luciferase reporter gene (PPM-Mill/luc; 1x10^6/mouse).

In the preventative model PPM-Mill/luc cells were preincubated with mAb TP41.2 or with isotype IgG control (200µg/1X10^6 cells/mouse) for 1 hour prior to i.p. injection and the mice were treated twice a week for four weeks. Tumor growth was significantly (P<0.0001) inhibited by mAb TP41.2 within 6 weeks, as compared to isotype IgG control (Figure 5-4A).
In the therapeutic model mAbs were administered to mice 5 days after injection of tumor cells, when tumors had successfully implanted on the peritoneal wall and the surrounding tissues, as assessed by IVIS (In vivo Imaging System). mAb TP41.2 strongly inhibited tumor growth; the inhibition was detectable as early as 7 days following the first administration of mAbs and became more marked during the first 2 weeks of treatment. In the following weeks, tumors continued to grow at a very slow pace. In contrast, tumors grew continuously and at a faster rate in the isotype IgG control group (p<0.0001; Figure 5-4B). Mice were tested for the survival rate and therefore maintained until spontaneous death occurred. At death a necropsy was performed and the tumors developed in the peritoneal cavity and on the surface of the surrounding organs were removed and macroscopically evaluated. Both in the preventive and in the therapeutic experiments the tumors collected from mice treated with mAb TP41.2 were consistently smaller than those from mice treated with the Isotype IgG control, in all five paired groups tested (Figure 5-5).
Figure 5-4: Role of CSPG4 in MM cell growth in immunodeficient mice.

A. Preventive experiment; CSPG4-positive PPM-Mill/luc cells (1x10⁶/mouse) were pre-incubated with mAb TP41.2 for 1 hour and injected i.p. in SCID mice. mAb TP41.2 was then administered i.p. twice a week for four weeks. The size of tumors was monitored weekly by IVIS™ after injection of luciferin. The luminescence signals are expressed as total flux of photons/sec.

B. Therapeutic experiment; CSPG4-positive PPM-Mill/luc cells (1x10⁶/mouse) were injected i.p. in SCID mice. After tumor establishment (5 days), mAb TP41.2 was administered i.p. twice a week for four weeks. The size of tumors was monitored weekly by IVIS™ after injection of luciferin. The luminescence signals are expressed as total flux of photons/sec.
Figure 5-5: Reduction by CSPG4-specific mAb TP41.2 of MM cell growth in immunodeficient mice.

PPM-Mill cells transduced with lentiviral vector induced recognizable tumors in SCID mouse. Necropsy of SCID mice inoculated with PPM-Mill/luc treated with (A) isotype IgG control or (B) mAb TP41.2. The differences in tumor masses of isotype IgG control and mAb TP41.2 treated animals are evident in every of the five mice of each group.

Mice survival was evaluated up to 150 days after xenografts injection both in the preventive (D1: immunotherapy from day 1) and in the therapeutic (D7: immunotherapy from day 7) experiments. The differences were calculated by the log-rank test and evaluated by Kaplan-Meier survival plot, which revealed a significant (p<0.05) increase in median survival of mAb TP41.2 treated mice, compared to isotype matched control treated animals: 131 days vs. 111 days.
respectively for the preventive (D1) experiment, and 125 days vs. 105 days respectively for the therapeutic experiment (Figure 5-6).

Figure 5-6: Kaplan-Meier survival plots of mice with MM xenografts. Survival curves of xenograft-bearing mice treated with CSPG4-specific mAb TP41.2 or isotype IgG control, in the preventative (D1) and therapeutic (D7) tests. Significance $p<0.05$. 
CHAPTER 6

Discussion

MM is an aggressive cancer that is usually associated with asbestos exposure. About 3000 cases of mesotheliomas are diagnosed each year in the US alone, and its incidence is expected to increase over the next decade in developing nations due to the continued use of asbestos worldwide. MM is renowned for its resistance to conventional therapies and the patients’ median survival is 1 year from diagnosis [56]. The high mortality rate from MM, underscores the need for novel targeted therapies for this deadly disease.

Currently, there is no effective standard therapeutic care for MM. Chemotherapy remains the mainstay of MM management and is based on the combination of Cisplatin and Pemetrexed that has yielded the best effect for malignant pleural mesothelioma in terms of median survival (12.1 months), median time to disease progression (5.7 months), and response rate (41%) in a phase III study [149]. Nevertheless, the major concerns of this treatment are short duration of response, rapid relapse and acute toxicity that can be prevented by supplementation of folic acid and vitamin B12.

Several novel second-line therapies have been proposed for MM, based on different approaches and against different targets. Small molecule inhibitors or monoclonal antibodies are being used to target angiogenesis and cell survival. De-regulated expression of growth factors or downstream effectors, like Vascular Endothelial Growth Factor (VEGF) Receptor, Insulin-like Growth Factor-1 (IGF-1) receptor, Hepatocyte Growth Factor (HGF) Receptor (c-Met), Epidermal Growth
Factor (EGF) Receptor, Platelet Derived Growth Factor (PDGF) Receptor β, is antagonized by tyrosine kinase inhibitors and monoclonal antibodies. Angiogenesis is targeted in MM by Bevacizumab (anti-VEGF) or by synthetic VEGF inhibitors. Attempts to disrupt cell survival pathways are ongoing by using tyrosine or serine kinase inhibitors (e.g. Sorafenib, Sunitinb, Imatinib) in Phase II trials. Also HDAC (Histone Deacetylase) inhibitors and proteasome inhibitors are also under clinical investigation. For a comprehensive review, see [150].

Also active immunotherapy is a potentially promising approach for MM care, based on the recognition of specific tumor antigens by the patient immunological system, leading to cytotoxicity specifically for cancer cells and generating immunological memory to insure long-term remission [151]. One recent example of a potentially effective immunotherapeutic approach is the use of tumor lysate-pulsed dendritic cells (DCs) that increases antitumor immunity, leading to a prolonged survival in MM [152].

Passive immunotherapy is also exploited as immunologic anti-cancer therapy, with the goal of providing immediate protection against tumor antigens that have functional activities associated with and responsible for the tumorigenic phenotype. Among different examples of tumor cell targets, validated for passive immunotherapy with monoclonal antibodies (mAbs) is CSPG4, a proteoglycan highly expressed in melanoma and other types of human carcinomas and sarcomas. Besides its expression in a broad range of transformed cells, this antigen is also expressed in pericytes, which are important in the angiogenic vasculature [2], and are associated with neo-vascularization \textit{in vitro} and \textit{in vivo}. 

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These findings, together with the availability of CSPG4-specific mAbs, led to the identification of this antigen as a specific melanoma diagnostic marker [154; 155] and as a target of passive antibody-based immunotherapy [156].

Over two decades ago, the cell surface CSPG4, has been proposed to be a potential target for monoclonal antibody (mAb)-based immunotherapy for melanoma, and just recently, other many types of cancer. The CSPG4-specific mAb alone or CSPG4-specific mAb conjugated with toxins effectively suppressed melanoma tumor growth and reduction of tumor vasculature pericytes in vivo [128; 134; 157]. More recent findings have also demonstrated the anti-tumor activity of CSPG4-specific mAb in triple-negative breast cancer xenografts [141], and when administered to patients with advanced melanoma, only minor clinical responses in a fraction of the cohort were observed [156; 158].

Also active, cell mediated immunity, induced by anti-idiotypic antibodies was proposed for melanoma therapy. The patients treated with anti-idiotypic antibodies developed both CSPG4-specific antibodies which correlated with significant survival prolongation [137] and regression of metastases [139] and cell mediated immunity by development of CSPG4-specific cytotoxic T lymphocytes [159; 160].

During these past two decades, attention has been drawn on T cell-based immunotherapy, in part because of the disappointing results of the antibody-based clinical trials conducted in the early 1980s and in part because of the postulated major role played by T cells in tumor growth control. However, more
recently, mAb-based therapies have secured a high demand due to their favorable outcome for their clinical and commercial success on a variety of malignant diseases [161].

Consequently, there has been an increased interest in identifying and characterizing antibody-defined tumor-associated antigens, with the understanding of the molecular events leading to the effective immune response.

Based on the reviewed recent information related to the distribution of CSPG4 on various types of tumors, its expression on pericytes in the tumor microenvironment [2], its role in cell biology as well as the potential mechanisms underlying the ability of CSPG4-specific immunity to control malignant cell growth, this study was initiated to examined the expression of CSPG4 in MM as a potential antigen for a diagnostic and therapeutic candidate.

Our results are the first to show that several primary MM cell lines and biopsies derived from primary human MM tissues express higher levels of CSPG4 protein on the cell surface as compared to HM or normal pleura. More than half of the lesions analyzed were positive for CSPG4 and all five sarcomatoid specimens were highly expressing the antigen (Figure 3-5). The high expression in cultured cells appears to be associated with high cell density (Figure 3-4). To elucidate the function of this antigen in MM we first used a siRNA strategy to silence its expression. Down-regulation of CSPG4 in MM cells (~40%) caused a reduction in tumor cell growth and motility. These effects were associated with a decrease in MM cell viability suggesting that CSPG4 could potentially function by maintaining the MM tumorigenicity.
Moreover, our findings demonstrated also that CSPG4 effect on MM cell survival is mediated through its interaction with components of the ECM. Previous studies also described the involvement of CSPG4 to interact with ECM components, such as fibronectin, laminin, and collagens, and to influence melanoma cell migration, invasiveness and tumor adhesion [162]. CSPG4 is highly expressed in melanoma cells, it binds ligands such as adhesion molecules, ECM, and integrins and modulating their activities [2], although some have speculated that it binds to chemokines, cytokines and growth factors as well [93]. CSPG4 may also promote cell polarization through Rho GTPase activation, stimulates $\alpha_4\beta_1$ integrin-mediated adhesion and spreading by recruiting and activating a signaling cascade such as CDC42, ACK1 and BCAR1, which activate FAK, Src and ERK1/ERK2 [110; 117].

Interestingly, CSPG4 played a similar role in MM cells. In this study, we tested three different CSPG4-specific mAbs [138] raised against the ectodomain of the CSPG4 protein. We first investigated their effects on cell survival in vitro. Among the antibodies mAb TP41.2 gave the best results in term of expression and inhibition on MM. On the other hand mAb 225.28 was more efficient on melanoma and breast cancer cells [142; 145], but it displayed lower avidity on MM in our experiments. In absence of any information, to the best of our knowledge, on possible polymorphisms of CSPG4 ectodomain, this difference cannot be even attributed directly to a differential glycosylation pattern among different tumor types, because the removal of the chondroitin sulphate component from the CSPG4 molecule has no effect on mAb binding [2]. This
suggests that the antigenic determinants of these antibodies are exclusively amino acids. However we cannot exclude that the different glycosylation may modulate, albeit it does not impair, mAb binding.

The mechanisms underlying the anti-apoptotic effect of CSPG4 are related to the interactions of MM cells with ECM. CSPG4-expressing cells treated with mAb TP41.2 reduced FAK phosphorylation (on Tyrosine 397) within the first 15 minutes (Figure 4-6), which was induced by adhesion to surfaces coated with FN. This result matches with the observation that CSPG4 enhances FAK activity in melanoma, where it plays an important role in tumor invasion, growth and survival [3]. Our results indicate that the domain within the extracellular portion of the CSPG4 core protein, specifically targeted by mAb TP41.2 and not by the other CSPG4-specific mAbs, is relevant to the enhancement of integrin-stimulated FAK phosphorylation. This may explain the different efficiency among the mAbs tested on diverse tumor types, which may have different ECM components, interacting with different domains of CSPG4.

Moreover, as recently shown in breast cancer [145], the treatment of CSPG4 expressing MM cells with mAb TP41.2 also decreased AKT phosphorylation (on Serine 473), with the consequent reduction of about 50% of cell survival. The reduction of AKT activity is a main pro-apoptotic marker and a hallmark for the reduction of migration and spreading ability. The fraction of cells surviving the mAb TP41.2 treatment, were still able to adhere and to spread especially on FN coating at the highest concentration. This suggests that other factors are involved in MM cell adhesion, in concert with CSPG4.
Previous studies have demonstrated that NG2, the rat counterpart of CSPG4, controls cell invasion and metastases. Recently, CSPG4 has been shown to exhibit similar functions in melanoma cells, both in vitro and in vivo [141]. CSPG4 expression is associated with membrane-type matrix metalloproteinases (MT-MMPs) expression that are activated at the sites of contact between melanoma cells and the underlying ECM components, suggesting its involvement in melanoma cell invasion [132]. Similarly, we observed that in MM cells CSPG4 is required for cell motility and wound-healing, since mAb TP41.2 impairs both activities.

Cell adhesion, motility and invasiveness are part of the full tumorigenic process, which includes epithelial-mesenchymal transition (EMT), neo-angiogenesis and anchorage independent growth [146]. Indeed, in a PCR array, specific for angiogenic pathways, significant differences between MM cells treated with mAb TP41.2 and with the isotype control were observed, indicating that pro-angiogenic signals, like vascular endothelial growth factor (VEGF), platelet-derived growth factor beta (PDGF-B), platelet-derived growth factor receptor beta (PDGF-Rβ), fibroblast growth factor (FGF) and others, are activated and sustained by CSPG4 in MM cells. The involvement of CSPG4 in the release of pro-angiogenic factors by MM cells suggest that the proteoglycan is a key regulator of the events occurring in the tumor microenvironment. This is in agreement with the role of CSPG4 and its NG2 homolog in promoting tumor vasculature, demonstrated by CSPG4 over-expression in pericytes [98], in mural cells [130] and in endothelial cells [122], as well as the efficacy of CSPG4
vaccine in targeting pericytes growth in melanoma [134].

Moreover, our results of the anchorage-independent growth assay (in soft agar) showed a remarkable reduction of the size and the growth rate of colonies in mAb TP41.2 treated cells. In absence of organized ECM, like in soft agar, loss of cell survival occurs because of the lack of outside-in signals by integrins upon interaction with ECM. In tumor cells, grown in these conditions, several deregulated pathways enable to escape tumor cell death. In MM cells, as in melanoma cells [129], CSPG4 overexpression is part of the escape mechanisms allowing tumor cell survival and its reduction by mAb treatment leads to a strong reduction in colony formation, possibly because of extensive cell death. The role of “survival factor” for CSPG4 in MM is confirmed also by the results of MTS curves and Annexin-V/PI cytograms.

Our data confirm that CSPG4 is required for and is truly participating to the carcinogenic process of mesothelial cells and in the maintenance of the transformed phenotype in MM cells, at least in vitro.

In vivo experiments are required to completely assess the tumorigenic process of a cell lineage. In our laboratory several mouse models were established for MM, including imageable xenografts in SCID mice, obtained by injection of human MM cells engineered for luciferase expression. With this system, the growth and spreading of human MM after and during anti-cancer treatments can be visualized in live mice by IVIS imaging. We used either a preventive or a therapeutic approach, by treating MM xenografts with mAb TP41.2 at concentrations adjusted for the in vivo conditions. As shown with a
similar approach for breast cancer [145] and for melanoma [128], we observed that CSPG4-specific monoclonal antibodies prevented or delayed MM tumor cell attachment and progression. These results correlate with the observed loss of attachment and tumor maintenance in vitro.

In conclusion, mAb TP41.2 is a good candidate for a therapeutic intervention based on the inhibition of the biological properties responsible for blocking the complex array of CSPG4 functions (cell growth, motility and survival), associated with the onset of malignant phenotype. Our data indicate that treatment of MM cells with mAb TP41.2 results in down-regulation of CSPG4 expression, accompanied with impaired signaling events, diminished growth rates and tumorigenic properties. In other words, CSPG4-specific mAbs induces an antibody-mediated passive immunological response against CSPG4 in MM expressing this antigen. Moreover, this antibody has shown in other tumors to have the potential of targeting pericytes in the tumor microenvironment controlling tumor neo-angiogenesis. Our preliminary PCR array survey suggest that this may occur also in MM. These characteristics make CSPG4 a universal antigen in cancer immunotherapy. We and others [128; 134; 145] by CSPG4 immunotherapy observed only a reduction in tumor growth rate in vivo, rather than a complete eradication. However, even if this antibody is not powerful enough to cause complete regression of all tumor lesions, it may enhance the sensitivity towards cytotoxic drugs. In addition, the known cooperation among cell surface proteins in controlling adhesive and invasive growth opens the possibility of combining this approach with immunotherapy or targeted therapy against other
antigens and with chemotherapy as well.

The preliminary analysis of CSPG4 expression was conducted on a low number of lesions, not equally distributed among the three histological subtypes and it was not sufficient to correlate CSPG4 with clinical parameters (diagnosis, prognosis, aggressiveness, survival rate) of patients. A further multivariate analysis, run on specimens from a well established cohort of patients, will reveal if CSPG4 may represent also a diagnostic and prognostic tool for MM.
CHAPTER 7

Future research

CSPG4 is a potential antigen for immunotherapy of tumor cells [128; 134; 142; 145] and tumor microenvironment [98; 122; 130]. Pericytes stabilize tumor blood vessels by cell-to-cell contact and provide vascular endothelial cells with survival signals. An interesting possibility is to target CSPG4 using mAb TP41.2 to reduce tumor angiogenesis in MM. CSPG4-mAb-based immunotherapy, by damaging pericytes, is expected to enhance the sensitivity of endothelial cells to cytotoxic drugs. This procedure is known as metronomic chemotherapy and the resulting anti-angiogenic effects can even inhibit the growth of tumor cells that do not express the targeted antigens [163].

Targeting pericytes in the tumor stroma might cause a certain degree of vasculitis that could promote the infiltration of the tumor by T cells specific to tumor associated antigens (TAAs) and improve the efficacy of cancer immunotherapies.

Convincing evidence indicates that an effective immunotherapy must counteract the escape mechanisms utilized by genetically unstable tumor cells [55] and cancer stem cells (CSC) [164; 165]. CSC are the subset of tumor cells that are highly tumorigenic, resistant to chemotherapeutic agents and radiation [166], and a major factor in tumor recurrence and/or metastasis.

Guided by this rationale, our strategy will combine antibody and cellular-based immunotherapy with metronomic chemotherapy to target MM cells,
including CSC, and genetically stable cells in the tumor microenvironment required for tumor growth.

Moreover, the current chemotherapeutic regimens are only marginally effective on tumor cells, which tend to be resistant to radiotherapy and/or chemotherapy. In MM this is the major cause of very frequent unsuccessful therapies [150; 167]. This behavior of MM cells is possibly due to the inappropriate gene expression resulting in unrestricted tumor growth and progression. CSPG4 is a good example of the effect of inappropriate protein expression and the CSPG4-specific antibody that has anti-tumor effect, may also contribute to the efficacy of chemotherapy in the treatment of MM. This will be a second option for future work and, based on the established use of chemotherapy in MM, the choice will focus on the combination of CSPG4 immunotherapy with Cisplatin and Pemetrexed [70].

A third possibility to improve the effects of mAb TP41.2 on MM therapy is to combine this treatment with a targeted therapy against a surface molecule that cooperates in CSPG4 signaling and tumorigenic effects. One of the member of wide group of proteoglycan, CD44 (hyaluronate receptor) has been found overexpressed with the family member NG2, murine homolog of CSPG4, in glioma, suggesting common functions in tracing and targeting the invading glioma cells [168]. CD44 is a main co-receptor of c-Met (HGF receptor) [169], which is a crucial tyrosine kinase receptor mediating epithelial-mesenchymal transition and invasive growth in a wide panel of tumors [170], including MM [30]. This opens the possibility to use monoclonal c-Met specific antibodies, which
have been demonstrated to block the invasive growth of melanoma in SCID xenografts [171], in combination with CSPG4 mAb TP41.2, blocking a possible co-receptor function, to reinforce the inhibitory effect of both monoclonal antibodies.

To our knowledge, no monoclonal antibody against MM antigen is in clinical use. CSPG4 has been extensively investigated as a target for monoclonal antibody based immunotherapy in melanoma, but whether this antigen can be exploited also in MM has not been determined yet. Our data support the hypothesis that CSPG4 proteoglycan may represent a useful target for antibody-based immunotherapy of MM. Most importantly, the results obtained through our studies provide the pre-clinical data necessary to translate an antibody-based therapeutic regimen to clinical trials for MM. With the lack of efficacious therapies available to treat patients with MM, the development of such regimens would have the potential to bring hope to patients who are diagnosed at the later stages of disease and currently have no recourse for treatment.
REFERENCES


[71] A.K. Nowak, M.J. Byrne, R. Williamson, G. Ryan, A. Segal, D. Fielding, P. Mitchell, A.W. Musk, B.W. Robinson, A multicentre phase II study of


[96] B.S. Wilson, G. Ruberto, S. Ferrone, Immunochemical characterization of a human high molecular weight--melanoma associated antigen identified


