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**Multiple Myeloma: The role of glycosylation in disease development
and progression.**

By

Siobhan Glavey

Doctor of Philosophy

January 2017

**Multiple Myeloma: The role of glycosylation in disease development
and progression.**

By

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A Thesis

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Doctor of Philosophy**

Supervisors

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ROLE OF CANDIDATE

Chapter 2: Candidate decided on the core concepts, which would be reviewed, with supervisors in order to provide a comprehensive context and background for the research proposed. Candidate performed the literature review, interpreted the literature and wrote the first and final drafts of the manuscript prior to submission for peer review and publication.

Chapter 3: Candidate was principal investigator for the research study and was successful in application for funding of this work (Health Research Board of Ireland, National Specialist Academic Fellowship Programme). Candidate developed the research question in collaboration with the supervisors. Candidate performed the genomic data analysis and Gene Set Enrichment Analysis. Candidate was lead scientist for all of the laboratory based bench top experiments described in the chapter. Candidate performed all of the animal studies documented in the chapter. Candidate supervised the imaging of animals required for this study. Candidate interpreted the results of the study in collaboration with supervisors and decided on further relevant experiments required as data became available. Candidate decided on the relevant statistical analysis required for the study and performed the statistical analysis. Candidate wrote the first and final drafts of the manuscript prior to the submission for peer review and publication.

Chapter 4: Candidate was principal investigator for the research study and was successful in application for funding of this work (Health Research Board of Ireland, National Specialist Academic Fellowship Programme). Candidate developed the research question in collaboration with the supervisors. Candidate designed the study and formulated the experimental plans. Candidate was the lead scientist for the laboratory-based work. Candidate performed the sequential extracellular matrix extractions from all samples in the study. Candidate directed the mass spectrometry, which was carried out in a core facility at Beth Israel Deaconess Medical Centre, Boston, MA, USA. Candidate collaborated with biostatisticians and experts in mass spectrometry to interpret the raw data. Candidate interpreted the analysed data, formulated the results, and wrote the first and final drafts of the manuscript.

DECLARATION

This is an article based thesis which adheres to the University Guidelines set out by the College of Medicine, Nursing and Health Sciences, National University of Ireland, Galway.

I the undersigned hereby declare that the work contained in this thesis is my own original work and has not been submitted for any degree or examination at the National University of Ireland, Galway or elsewhere. All the sources I have used or quoted have been indicated and acknowledged by means of completed references.

Signed: Siobhan Glavey

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DEDICATION

I would like to dedicate this PhD to my parents, Thomas and Kathleen Glavey.

THESIS ABSTRACT

Multiple myeloma (MM) is a clonal plasma cell disorder that is characterized by skeletal destruction, renal failure, anaemia and hypercalcaemia. MM cells are intimately associated with the bone marrow microenvironment, with an array of adhesive interactions taking place between the bone marrow extracellular matrix (ECM) and the surface of MM cells. The considerable heterogeneity in the behaviour of MM is partially governed by differences in gene expression. As MM progresses further alterations in gene expression ensue, drug resistance increases and some cases develop bone marrow microenvironmental independence with spread to extra-medullary sites, possibly due to changes in adhesion properties. Despite recent advances in therapy MM remains an incurable disease. In particular, little progress has been made in patients with high-risk disease, in whom median survival remains less than 3 years (1). Knowledge of the events that underlie disease progression in MM is incomplete. Using chromosomal analysis and high throughput (HTP) genomic approaches important prognostic information can be obtained, stratifying patients into low and high-risk groups. These technologies, however do not give an account of the entire milieu within which the malignant plasma cells survive and proliferate. Considerable information may be overlooked using these approaches alone, particularly regarding the impact of PTMs on the ability of plasma cell surface proteins to interact with the surrounding microenvironment. The composition of the bone marrow microenvironment is also an important consideration if we are to acquire a deep understanding of what factors are at play, which may influence MM cell proliferation and survival within this supportive niche.

PTMs are covalent, generally enzymatic, modifications that occur during or after the biosynthesis of proteins and serve to increase the functional diversity of proteins. These modifications include, but are not limited to, phosphorylation, ubiquitination, methylation, acetylation, glycosylation and proteolysis. This diverse array of protein modifications influences almost all aspects of normal and disease state cell biology. Several of these PTMs occur aberrantly in MM and have been implicated in the development of drug resistance (2-4). When considering MM cells within the bone marrow microenvironment it is important to consider PTMs that may functionally alter the properties of cell surface proteins, which act as an interface between the

malignant cell and other bone marrow niche cells. One such PTM is glycosylation, which results in the alteration of sugar moieties on cell surface proteins, which can in turn alter their adhesive properties.

Glycosylation is a stepwise process of covalent attachment of oligosaccharide chains to proteins, and alterations in this process have been associated with malignant transformation. Altered glycosylation is a universal feature of cancer cells and alterations in this process have been associated with a more aggressive phenotype in several solid and haematological malignancies. The glycosylation pattern of a cell can change rapidly depending on the cell-cell interaction, local microenvironment and immunological milieu. This process may significantly influence disease biology and risk of progression in MM plasma cells, where the cells are known to be dependent on bone marrow stromal cells and the bone marrow microenvironment for survival and proliferation signals. Many cytokines and adhesion molecules, critical for MM survival, are glycoproteins and depend on glycosylation for their normal function. The role of glycosylation in the interaction between the ECM and MM cells has not yet been defined. The study of the ECM in MM and other cancers has been slowed in the past by the lack of high throughput technologies that allow for rapid and detailed profiling of large insoluble ECM proteins. In MM adhesive interactions between MM cells and the ECM are known to confer drug resistance and may offer protection to MM cells from therapeutic agents. Therefore knowledge of the composition of the ECM is needed in order to further advance the identification of therapeutic targets to overcome these mechanisms.

The hypothesis of the work outlined in this thesis is that differential transcriptional regulation of glycosylation-associated genes, and consequent differences in glycosylation of plasma cell surface proteins, plays a role in the pathobiology of MM. The primary aim of this body of work is to explore the role of glycosylation in MM and to advance our understanding of the alterations that occur in this process in malignant plasma cells. A secondary aim of this work is to more clearly define the alterations that occur in the ECM of MM, as an important component of the bone marrow microenvironment, where glycosylated cell surface proteins are widely present

The work presented in this thesis demonstrates that altered sialylation influences homing and survival of MM cells in the bone marrow niche *in vivo*. Knockdown studies demonstrate that reduction in alpha 2,3 linked sialic acid on the surface of MM cells results in decreased tumour burden and prolonged survival in xenograft murine models. This study highlights the importance of altered glycosylation, particularly sialylation, in MM progression and metastasis. Further studies were undertaken to interrogate the bone marrow tumour microenvironment in MM, and a novel proteomics platform was applied, which facilitated the identification and annotation of ECM proteins in this disease. This work demonstrates that the tumour ECM is remodeled at an early stage in MM development in humans and this process continues as the disease progresses.

The body of work presented serves to advance the current knowledge of the MM bone marrow microenvironment and the role of glycosylation in this disease.

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CHAPTER 1

INTRODUCTION

MM is a malignancy of clonal plasma cells originating in the bone marrow, which accounts for 10% of malignant haematological disorders (1). Plasma cells are terminally differentiated B-lymphocytes that produce a single type of antibody and are long living mediators of humoral immunity (2). MM appears to arise from post germinal centre plasma cells which is supported by identification of somatic mutations in the variable region of immunoglobulin genes, occurring as an early event in MM (3). MM evolves from a pre-malignant condition, monoclonal gammopathy of undetermined significance (MGUS), which is usually asymptomatic and identified as an incidental laboratory finding (4). Although only identified in approximately 10% of patients, studies have found that MM is almost always preceded by MGUS with an annual risk of progression of approximately 1% per year (4, 5). Smouldering myeloma is an intermediate stage between MM and MGUS and progresses to MM at a rate of 10% per year (6). Ireland is amongst the countries in Europe with one of the highest incidence rates of MM at 7 per 100,000, making MM the second most common haematological malignancy (7). The mortality rate of MM in Ireland is estimated at 3.9 per 100,000, which is almost identical to that of malignant melanoma in this country (7). Therefore, it is evident that advances in therapy, and working towards a cure for this disease, are important goals, which will impact large numbers of the population.

Several new therapies have been approved for the treatment of MM in recent years, including novel proteasome inhibitors, immuno-modulators, histone deacetylase inhibitors and most recently monoclonal antibodies targeting cell surface molecules (8-11). However despite this remarkably rapid progress MM remains incurable with a median survival of 3 years in patients with high-risk disease (12). Many of these advances have arisen from a greater understanding of the molecular and cytogenetic events that culminate in the evolution of the malignant MM cell. Two distinct acquired chromosomal abnormalities are widely accepted to be involved in myelomagenesis; hyperdiploidy and heavy chain immunoglobulin gene (IGH) rearrangements, observed in approximately 55% and 40% of MM patients respectively (13-16). With the development of genetic techniques such as next generation sequencing (NGS) in the last number of years, a plethora of genetic data has confirmed the molecular heterogeneity of this disease and provided a clearer picture of disease pathogenesis. High throughput technologies, most notably NGS,

have resulted in a better understanding of the molecular basis of MM (17, 18). However MM is a heterogeneous condition both biologically and clinically - this is likely due to complex changes not only in the MM cell itself but also in the surrounding bone marrow milieu, which provides growth and survival signals to MM cells (19).

Taking a wider approach and interrogating the cancer microenvironment has led to the understanding that cancer cells closely interact with their surroundings where the repertoire of immune cells, stroma, vascular endothelium and the ECM are in play. In MM, the malignant plasma cells express cell surface adhesion molecules such as CD29/CDw49d (VLA-4), CD18/CD11a (LFA-1), C-X-C Motif Chemokine receptor 4 (CXCR4) and CD44 which facilitate localization to the bone marrow via specific adherence to both ECM proteins and to bone marrow stromal cells (BMSCs) (20). Adhesion of MM cells triggers interleukin-6 (IL-6) secretion by normal and MM BMSCs and promotes tumour cell growth (20). BMSCs are capable of inducing altered signalling in MM cells, including STAT3 phosphorylation, and also mediate resistance to apoptosis and drug therapies (21, 22). BMSC derived soluble factors, such as stromal cell-derived factor 1 alpha (SDF1), promote proliferation, induce migration and mediate drug resistance in MM cells. Additionally, SDF-1 up-regulates secretion of IL-6 and vascular endothelial growth factor (VEGF) in BMSCs, which promotes MM cell growth, survival and migration (23). Cell adhesion-mediated drug resistance (CAM-DR) is a problematic mechanism of therapy escape in MM. CAM-DR has been shown to be partially, mediated by Wnt/ β -catenin signalling and VLA-4 overexpression (24, 25). The mechanism by which MM cells become resistant to lenalidomide, via CAM-DR, is partially related to the overexpression of the hyaluronan binding protein CD44 and knockdown of CD44 in MM cells leads to reduced adhesion and sensitization to lenalidomide (26, 27). CD44 overexpression has been linked to unfavourable clinical prognostics in MM (28). CD44 is a receptor for hyaluronic acid and can also interact with other ligands, such as collagens and matrix metalloproteinases. Adhesion to fibronectin, a major ECM component, is also important in MM, as it has been repeatedly shown to contribute to drug resistance in MM cells (29-31).

The ECM itself is a major component of the tumour microenvironment in several cancers, contributing to the regulation of cell survival, proliferation, differentiation and metastasis (32, 33). Rather than playing a passive bystander, or facilitatory, role in MM, the ECM itself is an active participant in disease pathogenesis. Gene expression profiling has shown that genes encoding for ECM components are downregulated during tumour progression (34), and in MM, ECM components, such as integrins, have been shown to play an important role in drug resistance (25). MM plasma cells directly interact with the ECM via binding of syndecan-1 (CD138) and very late antigen-4 (VLA-4) to ECM proteins such as collagen type-1 and fibronectin. These adhesive interactions of MM cells result in upregulation of anti-apoptotic proteins and cell cycle deregulation (35). The ECM also contributes to the bone marrow milieu in MM and the interaction of MM cells with the ECM promotes MM development (36). Most studies to date have focused on the adhesion of MM proteins to ECM components, rather than on the nature and composition of the ECM itself. More recently the interrogation of the ECM in cancer has been facilitated by the genesis of high throughput proteomics using technologies that make these large and insoluble proteins amenable to detailed analysis (37). Recent studies have demonstrated upregulation of ECM proteins, receptors and modulating enzymes in MM and MGUS including matrix metalloproteinases and the growth factor periostin (38). Matrix metalloproteinases, such as CD147 (EMMPRIN), are known to be upregulated in MM which may be indicative of active ECM remodelling in this disease however an extensive profile of the ECM composition in MM has not been carried out to date (39).

Clearly adhesive interactions with BMSCs and fibronectin within the bone marrow niche are critical to MM cell survival and proliferation, however there has been a relative paucity of novel targets arising from this increasing body of literature. Over the last number of years several advances in high throughput technologies for genomics and proteomics in cancer have rapidly advanced our knowledge in MM. However, much of the complexity of bone marrow microenvironment-MM cell interaction remains beyond our reach using these approaches alone. This may be in part due to that fact that cell surface adhesion molecules and their receptors are dependent on PTMs for their function. PTMs are diverse and dynamic in their function and alter proteins via a number of mechanisms including acetylation,

phosphorylation, methylation and glycosylation (40). The very nature of these modifications makes building a profile of their functional contribution to the cancer microenvironment challenging. Encouragingly, recent advances in high throughput technologies have rendered PTMs amenable to rapid and detailed analysis. In the case of methylation this has led to large profiling studies in MM, which indeed have verified prognostic relevance of this PTM (41). Inhibition of methylation in MM is the subject of several on-going clinical trials and has led to the licensing of novel therapies such as panobinostat, a histone deacetylase inhibitor.

Glycosylation is a frequent PTM, which results in the addition of carbohydrate determinants, “glycans”, to cell surface proteins and lipids. These glycan structures form the “glycome” and play an integral role in cell-cell and cell-matrix interactions through modulation of adhesion and cell trafficking.

Glycosylation is increasingly recognized as a modulator of the malignant phenotype of cancer cells, where the interaction between cells and the tumour microenvironment is altered to facilitate processes such as drug resistance and metastasis (42, 43). Changes in glycosylation of cell surface adhesion molecules such as selectin ligands, integrins and mucins have been implicated in the pathogenesis of several solid and haematological malignancies, often with prognostic implications (43). However, to date, in comparison to the other frequent PTMs, glycosylation has been relatively under-investigated in MM, largely due to the lack of high throughput platforms to facilitate rapid analysis as outlined above. MM is a cancer in which cellular trafficking is known to occur continuously to multiple areas of the bone marrow niche and indeed MM itself has been proposed as a model for the study of metastasis in cancer (44). MM cells are thought to engage in the multistep invasion-metastasis cascade, which involves dissemination of cancer cells to anatomically distant permissive microenvironments (44, 45). This multistep process is characterized by (1) cell invasion, (2) egress via trans-endothelial migration into blood vessels, (3) circulation, (4) homing to distant tissues and (5) formation of micrometastasis (45, 46). In MM, as is the case in normal lymphocyte trafficking, this process is mediated via integrins, selectins and their ligands (47-50). Glycosylated proteins are involved in capture, rolling and firm adhesion of cancer cells, and consist for the most part of glycosylated selectin ligands (51). Of note, aberrant glycosylation, specifically altered sialylation of selectins, has been implicated as a metastatic driver in breast cancer (52).

Glycosylation is also implicated in several other aspects of MM pathobiology. For example, CD44 is a highly glycosylated trans-membrane protein and binding of CD44 to hyaluronan is dependent on N-linked glycosylation (53). Like CD44, several other cell surface adhesion molecules known to be active in MM cell adhesion are glycosylated and depending on glycosylation for their function, this includes CD29 (β 1 integrin), VLA-4, CD54, CD138 and CXCR4 (54-58). CD138 is a proteoglycan that requires heparin sulphate and chondroitin sulphate for its activity (59, 60) and functions as an integral membrane protein, which participates in cell proliferation, migration and cell-matrix interactions via its receptor for ECM proteins in both normal physiological states and cancer (61, 62). In MM major genes encoding for proteins involved in heparan sulphate and chondroitin sulphate synthesis have been implicated in the malignant transformation of cancer cells (63). Glycosylation is also likely to impact on CAM-DR in MM where integrin mediated adhesion to fibronectin confers survival and reduced apoptosis, as integrin mediated adhesion to fibronectin is known to be altered by glycosylation in other cancers (25, 31, 64-66).

Investigation of glycosylation in MM, based on its integrated role in MM pathobiology, is a necessary step in order to gain a greater understanding of the bone marrow milieu. The work described in this thesis focuses on the contribution of specific glycosylation changes that occur in MM and the functional significance of these changes on cell trafficking, metastasis and survival of MM cells. Additionally there is a specific focus on the ECM composition and remodelling in MM patients. Overall the work serves to provide new insights into the role of the altered bone marrow milieu in MM with a specific focus on glycosylation and the ECM.

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CHAPTER 2

GLYCOSYLATION AND CANCER: REVIEW OF THE CURRENT LITERATURE

Reference: Glavey SV, Huynh D, Reagan MR, Manier S, Moschetta M, Kawano Y, Roccaro AM, Ghobrial IM, Joshi L, O'Dwyer ME. The cancer glycome: carbohydrates as mediators of metastasis. *Blood Reviews*. 2015 Jul; 29(4): 269-79.

Abstract

Glycosylation is a frequent PTM, which results in the addition of carbohydrate determinants, “glycans”, to cell surface proteins and lipids. These glycan structures form the “glycome” and play an integral role in cell-cell and cell-matrix interactions through modulation of adhesion and cell trafficking.

Glycosylation is increasingly recognized as a modulator of the malignant phenotype of cancer cells, where the interaction between cells and the tumour microenvironment is altered to facilitate processes such as drug resistance and metastasis. Changes in glycosylation of cell surface adhesion molecules such as selectin ligands, integrins and mucins have been implicated in the pathogenesis of several solid and haematological malignancies, often with prognostic implications. In this review we focus on the functional significance of alterations in cancer cell glycosylation, in terms of cell adhesion, trafficking and the metastatic cascade and provide insights into the prognostic and therapeutic implications of recent findings in this fast-evolving niche.

Section I

Introduction

(I.i) Physiological role of glycosylation

Glycosylation is a PTM that occurs in the endoplasmic reticulum (ER) and results in the addition of carbohydrate motifs, “glycans”, to proteins and lipids that are, in most cases, destined for the cell surface. The resultant “glycoprotein” or “glycolipid” structures at the cell surface form a carbohydrate rich layer, which plays an integral role in the interaction of the cell with its surrounding environment. Of the more than 200 different types of protein PTMs, glycosylation occurs frequently and results in the addition of functional carbohydrate motifs to protein structures (1, 2). Glycans interact with carbohydrate binding proteins known as “lectins” that are specific for glycan moieties and are commonly used in purified form to study glycosylation *in vitro*. One of the main functions of lectins in mammalian cells is to mediate cell-cell interactions and therefore interactions of glycans with their respective lectins has major implications for cell trafficking.

Glycosylation of a given protein is achieved through a complex series of post-translational enzymatic steps that lead to the formation of protein-bound glycans with specific and diverse biological functions. These carbohydrate side chains are capable of modulating the interaction of the protein with its environment influencing key factors such as protein half-life, solubility, binding activity and specificity. Proteins with the same amino acid sequence can possess different glycan structures, producing different glycoforms of the same protein. These glycoforms can differ in key properties such as stability, folding, localization and ligand specificity (3) with consequent implications for physiological processes, including protein folding and trafficking, cell–cell and cell–matrix interactions, cellular differentiation and the immune response (4-6). Therefore, the glycosylation status of a protein can be used to differentiate protein glycoforms and molecular changes in glycosylation of proteins have been used to distinguish normal from disease states in humans. (7, 8). Furthermore, as cell communication, adhesion, and signalling also play a major role in cancer, changes in glycosylation of surface proteins on malignant cells can alter interactions between cancer cells and their surrounding environment (6) (9-11).

Glycosyltransferases are enzymes that regulate the process of glycosylation in humans where their action is dependent on the availability of precursor monosaccharide molecules and other parameters (12, 13). Glycosyltransferases work to add and subtract monosaccharides to and from glycan structures, examples of these enzymes include sialyltransferases and fucosyltransferases, which are responsible for the addition of sialic acid and fucose moieties, respectively. The intracellular sites of action of these enzymes include the ER, Golgi apparatus, cytosol and nucleus.

Two major types of glycosylation occur on proteins; 1) O-linked glycosylation refers to the addition of N-acetyl-galactosamine to serine or threonine residues by the enzyme UDP-N-acetyl-D-galactosamine transferase, this is then followed by the addition of other carbohydrates such as galactose, N-acetyl-D-glucosamine or sialic acid (Fig 1). ;2) N-linked glycosylation occurs in the ER and refers to the process by which an oligosaccharide chain is enzymatically attached to the amide group of an asparagine in the consensus sequence Asn-X-Ser/Thr, where X represents any residue except proline (Fig. 1). This sequence can be used to identify potential N-glycosylation sites in peptide sequences.

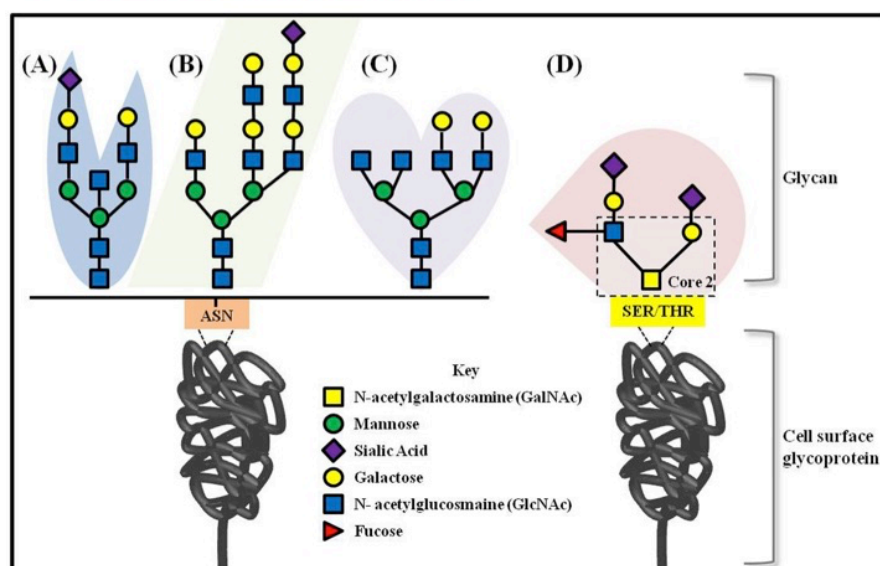


Figure 1. O and N-linked protein glycosylation: A-C: N-linked glycosylation; A - bisecting GlcNAc, B - Tri-antennary glycan; C - Tetra-antennary glycan; D – O-linked glycosylation, example shown is alpha 2, 3-sialylated glycan.

O-linked glycosylation also contributes to the production of proteoglycans by addition of glycosaminoglycan (GAG) chains to a core protein. GAGs consist of repeating disaccharide units composed of an N-acetylated or N-sulphated hexosamine and either a uronic acid (glucuronic acid or iduronic acid) or galactose. Examples of GAGs include hyaluronan, dermatan sulfate, keratan sulfate, chondroitin sulfate, heparin, and heparan sulfate. Heparan and chondroitin sulphate are linked to serine residues of core proteins by xylose and this process is mediated by a xylosyltransferase. Proteoglycans and their associated GAGs form essential components of the ECM, where they function in cell adhesion via interactions between the complex carbohydrate motifs (14).

It is clear that alterations in gene expression and protein expression are not the sole factors responsible for phenotype determination in cancer cells, where not only the cell itself is affected, but also the microenvironmental components such as the ECM. The impact of PTMs on proteins and lipids has identified a layer of complexity, beyond the amino acid sequence, which has the consequence of greatly altering the function and even the purpose of that protein in a given context. Although the protein sequence is governed by the relevant genomic code, many properties of functional cell surface proteins, and circulating glycoproteins, are governed by the modification of glycans and therefore consideration must be given to the glycosylation status of a protein when considering its activity within a biological system.

This rapidly developing field has provided new cancer biomarkers and potential targets recently in a variety of solid and haematological cancers (15-17). This review focuses on the enzymes involved in this process and the cell surface proteins that become modified as a result of their action, with an overall focus on the implications for cell trafficking and metastasis of cancer cells and the interaction of the ECM in this process.

Section II

Carbohydrates and the cancer cell

(II.i) Glycosylation and cancer

The normal process of glycosylation is disrupted during malignant transformation of cells (18, 19). These changes result in alterations in tumour cell surface glycans and therefore interactions with endogenous lectins are impacted, which influences the metastatic potential of the tumour cells. Complex carbohydrate structures that can be found attached to proteins and lipids on the surface of cancer cells have a major influence on their phenotype and the interactions that they have with the surrounding environment (20) (Fig. 2). In parallel with the changes in glycosylation, expression and levels of carbohydrate-binding proteins also change during malignant transformation leading to altered overall presentation of glycans and their cognate receptors, i.e. lectins.

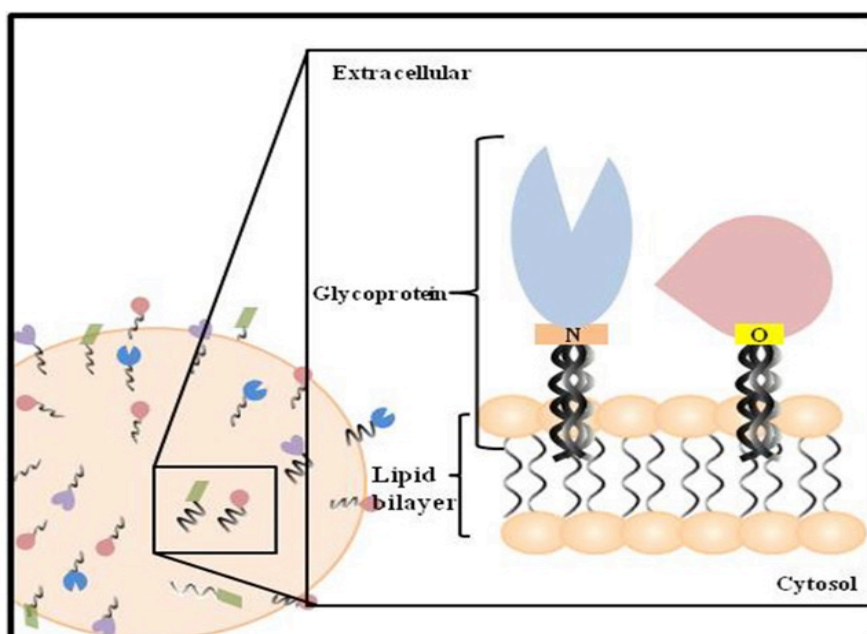


Figure 2. Presentation of glycans on cell surface proteins .Representative image of N- and O-linked glycosylation on cell surface proteins, adding a layer of complexity composed of glycan moieties.

Alterations in glycosylation of malignant cells can take a variety of forms, including changes in the amount, linkage and acetylation of sialic acids, changes in the branching of N-glycans mediated by glycosyltransferases, alterations in expression of glycosaminoglycans such as heparan sulphate, and altered glycosylation of mucins,

which are heavily glycosylated epithelial-derived proteins known to be implicated in certain cancers (14). Studies of the mechanisms by which alterations in glycans are able to bring about changes in cancer cell biology have been impeded by the complexity and heterogeneity of glycans, however recent advances in glycomics, including glyco-genome analysis, HPLC, mass spectrometry and lectin profiling have facilitated comprehensive characterization of the glycome of several tissues (21).

The mechanisms by which glycosylation changes mediate tumour metastasis and invasion are mostly unknown, however roles of specific cell surface glycoproteins and their carbohydrate motifs have emerged and will be reviewed in the following sections.

(II.ii) Implications of glycosylation in cellular metastasis

Both solid and haematological malignancies begin the process of metastasis from the primary niche by escaping to the systemic circulation, which is followed by extravasation to secondary sites and growth of distant metastatic lesions. Altered cell surface glycosylation has been implicated in this cascade where it supports tumorigenesis and metastasis (22, 23). During the process of transformation, from a normal cell to its malignant counterpart, cells acquire several mutations in proto-oncogenes and tumour suppressor genes giving rise to an altered phenotype. However, changes also occur at the cell surface, which alter the interaction of cancer cells with the surrounding environment, and facilitate participation in the multi-step process of metastasis. Alteration of tumour-cell-surface glycosylation changes the extracellular “velcro” layer and results in differential adhesive and invasive properties of these cells. Cancer cells in the circulation extravasate into tissues and form new metastatic niches using mechanisms that normally function to recruit leucocytes to sites of inflammation and injury (24). Adhesion molecules expressed on the surface of cancer cells play a crucial role in metastasis and the ability of cancer cells to metastasize is largely determined by their ability to interact with endothelium, which is mediated, at the initial phases, by integrins and selectins. Selectins are a family of three trans-membrane adhesion molecules that are expressed on the surface of leukocytes (L-selectin), platelets (P-selectin) and vascular endothelial cells (E and P-selectin) (25). Selectins are expressed on endothelial cells and interact with their ligands on cancer cells to play an important role in initiating the metastatic process by regulating the tethering and rolling of cancer cells to the vascular endothelium, a pre-requisite for

subsequent trans-endothelial migration across the vessel wall (Fig. 3). The interaction of selectins with their ligands is greatly influenced by glycosylation and some glycans such as sialyl Lewis X (SLe^x) and sialyl Lewis A (SLe^a) play a critical role in E-selectin ligand function.

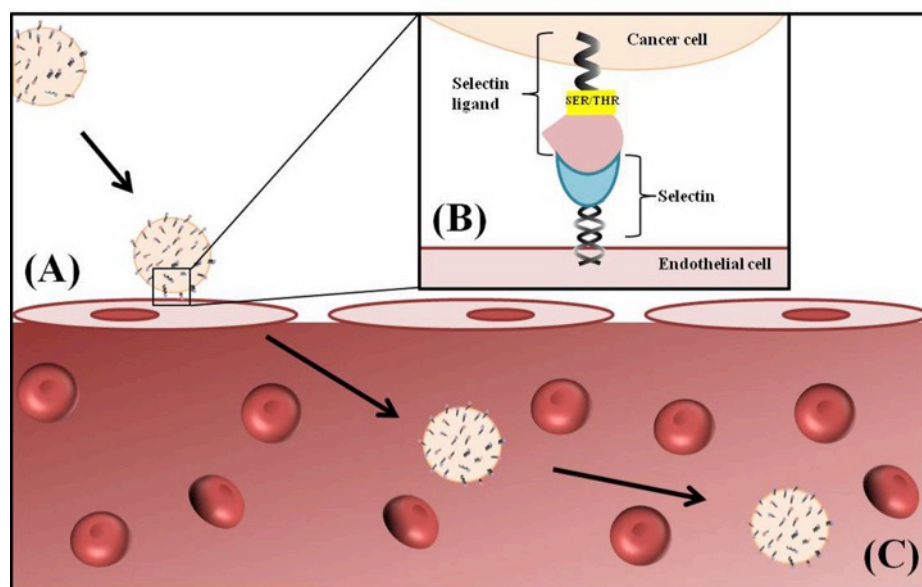


Figure 3. Selectins interacting with glycosylated ligands at the surface of cancer cells during the process of haematogenous metastasis. A) Cancer cell initial interaction with endothelial cells followed by tethering and rolling of the cell on endothelium. B) Critical interaction between the selectin ligand of a cancer cell and selectin on an endothelial cell mediated by sialylated glycans on selectin ligands. C) Trans-endothelial migration of cancer cell allowing entry into the blood stream and haematogenous metastasis.

Integrins are large trans-membrane glycoproteins that serve as cell-cell adhesion molecules and are responsible for mediating the interaction of cells with ECM components such as collagens, fibronectin and laminins. This review focuses on four important differentially glycosylated proteins in cancer; selectin ligands, integrins, mucins and galectins, and on the enzymes mediating these changes with a view to implications for cell trafficking and metastasis. The purpose of the review is to provide a comprehensive background, specifically related to aberrant glycosylation in cancer, and how this might be further interrogated in order to expand knowledge and facilitate the development of novel therapeutic strategies in this field.

Section III

Targets of glycan modifications in cancer

(III.i) Selectins

As previously mentioned, selectins are vascular cell adhesion molecules which mediate adhesion of leukocytes and platelets with the endothelium. There are three members of the selectin family: P-, E-, and L-selectins. P-selectin is present in the storage granules of platelets (α -granules) and endothelial cells (Weibel-Palade bodies), and rapidly translocates to the cell surface upon activation (26). L-selectin is expressed on the surface of almost all leukocytes. The physiological functions of selectins are well described in relation to immune response, inflammation, cell trafficking, and haemostasis (27) largely through the study of specific selectin knockout mouse models.

L-selectin mediates fast rolling of leukocytes on endothelium while P- and E-selectin support rolling at lower velocities within the vasculature (27). The initial steps in cell migration involve tethering and rolling of cells on the vascular endothelium, which is mediated by the interaction of selectins on the endothelial surface and their carbohydrate ligands. Structurally, selectin ligands consist of distinct glycan structures, which incorporate the terminal core tetrasaccharide structure SLe^x (example of O-linked glycosylation shown in Fig. 4) and SLe^a on a protein backbone. Selectins can bind to various classes of molecules including mucins, sulphated glycolipids and glycosaminoglycans, and most of these molecules are capable of acting as functional selectin ligands *in-vivo* (26).

of a glycan chain where structurally it contains a Gal β 1-4GlcNAc backbone. Attached to the Gal is a α 2-3NeuAc and the GlcNAc has a α 1-3Fuc attached. SLe^x synthesis is initiated by adding α 2-3NeuAc to the Gal of N-acetyl-lactosamine; this reaction is catalysed by β gal: α 2-3 sialyltransferases, of which there are several in humans. (38). Following this, α 1,3/4-fucosyltransferases catalyse the addition of α 1-3Fuc to the GlcNAc (39, 40). Once in place on a selectin ligand, SLe^x determines binding specificity and directs functionality of various selectin ligands; therefore alterations in this glycan structure have major implications for cell trafficking and metastasis.

P-selectin glycoprotein ligand-1 (PSGL-1) is the predominant physiologic ligand for P-selectin and L-selectin, but when modified by HECA-452 reactive glycans it can also serve as an E-selectin ligand (41, 42). HECA-452 is a monoclonal antibody that recognises SLe^x related structures that bind to E-selectin.

PSGL-1 plays a role in leukocyte trafficking and PSGL-1 mediated rolling is a prerequisite for integrin mediated firm adhesion and PSGL-1 upregulation in immune cells may be a mechanism of enhanced migration (43). PTMs of PSGL-1 are important for optimal selectin binding (44); to bind to P-selectin, PSGL-1 requires an α 2,3-sialylated and α 1,3-fucosylated core 2 O-glycan attached to a specific N-terminal threonine with tyrosine sulphation near the N-terminus optimizing the binding to P-selectin (44-46). To bind to E-selectin, PSGL-1 requires core 2 α 1,3-fucosylated and α 2,3-sialylated O-glycans, indicating a potential role for alpha 2,3 sialyltransferases in the function of PSGL-1 as both an E and P-selectin ligand (44, 47). PSGL-1 has previously been shown to regulate the adhesion and homing of MM cells to the bone marrow niche and regulates proliferation and development of drug resistance in MM cells (48). Furthermore PSGL-1 is critical for macrophage-mediated MM cell drug resistance (49), both of these studies outline an important role for PSGL-1 in MM biology.

This provides a basis for the concept that alpha 2,3 sialyltransferases may be important in generation of E-selectin ligands, which mediate homing and retention of MM cells in the bone marrow via interaction with E-selectin. Overcoming the interaction of E-selectin and its ligands, potentially by inhibiting the activity of alpha 2,3 sialyltransferases, could be a useful chemo-sensitizing strategy in MM and AML. Both normal hematopoietic stem cells and cancer stem cells are known to express E-selectin ligands (50). Recent data demonstrates that selectins and their ligands are

required for homing and engraftment of BCR-ABL⁺ leukemic stem cells in the bone marrow niche and adhesion of colon carcinoma cells to E selectin activates survival pathways, such as NFκB (51, 52).

(III.ii) Integrins

As mentioned, integrins are large complex trans-membrane glycoproteins that act as cell adhesion molecules. The integrin family comprises 24 members, that consist of a combination of 1 of 18 α- and 1 of 8 β-subunits (53). Integrins directly bind components of the ECM, such as laminin, fibronectin and collagen and themselves convey signals downstream, following binding to extracellular ligands. This occurs via a variety of cell signalling molecules such as focal adhesion kinase (FAK) and Src via activation of kinases, GTPases and the Ras/Rho pathways (54). Through these mechanisms integrins can modulate cell adhesion, migration and proliferation and have therefore been extensively investigated in cancer. The ubiquitous presence of integrins on tumour cells, blood components, vasculature, and stromal cells suggests that integrins contribute to the metastatic cascade. In cancer, integrins display altered branching of N-glycans, mediated by N-acetyl-glucosaminyltransferase III (GnT-III) and N-acetyl-glucosaminyltransferase V (GnT-V). Typically integrin glycosylation by GnT III inhibits cell migration while integrins glycosylated by GnT-V promote cell migration (55). In colorectal cancer, alterations to the N-glycan branching of integrins has been shown to contribute to a more invasive phenotype (56). Modification of integrin-associated glycans can also be mediated by sialyltransferases, which has been demonstrated in pancreatic cancer where ST3GAL-III transfected cells exhibited higher SLe^x and lower α2,6-sialic acid content on the glycans of their α2β1 integrin molecules and higher adhesive potential (57). Therefore, it is clear that altered integrin glycosylation has major implications for cell adhesion and metastatic potential, making this an important focus of glycosylation research in cancer.

(III.iii) Mucins

Mucins are large glycoproteins with a “bottle- brush” like conformation, which carry many clustered glycosylated serines and threonines in tandem repeat regions. The human mucin family (MUC) consists of 21 mucins (MUC1 – 21) that are further classified as secreted or transmembrane mucins and can be found expressed on normal and malignant epithelial cells (58, 59). In normal tissues, mucins serve to

protect cells from the microenvironment by formation of a protective layer (37). Mucins are aberrantly expressed in many cancers where they display differential patterns of glycosylation and many cancer-associated mucins (and their glycans) serve as circulating biomarkers with clinical utility (60). Specific O-glycans that are associated with mucins in cancer include the carbohydrate antigens Tn (GalNAc α 1-O-Ser/Thr), STn (NeuAc α 2-6GalNAc α 1-O-Ser/Thr), and T (Gal β 1-3GalNAc α 1-O-Ser/Thr) (61, 62). Aberrant glycosylation of mucins is a common feature of all adenocarcinomas and tumour cells are known to express mucins that are associated with the epithelium from which they are derived, alongside new cancer-associated mucin core structures and glycan structures (63). Mucins are generally expressed on the apical domain of epithelium with soluble mucins being secreted into the lumen. During malignant transformation there is disarray of correct mucin expression on endothelial cells which allows soluble mucins to enter the extracellular space and circulation (14). Due to their circulating nature and because they can be detected by monoclonal antibodies, mucins have been proposed as prognostic and diagnostic markers in several cancers including the well known markers CA-125 and CA19-9. In cancers of epithelial origin in particular, mucins appear to be the major carriers of altered glycosylation (14).

It is likely that cancer cells alter their mucin expression in order to allow them to interact appropriately with the tumour microenvironment and enhance survival signalling. SLe^x can be considered a mucin glycan as it is frequently associated with mucins and, as mentioned, it has been demonstrated in colonic carcinoma cell lines that 5-Azacitidine (5-Aza) treatment enhances SLe^x production on the mucin MUC1 by inducing the expression of ST3 β -galactoside α -2,3-sialyltransferase 6 (ST3GAL-VI) (64). 5-Aza is an inhibitor of DNA methyl-transferase, which leads to DNA hypomethylation, weakening the effects of natural gene silencing mechanisms. Following treatment, the colonic carcinoma cell line also showed a corresponding increase in the adherence of these cells to E-selectin under dynamic flow conditions. Knockdown of ST3GAL-VI in these cells reduced the level of SLe^x without affecting MUC1 expression. This points towards hypomethylation as a mechanism of regulation of ST3GAL-VI and consequently SLe^x expression on mucins (64).

In pancreatic cancer, differential glycosylation of mucins MUC1 and MUC4 is apparent as the disease progresses from early adenocarcinoma to the metastatic state and MUC1 has been shown to induce multidrug resistance gene expression (65-67).

Tn and STn are highly expressed in pancreatic cancer tissue however, sTn antigen on MUC1 is associated with the malignant state whereas Tn on MUC1 is observed in normal pancreatic ductal cells (67). The presence of high levels of Tn and sTn structures on cancer cells is well described and may in part be due to mutations in the Cosmc protein (68, 69), the core 1 synthase enzyme or as previously stated any of the enzymes involved in O-glycan extension. Cosmc is a chaperone protein that is necessary for core 1 activity and consequently the extension of Tn glycan into core 1 or core 2 structures, including the T antigen (67). It has recently been demonstrated that glycan elongation beyond the mucin-associated Tn antigen in pancreatic and breast cancer protects cancer cells from immune mediated killing by natural killer (NK) and T-cells, indicating that alterations in glycosylation can mediate cancer cell immune escape (70). This cancer specific glycoform signature has the advantage of facilitating specific targeting of cancer cells, potentially minimizing toxicity and increasing efficacy of therapeutic strategies directed to these targets. Recently this has been successfully applied to pancreatic cancer and T-cell leukaemia using genetically modified T cells expressing chimeric antigen receptors (CAR-T cells) that recognise the cancer associated Tn glycoform of MUC1 (71).

It has previously been shown that the extracellular portion of MUC1 binds to pro-tumorigenic factors such as galectin-3 (72); this interaction likely influences downstream signalling events as the cytoplasmic tail of MUC1 interacts with a number of receptor tyrosine kinases. MUC4 associates with the ErbB2 receptor to affect proliferation, apoptosis and epithelial mesenchymal transition (EMT) in cancer cells (73-75).

Therefore, it stands that differential glycosylation of mucins has a broad array of effects on the cancer cell glycome itself but also on immune escape of cancer cells, survival and proliferation.

(III.iv) Galectins

Galectins are a family of 15 immuno-regulatory lectins which bind to galactose that is either β 1,3 or β 1,4 linked to N-acetyl-glucosamine (63). Galectins are soluble proteins with both intracellular and extracellular functions and are expressed by a wide variety of cells including epithelial and immune cells where they are bound to proteins by both N-linked and O-linked glycosylation. They have a broad range of function

including the mediation of cell-cell interaction, cell-matrix adhesion, apoptosis regulation and suppression of T-cell receptor activation (76, 77).

Several studies point towards a role for galectins in regulating cancer cell functions such as adhesion, invasion and metastasis (78, 79). Galectin-3 has been extensively implicated in several cancers where its presence on cancer cells, or on endothelial cells, can help to promote adhesion and metastasis (80, 81). In colon cancer cells, homotypic interactions between Galectin-3 and MUC1, both present on the cell surface, increase the survival of tumour cells and promote embolism formation and dissemination of tumour cells (80). Similar homotypic interactions, mediated by Galectin-3, are also observed in highly metastatic breast cancer cells (81). These and other studies highlight the interplay of cancer glycome components in promoting metastasis through glycan specific interactions. Galectin-3 is also highly expressed in Diffuse Large B Cell Lymphoma (DLBCL) (82) where expression has been linked to a poor outcome for patients (83). Galectin-3 is also aberrantly expressed in many other types of cancer where it has been shown to inhibit apoptosis, possibly via Bcl-2 and in leukaemia cells increased Galectin-3 facilitates survival via stabilization anti-apoptotic Bcl-2 family members (84, 85). Furthermore, the gene encoding Galectin-3, LGALS3, is up-regulated in acute myeloid leukaemia where it has been shown to be independently associated with an unfavourable outcome in these patients (86).

Galectin-1 has also been reported to promote tumour growth by inducing apoptosis of tumour responsive activated T-cells following glycan specific binding to CD45 or CD43 on T-cells (63, 87, 88).

Galectins have been targeted in cancer using modified citrus pectins (MCP) which are complex carbohydrates capable of combining with the carbohydrate-binding domain of Galectin-3 (89). In MM one of these compounds was able to induce apoptosis in various MM cell lines, including those resistant to dexamethasone, melphalan, or doxorubicin. Interestingly this compound was able to overcome the growth advantage conferred by anti-apoptotic protein Bcl-2, heat shock protein-27, and nuclear factor- κ B, and blocks vascular endothelial growth factor-induced migration of MM cells (90). This same compound has recently been found capable of removing cell-surface -3 from CD45 rendering DLBCL cells susceptible to chemotherapeutic agents. This is regulated by C2GnT-1 glycosyltransferase (91). The need for development of

Galectin inhibitors, particularly Galectin-3 is apparent and efforts to address this unmet need in cancer are underway (92).

Section IV

Glycosyltransferases: Mediators of carbohydrate modifications in cancer

Glycosyltransferases are a large and diverse family of enzymes that are responsible for the assembly of monosaccharide moieties into linear and branched glycan chains. These enzymes tend to act sequentially so that the product of one enzyme prepares its acceptor as the substrate of the next enzyme in the process. Glycosyltransferases are specific for the type of linkage (α or β), and the linkage position of the glycoside bond formed [e.g. $\alpha(1\rightarrow3)$ or $\beta(1\rightarrow4)$]. Glycosyltransferases were initially considered to be specific for a single glycosyl donor and acceptor, which led to the “one enzyme-one linkage” concept (93). Subsequent observations have refuted the theory of absolute enzymatic specificity by describing the transfer of analogs of some nucleoside mono- or di-phosphate sugar donors and it is now clear that some glycans may be assembled by the action of any one of a number of highly related transferases (94). Sugar nucleotide donors for glycosyltransferases in humans are: UDP-glucose, UDP-galactose, UDP-GlcNAc, UDP-GalNAc, UDP-xylose, UDP-glucuronic acid, GDP-mannose, GDP-fucose and CMP-sialic acid.

As mentioned previously N-linked glycosylation begins in the ER with the synthesis of dolichol-linked GlcNAc residue which is then constructed into a precursor oligosaccharide and extended to a precursor glycan by the additions of 2 GlcNAc, 9 mannose and 3 glucose molecules (Dolichol-GlcNAc₂-Man₉-Glc₃). The precursor glycan is transported to a protein in the lumen of the ER where an oligosaccharyl-transferase recognizes the consensus sequence (Asn-X-Ser or Asn-X-Thr) of the polypeptide acceptor. The glycan is further processed in the ER by glycosidases I and II and a series of mannosidases. Following this, glycosyltransferases add sugar residues to the core glycan structure, giving rise to the three main types of N-glycans – bisecting GlcNAc, tri-antennary glycans and tetra-antennary glycans (Fig. 1)

While N-glycosylation is the most common glycosidic linkage, O-glycosylation also plays a key role in cancer biology, as previously highlighted. Mucin synthesis requires

O-glycosylation and it is also critical for the formation of proteoglycan core proteins. The linkage mechanism involved in O-glycosylation is not as complex as that of N-glycosylation and O-glycosylation also differs in that glycans are added one-at-a-time to serine or threonine residues (Fig. 1).

Proteins are O-glycosylated in the Golgi by N-acetylgalactosamine (GalNAc) transferase, which transfers a single GalNAc residue to the β -OH group of serine or threonine. Proteins can also undergo O-glycosylation with GlcNAc, fucose, xylose, galactose or mannose, depending on the cell and species. As with N-glycosylation, sugar nucleotides serve as monosaccharide donors for O-glycosylation, which then continues as various sugars are added to the growing glycan chain (13, 95).

Common categories of changes in N- and O-linked glycosylation occurring in cancer and specific glycosyltransferases involved are covered in the below sections.

(IV.i) Altered branching and truncation of glycans in cancer:

An important example of the action of glycosyltransferases in cancer is in relation to the branching of N-glycans on the surface of cancer cells. An increase in the size of N-glycans on the surface of cancer cells has been attributed to an increase in β 1–6 branching of N-glycans that results from enhanced expression of GnT-V. This glycosyltransferase is coded on MGAT5 and this gene is induced in several cancers, including hepatocellular cancer (HCC) (96). Cell lines with increased GnT-V expression show an increased frequency of metastasis in animal models and when this enzyme is lost cells lose this metastatic phenotype (97, 98).

GnT-V is currently being evaluated as a potential glyco-target and a compound that partially blocks MGAT-5, by diverting the biosynthesis pathway upstream of the enzyme, has shown activity against breast cancer in mice (99). Furthermore, Ma and colleagues demonstrated that inhibiting MGAT-5 expression in a murine breast cancer cell line significantly reduced breast cancer cell proliferation following a reduction in complex surface N-glycans which translated to reduced tumor progression *invitro* and *invivo* (100). Although it has been demonstrated in several models that MGAT-5 deficiency can reduce tumor growth and metastasis *invitro* and *invivo* the mechanism has not been fully defined. Morgan et al. proposed that GnT-V mediated N-glycosylation negatively regulates Th1 cytokine production (101).

There is also some evidence that GnT-V can affect integrin stability directly through modification of glycans. This was demonstrated in a study by Wang et al. (102) that showed attenuation of the number of β 1-6 GlcNAc branching structures on β 1 integrin in MGAT-5-inactive-mutant transfected hepatocellular cancer cell lines. Interestingly, there was decreased β 1 integrin expression in the inactive-mutant transfected cell line despite no significant change in the mRNA level, suggesting that the presence of β 1-6 GlcNAc branching contributed to the expression of a more mature and stably expressed form of β 1 integrin and as a functional consequence was more effective in promoting cell migration to fibronectin. GnT-V may also regulate expression of cytokine receptors as reported by Partridge et al. (103) who demonstrated that expression of MGAT-5 sensitized mouse cells to multiple cytokines through promoting the substitution of N-glycan with N-acetyl-lactosamine, the preferred ligand for Galectin-3. In this case MGAT-5 was shown to be rate-limiting factor for cytokine signaling and consequently for epithelial mesenchymal transition, cell motility and tumor metastasis (103).

Since certain glycan signatures can be linked to malignancy and metastasis, efforts have been made to assess the glycan profile of drug and chemotherapy resistant cancer cells. HT-29 colon cancer cells have been shown to have higher levels of α 2,3 and α 2,6 sialylated structures when methotrexate resistance develops (104). Various techniques have been employed to evaluate the cell surface glycan profile of therapy resistant cancer cells ranging from lectin binding studies to mass spectrometry. Down regulation of GnT-V has been shown to enhance chemosensitivity in breast cancer (100) and de-glycosylation with PNGase-F, which cleaves N-linked glycans from the proximal GlcNAc residue, also produced a similar effect. The glycan products of GnT-V act as tumor-associated glycan markers and they are commonly increased in a variety of malignancies where levels have been shown to correlate with disease progression (105-107). Also, β 1-6 branched oligosaccharides are increased in breast cancer, as demonstrated by Phaseolus vulgaris lectin-L (PHA-L) binding. PHA-L recognizes and binds specifically to terminal galactose, N-acetylglucosamine and mannose residues of complex glycans on mammalian glycoproteins, and in this study was shown to be predictive of a worse outcome (108).

Altered truncation of glycans in cancer is not restricted to N-linked glycans. Truncation of O-linked glycans such as T, Tn, sT, Tn on mucins of epithelial cancers, such as breast ovarian and colorectal cancer, has also been noted (109-112). Increased expression of sTn antigen in these cancers has been proposed as a biomarker due to its association with inferior outcomes in these cancers (113, 114).

(IV.ii) Sialyltransferases

Sialyltransferases are enzymes that transfer sialic acid from the activated cytidine 5'-monophospho-N-acetylneuraminic acid (CMP-NeuAc) to terminal positions on sialylated glycolipids (gangliosides) or to the N- or O-linked sugar chains of glycoproteins (Fig. 1). Sialyltransferases belong to the glycosyltransferase family with 29 members which comprises enzymes that can be classified according to the Carbohydrate-Active enZymes system (<http://www.cazy.org>) sialyltransferase; β -galactoside α -2,6-sialyltransferase; α -N-acetyl-galactose-aminide α -2,6-sialyltransferase; β -galactoside α -2,3-sialyltransferase; N-acetyl-lactosaminide α -2,3-sialyltransferase; (α -N-acetyl-neuraminy-2,3- β -galactosyl-1,3)-N-acetyl-galactosaminide, α -2,6-sialyltransferase; α -N-acetyl-neuraminide α -2,8-sialyltransferase; lactosylceramide α -2,3-sialyltransferase.

Sialyltransferases have been shown to be aberrantly expressed in several cancer models, most prominently reported in the literature are ST3GAL-I and ST3GAL-IV and ST3GAL-VI (115-117), additionally, our group has recently reported a role for ST3GAL-VI in migration and trafficking of MM cells *invitro* and *invivo* (118). Moreover, we showed that high levels of expression of ST3GAL-VI were independently associated with reduced survival in patients treated on the MRC Myeloma XI study (118). As previously mentioned, aberrant glycosylation is a recurring theme in breast cancer and consequently O-linked glycosylation has been extensively studied in this disease and sialylated core 1 chains are reported to be expressed at higher levels on breast cancer cells than in their normal mammary counterparts, where core 2 based O-linked glycans predominate (115). This has been attributed to the over-expression of ST3GAL-I (119, 120) which is up-regulated by cyclo-oxygenase-2 (COX-2) in breast cancer (115). COX-2 has been implicated in the induction of several malignancies (121, 122) where it may also be exerting its effect via alterations in glycosylation. A previous study by this group demonstrated that spontaneous Polyoma virus middle T antigen (PyMT) induced mammary tumours

developed earlier when human ST3GAL-I was expressed as a transgene driven by the MUC1 promoter to ensure expression of the sialyltransferase in the mammary gland (123).

One mechanism by which sialyltransferases may contribute to an enhanced metastatic phenotype in cancer cells is via the generation of SLe^x, which is known to serve as a selectin ligand and therefore has implications in the interaction of endothelial selectins with their ligands on the surface of cancer cells. Treatment of neutrophils with sialidases has produced evidence that sialic acids may play an important role in selectin ligand function (34, 124). There are six different sialyltransferases present in mammals that have the ability to generate α 2-3 sialic acid linkages on glycoproteins and glycolipids, all of which could therefore theoretically contribute to the generation of SLe^x and be implicated in the malignant phenotype of cells (125). Sperandio and colleagues have demonstrated that ST3GAL-IV-deficient mice have a defect in selectin ligand function *in vivo*, including a mild reduction in E-selectin–dependent rolling, an increase in E-selectin–dependent rolling velocity, and a decrease in L-selectin–dependent rolling during inflammation, which relies on P-selectin glycoprotein ligand-1 (PSGL-1), the predominant selectin ligand expressed on leukocytes. (34, 35). It was noted that ST3GAL-IV deficiency alone does not account for the full contribution of sialyltransferases to selectin ligand synthesis and *in vivo* studies were carried out to further evaluate this. ST3GAL-VI deficient and ST3GAL-IV/VI double-deficient mice were found to have a deficiency in P-selectin mediated leukocyte rolling in an *ex-vivo* chamber system. This was seen in the leukocytes from ST3GAL-VI deficient mice and was more pronounced in the double deficient mice where it was equivalent to that of sialidase treated leukocytes. The most pronounced effect of ST3GAL-VI function was apparent in P-selectin ligand formation. Neutrophil recruitment into the inflamed peritoneal cavity and lymphocyte homing to secondary lymphoid organs were impaired in ST3GAL-VI-null mice and more severely in double-deficient mice (125). This provided the first evidence of a coordinated role for these sialyltransferases in selectin ligand synthesis. Through a coordinated process these enzymes work to generate SLe^x or its sulphated form, 6-sulpho- SLe^x on glycoproteins or glycolipids with binding activity to selectins (126). The absence of one or more of these enzymes can alter leukocyte rolling with implications for cancer cell adhesion, homing and metastasis. Our group has

demonstrated that in MM, a cancer demonstrating widespread cell trafficking at diagnosis, knockdown of ST3GAL-VI, which is expressed at high levels at the mRNA level in cell lines and patients, results in reduced adhesion and trans-endothelial migration of MM cells *invitro* alongside a reduction in bone marrow homing *invivo* resulting in prolonged survival of xenograft mice (118). This provides evidence of functional consequences of alterations in cell surface glycosylation in MM cells, where α 2,3 sialylation may participate in selectin ligand formation and therefore impact cellular trafficking and metastasis.

The enhanced expression of carbohydrate ligands such as SLe^x is well established in several cancer models but the molecular mechanisms that lead to this are not well understood. Evidence is accumulating indicating an interaction between epigenetics and alterations in the human glycome (127), for example the accumulation of SLe^x in colon cancer cells may be a result of DNA hypomethylation. (64). It is likely however that this cannot explain the diversity of changes seen in the carbohydrate determinants on cancer-associated ligands. Another interesting proposed mechanism relates to alterations in sugar transportation and intermediate carbohydrate metabolism. Cancer cells exhibit a metabolic shift from oxidative to anaerobic glycolysis, this is known as the Warburg effect, which corresponds to increased gene expression of sugar transporters and glycolytic enzymes in cancer cells. These changes have been recently linked to induction of genes related to the expression of SLe^x in cancer (128). This includes ST3GAL-I and Fuc-T VII, which are induced when colon cancer cells are grown under hypoxic conditions; interestingly this is believed to be mediated by hypoxia inducible factor (HIF) (129). This leads to higher expression of SLe^x and SLe^a on cancer cells and is likely to at least partially explain the increased SLe^x determinant expression seen in some cancers which was accompanied, in this study, by a concomitant increase in E-selectin binding activity. This process refers to the “neosynthesis” hypothesis related to the mechanism of enhanced expression of carbohydrate determinants of selectin ligands in cancers (130, 131). This was further examined in a study looking at the association between SLe^x and SLe^a expression on colon cancer cells and EMT. Induction of EMT was shown to increase SLe^x and SLe^a expression and enhance E-selectin binding. In this study transcript levels of ST3GA-I/III/IV and FUT-III were significantly elevated and were regulated by C-MYC. This study outlines the role of SLe^x and SLe^a expression in mediating selectin binding during EMT (132).

(IV.iii) Fucosyltransferases

Fucosyltransferases are a family of enzymes that transfer L-fucose sugar from a GDP-fucose (guanosine diphosphate-fucose) donor substrate to an acceptor substrate, such as core GlcNAc (N-acetylglucosamine) sugar, in the case of N-linked glycosylation, or to a protein, in the case of O-linked glycosylation produced by O-fucosyltransferases. Along with having sialic acid as its terminal sugar, SLe^x also has fucose which is regulated by FUT-I-VII and FUT-IX (133, 134). So it stands, as for sialic acid, that manipulation of the fucose may influence selectin ligand synthesis and interactions in a similar manner to that of sialic acid; indeed this has been shown to be the case in several cancer models. In colon cancer SLe^x expression is not only regulated by sialyltransferases but is also regulated by the fucosyltransferases such as FUT-VI and FUT-III. Inhibition of FUT-III in colon cancer inhibits selectin mediated adhesion and metastasis (135) and FUT-TVI knockdown is associated with a reduction in SLe^x expression in colon cancer cell lines (136). Zandberg and colleagues adopted an interesting metabolic engineering strategy to inhibit the biosynthesis of SLe^x in cancer cells using per-acetylated 5-thio-L-fucose. Blockade of fucosyltransferases led to functionally significant impairments in SLe^x levels and selectin mediated adhesion (137). The above mentioned fucosylation relates to addition of fucose residues to the N- and O-linked terminus in an α 2,3 and/or 4 linkage pattern; however fucosylation of the core structure of N-glycans has also been reported to be altered in cancer cells (134). Core fucosylation is carried out by FUT-VIII and has been found to be elevated in breast, colon, ovarian and lung cancer (120, 138-142). The importance of fucosylation in the ability of cancer cells to migrate and metastasize therefore appears to be mainly related to their role in the synthesis of SLe^x and the implications of these changes for selectin ligands, targeting altered fucosylation in cancer cells is therefore an attractive therapeutic strategy given the importance of this process in cell adhesion and trafficking.

Section V

Mechanisms of regulation of glycosylation changes in cancer

Although it is known that glycans are altered in cancer, the mechanisms of regulation that govern the expression of the implicated genes are not well understood. It is likely that the genetic landscape of glycomics is not regulated by any one process but instead is an interplay of many factors, made more complex in the malignant state.

However progress in this area is being made and evidence is accumulating that these genes may be altered by hypoxic conditions in the local microenvironment or may also be regulated by methylation.

(V.i) Hypoxia and glycan expression

Under the poorly oxygenated conditions found in locally advanced tumors, hypoxia-resistant cancer cells survive by acquiring hypoxia tolerability through the HIF transcription factor, the nuclear translocation of which is facilitated by inactivation of tumor suppressors such as p53 (14). As mentioned above HIF induces transcription of several genes for glycan synthesis, leading to the significant alteration of glycan profiles, including enhanced sialyl Lewis^{x/a} expression in cancer cells (129).

Another very interesting study has also implicated hypoxia as a determinant of glycogene expression in colon cancer. Koike and colleagues demonstrated that hypoxic culture of colon cancer cells induced a marked increase in expression of selectin ligands, the SLe^x and SLe^a determinants at the cell surface, which led to a definite increase in cancer cell adhesion to endothelial E-selectin. HIF was increased in colon cancer where it induced transcription of four important glycoenzymes - FucT-VII (FUT7), sialyltransferase ST3GAL1, and UDP-galactose transporter-1 (UGT1), which are all known to be involved in the synthesis of the carbohydrate ligands for E-selectin (129).

(V.ii) Hypermethylation

DNA methylation and histone de-acetylation, the epigenetic mechanisms for suppression of normal gene transcription commonly observed in cancers, are proposed to underscore the aberrant expression of glycosylation related genes seen in some cancers (127). As previously mentioned, DNA hypomethylation leads to enhanced SLe^x production on MUC1 rendering colon cancer cells more favorable to liver metastasis. It has been shown that a change in cytosine methylation within the promoter of certain glycosylation related genes is responsible for the expression of cancer-associated carbohydrate antigens in gastrointestinal, colon, pancreatic, and breast cancer (143-145). Further work is needed to advance our understanding of the link between the cancer glycome and epigenetics.

Section VI

Clinical significance – diagnostics, prognostics and therapy:

Given the large body of evidence that has accumulated to definitively implicate changes in glycosylation in the development and progression of certain cancers, there has been a focus on clinically applicable glycan targeting for diagnostic and prognostic purposes (146). To date, this has taken the form of development of tumour-associated glycan markers as diagnostic and prognostic tools alongside a large focus on the development of vaccines in this area; however increasing attention is being focused on harnessing glycan specific changes in cancer to improve therapeutic strategies (20). This has led to variable success due to the inherent challenges faced when studying protein glycosylation related to the complexity and diversity of glycan structures and also, in the past, due to lack of reliable high throughput tools for detailed glycan analysis and profiling. An in-depth review of the technologies that are available or becoming available to evaluate and develop these markers is beyond the scope of this review, however there are several useful reviews of this topic (12, 20, 146). The mainstay of tools to study glycans remains the use of lectins that have an affinity for specific carbohydrate structures. Lectin based methods include immunohistochemistry, lectin blots, liquid chromatography and lectin microarrays. Despite much progress using lectins as analytical research tools, there has been an almost complete lack of clinically applicable high throughput tools to quantify serological glycan biomarkers.

Although several of the well-known “tumour markers” used clinically are glycoproteins, it has only been in recent years that these have been analysed for more specific glycoforms to increase the sensitivity and specificity of these tests. One such example is the identification of altered PSA glycosylation patterns in prostate cancer that can help to distinguish between significant and insignificant prostate tumours (147). Carbohydrate determinates on glycoproteins and glycolipids have been shown in the past to serve as useful serum diagnostic and prognostic markers in a variety of cancers. SLe^a is important in adhesion of colon, rectal and pancreatic cancer cells to the endothelium while SLe^x was found to play a role in adhesion of lung, breast and ovarian cancer cells (128).

Therapeutic strategies have, as already mentioned, started to focus on targeting cancer specific glycoforms of tumour associated proteins, However, given the accumulating

body of evidence implicating selectins, and their altered glycosylation, in cancer cell adhesion, migration and metastasis there is understandably a specific focus on this area in this literature. This is apparent in AML where it has been shown that the majority of primary patient AML blasts and leukaemia stem cells express an E-selectin ligand (148). E-selectin ligand expression appeared to be up-regulated in relapsed as compared to newly diagnosed patients. A glyco-mimetic selective E-selectin inhibitor, GMI-1271, was able to overcome adhesion mediated chemotherapy resistance of AML *invitro* and reduce the leukaemia burden of primary AML engrafted NODscid IL2Rgc^{-/-} mice in combination with chemotherapy agents daunorubicin and cytarabine. They found that adhesion of primary AML blasts to E-selectin caused upregulation of members of the Wnt and sonic hedgehog pathways, which could be inhibited by the small molecule E-selectin antagonist GMI-1271(148). Based on this data a phase I/II, trial of GMI-1271, as a chemo-sensitizing adjunct to standard chemotherapy in AML, is on-going and interim results have shown a promising complete remission rate of 45% in relapsed/refractory patients (149). Based on this high response rate GMI-1271 has received Fast Track designation from the US Food and Drug Authority in 2016.

Other approaches to inhibiting E-selectin:selectin ligand interactions include the use of sialyltransferase inhibitors and aptamers (150). Aptamers are oligonucleotide-based recognition molecules that have extraordinarily high sensitivity and selectivity towards their targets. First generation aptamers are currently in clinical trials as potential anti-cancer agents, anti-coagulants, anti-diabetic agents, and for treatment of macular degeneration, but so far only one aptamer (Macugen/pegaptanib) has been approved. Aptamers are selected using an *invitro* selection process, known as Systematic Evolution of Ligands by EXponential enrichment (SELEX) using an initial, highly diverse library of oligonucleotide sequences that was simultaneously developed by Gold and Szostak (151) (152). It is possible that DNA-based aptamers against the sialic acid *N*-acetylneuraminic acid (Neu5Ac) may have potential as an E-selectin inhibitors and remodelling of the glycome in myeloid cells using inhibitors of sialyl- and fucosyltransferases has already shown promise as it results in loss of selectin binding and impaired leukocyte rolling (153). Sialyltransferase inhibitors have also shown specific activity in cancer where they have been demonstrated to suppress tumour angiogenesis and cell metastasis in several models both *invitro* and

invivo (84) (154, 155).

The use of single lectins for specific detection of glycans associated with certain malignancies has been helpful in some cases such as the application of concanavalin lectin (ConA) and Wheat germ agglutinin lectin (WGA) reactivity to p185 in breast cancer (156) or the measurement of T-antigen in cervical cancer using peanut agglutinin lectin (PNA) (157). In the last decades the emergence of lectin arrays has made it possible to profile a glycoprotein and compare it with other samples in a high throughput manner. A variety of approaches have been used including application of lectins to an array for direct detection of glycans or lectin/antibody arrays where antibodies to potential glycoprotein markers are printed onto glass slides. These microarrays are hybridized against serum lectins to detect different glycan structural units on the captured glycoproteins in a sandwich assay format (158).

Mass spectrometry (MS) based methods remain the gold standard for identification and structural analysis of protein glycosylation. MS can also be used to quantify carbohydrates released from individual or multiple glycoproteins. This has been applied in breast cancer using a MALDI-MS based glycomic profile of permethylated glycans to detect biomarkers in patients' serum (1, 159). Focusing simply of glycosylation related genetic signatures in certain cancers has yielded interesting and potentially useful prognostic information. In MM distinct glycome of genes between normal and malignant plasma cells has been defined and this has been associated to distinct cytogenetic abnormalities in this disease (160).

As our understanding of the complex glycome of cancer cells increases glycome remodelling becomes an ever more attractive approach to manipulate the metastatic and immune evasion properties of these cells. Moving forward rapidly in this field requires additional advances in the reliable detection and quantification of glycan heterogeneity.

Summary

With recent advances in glyco-analytical technologies a greater understanding of the functional significance of seemingly minor changes in carbohydrate linkages on cell surface proteins and lipids has come to light. There has been renewed interest in glycosylation as a dynamic process that can evolve quickly and transiently to accommodate changes in the local microenvironment of the cell and facilitate adhesive and migratory interactions. Our understanding of changes in glycan determinants on cancer related proteins, such as mucins, selectin ligands and integrins, has uncovered a new layer of complexity, leading to a greater understanding of how this normal process is altered in cancer, and how these subtle alterations can have enormous implications for cancer cell metastasis, survival, proliferation and immune escape. This deeper understanding of the cancer glycome has led to the exploitation of glycosylation for therapeutic and prognostic applications in a wide array of solid and haematological malignancies and has the potential to greatly impact the field.

The current wave of novel emerging data in this field provides rationale for investigation of newly opened questions as well as revisiting of previously under-investigated topics using newly available tools. As mentioned, altered glycosylation of selectin ligands in cancer contributes to a metastatic phenotype, however much remains to be answered about the functional interplay of glycosyltransferase deregulation in the tumour microenvironment and research to date is just beginning to “scratch the surface”. In-depth understanding of carbohydrate remodelling in cancer will require detailed profiling of glycosylation patterns in the context of the tumour microenvironment.

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CHAPTER 3

THE SIALYLTRANSFERASE ST3GAL6 INFLUENCES HOMING AND SURVIVAL IN MULTIPLE MYELOMA

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Abstract

Glycosylation is a stepwise procedure of covalent attachment of oligosaccharide chains to proteins or lipids, and alterations in this process, especially increased sialylation, have been associated with malignant transformation and metastasis. The role of altered sialylation in MM cell trafficking has not been previously investigated. In the present study we identified high expression of beta-galactoside alpha-2, 3-sialyltransferase, ST3GAL6, in MM cell lines and patients. This gene plays a key role in selectin ligand synthesis in humans through the generation of functional SLe^X. In MRC-IX patients, high expression of this gene is associated with inferior overall survival. In this study we demonstrate that knockdown of ST3GAL6 results in a significant reduction in levels of alpha 2,3 linked sialic acid on the surface of MM cells with an associated significant reduction in adhesion to MM bone marrow stromal cells and fibronectin along with reduced trans-endothelial migration *invitro*. In support of our *invitro* findings we demonstrate significantly reduced homing and engraftment of ST3GAL6 knockdown MM cells to the bone marrow niche *in vivo* along with decreased tumour burden and prolonged survival. This study points to the importance of altered glycosylation, particularly sialylation, in MM cell adhesion and migration.

Introduction

Cell trafficking, glycosylation and sialyltransferases in Multiple Myeloma

MM is characterized by the presence of multiple lesions, indicating continuous trafficking of tumour cells to distant bone marrow niches (1, 2). The first step in cellular trafficking is the deceleration of blood cells on target endothelium to below the local blood flow rate, a process known as rolling (3). While rolling, leucocytes are activated by binding to selectins and by chemokines like CXCL12, leading to integrin activation, adhesion and subsequent migration. Selectin-based adhesion is mediated by the binding of selectins on endothelial cells to sialo-fucosylated proteins or lipids on selectin ligands. P selectin glycoprotein ligand 1 (PSGL-1) is highly expressed in MM and can bind to both E and P-selectin and the potential therapeutic benefit of selectin inhibition in MM has been demonstrated (4).

Functional selectin ligands require post-translational modification of scaffold proteins by glycosyltransferases and sulfotransferases (Fig. 1A). The minimal recognition motif for all selectins is sLe^x and its isomer sLe^a, which are tetrasaccharides that are synthesized by the combined action of alpha 1,3-fucosyltransferases, alpha 2,3-sialyltransferases, 1,4-galactosyltransferases and N-acetyl-glucos-aminyltransferases. Enhanced expression of sLe^x and sLe^a structures is frequently associated with cancer and elevated levels of sialyltransferases have been linked to increased risk of metastatic disease in solid tumours (5).

With this background we examined the altered expression of glycosylation related genes in MM and demonstrate, using *invitro* and *invivo* model systems, that knockdown of ST3GAL6 attenuates MM cell adhesion and migration *invitro* and reduces the ability of these cells to home to the bone marrow *invivo* resulting in a reduction in tumour burden and prolonged survival of xenograft mice. This study highlights the important role of SLe^x modifying enzymes in alterations of cell trafficking in MM.

Methods

Gene expression analysis

We performed gene set enrichment analysis (GSEA) to determine if the glycosylation related gene sets were enriched in MM patients [Fonseca et al GSE6477, (6)]. GSEA was performed following the developer's protocol (<http://www.broad.mit.edu/gsea/>)(7). Analysis of publically available gene expression data (GSE 6477) and data which was provided to us by collaborators (MRC-IX trial data (8)) was carried out to examine the expression of ST3GAL6 in MM patients and healthy controls. Where available, we examined the association between high levels of expression of ST3GAL6 and patient outcomes. Survival data was generated using Kaplan Meier survival analysis and Log Rank Test (GraphPad Inc, CA, USA)

Cell lines & primary cells

The RPMI-8226 and MM1S cell lines were purchased from ATCC (Manassas VA) and have been authenticated by Molecular Diagnostics Laboratory Research Services at Dana Farber Cancer Institute. Primary MM cells were obtained from bone marrow aspirates of individual MM patients using CD138+ micro-bead selection (Miltenyi Biotech, Auburn CA) as previously described (9). Bone marrow stromal cells (BMSCs) were obtained from healthy donor and MM patient fresh bone marrow samples and isolated as described (10). Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza. All human MM cell lines and BMSCs were cultured as in previous studies (4, 9). Green-fluorescent-protein and luciferase-positive (GFP-Luc+) MM1S cells were generated using lenti-virus infection, as previously described (11). Calcein-AM was obtained from Invitrogen (Carlsbad, CA). For hypoxia experiments, cells were cultured in 1% O₂ for 24 hours. Informed

consent was obtained from all patients in accordance with the Declaration of Helsinki. These studies were approved by the Dana-Farber Cancer Institute Institutional Review Board and the Research Ethics Committee, Galway University Hospital.

DNA synthesis assay & cell cycle analysis

Proliferation rate of MM1S cells (scrambled control and shST3GAL6 cells) were measured by cell counting using trypan blue exclusion and DNA synthesis using [3H]-thymidine uptake (Perkin Elmer, MA, USA) as previously described (12). For cell cycle analysis MM cells were stained with propidium iodide (PI; Sigma-Aldrich, MO, USA) and cell cycle was determined using flow cytometry as previously described (12). Cell survival was measured using MTT assays.

Flow cytometry

Expression of alpha 2,3 sialic acid at the cell surface was analysed by flow cytometry using biotinylated Maackia amurensis (MAA) lectin (Vector Labs, CA, USA) conjugated to streptavidin APC (BD Biosciences, NJ, USA). Expression of HECA-452 was analysed by flow cytometry using Cutaneous Lymphocyte Antigen (BD Biosciences, NJ, USA).

shRNA mediated ST3GAL6 gene silencing

To determine the role of ST3GAL6 in MM biology we established ST3GAL6 knockout RPMI-8226 and MM1S-GFP-Luc cell lines using a lenti-viral system as previously described (13). The efficiency of ST3GAL6 knockdown was assessed by real time quantitative PCR (qRT-PCR) using the following primers Forward Primer: TTG CCT CTC TGC TGA GGT TT, Reverse Primer: CCT CCA TTA CCA ACC ACC AC (14). The resultant stable ST3GAL6 knockdown (shST3GAL6) cell line was compared to the scrambled control cell line in all subsequent functional assays. The sense and antisense oligonucleotide sequence for construction of ST3GAL6 shRNA were as follows: clone no 10402; [NM_006100.2-1332s1c1, target sequence CCTTTGCACTACTATGGGAAT (clone A2), NM_006100.2-1110s1c1 target sequence CCAGCCTTAAACCTGATTTAT (clone A3)].

Adhesion assays

Assays for adhesion of MM cells to HUVECs and MM BMSCs were performed using 96-well plates. HUVECs or MM BMSCs (5×10^3 cells/well) were cultured to confluence overnight in 96-well plates to establish a monolayer. MM cells were serum starved for 4 hours, pre-labelled with calcein AM, added to the HUVECs or BMSCs and allowed to adhere for 2 hours at 37°C. Non-adherent cells were aspirated off, HUVECs or BMSCs were washed with PBS, and fluorescence intensity was measured using a fluorescent-plate reader (Ex/Em _ 485/520 nm). Fibronectin adhesion assays were performed using an *in vitro* adhesion assay plate coated with fibronectin following the manufacturer recommendations (EMD Biosciences, San Diego, CA) as previously described (15).

Trans-endothelial migration assays

Migration assays were performed as previously described (4). Briefly, HUVECs (5×10^3 cells/well) were incubated overnight in the upper chambers of transwell migration assay plates (pore size 8 μ m; Corning Life Sciences, Acton, MA). Migration ability of MM scrambled control cells vs. shST3GAL6 cells was assessed following 4 hours of serum starvation. MM cells were pre-labelled with calcein AM and then placed in the upper migration chambers. 500 μ L of BMSC conditioned DMEM media was added to the lower chambers. After 4 hours at 37°C cells that migrated to the lower chambers were counted on a fluorescent plate reader (Molecular Devices, USA).

Rolling assays

Rolling assays were performed using an *invitro* flow chamber assay system (Cellix, Dublin). In brief, MM cells (2×10^6 /ml, 40 μ L/channel) were pipetted into individual channels of p-selectin coated chips (15 μ g/ml) and shear stress of 1dyne.cm² was applied with live frame capture every 0.5 seconds for a minimum of 30 frames per repeat.

Immunoblotting

Immunoblotting was performed as previously described (16). Briefly, whole-cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad

Laboratories, Hercules, CA). The antibodies used for immunoblotting included anti-ST3GAL6 antibody (Sigma-Aldrich, MO, USA), anti-phospho-Src (p-Src; Tyr 416), anti-phospho-cofilin (p-cofilin) and anti-phospho-paxilin (p-paxilin) with anti-actin or anti- α -tubulin (Cell Signaling Technology, Danvers, MA) used as protein loading controls.

Immunohistochemistry

Bone marrow biopsies were obtained from 6 MM patients, 3 MGUS patients and 3 healthy subjects. Consecutive serial sections cut from each FFPET block were used for immunohistochemistry (IHC). Four-micrometre sections were cut onto poly-lysine slides, allowed to dry overnight at 40°C, dewaxed in xylene, and rehydrated through graded alcohol to water. Rabbit polyclonal anti-ST3GAL6 antibody was obtained from ATLAS antibodies (Stockholm, Sweden). Full details of the IHC protocol are available in the supplemental methods. Slides were dual stained with mouse monoclonal anti-human-CD138 (BD Biosciences, Dublin) and polyclonal rabbit anti-humanST3GAL6 antibody. For detection of HECA-452 expression bone marrow biopsies were dual stained with rat IgM, kappa anti-human HECA-452 (BD Pharmingen) and CD138.

Animals

Approval for animal studies was obtained from Dana-Farber Cancer Institute and Massachusetts General Hospital Institutional Animal Care and Use Committees. Female, 6 to 7 weeks old, severe combined immuno-deficient beige (SCID-Bg) mice were obtained from Taconic Laboratories. Anaesthesia was performed by intra-peritoneal injection of ketamine/xylazine (Lloyd Laboratories, Shenandoah, IA) at 12mg/kg body weight prior to *in vivo* imaging sessions. At study endpoints mice were sacrificed by inhalation of CO₂. For survival studies mice were followed until development of hind limb paralysis at which time they were sacrificed according to our institutional animal protocol.

Xenograft models

Tumours were established in mice using MM1s-GFP-Luc⁺ cells (5×10^6 / mouse), which were injected into the tail vein of SCID-Bg mice (n=3/group for homing, n = 12 for tumour burden of which 3/group were also assessed for engraftment, n = 14 for survival)

Bioluminescent imaging (BLI)

To detect tumour burden on a weekly basis mice were anesthetized and injected with 75 mg/kg luciferin (Xenogen, Hopkinton, MA) and imaged for bioluminescence 5 minutes after the injection.

***In vivo* confocal microscopy**

Using the SCID-Bg xenograft mice, homing and engraftment of MM cells to distant bone marrow niches was tracked *in vivo*, by using *in vivo* confocal microscopy (4, 17, 18). Briefly, MM cells that homed to the bone marrow 18 hours following IV injection, or engrafted 4 weeks following injection, were imaged *in vivo* using a Zeiss 710 confocal system (Carl Zeiss Microimaging, Jena, Germany) on an upright examiner stand with a custom stage. A skin flap was made in the scalp of the mice to expose the underlying dorsal skull surface. High-resolution images with cellular detail were obtained through the intact mouse skull at depths of up to 250µm from the surface of the skull using a 10x 0.45NA Plan-Apo objective (Carl Zeiss Microimaging). GFP was excited with the 488nm line on an Argon laser. Blood vessels were imaged using Evans Blue (100µL IV) (Sigma-Aldrich, St. Louis, MO) excited with a 633 nm laser. Emission signals were collected by the Zeiss internal confocal Quasar detectors.

Statistical analysis

Statistical differences between experimental groups were analysed using GraphPad Prism (GraphPad Software, CA, USA). T-tests were used to compare means (2-tailed; α 0.05). P values less than 0.05 were considered significant. Kaplan-Meier survival probability curves were constructed to compare groups graphically and Log-Rank analysis was used to test for significant differences in survival.

Results

GSEA confirms over-expression of glycosylation related signatures in multiple myeloma

Enrichment of glycosylation related signatures in MM were demonstrated by GSEA (7) using an MSigDB gene set and GSE6477. (Fig. 1B, heat map of the entire gene set Fig. S1). The false discovery rate (FDR) q-value was 0.24 and normalized enrichment score 1.14, with FDR values < 0.25 considered significant (7). ST3GAL6 emerged from this analysis as being one of the most significantly increased genes in MM patients compared to healthy donors, as shown in the heat-map in Figure 1C. Moreover further analysis of GSE6477 demonstrates significantly higher levels of ST3GAL6 expression as the disease progresses from MGUS through smouldering disease, to newly diagnosed MM (Fig. 1D & Table 1).

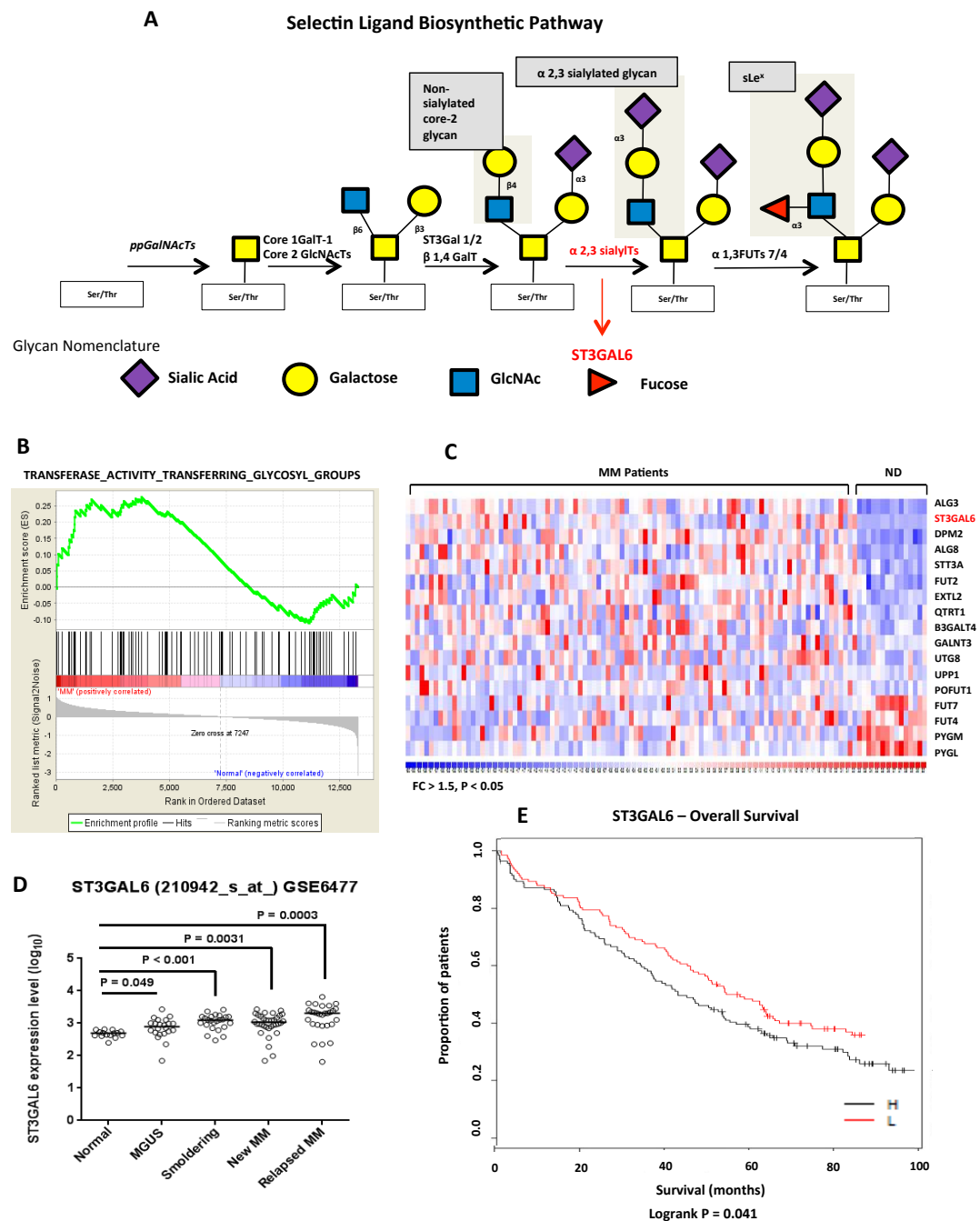


Figure 1. Glycosylation gene expression in MM. (A) Selectin ligand biosynthetic pathway, sialyltransferases, including *ST3GAL6*, transfer sialic acid from the activated cytidine 5'-monophospho-N-acetylneuraminic acid to terminal positions on sialylated glycolipids (gangliosides) or to the N- or O-linked sugar chains of glycoproteins. *ST3GAL6* contributes to the formation of selectin ligands and *sLe^x*, a carbohydrate, which is important in cell-cell recognition. (B) Gene Set Enrichment Analysis (GSEA) software analysed glycosylation related genes in MM patients from the previously published dataset GSE6477. Demonstrated graphically is enrichment of a publically available MSigDB

gene set (*TRANSFERASE_ACTIVITY_TRANSFERRING_GLYCOSYL_GROUPS*) in MM patients (left, red) in comparison to healthy controls (blue, right). False discovery rate (FDR) = 0.24 and Normalized Enrichment Score (NES) = 1.14. (C) Heatmap demonstrating significantly altered glycosylation gene expression pattern in MM patients vs. normal donors (ND) with fold change (FC) greater than 1.5 and $P < 0.05$. ST3GAL6 is highlighted in red as one of the most significantly enriched glycosylation genes in MM patients. (D) Analysis of ST3GAL6 expression in the GSE6477 dataset showing significantly increasing expression of ST3GAL6 with disease progression. Comparison of ST3GAL6 expression in healthy donors (Normal) to that in patients with MGUS, smouldering MM, newly diagnosed MM and relapsed MM shows increasing expression levels of this gene ($P = 0.049$, < 0.0001 , 0.0031 , 0.0003 , respectively, see Table 1). (E) Kaplan Meier survival proportions analysis of patient outcome data from the MRC-IX trial demonstrating high levels of ST3GAL6 expression associated with inferior overall survival; Low (L) = 48mths, High (H) = 36mths, Log Rank $P = 0.041$. $N(H) = 129$, $N(L) = 130$.

High expression of ST3GAL6 is associated with inferior survival in multiple myeloma patients

To further validate the influence of this gene in MM patients we assessed the effect of expression on survival of patients using GEP data from the MRC IX trial (Fig. 1E). Patients expressing higher levels of ST3GAL6 had a significantly reduced OS compared to those with lower expression levels (48 vs. 36 months, respectively, Log Rank $P = 0.041$). On multivariate analysis ST3GAL6 is an independent predictor of reduced OS after adjustment for known prognostic variables HR = 1.47 (Table 2). Subset analysis revealed that the adverse effect on survival of high ST3GAL6 expression was restricted to patients who did not receive either high dose therapy or thalidomide ($P = 0.0071$, $P = 0.0173$, respectively) (Fig. S2).

ST3GAL6 is expressed at high levels in CD138+ cells from MM patients

Comparing the expression of ST3GAL6 in primary CD138+ cells from MM patients in comparison to healthy donors we noted significantly higher levels of ST3GAL6 in newly diagnosed (n=3, $P = 0.0001$) and relapsed disease (n=3, $P < 0.0001$) with the highest levels of expression seen in patients with secondary plasma cell leukaemia (n=2) (Figure 2A, $P = <0.0001$). Immunohistochemistry staining for ST3GAL6 protein was carried out comparing healthy donors to that of MGUS (n=3) and MM (n=6) patients (Fig. 2B). This revealed ST3GAL6 expression in MGUS and MM cases, which was not apparent in healthy donors (Fig 2B). In some cases of MM, showing high levels of ST3GAL6, there were corresponding high levels of staining with the monoclonal antibody HECA-452 which binds to sLe^x and reacts with both Cutaneous Lymphocyte Antigen (CLA) and Hematopoietic cell E-/L-selectin ligand (HCELL) (19) (Fig 2C).

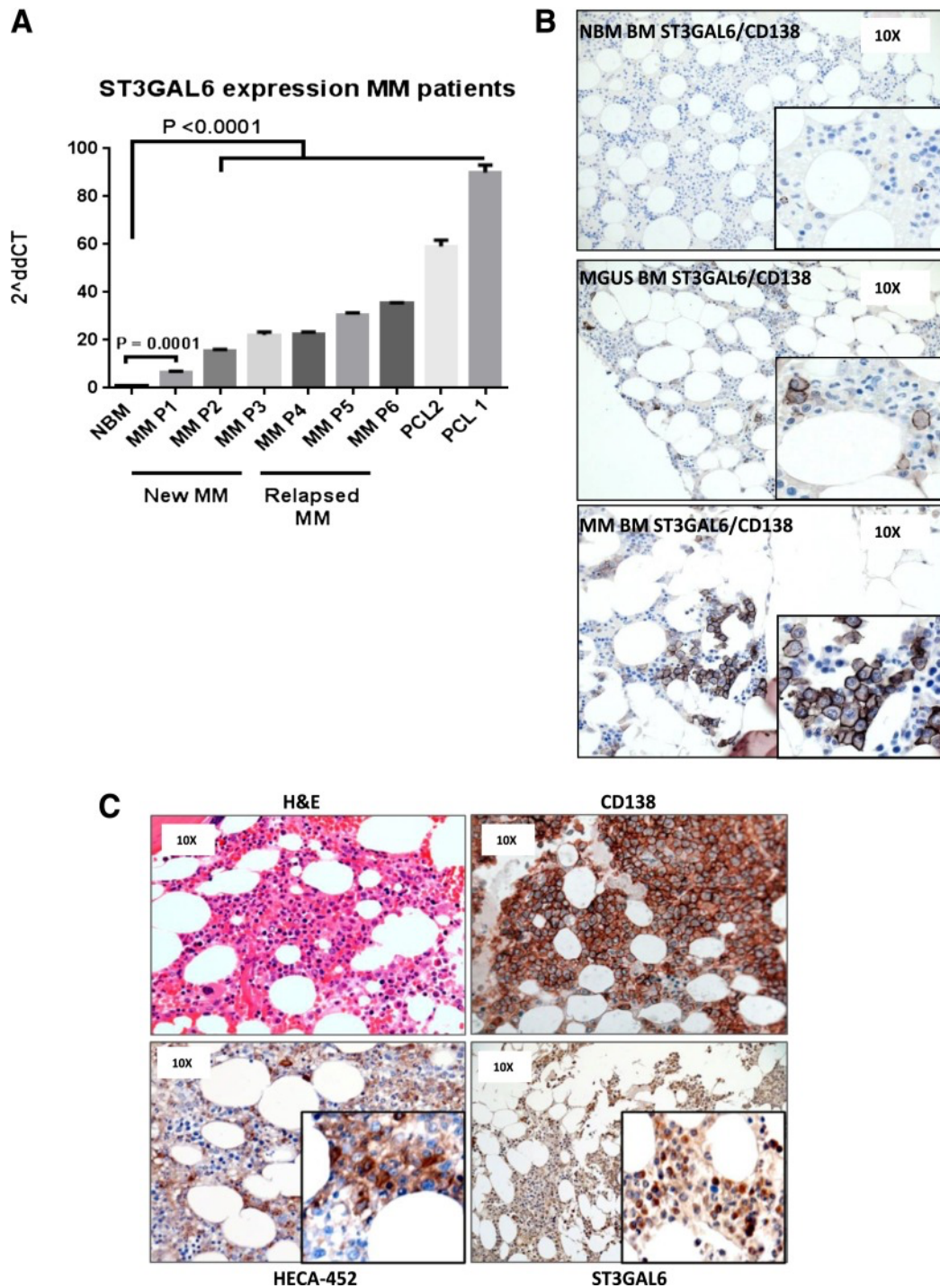


Figure 2. ST3GAL6 expression MM patients. (A) qRT-PCR demonstrating expression levels of ST3GAL6 mRNA isolated from CD138⁺ cells of MM patients; newly diagnosed (New MM) $n = 3$, relapsed MM $n=3$, or plasma cell leukaemia patients (PCL) $n=2$ in comparison to CD138⁺ cells isolated from normal healthy bone marrow (NBM). Newly diagnosed patients had a significantly higher level of ST3GAL6 mRNA and patients with relapsed MM and PCL demonstrated increasingly higher levels with disease progression; NBM vs. P1 $P = 0.0001$, NBM vs. all other patients $P < 0.0001$. (B) Immunohistochemistry (IHC): Dual staining for CD138 and ST3GAL6 demonstrating minimal expression of ST3GAL6/CD138 in healthy donor bone marrow (NBM, top panel, $N=3$). Positive expression of ST3GAL6 was noted in patients with MGUS (middle panel, $n= 3$) and in MM patients (bottom panel, $n=3$). ST3GAL6 cytoplasmic brown staining, CD138 membranous dark brown/black staining, blue counterstain.

(C) IHC 4 panels from an MM patient (n=6) demonstrating H&E (top left), CD138 (top right), HECA-452 (bottom left), which recognizes sialo-fucosylated glycans and SLe^x, and ST3GAL6 (bottom right), this demonstrated in this patient with high ST3GAL6 expression there is a corresponding high expression of HECA-452.

ST3GAL6 is expressed at high levels in MM cell lines

Screening of 5 MM cell lines for ST3GAL6 expression (MM1S, MM1R, U266, RPMI-8226 and H929) demonstrated significantly higher levels of ST3GAL6 mRNA and protein in comparison to healthy CD138⁺ cells (Fig. 3A & B). Expression of alpha 2,3 sialic acid, synthesis of which is contributed to by ST3GAL6, was also demonstrated to be higher at the surface of these MM cell lines in comparison to CD138⁺ cells from healthy donor bone marrow using flow cytometry for MAA lectin (Fig. 3C). Hypoxia is known to induce expression of sialyltransferases in cancer, to test if this may be occurring in MM we assessed gene expression following 24 hours of hypoxic culture, this demonstrated induction of ST3GAL6 in MM cell lines (Fig. S3F).

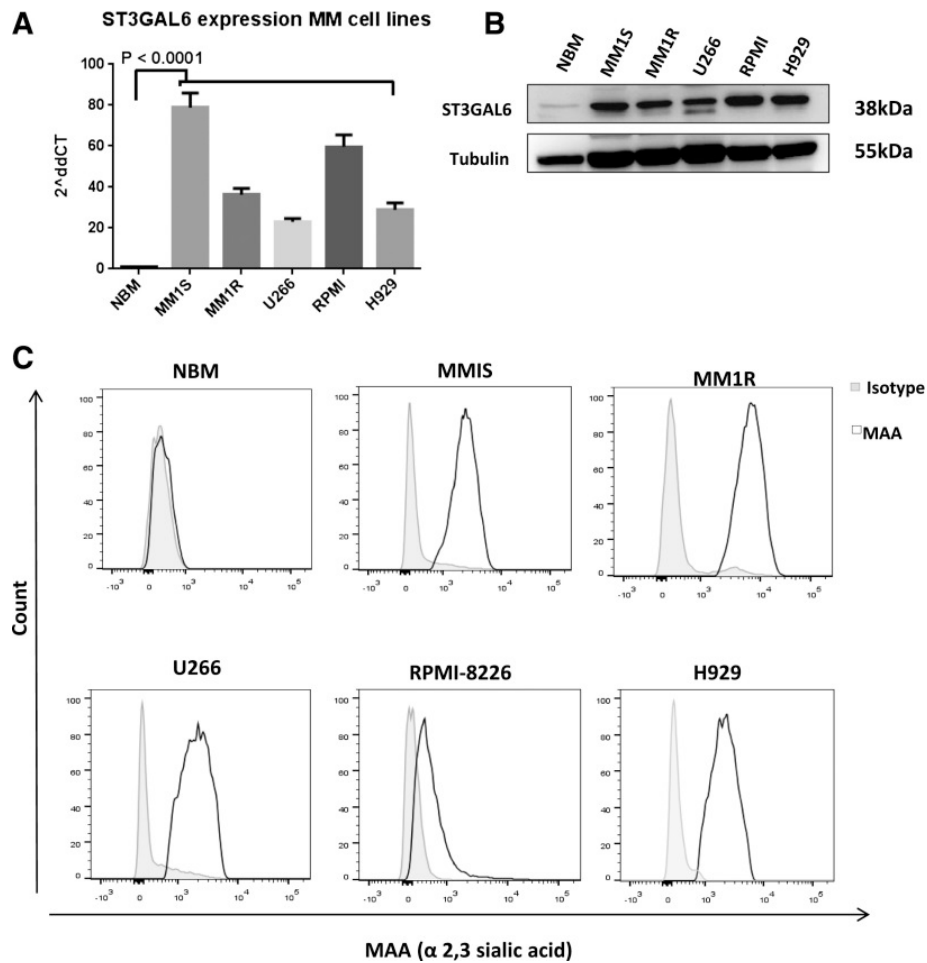


Figure 3. ST3GAL6 expression MM cell lines. (A) ST3GAL6 expression MM cell lines; qRT-PCR for ST3GAL6 gene expression in MM cell lines MM1S, MM1R, U266, RPMI-8226 (RPMI) and H929 in comparison to CD138 cells isolated from healthy donor bone marrow (NBM) $p < 0.0001$. (B) Western blot demonstrating expression level of ST3GAL6 protein in CD138+ cells isolated from the bone marrow of healthy controls (NBM) compared to MM cell lines MM1S, MM1R, U266, RPMI8226, H929. (C) Flow cytometry demonstrating surface expression of MAA lectin, which binds to α 2,3 sialylated glycans, at the surface of MM cell lines MM1S, MM1R, U266, RPMI8226, H929 in comparison to CD138+ cells isolated from healthy donor bone marrow (NBM). MAA is synthesized by ST3GAL6 and presence of this lectin at the cell surface is indicative of ST3GAL6 activity in these cells.

Knockdown of ST3GAL6 in MM cell lines

To investigate the functional role of ST3GAL6 in MM we performed lenti-viral mediated knockdown in two MM cell lines, MM1S and RPMI-8226. The efficiency of this knockdown is demonstrated in Figure 4 using qRT-PCR (4A) and immunoblotting (4B). Knockdown of ST3GAL6 in MM cell lines resulted in a significant reduction in the amount of alpha 2,3 sialic acid at the surface of the cells (Fig. 4C, MM1S Scrambled vs. MM1S A2 clone $P = 0.0006$, RPMI-8226 Scrambled vs. RPMI-8226 A2 clone $P = 0.001$), indicating that in these cells ST3GAL6 is contributing to the synthesis of this glycan, which is a component of selectin ligands. A proportion of MM cells from the MM1S and RPMI-8226 cell lines demonstrate positive staining for HECA-452, this was absent in the ST3GAL6 knockdown cells (Comparison of MFI's: MM1S Scrambled vs. MM1S clone A2 $P = 0.014$, Scrambled vs. MM1S clone A3 $P = 0.0059$; RPMI Scrambled vs. RPMI-8226 clone A2 $P = 0.005$, Scrambled vs. RPMI-8226 clone A3 $P = 0.001$.), importantly this indicates reduced reactivity with CLA and HCELL which are sialo-fucosylated glycoforms of PSGL-1 and CD44, respectively, which can bind E-selectin (Fig. 4D). Knockdown of ST3GAL6 did not affect MM cell proliferation, as assessed by thymidine uptake or cell counting methods (Fig. S3A & B), and there was no effect on cell cycle or apoptosis (Fig. S3 D & E)

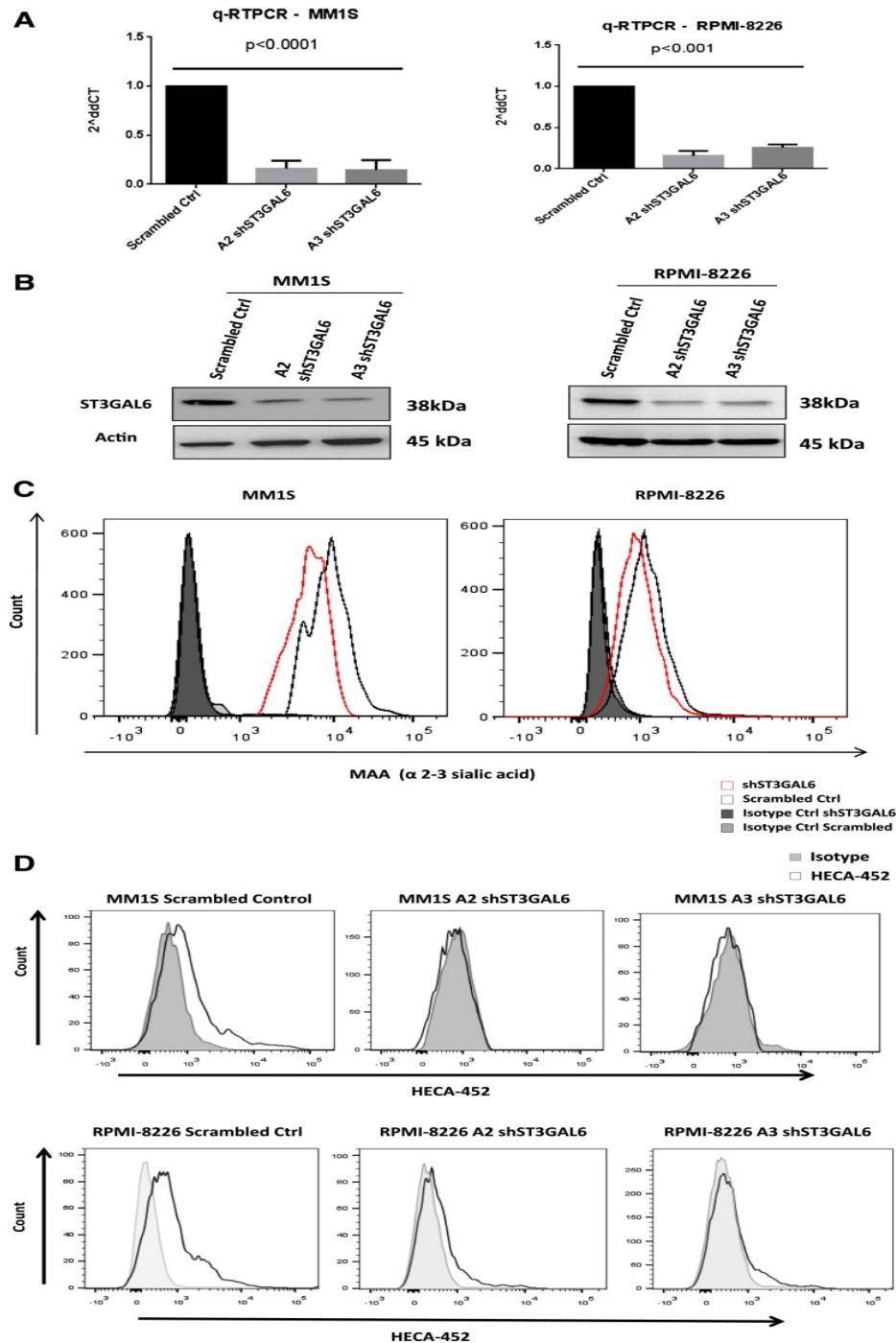


Figure 4. ST3GAL6 Knockdown MM cell lines. (A) qRT-PCR demonstrating efficiency of lenti-viral mediated knockdown of ST3GAL6 in MM1S (left) and RPMI-8226 (right) cell lines vs. corresponding scrambled controls. shST3GAL6 MM1S cells vs. scrambled control $P < 0.0001$, shST3GAL6 RPMI-8226 cells vs. scrambled controls $P < 0.001$. (B) Western blot for ST3GAL6 protein expression in MM1S and RPMI-8226 cell lines in comparison to scrambled controls demonstrating significant reduction of protein expression in knockdown cell lines. (C) Flow cytometry demonstrating reduced expression of MAA lectin on MM1S and RPMI-8226 cells following the knockdown of ST3GAL6 in these cell lines. Comparison of MFI's: MM1S Scrambled vs. MM1S A2 clone $P = 0.0006$, RPMI-8226 Scrambled vs. RPMI-8226 A2 clone $P = 0.001$. (D) Flow cytometry demonstrating reduced expression of HECA452 on MM1S and RPMI-8226 shST3GAL6 cells in comparison to scrambled control cells. Comparison of MFI's: MM1S Scrambled vs. MM1S clone A2 $P = 0.014$, Scrambled vs. MM1S clone

A3 $P = 0.0059$. RPMI Scrambled vs. RPMI-8226 clone A2 $P = 0.005$, Scrambled vs. RPMI-8226 clone A3 $P = 0.001$.

Knockdown of ST3GAL6 in MM cell lines reduces adhesion to MM BMSCs, HUVECs and fibronectin

Given the potential for knockdown of ST3GAL6 to result in an alteration of carbohydrate-mediated interactions with endothelial cells, and components of the bone marrow microenvironment, we performed *invitro* adhesion assays. A significant reduction in adhesion to BMSCs was demonstrated for MM1S and RPMI-8226 shST3GAL6 clones A2 and A3 (Fig. 5A, $P = 0.0263$, 0.0286 , 0.0023 , 0.003 , respectively). A reduction in adhesion to fibronectin-coated plates was also seen in shST3GAL6 cells (Fig. 5B) (MM1S $P = 0.0003$, RPMI-8226 $P < 0.0001$). We also assessed the ability of shST3GAL6 cells to adhere to endothelial cells. Knockdown of ST3GAL6 in both MM1S and RPMI-8225 cells resulted in reduced MM cell adhesion to HUVEC's *in vitro* in comparison to scrambled control cells Fig 5C (MM1S; $P = 0.0003$, < 0.0001 for clones A2 and A3 respectively; RPMI-8226; $P = 0.01$, $P = 0.003$ for clones A2 and A3 respectively).

ST3GAL6 influences MM cell trans-endothelial migration

Migratory ability was also affected in shST3GAL6 MM cells in comparison to scrambled controls, as shown by a reduced ability of these cells to migrate to BMSC conditioned media in a trans-endothelial cell migration assay incorporating HUVECs (Fig. 5D). When compared to scrambled control cells, MM1S and RPMI-8226 shST3GAL6 cells had a reduced ability to migrate (MM1S; Clone A2 vs. scrambled control $P < 0.01$, A3 $P < 0.01$; RPMI-8226 clone A2 $P = 0.0003$, A3 $P = 0.0001$).

ST3GAL6 knockdown attenuates Src activation in MM cells.

In order to assess the effect of ST3GAL6 knockdown on adhesion and migration related proteins we performed western blots following co-culture of shST3GAL6 MM1S cells or scrambled controls with primary BMSCs from MM patients. ST3GAL6 knockdown attenuated Src and paxilin activation with a corresponding increased phosphorylation and inactivation of cofilin (Fig. 5E).

Ability of MM cells to roll on p-selectin is impacted by ST3GAL6 knockdown.

Given the potential impact of a reduction in the availability of carbohydrate epitopes on selectin binding we assessed the ability of ST3GAL6 knockdown cells to roll on p-selectin *invitro*. There was a modest but significant reduction in the ability of shST3GAL6 cells to roll on p-selectin in comparison to scrambled control cells (P = 0.02 Fig. 5F).

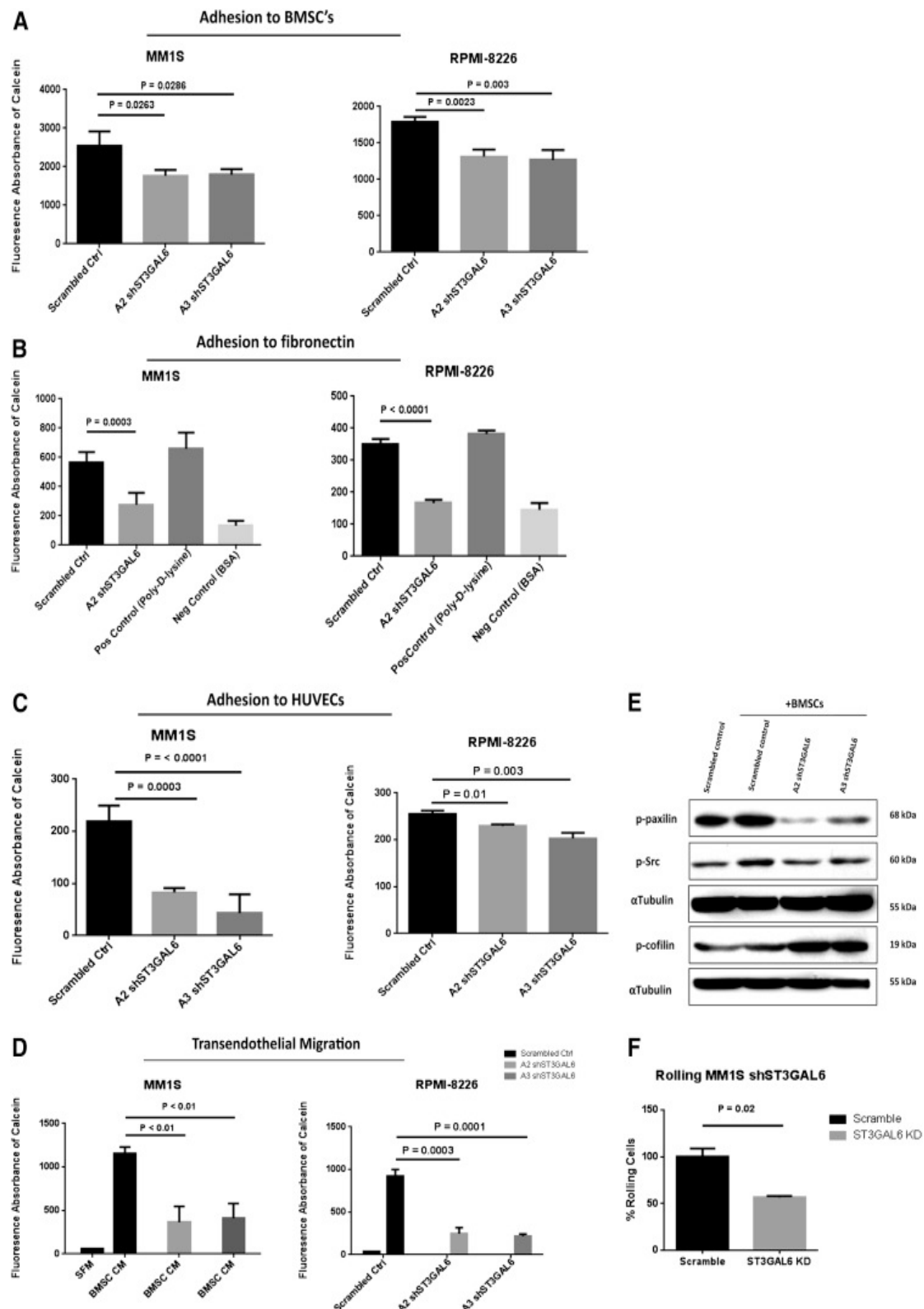


Figure 5. In vitro functional effect of ST3GAL6 knockdown in MM cell lines.

(A) Effect of ST3GAL6 knockdown on the ability of MM1S and RPMI-8226 cells to adhere to BMSC's isolated from the bone marrow of MM patients. MM1S clone A2 $P = 0.0263$, clone A3 $P = 0.0286$. RPMI-8226 clone A2 $P = 0.0023$, clone A3 $P = 0.003$. (B) Adhesion of ST3GAL6 knockdown cells to fibronectin invitro in comparison to corresponding scrambled control cells. MM1S A2 clone $P = 0.0003$, RPMI-8226 $P < 0.0001$. Similar results were obtained for MM1S and RPMI-8226 A3 clones,

data not shown. (C) Adhesion of MM1S and RPMI-8226 shST3GAL6 cells to HUVEC's in comparison to scrambled control cells. Both shST3GAL6 knockdown cells had a significantly reduced ability to adhere to HUVEC's invitro. MM1S clone A2 $P = 0.0003$, clone A3 $P = <0.0001$. RPMI-8226 clone A2 $P = 0.01$, clone A3 $P = 0.003$. (D) Migration of ST3GAL6 knockdown cells to BMSC conditioned media (BMSC CM) in a trans-endothelial migration assay. The ability of ST3GAL6 knockdown cells to achieve trans-endothelial migration was significantly reduced in comparison to scrambled control cells. MM1S clone A2 $P < 0.01$, clone A3 $P < 0.01$. RPMI-8226 clone A2 $P = 0.0003$, A3 $P = 0.0001$. (E) Western blot demonstrating reduced levels of adhesion related proteins in MM1S shST3GAL6 cells, which were co-cultured with the BMSC's from MM patients for 24 hours. Phosphorylated paxilin was reduced in shST3GAL6 in comparison to scrambled control cells cultured in the presence of BMSC's. Phosphorylation of cofilin was greater in the MM cells co-cultured with BMSC's. We also noted an induction of p-SRC in scrambled control cells upon co-culture with BMSC's, this induction was not apparent in the shST3GAL6 cells. α -tubulin was used as a loading control. (F) Rolling of shST3GAL6 cells on p-selectin in comparison to scrambled control cells demonstrates a reduced ability of these cells to roll on p-selectin ($P = 0.02$).

Knockdown of ST3GAL6 reduces MM cell homing and engraftment *in vivo*

Given the potential for knockdown of ST3GAL6 to affect functions of selectin ligands, and based on our *in vitro* data showing reduced adhesive and migratory properties of these cells, we assessed the ability of shST3GAL6 cells to home to the bone marrow *in vivo* and to engraft. Mice were assessed for the presence of MM cells the bone marrow of the skull 18 hours following injection of MM1S shST3GAL6 GFP+ cells or scrambled control cells (5×10^6 cells/mouse) using *in vivo* confocal microscopy (Fig. 6A). We noted a reduction in homing of shST3GAL6 GFP+ cells in the skull bone marrow of mice 18 hours following IV injection in comparison to mice that had received scrambled control cells, representative images are shown in Fig. 6B ($n=3$ /group). This reduction in homing was statistically significant for shST3GAL6 cells Fig 6C. $P = 0.0015$). Mice were again evaluated after 4 weeks in order to demonstrate sustained attenuation of colonization and engraftment of shST3GAL6 cells at this site. We noted a marked reduction in the presence of shST3GAL6 GFP+ cells in comparison to scrambled control cells indicating that the initial reduction in homing translated to fewer cells reaching the bone marrow and a reduced tumour burden over time. Representative images are shown in Fig. 6B ($n=3$ /group). This reduction in engraftment was statistically significant (Fig 6E. $P < 0.0001$).

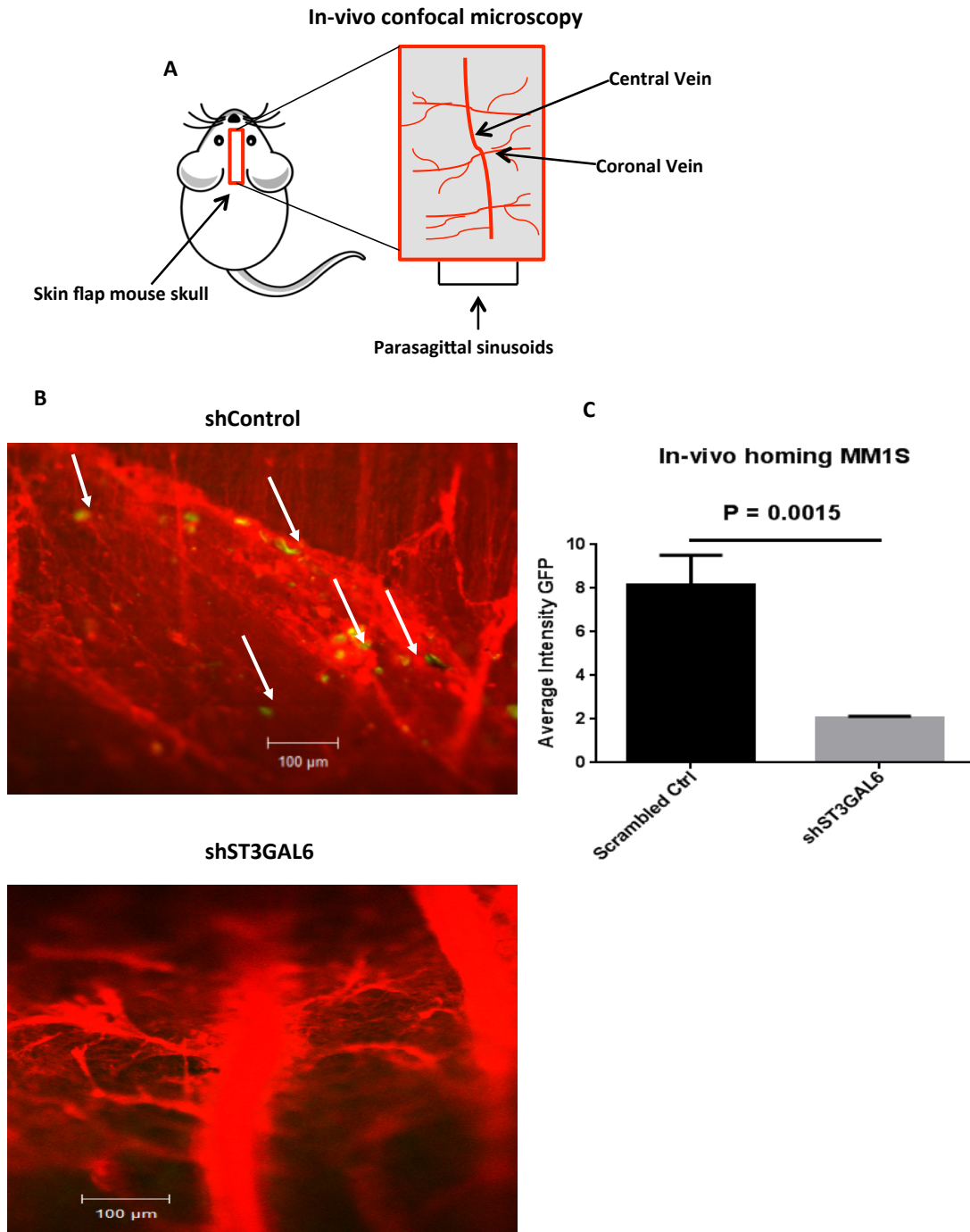


Figure 6. In-vivo ST3GAL6 knockdown. (A) Graphical representation of the anatomical region of the mouse skull examined using con-focal microscopy. (B) Representative invivo confocal microscopy images of scrambled control MM1S-GFP⁺ cells demonstrating homing to the bone marrow of SCID-Bg mice 18 hours following tail vein injection of 1×10^6 cells (left panel). In these mice GFP⁺ cells were visible within the vessels and in the surrounding bone marrow niche indicating their homing ability. The panel on the right demonstrates the corresponding vessels in mice that received shST3GAL6 cells; in these mice there was no evidence of GFP⁺ cells arriving to the bone marrow indicating an impaired ability of these cells to home. Images are representative of $n=3$ /group. (C) Quantification of invivo homing of shST3GAL6 or scrambled control cells GFP⁺ cells to the bone marrow invivo. shST3GAL6 cells demonstrated reduced homing ability $P = 0.0015$. (D) Representative

invivo confocal microscopy images demonstrating a reduction in engraftment and tumour growth of ST3GAL6 knockdown cells in the bone marrow niche of the skull 4 weeks following intravenous injection of 5×10^6 shST3GAL6 or scrambled control cells (n=3/group) (E) Quantification of invivo engraftment of shST3GAL6 cells vs. scrambled controls. shST3GAL6 cells demonstrated significantly reduced engraftment $P < 0.0001$.

Knockdown of ST3GAL6 decreases MM tumour burden and increases survival *invivo*

To examine the effect of ST3GAL6 knockdown on tumour progression *invivo* SCID-Bg mice (n=6/group) were injected with MM1S shST3GAL6 Luc + cells or scrambled control cells (5×10^6 cells/mouse) via tail vein and underwent weekly BLI to assess tumour growth. Mice receiving shST3GAL6 cells demonstrated a statistically significant reduction in tumour burden (Figures 7A & 7B, $P=0.005$). Following completion of the *invivo* study qRT-PCR of RNA extracted from MM cells isolated from the bone marrow of the mice confirmed preservation of knockdown of ST3GAL6 (Fig. S3G). A separate study was carried out to evaluate the effect of ST3GAL6 knockdown on the survival of xenograft mice (n=7/group) and Kaplan Meier analysis was performed (Fig. 7C) demonstrating prolonged survival for mice receiving shST3GAL6 cells vs. scrambled control cells. (Log Rank $P= 0.0014$).

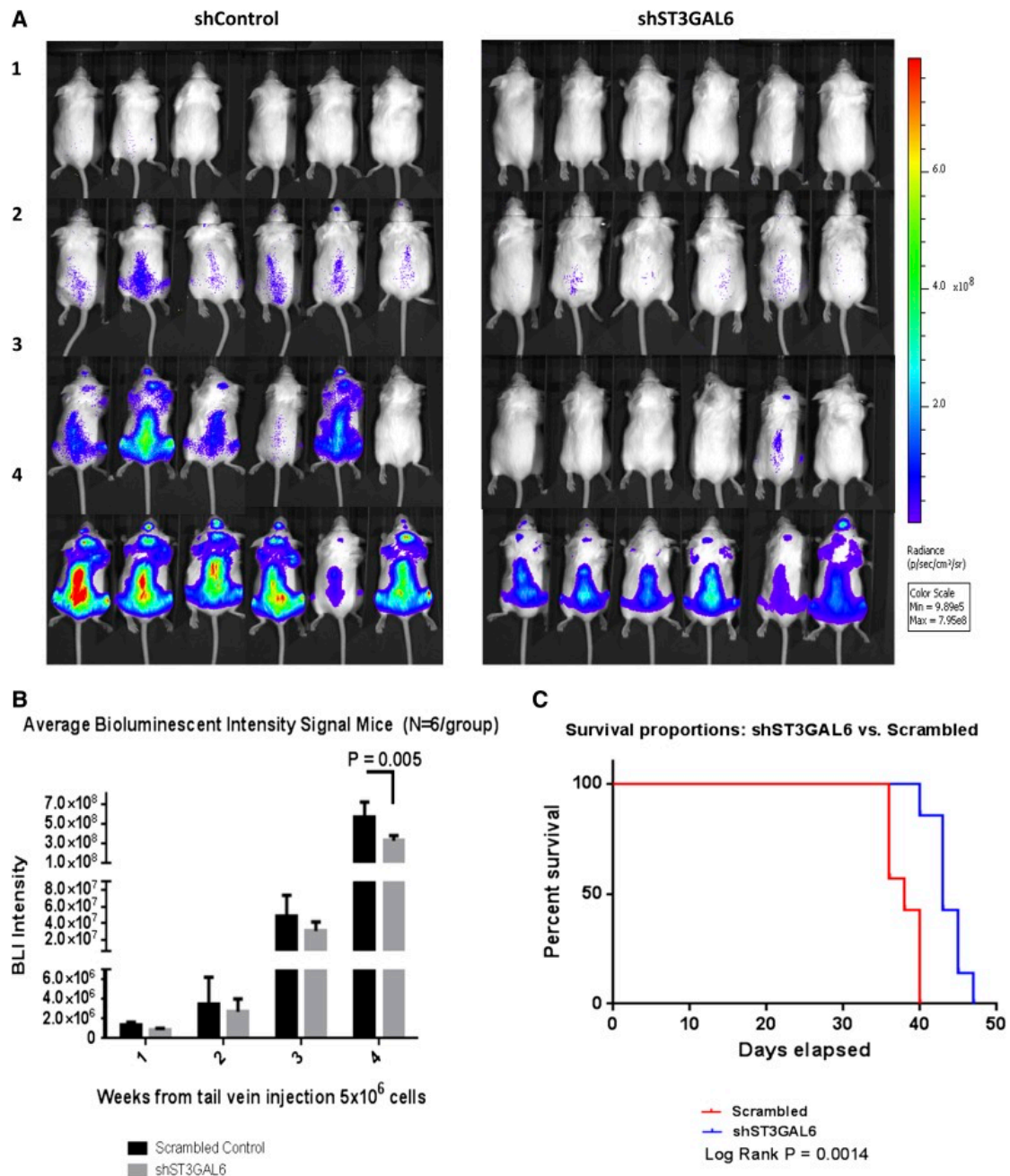


Figure 7. Effect of ST3GAL6 knockdown on tumour burden and survival in mice. (A) Tumour burden as assessed by bioluminescent imaging - weeks shown are 1, 2, 3 and final imaging at week 4 post injection of 5×10^6 cells ($n=6/\text{group}$). A reduction in tumour burden was noted in mice that received shST3GAL6 cells in comparison to scrambled controls. (B) Average BLI signal was quantified over the course of the 4-week study. This demonstrated a statistically significant reduction in tumour burden for mice that received shST3GAL6 cells in comparison to scrambled controls cells. $P = 0.005$. (C) Kaplan Meier survival probability curve; the effect of knockdown of ST3GAL6 in MM cells on survival of engraft mice was evaluated in a separate survival study. Mice injected intravenously with 5×10^6 shST3GAL6 cells had a superior survival to mice injected with scrambled control cells ($n=7/\text{group}$) Log Rank $P = 0.0014$.

Discussion

Until recently there has been little focus on the role of glycosylation in the biology and disease progression of MM. However, as in several solid malignancies (20-23), there is accruing evidence that altered glycosylation is important in MM (24-26). We were particularly interested in identifying alterations in glycosylation with the potential to impact cell trafficking in MM, given the crucial role that this process is known to play in cell-cell interactions and haematogenous metastasis of cancer cells (27). We report enrichment of glycosylation genes in MM and identified the sialyltransferase ST3GAL6 as being highly expressed. Knockdown of ST3GAL6 in our study resulted in a reduction in the adhesion and migratory abilities of MM cells *in vitro*, which was supported by a reduced ability of these cells to home to the bone marrow *in vivo*.

The sialyltransferases are enzymes that transfer sialic acid from the activated cytidine 5'-monophospho-N-acetylneuraminic acid (CMP-NeuAc) to terminal positions on sialylated glycolipids (gangliosides) or to the N- or O-linked sugar chains of glycoproteins (Fig. 1A). Studies performed in knockout mice, lacking either ST3GAL4 or ST3GAL6, have uncovered a critical role for ST3GAL6 in the generation of functional selectin ligands in mice (28). Indeed, in murine neutrophils deletion of ST3GAL6 results in a modest reduction only in MAA binding, along with a moderate reduction in both E and P selectin ligand expression. Our data suggests that ST3GAL6 may play a more critical role in the generation of E-selectin ligands in human MM cells. That we only see a modest reduction in MAA binding is likely due to on-going production of alpha 2,3 sialic acid by ST3GAL4, which may be incorporated into selectin ligands along with deposition of alpha 2,3 sialic acid on other glycoproteins and glycolipids by other alpha 2,3 sialyltransferases.

Selectin ligands, such as PSGL-1 have recently shown to play an important role in the pathobiology of MM (4, 21, 22). Studies of selectins and their ligands have highlighted the importance of posttranslational glycosylation in the process of leucocyte tethering, rolling and trans-endothelial migration, which are central to cellular trafficking (29-31). PSGL-1 is the predominant physiologic ligand for P-selectin and L-selectin, but when modified by HECA-452 reactive glycans (CLA) it can also serve as an E-selectin ligand (24). Posttranslational modifications of PSGL-1

are important for optimal selectin binding; to bind to P-selectin, PSGL-1 requires an alpha 2,3-sialylated and alpha 1,3-fucosylated core 2 O-glycan and to bind to E-selectin, PSGL-1 requires core 2 alpha 1,3-fucosylated and alpha 2,3-sialylated O-glycans, indicating a crucial role for ST3GAL6 in the function of PSGL-1 as both an E and P-selectin ligand (32, 33).

Therefore, our *invivo* findings of reduced tumour burden and prolonged survival of mice receiving shST3GAL6 cells may be related to a reduced ability of these cells to traffic to the bone marrow as a result of a reduction in the availability of functional selectin ligands in these cells. Alongside the potential effect on P-selectin binding, the reduced homing and engraftment of shST3GAL6 cells in the bone marrow *invivo* may be influenced by the loss of E-selectin binding, since CLA can serve as an E-selectin ligand and E-selectin is constitutively expressed by the bone marrow microvasculature (34). Loss of PSGL-1 in mice leads to moderate reduction in rolling on E-selectin *invivo*, while PSGL-1-deficient hematopoietic progenitors have reduced E-selectin-dependent bone marrow homing, thus E-selectin could conceivably play a dominant role trafficking of sLe^x expressing MM progenitors in the bone marrow niche (35, 36).

The mechanisms leading to increased ST3GAL6 gene expression in MM are unclear. It has previously been demonstrated in colon cancer that hypoxia induces expression of glycosylation genes resulting in an increase in selectin ligand expression and adhesion to endothelial selectin (37). In keeping with this finding we noted have noted an induction of ST3GAL6 expression in MM cells following hypoxic culture. It appears from our *invitro* data that silencing ST3GAL6 primarily mediates changes in migration and adhesion, in support of this we noted reduced phosphorylation of various proteins involved in adhesion and migration, including p-Src and p-Paxilin, this is in keeping with previous studies where sialyltransferase inhibition was shown to reduce the activation of adhesion related signalling in lung and breast cancer (38).

ST3GAL6 over-expression may have potentially important clinical implications. In our analysis of the MRC-IX GEP data we noted a reduced OS for patients with high levels of ST3GAL6 expression in the non-intensive treatment arm and for patients who did not receive thalidomide, this could potentially indicate that treatment intensification or the use of thalidomide or other immuno-modulatory drugs may be beneficial in patients with high levels of ST3GAL6, however further studies will be needed to address this question. Finally, patients with high levels of ST3GAL6, and or

other sialyltransferases involved in selectin ligand synthesis, may benefit from potential future therapeutic strategies such as the use of selectin or sialyltransferase inhibitors with the ultimate aim of preventing dissemination and achieving superior outcomes for MM patients (4, 38-40).

Tables

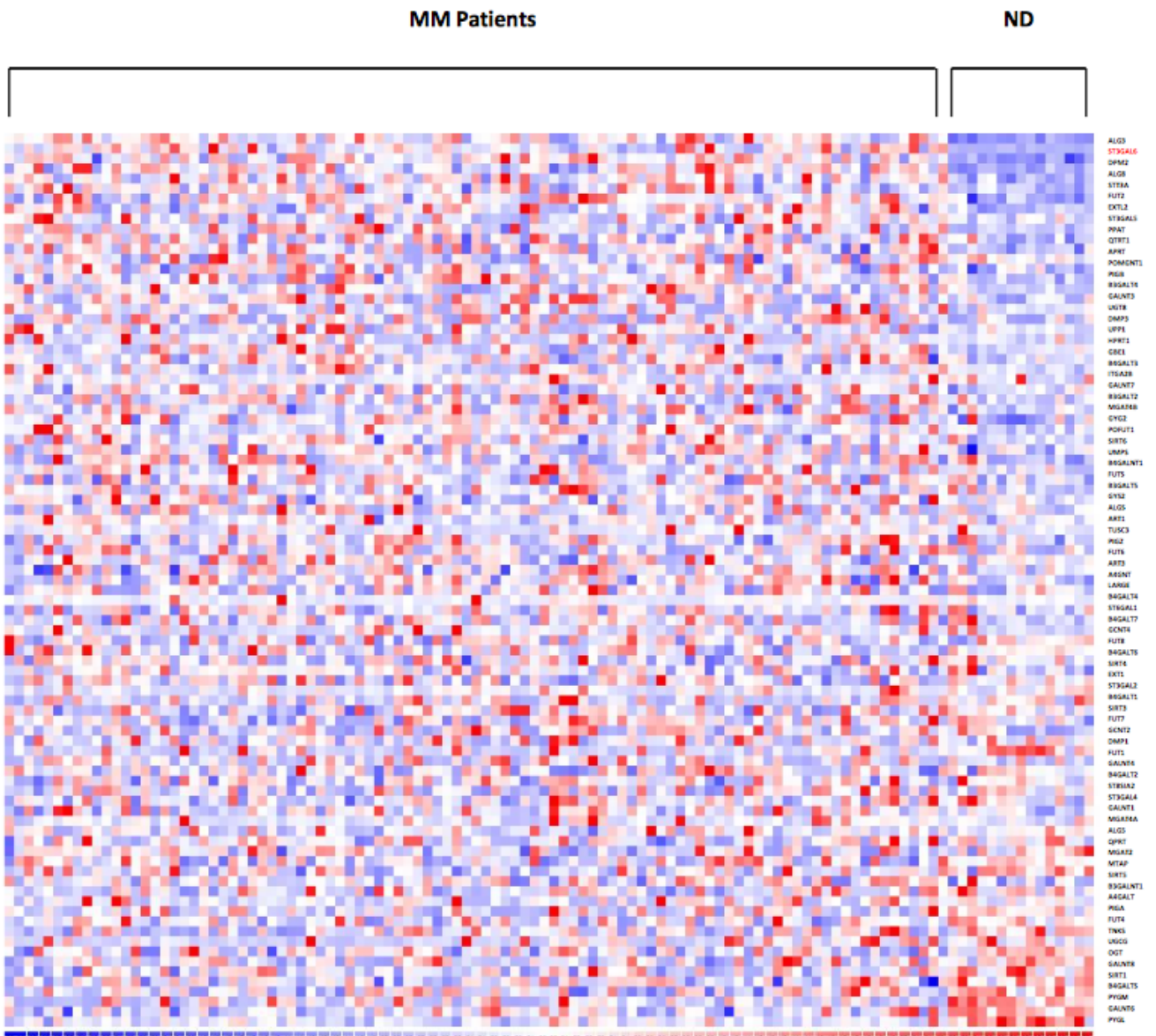
Table 1

	p-value	95% CI
Healthy vs. MGUS	0.049	(0.0008498 to 0.3576)
Healthy vs. Smouldering	< 0.0001	(0.2308 to 0.4988)
Healthy vs. New MM	0.0031	(0.1059 to 0.4901)
Healthy vs. Relapsed MM	0.0003	(0.2388 to 0.7260)

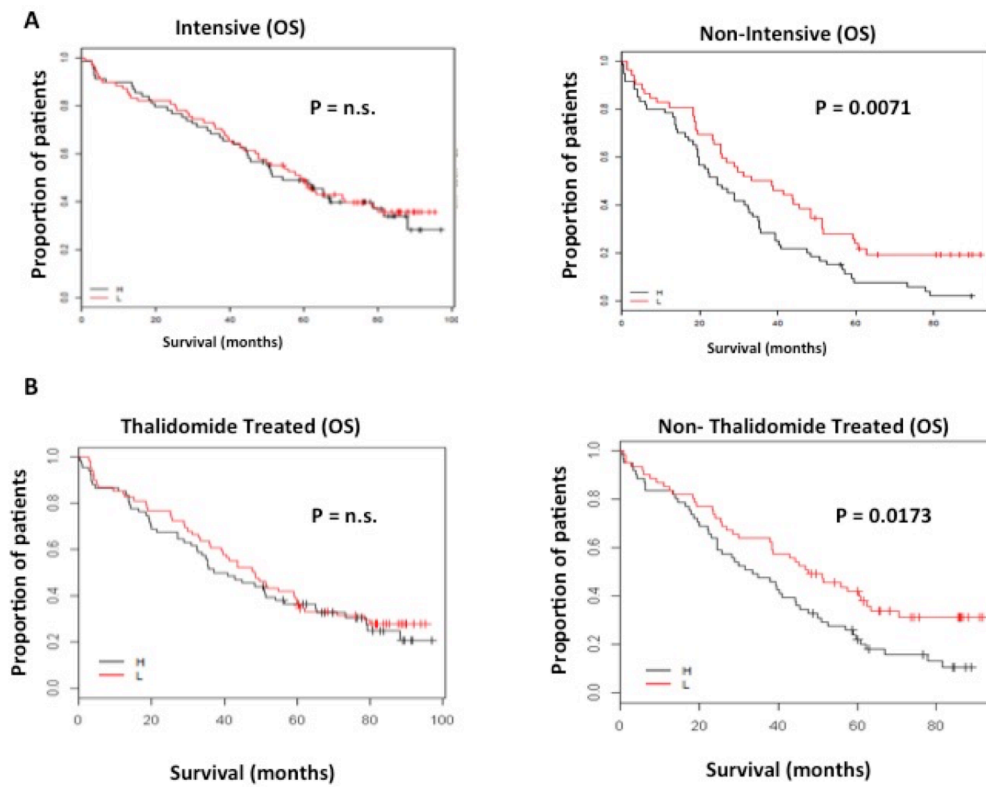
Table 2

	HR	HR-95% CI	p-value
High ST3GAL6	1.47	1.01-2.13	0.04
ISS stage	1.5	1.20-1.87	<0.001
Adverse IgH	1.5	1.0-2.37	0.05
Gain 1q	1.5	1.05-2.28	0.03

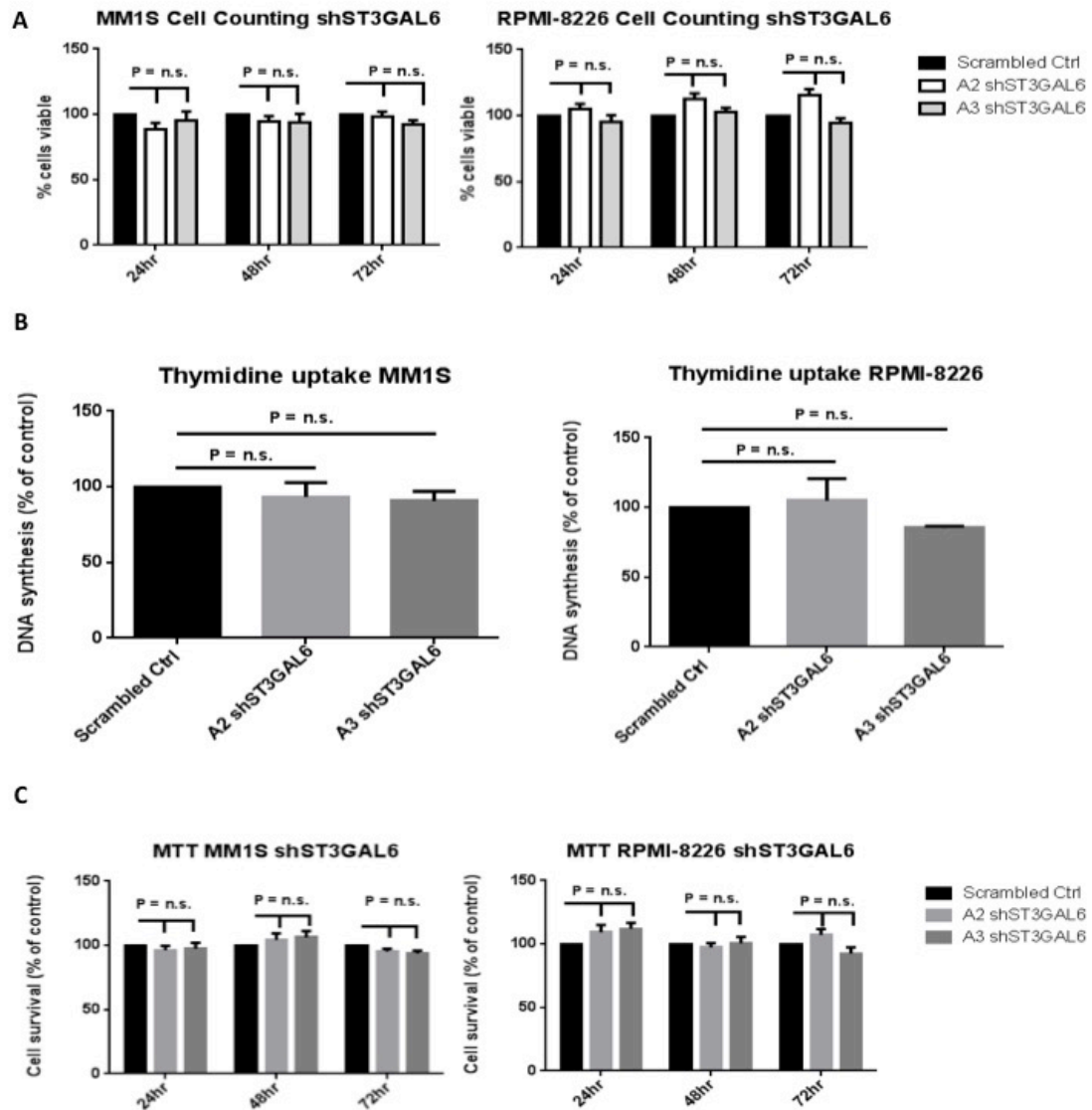
Supplemental Figures



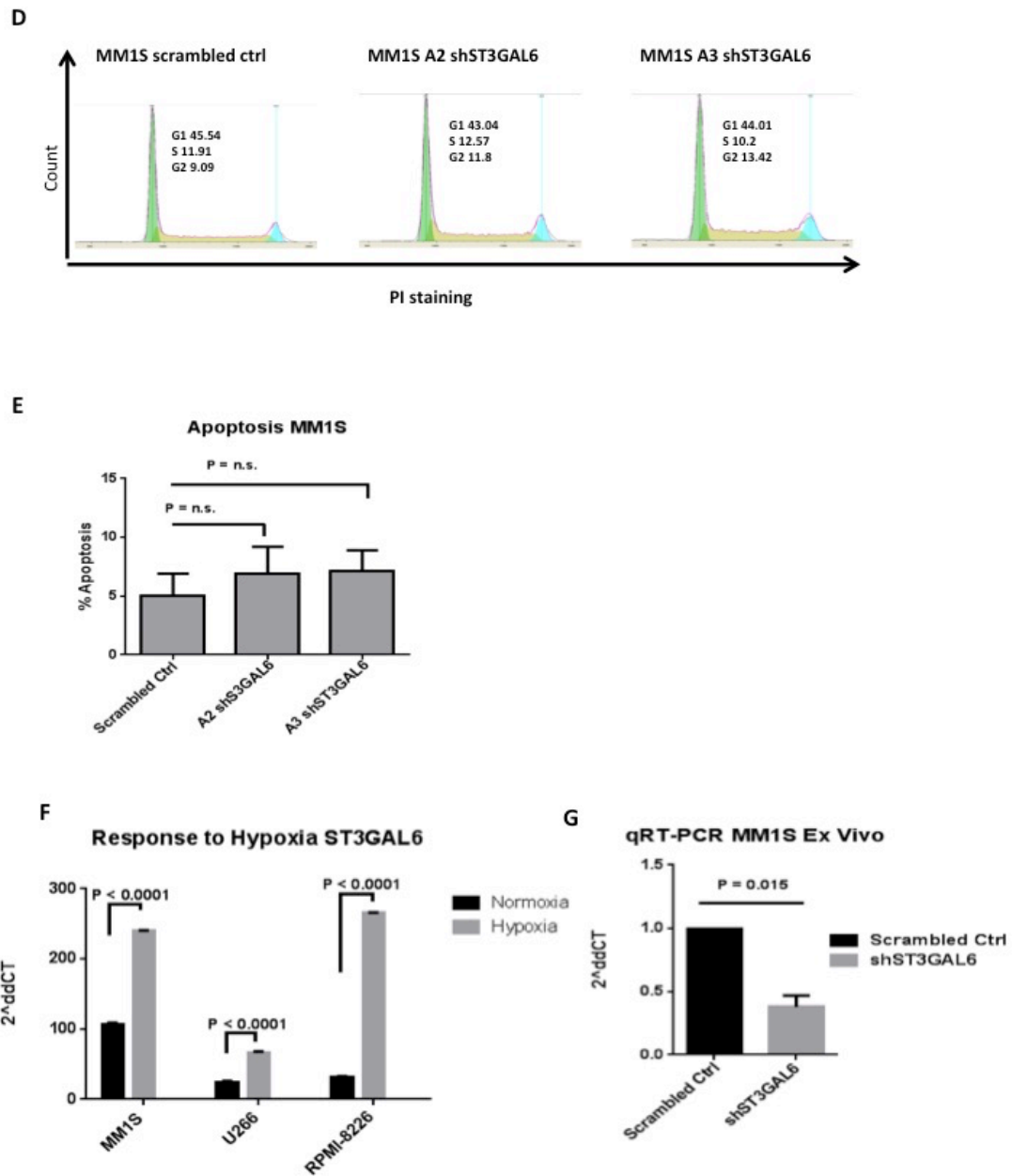
Supplemental Figure 1: Heatmap of entire MSigDB gene set signature in the GSE6477 dataset.



Supplemental Figure 2. OS according to ST3GAL6 expression for MRC-IX arms.



Supplemental Figure 3 continued



Supplemental Figure 3. Cell proliferation DNA synthesis, cell cycle analysis, apoptosis, response to hypoxia and ex-vivo-qRT-PCR.

Supplemental figure legends:

Supplemental Figure 1: Heatmap of entire MSigDB gene set signature in the GSE6477 dataset.

Heat Map and global gene expression analysis: Unsupervised hierarchical clustering of glycan gene expression. Individual patient samples are shown in columns and genes in rows. Expression is displayed in red color and blue color depending on expression above or below median expression level. Color intensity is dependent on the degree of deviation from median. Sample type is highlighted above the heatmap; left side MM patients, right side Normal Donors (ND)

Supplemental Figure 2. OS according to ST3GAL6 expression for MRC-IX arms.

(A) Intensive and non-intensive arm patient median OS according to ST3GAL6 expression, using median intensity expression values as cutoff; Median OS H = 54mths, L = 59.1mths, N high = 69, N low = 78, P = n.s. Non Intensive OS Median H = 24.4, L = 36.5, N high = 60, N low = 52, P = 0.0071

(B) Thalidomide treated and non-thalidomide treated patient median OS according to ST3GAL6 expression, using median intensity expression values as cutoff; (months); H = 39.1, Median L = 48.3, N high = 58, N low = 69, P = n.s. Non-Thalidomide treated Median OS; H = 33.4, Median L = 47.6, N high = 61, N low = 61, P = 0.0173

Supplemental Figure 3. Cell proliferation DNA synthesis, cell cycle analysis, apoptosis, response to hypoxia and ex-vivo-qRT-PCR

(A) Cell counting by trypan blue exclusion comparing shST3GAL6 MM cell lines to their respective controls demonstrates that knock down of ST3GAL6 does not induce a change in proliferation in these cell lines at 24, 48 and 72 hours.

(B) DNA synthesis assay comparing shST3GAL6 cells to their respective scrambled controls. No significant difference in [3H]-thymidine uptake was noted for MM1S or RPMI-8226 knockdown cells in comparison to scrambled controls at 24hours.

(C) MTT assay demonstrating no changes in MM cell survival following knockdown of ST3GAL6 at 24, 48 and 72 hours.

(D) Cell cycle analysis. No significant difference in cell cycle phase proportions was noted between MM1S shST3GAL6 cells and scrambled control cells.

(E) Apoptosis as assessed using flow cytometry shows no change between shST3GAL6 MM MM1S cells and scrambled controls.

(F) Response of ST3GAL6 expression in MM cell lines MM1S, U266 and RPMI-8226 to hypoxia. A significant induction of ST3GAL6 expression is noted in all three cell lines. $P < 0.0001$ for all cell lines in comparison to their respective normoxic controls.

(G) qRT-PCR comparing expression of the ST3GAL6 gene in shST3GAL6 cells recovered from the bone marrow of mice following sacrifice at the end of *in vivo* study, this demonstrates sustained knockdown of ST3GAL6 in comparison to cells isolated from the bone marrow of scrambled control mice $P = 0.015$.

Supplemental methods

Immunohistochemistry:

Bone marrow biopsies were obtained from 6 MM patients, 3 MGUS patients and 3 healthy subjects. Four-micrometer sections were cut onto polysine slides, allowed to dry overnight at 40°C, dewaxed in xylene, and rehydrated through graded alcohol to water.

Single antigen labeling

Heat-induced epitope retrieval (HIER) was performed using a microwave oven in 10mM citrate buffer pH 6.0 (7.5 min. for ST3GAL6 and CD138). After HIER the slides were allowed to cool at RT, endogenous peroxidase activity was inactivated by incubation with 0.3% H₂O₂ in methanol for 40 min.; and then blocked with blocking solution (1% BSA and 2% FBS in PBS) for 60 min at RT. The sections were incubated OVN with each antibody diluted in blocking solution using the following concentrations: ST3GAL6 (rabbit polyclonal antibody, ATLAS antibodies #HPA018792, Lot#R07790 at 0.4mg/ml, dilution 1:100), CD138 mouse monoclonal (Clone CRCT2.1, AbD serotec #MCA2459GA, batch 0410 at 0.1mg/ml, dilution 1:100). The sections were washed with PBS and incubated in ImPress universal Anti-Mouse/Anti-rabbit antibody reagent (Vector. Catal # MP-7500) for 30 min. Additional washing with PBS was performed. Immuno-reactivity was visualized by incubation of sections with ImmPACT DAB peroxidase substrate (Vector Catal# SK-4100). Sections were counterstained with light Mayer's hematoxylin, dehydrate in

alcohol, cleared in xylene and mounted in DPX. MT-TMA sections were stained to control tissue specificity and non-specific binding or non-specific background staining by sources other than primary antibody; either with or without primary antibody (negative control). Stomach was used as an internal control for ST3GAL6. It contains tissue elements either strongly expressing the antigen (i.e., glandular stomach cells) other tissue elements no expressing the antigen (i.e., fibroblasts). Skin was used as internal control for CD138 with stratified squamous epithelium expressing the antigen. Protein expression was assessed using a light microscope and photographs taken using Olympus BX61 Microscope.

Double antigen labeling

Heat induced epitope retrieval (HIER) was performed using a microwave oven in 10mM citrate buffer pH 6.0 for 7.5 min. After HIER the slides were allowed to cool at RT, endogenous peroxidase activity was inactivated by incubation with 0.3% H₂O₂ in methanol for 40 min.; and then blocked with blocking solution (1% BSA and 2% FBS in PBS) for 60 min at RT. The sections were incubated overnight with ST3GAL6 (rabbit polyclonal antibody, ATLAS antibodies #HPA018792, lot#R07790 at 0.4mg/ml, dilution 1:100 in blocking solution). The sections were washed with PBS and incubated with ImPress universal Anti-Mouse/Anti-rabbit antibody reagent (Vector. Catal # MP-7500) for 30 min. Immuno-reactivity for ST3GAL6 was visualized by incubation of sections with NovaRed (Vector SK-4800). This was followed by washes with PBS, peroxidase blocking with 0.3% H₂O₂ in methanol for 40 min and blocking solution (1% BSA and 2% FBS in PBS) for 60 min at RT. The sections were incubated overnight with CD138 mouse monoclonal (Clone CRCT2.1, AbD serotec #MCA2459GA, batch 0410 at 0.1mg/ml, dilution 1:100), followed by PBS washes and incubation with the secondary antibody (ImPress universal Anti-Mouse/Anti-rabbit antibody reagent) for 30 min at RT. Immuno-reactivity for CD138 was visualized by incubation of sections with DAB+Ni (Vector SK-4100). Sections were counterstained with light Mayer's hematoxylin, dehydrate in alcohol, cleared in xylene and mounted in DPX. Consecutive sections of a MT-TMA were used to control tissue specificity and one section was incubated without primary antibody as a negative control. Glandular stomach cells were strongly stained and were used a control of positivity and tissue specific for ST3GAL6. Protein expression was

assessed using a light microscope and photographs taken using Olympus BX61 Microscope.

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CHAPTER 4

PROTEOMIC CHARACTERIZATION OF THE MULTIPLE MYELOMA BONE MARROW EXTRACELLULAR MATRIX

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Reference Proteomic characterization of human multiple myeloma bone marrow extracellular matrix.

Glavey SV, Naba A, Manier S, Clauser K, Tahri S, Park J, Reagan MR, Moschetta M, Mishima Y, Gambella M, Rocci A, Sacco A, O'Dwyer ME, Asara JM, Palumbo A, Roccaro AM, Hynes RO, Ghobrial IM.

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Abstract

The ECM is a major component of the tumour microenvironment, contributing to the regulation of cell survival, proliferation, differentiation and metastasis. In MM, interactions between MM cells and the bone marrow microenvironment, of which the ECM forms a major component, are critical to the pathogenesis of the disease and the development of drug resistance. Nevertheless, composition of the ECM in MM, and its role in supporting MM pathogenesis, has not been reported in detail. This work applies a novel proteomics based strategy and defines the bone marrow ECM composition in patients with MGUS, newly diagnosed and relapsed MM, compared to healthy donor-derived bone marrow ECM. This study demonstrates that the tumour ECM is remodelled at the mRNA and protein level in MGUS and MM to allow development of a permissive and immune suppressive microenvironment. ECM proteins ANXA2 and LGALS1 are upregulated in MM and high expression is associated with an inferior overall survival in MM patients. This study points to the importance of ECM remodelling in MM and provides a novel proteomics pipeline for ECM interrogation in cancers with bone marrow tropism.

Introduction

MM is a plasma cell malignancy which accounts for approximately 10% of all haematological cancers (1). MM provides a model for the study of cancer cell metastasis as MM cells are known to traffic to distant bone marrow niches via hijacking the normal processes of cellular metastasis (2). Progression of the disease is mediated by intrinsic factors of the clonal cells along with factors that mediate a permissive tumour microenvironment (3). The MM tumour niche comprises the components of the bone marrow microenvironment - cellular (stromal cells, osteoblasts, osteoclasts, endothelial cells and immune cells) and non-cellular (ECM) (4, 5). Despite advances in therapies in recent years, MM remains an incurable disease with a median survival of approximately 5-7 years for newly diagnosed patients (6). Identification of the molecular mechanisms leading to MM has the potential to lead to the development of novel prognostic tests and therapies for MM patients (7)(8).

The ECM is a complex meshwork of proteins that was traditionally thought to serve as an amorphous scaffold for cells, providing anchorage and support to the surrounding environment. It is now more widely understood to actively participate in cell functions such as proliferation, migration and survival via cell-to-matrix interactions (9, 10). The ECM is also rich in growth factors and cytokines thus supporting cell biology (9); therefore it follows that alterations in the ECM may be expected to occur in disease states such as inflammation and cancer. Indeed, this is the case in several tumour types where ECM alterations have been associated with changes in metastatic potential of tumour cells *in vivo* and in clinical outcomes (11, 12). The role of the ECM as a metabolic regulator of cell function also has implications for malignant processes (13) and molecular signals of metastasis have implicated ECM components and their receptors in tumour progression (14).

It is however, necessary to further enhance our understanding of matrix biology, in order to better uncover clinically meaningful targets within this complex and dynamic microenvironment. In the past, attempts to systematically characterize the ECM were challenged by the vast diversity and number of ECM proteins and also by their biochemical nature. A new perspective on proteomic analysis of the ECM, on a relatively high throughput scale, came with the definition and *in vivo* characterization of the “matrisome” (8). The term “matrisome”, initially used by Martin and

collaborators in 1984 was updated and refined by Naba et al in 2012. This study revealed tissue specific signatures of ECM proteins and importantly provided a method to determine the origin (tumour cell or stroma) of individual matrix proteins. The “matrisome” itself is defined as the components constituting the ECM “core matrisome” and all associated components “matrisome associated”. This method has yielded important insights into the composition of several solid tumour matrices along with potential novel therapeutic and prognostic targets (11, 15).

ECM alterations that influence cellular metastasis have implications not only for solid tumours but also for haematological malignancies, where the process of cell migration from the primary niche is largely dependent on the tumour microenvironment (16-18). Genomic and other comprehensive profiling tools have been applied to MM in order to uncover new targets, however to date there is a paucity of data related to the tumour ECM in MM, largely due to the reasons outlined above. This study applies a proteomics pipeline to study the tumour ECM from the bone marrow of MM patients, by using mass spectrometry. This serves to characterize the unique extracellular matrices of patients with the pre-myeloma condition, MGUS, newly diagnosed and relapsed MM patients and compare this ECM signature to that of healthy human donor bone marrow. The results demonstrate ECM remodelling with disease progression, which is evident even at the MGUS stage. Annexin-A2 (ANXA2) and Galectin-1 (LGALS1) are identified in this study as two ECM-associated proteins with prognostic significance for MM-patient overall survival.

Materials and methods.

Human bone marrow aspirates. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki. These studies were approved by the Dana-Farber Cancer Institute Institutional Review Board and the University of Torino, Italy. Whole bone marrow was obtained from healthy human donors (n=10), MGUS patients (n=3), newly diagnosed MM patients (n=7) and patients with relapsed MM (n=6). Fresh bone marrow samples (20mls/patient) were filtered and underwent red cell lysis, followed by centrifugation at 2500rpm for 10 minutes (see supplemental methods 1 for full protocol). The isolated pellet was then processed through the sequential extraction methods outlined below.

ECM protein enrichment and SDS gradient gel separation. Sequential extractions of whole bone marrow samples were performed using the CNMCS (Cytosol/Nuclear/Membrane/Cytoskeletal) Compartmental Protein Extraction Kit (Cytomol, Union City, CA). ECM proteins were enriched from bone marrow samples obtained from MM patients and normal donors according to previously published methods (8). In brief, whole bone marrow samples were homogenized and subjected to sequential extraction methods to remove (1) cytosolic proteins, (2) nuclear proteins, (3) membrane proteins and (4) cytoskeletal proteins, leaving a final insoluble fraction enriched for ECM proteins.

Immunoblotting. For validation of the extraction process, the different fractions were separated on SDS-polyacrylamide gradient gels, transferred to nitrocellulose membranes and probed with relevant subcellular compartmental antibodies to confirm elimination of intracellular proteins and enrichment of ECM proteins. The antibodies used for immunoblotting included anti-GAPDH, anti-Histone (Cell Signaling Technology, Danvers, MA), anti-transferrin (Invitrogen, Camarillo, CA), anti-vimentin (Cell Signaling Technology, Danvers, MA), anti-fibronectin (Abcam, Cambridge, MA), anti-actin (Santa Cruz Biotechnology, Dallas, TX) or anti- α -tubulin (Cell Signaling Technology, Danvers, MA).

Protein digestion in peptides. The ECM-enriched protein fractions obtained after decellularization were solubilized and reduced in a solution of 8M urea in 100mM

ammonium bicarbonate containing 10mM dithiotreitol at 37°C for 30 minutes. The solubilized ECM proteins were then separated on 4-20% SDS-polyacrylamide gradient gels. Gels were stained with Gel Code Blue (Thermo Scientific) and washed in dH₂O. Gels were then cut into 2 or 3 pieces using a clean scalpel and gel pieces were transferred into clean microcentrifuge tubes. Gel bands were washed twice with 200ml of 50% acetonitrile for 15 min. Protein gel samples were reduced with 10mM dithiothreitol (DTT), alkylated with 55mM iodoacetamide and digested overnight with 100ng of sequencing grade L-(tosylamido-2 phenyl) ethyl chloromethyl ketone (TPCK) modified trypsin (Promega) at pH8.3. Samples were acidified with 0.5% trifluoroacetic acid and desalted using C18 zip tips (Millipore) and concentrated to 10µL.

Liquid chromatography and Tandem Mass Spectrometry. 2 to 4µL of peptides were analysed by microcapillary liquid chromatography (C18) tandem mass spectrometry (LC-MS/MS) using an EASY-nLCII nanoflow HPLC (Thermo Fisher Scientific) coupled to a hybrid Orbitrap Elite high-resolution mass spectrometer (Thermo Fisher Scientific) in Top 6 data-dependent acquisition positive ion mode at a flow rate of 300 nl/min.

Protein and peptide identification. The LC-MS/MS datasets were analysed with the Spectrum Mill software package, v 5.0 pre-release (Agilent Technologies, Santa Clara, CA). MS/MS spectra were searched against UniProt databases containing reference proteome sequences (including isoforms and excluding fragments) downloaded from the UniProt website on October 17, 2014. Redundant sequences were removed, and a set of common laboratory contaminant proteins (150 sequences) was appended. The human database comprised 59,079 entries. Search criteria, described in the Supplementary Methods, yielded target-decoy-based false-discovery rate (FDR) estimates for each patient's dataset of <0.8% at the peptide-spectrum match level and <1.3 % at the distinct peptide level. Across all datasets together the peptide-level FDR was 1.9%. Peptide-spectrum matches from all datasets together were assembled into proteins, and each protein was annotated as ECM-derived or not, as previously described (8, 19).

Analysis of gene expression profiling and survival analysis. Publicly available gene expression profiles (GEP) were analysed to evaluate the expression level of ANXA2 and LGALS1 in MM patients and cell lines (GSE6477). The Cancer Cell Line Encyclopedia (CCLE) database (<http://www.broadinstitute.org/ccle/home>) was used to determine the expression level of ANXA2 and LGALS1 across over 1,000 cell lines, expressed by Robust Multi-array Average (RMA) values. GEP was further studied to assess the differential expression of ANXA2 and LGALS1 in MM vs. normal plasma cells – GSE6477. Gene levels were expressed by normalized expression values and 2-tailed t test were calculated to compare the 2 groups. Finally a large GEP dataset was analysed - GSE2658 – that enrolled 350 patients at diagnosis of MM. A Kaplan-Meier analysis was performed to compare overall survival (OS) of patients with low vs. high expression level of ANXA2 or LGALS1 based on the median expression of the cohort.

Gene set enrichment analysis (GSEA) GSEA was performed (GSEA)(20) to determine whether the identified ECM gene sets followed the same pattern at the mRNA level in MM patients compared to healthy donors. GSEA was performed following the developer's protocol (<http://www.broad.mit.edu/gsea/>) using the GSEA6477 dataset (21).

Results

ECM enrichment from normal and diseased whole bone marrow samples.

Murine and human bone marrow was examined, using Masson's trichrome to stain fibrillar collagens, as an assessment of ECM content (22) (Fig. 1). This identified ECM within healthy murine bone marrow niches (Fig. 1A). The normal architecture of the ECM became disorganized in MM murine bone marrow but was still clearly present (Fig. 1B). Similarly, ECM was also identifiable within the bone marrow of healthy human donors (Fig. 1C) and in MM patients (Fig. 1D), where morphology was again perturbed.

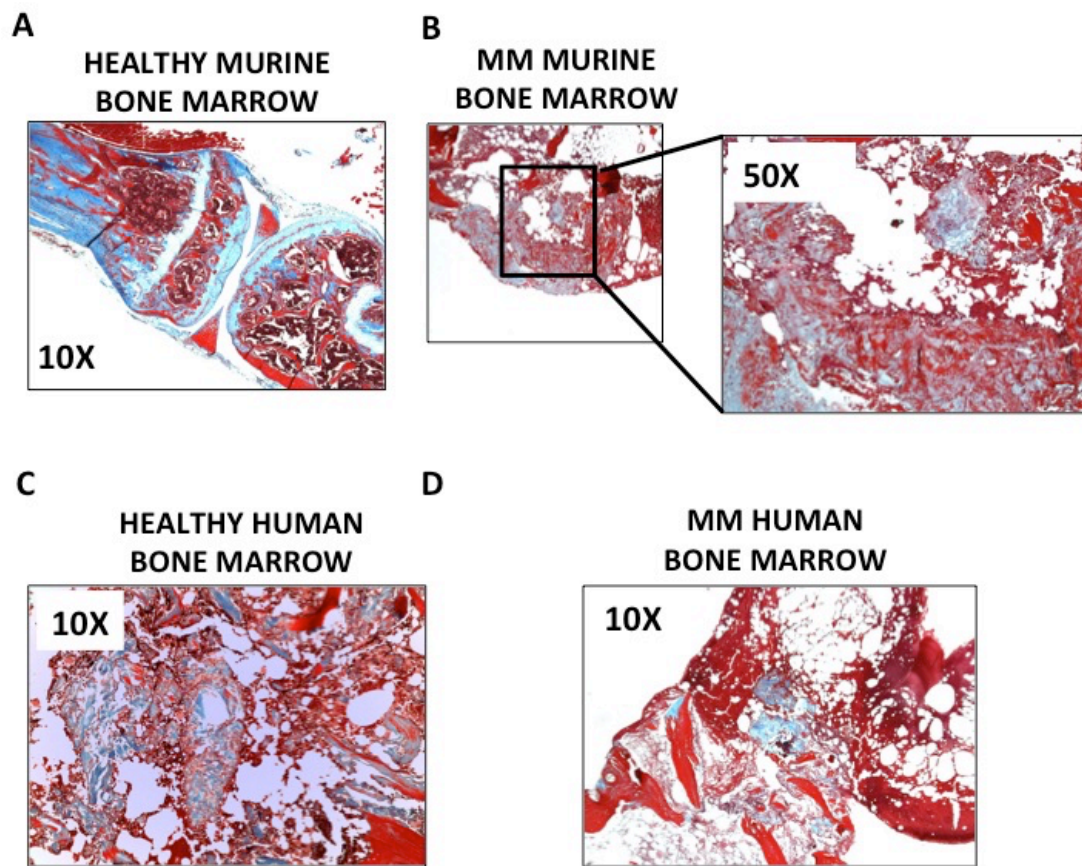
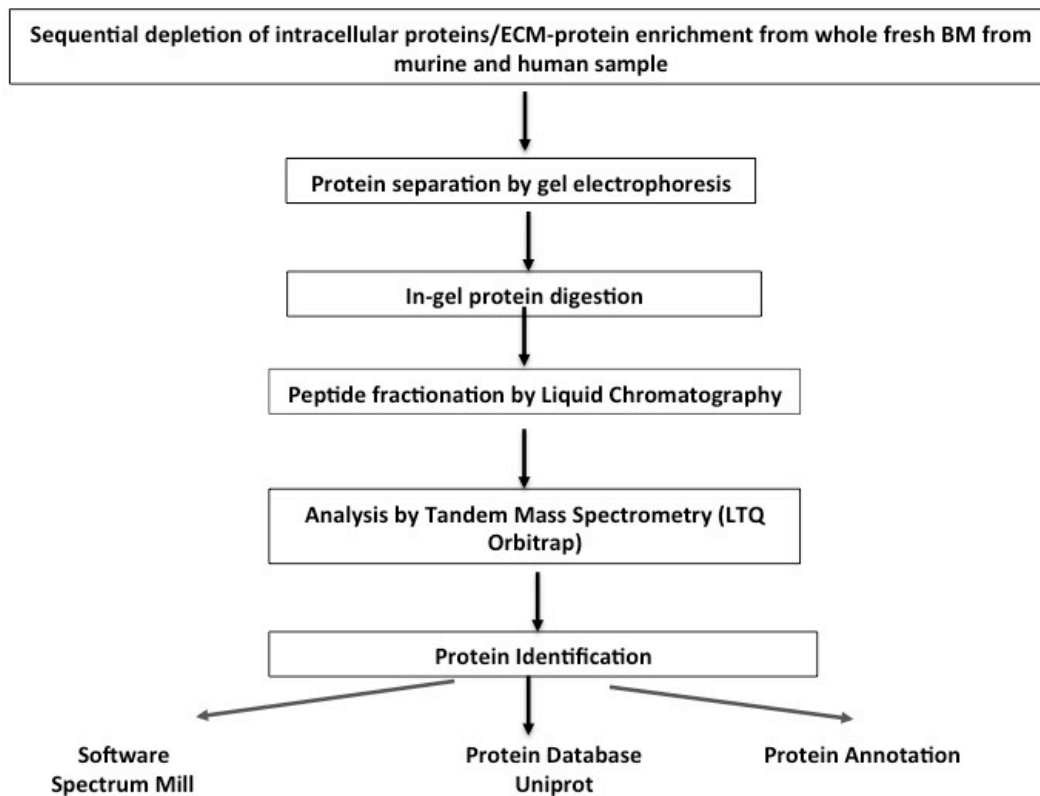


Figure 1: ECM content assessment Masson's trichrome stain demonstrating presence of ECM (blue) in A – healthy murine bone marrow, which becomes disorganized but is still present in murine MM bone marrow. C – demonstrates the presence of ECM in human MM patient bone marrow.

In order to isolate and characterize this ECM, a protocol was developed based on the previous work by Naba et al (8) specifically tailored to extract ECM proteins from the liquid marrow (Fig. 2A). Antibody markers, including anti-GAPDH, -histone, -transferrin receptor, and -vimentin antibodies were used for the cytosolic, nuclear, membrane and cytoskeletal compartment in order to confirm enrichment at each step. In addition, an anti-fibronectin antibody was used to monitor the behaviour of one exemplary ECM protein during the de-cellularization of bone marrow samples. Although a portion of fibronectin was observed to be partially depleted at each step, it was present in the final fraction (Fig. 2B left panel)

The protein fraction that remains insoluble after de-cellularization was then separated using gel electrophoresis, gels were stained using Coomassie blue stain (Fig. 2B, right panel). Gel bands were further cut from the gels and proteins were subjected to in-gel protein digestion to generate peptides to be separated by liquid chromatography and analysed by mass spectrometry.

A



B

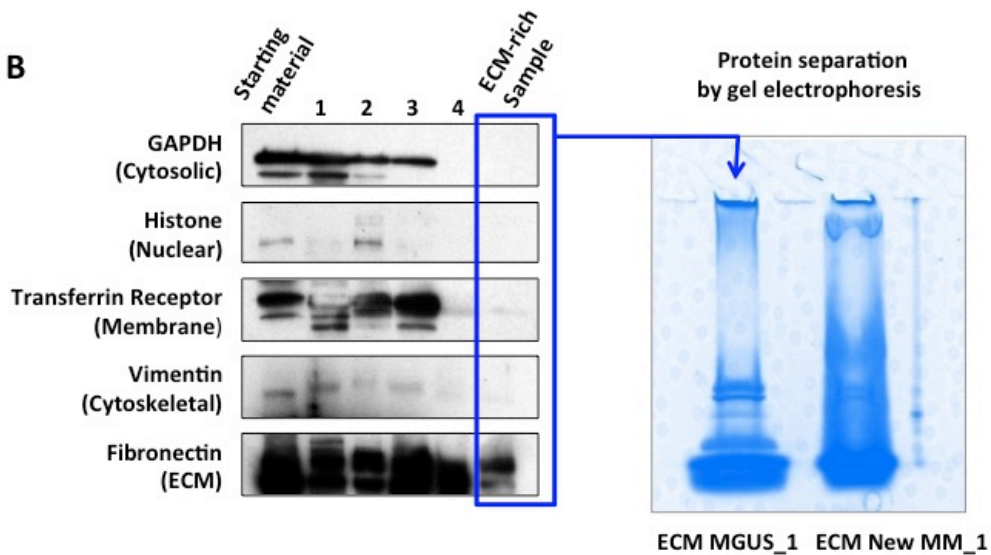


Figure 2: *A Experimental strategy deployed to characterize the composition of the extracellular of healthy bone marrow and MM-patient derived bone marrow B Western blot shows the depletion of intracellular components (GAPDH, histones, Transferrin receptor and vimentin) during the four-step de-cellularization process. Coomassie-stained gel shows the pattern of migration of proteins from the ECM-enriched samples.*

Proteomic characterization of healthy human donor, MGUS and MM ECM.

To more clearly define the proteomic signature of bone marrow ECM, bone marrow was obtained from patients with MGUS and MM, and compared to healthy donor-derived bone marrow. Liquid chromatography and tandem mass spectrometry were performed using proteins from the insoluble ECM enriched fractions. A summary of the independent samples from healthy donors, MGUS patients and MM patients is shown in supplemental Figure S1. Peptide abundance, spectral count, number of unique peptides and the number of proteins identified within the whole bone marrow were analysed for both healthy donors and patients with MGUS or MM, either at first diagnosis or at relapse (Fig. 3). In a representative healthy donor-derived sample, 24% of total spectral count signals were core matrisome or matrisome associated with 43% of the total precursor ion intensity corresponding to proteins defined as ECM (Fig. 3A).

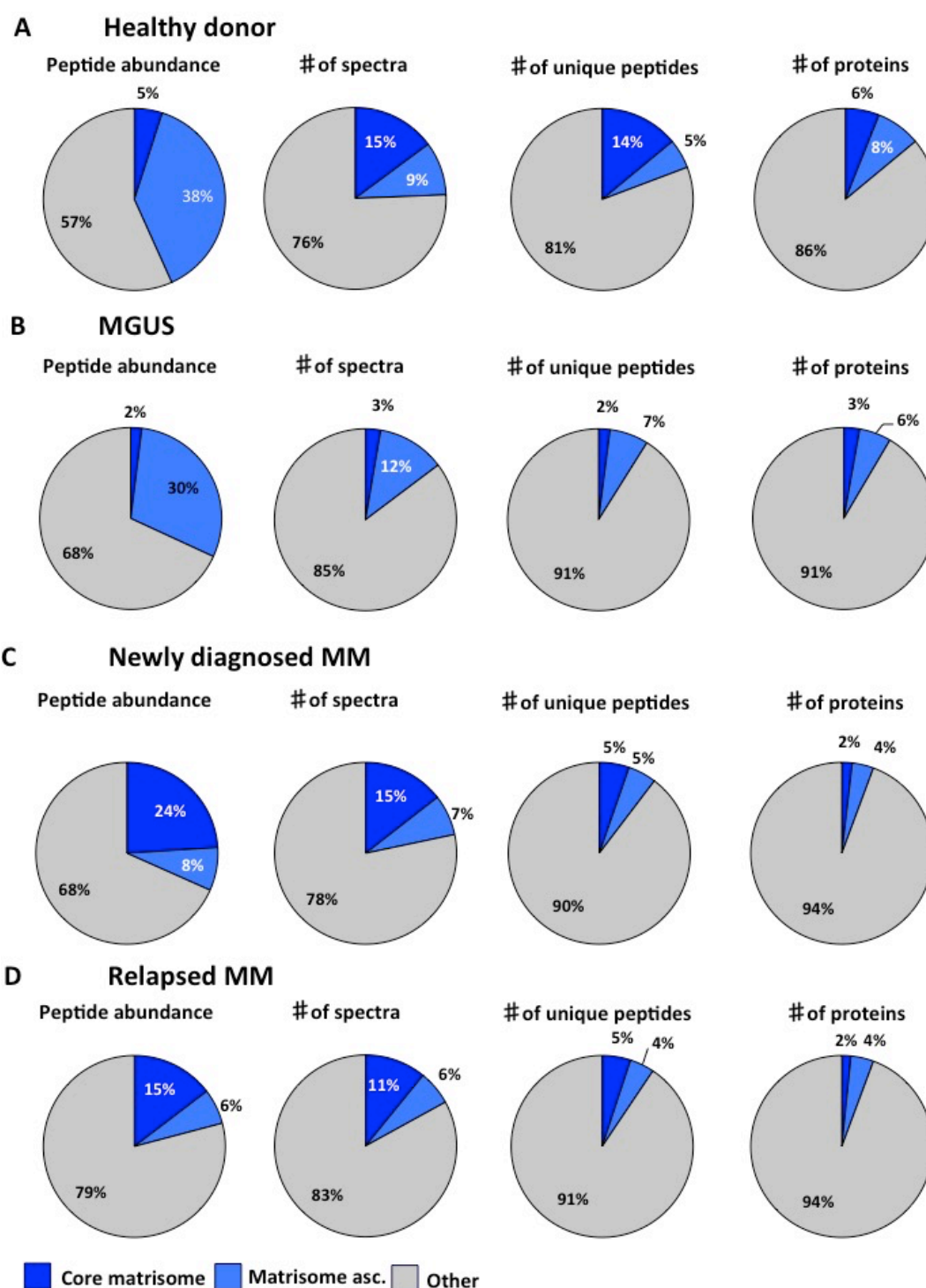
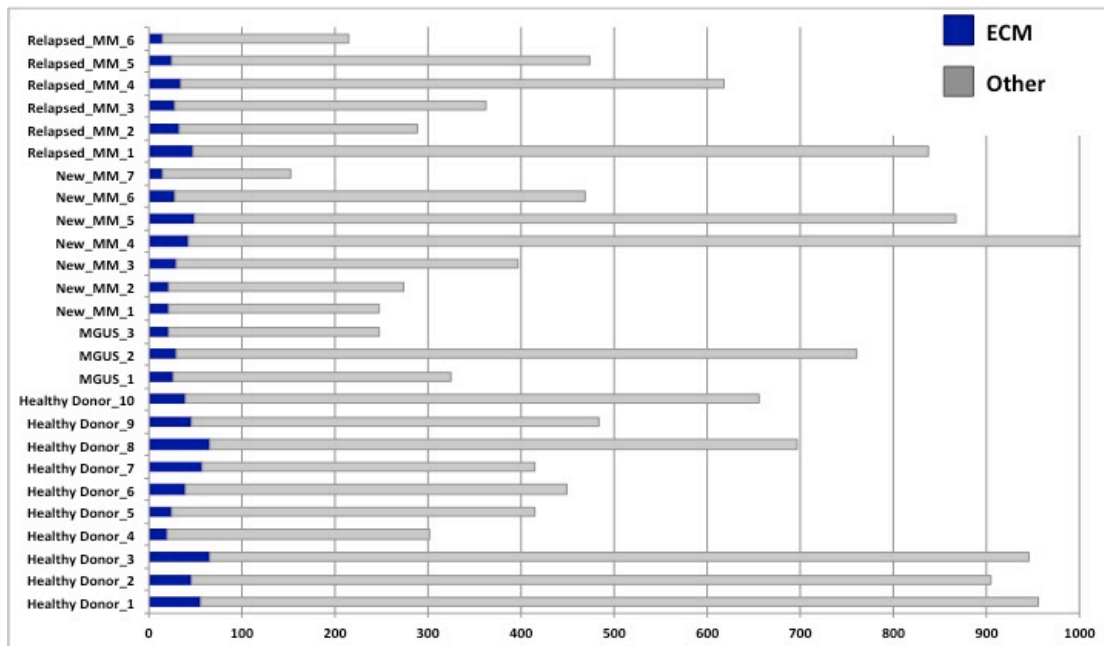


Figure 3: Characterization of healthy human donor, MGUS patient, and multiple myeloma patients ECM in both newly diagnosed and relapsed patients. The pie charts represent data from the human bone marrow that has been processed through the proteomics pipeline. Left panel shows the peptide abundance (precursor ion MS signal corresponding to matrisome or non matrisome (intracellular) proteins. Other panels represent, from left to right, the distributions in terms of number of spectra, unique peptides and proteins (right panels).

In previous studies conducted on solid tumours (melanoma or mammary tumour xenografts, and human metastatic colorectal carcinomas), it has been reported that the majority, typically (>75%) of the total precursor-ion intensities, correspond to matrisome proteins (8, 11, 15). This may reveal that the bone marrow ECM is more soluble and more readily extractable during the de-cellularization process than the ECM of normal tissues and solid tumours. In addition, it is worth noting that the inter-patient variability was greater than previously observed in solid tumours. Starting with 20 ml of bone marrow aspirate for each donor or patient, the proportion of the total precursor-ion intensities corresponding to matrisome proteins ranged from 10% to nearly 50% (Fig. 4 & Figs. S2A-B-C).

A Number of proteins



B

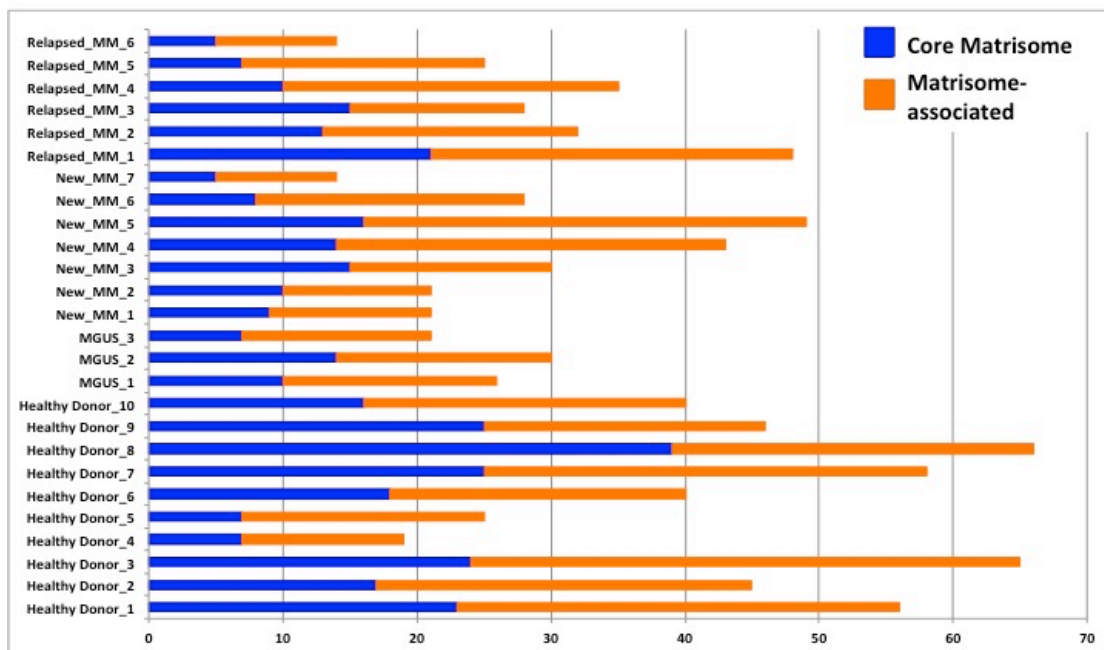


Figure 4: Bar charts represent the number of ECM vs. non-ECM proteins (top panel) and for core matrisome and matrisome-associated proteins (lower panel)

Characterization of the matrisome from healthy donor bone marrow.

The matrisome of healthy donor-derived bone marrow was identified as the ensemble of proteins detected in three independent biological replicates (i.e patient/donor) and by at least three peptides in one of the replicates. According to this definition the bone marrow ECM from 10 independent healthy donors was composed of 62 proteins (Table 1A). As previously, proteins were classified as part of the core matrisome, which comprises ECM glycoproteins, collagens, and proteoglycans, or being matrisome-associated proteins (ECM-affiliated proteins, ECM regulators, and ECM-associated secreted factors) (8, 19). Using this method, thirty proteins were core matrisome and 32 were matrisome associated. The interrogation of the MatrisomeDB database revealed the identification of 11 proteins (Table 1A) that have not been previously detected in any of the human tissues included in the database (23). As human bone marrow has not been previously subjected to this type of method, these proteins may be specific to this tissue.

Characterization of the matrisome from MGUS and MM patients.

To define the proteomic signature for bone marrow ECMs derived from MGUS patients, the same criteria as outlined above was used. Eleven proteins were identified, two of which were core matrisome proteins, namely Bone Marrow Proteoglycan 2 (PRG2) and Bone Marrow proteoglycan 3 (PRG3).

Nine of the proteins identified in the MGUS patient bone marrows were matrisome associated, including the ECM-affiliated protein ficolin 1 (FCN1), the ECM-remodelling enzymes CTSG, Serpins, and the secreted factors HRNR, S100A8 and S100A9 (Table 1B).

Analysis of the ECM signature of MM bone marrow identified 32 proteins (Table 1C). Ten of these proteins were core matrisome proteins, with 22 being matrisome associated. Interestingly two proteins were identified as matrisome associated, ANXA2 and LGALS1, which were not identified at a significant level in healthy donor or MGUS bone marrow. The ECM of patients with relapsed MM comprised of 25 proteins, 10 of which are core matrisome proteins and 15 of which are matrisome associated (Table 1D).

The analysis of the ECM identified signatures of MGUS and progressive MM.

By comparing the matrisomes of healthy donor bone marrow, MGUS and progressive stages of MM, 11 ECM proteins were identified that were expressed by all four groups (Fig. 5A-B).

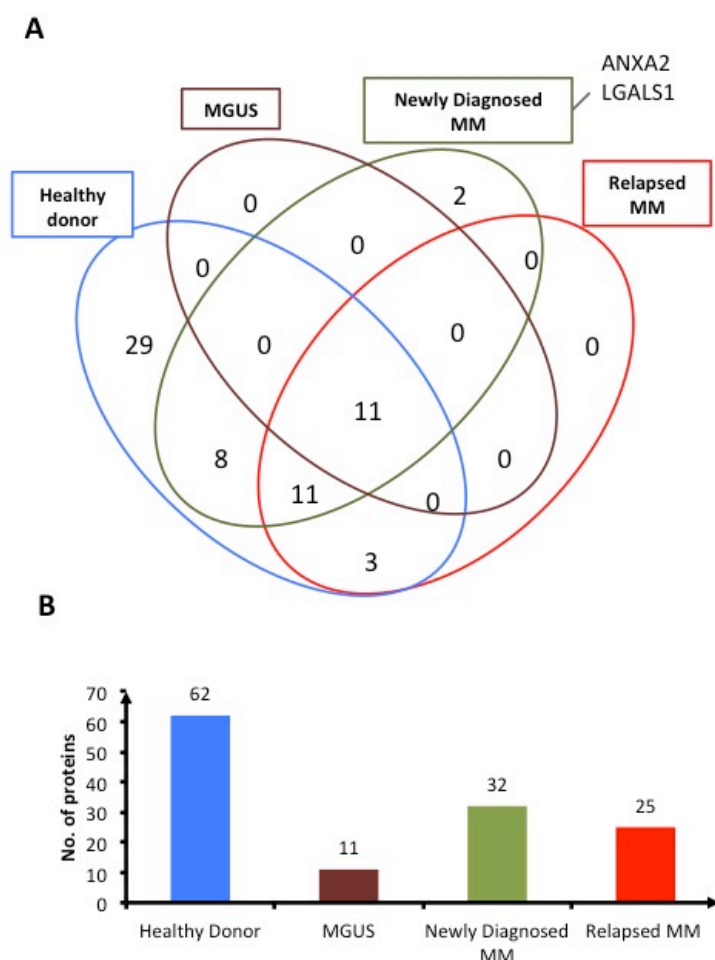


Figure 5: (A) Venn diagram shows the overlap and differences between the matrisome of health bone marrow, of the bone marrow from newly diagnosed MM patients and the bone marrow of relapsed MM patients. The matrisome of each tissue type was defined as the ensemble of proteins identified in at least 3 of the samples profiled. Matrisome of healthy bone marrow = proteins detected in at least 3 out of the 10 independent samples profiled Matrisome of MGUS patients = proteins detected in at least 3 out of the 3 independent samples profiled Matrisome of newly diagnosed patients = proteins detected in at least 3 out of the 7 independent samples profiled Matrisome of relapsed patients = proteins detected in at least 3 out of the 6 independent samples profiled (B) Bar graph demonstrating the numbers of proteins identified in each group. Complete list of proteins provided in Table 2 with corresponding spectral counts

Examples of these proteins are cathepsin-G (CTSG) and neutrophil elastase (ELANE). It was noted that the normal-bone-marrow matrisome contained more proteins than diseased bone marrow samples. Because the proteomic pipeline employed analysed only proteins that remained insoluble after de-cellularization, one hypothesis is that the ECM proteins in MGUS or MM bone marrow samples are more soluble. In agreement with this hypothesis, the loss of several collagens and other fibrillar ECM glycoproteins was observed, such as fibronectin in MGUS and MM samples (Table 2). We also observed that some collagens and matrix metallo-proteinases (MMPs) found in healthy donor bone marrow and not detected at the MGUS stage are detected later in the disease progression pathway. This includes, among others, COL1A1, COL1A2, COL3A1, COL5A1, MMP8 and MMP9 (Table 3).

It is possible that the consistent loss of these collagens in the bone marrow microenvironment seen in MGUS, newly diagnosed MM and relapsed MM suggests that their absence is a deliberate remodelling within the bone marrow. An inverse pattern of remodelling may also be present, where some collagens and matrix metallo-proteinases found in healthy donor bone marrow are not detected at the MGUS stage but are seen to re-emerge at lower abundance later in the disease progression pathway. When comparing newly diagnosed MM to healthy donor bone marrow and that of MGUS, Interestingly, two proteins, Annexin A2 and Galectin-1 are seen to emerge that are not present in earlier stages of the disease (Table 2).

MM cells present with an enrichment for ECM-related genes.

Given that remodelling of the bone marrow ECM was evident at the protein level in MM, a validation analysis was performed at the corresponding mRNA level to ensure that this was truly reflective of an altered ECM signal, and not the result of tumour invasion with consequent local ECM destruction. Using the signature of ECM proteins found to be present in the normal ECM, and absent in the MM tumour ECM, GSEA was performed to assess for enrichment of this gene signature in healthy donors vs. MM patients. Enrichment of the corresponding ECM gene signature was demonstrated in healthy donors compared to MM patients (Figure 6A-B; FDR<0.25). It has been shown that the majority of the tumour ECM is derived from the tumour cells in the main part, with stromal contribution at different stages of metastasis (15). Therefore, our findings suggest the ability of tumour cells to contribute to microenvironmental remodelling.

Annexin A2 and Galectin-1 and their prognostic relevance in MM.

ANXA2 and LGALS1, two ECM-affiliated proteins, were identified in newly diagnosed MM bone marrow-ECMs, and not in healthy donor or MGUS bone marrow-ECMs. Therefore the expression of ANXA2 and LGALS1 in MM cell lines was analysed, using the Cancer Cell Line Encyclopedia (CCLE); and it was found that both proteins were expressed in all MM cell lines (Fig. 6C). These findings, were further corroborated, by demonstrating the higher expression of both ANXA2 and LGALS1 in MM- as compared to healthy donor-derived plasma cells (GSE2658 and GSE6477; Fig. 6D). (21) (24)

Specifically, while ANXA2 levels were consistently higher in any given MGUS, smouldering-MM, MM newly diagnosed or MM relapsed patient as compared to healthy donors, LGALS1 was significantly higher in MGUS and newly diagnosed MM patients as compared to healthy individuals (Fig. 6D).

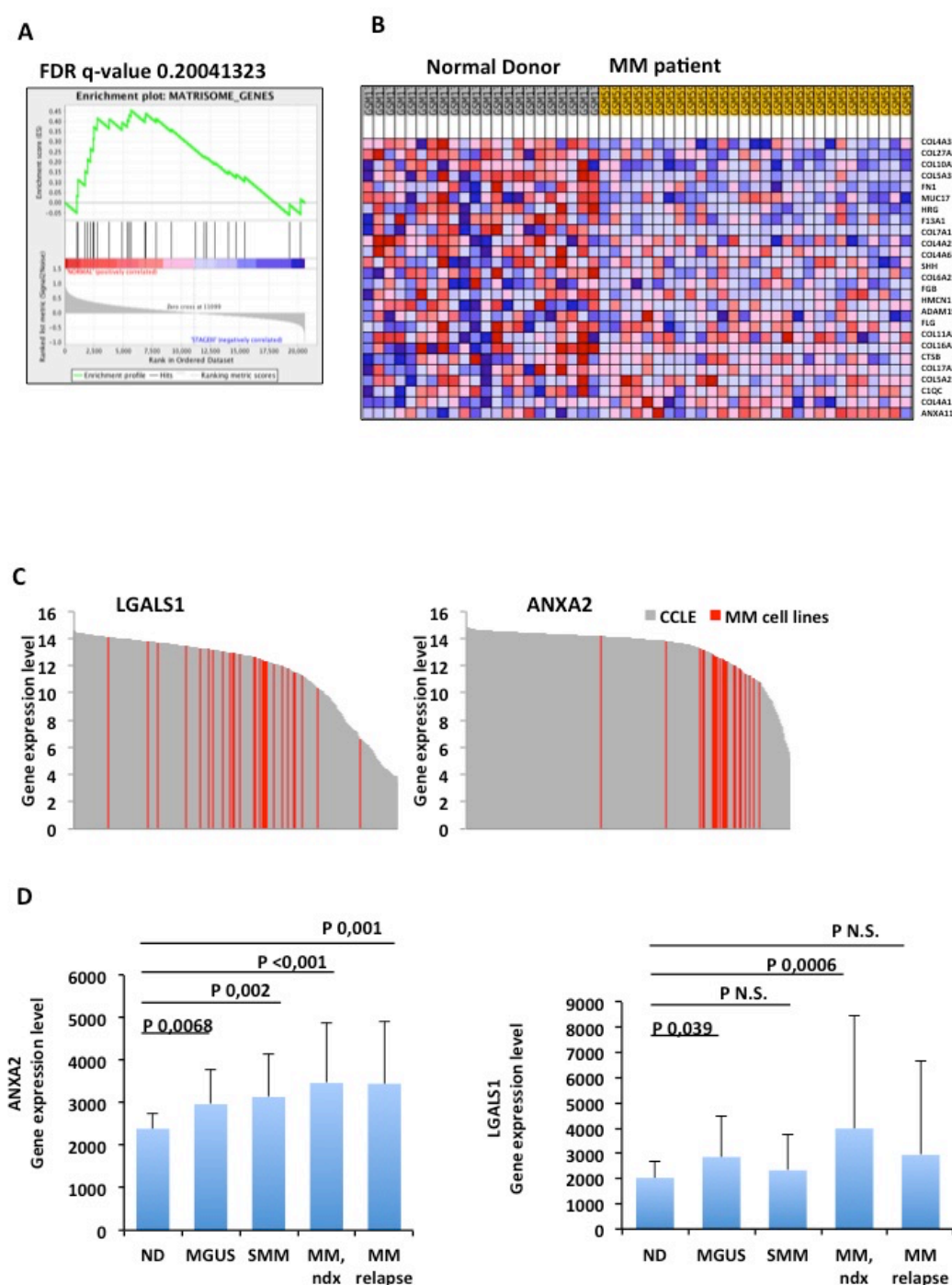


Figure 6 Matrisome gene expression in multiple myeloma (A) Gene set enrichment analysis (GSEA): demonstrating that ECM proteins seen to be depleted in the bone marrow matrix with disease progression were also significantly down regulated at the gene level in normal donors (left) compared to MM patients (right). (B) Heatmap demonstrating enrichment profile of relevant ECM genes in healthy donors vs. MM patients. (C) Analysis of CCLE MM cell lines (red bars) demonstrating the expression of LGALS1 and ANXA2 in MM cell lines at the mRNA level. (D) ANXA2 is significantly over-expressed at the mRNA level in MGUS, soldering MM (SMM), newly diagnosed MM (MM, ndx) and relapsed MM (MM relapse) in comparison to normal donors (ND). LGALS1 is also significantly over-expressed at the mRNA level in newly diagnosed MM patients compared to normal donors. Dataset reference: Cancer Res. 2007 Apr 1;67(7):2982-9. (GSE6477)

In order to further understand if these targets may be interacting, leading to a permissive tumour microenvironment in MM, the Mulligan dataset (25) was analysed for co-expression of these genes in MM using the publically available Oncomine resource (www.oncomine.org). This revealed ANXA2 as one of the most highly co-expressed genes with LGALS1 in 264 MM patients (Fig. 7A, Correlation co-efficient = 0.522).

To interrogated the influence of both ANXA2 and LGALS1 in modulating survival in MM patients GEP data was analysed GSE2658 (26), which confirmed that patients expressing higher levels of ANXA2 had a significantly reduced overall survival (OS) compared with those with lower expression levels (Figure 7B; Log-rank $P = 4.9\text{e-}05$). Similarly, high levels of LGALS1 were also associated with an inferior OS (Fig. 7C Log-rank $P = 0.05$)

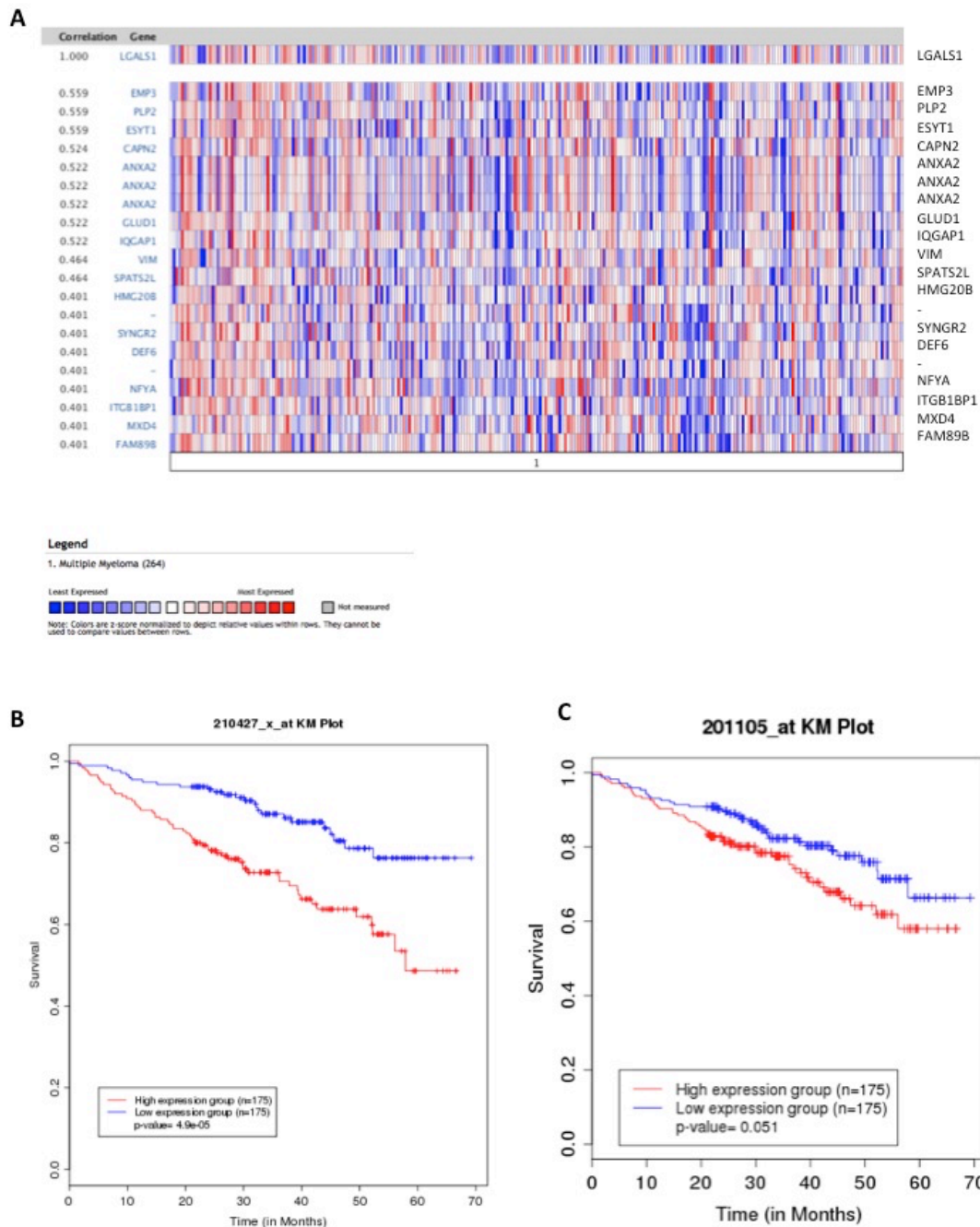


Figure 7: Expression of ECM proteins in MM patients and impact on overall survival.

(A) Co-expression of *LGALS1* and *ANXA2* in MM patients – data sourced from Oncomine (www.oncomine.org) Mulligan MM gene expression Blood. 2007 Apr 15;109(8):3177-88. Epub 2006 Dec 21. (B) Kaplan-Meier survival proportions analysis of patient outcome data from the GSE2658 MM GEP dataset (Leukemia. 2006 Jul;20(7):1288-90.) demonstrating high levels of *ANXA2* expression associated with inferior overall survival. Log-rank $P = 4.9e-05$ (C) Kaplan-Meier survival proportions analysis of patient outcome data from the GSE2658 MM GEP dataset (Leukemia. 2006 Jul;20(7):1288-90.) demonstrating high levels of *LGALS1* expression associated with inferior overall survival. Log-rank $P = 0.051$.

Discussion

In this study the bone marrow ECM of normal human bone marrow was interrogated using a mass spectrometry pipeline that has not been previously applied to this tissue. This was used to compare the bone marrow ECM signature of patients with MGUS, newly diagnosed MM and relapsed MM.

The results demonstrate that significant remodelling of the bone marrow ECM is evident even at the MGUS stage, where loss of collagens and fibronectin (COL4A1-A3, COL4A6, COL5A2-3, COL6A2-3 COL10A1, COL11A2, COL16A1, COL27A1 and FN1) is evident. The absence of these proteins was consistent throughout the spectrum of MM from newly diagnosed to relapsed disease suggesting that the loss of these ECM components may contribute to a permissive tumour microenvironment supportive of the development and progression of MM. Indeed it has been shown that fibroblasts from patients with MGUS and MM demonstrate altered ECM profiles in comparison to those of healthy donors, supporting the hypothesis of early ECM remodelling (27).

Similarly, some collagens and matrix metallo-proteinases found in healthy donor bone marrow are not detected at the MGUS stage but are seen to re-emerge at lower abundance later in the disease progression pathway indicating that they may be more supportive in aggressive phenotypes. This includes, among others, COL1A1, COL1A2, COL3A1, COL5A1, MMP8 and MMP9.

Interestingly, two proteins, ANXA2 and LGALS1 emerge in newly diagnosed MM but are not detected in healthy donors or MGUS patients. These proteins are expressed in MM cell lines at the mRNA level in CCLE datasets. It is reasonable to hypothesize that these proteins may be required for myelomagenesis and therefore further examination their presence in MM patient bone marrow was carried out. GEP profiles reveal that these proteins are highly expressed in MM bone marrow plasma cells in comparison to healthy donor-derived plasma cells. This may indicate that these proteins are tumour cell derived within the bone marrow niche and are specific to the malignant phenotype. If this is the case, then plasma cells themselves may contribute to bone marrow ECM remodelling as has been shown in other tumours (15) Indeed, the galectins, of which LGALS1 is a member, are a family of beta-galactoside-binding proteins implicated in modulating cell-cell and cell-matrix interactions and recently LGALS1 suppression has been shown to inhibit MM

induced angiogenesis and tumour growth *in vivo* (28). ANXA2 is a calcium dependent phospholipid binding protein, which plays a role in cell growth and heightens osteoclast formation and bone resorption. Osteoclasts are known to contribute to an immune suppressive microenvironment in MM and are widely known to be associated with MM pathogenesis (29). Also, ANXA2 has previously been shown to promote MM cell growth and reduce apoptosis in MM cell lines (30). Identification of these targets, known to play a role in MM biology, helps to provide a wider validation of the remodelling of the ECM signature that has emerged using this novel strategy.

To further understand the pathobiology of bone marrow remodelling in MM patients analysis was performed to examine if there might be a common expression pattern for these two proteins within the MM bone marrow niche. Using data from Oncomine, 264 MM patients were analysed and it was found that ANXA2 is one of the most highly co-expressed genes with LGALS1 in these patients. This leads to hypothesis that the bone marrow re-modelling seen in MM is likely to be an organized and deliberate process that occurs as a result of multiple ECM protein switches being turned on and off within the bone marrow niche. Both of these markers have previously been identified as being important in MM, and this study provides further validation that they are active in an overall ECM remodelling process within the bone marrow.

The prognostic relevance of our findings was determined using GEP datasets, which provided survival outcomes for MM patients. This demonstrated that patients expressing higher levels of ANXA2 had a significantly reduced OS compared with those with lower expression levels. Similarly, high levels of LGALS1 were also associated with an inferior OS. This finding demonstrates that the bone marrow ECM plays an important role in MM and suggests that ECM remodelling is an active process within the bone marrow niche that has prognostic implications for MM patients. This previously under-evaluated area provides a potential resource for the identification of novel targets in this disease which may lead the way to preserving the normal ECM architecture and provide a less permissive niche.

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Tables

Table 1A: The matrisome of normal human bone marrow: Defined as the ensemble of ECM and ECM-associated proteins detected in at least 3 samples with a spectral count ≥ 1 per replicate.

The interrogation of the MatrisomeDB database revealed the identification of 11 proteins (marked with *) that have not been previously detected in any of the human tissues included in the database.

Normal human bone marrow matrisome (62 proteins)

Core matrisome			Matrisome-associated		
ECM Glycoproteins	Collagens	Proteoglycans	ECM-affiliated Proteins	ECM Regulators	Secreted Factors
FGA	COL1A1	PRG2	ANXA1	ADAM19*	FLG*
FGB	COL1A2	PRG3	ANXA11	CTSB	HRNR*
FGG	COL2A1		ANXA6	CTSC	PF4*
FN1	COL3A1		C1QC	CTSD	S100A8
HMCN1	COL4A1		CLC*	CTSG	S100A9
MXRA5	COL4A2		FCN1	CTSS	SHH*
THBS1	COL4A3		MUC17*	CTS2	
ZP3*	COL4A5			ELANE	
	COL4A6			F13A1	
	COL5A1			HRG	
	COL5A2			MMP23B*	
	COL5A3			MMP8*	
	COL6A2			MMP9	
	COL6A5			SERPINA1	
	COL7A1			SERPINA3	
	COL10A1			SERPINB1	
	COL11A2			SERPINB6	
	COL16A1			SERPINB8*	
	COL17A1			SERPINB9	
	COL27A1				

* indicates ECM or ECM-associated proteins not detected before in any human tissue matrisome

Table 1B: The matrisome of MGUS patient bone marrow Defined as the ensemble of ECM and ECM-associated proteins detected in at least 3 samples with a spectral count ≥ 1 per replicate.

The interrogation of the MatrisomeDB database revealed the identification of 2 proteins (marked with *) that have not been previously detected in any of the human tissues included in the database.

MGUS human bone marrow matrisome (11 proteins)

Core matrisome		Matrisome-associated		
		ECM-affiliated Proteins	ECM Regulators	Secreted Factors
Proteoglycans		FCN1	CTSG	HRNR*
	PRG2		ELANE	S100A8
	PRG3		SERPINB1	S100A9
			SERPINB8*	
			SERPINB9	

* indicates ECM or ECM-associated proteins not detected before in any human tissue matrisome

Table 1C: The matrisome of newly diagnosed MM patient bone marrow Defined as the ensemble of ECM and ECM-associated proteins detected in at least 3 samples with a spectral count ≥ 1 per replicate.

The interrogation of the MatrisomeDB database revealed the identification of 4 proteins (marked with *) that have not been previously detected in any of the human tissues included in the database.

Newly diagnosed MM human bone marrow matrisome (32 proteins)

Core matrisome			Matrisome-associated		
ECM Glycoproteins	Collagens	Proteoglycans	ECM-affiliated Proteins	ECM Regulators	Secreted Factors
FGA	COL1A1	PRG2	ANXA1	CTSC	HRNR*
THBS1	COL1A2	PRG3	ANXA2	CTSD	PF4*
	COL2A1		ANXA6	CTSG	S100A8
	COL3A1		FCN1	CTSS	S100A9
	COL4A5		LGALS1	ELANE	
	COL5A1			MMP8*	
				MMP9	
				SERPINA1	
				SERPINA3	
				SERPINB1	
				SERPINB6	
				SERPINB8*	
				SERPINB9	

* indicates ECM or ECM-associated proteins not detected before in any human tissue matrisome

Table 1D: The matrisome of relapsed MM patient bone marrow: Defined as the ensemble of ECM and ECM-associated proteins detected in at least 3 samples with a spectral count ≥ 1 per replicate.

The interrogation of the MatrisomeDB database revealed the identification of 5 proteins (marked with *) that have not been previously detected in any of the human tissues included in the database.

Relapsed human MM bone marrow matrisome (25 proteins)

Core matrisome			Matrisome-associated		
ECM Glycoproteins	Collagens	Proteoglycans	ECM-affiliated Proteins	ECM Regulators	Secreted Factors
FGG	COL1A1	PRG2	CLC*	CTSG	HRNR*
MXRA5	COL1A2	PRG3	FCN1	ELANE	PF4*
THBS1	COL2A1			MMP8*	S100A8
	COL3A1			MMP9	S100A9
	COL5A1			SERPINA3	
				SERPINB1	
				SERPINB6	
				SERPINB8*	
				SERPINB9	

* indicates ECM or ECM-associated proteins not detected before in any human tissue matrisome

Table 2: Semi-quantitative comparison of proteins identified in each group: n = number per group, Spec count = spectral count, used as a semi-quantitative representation of abundance. Proteins shown were present in at least 3 independent samples/group.

Comparison of spectral counts between groups

	Healthy Donor		MGUS		New Dx MM		RelapsedMM	
	n	Spec Count	n	Spec Count	n	Spec Count	n	Spec Count
CTSG	10	3072	3	229	7	518	6	374
ELANE	10	1380	3	390	7	801	6	476
FCN1	10	140	3	16	5	42	5	23
HRNR	7	28	3	28	4	19	5	25
PRG2	10	463	3	229	7	308	6	323
PRG3	10	149	3	73	7	90	6	107
S100A8	10	328	3	32	7	167	6	106
S100A9	10	814	3	155	7	318	6	291
SERPINB1	10	845	3	62	7	305	6	141
SERPINB9	10	61	3	5	5	17	5	9
SERPINB8	10	61	3	5	5	17	5	8
COL1A1	8	533			5	49	4	66
COL1A2	6	326			5	23	4	34
COL2A1	7	85			3	3	4	9
COL3A1	6	66			3	8	4	22
COL5A1	6	13			3	4	3	3
MMP8	9	51			4	12	5	8
MMP9	10	420			6	216	6	89
PF4	5	55			4	26	5	18
SERPINA3	9	59			3	43	4	11
SERPINB6	9	60			4	22	4	11
THBS1	6	58			5	29	5	14
ANXA1	7	23			3	5		
ANXA6	6	43			3	4		
CLC	9	60					3	33
COL4A5	6	8			3	4		
CTSC	3	21			3	9		
CTSD	5	60			3	34		
CTSS	5	39			3	18		
FGA	7	143			3	449		
FGG	7	142					3	237
MXRA5	3	3					3	3
SERPINA1	7	117			4	99		
ADAM19	3	4						
ANXA11	6	26						
ANXA2	<3	<3			4	7		
CIQC	4	9						
COL10A1	3	6						
COL11A2	4	5						
COL16A1	5	7						
COL17A1	5	5						
COL27A1	3	3						
COL4A1	3	22						
COL4A2	4	19						
COL4A3	4	4						
COL4A6	3	4						
COL5A2	4	26						
COL5A3	3	6						
COL6A2	3	11						
COL6A5	5	6						
COL7A1	8	14						
CTSB	3	5						
CTSZ	5	6						
F13A1	4	41						
FGB	7	159						
FLG	3	3						
FN1	9	389						
HMCN1	3	5						
HRG	3	8						
LGALS1	<3	<3			3	4		
MMP23B	3	3						
MUC17	4	8						
SHH	3	4						
ZP3	3	4						

1000-1500	
500-1000	
100-500	
<100	

Table 3: Proteins absent in MGUS and re-emerging in later stages of MM: Catalog of the proteins which were absent in MGUS patient bone marrow but present in normal bone marrow and found to be present in either newly diagnosed MM patient bone marrow, relapsed MM patient bone marrow or both.

Proteins absent in MGUS and re-emerging in later stages of MM

	Healthy		MGUS		New Dx MM		RelapsedMM	
	NumPat	Spec Count	NumPat	Spec Count	NumPat	Spec Count	NumPat	Spec Count
COL1A1	8	533	<3	<3	5	49	4	66
MMP9	10	420	<3	<3	6	216	6	89
COL1A2	6	326	<3	<3	5	23	4	34
FGA	7	143	<3	<3	3	449	<3	<3
FGG	7	142	<3	<3	<3	<3	3	237
SERPINA1	7	117	<3	<3	4	59	<3	<3
COL2A1	7	85	<3	<3	3	3	4	9
COL3A1	6	66	<3	<3	3	8	4	23
SERPINB6	9	60	<3	<3	4	22	4	11
CLC	9	60	<3	<3	<3	<3	3	33
CTSD	5	60	<3	<3	3	34	<3	<3
SERPINA3	9	59	<3	<3	3	43	4	11
THBS1	6	58	<3	<3	5	29	5	14
PF4	5	55	<3	<3	4	26	5	18
MMP8	9	51	<3	<3	4	12	5	8
ANXA6	6	43	<3	<3	3	4	<3	<3
CTSS	5	39	<3	<3	3	18	<3	<3
ANXA1	7	23	<3	<3	3	5	<3	<3
CTSC	3	21	<3	<3	3	9	<3	<3
COL5A1	6	13	<3	<3	3	4	3	3
COL4A5	6	8	<3	<3	3	4	<3	<3
MXRA5	3	3	<3	<3	<3	<3	3	3

SAMPLES

Human samples:

Healthy donor (Normal bone marrow – NBM) n= 10

MGUS n = 3

Newly diagnosed MM patients n= 7

Relapsed MM patients n= 6

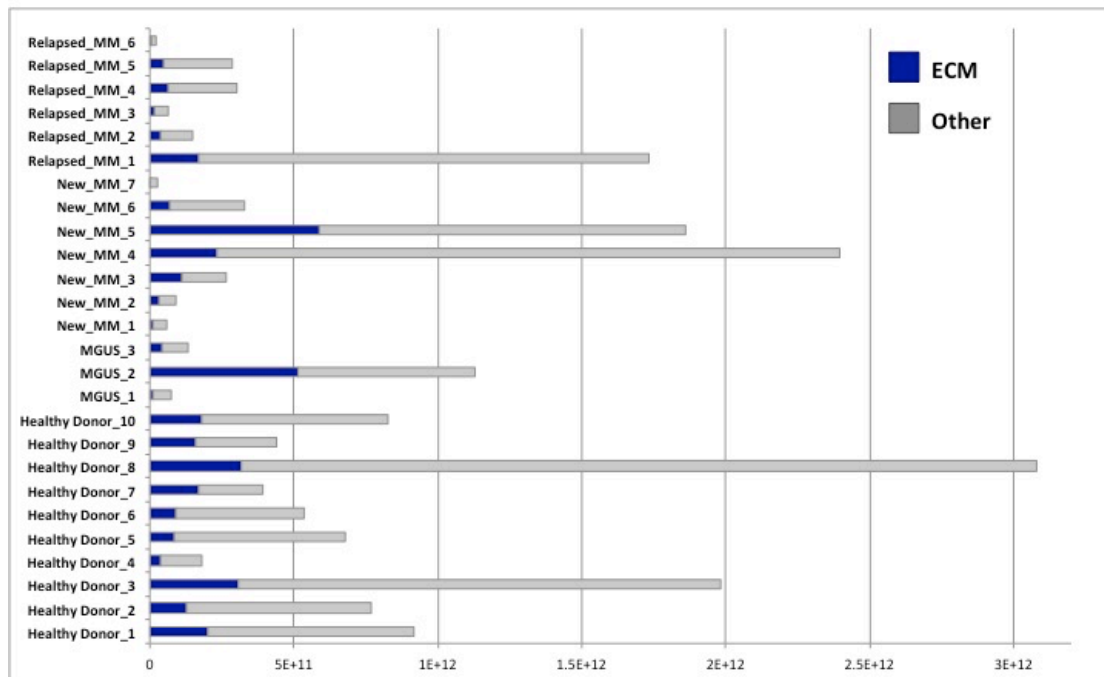
Murine samples:

Early – pooled bone marrow of 3 mice harvested 2 weeks post injection of MM1S-GFP-Luc⁺ cells (5×10^6).

Late - pooled bone marrow of 3 mice harvested 5 weeks post injection of MM1S-GFP-Luc⁺ cells (5×10^6).

Supplemental Figure S1: List of human and murine samples used for this study.

Peptide abundance (Total Intensity)



Peptide abundance (Total Intensity) - *Matrisome only*

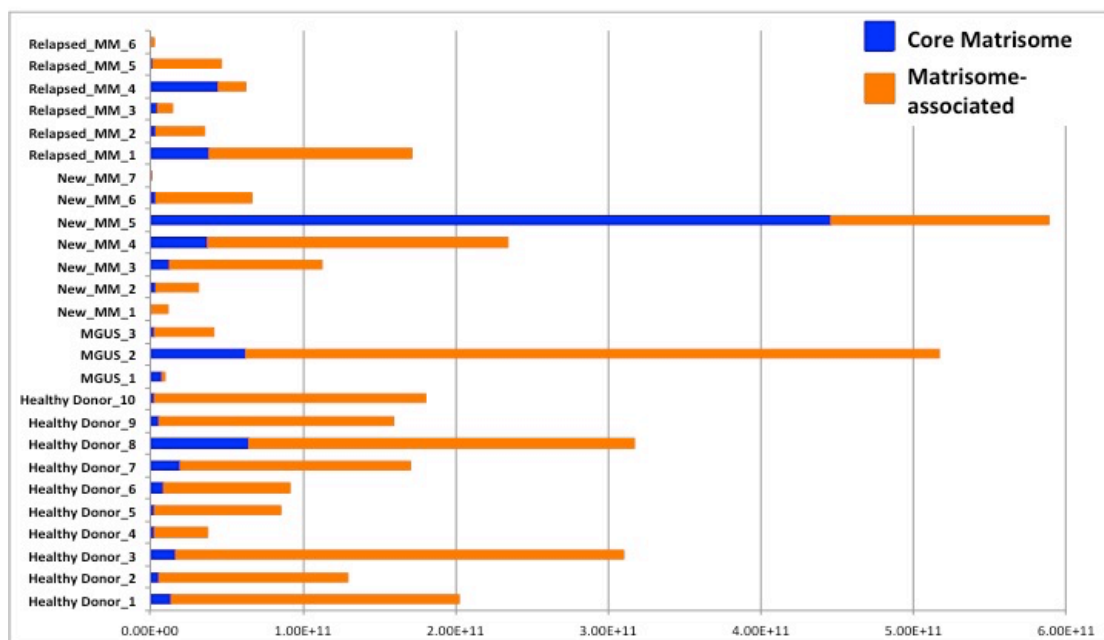
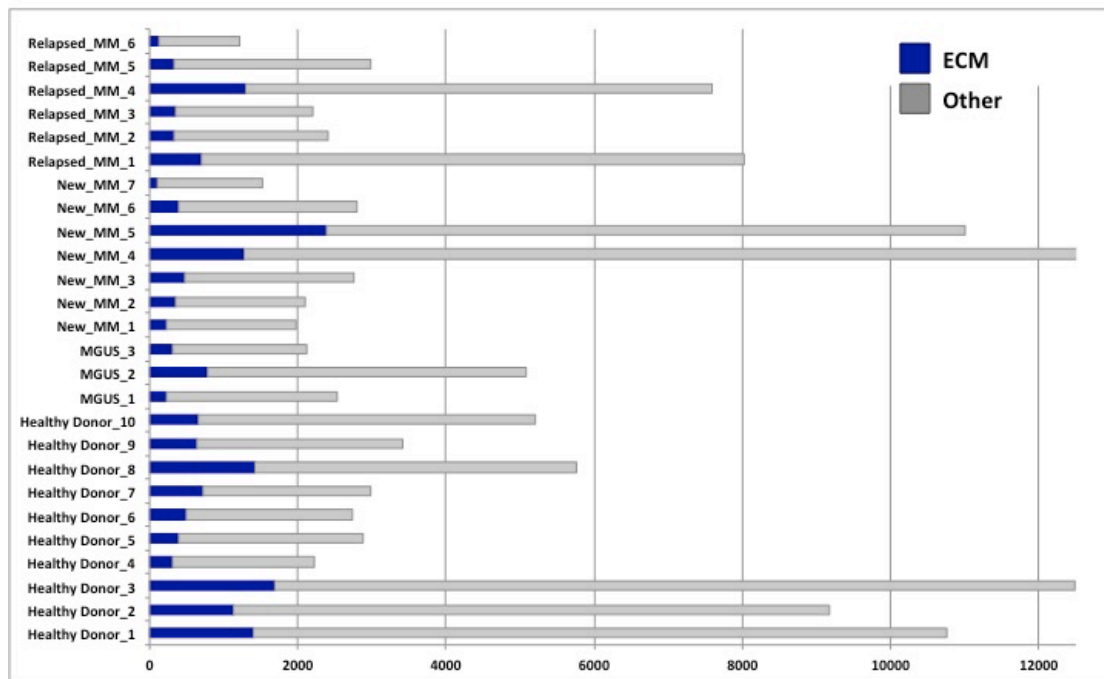


Figure S2A: Bar charts represent the peptide abundance for ECM vs. non-ECM proteins (top panel) and for core matrisome and matrisome-associated proteins (lower panel).

Number of Spectra



Number of Spectra - *Matrisome only*

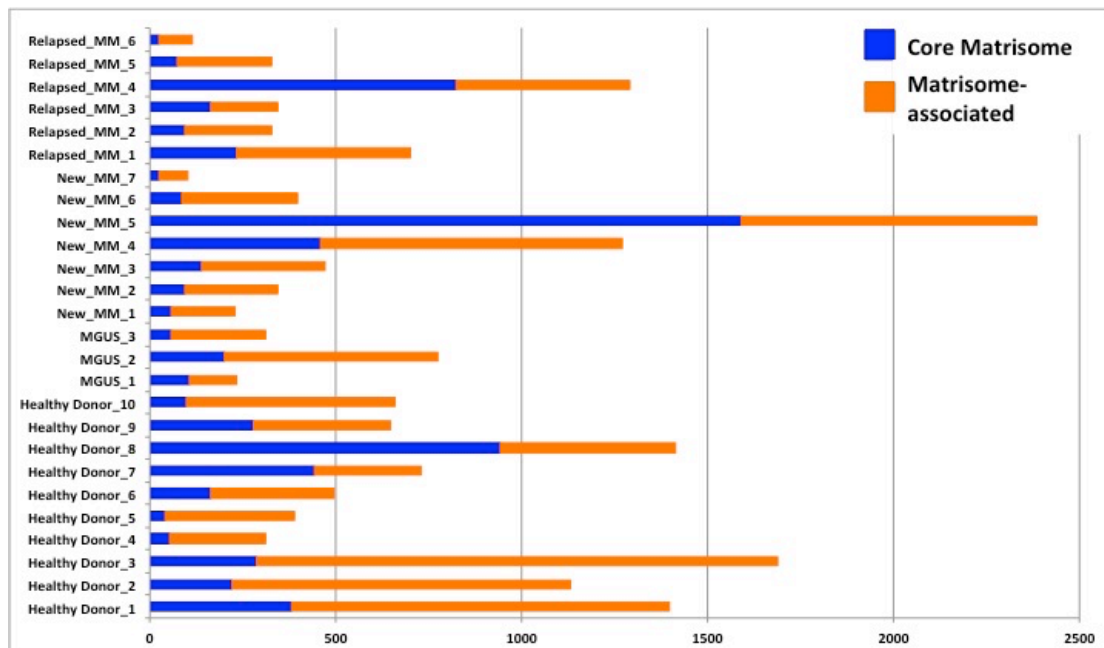
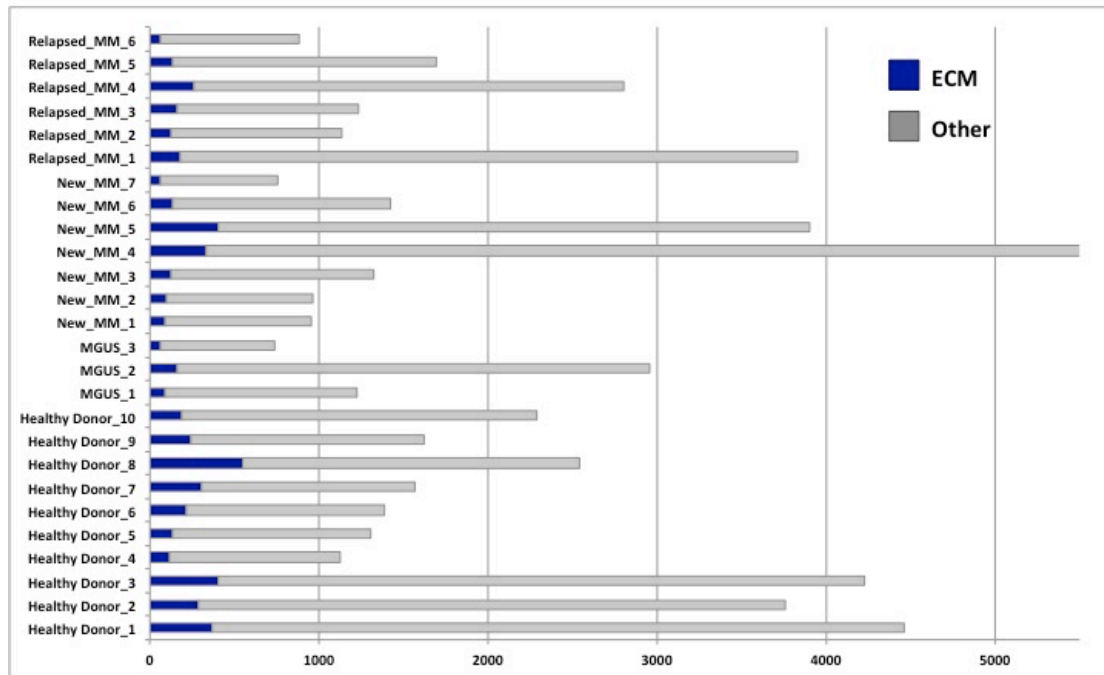


Figure S2B: Bar charts represent the number of spectra for ECM vs. non-ECM proteins (top panel) and for core matrisome and matrisome-associated proteins (lower panel).

Number of Unique Peptides



Number of Unique Peptides - *Matrisome only*

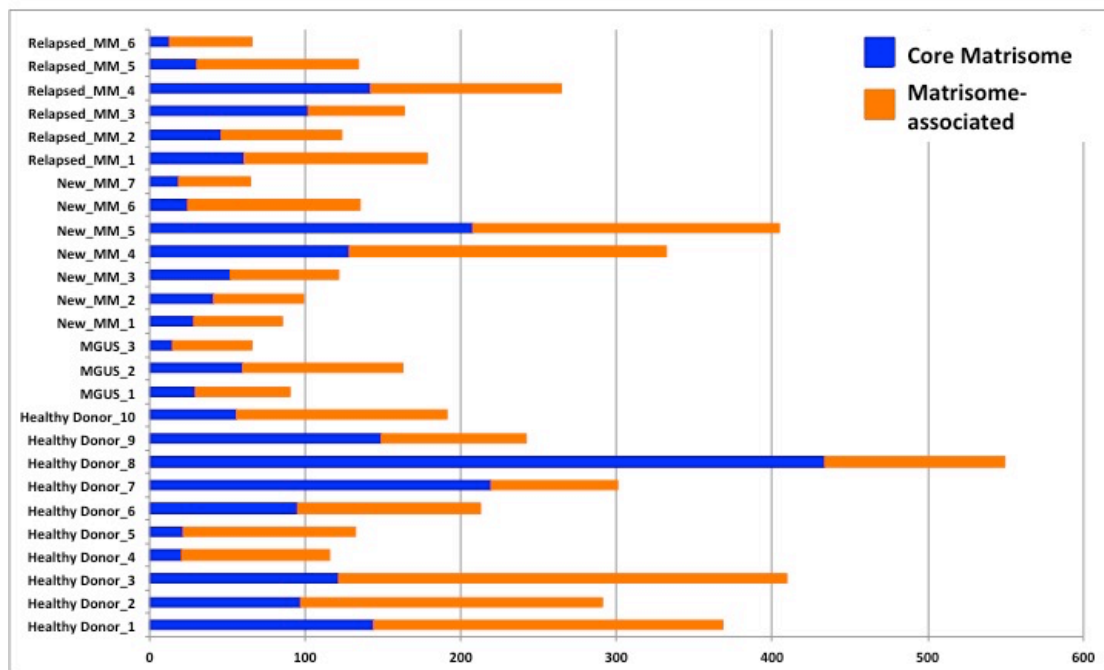


Figure S2C: Bar charts represent the number of unique peptides for ECM vs. non-ECM proteins (top panel) and for core matrisome and matrisome-associated proteins (lower panel).

CONCLUSIONS AND FUTURE DIRECTIONS

Our understanding of the pathobiology of cancer has been gleaned, for the most part, from genomic and proteomic studies that have focused on the tumour cell itself. While this has provided pivotal insights into tumour development and progression it is increasingly recognised that these cells operate within the context of a complex and dynamic microenvironment. Within this environment crosstalk between cancer cells, stroma, ECM components and immune cells create a supportive niche.

In MM direct cell-cell contact and soluble mediators such as cytokines regulate the growth, survival and homing of MM cells (1) while the bone marrow provides protective mechanisms for the development of micrometastasis and drug resistance (2-4). Glycoproteins such as selectin ligands have been implicated in the interaction between MM cells and the bone marrow microenvironment (4) however the glycan elements of these proteins, which largely determine their function, were previously not well defined in MM. This work provides insight into an additional layer of complexity within the bone marrow niche, which previously had not been explored extensively in MM. New insights are gained from this work in relation the importance of aberrant glycosylation in MM and global ECM remodelling.

An important focus of this work was to more clearly define the effect of altered glycosylation on the ability of MM cells to adhere and traffic within the bone marrow niche. This work identifies de-regulation of glycosyltransferases in MM and demonstrates that altered sialylation is apparent on the surface glycome of MM cells. Specifically the sialyltransferase ST3GAL6 is up-regulated in this disease and knockdown of this gene in MM cells reduces adhesion and migration, which is central to the process of metastasis. The ability of tumour cells to home to the bone marrow was also inhibited by knock down of ST3GAL6. This further validates the functional significance of seemingly minor changes in sugar moieties on the surface of cancer cells as being critical to tumour metastasis. Selectin ligands are dependent on sialylation for their normal function in the processes of leucocyte rolling, which is pivotal to cellular trafficking (5-7). The high expression of this sialyltransferase was also seen to directly impact on the survival of MM patients, with patients who have a higher expression of ST3GAL6 surviving, on average, 14 months less than those that have a low expression of this gene (Log Rank $P = 0.041$). This indicates that therapies that inhibit ST3GAL6 in MM may have a meaningful impact on MM patient survival and provides rationale for clinical studies in this area. It is feasible that using inhibition of ST3GAL6 in MM may result in clinical benefit for patients. ST3GAL6

knockdown resulted in reduced homing of MM cells to the bone marrow *in vivo*, which may be due to the reduced availability of functional selectin ligands on these cells. Conceptualization around this topic requires a deep understanding of the role of selectin ligands in the process of cancer cell bone marrow homing. A recent study has shown that in breast cancer E-selectin is critical to allow trafficking of cancer cells into the bone marrow, while the SDF-1/CXCR4 interaction anchors these cells within the bone marrow niche (8). Within the bone marrow these cells may reside in a dormant state where they may become resistant to systemic therapies that target proliferating cells and therefore persist as minimal residual disease (MRD), only to re-emerge as a metastatic clone (9, 10). The afore mentioned study provides rationale for the combination of CXCR4 and E-selectin ligand inhibition in breast cancer in order to drive breast cancer cells from the protective bone marrow niche (8).

In MM relapse of the disease post treatment is inevitable in most cases. This is thought to be due to the emergence of MM clones that have persisted in the bone marrow as MRD and are predictive of relapse in this disease (11). Strategies that target the MM cell directly have improved survival of MM patients, however eradication of MRD remains an outstanding goal (12). The therapeutic application of inhibitors of selectin ligand function, such as inhibitors of ST3GAL6, would ideally harness the potential ability of this strategy to reduce the homing of MM cells to the bone marrow. This could potentially result in reduced tumour burden, as seen in the *in vivo* models in this study, but it may also make dormant tumour cells more amenable to cytotoxic therapies by limiting the ability of these cells to migrate to the protective bone marrow niche.

Further interrogation of the bone marrow niche in MM was also carried out in this work in the form of ECM profiling at different stages of the disease. The ECM is a major component of the tumour microenvironment in several cancers, contributing to the regulation of cell survival, proliferation, differentiation and metastasis (13, 14). It is widely accepted that ECM components, such as integrins, are involved in MM cell interactions within the bone marrow, however a detailed profile of the ECM in MM had not been performed. This work applies a novel pipeline and annotation system to the ECM in MM in order to identify changes occurring at the proteome level as the disease evolves from the normal state progresses. The findings are in keeping with other studies that have demonstrated ECM remodelling activity early in the disease at

the MGUS stage (15). This supports the concept that not only does the ECM contribute to the permissive tumour microenvironment for malignant plasma cells but also participates in the pre-metastatic niche. Preparation of the metastatic niche is potentially mediated by factors such as matrix metallo-proteinases and lysyl-oxidase with consequent modulation of collagens and elastins at the pre-metastatic site. The concept of release of growth factors and cytokines by the tumour cells in preparation for distant site engraftment is now emerging. One such example is bone marrow-derived hematopoietic cells that express VEGFR1, which home to tumour specific pre-metastatic sites and form cellular clusters before the arrival of tumour cells, this is coincident with the upregulation of fibronectin at these sites providing a permissive niche for arriving tumour cells (16). However, it is increasingly clear that not only does the ECM form an important part of the permissive tumour microenvironment for malignant plasma cells, it also contributes to the pre-metastatic niche with alterations in the ECM evident at the MGUS phase. A recent proteome profiling study of primary BMSCs from MM patients, MGUS patients and non-neoplastic control patients revealed a group of ECM proteins, ECM receptors and ECM modulating enzymes that are up-regulated in a stepwise fashion from MGUS to MM. This includes proteins such as laminin $\alpha 4$, lysyl-hydroxylase 2, integrin $\alpha 5\beta 5$ and matrix metalloproteinase-2 (MMP-2). This indicates that matrix remodelling in MM is already present at the MGUS phase and may provide rationale for ECM directed targeting of the pre-metastatic niche in MM (15). This study also identified ECM proteins that were specific to MM and not identified in the bone marrow of healthy patients; ANXA2 and LGALS1. The confirmation of expression of the corresponding genes may indicate that plasma cells are themselves capable of altering the ECM composition in MM, which has been shown to be the case in other cancers (17, 18).

Future directions in the field of MM should continue to advance our understanding of the plasma cell in the context of the bone marrow microenvironment. Current therapies have focused with varying success on the elimination of plasma cells through direct or indirect cytotoxicity. This approach impacts on the “seed” in MM however the effects of these therapies on the “soil” are not fully known. As technologies rapidly advance our ability to interrogate the interactions between the plasma cell and its surrounding microenvironment therapies for this incurable disease can be further refined.

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