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GLYCOMIC INSIGHTS INTO MICROVESICLE BIOGENESIS

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GLYCOMIC INSIGHTS INTO MICROVESICLE BIOGENESIS

by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

August 2011

Dedication

I dedicate this dissertation to my mother and father. Thank you for teaching me the value of hard work and always believing in me.

Acknowledgements

I thank my advisor, Dr. Lara K. Mahal for her support, patience, guidance and encouragement. I would also like to thank my committee members, Drs. David Stein, Vishwanath Iyer, Christopher Sullivan and Hung-Wen Liu for their guidance. I thank Drs. Carlos F. Gonzalez and Amanda Engledow for sparking my initial interest in biological research. I am grateful to all the past and present members of the Mahal Lab for the intellectual support they have provided but most importantly for their friendship. I am forever indebted to my family for their love and support. My husband, Alex Gonzalez, is the reason I survived this crazy ride. I owe him my sanity and my PhD.

Glycomic insights into microvesicle biogenesis

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The University of Texas at Austin, 2011

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Cells can mediate intercellular communication by the secretion and uptake of microvesicles, nano-sized membranous particles that carry signaling molecules, antigens, lipids, mRNA and miRNA between cells. The biological function of these vesicles is dependent upon their composition and cellular origin which is regulated by mechanisms that are not well understood. Based on their molecular content, microvesicles may play a role in immune regulation, cancer progression, the spread of infectious agents and numerous other important normal and pathogenic processes. The proteomic content of microvesicles from diverse sources has been intensely studied. In contrast, little is known about their glycomic content. The glycosylation pattern of a protein or lipid plays a key role in determining its functional properties in several ways. Glycans can determine the trafficking of a protein to particular regions of the cell as well as the protein's half life. In addition, the glycan-dervied oligomerization of glycolipids and glycoproteins is a known mechanism for the activation of receptors and recognition of ligands on the surface of the cell. Glycomic analysis may thus provide valuable insights into microvesicle function.

I utilized lectin microarray technology to compare the glycosylation patterns of microvesicles derived from a variety of biological sources. When compared to cellular membranes, microvesicles were enriched in high mannose, polylactosamine, α2-6 sialic acid, and complex *N*-linked glycans but exclude terminal blood group A and B antigens.

The polylactosamine signature in microvesicles from different cell lines derives from distinct glycoprotein cohorts. After treatment of Sk-Mel-5 cells with lactose to inhibit lectin-glycan interactions, secretion of microvesicle resident proteins was severely reduced. Taken together, this work provides evidence for a role of glycosylation in microvesicle-directed protein sorting.

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Chapter 1: Introduction

1.1 Overview

Microvesicles are a diverse group of cellular messengers, influencing their environments in critical ways that scientists have only just begun to appreciate [1]. Despite the fact that glycans play a fundamental role in many aspects of cellular biology and communication, the glycosylation of these particles is an overlooked and underrepresented aspect of microvesicle research. The focus of my dissertation is to comprehensively examine and compare the carbohydrate profiles of extracellular microvesicles and investigate whether glycosylation influences microvesicle content. To this end, I have isolated and characterized secreted vesicles from a diverse panel of biological sources and utilized lectin microarray technology as a tool for comparative analysis of microvesicle glycosylation. Microvesicles from a diverse panel displayed a conserved carbohydrate signature suggesting the existence of an active microvesicle-directed sorting mechanism. The final chapter describes steps taken to investigate whether glycan-lectin interactions are involved in microvesicle protein sorting.

This introductory chapter gives a brief background on the biogenesis of microvesicles and discusses some of the confusion in the literature. In addition, some examples of their biological and clinical importance are provided with an emphasis on how cellular origin and content can vastly influence function. Due to their biogenesis, microvesicle membranes are a derivative of the plasma membrane [2]. As such, they contain proteomic, lipidomic and glycomic features that are relative to cellular membranes [3-5]. Therefore, the typical glycans of mammalian cellular membranes are introduced, with examples of some of the important roles ascribed to glycans. In addition, central aspects of glycobiology are discussed, such as the tools used to study glycans and

the challenges that arise from their inherent properties. Finally, I will explain the concept of lectin microarrays and why this particular technology is well suited for comparative analysis of multiple samples.

1.2 MICROVESICLES

It is now widely accepted that cells can mediate intercellular communication by the release of microvesicles that can shuttle biologically active molecules to recipient cells. Microvesicles are composed of cytosolic content enclosed by a phospholipid bilayer with the same outside orientation as the cell membrane. They are essentially small cellular packages, exporting an assortment of soluble and membrane-bound proteins, lipids, small molecules and nucleic acids to the extracellular space [1, 6-8]. First described in 1963 as "dust" produced by platelets, secreted vesicles were considered at that time to be inert cellular debris [8]. Research from the past three decades strongly supports the currently held view that vesicle secretion is a biologically important cellular process conserved from eukaryotes to humans. Since first identified, they have been isolated and studied from the *in vitro* cultures of numerous species and cell types as well as from physiological fluids such as urine, blood, saliva, seminal fluid, breast milk, and tumor effusions [4, 8, 9]. Through transport in biological fluids they can potentially communicate with other cells at considerable distances in a manner analogous to the endocrine system.

Nomenclature

Microvesicles have been the focus of intense research due to their promising pontential for clinical applications, implications in various pathogenesis processes and

possible role in a number of intercellular signaling events [10-14]. Nevertheless, the scientific community has not reached a consensus on the terminology used for this heterogeneous group of organelles. Researchers tend to classify these particles based on several different categories: 1) their phenotypic characteristics, such as size and content, 2) their cellular origin, 3) their biological impact and 4) their biogenesis or cellular exit pathway. For example, scientists have used the terms microvesicles and nanovesicles to define two broad categories of vesicles based on size [15]. With regard to cellular origin, content or biological impact, it can be said that these are heavily intertwined, with cellular origin dictating vesicle content which in turn has an effect on its biological role. Thus, the terms oncosomes, prostasomes, melanosomes, tolerosomes, prominosomes and dexosomes are just some of the many descriptive names given to secreted vesicles [16]. The popular term, microparticles, typically refers to procoagulant vesicles found in the bloodstream and may originate from platelets, granulocytes, monocytes, endothelial cells, smooth muscle cells, and tumor cells [17].

Extracellular vesicles are categorized by one of three known biogenesis pathways, however, the possible existence of other mechanisms cannot be ruled out. As the name implies, apoptotic blebs/bodies/vesicles are the result of systematic cellular breakdown from apoptosis. To make matters more confusing, the term apoptosome refers to a large protein structure formed at the onset of apoptosis and despite similarities with the way in which other vesicles are named, is not used to describe secreted apoptotic vesicles [18]. The size distribution of apoptotic vesicles has not been systematically identified but generally these particles are heterogeneous in size and can be greater than 1 μm in diameter. Apoptotic vesicles are known to display phosphatidyl serine (PS) on the outer leaflet of their membranes and contain cellular material including genomic DNA and intact organelles [19]. The process of clearing these vesicles from the body is performed

by macrophages and other phagocytes [20]. In the absence of clearance, such as in impaired immunity or *in vitro* cell cultures, apoptotic vesicles may interact with and influence other cells.

A second biogenesis mechanism involves the outward budding and subsequent expulsion of vesicles directly from the cellular membrane surface. As in the previous category, many terms have been coined to describe membrane shed vesicles including shedding microvesicles, ectosomes, membrane microvesicles and microparticles [8, 21]. These vesicles are sometimes referred to as ectosomes in the literature. The term ectosome is used in reference to the process of ectocytosis which is the shedding of proteins from the ectodomain of cells. Ectosomes are heterogeneous in size ranging from 100 nm to several microns, but smaller vesicles that are 50-80 nm in size have also been observed [22]. They are commonly characterized by the presence of lipid-raft domains and exposure of PS on the outer surface [23]. However, whether these diverse vesicles all share the same properties is unknown. Budding of vesicles from the cell membrane has been observed in numerous cell types as either a constitutive process or by activation of the cell through various means.

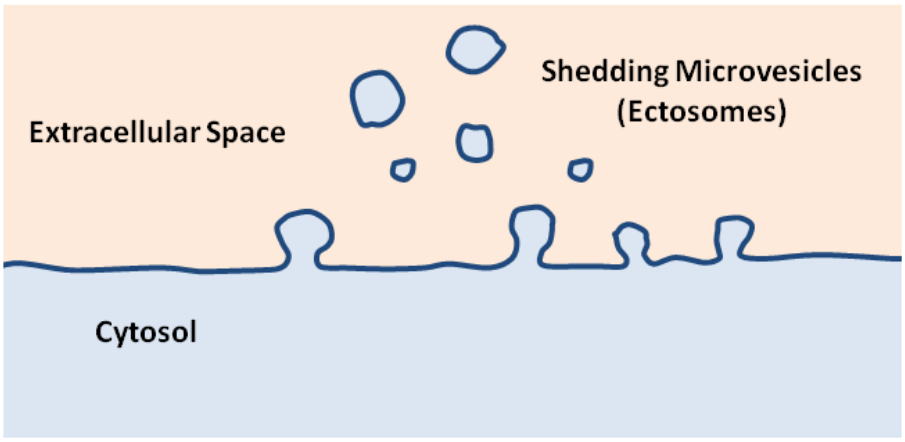


Figure 1.1: Schematic representation of microvesicles that are shed from the outward budding of the plasma membrane (ectosomes). They contain cytosolic material and are enveloped by a phospholipid bilayer.

Exosomes are vesicles of endosomal origin. After endoctyosis, cargo is sorted either to the lysosome or to late endosomal compartments. Smaller vesicles are then formed from the inward budding of the limiting membranes of multivesicular bodies (MVB) and release happens upon fusion of the MVB with the plasma membrane. These vesicles are reportedly more homogeneous in size and appearance, with a size range of about 30 to 100 nm and a cup-shaped morphology seen by electron microscopy [1, 15]. Of the three broad categories of extracellular vesicles, exosomes have been the most widely studied in terms of their proteomic and nucleic acid content. Consequently, proteomic studies have identified several commonly incorporated exosomal proteins including members of the tetraspanins and heat shock proteins. These proteins, considered exosomal markers, are often used to identify exosomes and differentiate them from shedding vesicles [1, 4].

One of the main problems in the scientific literature is that the terms used to describe extracellular vesicles can often have several opposing definitions. For example, the word microvesicle is at times used to encompass all secreted vesicles including exosomes and apoptotic vesicles [24]. On the other hand, some researchers use this term to refer exclusively to the larger shedding vesicles [8]. Similarly, many articles claim to be studying exosomes based solely on reports of size, density or the presence of one or two "exosomal markers" with no other proof that the vesicles are of endosomal origin. The problem with this is that when comparing the numerous exosome proteomic studies, there is not one single marker that is consistently present in all of them [1]. The question remains whether this is due to the lack of a true marker, or whether all of these studies were indeed done on *bona fide* exosomes. As mentioned previously, vesicles with a size and density similar to exosomes have been reported to arise from the direct budding of the cell membrane of neural progenitor and epithelial cells. These vesicles were obtained as a heterogeneous mixture with exosomes but there was a clear distinction in their protein content and they did not contain the marker CD63 whereas the exosomes did [25, 26]. On the other hand, vesicles have also been observed to bud directly from the tetraspanin-enriched domains of T-cell plasma membranes [27]. The idea that two separate vesicle populations exists containing larger membrane shed ectosomes and smaller endosomal exosomes positive for CD63 came from studies done on platelets [28]. However, it is possible that these absolute distinctions cannot be universally made across all cell types. It is clear that we need to have a better understanding of these extremely diverse extracellular messengers.

For the purposes of this manuscript, I will use the term microvesicles to refer to the secreted vesicles of endosomal and plasma membrane origin from all biological sources with the exclusion of apoptotic vesicles. I will distinguish between them when

referring to literature that clearly makes the distinction. The microvesicles obtained in the work described in this dissertation are 40 – 100 nm in size and contain the exosomal markers, CD63 and CD81. However, because the biogenesis of these microvesicles was not defined, I will not refer to them as exosomes.

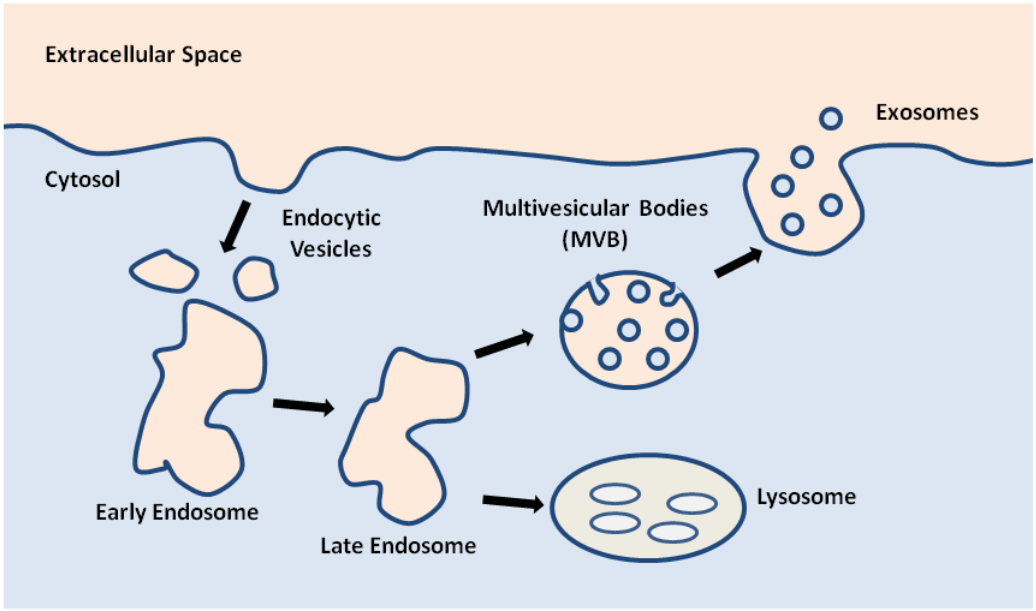


Figure 1.2: Schematic representation of the biogenesis of exosomes. Endocytic vesicles are targeted to the early endosome where proteins are either trafficked back to the plasma membrane or sent to the late endosome. In the late endosome, cargo is sorted to the lysosome for degradation or to multivesicular bodies. Exosomes are formed from the inward budding of limiting membranes. The MVB fuses with the cell membrane and releases exosomes into the extracellular space.

Biological and clinical significance

Microvesicles have been implicated in a myriad of normal and pathogenic cellular processes. Although they are known to share many similar characteristics, the diversity of their biological impact stems from the unique properties inherited from parent cells as well as the properties of the recipient cells. Several studies have shown that microvesicles can interact with cells in many different ways including fusion and subsequent release of their content [29]. Because of their many components, including membrane bound receptors, soluble ligands, mRNA and microRNA, this can lead to pleiotropic consequences for the recipient cell (Figure 1.3). Those consequences vary from cell to cell and are dependent on many factors, the arguably most important of these is the molecular content of the vesicle.

A simple explanation of the role of microvesicles is that they advance the agenda of the parent cell. This can be seen in numerous examples. For instance, platelet microvesicles have procoagulant properties [30, 31] whereas dendritic cell vesicles stimulate the immune system by activating T-cells and microglia [32, 33]. The latter has led to enthusiasm for the potential to utilize immuno-stimulatory microvesicles as anti-cancer agents. Anti-tumor responses were upregulated in mouse models upon treatment with microvesicles from dendritic cells pulsed with tumor antigens as well as tumor-cell derived microvesicles [12, 14]. In addition, it is believed that microvesicle secretion is exacerbated with the onset of cancer given that greater quantities are found in the fluids of cancer patients [8, 34]. This increase has also been associated with conditions such as atherosclerosis [31, 35], diabetes [36], preeclampsia [37], and other inflammatory disorders [23]. Differences in proteomic and nucleic acid content can be detected when comparing normal and diseased vesicles. As a result, the use of circulating microvesicles

as biomarkers for the non-invasive detection of various diseases is under investigation [38-40]. Thus, there are many promising therapeutic uses currently being explored.

In contrast to their proposed stimulatory role, microvesicles can also be immuno-suppressive in their function, promoting immune tolerance to cancerous tumors, to food antigens in the gut [41], during pregnancy [42-45] and breast feeding [46]. One of the ways in which both cancer cell and placenta microvesicles are thought to achieve this is by presentation of the Fas ligand on the vesicle surface which leads to the activation of T-cell apoptosis [47]. In addition to evasion of the immune system, tumor derived microvesicles are implicated in disease progression by several other methods. These include epigenetic transformation of neighboring cells [19, 48], promotion of angiogenesis [49], and the creation of a pre-metastatic niche [50]. The propensity of microvesicles to expose recipient cells to regulatory and signaling molecules is a powerful tool used for the propagation of cancer and other diseases. The above examples are by no means comprehensive, as microvesicles have been described in countless other important biological roles.

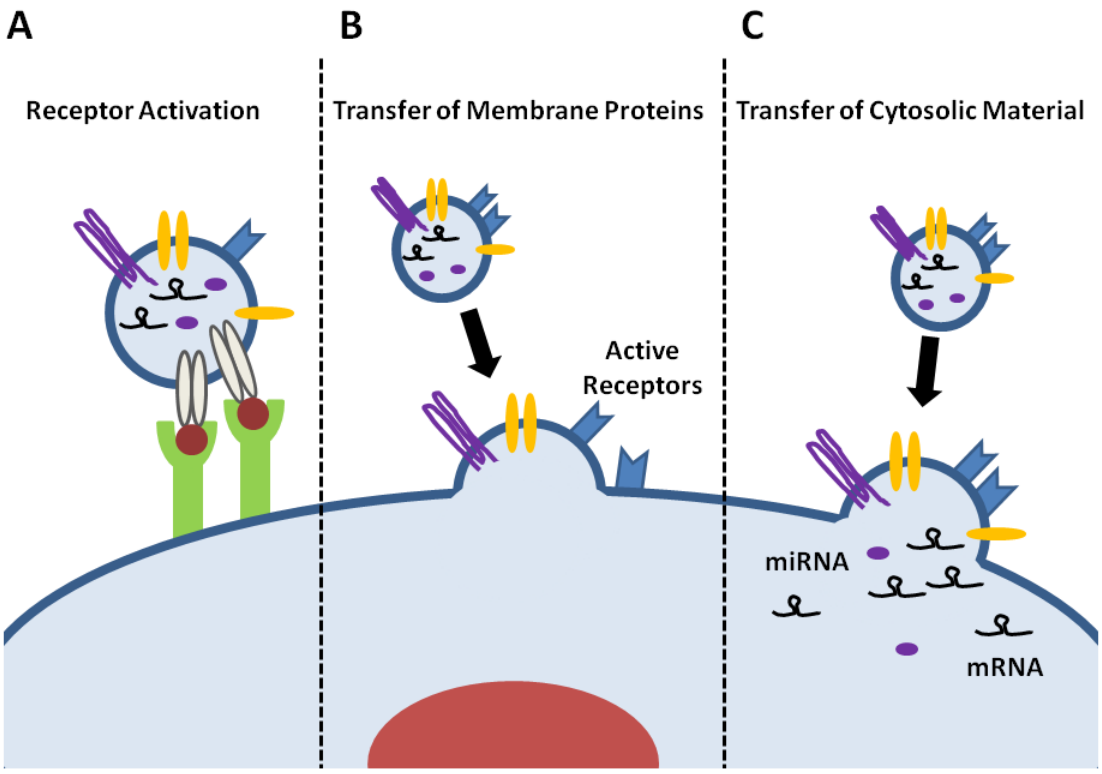


Figure 1.3: The interaction of microvesicles with acceptor cells can have pleiotropic consequences. (A) Exposed ligands on the microvesicle surface leads to receptor activation of the recipient cell and activation of cell signaling. (B) Fusion of the microvesicle with the cell leads to incorporation of membrane bound proteins. This can lead to cellular transformation as seen in the transfer of the mutant, hyper-active form of EGFR from cancer cell microvesicles to normal cells. (C) Uptake of microvesicles also leads to transfer of cytosolic material such as soluble proteins, small molecules and RNA. The transfer of miRNA can lead to various epigenetic reprogramming of the recipient cell.

1.3 GLYCOBIOLOGY

The diversity of proteins and lipids found in organisms can be amplified by the addition of carbohydrate moieties which can alter both structure and function. This process of glycosylation is conserved across all domains of life and is the most abundant post-translational modification found in nature [51]. Glycans are involved in countless important biological processes including protein maturation and stability, cellular adhesion, receptor activation, immunity and host-pathogen recognition [52]. In fact most extracellular events are the product of some form of glycan interaction. As a result, all cells from bacterial to mammalian are densely covered in carbohydrates. Given their importance, it seems odd that glycobiology, the study of the biological role of carbohydrates, is an understudied field [53].

The glycosylation of mammalian cell membranes

Glycans, from single monosaccharides to highly complex oligosaccharide structures can exist in free form or covalently attached by a glycosidic bond to lipids and proteins. The most common and diverse type of glycolipids found in animals are glycosphingolipids, in which the glycan is attached to the hydroxyl group of a ceramide. As seen in the myelin sheath of neurons, they can account for a substantial portion of the cell membrane content. They are typically found in clusters within lipid raft domains and are involved in the activation of various cell signaling events [54]. A second group of glycolipids are the glycosylphosphatidylinositol (GPI) anchors, composed of phosphatidylinositol and ethanolamine linked by a glycan bridge and attached to the carboxy terminus of proteins via the ethanolamine [55]. GPI anchors are diverse because the properties of both the lipid and glycan can vary and they may be attached to one of many different proteins or no protein at all. Like glycosphingolipids, GPI anchored

proteins associate with lipid rafts and are involved in cell signaling [56]. In addition, GPI anchored proteins are known to be enriched in microvesicles [57].

The two major classes of membrane glycoproteins are formed from the glycosidic linkage of oligosaccharides to either the nitrogen of asparagines (*N*-linked glycans) or the hydroxyl oxygen of serines and threonines (*O*-linked glycans) on proteins. Processing of *N*-linked glycans occurs in the lumen of the ER and golgi by the sequential action of numerous glycosidases and glycosyltransferases. All *N*-linked glycans are attached to the protein via a conserved pentasaccharide core structure composed of $Man_3GlcNAc_2$ linked to asparagine. The fully formed *N*-linked glycans are classified into one of three categories: high mannose (oligomannose), complex and hybrid (Figure 1.4). These diverse glycans can be rather complex, with highly branched tri- and tetra-antennary structures composed mostly of mannose (Man), galactose (Gal) and *N*-acetyl-D-glucosamine (GlcNAc) and typically capped with *N*-acetyl-D-galactosamine (GalNAc), fucose (Fuc) or sialic acid (NeuAc) [58].

One of the most well known roles of *N*-glycosylation is to aid in the maturation and quality control of proteins. However, the abundance of this modification on membrane bound and secreted glycoproteins implies an essential role in numerous other extracellular processes. Studies based on *N*-linked glycosylation mutants have shown that the absence or aberration of this modification can have profound effects on cellular development and can even be lethal in embryonic mice [59]. These adverse effects are due to changes in one or several features of normal proteins leading to changes in protein function. An example of this is highlighted in studies of the neurotransmitter transporter, GAT1, where different glycosylation mutants led to either decreased protein stability, an arrest in cell membrane trafficking or a decreased affinity for the substrate. In all cases, changing the *N*-linked glycosylation of GAT1 essentially inhibited its function [60].

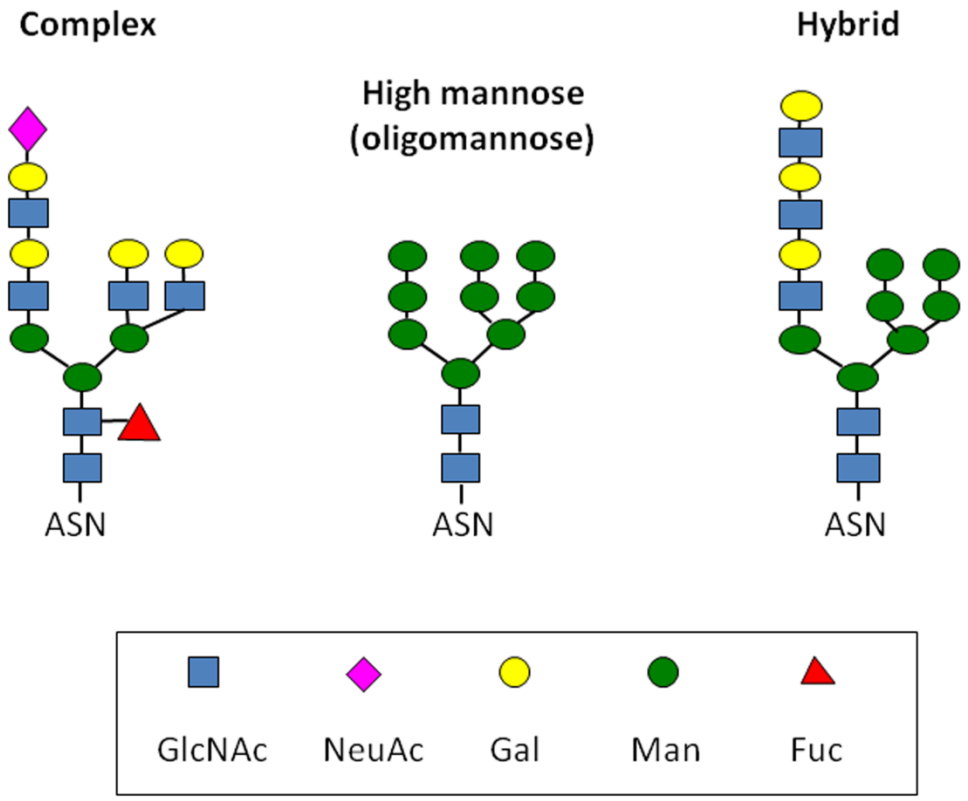


Figure 1.4: The three categories of *N*-linked glycans are complex, high mannose and hybrid. Examples of each are shown.

The most abundant type of *O*-glycosylation is initiated by the addition of GalNAc linked α to serines or threonines. Proteins containing high amounts of this type of modification (called the Tn antigen) and extensions thereof are known as mucins and mucin-like glycoproteins. Extension of the Tn antigen occurs by the addition of Gal, GlcNAc, Fuc and NeuAc to form linear or highly branched polymers. In contrast to *N*-glycoproteins, which all have the same core, mucin glycans can contain one of eight different core structures, the most common being the core 1 or T antigen. Mucins are heavily glycosylated secreted and cell surface proteins. As the name implies, they are

responsible for the protective and hydrating mucous secretions of epithelial cells [61]. O-GalNac glycans are similar to *N*-linked glycans in that they can be important for protein stability, trafficking and function [62]. Additionally, *O*-linked glycans are involved in immunity, tissue development, cell adhesion and other cellular interactions. Studies of mice deficient in the transferase gene responsible for the addition of the core 1 had defective angiogenesis and died by embryonic day 14 [63]. This demonstrates the importance of *O*-linked glycosylation for normal development.

Examples of the different types of glycan modifications described above are depicted in Figure 1.5.

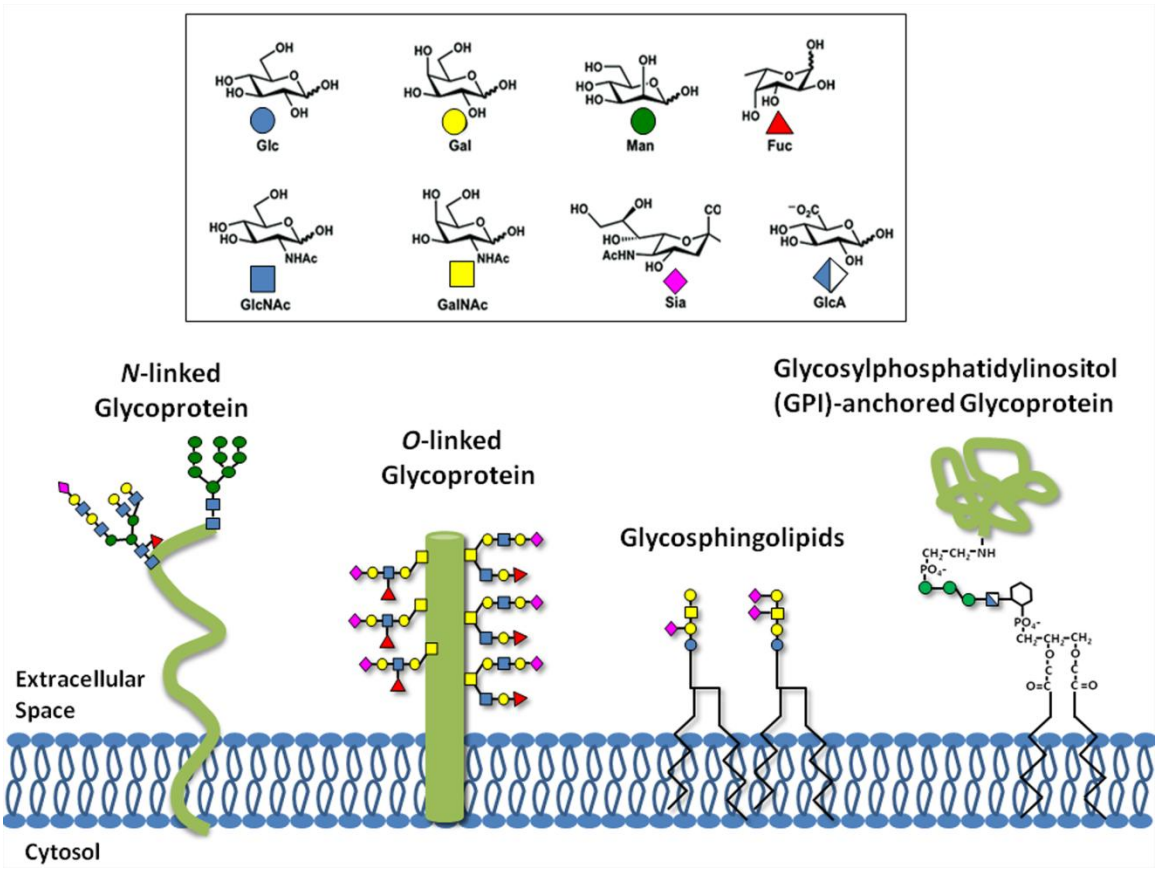


Figure 1.5: Examples of common glycan modifications found on plasma membrane proteins and lipids.

The complexity of glycans leads to challenges in the field

In order to gain a greater understanding of the role of glycosylation on biological processes, researchers must investigate how the diverse properties of glycans can influence protein function. The field of glycobiology is faced with several challenges due to the inherent properties of carbohydrates. In a glycosidic bond, a monosaccharide can form either an α or β linkage to one of several hydroxyl groups on another monosaccharide. In addition, monosaccharides can form more than two glycosidic bonds to form branched polymeric structures with multiple possible combinations of glycosidic

linkages [64]. This is in contrast to the linear polymers formed from the single possible linkage of amino acids and is one of the reasons for the greater complexity and diversity of carbohydrate structures. This structural diversity is what allows carbohydrates to be the determinants of intercellular recognition and communication.

Knowledge of the sequence, glycosidic linkage and overall structure of a carbohydrate, requires the application of several analytical tools. Obtaining the monosaccharide composition of a glycan through means such as mass spectrometry, high performance liquid chromatography (HPLC) or gas chromatography (GC) does not provide information of the anomeric glycosidic linkages. These technologies are often used in conjunction with the use of glycosidases or chemical techniques that cleave oligosaccharides based on well characterized linkages. Although, mass spectrometry has been an invaluable tool for glycomics, the stereoisomeric nature of carbohydrates makes it difficult to apply mass spec as a stand-alone method for the identification of monosaccharides. Therefore, the specific monosaccharides must be deducted from prior knowledge of existing glycans, metabolic pathways and expression profiles. Even after high quality sequence and linkage analysis, to obtain structural information, additional methods such as nuclear magnetic resonance (NMR) and X-ray crystallography must be employed [65].

Unlike proteins that are the products of a highly conserved genetic code, there is no known similar code that reliably dictates the addition of monosaccharide units on glycans. Instead, glycosylation is influenced by dynamic factors such as the metabolic state of the cell or the expression and availability of the enzymes responsible for glycan synthesis. Quite often, different glycan processing enzymes will compete for substrates. This leads to differences in glycosylation within a single glycan attachment site on a protein, a phenomenon called microheterogeneity [66]. Predictive glycosylation sites

based on amino acid sequences are known, however, microheterogeneity makes it difficult to predict which glycan if any will be added to that site. An analysis of the site specific glycosylation of γ-glutamyl transpeptidase in human renal tissue identified 15 different glycans [67]. Moreover, proteins that contain several glycosylation sites can display macroheterogeneity by the occupation of disparate glycans. For example, the Notch receptor is known to contain both *N*- and *O*-linked glycans [68]. As discussed previously, differences in the glycoforms of a given protein can lead to changes in cellular location, half-life, and binding partners. Regardless of the challenges, protein glycosylation is an important aspect of protein and cellular diversity that must be carefully studied. For these reasons, glycobiologists are constantly trying to develop new methods to more efficiently study the glycosylation of biological samples. Some of the common methods were discussed above. The next section focuses on the use of glycan-binding proteins to study carbohydrate structure and function.

1.4 LECTINS

Carbohydrates, like DNA, are found in all existing life forms. Hence, all life forms also contain the proteins that recognize and bind to carbohydrates. One such category of proteins are the lectins, carbohydrate-binding proteins that are non-enzymatic and of non-immune origin (i.e. not antibodies). Lectins from plant sources were initially studied for their ability to agglutinate bloods cells. It was almost a half a century later when their hemagglutination properties were linked to carbohydrate binding and another ten years before their blood group specificity was described in the 1940s. Since then, glycan-lectin interactions have been shown to be important for protein trafficking, immunity, cellular recognition and homing. Pathogens such as viruses and bacteria utilize

glycan-lectin interactions for host recognition and cellular adhesion [69-71]. As seen in the secretion of the highly toxic ricin from the castor oil plant *ricinus comunis,* the secretion of lectins can also serve as a protective mechanism.

Lectins are classified into different families based on amino acid sequence homology of their carbohydrate recognition domains (CRDs). Members of the same family typically have an affinity for the same or similar carbohydrate structures but there are many cases where homologous CRDs recognize dissimilar structures. Multiple X-ray crystallography studies exist for lectins with their known binding partners. In general, the CRD of lectins that recognize *N*- and *O*-linked glycans consist of shallow binding indentations on the surface of the protein [72]. Therefore, lectins tend to bind to the terminal carbohydrate residues on the glycan with relatively low affinities (K_d values can be in the low millimolar range). However, because lectins often contain several CRDs or are multimeric, their multivalency serves as a mechanism for forming biologically relevant interactions [73]. Some lectins can be very specific whereas others may be more promiscuous in their glycan recognition. A well-known example of a highly specific lectin is the bird influenza virus hemagglutinin, which preferentially binds to NeuAcα2-3Gal linkages and therefore does not typically infect human tracheal epithelia, which are rich in terminal NeuAcα2-6Gal epitopes [74].

Lectin Microarray Technology

Traditionally, lectins have been used in assays such as histochemical staining, affinity chromatography and cell typing. However, given the innate heterogeneity and complexity of most biological samples, a more extensive approach to studying these samples is needed. Taking a cue from DNA and antibody microarray technology, our lab

and a few others independently developed the lectin microarray to study the glycosylation patterns of biological samples in a more comprehensive and high-throughput fashion. In this method, multiple lectins with diverse specificities are covalently attached to a solid substrate in a microarray format which can then be probed with glycosylated samples [75]. Our lectin microarray consists of approximately 80 commercially available plant lectins and 10 recombinantly produced bacterial lectins printed onto a hydrogel slide in a 24-subarray format. Each subarray is printed with 3 replicate lectin spots with each spot containing picoliter amounts of concentrated lectin solution. One microarray slide can therefore be incubated with 24 different glycosylated samples (Figure 1.6). After rinsing away any unbound material, the slide is scanned and the different patterns of flourescent spots correspond to the lectin binding and therefore glycosylation patterns of the samples [76-78]. The spots not only give a positive or negative output if a particular glycan is present or absent, but we can also semi-quantitatively compare carbohydrate epitope amounts. This technology has been successfully used in our lab and by others to analyze the glycomic content of pathogenic bacteria, HIV, mammalian cells and tissues, and other biologically relevant samples [79-82].

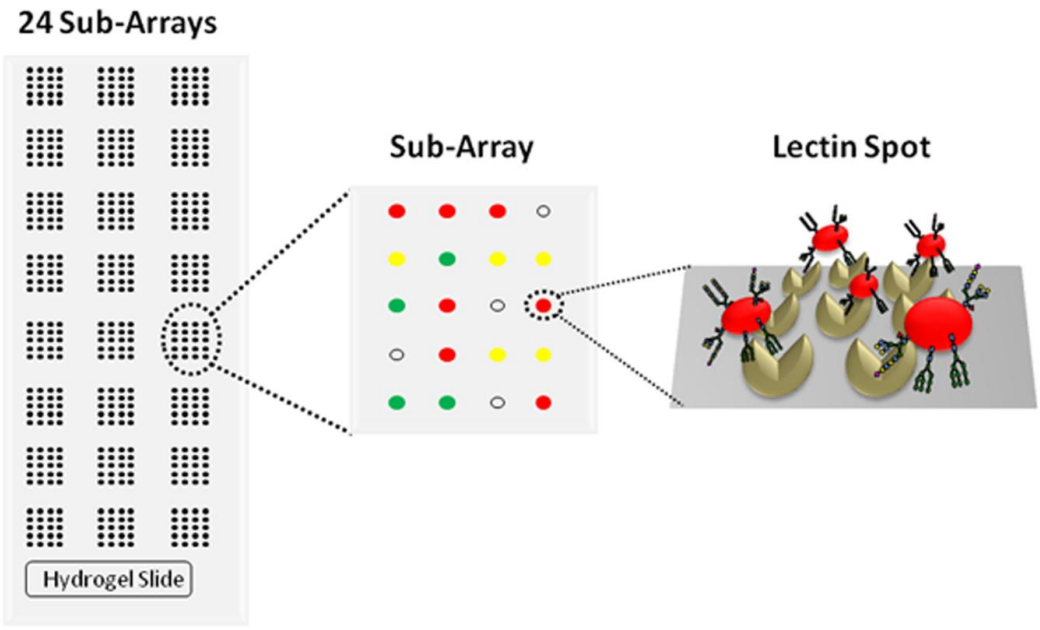


Figure 1.6: Schematic of the current lectin microarray technology. The lectins are printed on a hydrogel slide in a 24 subarray format so that 24 samples may be probed on one slide. Fluorescently labeled glycosylated samples are incubated on the array. After washing to removing unbound material, slides are scanned. The lectin binding pattern reveals the glycosylation profile of the sample.

There are two kinds of methods typically applied in our lab for lectin microarray analysis. In the first and simpler method, single color analysis, we label our samples with a fluorescent dye such as cyanine-3 and probe one sample per subarray. Although the resultant lectin binding patterns provide us with information about the glycomic content of our samples, to do a more comparative analysis between samples, we use the ratiometric dual-color approach. In this method two different orthogonally labeled samples (cyanine-3 and cyanine-5 labeled) are incubated on the same array. These two samples can then competitively bind with the lectins on the array providing a ratiometric output between the two channels. Because slight differences in individual lectin activity

and local background between arrays would not affect the ratio, we use this method for comparative analysis across multiple samples by using the same orthogonally labeled reference. An example of both a single-channel and dual-channel probed lectin array is provided in Figure 1.7. Thus, the ability to probe many samples at once and semi-quantitatively measure glycomic content makes lectin microarray technology quite suited for a comparative glycomic analysis of a diverse set of microvesicles

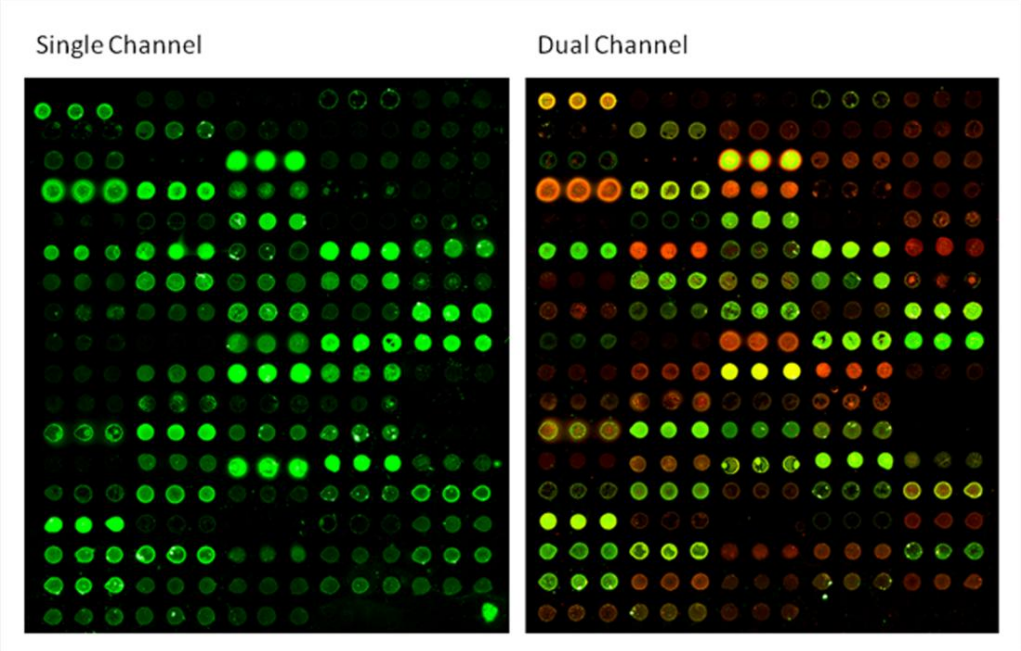


Figure 1.7: Example of a single-color and dual-color lectin microarray experiment. For both, 1.5 µg (by protein) of Cy-3 labeled Sk-Mel-5 microvesicles were probed. An equal amount of Cy-5 labeled H9 membrane was incubated with the microvesicles as the reference in the dual color experiment.

Chapter 2: Isolation and characterization of microvesicles

2.1 INTRODUCTION

Overview

Previously, researchers in our lab effectively utilized lectin microarray technology to compare the glycosylation profiles of microvesicles, HIV particles and cell-membranes derived from three different human T- cell lines. From this analysis, Krishnamoorthy et al. identified a distinct glycomic signature in T-cell microvesicles that closely resembled the HIV glycome [79]. We wanted to know if this glycomic signature was typical of all or most human microvesicles or was exclusive to T-cell or hematopoietic cell vesicles. Identifying a pervasive microvesicle glycomic profile would have important implications for the role of glycans in microvesicle biogenesis and/or function. To investigate this, my work focused on examining the glycomic content of microvesicles derived from a diverse panel of biological sources. This chapter describes the characterization and isolation of microvesicles and is the first comparative analysis of the microvesicle yields obtained from multiple different cell lines.

Methods for Microvesicle Isolation and Characterization

It is now widely accepted that cells secrete various types of lipid-bilayer enclosed microvesicles that have the ability to deliver cellular content to surrounding recipient cells. These different particles are defined by physical characteristics such as size, density, lipid and protein content although the lines of classification are often blurred [83]. In general, there are two basic categories of cell secreted microvesicles defined by what is believed to be their mechanism of biogenesis. Exosomes are described as having an endosomal origin and are thought to be released after the fusion of multivesicular

bodies with the plasma membrane. There is no consensus on the size of exosomes but they are generally reported as being within the range of 20-200 nm in diameter [1]. A second type of microvesicle, most often called shedding microvesicles, is released to the extracellular environment by the outward budding of the plasma membrane. Shedding microvesicles (ectosomes) are typically described as being larger in size (up to 1 μm) than exosomes. However, several researchers have reported observing exosome-sized vesicles that bud from the plasma membrane [8]. A more detailed discussion on the biogenesis and properties of exosomes and shedding microvesicles is given in Chapter 1.

The various physical characteristics of microvesicles have led to the adoption of multiple protocols for their isolation from cell culture and physiological fluids. These methods make use of the known size, density, shape and macromolecular content of microvesicles. To separate vesicles by size, researchers often perform a series of differential filtration or centrifugation steps or a combination of both. Vesicles smaller than 200 nm can be obtained by passage through a 0.2 or 0.1 μm filter or pelleted by ultracentrifugation at 100,000 x g while larger vesicles are obtained at slower speeds such as 10,000 x g [28]. Concerns about the integrity of microvesicles after applying force through a filter, has led to the adoption of filtration devices that makes use of both a centrifuge and filters for separation of vesicles by size [84]. There are, however, potential pitfalls to both of these isolation methods. The main problem with the ultracentrifugation methods is the high cost of equipment and maintenance. While filtration may seem to be a suitable alternative, there is a greater loss of sample associated with the use of filters for microvesicle isolation due to adherence of proteins to the filter membrane [8]. Another potential problem is the contamination of the sample with unwanted material such as vesicles of a larger or smaller size and precipitated proteins. Two common methods for obtaining a more homogeneous microvesicle population are the use of either sucrose

cushion or density gradient ultracentrifugation to separate vesicles by their density [85]. Although this leads to purer samples, there is substantial reduction in microvesicle yield. Lastly, researchers make use of known surface markers such as the tetraspanins, CD63 and CD81. These proteins are typically found in exosomes and can be used to exclusively pull-down and isolate microvesicles containing exosomal markers.

After isolation, microvesicles can be characterized by several methods. Since nano-sized microvesicles are too small to be imaged by most commonly used fluorescence and light microscopes, the gold standard for examining microvesicles is transmission or scanning electron microscopy. With electron microscopy images, the size and shape of the microvesicles can be noted as well as the identification of markers by immunogold labeling. Obtaining TEM images can be quite costly, and researchers most commonly look for a set of microvesicle markers by western blot analysis. Like the isolation and characterization methods, quantification of microvesicle amounts can be done in several ways. Often, microvesicles are quantified by protein or nucleic acid amount depending on the molecule or mechanism of interest. Although quantification by such methods is convenient, the assumption is made that microvesicles contain consistent amounts of protein or RNA. Alternatively, dynamic light scattering can be effectively used to quantify as well as measure the average size of microvesicles [86]. Fluorescence-activated cell sorting (FACS) is another method of quantification, however, most flow cytometers can only accurately measure particles that are greater than 0.5 μm in diameter [87, 88].

2.2 RESULTS AND DISCUSSION

Isolation of microvesicles from a diverse panel

To identify a microvesicle glycomic signature we needed a large and diverse panel. For this reason, we focused on members of the NCI-60, a set of 59 cancer cell lines obtained from human tissues that is maintained by the National Cancer Institute. We chose this set because of the diversity of cell types and their extensive characterization from numerous studies. In addition, we had these cell lines readily available due to a large-scale NCI-60 glycomic and genomic project currently being undertaken in our laboratory. The normal skin cell lines, and the human serum were chosen to rule out the possibility of a cancer specific glycan signature. The initial dataset for microvesicle isolation contained one renal cancer, two colon cancers, one breast cancer, two melanomas, three T-lymphomas, two normal skin cell lines, and human serum. Microvesicle yields were quantified by protein concentration. Approximately equal amounts of cell culture resulted in vastly different yields for the different cell lines, with Sk-Mel-5 producing the greatest amount of microvesicle protein per milliliter of culture (Table 2.1). Cancer and normal cell lines were cultured and microvesicles isolated from at least three or two passages, respectively.

Cell Line	Description	Average Yield (µg protein per ml of culture)	Std Dev
ACHN	renal carcinoma	120.0	42.2
H9*	T-cell lymphoma	NA	
Jurkat *	T-cell lymphoma	NA	
SupT1*	T-cell lymphoma	NA	
HCT-15	colon adenocarcinoma	154.7	4.0
HT-29	colon adenocarcinoma	142.7	48.4
MCF-7	breast carcinoma	111.0	8.5
Sk-Mel-5	melanoma	484.7	161.6
Sk-Mel-28	melanoma	128.0	81.7
Hs 895.Sk	normal skin	75.0	2.0
TE 353.SK	normal skin	99.0	4.2

Table 2.1: Isolation yields from panel of cell-derived microvesicles. * T-cells were cultured and microvesicles isolated by Dr. Lakshmipriya Krishnamoorthy. NA: Data not available.

Because previous studies from our lab suggested that microvesicles may emerge from cell membrane microdomains defined by carbohydrates [79], we decided to compare the microvesicles to their parent cell membranes as well. The procedure for microvesicle isolation and cell membrane preparation is outlined in Figure 2.1. Large cell cultures of over 300 ml are needed to obtain usable (µg) amounts of microvesicles for most cell lines. The conditioned media or physiological fluid is taken through a series of differential centrifugation and/or filtration steps and microvesicles are obtained after ultracentrifugation at 100,000 x g. After removal of conditioned media, cells are washed to remove traces of microvesicles, harvested and lysed by sonication. Sonication causes disruption of the cellular membranes which then form smaller sized liposomes in solution. The liposomes are isolated from the cellular lysate by ultracentrifugation.

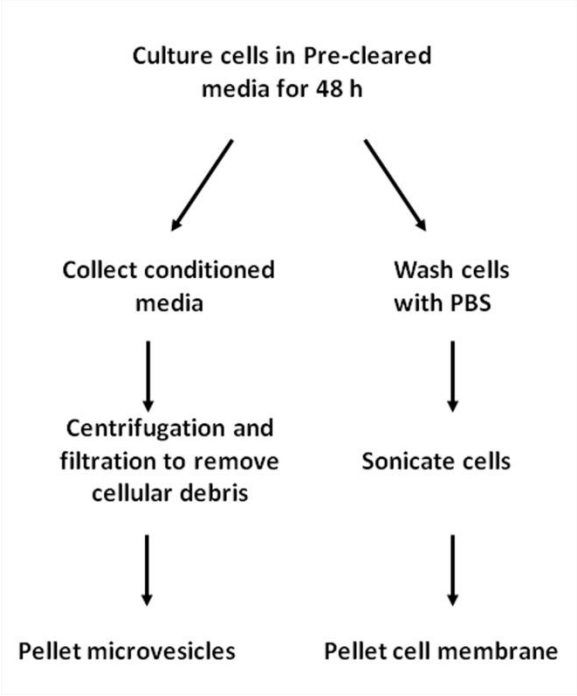


Figure 2.1: Schematic of microvesicle isolation and cell membrane liposome preparation. Microvesicles and cell membrane are obtained from a matched set.

Electron microscopy images show that the sonication process produces liposomal structures that range in size from approximately 50 to 200 nm in diameter, a size equivalent to the range of the microvesicles obtained from HT-29 and Jurkat cells (Figure 2.2). The HT-29 and Jurkat microvesicles are within the reported size range of exosomes. Many of the vesicles display the typical cup-shaped morphology described in the literature for exosomes [85].

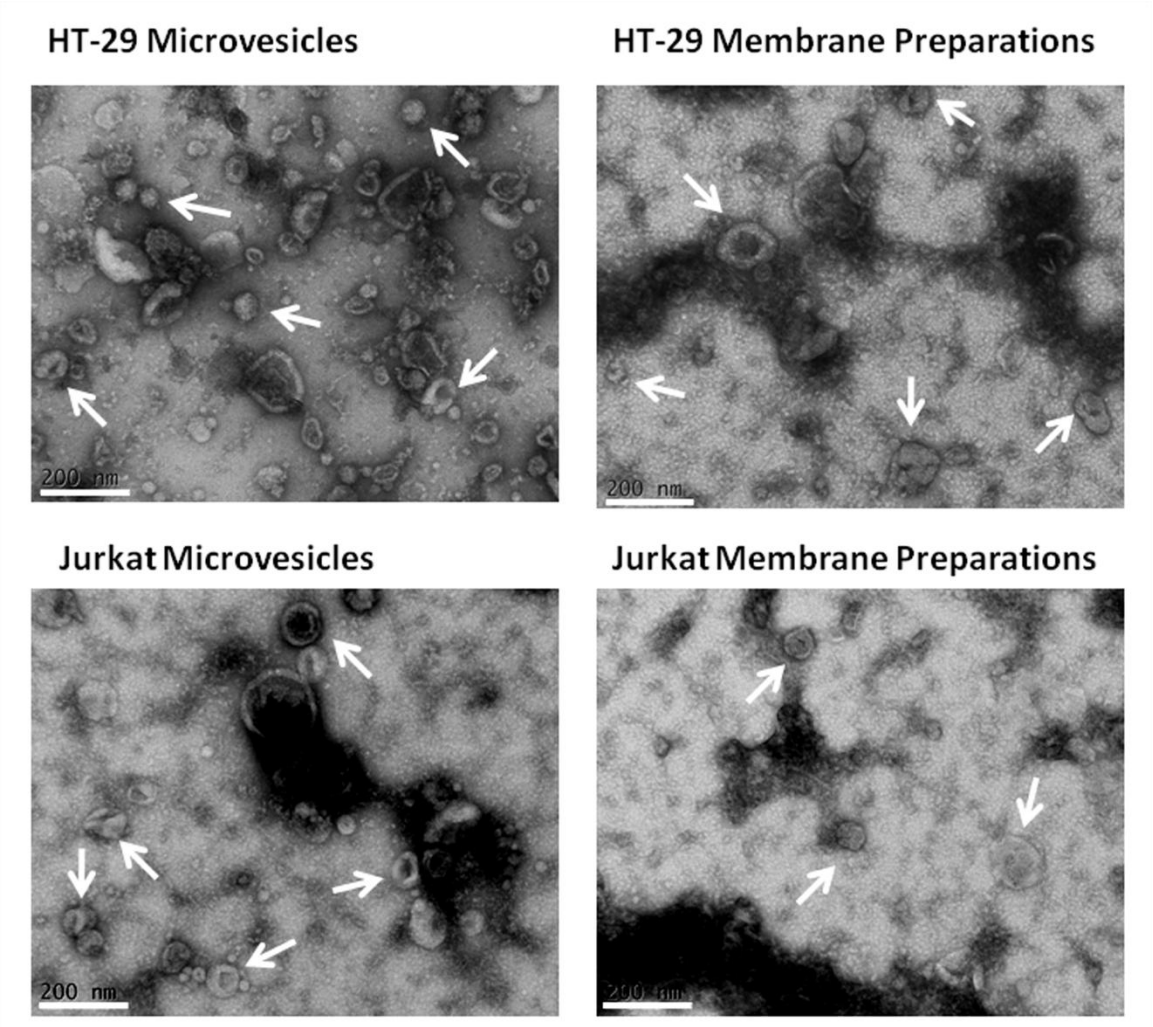


Figure 2.2: Transmission electron images of microvesicles and membrane preparations isolated from HT-29 and Jurkat cells. The membrane preparation protocol generates liposomes within the size range of the microvesicles (arrows). Scale bar for all images is 200 nm.

In order to ensure that we were comparing analogous types of vesicles, the pellets from the conditioned media (the pelleted microvesicles) were probed for the presence of CD81 by western blot. The tetraspanin, CD81, is often observed in proteomic studies of exosomes and is therefore commonly used as an exosomal marker [4, 85]. The marker was present in the probed microvesicle samples (Figure 2.3). A problem with our isolation protocol (discussed below) was discovered before all samples were probed. We therefore focused our efforts on correcting this problem before further analysis of the microvesicles.

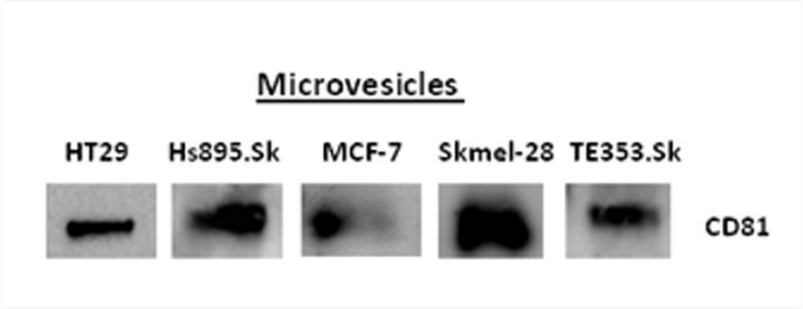


Figure 2.3: Microvesicles contain the exosomal marker CD81. Pellets obtained from cell conditioned media were probed for the presence of CD81 by western blot.

Contamination of microvesicle samples with bovine serum glycans

To isolate microvesicles, we followed an often cited protocol that recommends the cells be grown in "pre-cleared" growth medium before microvesicle isolation. This pre-clearing step, overnight ultracentrifugation of fetal bovine serum (FBS) containing media at 100,000 x g, should result in growth media that does not contain contaminating bovine vesicles or other protein products that may pellet with the cell-derived microvesicles [85]. After isolation and characterization of the panel of microvesicles and

cell membranes, samples were labeled with NHS-cyanine3 and incubated on the lectin microarray against an equal amount (1.5 µg protein) of NHS-cyanine5 labeled H9 cell membrane (Figure 2.4). H9 was chosen as a common biological reference because the cells are easily cultured in large quantities, enough to use on numerous arrays and the glycosylation profile of H9 gave a sufficient positive lectin binding pattern to be functional as a reference. In addition, H9 was previously used as the common reference for the T-cell microvesicle study and would provide a basis for comparison of the two studies.

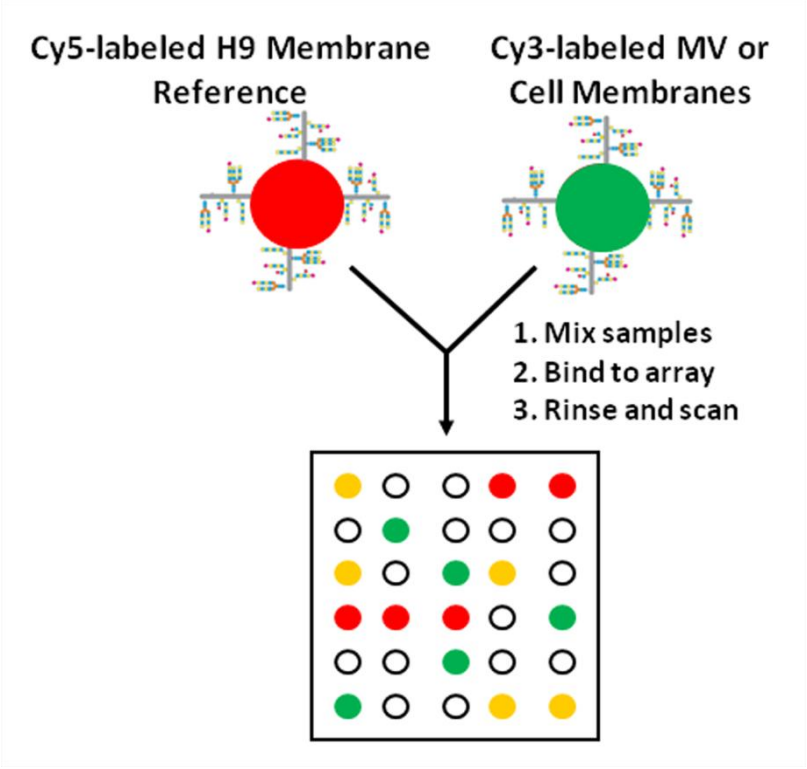


Figure 2.4: Schematic describing the lectin microarray experiment for comparison of the panel of microvesicles and cell membranes using H9 membrane as a common reference. All samples are Cy-3 labeled and mixed with the Cy-5 labeled reference for analysis on the array.

As a control, 300 ml of unconditioned pre-cleared media was subjected to a mock isolation, the pellet obtained was fluorescently labeled and incubated on the array in addition to the microvesicles and parent cell membranes. Hierarchical clustering of the arrays revealed a problem with our initial dataset. Hierarchical clustering of the cell derived microvesicles revealed a prevailing lectin binding pattern ($r = 0.53$, $N = 72$, $P < 0.001$). However, the pellet from the pre-cleared media showed a similar glycomic signature to the panel of microvesicles (Figure 2.5). We therefore could not be sure if the similarities between the cell-derived microvesicles were due to a contamination of bovine glycans. It is unknown whether the source of contamination is a result of large precipitates of soluble glycoproteins or from bovine vesicles that did not pellet with the overnight ultracentrifugation of the media. Because the lectin microarray cannot distinguish bovine from human glycans, the microvesicle samples isolated from pre-cleared FBS containing media would not be useful for glycomic analysis.

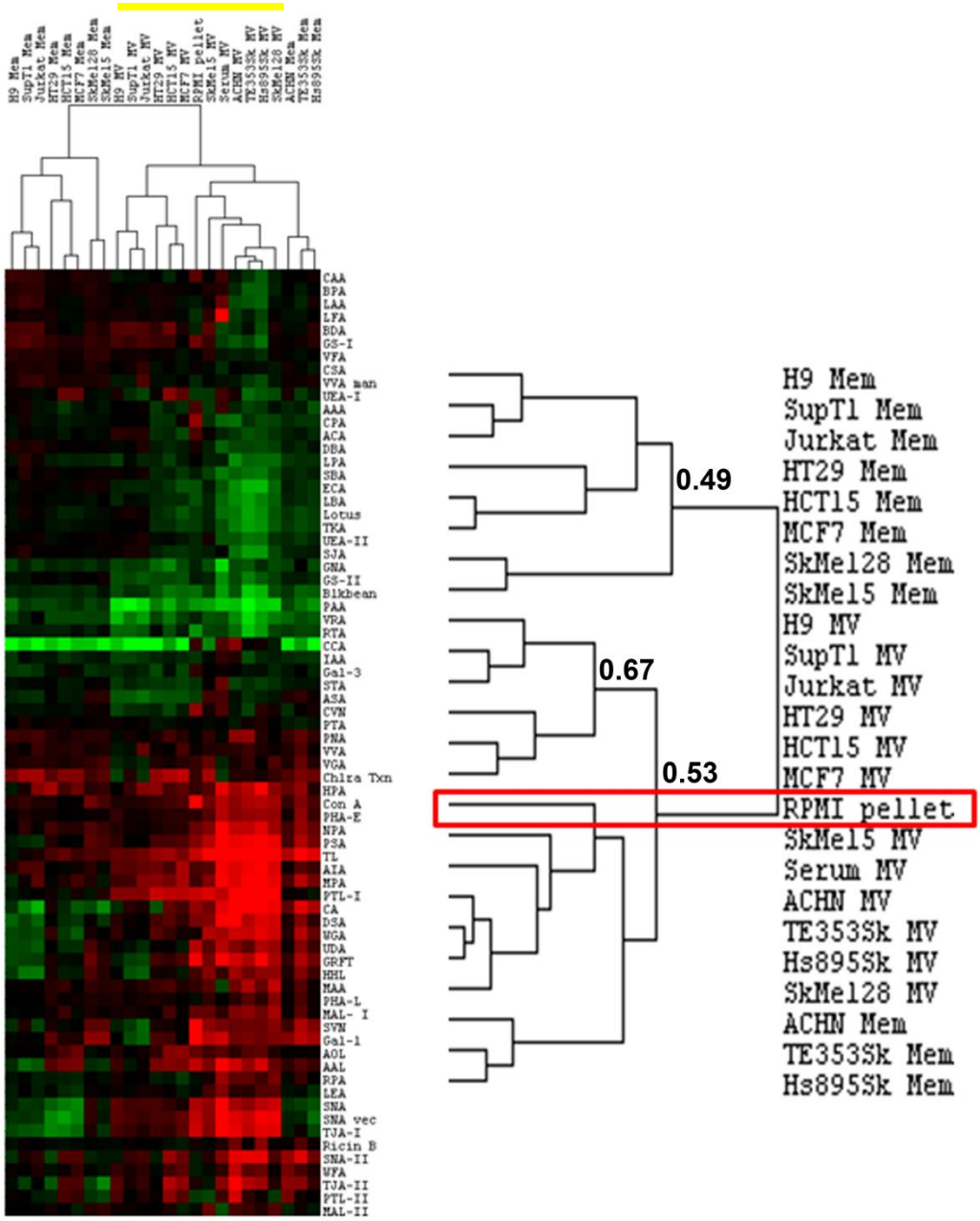


Figure 2.5: Dual-color analysis reveals a contamination of the microvesicles with bovine serum glycoproteins. Microvesicles (MV, yellow bar) and their corresponding membranes (Mem) were analyzed with H9 as the common reference. Pre-cleared growth media was subjected to mock microvesicle pelleting, the pellet was examined on the array. Arrays and lectins were hierarchically clustered using the Pearson correlation coefficient (shown for selected clusters) with average linkage analysis.

Isolation and characterization of microvesicles from serum-free cell cultures

As a result of the issues with pre-cleared FBS containing media, we decided to modify our isolation protocol so that contamination from serum would not be an issue and repeat our analysis to confirm the presence of a microvesicle glycomic signature. There are several alternatives to FBS that can be used as a supplement for growth media. However, these alternatives involve the use of a select number of growth factors which can be glycosylated and could in theory precipitate and pellet with microvesicles. To avoid the possibility of contamination, microvesicles were isolated from serum- and additive-free media. Previously, conditioned media was collected 48 hours post addition. However, because of concerns about cell stress in completely additive-free medium, conditioned media was collected 18-20 hours post addition. Even though cultures were increased from 300 ml to 360 ml, some cell lines (ACHN, MCF-7, Sk-Mel-28, Hs895.Sk) did not produce detectable or sufficient amounts of microvesicles. This is most likely due to the shortening of conditioning time from 48 hours to less than 24 hours as well as the decrease in protein content from the contamination of serum proteins. However, the possibility that factors are present in serum that may activate microvesicle production and secretion cannot be ruled out. Isolation of microvesicles from the normal skin cell line, TE353.Sk, was not attempted. Because of their slow doubling time and the lack of MV produced from the other normal skin cell line, Hs895.Sk, it was therefore assumed the TE353.Sk would not produce usable amounts of MV in a reasonable amount of time.

Biological replicates for our samples were obtained by culturing three different passages of the cells and isolating MV and cell membrane from each passage separately. Microvesicles obtained from all cell lines and replicates were probed for the exosomal marker, CD81, as done previously. In addition, we probed for a second exosomal marker, CD63. Western blot images reveal that the microvesicles contain variable amounts of the

exosomal markers (Figure 2.6). Microvesicles from every cell line contained the exosomal markers. For consistency, passages that contained low amounts of the markers were not included in our glycomic analysis. Serum-derived exosomes lacked detectable amounts of CD63 and CD81 and were not included in glycomic analysis. Because of this, microvesicles from the physiological fluid, breast milk, were isolated to include a non-culture and non-cancer element to the study. Breast milk microvesicles contained CD81 and CD63 (Figure 2.6). The revised panel to be used for glycomic analysis is listed in Table 2.2 Transmission electron microscopy images of microvesicles and membrane preparations from serum-free cultured Sk-Mel-5 (Figure 2.7) reveal that they are in the range of 50 to 150 nm in diameter as previously observed for the HT-29 and Jurkat microvesicles and membranes isolated from pre-cleared media (Figure 2.2).

It was surprising to find that our human serum pellets did not contain CD81 and CD63 as there are numerous research studies that claim that exosomes are present in human serum. Upon further investigation, I came across a research article in which vesicles were absent from the 100,000 x g pellets of human and mouse serum [89]. Other examples have since emerged indicating that certain circulating proteins and even microRNA, previously believed to be derived from microvesicles, are actually independent of vesicles [90]. This highlights the importance of a thorough characterization of pelleted samples, however I do not believe that exosomes are absent from all human sera. It has been found that cancer and immune disease elevate the levels of microvesicles found in blood so it is possible that our sample, obtained from a healthy donor, contained only trace amounts of microvesicles. Further studies are needed to determine the content of the pellets obtained from our human serum samples. It was interesting to note that despite the absence of CD63 and CD81, the human serum

microvesicles displayed a similar lectin binding pattern to all the other microvesicles in the panel (Figure 2.5).

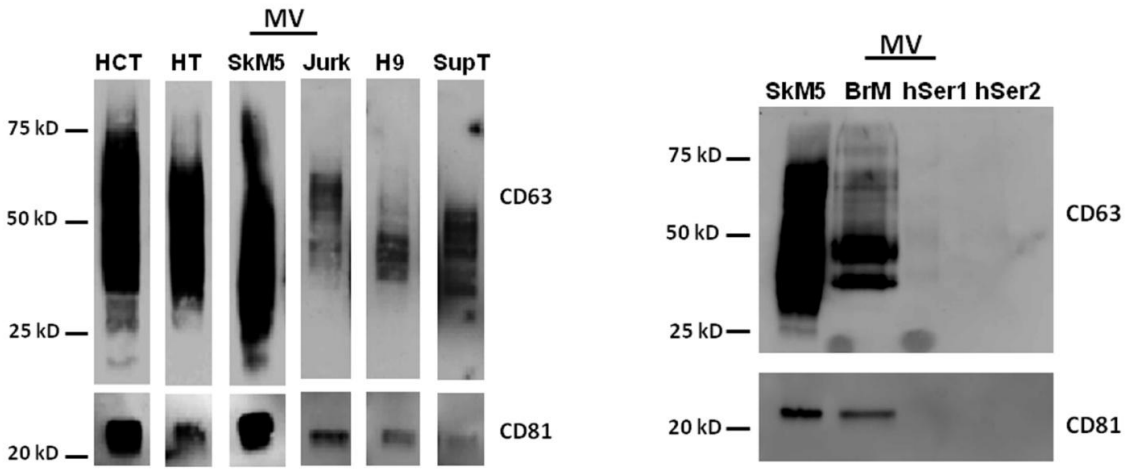


Figure 2.6: Western blot analysis of the pelleted serum-free cell conditioned media and physiological fluids reveals the presence of exosomal markers CD63 and CD81 in HCT-15 (HCT), HT-29 (HT), (Sk-Mel-5 (SkM5), Jurkat (Jurk), H9, SupT1 (SupT) and breast milk (BrM) derived microvesicles. Human serum pellets (hSer) from two different isolations did not contain the markers.

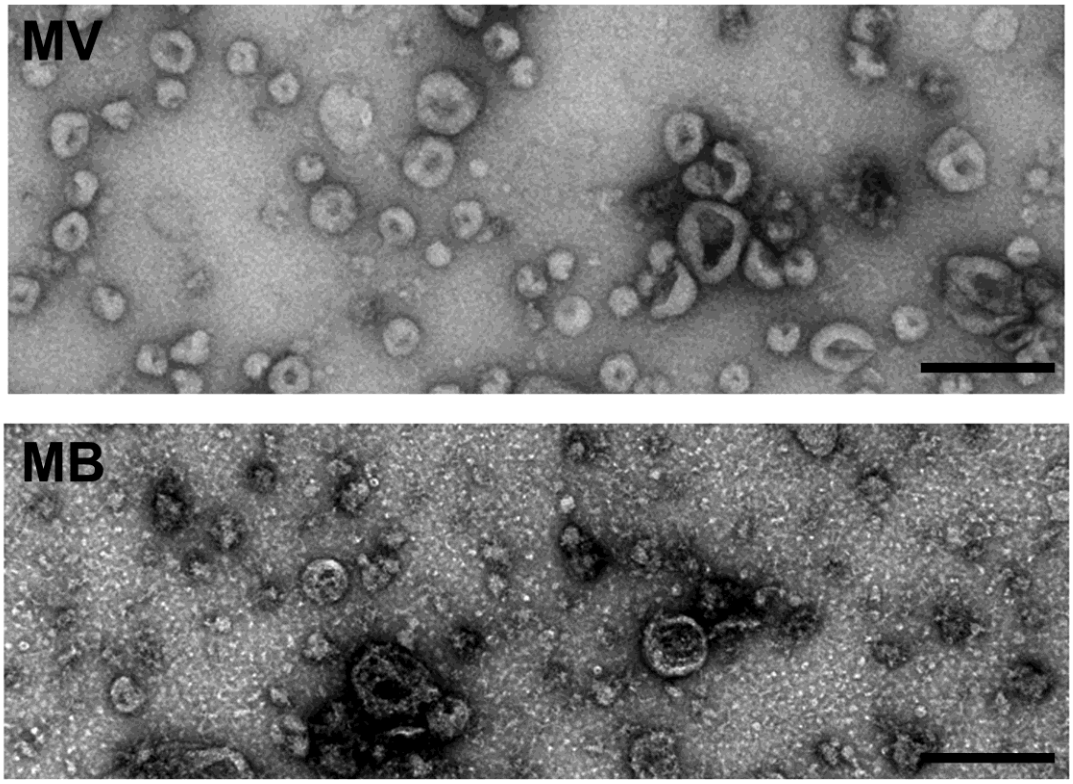


Figure 2.7: Transmission electron microscopy images of microvesicles and membrane preparations from serum-free cultured Sk-Mel-5 cells. Microvesicles have the typical size and morphology of exosomes. Cell-membrane liposomes from serum-free Sk-Mel-5 are similar in size and shape to the previously observed HT-29 and Jurkat liposomes derived from pre-cleared FBS-containing cell cultures. Scale bar is 200 nm.

Source	Description
H9*	T-cell lymphoma
Jurkat *	T-cell lymphoma
SupT1*	T-cell lymphoma
HCT-15	colon adenocarcinoma
HT-29	colon adenocarcinoma
MCF-7	breast carcinoma
Sk-Mel-5	melanoma
Breast Milk	Physiological fluid

Table 2.2: Revised panel of microvesicle sources for glycomic analysis. All samples contained exosomal markers CD63 and CD81. Cell conditioned media was free of FBS. * T-cells were cultured by William Eng.

Comparison of pre-cleared and serum free cultured microvesicles

We wanted to know to what extent FBS contamination was influencing the glycomic profiles of microvesicles. To this end, we compared serum-free and pre-cleared derived samples by dual-color microarray analysis using H9 membrane as the common reference. Microvesicles were compared by hierarchical clustering using the Pearson correlation coefficient as the distance metric between all samples. Cluster analysis (Figure 2.11) shows that the microvesicles as a whole are statistically similar ($r = 0.554$, $N = 88$, $P < 0.0001$, two tailed *t*-test). However, only the vesicles from HCT-15 and Sk-Mel-5 are highly correlated ($r > 0.85$) with their serum-free (SF) counterparts. Biological replicates of microvesicles and cell-membranes are usually tightly clustered with a Pearson correlation coefficient of 0.85 or greater. For this reason, the panel of cell-derived microvesicles to be used for further glycomic analyses will be derived from serum-free media.

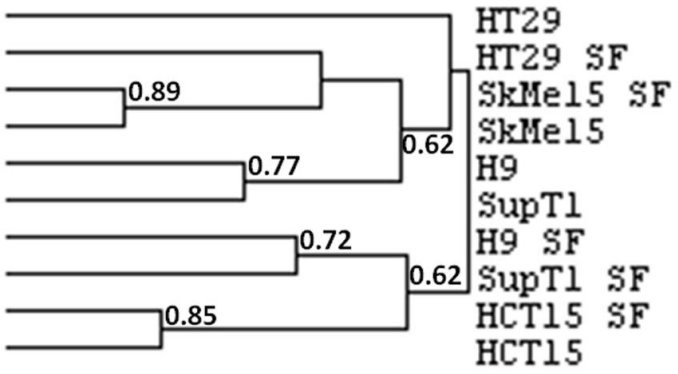


Figure 2.8: Hierarchical clustering of microvesicles from serum free (SF) and pre-cleared media after dual color lectin microarray analysis using H9 as the reference.

2.3 CONCLUSIONS

This chapter describes the isolation and characterization of microvesicles from a diverse panel of sources for the purpose of studying their glycomic content. After initial setbacks due to potential contamination from FBS, our panel was ultimately smaller than previously projected, with less cell lines and only one biological fluid. Microvesicles were nonetheless successfully obtained from two colon, three T-cell, and two skin cancer cell lines as well as breast milk from a healthy mother for glycomic analysis. Electron microscopy images demonstrate that our isolation protocol rendered microvesicles that are within the typical size range described of exosomes. With the exclusion of human serum the microvesicle panel contained the exosomal markers CD81 and CD63. However, further studies are needed before any conclusion of endosomal origin can be made as several labs including our own have produced evidence that exosome-sized particles may bud directly from the plasma membrane [25-27, 79]. In addition, there are considerably fewer proteomic studies of shedding microvesicles and therefore the

possibility cannot be ruled out that these particles may also contain CD81 and CD63. Vesicles budding directly from tetraspanin enriched plasma membrane domains have been observed [27].

2.4 MATERIALS AND METHODS

Cell culture

The ACHN, HT29, HCT-15, MCF-7, Sk-Mel-5 and Sk-Mel-28 cell lines were purchased from the National Cancer Institute (Frederick, MD). The normal skin cell lines, Hs895.Sk and TE353.Sk, were purchased from ATCC. H9 and SupT1 were obtained from J. Bess (AIDS Vaccine Program, NCI, Frederick, MD). Jurkat-Tat-CCR5 cells were obtained from Q. Sattentau (University of Oxford). Primary skin cells were cultured in DMEM with 10% (v/v) FBS (ATCC), all other cells were cultured in RPMI 1640 (Lonza) with 2 mM L-glutamine and 10% (v/v) FBS (Atlanta Biologicals, Lawrenceville, GA). All cells were cultured at 37˚C with 5% CO_2. Penicillin-streptomycin (Mediatech, Manassas, VA) was added to the non-adherent T-cell lines which were cultured by William Eng and Dr. Lakshmipriya Krishnamoorthy.

Breastmilk Collection

Our collaborator, Dr. Karen D. Hendricks-Muñoz obtained human milk samples from a healthy 34 year old Caucasian mother who delivered a preterm infant at 30 weeks gestational age. This was the mother's third pregnancy and delivery. The mother was rubella immune and negative for Hepatitis, HIV and Chlamydia infections with a history of infertility due to uterine adhesions. The sample used in this analysis was obtained by mechanical expression at 27 days post-partum, immediately frozen and stored at −20°C

until analysis. This research was performed with NYU School of Medicine Institutional Review Board approval and the mothers' written informed consent.

Microvesicle Isolation

Cells were rinsed twice with Hank's buffered salt solution (HBSS) (Mediatech, Manassas, VA) to remove traces of FBS and growth medium was replaced with either serum-free medium or pre-cleared growth medium. To pre-clear the FBS, media containing 20% FBS was ultracentrifuged at 100,00 x g for 16 h at 4˚C, serum free media was added to the supernatant to achieve a final concentration of 10% FBS. Media was then filter sterilized. After 48 h (pre-cleared) or 24 h (serum free), conditioned media was collected and microvesicles were isolated by differential centrifugation as described previously. Briefly, cell debris and larger vesicles were pelleted out of the media by sequential centrifugation (300 x g, 10 min, 2,000 x g, 20 min and 10,000 x g, 30 min; Beckman Coulter). The cleared supernatant was subjected to ultracentrifugation at 100,000 x g for 1 h to obtain a microvesicle pellet. Microvesicles were resuspended in either PBS (0.1 M phosphate buffer, 0.15 M NaCl, pH 7.4) or Cy-labeling buffer (0.1 M $NaHCO_3$, pH 9.3). A slightly altered protocol was utilized for the isolation of microvesicles from human breast milk and serum. Differential centrifugation (as above) of 2 ml of breast milk and varying amounts (14 -50 ml) of serum was followed by sequential filtration through 0.45 μm and 0.22 μm filters. The filtrates were overlayed onto a 30% sucrose cushion and centrifuged at 100,000 x g for 1 h. The pellets were resuspended in PBS and centrifuged at 100,000 x g for 1 h. The washed pellet were then diluted in Cy-labeling buffer. The protein levels of all microvesicle preparations were

quantified using the micro-BCA assay (Thermo Scientific). Normal human serum was purchase from Lonza (catalog #14-402E).

Cell membrane preparation

Labeled cell membranes were prepared as described previously with some exceptions. After collecting conditioned media, cells were washed twice in cold PBS and either scraped (adherent cells) or resuspended (non-adherent cells) in cold PBS containing protease inhibitor cocktail. Cells were then sonicated on ice (3 x 5 s, 70% power, Branson sonicator) to disrupt cell membranes. Membranes were pelleted by ultracentrifugation at 100,000 x g for 1 h. The pellet was resuspended in Cy-labeling buffer and homogenized by sequential passing through 18- and 24- gauge needles. We determined the protein concentration using the DC protein assay (Bio-Rad).

Cy3- and Cy5- labeling

Samples were fluorescently labeled with 60 mg of Cyanine 3 (Cy3) or Cyanine 5 (Cy5) mono reactive-NHS per mg of protein in Cy-labeling buffer for 30 min at room temperature with gentle rocking (GE Life Sciences). The labeling reaction was quenched by the addition of a Tris-buffered saline stock solution (2 M Tris-HCL, 1.2 M NaCl, pH 6.8) to a final concentration of 250 mM Tris-HCl and 150 mM NaCl (final pH ~7.6-7.8) for 30 min. Microvesicle samples were used without further purification from the quenched excess dye. For cell membrane samples, excess dye was removed by dialyzing into PBS overnight at 4˚C. This prevented the high background observed with these samples when used after quenching alone, which is most likely due to the exposure of

hydrophobic lipids in unsealed membrane bilayers following the cell disruption. Protein concentrations for membrane samples were obtained after dialysis.

Lectin Microarray Print.

All lectins were purchased from either EY Laboratories (San Mateo, CA) or Vector Labs (Burlingame, CA) with the following exceptions: cyanovirin (CVN), scytovirin (SVN) and griffithsin (GRFT) were gifts from Dr. B. O'Keefe (NCI-Frederick); galectin-9 was a gift from Dr. L. Baum (UCLA Medical School); and Gaf-D, PA-IL, PA-IIL, PapGII, PapGIII and RS-IIL were made recombinantly as previously described. Three spots per lectin were printed using a Nanoplotter 2.1 piezoelectric printer (GeSIM, Germany) at 14˚C and 45% humidity. See Table 31. for lectin list, print concentrations and buffers.

Microarray Hybridization and Analysis.

Microarray slides were submerged in blocking buffer (50 mM ethanolamine, mM sodium borate ph 8.5) for 1 h followed by 3 washes with PBST (PBS with 0.005% Tween) and a final wash with PBS. The microarray slides were fitted to a 24-well frame (ArrayIt, Sunnyvale, CA). Equal amounts (1.5 µg) of Cy3-labeled microvesicles were incubated with equal amounts of Cy5-labeled H9 reference membrane in 100 µl total volume PBST. Samples were hybridized to the lectin microarrays for 2 h at room temperature with gentle rocking. The individual subarrays were then washed with PBST for 5 x 5 min with a final 10 min wash in PBS. Slides were scanned and analyzed using a GenePix 4300B fluorescent slide scanner (Molecular Devices, Sunnyvale, CA) with GenePix Pro 7 software at a resolution of 5 µm. The photomultiplier (PMT) gain settings

were 500 for the Cy3 channel and 450 for the Cy5 channel. For each channel, the background subtracted median fluorescence of the three replicate spots per lectin was tested for outliers using the Grubbs outlier test with alpha = 0.05. Arrays and lectins were hierarchically clustered by the $\log_2$ ratios using the Pearson correlation coefficient with average linkage analysis (Cluster 3.0). Clusters were visualized with Java Treeview. If the Pearson correlation values were statistically significant ($P < 0.0001$ for a two tailed *t* test, DF = N-2, calculated using http://faculty.vassar.edu/lowry/tabs.html#r) then arrays were considered to be statistically similar.

Western Blots

Microvesicle and cell membrane proteins (3 µg of protein) were separated by SDS-PAGE under non-reducing conditions and transferred to nitrocellulose membrane. Samples were probed with antibodies to CD63 (1:500 dilution) (RFAC4, Millipore, Billerica, MA) and CD81 (1: 500 dilution) (H-121, Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with the appropriate HRP-conjugated secondary antibody (1:10,000 dilution) (Bio-Rad, Hercules, CA). Blots were visualized using Supersignal West Pico or Supersignal West Femto Chemiluminescent Substrate (Thermo Scientific) and a GBox gel imaging system (Syngene).

Electron Microscopy

Images were obtained from the New York University Langone Medical Center Office of Collaborative Sciences Microscopy Core. Samples were negatively stained with 1% Uranyl Acetate in ddH2O on 400 mesh Cu grids coated with a Carbon membrane.

Grids were viewed on a Philips CM12 tungsten emission TEM at 120kV and imaged with a Gatan 4k x 2.7k digital camera.

Chapter 3: Glycomic Profiling of Microvesicles

3.1 INTRODUCTION

Overview

Our initial glycomic analysis of microvesicles using lectin microarrays hinted at the possibility of the presence of a conserved glycomic signature for these particles. However, the discovery of contamination from FBS derived glycans complicated this analysis and required a different method of generating vesicles. After successful isolation and characterization of a panel of serum-free derived microvesicles, this chapter focuses on the analysis and comparison of their glycomic content using lectin microarrays.

Glycosylation of microvesicles

The knowledge that cells release vesicles that carry and transfer bioactive cellular content has led to enthusiastic interest in these particles for their diagnostic and therapeutic potential [91]. Because of this, there has been a surge of studies that profile the proteomic and RNA content of microvesicles from numerous sources. Many of the findings from these studies are compiled in Exocarta, an open source database of exosomal proteome and RNA data [92]. In contrast to the wealth of proteomic and RNA information gathered from multiple studies, very little is known about the glycomic content of these vesicles. Much of what is known about microvesicle glycosylation derives from research done on individual secreted glycoproteins. Interestingly, several of these studies have demonstrated that microvesicles contain proteins with distinct glycoforms than those found intercellularly. For instance, the secreted protease ADAM-10 was found to display an enrichment of complex *N*-linked glycans in the exosomal fraction when compared to the cellular enzyme [93]. This and other glycoprotein studies

have led to the belief that glycosylation may be a determining factor for trafficking of glycoproteins to particular secreted vesicles. In order to make this a more compelling argument, a comprehensive glycomic and glycoproteomic study is needed.

Not suprisingly, researchers are beginning to find that glycosylation on microvesicles can have an effect on biological function. For example, when investigating the role of microvesicle-secreted synapsin on neurite outgrowth and neuronal survival, Wang et al. found that this process was dependent on the presence of oligomannose structures on synapsin [94]. In another study, human tracheobronchial derived vesicles revealed the importance of α2-6 sialylated mucins for neutralizing influenza virus [95]. Recently, Escrevente et al looked for the presence of glycans in microvesicles from three different cell lines, ovarian carcinoma, embryonic kidney and neuroglioma cells, using the lectins SNA, MAL and ConA and demonstrated the presence of α2-6 and α2-3 sialic acid and α-mannosyl branched glycans. In addition, they found that pre-treatment of SKOV3 cells with mono- and disaccharides decreased the cellular uptake of the vesicles. However, desialylation of microvesicles using Neuraminidase did not significantly reduce uptake [96]. This and other studies like it hint at the possibility that glycans may be involved in cellular recognition or uptake, much like viruses, which have similar characteristics to microvesicles, are known to utilize glycan-lectin interactions for these processes [97]. Using lectin microarrays, our lab previously described the glycosylation profiles of T-cell derived microvesicles, membranes and HIV. Comparing the three profiles revealed many similarities between the virus and microvesicles [79]. This study is yet another clue that microvesicles may share cellular exit or entry mechanisms or both that make use of glycan-lectin interactions. Given the purported role of microvesicles in intercellular communication and their potential for use in diagnostics, it is important to learn more about the glycosylation of these particles.

3.2 RESULTS AND DISCUSSION

Single color analysis

The panel to be analyzed on the lectin microarray, a total of 21 samples, was comprised of microvesicles from serum-free cell culture, their parent cell membranes and breast milk microvesicles. As a quality control to detect any lectin print issues or anomalous lectin binding, the first slide of every print batch is tested by incubation with a panel of fluorescently labeled glycoproteins. If the glycoprotein-lectin binding pattern is adequate, each slide thereafter is incubated with at least two glycoproteins. A total of two slides were used for the single color glycomic analysis of the panel. It is important to determine if differences in lectin activity exist between slides, particularly for single color analysis because this method is more sensitive to these types of issues. To test the slides for consistency in lectin printing and activity, the glycoprotein data were hierarchically clustered using the uncentered variant of the Pearson correlation and average linkage analysis. All replicate glycoprotein samples had a Pearson correlation coefficient of above 0.95 (Figure 3.1). This high correlation between all arrays gave us confidence that the single color samples could be compared using two slides.

For the single color analysis, equal amounts of Cy3-labeled microvesicles and cell membranes, 1.5 μg based on protein concentration, were probed on the lectin array. An example of the raw single color data for Sk-Mel-5 microvesicles and membranes is given in Figure 3.2. Some of the notable differences and similarities between the samples are clearly visible by eye and highlighted in the figure. However, there are some inconsistencies with the lectin binding data. In SkMel-5, WGA, TJA-I (sialic acid binding lectins), NPA (a high mannose lectin), and DSA (a polyLacNAc binder) appear to bind microvesicles and the corresponding cell membrane preparations at similar levels as measured by median fluorescence intensity. In contrast, lectins with similar specificity

profiles (α2-6 sialic acid: SNA; high mannose: HHL, PSA) have a preference in binding to the microvesicles. This suggests that the former lectins cannot distinguish differences between microvesicles and membranes because they are completely saturated and are outside the linear range of detection. This is a problem that can sometimes occur because it is difficult to predict what concentration will be within the linear binding range for all lectins and samples. A way to circumvent this problem is by the ratiometric dual-color approach, in which competitive binding between the sample and a common reference enables comparison of the relative binding levels. This method enhances sensitivity to differences as well as true similarities between samples. Because of this, the microvesicles were analyzed by the dual-color approach as well and this analysis is discussed in the next section.

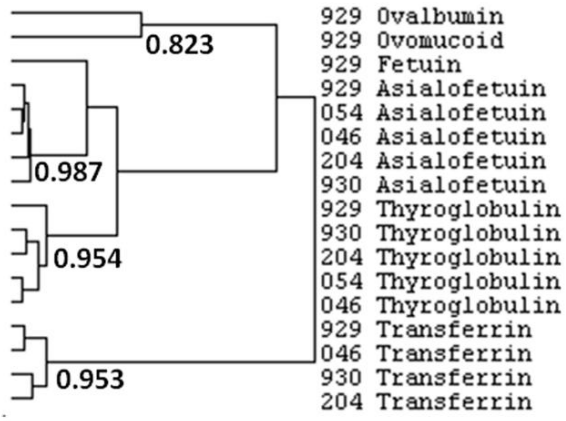


Figure 3.1: Assessment of slide to slide variations in lectin quality by incubation with glycoprotein standards. Arrays were analyzed by hierarchical clustering using uncentered correlation and average linkage analysis. Correlation coefficients are shown. Slides are numbered according to their bar code.

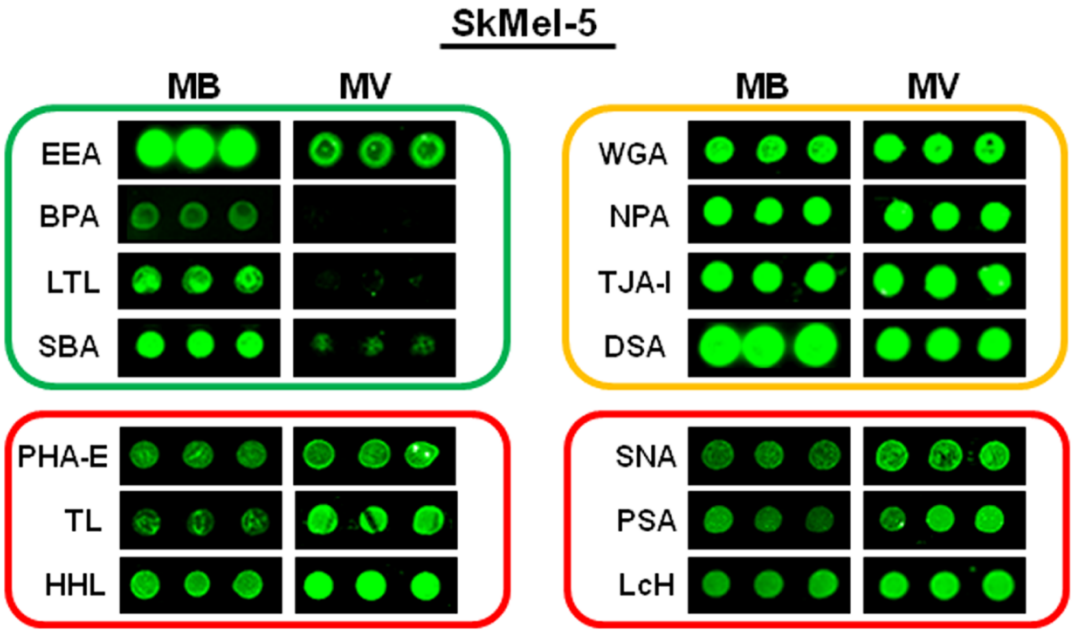


Figure 3.2: Comparison of SkMel-5 MV and MB on the lectin microarray demonstrates exclusion (green block) and enrichment (red blocks) of glycan epitopes in MV as well as lectins that bind equally to both (yellow block). Although it may appear that DSA preferentially binds to the membrane, fluorescence intensities for the two samples are approximately the same since spot circumference does not heavily influence the median fluorescence calculated by the Genepix program.

To observe the glycosylation profiles of the entire panel, the lectin values were clustered using the program Cluster 3.0 but arrays were manually arranged so that microvesicles and membranes were segregated (Figure 3.3) [98]. Biological triplicates (three different passages) were incubated for the two cell lines from the panel that produced the highest amount of microvesicles, Sk-Mel-5 and HCT-15. The data was not normalized so that an unaltered representation of the lectin binding pattern is shown. The heat map displays actual fluorescence intensities of the bound samples to the lectin spots. The heat map reveals that the microvesicles are generally enriched in high mannose

(HHL, PSA, NPA, SVN, UDA), complex *N*-linked glycan (Calsepa, LcH, PHA-E, TL), α2-6 sialic acid (SNA, TJA-I) and polyLacNAc (LEA, DSA) epitopes but do not contain substantial amounts of terminal GalNAc (CAA, DBA, SBA, SJA, BPA), blood group A or B (EEA, LTL) compared to the cell membranes.

The breast milk microvesicles displayed overall lower fluorescence intensities than the rest of the panel (Figure 3.4). This may be due to breast milk microvesicles having lower glycosylation levels, however, I do not believe this to be the case. As mentioned previously, equal amounts of microvesicles are hybridized to the array based on protein concentration. Because the yields from microvesicle sedimentations are typically less than 100 μg, the sample is conserved by obtaining protein concentrations from diluted samples. Concentrations are determined by the Pierce Micro BCA protein assay which can accurately detect low μg/mL concentrations of protein samples in a microplate format within the linear range of 2 - 20 μg/mL. The concentration of the breast milk microvesicles (assay performed in triplicate) was 2.0 μg/mL with a standard deviation of 0.165. With the standard deviation, the concentration of the sample was below the linear range of the standard curve. This was the only sample that fell below the linear range. Therefore it is quite possible that the Micro BCA assay gave an innacurate protein concentration for the breast milk microvesicles. Despite the overall lower fluorescence intensities of the lectin spots, the breast milk microvesicles display a binding pattern that is similar to the cell derived vesicles (Figures 3.3 and 3.4) with recognition of α2-6 sialic acid (SNA, SNA-I, TJA-I), complex *N*-linked glycans (LcH, calsepa), polylactosamine (DSA) and high mannose glycans (NPA, PSA). However, not all of the lectins that recognize these epitopes were visibly positive (yellow on the heat map) for binding to the breast milk sample. I believe this inconsistency is due to the possible lower than calculated protein amount that was hybridized to the array.

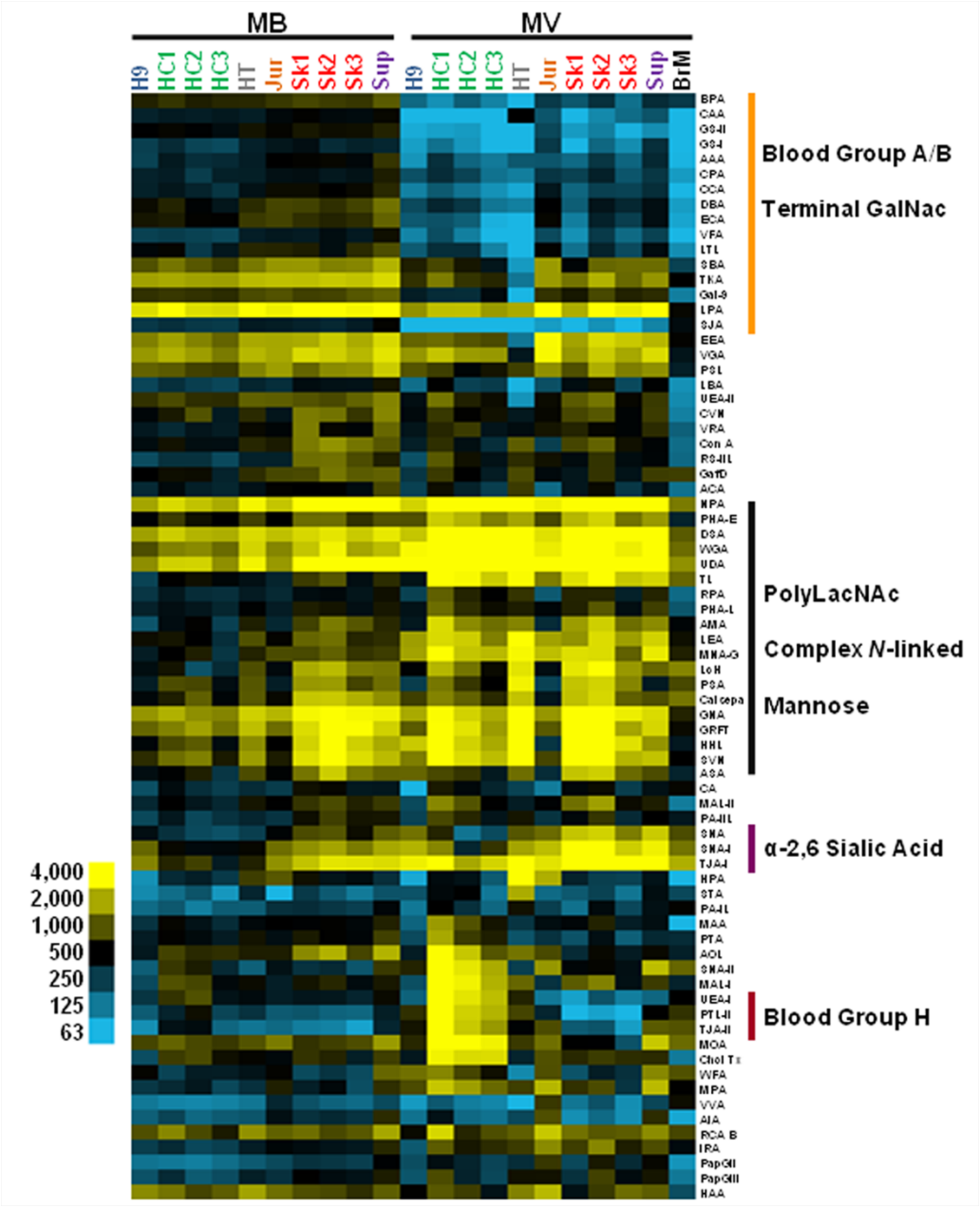


Figure 3.3: Microvesicles and cell-membranes have discrete glycomic profiles. Equal amounts (1.5 µg by protein) of Cy3-labeled samples were added to the lectin microarray. Lectins were hierarchically clustered by using the Pearson correlation coefficient as the distance metric and average linkage analysis (n = 74 lectins). Heat map is shown. Yellow indicates median fluorescence units > 500 and blue indicates median fluorescence < 500. Abbreviations: GalNAc (*N*-acetylgalactosamine), PolyLacNAc (poly-*N*-acetyllactosamine)

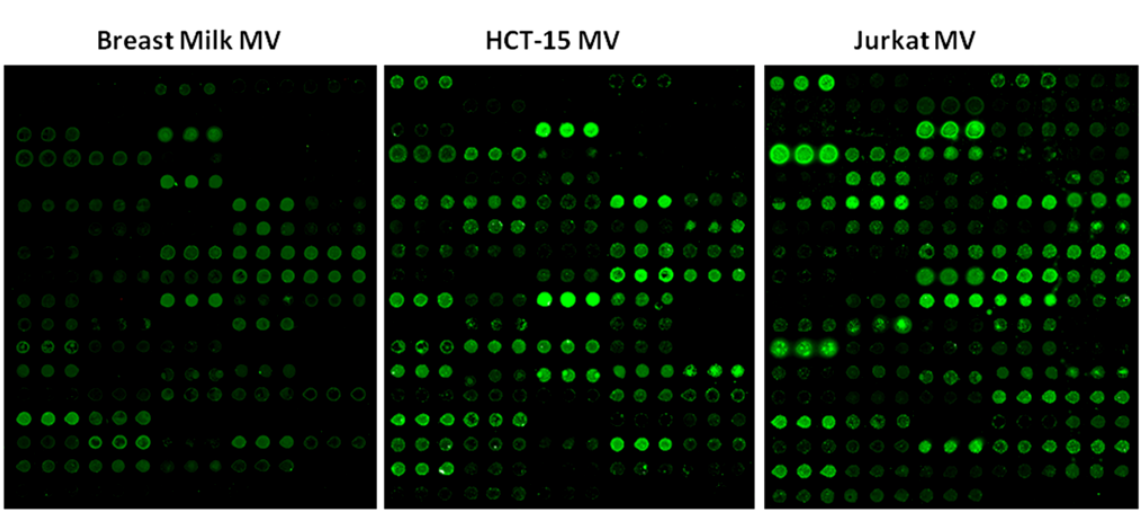


Figure 3.4: Lectin binding pattern of Cy3-labeled microvesicles from breast milk, HCT-15 and Jurkat. Protein concentration was determined by Pierce Micro BCA protein assay and 1.5 µg of protein were probed. Breast milk microvesicle binding pattern has overall lower fluorescence intensity.

Lectin binding on the array is carbohydrate specific

The labeled glycosylated samples probed on the arrays produced lectin binding patterns. To test if these patterns are the result of specific carbohydrate-lectin interactions, several control experiments were performed. The Sk-Mel-5 cell line was chosen for control experiments because this cell line produced the greatest quantity of microvesicles. The arrays were pre-treated with mono- and disaccharides before addition of labeled Sk-Mel-5 microvesicles. The final concentration of the sugars after the addition of the sample was 100 mM which is the concentration that researchers typically use in lectin inhibition experiments. Lectin inhibition was determined by percent decrease in spot fluorescence intensity. The heat map in Figure 3.5 shows the carbohydrate-based inhibitions of the Sk-Mel-5 binding lectins. For ease of interpretation the lectins are

ordered on the heat map based on their broad mono- and disaccharide specificities. Complete inhibition was not expected as lectin affinity and avidity for carbohydrate moieties in a cellular or biological context is typically higher than for mono- and disaccharides. Generally, lectins were inhibited according to their specificities. Several lectins were inhibited by more than one sugar, for example, Calsepa, ConA, LcH and UEA-II, these lectins are known to bind more than one carbohydrate epitope. The lectins PHA-E, TL and AMA recognize branched, bi- and tri-antennary structures and, as expected, were very weakly or not at all inhibited. These results confirm that the lectins on the array are binding to the samples based on carbohydrate interactions.

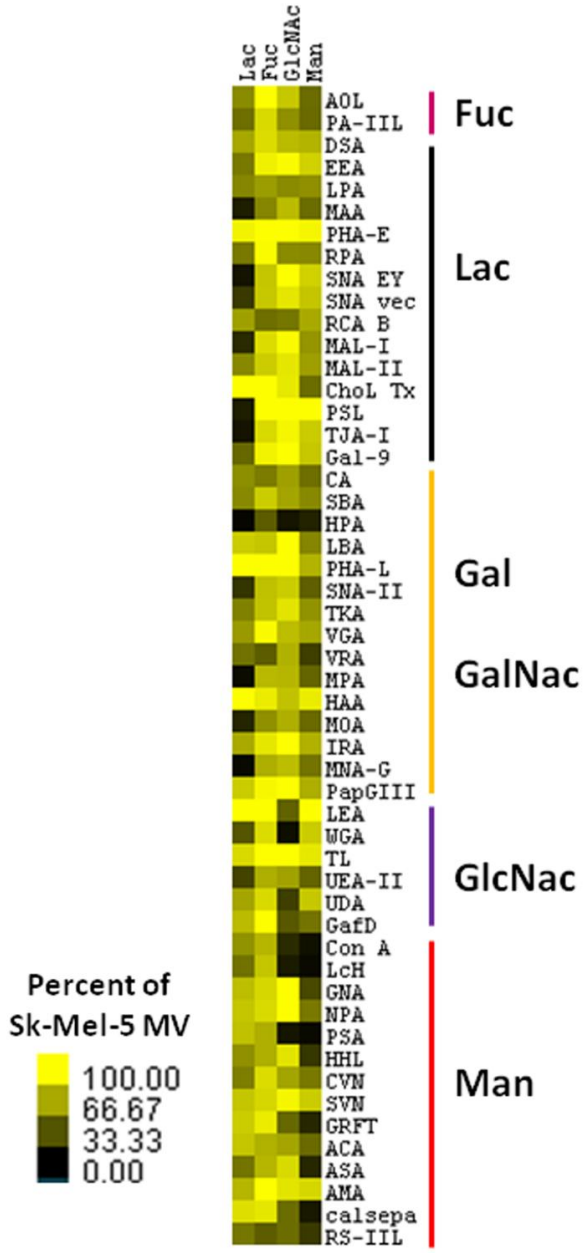
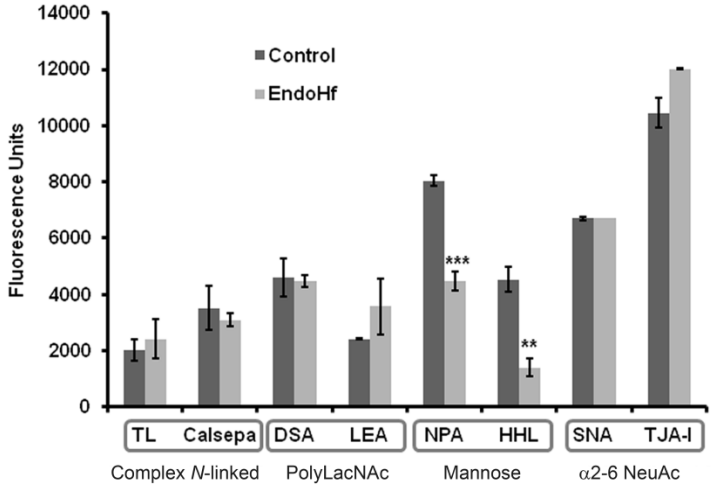


Figure 3.5: Mono- and disaccharide inhibition of lectins on the array shows lectin binding is specific. Lectin arrays were preincubated with 200 mM GlcNAc, fucose, mannose or lactose for 30 min before addition of labeled SkMel-5 MV (final sugar concentration was 100 mM). Heat map shown as a percentage of the untreated Sk-Mel-5 fluorescence values.

Several other control experiments were performed to confirm the specificity of lectin binding. Labeled Sk-Mel-5 microvesicles were treated with one of three glycosidases: EndoH_f, which cleaves within the core of high mannose and hybrid *N*-linked glycans, PNGaseF, which removes all *N*-linked glycans and Neuraminidase, which cleaves sialic acids linked α2-3, α2-6, or α2-8. Typically, glycoproteins are first denatured before treatment with glycosidases to allow for better access to the cleavage site. However, to keep the vesicles intact, the denaturing step was omitted and the samples were enzymatically treated for a longer period of time (18 h). Because the samples were not denatured before treatment, it is possible that not all cleavage sites were accessible to the enzymes. After glycosidase treatment, microvesicles were probed on the lectin microarray and compared to untreated controls. Figures 3.6 and 3.7 show the effects of enzyme treatment by a representative panel of lectins that preferentially bind to microvesicles. Treatment of SkMel-5 microvesicles with Endo H_f decreased binding to mannose lectins (NPA, HHL) but did not significantly affect binding of lectins with a specificity for complex structures (TL, Calsepa). In contrast, PNGase F significantly reduced the binding of lectins to complex, polyLacNAc, mannose and α2-6 sialic acid epitopes demonstrating that these lectins are binding to *N*-linked glycoproteins. The lectin LEA, which recognizes polyLacNAc containing glycans, did not show a great decrease in binding to the microvesicles after PNGaseF treatment. Therefore, the possibility cannot be ruled out that there are multiple sources of polyLacNAc glycans in the vesicles: complex *N*-linked as well as glycolipids or *O*-linked glycoproteins.

A



B

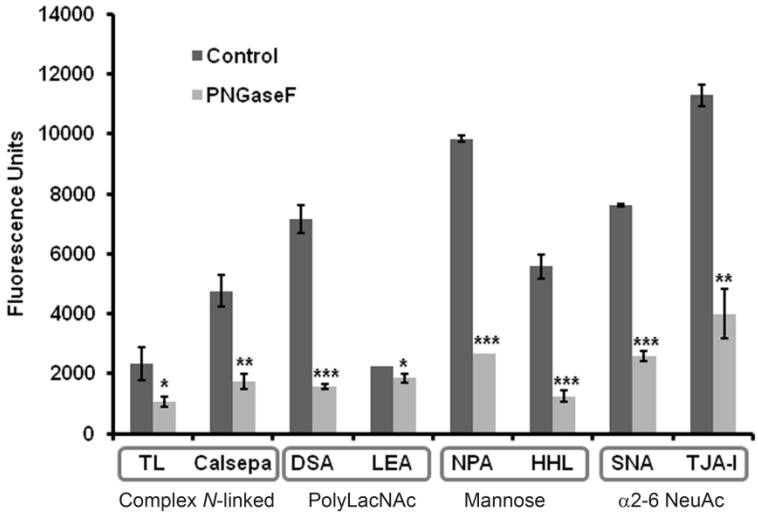


Figure 3.6: Glycosidase treatment of microvesicles confirms specificity of lectins. Cy3-labeled SkMel-5 MV were treated with (A) EndoHf and (B) PNGaseF prior to incubation on the array. Values are expressed as a mean ± SD of replicate spots. Statistically significant differences are indicated by their p-values: * $p < 0.05$; **$p < 0.001$; *** $p < 0.0001$ calculated using the unpaired t-test.

Because of their affinity for negatively charged structures, many sialic acid binding lectins bind to sulfated carbohydrates as well. To test if the signature seen for microvesicles was due to the presence of α2-6 sialic acid, labeled Sk-Mel-5 vesicles were treated with neuraminidase before incubation on the array. Fluorescence intensity significantly decreased in α2-6 sialic acid binding lectins (Figure 3.7) but there was no change in α2-3 sialic acid binding lectins. This tells us that the α2-6 specific lectins are binding to sialic acid but the lectins that recognize α2-3 are possibly binding non-specifically. Additionally, while most other non-sialic acid binding lectins on the array were unaffected by neuraminidase treatment (data not shown) there were some Gal and GalNac specific lectins (represented by VVA and WFA) that increased in binding. This is most likely due to exposure of these epitopes after the removal of sialic acid.

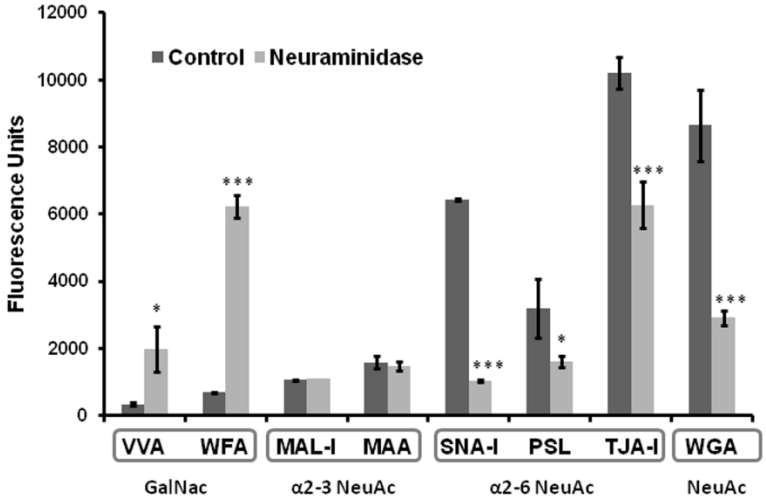


Figure 3.7: Neuraminidase treatment of SkMel-5 MV demonstrates specificity of sialic acid binding lectins. Labeled MV were treated with Neuraminidase prior to incubation on the array. Values are expressed as a mean ± SD of replicate spots. Statistically significant differences are indicated by their p-values: * $p < 0.05$; **$p < 0.001$; *** $p < 0.0001$ calculated using the two-tailed t-test.

Dual color analysis using a common reference

Although single color analysis provided information about the presence or absence of certain glycans in microvesicles and cell membranes, for a true comparison of the relative binding levels between samples we implemented dual color analysis using a common reference. In this method, competitive binding of the sample and a common reference gives a ratiometric output. This increases sensitivity to glycomic differences among samples and decreases sensitivity to variations between arrays. The biological reference used for comparative analysis was Cy5-labeled H9 cell membrane preparations. Glycoprofiles of the panel were obtained by incubation of equal protein amounts of Cy3-labeled sample against an equal amount of reference. All samples were normalized by setting the median equal to zero for each channel (median centering). This was done for several reasons. First, as discussed previously, the fluorescence pattern of breast milk microvesicles was less intense compared to the rest of the samples. Second, because of the limited quantities of microvesicles produced from most cell cultures, loss of sample is avoided by quenching the labeling reaction with tris base instead of removing excess dye by dialysis or filtration. This means that a dye to protein ratio of the labeled microvesicles cannot be accurately calculated and controlled for all samples. Finally, to account for dye bias between the Cy3 and Cy5 channel, we typically hybridize a dye swapped pair on the array and then calculate the yang correlation for that pair [80]. However, again because of the quantities of the microvesicles, this analysis could not be done with a dye swapped pair. For these reasons, the data were median centered to accurately compare the samples. This type of global normalization is typical of microarray analysis and addresses systematic variations that are not biologically relevant [99].

Hierarchical clustering of the arrays using the Pearson correlation revealed that all but the Jurkat microvesicles clustered together and away from their corresponding cell

membranes (Figure 3.8). The cluster was statistically similar by two-tailed statistical analysis of the Pearson correlation value ($r = 0.64$, $N = 75$, $P < 0.0001$). The reason that Jurkat microvesicles cluster more closely with the parent cell membrane is unknown. One possibility is that since Jurkat cells have truncated *O*-glycosylation synthesis pathways, this may account for the differences between Jurkat microvesicles and the rest of the panel [100]. Upon clustering the microvesicles and cell membranes separately (Figure 3.9), a conserved lectin binding pattern for the entire panel of microvesicles is revealed ($r = 0.57$, $N=75$, $P < 0.0001$). In contrast, clustering of the parent cell membranes did not show a conserved glycomic signature ($r = 0.25$, $N = 75$, $P = 0.03$). Giving confidence to the reproducibility of our data, all biological replicates tightly clustered with a correlation coefficient of $r = 0.91$ or greater. Included within the microvesicle cluster are the breast milk derived samples. Because this sample was obtained from human physiological fluid, this suggests that the conserved glycomic pattern observed is neither specific to cancer derived vesicles nor a phenomenon of the cell culture conditions.

Dual color analysis improved the sensitivity of some of the lectins that were previously outside of the linear range of detection. Whereas the single color analysis portrayed binding to WGA, TJA-I, DSA and NPA to be approximately equal between Sk-Mel-5 microvesicles and membranes, ratiometric analysis revealed the expected differences between the two due to the enrichment of α2-6 sialic acid, polylactosamine and high mannose epitopes in the microvesicles. In addition, dual color analysis revealed two sets of GalNAc-specific lectins with divergent behaviors. The first set (DBA, BPA, SBA, VVA), bind to terminal α- and β-GalNAc epitopes such as Blood Group A and were previously identified in single color analysis as displaying lower binding to the microvesicles than the membranes. A second group of GalNAc binders (MNA-G, MPA, SNA-II, IRA, HPA, AIA) identified by dual color analysis revealed the enrichment of

terminal α-GalNAc structures, such as the Tn antigen, in most microvesicles. Overall, the conserved microvesicle signature consisted of an enrichment of high mannose, complex *N*-linked glycans and polyLacNAc as well as a subset of terminal αGalNac containing epitopes .

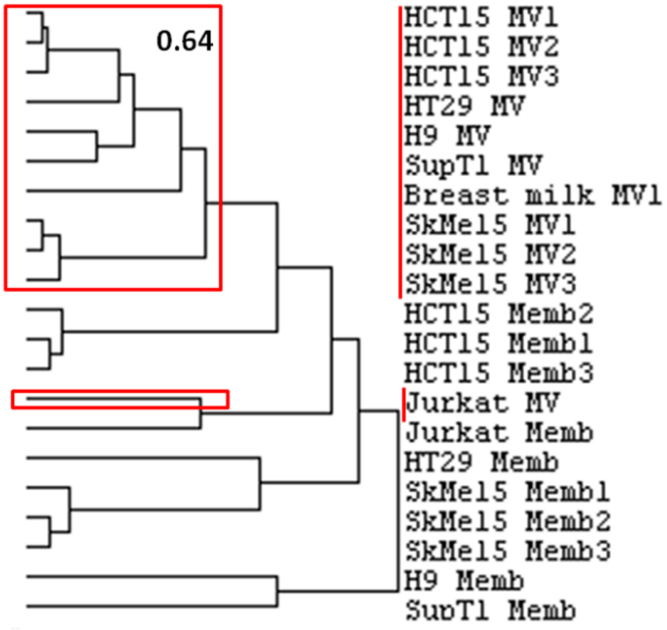


Figure 3.8: Hierarchical clustering of the microvesicle and membrane glycomes using the Pearson correlation with average linkage analysis.

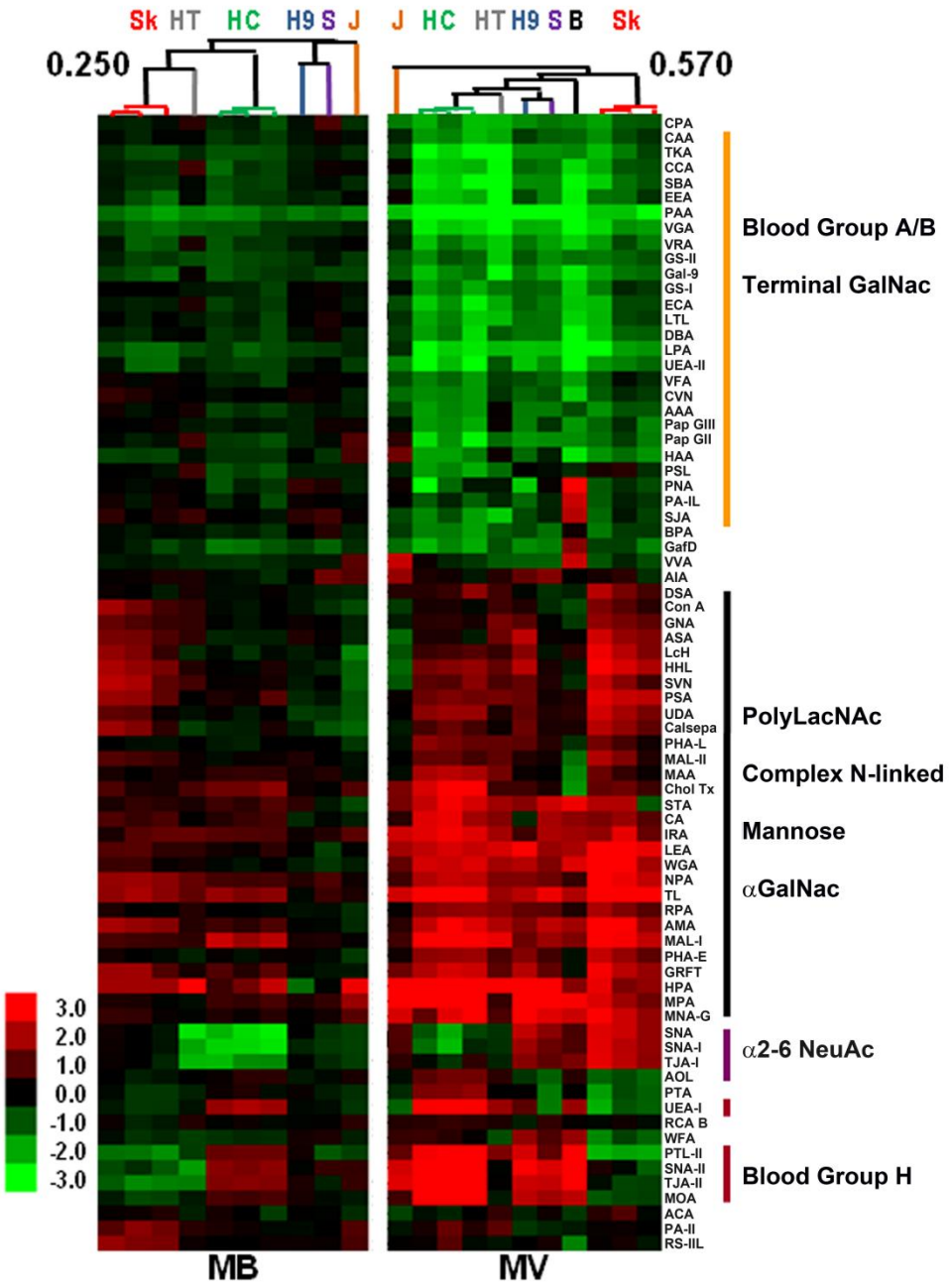


Figure 3.9: Ratiometric lectin microarray comparison using H9 membrane as a reference. Arrays were hierarchically clustered using the Pearson correlation coefficient as the distance metric and average linkage analysis (n=75 lectins). Heat map is shown. Red indicates greater than the median; green indicates less than the median. Select Pearson correlation values are shown on the tree. SkMel-5 (Sk), HT29 (HT), HCT-15 (HC), SupT1 (S), Jurkat (J), breast milk (B).

In addition to the conserved signature, microvesicles displayed some differences in their glycosylation (Figure 3.10). For the cell culture derived microvesicles, these differences are clearly a reflection of the parent cells. For example, HCT-15 and HT29 cells have lower levels of α-2,6 sialic acid in their membranes when compared to the entire panel of cell lines and consequently display lower levels overall of this epitope in their cognate microvesicles (SNA, SNA-I and TJA-I). Additionally, SkMel-5 membranes did not bind to blood group H specific lectins (PTL-II, TJA-II, AOL, UEA-I) and the absence of this epitope is also seen in the microvesicles. However, blood group H is clearly abundant in both HCT-15 membranes and microvesicles and seems to be enriched in all but the Sk-Mel-5 vesicles. Breast milk microvesicles also displayed differences in their glycosylation profiles with binding to some Gal (PNA, PA-IL), GlcNAc (GafD) and GalNAc (SJA, VVA) specific lectins.

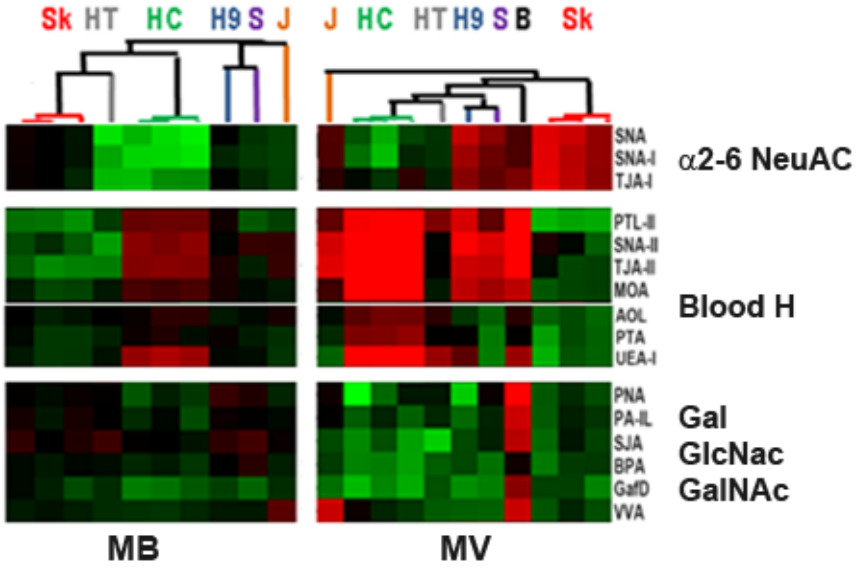


Figure 3.10: Microvesicles display some differences in glycosylation. Arrays were analyzed as previously described in Figure 3.9. SkMel-5 (Sk), HT29 (HT), HCT-15 (HC), SupT1 (S), Jurkat (J), breast milk (B).

3.3 CONCLUSIONS

Using lectin microarray technology, I was able to characterize and compare the glycosylation profiles of microvesicles from a diverse panel of sources. In doing so, I found that the microvesicles were enriched in many of the same types of carbohydrate epitopes. If making an analogy between the glycome and proteome of these particles, one could assume that microvesicles from diverse sources share certain typical traits but are largely a miniature copy of the parent cell. However, this did not seem to be the case in our glycomic comparison as most microvesicles, with the exception of Jurkat derived, were quite divergent from their parent cell membranes. Glimpses of the glycosylation profiles of microvesicles had been previously observed for individual glycoprotein studies, for ovarian microvesicles using three lectins, and on T-cells using the lectin array. Our work corroborated with many of these studies by identifying the presence of a high mannose, polylactosamine, α2-6 sialic acid and complex *N*-linked glycan signature in microvesicles. Although the reason for this conserved signature is unknown, it is very likely that the glycosylation profiles of microvesicles serve a biologically significant purpose. The presence of α2-6 sialic acid is known to increase the serum half-life of many circulating proteins such as follicle stimulating hormone (FSH) and acetyl choline esterase (AChE) [101, 102]. Since microvesicles are found in various physiological fluids, they could potentially be utilizing sialic acids to prolong their half-life as well. It will be interesting to investigate if the biological relevance of this conserved glycome is seen for individual glycoproteins.

The lectin microarray allowed for the analysis of multiple samples in small quantities and was a useful tool for the comparative glycomic analysis of microvesicles. However not all of the analysis of these particles was completely clear. For example, the reason for the divergent behavior of certain αGalNAc binding lectins is unknown. This is

most likely due to the more intricate binding preferences of the lectins together with the subtle differences in glycosylation of the samples. Based on the fact that we see αGalNAc enriched in the microvesicles, the epitope that is excluded is most likely βGalNAc. The fact that many of the lectins on our array have somewhat promiscuous binding specificites can also be a problem. Our lab is currently working on solutions to these issues by implementation of recombinant lectin technology [103, 104].

Exosomes, and to a lesser known extent, ectosomes, have been shown to contain certain protein markers found in all or most studied samples. However, multiple proteomic studies and meta-analyses have revealed an immense variability in protein content, even within vesicles of the same type (e.g. urine microvesicles). This is one of the challenges for clinicians interested in the use of microvesicles as biomarkers. Considering the diversity of the proteomic content of microvesicles as well as the cell type differences in plasma membrane glycosylation, one would expect the same level of variability in the glycome. In this regard, the conserved glycomic profile of microvesicles is surprising and may imply that glycosylation is a signal for sorting of glycoproteins and glycolipids to these particles. The focus of the next chapter is to investigate the origins of this conserved microvesicle glycan signature.

3.4 MATERIALS AND METHODS

Microvesicle isolation and membrane preparations

All samples for lectin microarray analysis were obtained as previously described in Chapter 2.

Lectin microarray print

All lectins were purchased from either EY Laboratories (San Mateo, CA) or Vector Labs (Burlingame, CA) with the following exceptions: cyanovirin (CVN), scytovirin (SVN) and griffithsin (GRFT) were gifts from Dr. B. O'Keefe (NCI-Frederick); galectin-9 was a gift from Dr. L. Baum (UCLA Medical School); and Gaf-D, PA-IL, PA-IIL, PapGII, PapGIII and RS-IIL were made recombinantly as previously described. Three spots per lectin were printed using a Nanoplotter 2.1 piezoelectric printer (GeSIM, Germany) at 14˚C and 45% humidity. See Table 3.1 for lectin list, print concentrations and buffers.

Microarray sample incubation

Microarray slides were submerged in blocking buffer (50 mM ethanolamine, mM sodium borate ph 8.5) for 1 h followed by 3 washes with PBST (PBS with 0.005% Tween) and a final wash with PBS. The microarray slides were then fitted to a 24-well frame (ArrayIt, Sunnyvale, CA). For single color experiments, we added 1.5 µg (by protein concentration) of labeled sample in a final volume of 100 µl in PBST to each subarray. For dual color experiments, 1.5 µg of Cy3-labeled microvesicles were incubated with equal amounts of either Cy5-labeled H9 reference or Cy5-labeled parent membrane in 100 µl total volume PBST. The order of the samples was randomized on a total of two slides for all dual color assays. Labeled glycoprotein standards were hybridized on all slides as controls and no statistical difference in lectin activity was observed between slides. Samples were hybridized to the lectin microarrays for 2 h at room temperature with gentle rocking. The individual subarrays were then washed with PBST for 5 x 5 min with a final 10 min wash in PBS. Slides were dried spinning for 1 min on a slides spinner. Slides were scanned and analyzed using a GenePix 4300B

fluorescent slide scanner (Molecular Devices, Sunnyvale, CA) with GenePix Pro 7 software.

Microarray analysis

The background subtracted median fluorescence of the three replicate spots per lectin was tested for outliers using the Grubbs outlier test with alpha = 0.05 (Microsoft Excel). For single color analysis, the average fluorescence of the replicate spots was calculated. For dual color analysis, the $\log_2$ values of the average fluorescence of the 3 replicate spots were median centered over the array in each channel to account for differences in labeling efficiency. Misprints and lectins with signal to noise ratios (SNR) of less than 5, as determined by the GenePix Pro 7 software, in 90% or more of samples were excluded from the cluster. The $\log_2$ values of the average fluorescence of the 3 replicate spots were median centered over the array in each channel to account for differences in labeling efficiency. The resultant datasets (arrays and lectins) were hierarchically clustered by the $\log_2$ ratios using the Pearson correlation coefficient with average linkage analysis using Cluster 3.0 [98] and visualized with Java Treeview [105]. Arrays were considered to be statistically similar if $P < 0.0001$. P values were obtained by employing a two tailed t test (N = number of lectins, r = Pearson correlation coefficient, Df = N-2) using the online statistical calculator: http://faculty.vassar.edu/lowry/tabs.html#r.

Mono- and disaccharide inhibitions

Subarrays were incubated with 50 µl of 200 mM fucose, lactose, mannose or GlcNAc in PBS for 30 minutes with gentle agitation. Negative controls were incubated in

50 µl PBS. After incubation 1.5 µg of Cy-3 labeled Sk-Mel-5 microvesicles in 50 µL of PBST 0.01% were added so that the final concentration of sugar was 100 mM in PBST 0.005%.

Glycosidase treatment

All enzymes were purchased from New England Biolabs. Cy3-labeled microvesicles (3 µg) were treated with either 1.5 kU EndoHf cloned from *Streptomyces plicatus*, 100 U Neuraminidase cloned from *Clostridium perfringens* or 1 kU PNGaseF purified from *Flavobacterium meningosepticum* per µg of microvesicle protein in the manufacturer provided buffer without the addition of detergent at 37˚C for 18 h. Negative controls were mock treated with enzyme buffer and incubated at 37˚C for 18 h.

Lectin		[Print] (ug/ml)	1mM Sugar‡	Specificity
AAA	*Anguilla anguilla*	1000	Fuc	α-Fuc
PNA	*Arachis hyogaea*	500	Gal	Terminal β-Gal
AIA	*Artocarpus intergrifolia (Jacalin)*	500	Gal	α-GalNAc not substituted at C-6
BPA	*Bauhinia purpurea*	500	Gal	Galβ1-3 or GalNAcβ1-4 more weakly
Con A	*Canavalia ensiformis*	500	Man	Branched and terminal mannose, terminal GlcNAc
CCA	*Cancer antennarius*	500	Lac	9-*O*-Acetyl NeuAc and 4-*O*-Acetyl NeuAc
CAA	*Caragana arborescens*	500	Gal	GalNAc or Gal
CPA	*Cicer arietinum*	1000	Lac	Complex
CA	*Colchicum autumnale*	500	Gal	Terminal β-Gal, α- and β-GalNac
DSA	*Datura stramonium*	500	Lac	GlcNAcβ1-4GlcNAc oligomers, polyLacNAc
DBA	*Dolichos biflorus*	500	Gal	α- and β-GalNAc (particularly sialylated branched structures)
ECA	*Erythrina cristagalli*	500	Gal	GalNAcβ1-4GlcNAc, Galβ1-4GlcNAc
EEA	*Euonymus eurpaeus*	1000	Lac	Blood Groups B and H
GNA	*Galanthus nivalis*	1000	Man	Terminal Manα1-3
SBA	*Glycine max*	500	Gal	Terminal α- or β-GalNAc
GS-I	*Griffonia simplicifolia I*	500	Gal	a-Gal, some GalNAc
GS-II	*Griffonia simplicifolia II*	500	GlcNAc	Terminal GlcNAc
HPA	*Helix pomatia*	500	Gal	Terminal α-GalNAc
LcH	*Lens culinaris*	1000	Man	Complex and high mannose
LFA	*Limax flavus*	500	Lac	α-NeuAc (O-glycans)
LTL	*Lotus tetragonolobus*	500	Fuc	Lewis ^{x}, Fucα1-2Gal, Fucβ1-3 GlcNAc
LEA	*Lypersicon esculentum*	500	GlcNAc	β1-4GlcNAc oligomers, polyLacNAc
LPA	*Limulus polphemus*	500	Lac	α-NeuAc
MAA	*Maackia amurensis*	500	Lac	NeuAcα-2,3
NPA	*Narcissus pseudonarcissus*	1000	Man	Terminal and internal Man
LBA	*Phaseolus lunatus*	1000	Gal	GalNAcα1-3[Fucα1-2]Gal (Blood A)
PHA-E	*Phaseolus vulgaris-E*	500	Lac	Complex *N*-linked (bisecting GlcNAc)

PHA-L	*Phaseolus vulgaris-L*	500	Gal	Complex trianntenary N-linked glycans
PSA	*Pisum sativum*	1000	Man	Man
AOL	*Aspergillus oryzae*	1000	Fuc	Fucα1-6 (core fucosylation), Fucα1-2Gal
PTA	*Psophocarpus tetragonolobus*	500	Gal	Gal, GalNAc
RPA	*Robinia pseudoacacia*	1000	Lac	Complex
SNA	*Sambucus nigra*	500	Lac	NeuAcα2-6, (Lac core)
SNA-II	*Sambucus nigra-II*	500	Gal	Gal-NAcα linked to C-2, C-3, or C-6 hydroxyl group of Gal
SNA-I	*Sambucus nigra-I*	500	Lac	Neu5Acα2-6, (Lac core)
STA	*Solanus tuberosum*	500	GlcNAc	GlcNAc oligomers (LacNAc,or LacdiNAc)
SJA	*Sophora japonica*	1000	Gal	GalNAc
TKA	*Trichosanthes kirilowii*	500	Gal	β-Gal, Neu5Acα2-3/6Galβ1
WGA	*Tritiicum vulgare*	1000	GlcNAc	β-GlcNAc, sialic acid, GalNAc
TL	*Tulipa sp.*	1000	GlcNAc	GlcNAc, Man, biantennary complex *N*-linked glycans
UEA-I	*Ulex europaaeus* I	500	Fuc	Fucα1-2Galβ1-4GlcNAc
UEA-II	*Ulex europaaeus* II	1000	GlcNAc	Fucα1-2Galβ1-4Glc(Nac), Chitin
UDA	*Uritica dioica*	1000	GlcNAc	High Man, GlcNAcb1-4GlcNAc oligomers
VGA	*Vicia graminea*	500	Gal	O-linked Galβ1-3GalNAc clusters
VVA	*Vicia villosa*	500	Gal	GalNAc
VVA (man)	*Vicia villosa*	500	Gal	Man
VRA	*Vigna radiata*	500	Gal	α and β-Gal
VFA	*Vicia fava*	500	Gal	Man, Glc, GlcNAc
WFA	*Wisteria floribunda*	500	Gal	GalNAc
RCA B	*Ricinus communis* agglutinin B	1000	Lac	Terminal β-Gal, terminal LacNAc
HHL	*Amarylis Lectin*	1000	Man	Manα1-3 and 1-6
MAL- I	*Maackia amurensis* -I	1000	Lac	NeuAcα2-3LacNAc
MAL-II	*Maackia amurensis* -II	500	Lac	NeuAcα2-3LacNAc
PTL-I	*Psophocarpus tetragonolobus*	1000	Gal	αGalNAc, Blood A and B, Galα1-3GalNAcα
PTL-II	*Psophocarpus tetragonolobus*	1000	Gal	βGalNAc, Type II Blood H
CVN	Cyanovirin	1000	Man	High Man
SVN	Scytovirin	500	Man	High Man

GRFT	Griffithsin	500	GlcNAc	High Man
Chol Tx	Cholera Toxin	2000	Lac	Ganglioside (GM1)
ACA	*Amaranthus Caudatus*	1000	Man	GalNAc, Galβ1-3GlcNAc
ASA	*Allium sativa*	1000	Man	High man (Man9-GlcNAc2)
MPA	*Macluria Pomifera*	500	Gal	Tn and Tα antigens
PSL	*Polyporus Squamosus*	1000	Lac	NeuAcα2-6LacNAc
TJA-I	*Trichosanthes japonica*	1000	Lac	NeuAcα2-6LacNAc or 6-Sulfo LacNAc.
TJA-II	*Trichosanthes japonica*	500	Lac	Fucα1-2Galβ1-3/4GlcNAc, GalNAcβ1-4Galβ1
Gal-9	Galectin-9	500	Lac	GalNacα1-3GalNacβ1-3Galα1-4Galβ1-4Glc
AMA	*Arum maculatum*	1000	Man	Biantennary core (GlcNAc), high man, LacNAc
Calsepa	*Calystegia sepium*	500	Man	Complex *N*-linked with bisecting GlcNAc
HAA	*Helix aspersa*	1000	Gal	Terminal GalNAc
MOA	*Marasmium oreades* agglutinin	1000	Gal	Galα1,3Gal, Blood B
IRA	*lris hybrid*	1000	Gal	GalNAc
MNA-G	*Morus nigra*	1000	Gal	Tn and Tα antigens
GafD†	*Escherichia coli* F17 fimbriae	500	GlcNAc	β-GlcNAc
PA-IL†	*Pseudomonas Aeruginosa Lectin* II	500	Gal	Terminal α-Gal
PA-IIL†	*Pseudomonas Aeruginosa Lectin* III	500	Fuc	α-Fuc, high Man
PapGII†	*Escherichia Coli Pap Adhesin*	500	Gal	Globotetraose
PapGIII†	*Escherichia Coli Pap Adhesin*	500	Gal	Globopentaose
RS-IIL†	*Ralstonia solanacearum* Non-fimbriae	500	Man	Man, Fuc

Table 3.1: Lectin printlist and specificities. Print Buffer: 0.005% Tween in PBS pH 7.6. † Print Buffer for Oriented Lectins: 100 mM Glutathione in 50 mM sodium borate pH 8.5.

Chapter 4: Investigating the role of lectin-glycan interactions in microvesicle protein sorting

4.1 INTRODUCTION

Overview

In the previous chapter I demonstrated that microvesicles from diverse sources display a common glycomic signature, in contrast to the cell membranes, which had significant differences in their glycosylation profiles based on cell type. Since glycosylation is a known cellular trafficking signal for proteins, this leads to the hypothesis that it may also play a role in sorting glycoproteins to microvesicle bound domains. This chapter explores the possibility that lectin-glycan interactions are responsible for the conserved microvesicle glycome.

Determinants for microvesicle protein incorporation

There are many lines of evidence that point to active sorting mechanisms for the incorporation of molecular content of microvesicles. For example, microvesicles are known to contain distinct sets of proteins (i.e. CD63 and CD81), lipids (cholesterol rich lipid rafts in ectosomes) and RNA, implying that the molecular content of microvesicles is not simply a random assortment of cellular material. Although some progress has been made towards understand this process, the mechanisms that drive microvesicle biogenesis are still largely undefined.

The endosomal sorting complex required for transport (ESCRT) machinery sorts ubiquitinated proteins to multivesicular bodies (MVB). Since exosomes are said to originate from MVB or MVB-like structures, the ESCRT machinery was thought to be important for cargo sorting to exosomes. However, neither ubiquitination nor the ESCRT

machinery is required for inclusion of proteins into secreted vesicles [106]. An alternative theory is that oligomerization is a key sorting determinant of membrane-bound proteins. There is increasing evidence that the formation of membrane-bound highly oligomeric protein structures may be a sorting determinant. Evidence for this theory comes from several sources. Antibody- and lectin-induced crosslinking of TfR and AChE led to increased secretion of these proteins in exosomes from reticulocytes [107]. Additionally, Gould *et al*. determined that highly oligomeric cytosolic proteins could be directed to exosomes upon addition of an acyl membrane anchor [108]. In T-cells, the secretion of MHC II via exosomes was characterized by association with CD9-containing, detergent-resistant complexes. Since the oligomeric membrane structures were formed artificially, it is still unclear whether these structures lead to microvesicle incorporation under natural cellular conditions. At this point, the cellular factors that deliver proteins to microdomains or that aid in microvesicle membrane budding are still unclear.

Lectins in protein trafficking

The modification of proteins with *N*- and *O*-linked glycans is a well-known marker for trafficking of glycoproteins to different destinations in the cell. This process involves the recognition of specific glycans by lectins that sequester and cluster the glycoproteins into membrane microdomains. A classic example of this is the sorting and trafficking of *N*-linked glycoproteins that occurs after post-translational processing in the ER and Golgi. The removal of properly folded proteins from the ER for entry into the Golgi is facilitated by mannose-binding lectins, such as ERGIC-53, VIP36, and VIPL [109]. Misfolded proteins are recognized by the lectin EDEM after the removal of one mannose residue, and sorted to the ER-associated degradation pathway [110]. In addition,

sorting of lysosomal hydrolases from the golgi to the lysosome occurs after the proteins are tagged with phosophomannosyl moieties and subsequently recognized by mannose-6-phosphate receptors (MPR). The receptor-ligand complexes are sequestered into tubular structures at the trans-golgi network and eventually packaged into clathrin-coated vesicles that are destined for the lysosome [111].

The proper function of polarized epithelial cells requires the differential transport of membrane bound receptors and transporters to either the apical or basolateral domains of the cell. Sorting into apical and basolateral bound vesicles is done in the trans-Golgi network followed by transport to the appropriate plasma membrane regions. Both *N*- and *O*-linked glycosylation have been identified as apical sorting signals, whereas basolateral sorting signals are confined to the cytoplasmic domain of proteins. This has been demonstrated both by addition of glycans to normally non-polarized proteins as well as the disruption of apical transport after glycosylation inhibition. Members of the galectin family have been implicated in apical sorting of glycoproteins and glycolipids in polarized cells [112]. Galectins are a family of 14 β-galactoside-binding mammalian lectins that are defined by a homologous carbohydrate recognition domain. They are found intracellularly in the nucleus and cytoplasm but they can also be secreted to the extracellular space via a non-classical secretion pathway. Multiple proteomic studies have identified galectins in microvesicles [11, 92, 113].

All galectins can form multimers, allowing for crosslinking of glycans and the formation of glycoprotein clusters. This multivalency of galectins is an important aspect of their cellular function [114]. In Madin-Darby canine kidney cells and mouse intestines, the carbohydrate-directed sorting of numerous membrane glycoproteins is aided by galectin-3. This multivalent lectin recognizes specific *O*- and *N*-linked carbohydrates and can form high molecular weight glycoprotein complexes which will then be trafficked to

the apical membrane [115, 116]. In the polarized colon cancer cell line, HT-29, binding of galectin-4 to sulfated glycosphingolipids leads to incorporation into "superaft" membrane domains and apical delivery [117].

4.2 RESULTS AND DISCUSSION

Direct comparison of microvesicles and cell membranes

As discussed in chapter 3, we observed a conserved glycan signature, with both enrichment and depletion of certain glycans compared to the parent cells. We hypothesized that this could be the result of a carbohydrate-based sorting process for glycoprotein incorporation into microvesicles. To look more closely at the glycomic differences between the parent and microvesicle membranes, I directly compared them on the lectin microarray using the dual-color assay. In a direct comparison, competitive binding between the microvesicles and the parent membranes clearly designate glycans that are differentially expressed on the microvesicle surface. Cy3-labeled microvesicles and an equal protein amount of Cy5-labeled membranes were mixed and incubated on the lectin array (Figure 4.1A). In this experiment, there was a consistent difference in the overall fluorescence intensities between the microvesicle and membrane samples that I had not previously encountered. This may be due to uneven dye degradation between the Cy3-labeled microvesicles and the Cy5-labeled membranes. This is surprising as Cy3 is typically more stable than Cy5, however, their storage in different buffers may account for the uneven dye degradation. Membrane preparations are dialyzed in PBS after labeling to remove excess dye. Because of the limiting amounts of microvesicles and the potential for loss from dialysis, the microvesicles are not dialyzed after labeling. Instead, the NHS-Cy3 labeling reaction is quenched by the addition of 200 mM Tris buffered

saline (TBS) ph 6.7 bringing the pH to 7.6. Therefore, Cy3 stored in a mixture of bicarbonate buffer and TBS may degrade more rapidly over time than Cy5-labeled membranes stored in PBS. To account for discrepancies in the intensities of the membranes and the microvesicles, the array data were median-centered. Although there was no common biological reference, heirarchical clustering using the Pearson correlation and average linkage analysis again showed a clear glycopattern ($r = 0.66$, $N = 72$ lectins, $P < 0.0001$). The previously observed pattern of enrichment (polyLacNAc, high mannose, complex *N*-linked glycans) and depletion (terminal GalNAc, blood group A or B antigens) of specific glycans was prominently displayed (Figure 4.1B). This implies that the glycan signature is due to an active sorting mechanism for incorporation of glycoproteins and/or glycolipids to microvesicles.

To test for the appropriateness of our global normalization method, I performed several lectin blots to compare Sk-Mel-5 microvesicles and membranes. Equal protein amounts were separated by gel electrophoresis and probed with TKA, ECA, TJA-I, NPA and DSA. Based on the array data, the lectins ECA and TKA (terminal Gal and GalNAc) preferentially bind to the membranes, whereas NPA (oligomannose) and TJA-I (α2-6 sialic acid) preferentially bind to the microvesicles. The ECA and TKA lectin blots confirmed the scarcity of their binding epitopes in the microvesicles in accordance with the array data (Figure 4.2). The DSA blot shows a clear enrichment of polyLacNAc in the microvesicles. However, the TJA-I and NPA blots were more difficult to interpret and did not completely correlate with the enrichment seen on the arrays for the microvesicles. This is because different sets of bands were more pronounced in both the microvesicles and the membranes. For TJA-I, the reason for this may be that the sialic acid signal seen in the arrays was in part due to sialylated glycolipids that are abolished after gel electrophoresis and therefore not detected by the lectin blot assay. This would not explain

the discrepancy in NPA binding, because glycolipids are not known to contain oligomannose residues. Because certain lectins only recognize higher ordered polyvalent structures, the lectin array may be a more suitable platform to measure these interactions than by separation on a gel. Nevertheless, several TJA-I and NPA reactive proteins were clearly present in the microvesicles.

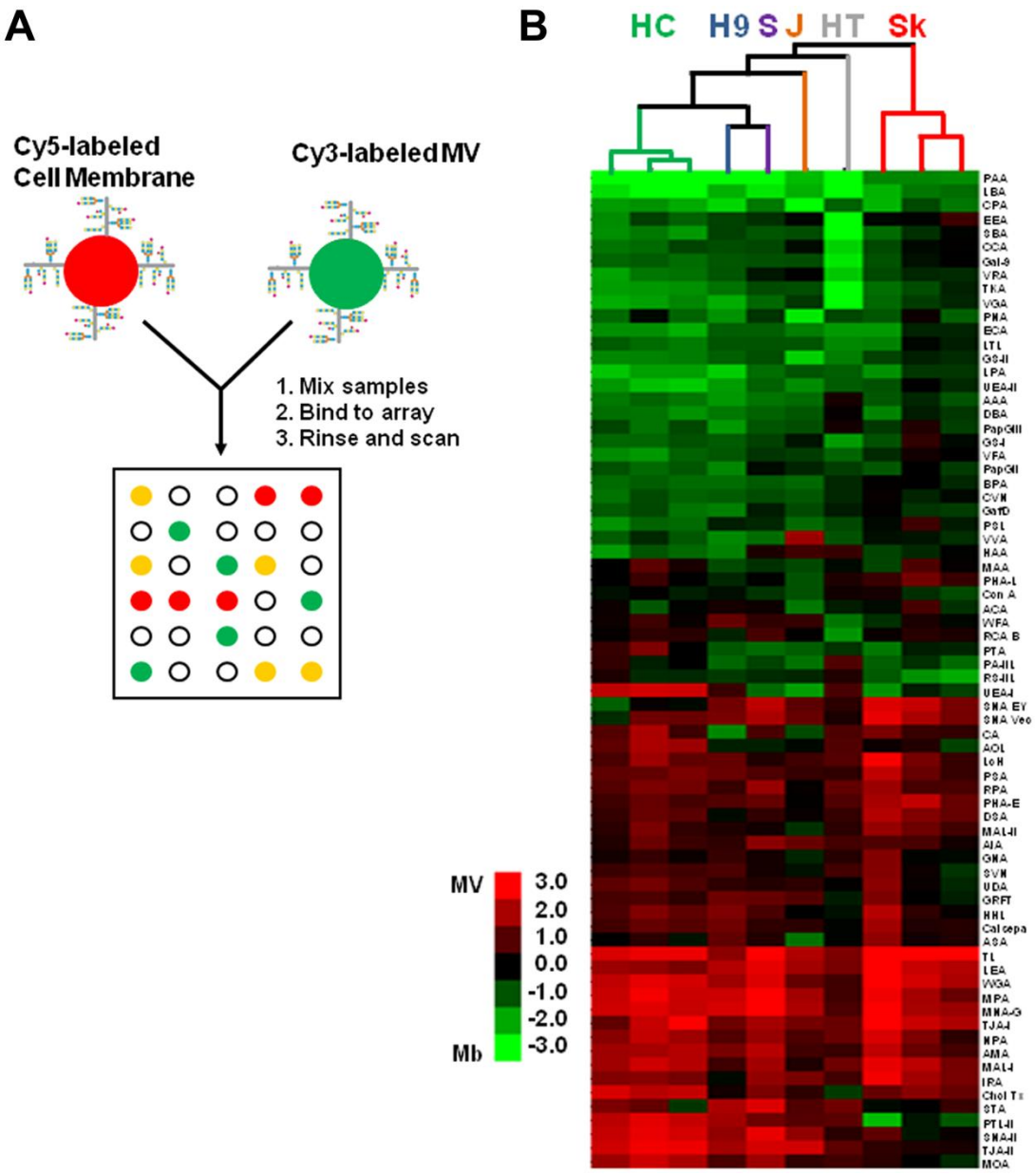


Figure 4.1: Direct comparison of microvesicles with their parent cell-membranes. (**A**) Experimental schematic. Equal amounts of Cy3-labeled microvesicles (MV) were analyzed against Cy5-labeled parent membranes (MB) on the lectin microarray. (**B**) Arrays were hierarchically clustered using the Pearson correlation coefficient as the distance metric and average linkage analysis (R = 0.66, N=72 lectins, P < 0.0001, students two tailed *t* test). Heat map of the median-centered data is shown. Red indicates greater binding to MV; green indicates greater binding to MB. HCT-15 (HC), SupT1 (S), Jurkat (J), HT29 (HT), SkMel-5 (Sk).

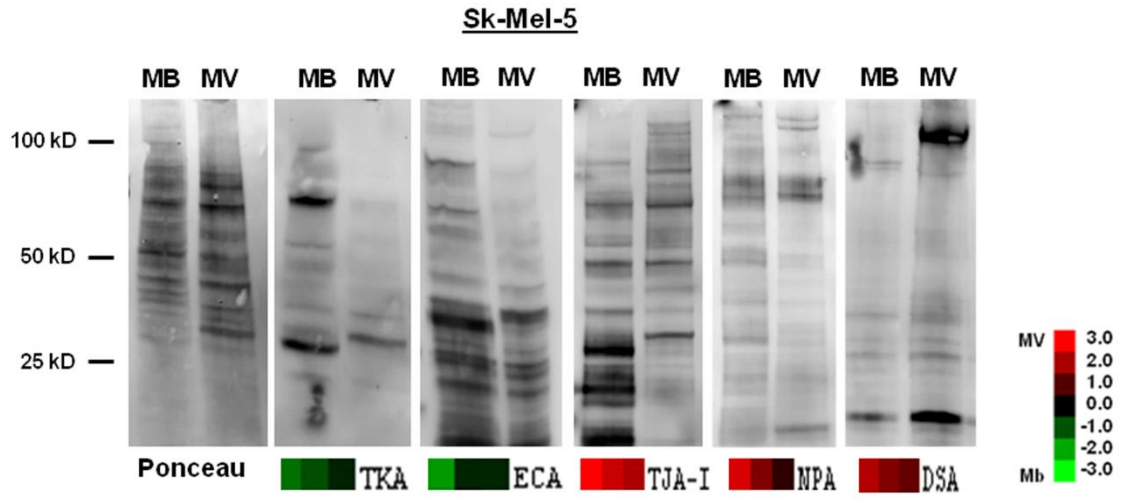


Figure 4.2 Lectin blot comparison of Sk-Mel-5 MB and MV. Equal protein amounts of were probed by the lectins TKA, ECA, TJA-I and NPA. Lectin microarray data from the direct comparison of MV and MB are shown for comparison. A representative ponceau stain from the TKA blot is shown as evidence of equal protein loading.

DSA blots identify distinct glycoprotein cohorts

The conserved glycosylation profiles could be the result of a carbohydrate mediated sorting mechanism analogous to those of apical glycoprotein sorting. Alternatively, the observed glycome could simply arise from the presence of predominant conserved proteins or lipids that contain the same glycoforms but are sorted by glycosylation independent mechanisms. For example, it was previously determined (Chapter 2) that CD63, a protein commonly associated with exosomes, was present in all of the vesicles anlayzed on the array. CD63 is a highly glycosylated protein known to contain complex *N*-linked polyLacNAc glycans [118] and is highly enriched in microvesicles when compared to cell membranes (Figure 4.3). Thus, this protein alone could potentially account for the enrichment of the polylactosamine component of the

microvesicle glycan signature. Using lectin array data alone, we cannot distinguish between these two possibilities. Therefore, I performed a lectin blot assay with DSA so that individual glycoprotein bands could be visible. The binding epitope of DSA is polyLacNAC found in complex *N*-linked glycans, which is one of the epitopes that is enriched in microvesicles. Equal amounts (1.5 µg protein) of microvesicles and membranes from three cell lines, SkMel-5, HT-29 and Jurkat, were probed. The microvesicles from each cell line showed a distinct pattern of DSA-positive bands. This provides preliminary evidence that different glycoproteins are responsible for DSA-reactivity in microvesicles from distinct cell lines (Figure 4.4A). To confirm enrichment of DSA epitopes in the microvesicle, the lectin blot lanes were quantified. The levels of enrichment observed in the microvesicles from the three cell lines were correlated with the microarray data with Sk-Mel-5 microvesicles displaying the most enrichment and Jurkat microvesicles displaying the least (Figure 4.4B).

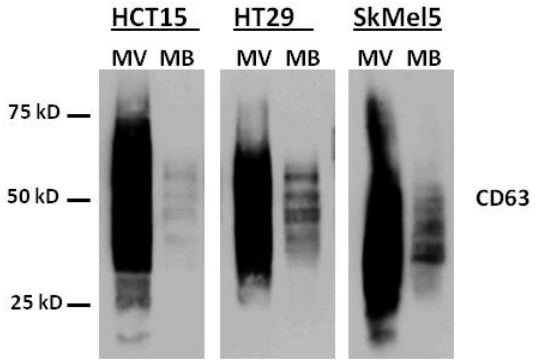


Figure 4.3 Microvesicles are highly enriched in CD63, a glycoprotein known to contain *N*-linked polylactosamine glycans. Equal protein amounts of MV and MB from the cell lines, HCT-15, HT-29 and Sk-Mel-5, were probed with anti-CD63.

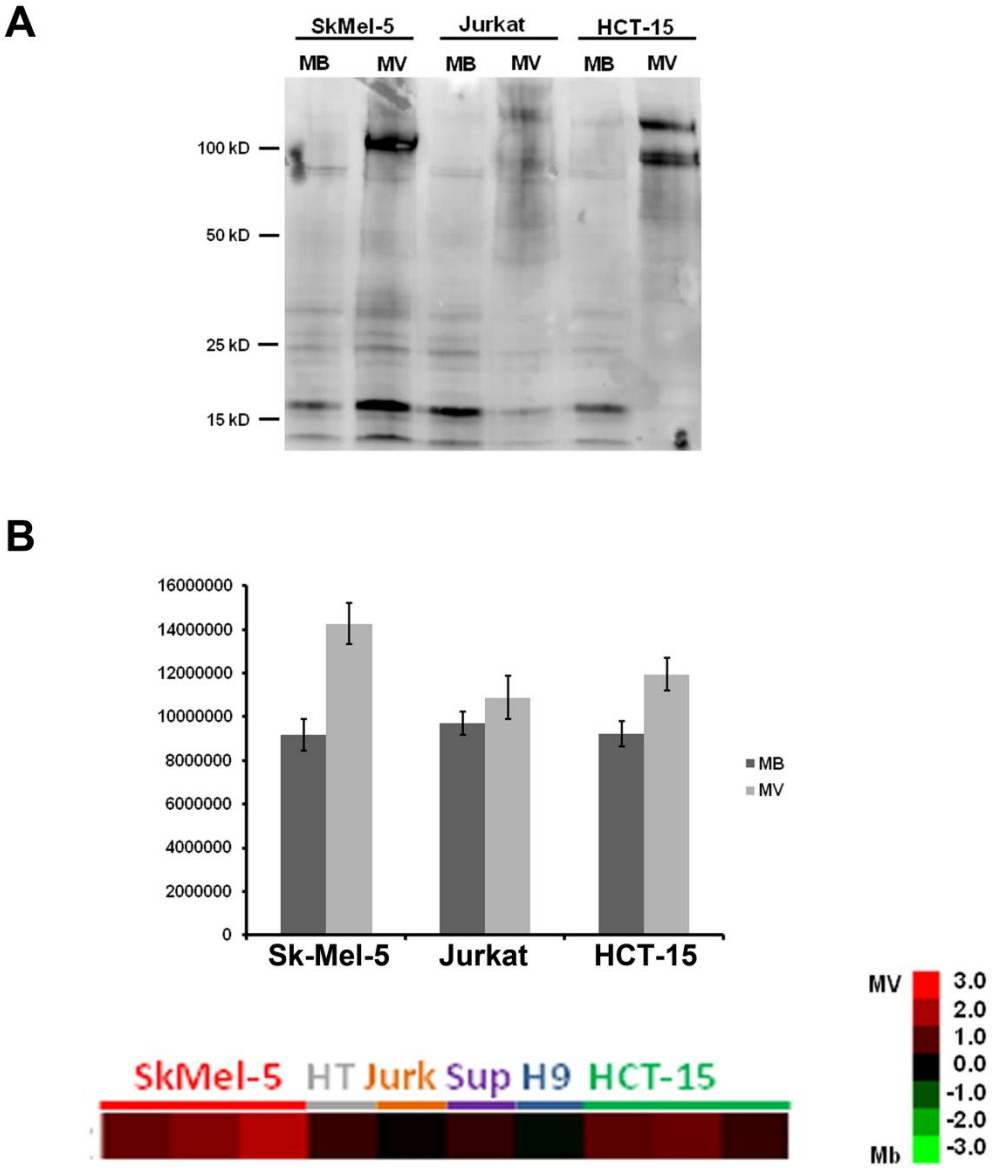


Figure 4.4 (**A**) DSA lectin blot of equal protein amounts of SkMel-5, Jurkat and HCT-15 MB and MV shows enrichment of DSA epitopes on multiple proteins in MV. (**B**) Lanes were quantified using NIH image by drawing 5 vertical lines through each lane and plotting the mean luminescence units for each lane ± SD. This was done to avoid bias due to lane imperfections. The level of enrichment in DSA binding to the microvesicles correlated with the lectin microarray data (shown for DSA).

A potential role for galectins in microvesicle-directed protein sorting

As previously mentioned, several labs have shown that oligomerization is sufficient to target membrane bound proteins to microvesicles [107, 108, 119]. Thus, the propensity for lectins and glycans to form highly clustered oligomeric complexes through their multivalent interactions makes lectins ideal candidates for microvesicle directed protein sorting. The presence of polylactosamine as a conserved feature of the microvesicle glycome, led us to the hypothesis that galectins may be involved in recognition and sorting of microvesicle bound proteins. Many members of the galectin family of lectins have an affinity for polyLacNAc epitopes. In addition, proteomic studies have identified galectins in microvesicles from various sources including galectin-5 in rat reticulocytes, galectin-7 in human parotid glands, galectin-3 in colorectal cancer cells and mouse dendritic cells and galectin-4 in HT-29 [92, 113, 120, 121].

I tested for the presence of galectins-3 and -4 in our panel of microvesicles by Western blot analysis. These lectins are known to be involved in apical trafficking of glycoproteins in polarized epithelial cells[112]. Figure 4.5 shows the presence of galectin-3 in SkMel-5, HCT-15 and HT-29 microvesicles. Galectin-4 was present in the microvesicles of the T-cell line H9 and the colon cancer cell line HT29. Because of the limiting amounts of HT-29 microvesicles, the western blot was not repeated to confirm the presence of galectin-3 in microvesicles from this cell line. Microvesicles from the T-cell lines Jurkat and Sup-T1 did not contain visible amounts of either of the two galectins. Expression of galectins is cell line dependent, with distinct galectin members differentially expressed in different cells. Therefore, it is possible that other galectins are present in Jurkat and Sup-T1. The multiple examples of the presence of galectins in microvesicles suggest that they play an important role in microvesicle biology. In

particular, the presence of galectins-3 and -4, which are known to be mediators of apical glyan sorting, implies that these lectins may have an analogous function in microvesicles.

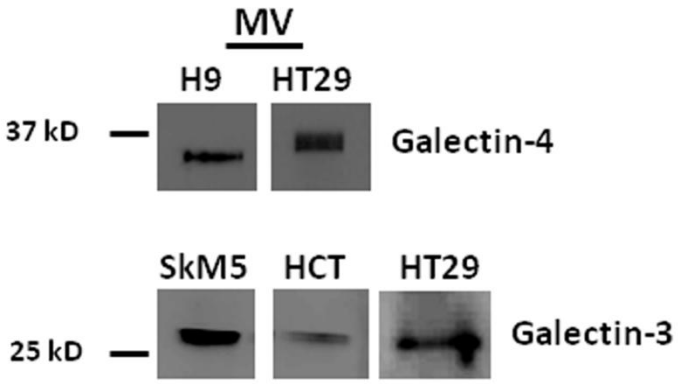


Figure 4.5 The panel of cell derived microvesicles were probed for galectin-3 and galectin-4 by western blot analysis. The microvesicles that are positive for the galectins are shown.

Galectins form intricate complexes called galectin-glycan lattices on the cellular surface through their multivalent interactions with cell surface glycoproteins (Figure 4.6). By this clustering mechanism, they regulate important cellular events such as apoptosis, differentiation and immunity [122, 123]. A common way that researchers disrupt the formation of galectin lattices is by inhibiting galectin with lactose. To investigate if galectin-glycan interactions are important for microvesicle formation, Sk-Mel-5 cells were treated with 100 mM lactose in serum-free media. After 15 h, conditioned media was ultracentrifuged for microvesicle isolation and the resulting pellets were probed for the presence of the glycoprotein, CD63. Lactose treatment of the cells resulted in a drastic reduction in CD63 compared to the microvesicles from the untreated controls (Figure 4.7) Even though CD81 is not glycosylated, a similar reduction was also seen

after lactose treatment. Both CD81 and CD63 are known to have multiple binding partners and recruit proteins to tetraspanin enriched microdomains, sometimes called the tetraspanin web [124]. Therefore, recruitment of CD81 to microvesicle may have been inhibited after lactose treatment due to its interaction with a glycoprotein.

Although many more studies are needed to test this, treatment of the cells with a high concentration of lactose could potentially be inhibiting important lectin-glycan interactions that are needed for incorporation into microvesicles. If this is true, one would expect a decrease in protein yield from the conditioned media after lactose treatment. In fact, the protein yield from untreated cells was 46 µg and from lactose treated cells was 30 µg. However, this experiment has not been replicated and therefore it is not yet known whether the difference in protein amounts is significant.

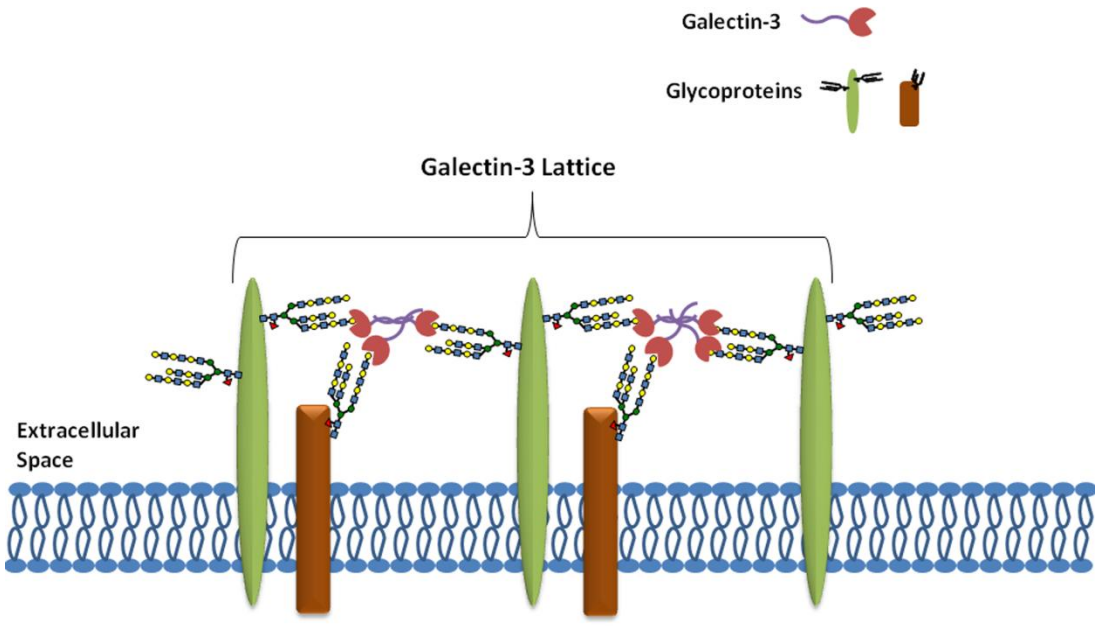


Figure 4.6 Schematic representation of a galectin-3 lattice structure on the cellular surface.

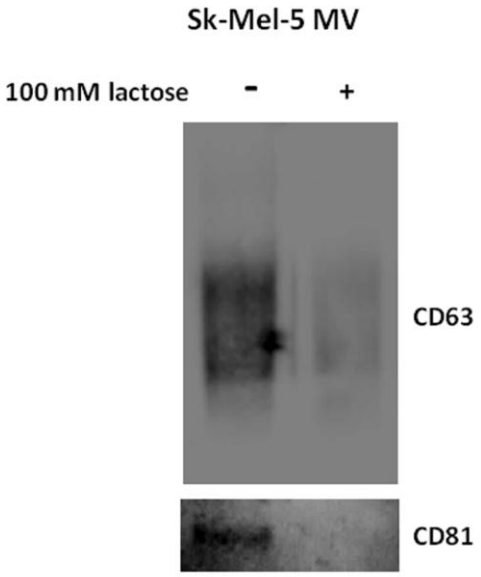


Figure 4.7 Lactose treatment of Sk-Mel-5 cells causes a decrease in secretion of CD63 and CD81. Sk-Mel-5 cells were treated with 100 mM lactose or no lactose in serum-free media for 15 h. Microvesicles were isolated and 5 μg of protein were probed with antibodies to CD63 and CD81.

Transfection of Sk-Mel-5 with galectin-3 shRNA

Compared to the rest of the cell lines used for our glycomic comparison, Sk-Mel-5 produced the greatest microvesicle protein yield. In addition, Sk-Mel-5 microvesicles contained galectin-3 making this cell line ideal for further analysis. Initial studies suggest that CD63 and CD81 incorporation into microvesicles is inhibited by lactose in Sk-Mel-5. Since galectin-3 is a known mediator of protein trafficking and can be inhibited by lactose, we hypothesize that it may be influencing protein trafficking to Sk-Mel-5 microvesicles. To investigate this, cells were transfected with a plasmid that contains galectin-3 shRNA, red fluorescent protein (RFP) as a transfection marker and a puromycin resistance marker. Figure 4.8 shows the cells with approximately 50%

transfection efficiency. Future work requires the creation and propagation of stable cell lines containing the galectin-3 shRNA plasmid for the production of microvesicles. Changes in the proteomic content of microvesicles obtained from these cell lines should provide insights into the importance of galectin for protein incorporation.

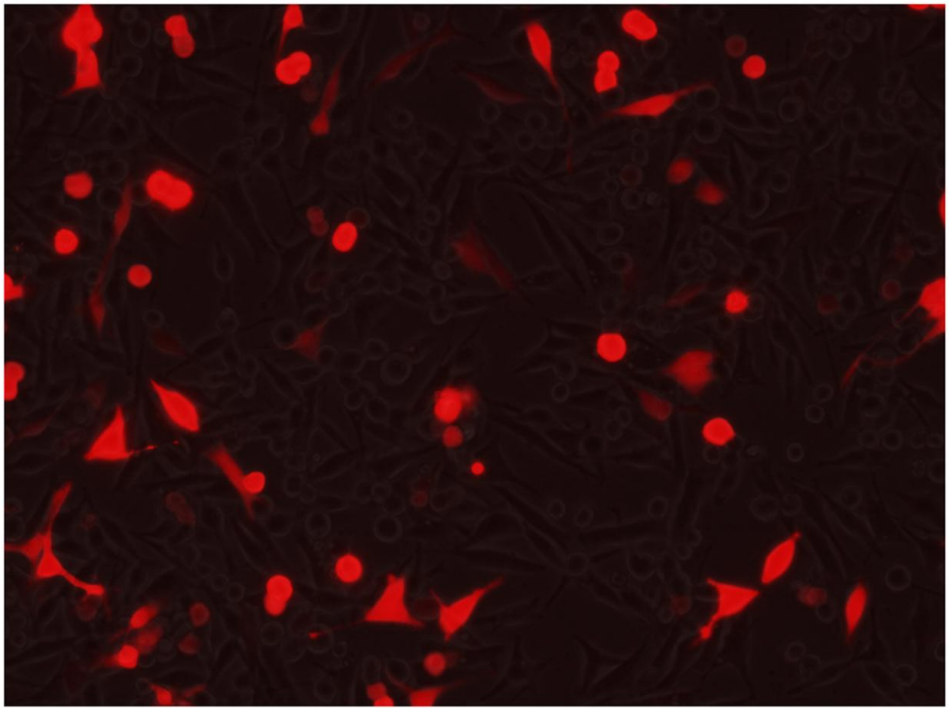


Figure 4.8 Transfection of Sk-Mel-5 cells with galectin-3 shRNA plasmid. Cells were transfected with Transit 2020 transfection reagent.

4.3 CONCLUSIONS

By comparing lipidomic, proteomic and transcriptomic microvesicle studies, researchers hypothesize that incorporation of microvesicle content is not a random process, but rather, incorporation occurs through an active sorting mechanism [4]. My work adds more evidence to this hypothesis through the identification of a conserved microvesicle glycome that is defined by both the enrichment and exclusion of cell membrane glycans. This glycan signature could be the result of a conserved set of microvesicle glycoproteins. However, by lectin blot analysis I observed distinct sets of protein bands in microvesicles from three different cell lines. This suggests that the conserved signature is more likely due to a glycan sorting mechanism, resulting in the enrichment of polylactosamine, high mannose, and complex *N*-linked glycan-containing proteins in microvesicles. Galectins are candidates for recognition and sorting of proteins that contain polylactosamine. Galectins-3 and -4 are known protein trafficking agents that were observed in several of our microvesicles. After cells were incubated in the presence of lactose for 20 hours, there was a considerable decrease in secretion of exosomal markers. This is further evidence that galectins may involved in microvesicle protein incorporation.

Pioneering work from the Gould lab has demonstrated by several exogenous means that the crosslinking of proteins at the cellular membrane can lead to an increase in microvesicle incorporation [108, 119]. My work puts forth compelling evidence for our hypothesis that galectins may be one of the responsible crosslinking agents that target microvesicle incorporation *in vivo*. Formation of a galectin lattice on the cellular surface may be a precursor to membrane budding. The work of Linda Baum and several other labs indicates that association with galectin lattices retains glycoproteins at the cellular surface by preventing endocytosis [122]. For example, galectin-9 lattices serve to retain

glucose transporters at the cellular surface and the absence of galectin-9 leads to diabetes in mice models [125]. In addition, galectin-3 lattices can inhibit the endocytosis of cytokine receptors leading to retention of tumor growth factor-β receptor on the surface of tumor cells [126].

If the same is true for Sk-Mel-5, this would have implications for the biogenesis of microvesicles in these cells. Endocytosis is said to be the process by which proteins are sorted through to the endosome and eventually to exosomes. Since secretion of CD63 and CD81 is decreased upon lactose treatment, presumably disrupting the formation of galectin lattices and increasing exocytosis, this would suggest that tetraspanin-containing vesicles are budding directly from the cell surface. Although this is not thought of as the typical biogenesis pathway for tetraspanin containing vesicles, outward microvesicle budding of CD63-enriched domains has been previously observed at the cellular surface [27].

4.4 MATERIALS AND METHODS

Direct comparison of microvesicles and cell membranes on the lectin microarray

Labeled samples used for direct comparison of microvesicles and parent cell membranes were procured previously from serum free conditioned media. Details of the cell culture conditions, isolation of microvesicles, membrane preparations, dye labeling and lectin microarray protocols are described in chapter 2.

Equal amounts (1.5 μg) of Cy3-labeled microvesicles were incubated with equal amounts of Cy5-labeled parent cell membranes in 100 μl total volume PBST. Samples were hybridized to the lectin microarrays for 2 h at room temperature with gentle rocking. The individual subarrays were then washed with PBST for 5 x 5 min with a final

10 min wash in PBS. Slides were scanned and analyzed using a GenePix 4300B fluorescent slide scanner (Molecular Devices, Sunnyvale, CA) with GenePix Pro 7 software at a resolution of 5 µm. The photomultiplier (PMT) gain settings were 500 for the Cy3 channel and 450 for the Cy5 channel. For each channel, the background subtracted median fluorescence of the three replicate spots per lectin was tested for outliers using the Grubbs outlier test with alpha = 0.05. The $\log_2$ values of the average fluorescence of the 3 replicate spots were median centered over the array in each channel to account for differences in labeling efficiency. The resultant datasets (arrays and lectins) were hierarchically clustered by the $\log_2$ ratios using the Pearson correlation coefficient with average linkage analysis (Cluster 3.0). Clusters were visualized with Java Treeview. Arrays were considered to be statistically similar if $P < 0.0001$. P values were obtained by employing a two tailed t test (N = number of lectins, r = Pearson correlation coefficient, Df = N-2,) using the online statistical calculator: http://faculty.vassar.edu/lowry/tabs.html#r.

Lectin blots

Microvesicles and membranes were procured previously from serum free conditioned media. Details of the cell culture conditions, isolation of microvesicles and membrane preparation protocols are described in chapter 2. Proteins (10 µg) were separated by SDS-PAGE (precast 4-20% polyacrylamide gel, Thermo Scientific) and transferred to nitrocelluse. Biotin-conjugated lectins (EY Laboratories, San Mateo, CA) were diluted in 5% bovine serum albumin (BSA) in PBST (phosphate buffered saline with 0.05% tween) to a final concentration of 1 µg/ml. Membranes were incubated with the lectins for 1 hour at room temperature. After washing in PBST, lectins were incubated

with streptavidin-HRP (Jackson ImmunoResearch, West Grove, PA) diluted 1:500 in 5% BSA/PBST. Blots were visualized using Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific) on a G:Box gel imaging system (Syngene).

Western blots

Microvesicle and cell membrane proteins (3 µg of protein) were separated by SDS-PAGE under non-reducing conditions and transferred to nitrocellulose membrane. Samples were probed with antibodies to CD63 (1:500 dilution) (RFAC4, Millipore, Billerica, MA) and CD81 (1: 500 dilution) (H-121, Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with the appropriate HRP-conjugated secondary antibody (1:10,000 dilution) (Bio-Rad, Hercules, CA). Blots were visualized using Supersignal West Pico or Supersignal West Femto Chemiluminescent Substrate (Thermo Scientific) on a G:Box gel imaging system (Syngene).

Lactose treatment of Sk-Mel-5 cells

Sk-Mel-5 cells were cultured in normal growth media (RPMI with 10% fetal bovine serum and 2 mM L-glutamine) in 10, 15 cm dishes until cells reached 80% confluency. Cells were gently rinsed twice with 5 mL serum-free media before addition of serum-free media with 100 mM D-lactose or serum-free media alone. Conditioned media was obtained 20 h post treatment and microvesicle isolation protocol was followed as previously described in chapter 2.

Transfection of Sk-Mel-5 cells with galectin-3 shRNA

The galectin-3 shRNA vector (pRFP-C-RS) was obtained from Origene Technologies and the Transit 2020 transfection reagent was from MirusBio. Sk-Mel-5 cells were cultured in 6 well dishes and transfection was done according to the manufacturer's instructions. Cells were imaged 24 h post transfection to evaluate transfection efficiency.

Chapter 5: Conclusions

The original goal of my research was to examine the glycosylation profiles of microvesicles. These cell-membrane derived extracellular vesicles can interact with other cells and are a vehicle for intercellular communication and transport. Glycosylation is often the first point of contact between a cell and its environment. Because of this, glycosylation plays a crucial role in numerous extracellular events including receptor activation, circulation half-life, homing to specific tissues and cell-cell recognition. Therefore, determining the glycomic content of microvesicles would provide important insights into their function and modes of cellular interaction. I chose to study microvesicles from multiple diverse sources so that I could compare their glycomes. This included microvesicles derived from human T-cell, colon and skin cancer as well as human breast milk from a healthy donor. Despite this diversity, a conserved glycomic signature for microvesicles was observed. Based on this data, the use of glycosylation as a marker for differentiating microvesicles from normal and healthy cells may not be possible. This has important implications for the utilization of glycans as markers in microvesicle diagnostics. On the other hand, the conserved glycome could potentially lead to glycan-based microvesicle isolation methods from biological fluids and cell culture media.

The identification of a conserved glycome defined by the enrichment and exclusion of certain glycan epitopes led us to hypothesize that glycosylation could potentially be a signal for sorting of glycoproteins and glycolipids to microvesicles. The second aim of my project was to investigate if lectin-carbohydrate interactions played a role in microvesicle-directed sorting of proteins. To this end, I identified galectin-3 and galectin-4, known glycoprotein trafficking agents, in several of the microvesicle samples.

These galectins are known to recognize polylactosamine, an epitope that was highly enriched in the microvesicles. This work therefore serves to identify galectins as candidates for microvesicle trafficking agents. Although further work is needed, initial data suggests that inhibition of galectin-3 lattices may interfere with protein incorporation into microvesicles. Future work would require a closer examination of the role of galectin-3, and other galectins, in microvesicle protein sorting.

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Vita

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