PROTEOMIC-BASED INVESTIGATION OF CELL SURFACE AND CELL SURFACE-ASSOCIATED PROTEINS OF THE HUMAN HEART

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science

Department of Physiology
University of Toronto

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2010

ABSTRACT

Plasma membrane (PM) proteins are at the interface between the cell and the external environment and are therefore the most accessible to therapeutic drugs. I utilized cationic silica beads and mass spectrometry (MS)-based proteomics to enrich for PM proteins of human cardiomyocytes, coronary smooth muscle cells, and coronary endothelial cells. The enrichment of PM proteins was confirmed and 1006 proteins were specifically filtered and enriched into a set of known and novel cardiomyocyte PM-associated proteins of which 42% had PM-associated gene ontology annotations and/or predicted transmembrane helices. Two novel candidates, namely popeye domain-containing protein 2 (POPDC2) and protein kinase C and casein kinase substrate in neurons protein 3 (PACSIN3) were selected and found to have confirmed PM localization. In conclusion, silica bead membrane extraction combined with MS-based proteomics successfully enriched for PM proteins of the human heart of which two novel candidate proteins were shown to have confirmed PM localization.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>2D-PAGE</td>
<td>Two-dimensional polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>alpha COP</td>
<td>Alpha subunit of coatamer protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATP5b</td>
<td>Mitochondrial F1 ATP synthase</td>
</tr>
<tr>
<td>CAV1</td>
<td>Caveolin-1</td>
</tr>
<tr>
<td>CC</td>
<td>In vitro and in vivo cardiomyocyte datasets combined</td>
</tr>
<tr>
<td>CS</td>
<td>Cell surface</td>
</tr>
<tr>
<td>DHPR</td>
<td>Dihydropyridine receptor</td>
</tr>
<tr>
<td>DRP-3</td>
<td>Dihydropyrimidinase-related protein 3</td>
</tr>
<tr>
<td>ECR</td>
<td>Extracellular region</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehydes-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-Protein coupled receptor</td>
</tr>
<tr>
<td>hcEC</td>
<td>Human coronary endothelial cells</td>
</tr>
<tr>
<td>hCM</td>
<td>Human cardiomyocytes</td>
</tr>
<tr>
<td>hcSMC</td>
<td>Human coronary smooth muscle cells</td>
</tr>
<tr>
<td>hfVC</td>
<td>Human fetal ventricular cells</td>
</tr>
<tr>
<td>ICAM1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>ILVBL</td>
<td>Acetolactate synthase-like protein</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>MBS</td>
<td>MES-buffered saline</td>
</tr>
<tr>
<td>MCM</td>
<td>Mouse cardiomyocyte</td>
</tr>
<tr>
<td>MD-fraction</td>
<td>Membrane depleted fraction</td>
</tr>
<tr>
<td>MES</td>
<td>2-[N-Morpholino]ethanesulfonic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MuDPIT</td>
<td>Multi-dimensional protein identification technology</td>
</tr>
<tr>
<td>MYADM</td>
<td>Myeloid-associated differentiation marker</td>
</tr>
<tr>
<td>Na/K ATPase</td>
<td>Sodium-potassium ATPase</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium-calcium exchanger</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysucinimide</td>
</tr>
<tr>
<td>NRP1</td>
<td>Neuropilin 1</td>
</tr>
<tr>
<td>PACSIN3</td>
<td>Protein kinase C and casein kinase substrate in neurons protein 3</td>
</tr>
<tr>
<td>PDI</td>
<td>Protein disulfide isomerase</td>
</tr>
<tr>
<td>PECAM1</td>
<td>Platelet endothelial cell adhesion molecule 1</td>
</tr>
<tr>
<td>P-fraction</td>
<td>Plasma membrane-enriched fraction</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PMCA</td>
<td>Plasma membrane calcium ATPase</td>
</tr>
<tr>
<td>POPDC2</td>
<td>Popeye domain-containing protein 2</td>
</tr>
<tr>
<td>PPS</td>
<td>PPS silent surfactant</td>
</tr>
<tr>
<td>pTMH</td>
<td>Predicted transmembrane helix</td>
</tr>
<tr>
<td>RYR2</td>
<td>Ryanodine receptor 2</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate- polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SMαA</td>
<td>Smooth muscle alpha actin</td>
</tr>
<tr>
<td>TX100</td>
<td>Triton X-100</td>
</tr>
<tr>
<td>UCHL1</td>
<td>Ubiquitin carboxyl-terminal esterase L1</td>
</tr>
<tr>
<td>VDAC1</td>
<td>Voltage dependent anion channel 1</td>
</tr>
</tbody>
</table>
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CHAPTER ONE: INTRODUCTION

I. THE MAMMALIAN PLASMA MEMBRANE

A. Overview of the Plasma Membrane

The plasma membrane (PM) is the initial barrier of the cell to the external environment and thus, it is an integral part of all interactions with the external environment and responsible for maintaining a unique intracellular composition. The PM is an asymmetrical bilayer composed of three different amphipathic lipids: phospholipids, glycolipids and cholesterol. The most abundant lipids are phospholipids of which there are four predominant types in mammalian plasma membranes, namely phosphatidylcholine, sphingomyelin, phosphatidylserine and phosphatidylethanolamine. The various phospholipids interact with different proteins to promote their activity. Proteins make up over 50% of the mass of the plasma membrane. The proteins and the lipids in the plasma membrane can diffuse laterally within the membrane, however many proteins and lipids can be confined to domains within the plasma membrane. Lipid domains are abundant in cholesterol, sphingolipids and membrane proteins, which are stabilized by condensed packing of sphingolipids and phospholipids. Protein domains can form by direct interaction of the functional components between proteins to develop into complexes or can be maintained by protein scaffolds. Cell junctions immobilize protein domains in a cell and facilitate cell-cell and cell-matrix interactions. Cell junctions can be classified as tight junctions, anchoring junctions or communicating junctions. Tight junctions are found between epithelial cells and prevent the leakage of molecules from one side of the sheet to another and the movement of proteins within the plasma membrane of each epithelial cell. Anchoring junctions connect the cytoskeleton of a cell to those of a neighbouring cell or the extracellular matrix. Anchoring junctions are composed of intracellular attachment proteins, which connect to the cytoskeleton, and transmembrane linker proteins, which bind the intracellular attachment proteins and transverse the plasma membrane to bind either the matrix or the linker protein of another cell. Communication junctions facilitate the movement of chemicals or electrical signals from one cell to its neighbour. The plasma
membrane of many cells is coated by a glycocalyx which is composed of carbohydrates covalently linked to proteins and lipids of the plasma membrane. 

**B. Plasma Membrane Proteins**

PM proteins assume a variety of key cellular functions such as migration, cell adhesion, cell-cell communication, signal transduction and interactions of the cell with the external environment. Proteins involved in these vital biological processes include receptors and their associated signalling proteins, transporters, channels, and linker proteins. Receptors are often transmembrane proteins with an extracellular domain that activates by changing conformation upon ligand binding to initiate a response within the cell. An important class of receptors essential in many signal transduction pathways, for example, include the G-protein coupled receptor (GPCR) family. These proteins activate when an extracellular signal binds to the receptor which transmits that signal through seven transmembrane helices. The helices are linked to a G-protein connecting the receptor to effector molecules by the activation of a Gα subunit, which interacts with the cytoplasmic face of the PM. Some receptors can be inside the cell and so an extracellular ligand has to either be small and have a high degree of hydrophobicity to cross the PM or cross via a membrane transporter. Transporters carry solutes and ions across the membrane and can be split up into adenosine triphosphate (ATP)-dependent pumps, which are involved in the active transport of ions, and carriers, which are involved in passive transport by changing conformation upon solute or ion binding. The sodium-potassium ATPase (Na/K ATPase) is an essential PM protein that maintains membrane potential and cell volume and facilitates secondary active transport of solutes. It functions by actively pumping sodium out of the cell and potassium into the cell against their respective electrochemical gradients with the hydrolysis of ATP. Carrier proteins also transport hydrophilic solutes across the PM down its electrochemical gradient or against its gradient by coupling it with the passive transport of ions. An additional method of transport for ions can occur through a transmembrane channel. Channel proteins change conformation in response to a signal to allow specific ions to passively travel across the membrane at a faster rate than carrier proteins.
Collectively, PM channels, receptors and transporters are vital for cell communication, signal transduction and cell homeostasis.

II. CELL SURFACE PROTEINS OF THE HUMAN HEART

A. Function and Dysfunction of Cardiac Cell Surface Proteins

1. Ion Channels and Transporters in Cardiac Health and Disease

Cell membrane proteins of human cardiomyocytes are vital to the spread of an action potential that excites the heart muscle\(^\text{20}\). In cardiomyocytes an electrochemical gradient is maintained across the cell membrane due to the Na/K ATPase\(^\text{21}\). During rest the cardiac cell is approximately at a -90mV resting potential as compared to the extracellular environment\(^\text{22}\). At the onset of an action potential the membrane becomes permeable to sodium ions due to the rapid opening of sodium channels giving an initial upstroke of positive current\(^\text{23}\). The cardiac sodium channel, Nav1.5, is composed of a pore forming alpha subunit and a modulatory beta subunit\(^\text{24}\). Mutations in the prominent sodium channel gene, SCN5A, which encodes the alpha subunit of Nav1.5, has been implicated in a number of cardiac diseases such as Brugada syndrome\(^\text{25}\), cardiac conduction defects\(^\text{26}\), and dilated cardiomyopathy\(^\text{27}\). For example, in Brugada syndrome a missense mutation in the SCN5A gene has been shown to cause decreased expression of the protein and as a result a significant reduction in the sodium current\(^\text{28}\). This drastic reduction in sodium current has been associated with sudden cardiac death due to ventricular fibrillation\(^\text{29}\).

The rapid depolarization is followed by a brief and partial repolarization caused by the activation of voltage-gated, inward rectifier potassium channels, which produces a transient outward potassium current\(^\text{22}\). This current can be reduced by phosphorylation of the potassium channels, and the reduction of the channel’s expression caused by chronic alpha-adrenergic stimulation and angiotensin II\(^\text{22}\). This reduction has been shown to produce faster repolarization in mammals\(^\text{22}\). The membrane potential following the
partial repolarization is maintained by an inward calcium current produced by the opening and slow inactivation of the L-type calcium channel coupled to the slow opening of outward potassium channels\textsuperscript{30}. The L-type calcium channel is composed of 5 subunits\textsuperscript{22}. Mutations in the cardiac specific subunit of the L-type calcium channel, Cav1.2, can for example cause Timothy syndrome, which is a multisystem disorder with symptomatic long QT syndrome and sudden cardiac death\textsuperscript{31}. The mutation opens a serine residue to phosphorylation which delays the closing of the calcium channel and promotes increased entry of calcium, thus prolonging cardiomyocyte excitability and preventing complete repolarization\textsuperscript{32}.

The influx of calcium ions also initiates the calcium induced calcium release phenomenon\textsuperscript{21}, which causes the opening of the ryanodine receptor that releases calcium from the sarcoplasmic reticulum, thus raising the intracellular calcium concentration\textsuperscript{21}. Calcium then binds to troponin C which subsequently causes the movement of the tropomyosin complex off of the actin binding site allowing the myosin head to bind to actin and thus initiating a cardiomyocyte contraction\textsuperscript{21}. Relaxation occurs when intracellular calcium declines causing calcium to dissociate from troponin\textsuperscript{21}. Intracellular calcium is removed from the cytoplasm by the sodium-calcium exchanger (NCX) and the plasma membrane calcium ATPase (PMCA), and is re-sequestered into the sarcoplasmic reticulum by the sarcoplasmic reticulum calcium ATPase\textsuperscript{21, 33}.

Repolarization of cardiomyocytes is mediated by the rapid delayed rectifier potassium currents that are conducted by hERG potassium channels\textsuperscript{30}. Mutations in the alpha-subunit of this channel have been shown to cause long QT syndrome, which can lead to lethal ventricular fibrillation\textsuperscript{30}. The mutations cause reduced outward potassium conductance that slows the rate of repolarization of the cardiomyocyte\textsuperscript{30}.

2. GPCR Pathways in Cardiac Health and Disease

Cardiac contraction can be regulated by the sympathetic\textsuperscript{34} and parasympathetic\textsuperscript{35} system. PM proteins are also vital to these regulatory pathways because circulating hormones bind to cell surface GPCRs that activate the protein cascades necessary to modulate contraction\textsuperscript{34, 35}. The beta-adrenergic receptors regulate the inotropic and
chronotropic functions of the heart through activation of the Gαs pathway that leads to the activation of adenylyl cyclase. Adenylyl cyclase then initiates the production of adenosine 3’,5’ monophosphate which activates protein kinase A. This kinase phosphorylates 1) the L-type Ca^{2+} channel, which increases Ca^{2+} entry into cells 2) phospholamban, which increases the rate of Ca^{2+} sequestration into the sarcoplasmic reticulum and thus accelerates cardiac relaxation and 3) troponin I and C, which reduce myofilament Ca^{2+} sensitivity. There are three main beta-adrenergic receptors, namely beta1, beta2 and beta3. The beta1 receptor is the most predominant beta receptor in the heart accounting for approximately 80% of the beta receptors in the heart. The beta2 receptor makes up approximately 20% of the beta receptors in the heart while the beta3 receptors are the least abundant. It has been shown that chronic activation of the Gαs pathway in transgenic mice progressively developed myocardial damage and cellular hypertrophy and death. Similarly, increased catecholamine stimulation during heart failure promoted the downregulation of beta1 receptors and the uncoupling of beta2 activation to adenylyl cyclase activation, which together caused diminished contractility during beta-adrenergic stimulation.

The Gαs pathway is opposed by the Gι pathway, which inhibits adenylyl cyclase and thus decreases the inotropic and chronotropic response of the cardiomyocyte. The adenosine-1 receptors, the muscarinic-2 receptors and the α2-adrenergic receptors signal via this GPCR pathway. Constant activation of this pathway by genetic overexpression of the Gι coupled receptor was shown to produce bradycardia and cardiomyopathy.

Another important GPCR pathway in the heart involves the activation of Gαq that recruits phospholipase C β which then hydrolyzes phosphatidylinositol 4, 5 biphosphate into diacylglycerol and inositol 1, 4, 5-triphosphate. The latter binds to receptors on the sarcoplasmic reticulum to release calcium. Diacylglycerol activates protein kinase C which was shown to be involved in cardiomyocyte growth and death. GPCRs that affect changes to the heart via the Gαq pathway include the angiotensin receptor, endothelin receptor and the α1-adrenergic receptors. It was found that high overexpression of this GPCR pathway in mice promoted hypertrophy, heart failure, and death.
B. Therapies of Cardiovascular Disease that Target PM Proteins

Approximately 50% of drugs target membrane proteins such as GPCRs, ion channels and transporters\textsuperscript{42}. Of these proteins, GPCRs make up the largest class of proteins targeted by drugs\textsuperscript{42} and targeted for the treatment of cardiovascular disease\textsuperscript{43}. For example, beta-adrenergic antagonists, which target the PM beta-adrenergic receptors, are widely used antiarrhythmic drugs\textsuperscript{44}. Also, angiotensin II receptor blockers are a common therapy to target heart failure\textsuperscript{45}. Channel proteins have been shown to be essential targets for antiarrhythmic drugs. Several conventional antiarrhythmic therapies include drugs that block PM sodium channels, potassium channels, and calcium channels\textsuperscript{44}.

III. PLASMA MEMBRANE PROTEOMICS OVERVIEW

PM proteins are essential to normal cardiac function and elucidating novel plasma membrane proteins in the human heart can lead to a greater understanding of cardiac cell function and disease. However, a comprehensive proteomic analysis of PM proteins of the human heart has been challenging in the past because it has been difficult to obtain a homogenous and highly enriched PM fraction and it is hard to solubilise hydrophobic proteins in aqueous solution thus making it difficult to identify via mass spectrometry (MS)\textsuperscript{11, 46}. Commonly, the analysis of plasma proteins requires the isolation, enrichment and solubilisation of PM proteins followed by the separation, identification and characterization of these proteins\textsuperscript{11}. This proteomic strategy to enrich and identify PM proteins is summarized in Figure 1.
Figure 1. Common proteomic strategies used to enrich and identify hydrophobic plasma membrane proteins. Proteins are initially isolated from the plasma membrane using one or a combination of biochemical techniques. The proteins must then be solubilised in aqueous solution using an appropriate buffer such as TritonX-100 (TX-100), CHAPS, PPS Silent Surfactant or Rapigest for example. The complex mixtures of membrane proteins must be separated by 2D-PAGE and then digested, or directly digested and separated the peptides by high performance liquid chromatography (HPLC). If the proteins are separated by 2D-PAGE, they are subjected to in-gel protein digestion. Subsequently peptide samples are analyzed by mass spectrometry.
IV. BIOCHEMICAL PURIFICATION OF PLASMA MEMBRANE PROTEINS

A. Differential Centrifugation and Density Gradient Centrifugation

There are many methods to isolate PM proteins based on the experimental problem being investigated\textsuperscript{11}. A classical method of PM isolation involved the disruption of cells and fractionation of cellular components by either differential centrifugation or density gradient centrifugation, or a combination of both\textsuperscript{48}. For example, these methods have been used to isolate PM proteins of neutrophils\textsuperscript{49}, intestinal epithelial cells\textsuperscript{50}, and human placental syncytiotrophoblast microvillus membrane and basal membrane\textsuperscript{51}.

Differential centrifugation of subcellular fractions involves a sequential centrifugation of the cell lysate homogenate in a medium at varying centrifugation speeds and times\textsuperscript{52}. The pelleting of each cellular component is based on its sedimentation coefficient which takes into account the density, shape, and volume of the particle as well as the density and viscosity of the gradient medium\textsuperscript{52}. To achieve a successful separation of subcellular contents the combination of the gravitational force and time of centrifugation that will separate each subcellular fraction must be determined\textsuperscript{52}. However the sedimentation coefficient between subcellular components is not great enough to allow the clean separation of these components and thus contributes to intracellular contamination in the final membrane pellet\textsuperscript{52}. Density gradient centrifugation allows the separation of cellular contents by allowing each subcellular component to come to rest in a section of the gradient that corresponds to its own density\textsuperscript{52}. The density gradient can either be continuous or discontinuous and spans the range of densities of the subcellular components\textsuperscript{52}. The drawbacks of the density gradient centrifugation method of PM isolation include the contamination of organelle membranes in PM fractions\textsuperscript{53}. 
B. Aqueous Two-Phase Partitioning

Aqueous two-phase partitioning takes advantage of the fact that the majority of aqueous mixtures of distinct water-soluble polymers will separate at a specific concentration called the critical concentration. Plasma membranes and its proteins separate in this system based on hydrophobicity, commonly using dextran and poly(ethylene glycol), where the plasma membranes have a higher affinity to the hydrophobic top phase. Aqueous two-phase partitioning was recently used to separate the plasma membranes of rat liver where approximately 67% of the identified proteins were classified as integral membrane proteins or membrane-associated proteins. This system was also successful in enriching plasma membranes of minute samples of the cerebellum and dorsal root ganglia of rat brains. Investigators reported that approximately 26% and 22% of identified proteins from the cerebellum and dorsal root ganglia experiments respectively were annotated as PM. However, all of these studies confirm contamination of cellular organelles and even with a combination of differential or density centrifugation as well as washing with sodium carbonate.

C. Silica Bead Plasma Membrane Isolation

The colloidal silica bead procedure was first developed in 1983 and exploits the anionic nature of plasma membranes. As depicted in Figure 2, intact harvested cells or cultured monolayers are incubated with cationic silica beads that bind to the anionic PM. The beads are cross-linked to each other and to the membrane using polyacrylic acid. The cells are lysed and centrifuged, and the crude PM pellet is separated from the membrane-depleted intracellular contents. Since the crude membrane pellet will still have many intracellular proteins it is further purified in a discontinuous nycodenz gradient. The resulting PM pellet is then subjected to a solubilisation agent to elute the PM proteins off of the beads. Chaney & Jacobson showed, using scanning electron microscopy, that cells are continuously coated with silica and significant changes in morphology are not observed, which was also later confirmed for endothelial cells cultured in a monolayer. However, cells that are prone to rupture or leakage can cause significant contamination to the PM
fraction because the presence of multivalent anions and soluble proteins can cause the silica beads to precipitate\textsuperscript{58}.

Figure 2. Silica bead membrane isolation procedure used to isolate plasma membrane proteins.

Plasma membrane fractions are isolated using cationic silica-beads that bind to the anionic plasma membrane. Cells are then incubated in polyacrylic acid to cross-link the silica beads to the membrane. The cells are lysed and centrifuged and the intracellular homogenate fraction (H) is separated from the crude membrane pellet. The pellet is then purified in a nycodenz gradient and the plasma membrane proteins (P) bound to the silica beads are then eluted using solubilising agents. In my study either 1% Triton-X-100 (TX-100), 8M Urea or 0.2% PPS Silent Surfactant (PPS) were used to solubilise and elute the cell surface associated proteins.

Many studies have used the silica bead procedure to isolate PM proteins of several cell types. Recently, silica bead membrane extraction has been used to isolate the plasma membrane and its associated proteins of rat lung endothelial cells \textit{in vitro} and \textit{in vivo}\textsuperscript{61}, cancer cell lines\textsuperscript{62} and placental cells\textsuperscript{63}. All of these experiments showed significant enrichment of PM proteins with 50% of total proteins being annotated as PM in the cancer cell investigation\textsuperscript{62} to 80% in the rat lung endothelial cell experiments\textsuperscript{61}. The isolation of plasma membranes of bovine aortic endothelial cells using silica beads, had approximately a 4- to 10-fold enrichment of the known cell-surface marker Na\textsuperscript{2+}/K\textsuperscript{+}-ATPase and a 5- to 15-
fold enrichment of the known PM marker angiotensin-converting enzyme. Furthermore, Schnitzer et al. showed that this technique could isolate endothelial caveolae, which are found on the cytoplasmic side of the membrane and are ripped off due to the shearing force applied during homogenization. Consequently, this PM isolation procedure is a comprehensive methodology used to isolate proteins from all facets of the PM.

D. Biotinylation

A more recent method to isolate PM proteins, called surface biotinylation, takes advantage of the strong affinity that the vitamin, biotin, has for avidin. This method is illustrated in Figure 3 and entails the incubation of cells with a biotin reagent that has a covalent modification, which allows it to bind to primary amines of proteins. The modified biotin molecule binds to proteins exposed at the cell surface as well as any extracellular proteins. The cells are then lysed and incubated in avidin beads where biotin and any bound proteins form a complex with avidin, whereas the remaining cellular contents flow through. The avidin-biotin-protein complex can be washed with strong detergents and salts to remove any non-specifically bound proteins. Theoretically, proteins exposed to the extracellular face of the PM can then be eluted using physical or chemical means. Proteins without an extracellular protein domain and those bound peripherally to the cytoplasmic face of the PM will not be isolated, which could be a limitation for studies aiming to complete a comprehensive analysis of plasma membrane proteins.

Many recent studies have used two main homologs of sulfo-N-hydroxysuccinimide (NHS)-biotin for cell surface labelling, namely sulfo-NHS-long chain-biotin and sulfo-NHS-SS-biotin. However, the NHS-long chain-biotin tends to interact with and become surrounded by the hydrophobic regions of proteins which inhibits the formation of the biotin-avidin complex. Also, it has been shown that the NHS-long chain-biotin can permeate biological membranes and therefore may not be the best choice for cell-surface labelling. All of these recent studies show a significant enrichment of PM proteins however many intracellular proteins were also detected such as cytoplasmic and cytoskeletal proteins. Contamination of intracellular proteins may occur due to intracellular protein leakage, permeation of biotin into the cell, and strong interactions.
between intracellular proteins and the PM\textsuperscript{66}. Yet it has been shown that proteins annotated as intracellular were experimentally also found associated with the PM\textsuperscript{69}.

### Figure 3. Isolation of PM proteins using surface biotinylation.

Cells are incubated with a modified biotin reagent, which bind to amino acids that have a primary amine group. The cells are then homogenized and the resulting lysate is incubated in neutravidin (or streptavidin) beads. The biotin, with any bound PM proteins, binds tightly to neutravidin whereas the intracellular flow-through is centrifuged out. The crude neutravidin-biotin-PM protein complex is washed with strong detergents and salts to remove any non-specifically bound intracellular contaminants. The proteins are eluted using a suitable reagent and in the case of my study 5% beta-mercaptoethanol (BME) was used to break the disulphide bond linking biotin to the protein.

### E. Glycocapture

It has been predicted that there are approximately 3094 membrane glycoproteins currently annotated in the UniProt database\textsuperscript{71}. Glycosylated proteins can be either O-linked (linked to serine or threonine residues) or N-linked (linked to asparagine residues). N-linked glycosylation is predominant in proteins destined for extracellular environments such as proteins with an extracellular domain, secreted proteins and proteins in body fluids\textsuperscript{72}. Many clinical markers and drug targets are glycoproteins such as the Her2/neu in breast cancer and alpha-fetoprotein in germ cell tumors\textsuperscript{72}. In the glycocapture procedure employed by Zhang et al., the initial step oxidized carbohydrates to convert cis-diol groups into aldehydes so that the carbohydrates could be linked to biocytin hydrazide\textsuperscript{72}. This step was followed by affinity enrichment of biocytin hydrazide-labelled peptides, enzymatic peptide release using PNGase
F, and mass spectrometry identification\(^73\). This methodology can identify PM proteins that have N-linked glycosylation sites on the extracellular surface of the cell and can separate them from the rest of the intracellular contents\(^73\). Wollscheid et al. showed that labelling T-lymphocytes with biocytin hydrazide and subsequent streptavidin fluorescent staining indicated that this molecule could label the cell surface without entering the cell\(^73\). Wollscheid’s group also showed that this glycocapture technology can identify single- and multi-transmembrane proteins\(^73\). A drawback of this procedure is that PNGase F can not release N-linked oligosaccharides containing core fucosylation and it will not remove intact O-linked sugars\(^72\). Also, a large number (1 x 10\(^8\)) of cells is required to complete one biological repeat\(^74\) and consequently cells that can only be passaged a few times and those that multiply very slowly may not be suitable for this procedure. Furthermore, this methodology only focuses on proteins that have an N-linked glycosylated extracellular domain and thus peripheral PM proteins found on the cytoplasmic side and proteins without an N-linked glycosylation will not be identified.

F. Cell-shaving

One of the major problems of identifying membrane proteins is their hydrophobic nature, which makes them difficult to solubilise in an aqueous solution that is required for mass spectrometry analysis\(^11\). The ‘cell-shaving’ methodology to isolate membrane proteins avoids resolving the entire membrane protein and only focuses on the protein domain that is exposed to the aqueous environment and is thus hydrophilic\(^75\). Isolating only the hydrophilic protein domain of a hydrophobic protein effectively avoids the loss of those hydrophobic proteins which are difficult to resolubilise in solution after they are removed from the PM\(^75\). Isolation of the hydrophilic protein domain also provides information about which domains of integral membrane proteins are exposed to the extracellular environment\(^11, 75\). The method involves exposing intact cells to the nonspecific protease, proteinase K, which cleaves the soluble domains from integral membrane proteins and other extracellular proteins\(^75\). These peptides are collected and then analysed by MS\(^75\). Challenges associated with this cell-shaving methodology include the instability cells experience when they are exposed to protease treatment\(^11\). Cell lysis can occur due to cell instability and can cause intracellular
contamination in the membrane fraction. The procedure was marginally successful in bacterial cells with cell walls, yet intracellular contamination was still present. In mammalian cells, Speers et al. used a fractionation technique to isolate the PM fraction and then used a combination of high pH and temperature with protease treatment to separate soluble protein domains from integral membrane proteins. Contamination from the fractionation technique was avoided by washing with sodium carbonate at high pH which opens up the membranes into sheets and washes away any non-specifically bound and peripheral proteins. However, this procedure only allows for the enrichment of PM proteins with an exposed surface domain and extracellular proteins. Any proteins within the lipid bilayer or on the inner surface of the bilayer will not be isolated.

V. SOLUBILIZATION AND SEPARATION OF PLASMA MEMBRANE PROTEINS

A. Solubilisation of Membrane Proteins

Many membrane proteins, especially integral membrane proteins, are highly hydrophobic. In addition, membrane proteins may still be found within the lipid bilayer following protein isolation. Membrane proteins must be delipidated and brought up into aqueous solution for their analysis by MS. However, one of the greatest issues facing the field of membrane proteomics is the solubilisation of hydrophobic membrane proteins in aqueous solution. Traditionally, chaotropes such as urea or guanidine hydrochloride or detergents such as sodium dodecyl sulphate, CHAPS or Triton-X100 (TX100) have been used to solubilise membrane proteins. Most often high concentrations of these reagents are necessary to solubilise membrane proteins that contain many transmembrane domains. The chaotropes denature proteins and, at high concentrations, make subsequent protein digestion difficult. Denaturation also promotes the exposure of the proteins’ hydrophobic amino acids to the aqueous solvent and can thus increase the occurrence of hydrophobic interactions, which causes the formation and precipitation of protein aggregates.
Detergents that have amphipathic properties are often successful in solubilising membrane proteins.\(^{81}\)

Chaotropes and detergents must be removed because they can interfere with downstream MS steps such as liquid chromatography (LC) or may introduce noise during MS analysis.\(^{11}\) To remove these solubilising agents and lipids the proteins need to be precipitated using a precipitation solvent such as, trichloroacetic acid, organic solvents such as acetone, or a combination of chloroform and methanol.\(^{11}\) Following precipitation, the precipitation solvent is removed and the proteins are resolubilized in an MS compatible solution. Any residual precipitation solvent that has not been removed will continue to cause protein precipitation thus leading to the loss of analyte.\(^{82}\) If the pellet is dried too extensively, in an attempt to remove all of the volatile precipitation solvent, then it may be impossible to resolubilise, thus causing a significant loss in protein.\(^{82}\) There is also a potential for the loss of highly hydrophobic proteins that may not resolubilise in the MS compatible solution.\(^{11}\)

Recently, MS compatible detergents have been developed, such as Rapigest (Waters), PPS Silent Surfactant (PPS; Protein Discovery) and Invitrosol™ (Invitrogen). PPS and Rapigest, for example, can be cleaved under acid conditions and removed by centrifugation. Therefore these detergents do not need to be removed which avoids the protein precipitation step that can cause the loss of proteins. Furthermore, all three detergents have shown to have greater solubilising capabilities than 2M Urea in Tris-\(\text{HCl}\).\(^{83}\) However, these detergents are very expensive compared to the MS-incompatible detergents and chaotropes mentioned above.

B. Separation of Membrane Proteins

Proteins from a membrane proteome study are complex and must be fractionated before MS analysis.\(^{46}\) A classical method to separate proteins is two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) where proteins are separated in the first dimension based on their isoelectric point and in the second dimension according to their mass.\(^{84}\) However, highly hydrophobic proteins are difficult to resolve with 2D-PAGE because their solubility is low at their isoelectric point and as a result they tend to precipitate.\(^{47}\) There have been improvements made to this method to increase the solubility and resolution of hydrophobic
proteins, such as applying better suited detergents. Alternatives to this method include replacing the anionic sodium dodecyl sulfate detergent with the cationic benzyldimethyl-n-hexadecylammonium chloride. The use of a cationic detergent has shown to increase membrane protein resolution possibly due to the fact that membrane proteins have an alkaline isoelectric point and therefore a cationic detergent will better solubilise these proteins than anionic detergents. After a protein is resolved on a gel it is then subjected to an in-gel digestion and the resulting peptides are analysed by MS. Another in-gel methodology that has shown to improve recovery of hydrophobic proteins is 2D-PAGE of peptides from a membrane protein sample that has been digested before separation. A disadvantage of this method, as with other in-gel methods, is the potential loss of analytes following sample extraction from gels.

The most common method of separation involves the digestion of proteins, usually by trypsin, and separating the resulting peptides by high performance LC. This method of separation has been successful in many membrane protein experiments such as the glyco-capture of human T-cell and mouse myoblast PM proteins, surface biotinylation of human umbilical vein endothelial cells and human embryonic kidney cells, and cell-surface shaving of Staphylococcus aureus PMs. Recently, two dimensional LC combined with mass spectrometry, called multidimensional protein identification technology (MudPIT), has greatly increased the resolution of peptides. It is based on the separation of peptides by strong cation exchange chromatography followed by reversed-phase chromatography, usually coupled directly with tandem MS. This methodology has worked well for rat brain membranes from a cell-shaving isolation and in vivo rat lung microvascular endothelial cell PMs from a silica bead membrane extraction.
V. STATEMENT OF INTENT

Plasma membrane proteins are essential to cardiomyocyte function and are major therapeutic targets of cardiovascular disease. The aim of this study is to isolate cell-surface associated proteins that are enriched in the human cardiomyocyte and identify novel plasma membrane proteins that may be essential to cardiac function. The overall objective of this study is to utilize comprehensive biochemical fractionation techniques, called silica bead membrane extraction and surface biotinylation, combined with liquid chromatography tandem mass spectrometry to enrich and identify the plasma membrane proteins from the major cell types of the human heart.

Specifically, I want to:
2. Enrich for cardiomyocyte cell-surface associated proteins that may be vital to cellular function.
CHAPTER TWO: MATERIALS AND METHODS

I. CELL CULTURE

A. Primary Cells

Cryopreserved primary human cardiomyocytes (hCM; cat.-no.: C-12810), human coronary smooth muscle cells (hcSMC; cat.-no.:C-12511) and human coronary endothelial cells (hcEC; cat.-no.: C-12221) were acquired commercially from PromoCell (Heidelberg, Germany). The cells were quickly thawed in a 37°C bath and cultured on 100mm plastic plates with corresponding growth media supplied by PromoCell in a 37°C, 5% carbon dioxide incubator. The cells were passaged at 85-95% confluence.

B. In Vivo Cells

Since cells in culture contain different properties than \textit{in vivo} cells, experiments were performed using human left ventricular cells (hfVC) isolated from 22 week-old fetuses in collaboration with Dr. Robert Hamilton (The Hospital for Sick Children). Whole left ventricles were cut into pieces of approximately 3mm in diameter and gently rocked overnight at room temperature in 1% collagenase diluted in Hank’s solution (136mM NaCl, 4.16 mM NaHCO$_3$, 5.36mM KCl, 0.34mM NaH$_2$PO$_4$, 0.44mM KH$_2$PO$_4$, 5.55mM Dextrose, 5mM Hepes). The tissue was removed from the 1% collagenase solution and placed in a digesting solution (Hank’s solution, 0.1mM EGTA, 1% BSA, 10mM Taurine, 5mM BDM and 1% collagenase) and digested for 20 minutes at 37°C. The dissociated cells were resuspended in digesting solution and centrifuged at 1000g for 5 minutes. The resulting pellet, containing dissociated cardiomyocytes, was then resuspended in appropriate buffer in preparation for membrane extraction experiments and allowed to settle for 10 minutes. The supernatant, which contained cellular debris from lysed cells and red blood cells, was removed. The ventricular cell pellet was gently resuspended by slowly inverting the tube.
II. PLASMA MEMBRANE ISOLATION

A. Cationic-Silica Bead Membrane Extraction

The cationic silica bead membrane extraction procedure established by Jacobson et al.\textsuperscript{58} was modified and applied to cells in culture. As depicted in Figure 2, primary cells, cultured in a dish, were initially washed three times with 2-[N-Morpholino]ethanesulfonic acid (MES)-buffered saline (MBS) (25 mM MES, pH 6.5, and 150 mM NaCl). The cells were then washed with a 1% cationic silica bead solution dissolved in MBS. The beads bound to the PM of intact cells\textsuperscript{58} and these beads were subsequently cross-linked to each other and the cell-surface using 0.1% polyacrylic acid dissolved in MES-buffered saline. The cells were isolated in a lysis buffer of sucrose/HEPES (250mM sucrose, 25mM HEPES, 20mM KCl, pH7.4) with 1x protease inhibitor and centrifuged at 1000g for 5 minutes. The supernatant, which contained majority of the intracellular proteins, was removed and labelled as the membrane-depleted (MD) fraction. The crude membrane pellet, which contains the high density PM and some of the remaining dispersed intracellular proteins, was re-suspended in the lysis buffer. To enrich the PM further, the crude membrane pellet was placed on top of a discontinuous nycodenz gradient (27.5-40%) and spun at 32,000 rpm at 4°C. The high density PM travelled to the bottom of the gradient leaving the intracellular contents in the supernatant. The enriched plasma membrane (P) was then eluted from the beads by redissolving the P pellet in either two different elution solutions, either 200µL of 1% TX-100 buffer (400 mM NaCl, 25 mM HEPES pH 7.4, 1% TX-100) or 200µL of 8M Urea. Three biological replicates were used for each membrane extraction at approximately 95% confluency (two 100mm plates per biological repeat).

Fetal hfVCs in solution were washed in MBS and centrifuged at 2500rpm for 5 minutes. The pellet was then re-suspended in a 1% silica bead solution and gently rocked for 10 minutes. Centrifugation was repeated and the resulting supernatant, containing the excess silica, was removed. The pellet was dissolved in a 0.1% polyacrylic acid solution. After gentle rocking for 10 minutes the solution was centrifuged and the cells were lysed by sonocation. Following centrifugation at 14000rpm for 20min, the resulting supernatant, containing the MD-fraction was removed and the membranous pellet was spun at 32000rpm
for 1 hour in a discontinuous nycodenz gradient (27.5-40%). The membrane-enriched pellet was then dissolved in 1% TX-100 buffer or 0.2% PPS-silent surfactant (Protein Discovery, cat.no. 21011) to elute the PM proteins.

B. Biotinylation

A previously established biotinylation procedure was modified and used to isolate cell surface proteins and obtain a more comprehensive PM protein profile. To biotinylate the exposed cell membrane proteins of cultured primary cells, 10mL of a 150 µM solution of Sulfo-NHS-SS-Biotin (Thermo Scientific; cat.no. 21331) was added to cells. This reaction was terminated by adding 10mL of 150mM Tris-HCl pH 7.4. The cells were then harvested by adding 10mL of washing buffer (150µM glutathione dissolved in PBS) to each plate with subsequent scraping. The cell solution was centrifuged at 1000g for 5 minutes and the pellet was washed with 10mL of washing buffer. The cells were pelleted at 1000g for 5 minutes and then lysed by adding 1mL of lysis buffer (2% NP-40, 2% sodium dodecyl sulfate, 100µM oxidised glutathione, 1x protease inhibitor) with a 30 minute incubation on ice. The solution was vortexed and the lysate was added to a 500mL slurry of neutravidin beads (Thermo Scientific; cat.no. 21011) and rotated for 2 hours and then another set of 500uL of beads overnight. The lysate solution in neutravidin was centrifuged at 1000g for 5 minutes and the supernatant was removed as the membrane-depleted (MD)-fraction. The beads were washed twice with buffer A (1% NP-40, 0.1% sodium dodecyl sulfate, 20mM oxidised glutathione in 1x PBS), twice with buffer B (2M NaCl, 1% NP-40, 20mM oxidised glutathione in 1x PBS), and twice with buffer C (50mM Tris-HCl pH 8.0) to remove any non-specifically bound proteins. The membrane-enriched (P)-fraction was then eluted by rotating the beads in 200uL of 5% beta-mercaptoethanol for 30 minutes at 30°C.
III. IMMUNOBLOT AND IMMUNOSTAINING ANALYSIS

A. Immunoblot Detection

Protein concentrations were elucidated for each protein fraction from each membrane extraction experiment. Approximately 10µg of protein from each protein fraction were resolved on a 10% sodium dodecyl sulfate -polyacrylamide gel (water, 37.5:1 Acrylamide/Bis Mix (BIO-RAD), 1.5 M Tris (pH 8.8), 10% sodium dodecyl sulfate (EMD), 10% Ammonium Persulfate (VWR International), TEMED (EMD)) by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Proteins were then transferred from the polyacrylamide gel to a nitrocellulose membrane. The membrane was blocked in 1x PBS with 0.2% Tween 20 (Sigma) (PBS-T) and 5% milk for 30 minutes with shaking at room temperature. This step was followed by incubating the membrane with primary antibody diluted in 5% milk-PBS-T solution overnight at 4°C on a shaker. Three 15 minutes washes with PBS-T were performed the following day, and the membrane was incubated with HRP-conjugated secondary antibody diluted in 5% milk-PBS-T solution for 1 hour at room temperature with shaking. Subsequently three 15 minutes washes with PBS-T were performed. The blots were treated with SuperSignal West Pico Chemiluminescent Substrates (Pierce) for 5 minutes and then either imaged using Fluoro-S™ Multi Imager (Bio Rad) or exposed to film in a dark room setting, which was subsequently developed. All blots were probed using commercially available antibodies: mouse monoclonal biotin (Jackson Laboratories; 1:500), mouse monoclonal sodium-potassium ATPase (α6F, Developmental Studies Hybridoma Bank, 1:500), rabbit polyclonal ubiquitin carboxyl-terminal esterase L1 (UCHL1; U5383, Sigma, 1:2000), mouse monoclonal glyceraldehydes-3-phosphate dehydrogenase (GAPDH; sc-47724, Santa Cruz; 1:500), mouse monoclonal transportin-1 (ab10303, Abcam; 1:1000), mouse monoclonal DHPR alpha-2 (MA3-921, Affinity Bioreagents; 1:1000, mouse monoclonal platelet endothelial cell adhesion molecule (PECAM1, BBA7; R&D Systems, 1:500 dilution), mouse monoclonal PMCA (generous gift from Dr. Mansoor Husain; 1:500 dilution), rabbit polyclonal protein disulfide isomerase (PDI, 539229, Calbiochem, 1:2000 dilution), rapid polyclonal estrogen receptor beta
(ab3576-100, Abcam, dilution 1:1000) and rabbit polyclonal alpha COP (PA1-067, Affinity Bioreagents, 1:1000).

B. Immunofluorescent Analysis

Cells were cultured on glass slides coated with gelatin (Sigma-Aldrich) in a 6-well culture plate. Cells were fixed by incubating the slides for 30 minutes in 1mL ice-cold 1x PBS followed by a 30 minute incubation in 2% paraformaldehyde (made in 1xPBS, pH 7.4), both at 4°C. The slides were washed with 1mL of fresh permeabilization buffer (0.2% Tween-20, 0.5% Triton X-100 in 1x PBS) at 4°C for 15 minutes each. The washed cells were then incubated in 1 mL of blocking buffer (5% FBS, 0.2% Tween-20, 0.5% Triton X-100 in 1x PBS) for 30 minutes at room temperature and then labelled with primary antibody diluted in blocking buffer overnight at 4°C. The following day the slides were washed in 1mL of permeabilization buffer 3 times for 15 minutes each and then incubated with fluorescent secondary antibody diluted in blocking buffer in the dark for 1 hour at room temperature. Subsequently, three 15-minute washes were performed with 1mL of 1x PBS in the dark at room temperature, before mounting in Fluoromount™ medium (Sigma). Images were collected by using a Leica DM IRBE inverted microscope equipped with a Leica TCS SP laser scanning confocal system. Primary antibodies used for immunofluorescent analysis were obtained from collaborators or commercially: mouse monoclonal α-actinin (Hybridoma bank, α6F; 1:500), rabbit polyclonal smooth muscle specific α-actin (gift from Dr. Gordon Keller; 1:500), mouse monoclonal PECAM-1 (R&D Systems, BBA7; 1:500), mouse monoclonal Biotin (Jackson Laboratories, 200-002-211; 1:500), rabbit polyclonal Biotin (Abcam, ab53494-1; 1:500), mouse monoclonal GAPDH (Santa Cruz, sc-47724; 1:500), rabbit polyclonal dihydropyrimidinase-related protein 3 (DRP-3; Chemicon International, AB5454; 1:5000). Secondary antibodies used for immunofluorescent analysis were obtained commercially: Alexa 488 1:500 and Alexa 633 anti-mouse (Invitrogen) secondary antibodies 1:200, and Alexa 488 1:500 and Alexa 633 anti-rabbit (Invitrogen) secondary antibodies 1:200.
IV. IDENTIFICATION OF PLASMA MEMBRANE PROTEINS

A. Sample Preparation for Mass Spectrometry Analysis

1. Trypsin Digestion of Triton- X 100 and Urea Eluted Samples
   Equal concentrations of the homogenate and membrane protein fractions were precipitated in 10% trichloroacetic acid in 5 times the sample volume of 100% acetone, re-solubilized in 8M Urea and reduced with 2mM dithiothreitol and alkylated with 8mM iodoacetamide. The sample was then diluted with a 100mM Tris-HCl pH8.5 to reduce the concentration of 8M Urea to 2M. Calcium chloride was added to the buffer to a final concentration of 1.8mM to facilitate trypsin digestion. Following alkylation the samples were digested with trypsin.

2. Trypsin Digestion of PPS Eluted Samples
   PPS solubilised samples were reduced with 5mM dithiothreitol and incubated at 50°C for 30 minutes. The samples were then cooled to room temperature and alkylated with 15mM iodoacetamide in the dark at room temperature for 30 minutes. The samples were then trypsinized overnight at 37°C. The silica beads were removed by centrifugation at 8000rpm for 5 minutes and the PPS was cleaved with hydrochloric acid to a final concentration of 200mM for 45 minutes at 37°C. The samples were spun at 14000rpm for 10 minutes at 4°C and the resulting supernatant was removed for further purification by solid phase extraction.

3. Solid Phase Extraction
   The peptide samples were then purified via solid phase extraction in which the hydrophobic solid phase retained the peptides while the contaminating polar solutes and salts were washed out in the liquid phase. The column was then washed with a hydrophilic buffer (0.1% trifluoroacetic acid) to further remove remaining polar solutes. The peptides were eluted with a concentrated hydrophobic volatile buffer
(70% acetonitrile/0.1% trifluoroacetic acid). The sample was then speed-vacuumed effectively removing the volatile solvent and leaving behind the peptides that were subsequently stored in a hydrophilic buffer (0.1% formic acid/water).

B. Protein Analysis and Identification

Triplicate samples were analyzed, according to the strategy depicted in Figure 4, by two-dimensional liquid chromatography tandem mass-spectrometry runs using an LTQ Orbitrap mass spectrometer for the in vitro samples and an LTQ linear ion trap mass spectrometer for the in vivo samples by Dr. Thomas Kislinger. Samples were initially loaded onto separate microcapillary fused silica columns containing strong cation exchange resin and reverse-phase resin. Peptides were eluted from the columns by way of a 9-step x 120min salt/water acetonitrile gradient for samples run on LTQ Orbitrap and an 8-step x 120min salt/water acetonitrile gradient for samples run on LTQ linear ion trap. The resulting spectra were searched using the X!Tandem\textsuperscript{91} algorithm against the human IPI (International Protein Index; \url{http://www.ebi.ac.uk/IPI}) protein sequence database (version 3.54). A rigorous peptide quality control strategy was applied to effectively minimize false positive identifications, as recently described\textsuperscript{92, 93}. The value of total reverse spectra to total forward spectra was set to 0.5%. Furthermore, only proteins identified with two unique peptides per analyzed fraction were accepted into the final set of proteins.
Figure 4. Schematic diagram of the applied work-flow for protein identification.  
Cells were incubated with cationic silica beads and the membrane was isolated from the cell lysate. Three biological repeats of the membrane-enriched fractions and the membrane-depleted fraction were analyzed by multidimensional protein identification technology (MudPIT)-based proteomics. The resulting peptides were then searched against a human protein database using the X!Tandem algorithm and proteins were accepted into the dataset if they had a false discovery rate of 0.5% and had 2 or more unique peptides per fraction.

C. Data normalization and filtering

Data from each silica bead extraction of each cell type was normalized and filtered to obtain a set of proteins that were enriched in membrane-enriched fraction and designated the CS-enriched dataset. Data was normalized similar to the scheme found in Sodek et al.\textsuperscript{93}. In short, spectral counts for each protein in each fraction were normalized by dividing the spectral count by the sum of all the spectral counts for that fraction. This value was then multiplied by the global average of all spectral counts. Data was filtered, as depicted in Figure 5, to obtain a membrane-enriched dataset. Proteins that were found in both the membrane-depleted and membrane-enriched fractions were accepted as PM enriched if
found in two or more MS runs and with a 2-fold increase in peptide spectra found in the membrane fraction. Proteins found only in the membrane fraction were accepted if found in two or more MS runs and with ≥ 5 spectral counts.

![Schematic diagram of data filtering strategy to obtain a cell surface-enriched dataset.](image)

**Figure 5.** Schematic diagram of data filtering strategy to obtain a cell surface-enriched dataset.

All proteins accepted had to be found in 2 or more mass spectrometer runs. Proteins found in both the membrane-depleted (MD) and membrane-enriched (TX100 or Urea/PPS) fractions were accepted if they had 2-fold or more peptide spectra in the membrane fraction than the MD-fraction. Proteins found only in the membrane-enriched fraction were accepted if it had 5 or more spectra.

V. PROTEOMIC DATA ANALYSIS

A. Hierarchical Clustering

The set of proteins acquired for the membrane-enriched (P)-fractions were compared to the membrane-depleted (H)-fraction using a hierarchical clustering analysis. Hierarchical clustering of data was performed by the program Cluster 3.0 available online ([http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm](http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm)). A student’s T-test was used to calculate the significance between the TX100 and urea, and TX100 and PPS predicted transmembrane helices and proteins.
B. Heat Map Generation

Clustered data was visually represented in a heat-map using the open source program Java TreeView available online (http://jtreeview.sourceforge.net/).

C. Subtractive Proteomic Comparison

Different subsets of data were compared in a Venn diagram that depicts the proteins in those subsets that are similar and those that are unique.

D. Bioinformatics

Proteins were analysed using the TMHMM 2.0 online program (http://www.cbs.dtu.dk/services/TMHMM/TMHMM2.0b.guide.php) to predict the number of predicted transmembrane helices a protein possesses and the number of proteins predicted to be transmembrane proteins based on a mathematical model\(^4\). A Gene Ontology (GO)-term analysis was applied to predict which proteins have a previously annotated subcellular localization of ‘membrane’, ‘plasma membrane’, ‘organelle membrane’, ‘cell-surface’, and ‘extracellular surface’ as well as ‘mitochondria’, ‘nucleus’, and ‘vesicle’ and what the biological processes each protein may potentially be involved in. The GO database consists of a controlled vocabulary to describe the cellular component, molecular function or biological process a gene may be involved in (http://www.geneontology.org/\(^5\)). Annotations in the database are attributed to a source and are inferred from experimental evidence, a computational analysis, another database or a judgement made by a curator. ArrayTrack is the open source program that was used to link genes to their gene ontology (http://www.fda.gov/ScienceResearch/BioinformaticsTools/Arraytrack/default.htm).

Proteins were also linked to their biological process gene ontology using another open source program called PANTHER (http://www.pantherdb.org/). PANTHER classifies genes based on published experimental evidence and predicts classifications based on evolutionary relationships.
E. Integrative Data Mining for Novel Protein Candidates

1. Potentially Vital Cardiomyocyte and Membrane Enriched Proteins

The CS-enriched data was further mined as shown in Figure 19 to obtain a set of candidate proteins that may be vital cardiomyocyte and cell surface associated proteins. All the proteins present in the CS-enriched dataset of the in vitro human hCMs and the in vivo human hfVCs were accepted so to enrich for proteins found in human cardiomyocytes (Figure 19, Step 1). The mouse cardiomyocyte proteome was then compared to the human data and any proteins that were not also found in the mouse study were removed (Figure 19, Step 2). To focus on cell surface associated proteins, proteins were removed if they did not have a GO-term of plasma membrane, cell surface, extracellular surface or a predicted transmembrane domain (Figure 19, Step 3). The data obtained from the coronary endothelial and smooth muscle dataset was then used to filter out proteins that were enriched in these subsets. An hcEC enrichment factor ratio was calculated by comparing the total spectra for each protein isolated from the hcEC CS-enriched data to 1) the average spectral count of the same protein found in the combined hfVC and hCM CS-enriched data if found in both datasets or 2) the CS-enriched spectral count of the hCM or hfVC if found in only one of the datasets. An hcSMC enrichment factor was elucidated in the same manner, where a ratio comparing the total spectral count found in the hcSMC CS-enriched fractions to the average spectral count of the hfVC and hCM CS-enriched fractions if found in both cardiomyocyte datasets or to the CS-enriched spectral count of either the hfVCs or the hCMs was calculated. Any proteins that had an hcEC enrichment factor above 1 were removed (Figure 19, Step 4) and any proteins with an hcSMC enrichment factor above 3 were removed (Figure 19, Step 5). This mining strategy allowed the removal of proteins that were not enriched in cardiac muscle cells, however by loosening the filtering for hcSMC-enriched proteins, proteins that may be important to contractile cells, in general, were still included. Proteins were then filtered based on the total spectral count of the hfVC and hCM membrane enriched fractions. Any proteins with a combined hfVC and hCM CS-enriched spectral count below 5 were removed (Figure 19, Step 6).
2. **Candidate Selection Based on Bioinformatics and Literature**

Candidates were chosen for further PM validation based on their degree of novelty, enrichment in cardiac tissue and potential as cell surface associated proteins as diagrammed in Figure 20. Therefore any proteins that had any intracellular GO annotations such as cytoskeleton, mitochondrion, endoplasmic reticulum and/or nucleus were removed (Figure 20, Step 1). Proteins that had cell surface annotations as well as an intracellular annotation were also removed. The open source BioGPS program\textsuperscript{96} (http://biogps.gnf.org) that provides gene annotations based on available databases was used to describe the human mRNA expression levels of the proteins in 91 tissues, organs and cell lines\textsuperscript{97}. Any proteins that were found to be enriched (had a value that was 3 times greater than the median expression value) in heart or cardiomyocyte from this MicroArray database was accepted into the final list (Figure 20, Step 2). An extensive literature search was performed to find if the remaining proteins were extensively studied in heart tissue in the past. If a PubMed (http://www.ncbi.nlm.nih.gov/pubmed) search of “heart’ AND ‘the protein name’” resulted in more than 10 peer reviewed journal articles the protein was not considered novel to cardiac research and thus removed from the final candidate set (Figure 20, Step 3). Finally, to increase the specificity for proteins enriched in cardiac tissue, proteins were removed from the final candidate list if they were found enriched (3 times greater than the median expression value in the MicroArray database) in 10 or more non-cardiac tissues or cells (Figure 20, Step 4).
VI. TAGGING OF CANDIDATE cDNA AND TRANSFECTION INTO HUMAN EMBRYONIC KIDNEY CELLS

A. Amplification of ORFeome Clones

The cDNA constructs for candidate proteins and controls were acquired from Open Biosystems’ Human ORFeome Collection, version 1. All cDNAs were provided in the pDONR223 Entry Vector. For each clone of interest, an inoculum was streaked onto LB agar plates with 50 µg/mL of spectinomycin and incubated overnight at 37°C. Individual colonies were grown in 2 mL of sterile 2x YT media with 50 µg/mL of spectinomycin. The amplified clones were purified using the QIAGEN Miniprep protocol and kit.

B. Gateway Cloning of cDNAs into Tagged Destination Vector

The cDNA insert from the pDONR223 entry vector was transferred to the V5 epitope and 6x His (V5/6His) tag encoding pEF-DEST51 destination vector using the LR Clonase reaction (Invitrogen). To perform the LR Clonase reaction 1 µL of pEF-DEST51 destination vector (150ng/µL), 5 µL of entry clone (20ng/µL) and 3 µL of TE buffer (pH 8.0). The LR Clonase™ II enzyme mix was thawed on ice and briefly vortexed. 2 µL was then added to the mixture and it was incubated at 25°C for 1 hour. To terminate the reaction, 1 µL of Proteinase K solution was added to the mixture and the samples were then incubated at 37°C for 10 minutes.

C. Amplification and Purification of V5/6xHis Tagged cDNA Constructs

The cloned DNA was transformed into DHF-α cells and plated on LB agar ampicillin plates overnight. The following day, 2 mL of sterile 2x YT bacterial growth media with
ampicillin was inoculated with cells from individual colonies overnight. To confirm appropriate swapping of cDNA in pEF-DEST51 vector, 2 mL of sterile 2x YT with chloramphenicol resistance was inoculated from identical colonies. DNA was purified by minipreparations from culture sets which displayed growth in ampicillin but not in chloramphenicol. Purified DNA was sequenced by ACGT Corporation using the T7 Forward Primers.

D. Culturing of Human Embryonic Kidney Cells

HEK-293 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) H21 (Tissue Culture Media Facility at University Health Network, Toronto) in a 37°C, 5% CO2, humidified incubator. The DMEM H21 media was supplemented with 10% fetal bovine serum (Gibco), 1x MEM Non-Essential Amino Acids Solution (Gibco), and 2.5µg/mL amphotericin-β (Sigma-Aldrich). Stock cultures were maintained in 75 cm² cell culture flasks (BD Falcon), in 13 mL of media. Confluent (80-100%) flasks of HEK-293 cells were plated into ten 100mm plates at a dilution of 1 into 5 to ensure 50-70% confluency of cells the next day for transfection. For immunofluorescent experiments, confluent flasks of cells were plated at a dilution of 1 in 20 on glass coverslips coated with gelatin in 6-well plates.

E. Transfection of Tagged cDNA Constructs

Transfection of cells was performed the next day using the calcium phosphate transfection method. For each plate of HEK-293 cells, at 50-70% confluency, a 1 mL solution was prepared with 61 µL of 2M CaCl₂, 10 µg of DNA, and 430 µL of sterilized water. The solution was lightly mixed before being added drop wise to 500 µL of 2x HEPES buffer (274 mM NaCl, 1.4 mM Na₂HPO₄·7H₂O, 54 mM HEPES, pH 7.0) and let stand 20 minutes at room temperature. For each well of HEK-293 cells in a 6-well plate, a 336uL solution was prepared with 20.3 µL of 2M CaCl₂, 5 µg of DNA, and 143 µL of sterilized water. The solution was lightly mixed before being added drop wise to 167 µL of 2x HEPES buffer and let stand 20 minutes at room temperature. The final solution was then
added drop wise to each plate or well. The cells received fresh media 18-24 hours later. The transfection procedure was repeated 24 hours after media change.

F. Harvesting of Cells and Sucrose Gradient Fractionation of Lysate

Three plates of HEK-293 cells were harvested and the cell lysate was layered on top of a 20-60% gradient as previously described by Sharma et al. The cells were suspended in a low ionic strength lysis buffer (10mM Tris-HCl pH 7.5 and 0.5mM MgCl\(_2\)) and lysed with 40 strokes in a dounce homogeniser on ice. An equal volume of buffer A (0.5 M sucrose, 10mM Tris-HCl pH 7.5, 40 µM calcium chloride, and 300mM KCl, 1mM PMSF, and 20µg/mL aprotinin) was added and the cells were further homogenized with 20 strokes. The sample was centrifuged for 15 minutes at 6000rpm and the supernatant was collected and layered on top of a 20-60% linear sucrose gradient (sucrose, 10mM Tris-HCl pH 7.6, 10mM EDTA and 1x protease inhibitors). Samples were centrifuged at 32000 rpm for 20 hours in a SW40Ti swinging bucket rotor. Thirteen 1mL fractions were collected from the bottom of the tube. Three biological repeats were completed for each candidate and were analyzed by SDS-PAGE gel electrophoresis followed by immunoblotting. The density/mm\(^2\) was measured for each immunoblot assay and scaled to a percentage of the maximum intensity. The mean density and standard error were calculated for each fraction from the three biological repeats, and plotted on a bar graph.
CHAPTER THREE: RESULTS

I. CHARACTERIZATION OF CELL-TYPES OF INTEREST

A. Immunofluorescent Staining and Cell Morphology

Primary human cardiomyocytes (hCM), primary coronary smooth muscle cells (hcSMC) and primary coronary endothelial cells (hcEC) were obtained from PromoCell (Germany) and cultured. In vivo fetal human left ventricular cardiomyocytes (hfVC) were obtained in collaboration with Dr. Robert Hamilton (The Hospital for Sick Children). Each cell-type was stained with a cell specific antibody and imaged using confocal microscopy (Figure 6). Approximately 85% of the in vitro hCMs (Figure 6A) and 95% of the in vivo hfVCs (Figure 6B) showed positive staining for α-actinin. However, the hCMs did not have prominent sarcomeric striations as seen in the hfVCs. The cultured cardiomyocytes had a more spindle-like appearance as compared to the ventricular cells that have a brick-shaped morphology. The endothelial specific membrane marker platelet endothelial cell adhesion molecule-1 (PECAM1) stained the PM of approximately 90% of the pebble-shaped coronary endothelial cells, as illustrated in Figure 6C. Approximately 85% of the spindle-shaped coronary smooth muscle cells were stained for smooth muscle specific alpha actin (SMαA) and as shown in Figure 6D, there were strong SMαA striations present in the cells.
Figure 6. Immunofluorescent staining of human heart cells with cell specific antibodies.

(A) The *in vitro* human cardiomyocytes (hCM) were stained positive for α-actinin, however intact sarcomeres were not present. (B) The *in vivo* human fetal ventricular cells (hfVC) were also stained with α-actinin which displayed a striated pattern indicating the presence of sarcomeres. (C) The coronary smooth muscle cells were stained positive for smooth muscle specific α-actin (SMαA). (D) The endothelial cells (hcEC) were stained for platelet endothelial cell adhesion molecule-1 (PECAM-1) which localized to the cell surface.
II. ISOLATION OF PLASMA MEMBRANE PROTEINS

A. Plasma Membrane Biotinylation

Cell surface proteins of all four cell types were also isolated using a previously described biotinylation procedure to focus on proteins with an extracellular domain. Cells were biotinylated, lysed and washed on neutravidin beads and cell surface proteins were eluted off of the beads using 5% beta-mercaptoethanol, as depicted in Figure 3. To validate that the biotin bound only to the plasma membrane and did not enter the cell upon biotinylation, each cell type in culture was biotinylated and then probed with an anti-biotin antibody (Figure 7, left panel). The cells were co-labelled with a cytoplasmic protein. The in vitro cardiomyocytes and coronary smooth muscle were co-labelled with the cytosolic protein dihydropyrimidinase-related protein 3 (DRP-3) and the coronary endothelial cells were co-labelled with GAPDH. Control cells were not biotinylated and co-stained with biotin and DRP-3 (hCMs and hSCMs) or GAPDH (hcECs) (Figure 7, right panel). Biotinylated cells showed prominent biotin staining on the cell surface of all three cell types. However, the numerous cell projections made it difficult to visualize individual cells with distinct biotin staining surrounding the cell. The insets at the top left more clearly showed green biotin staining on the surface of the cell with cytoplasmic red staining on the inside of the cell (Figure 7, left panel). Biotin staining was completely absent in the control cells with prominent intracellular staining (Figure 7, right panel).

B. Plasma Membrane Protein Enrichment via Biotinylation

To validate that PM proteins were isolated, a western blot analysis was carried out and the MD-fraction and the P-fraction were probed for the known PM protein, Na/K ATPase, and the cytoplasmic protein, GAPDH. As shown in Figure 8A, there is a strong GAPDH and Na/K ATPase signal in the MD-fraction, which was absent in the P-fraction. The lack of a GAPDH signal in the P-fraction could indicate that it was depleted by the biotinylation procedure. However, since the Na/K ATPase signal was present in the MD-fraction and absent in the P-fraction it indicated that the biotinylation procedure did not isolate PM proteins.
C. Assessment of Neutravidin Saturation

Since the plasma membrane protein, the Na/K ATPase, was found in the cytosolic flow-through fraction the same fractions were then probed for biotin to assess whether biotin did not bind to the neutravidin beads. Figure 8B (left) illustrates that a significant amount of biotin was found in the H-fraction. The presence of biotin in the flow-through may have been due to super-saturation of the neutravidin beads with biotin and so the experiment was performed again with the inclusion of double the amount of beads with an overnight incubation of the cell lysate in the neutravidin slurry. Increasing the volume of neutravidin beads used caused a substantial decrease in the amount of biotin coming through with the MD-fraction (Figure 8B, right) indicating that most of the biotin was found on the beads. These new fractions and the beads were then probed for GAPDH and Na/K ATPase (Figure 8C). A strong signal of GAPDH was evident in the MD-fraction and on the beads but was depleted in the P-fraction. The MD-fraction also had a slight Na/K ATPase signal with a similar signal found on the beads, yet this signal was completely absent in the P-fraction.
Figure 7. Immunofluorescent validation of biotinylated plasma membranes.

Primary human cardiomyocytes (hCM), coronary smooth muscle cells (hcSMC) and coronary endothelial cells (hcEC) were biotinylated and co-stained with biotin (green) and DRP-3 (red) for the hCMs and hcSMCs, and biotin (green) and GAPDH (red) for the hcECs (left panel). Intracellular DRP-3 (hCM and hcSMC) and GAPDH (hcEC) staining was present with prominent biotin staining on the cell surface. This cell surface staining pattern can be more clearly visualized by the zoomed-in insets at the top left corner of the biotinylated images. Control cells were not biotinylated and then stained for biotin and DRP-3 in the hCMs and hcSMCs, and GAPDH in hcECs (right panel). Control cells had intracellular staining but PM biotin stain was absent.
Figure 8. Immunoblot analysis of biotinylation procedure.
(A) The intracellular homogenate fraction (H), and the membrane enriched pellet fraction (P) were probed for a known cytosolic (GAPDH) and plasma membrane (Na/K ATPase) protein. Plasma membrane protein enrichment was absent in the membrane enriched fraction. (B) The same fractions were probed for biotin and a significant amount of biotin was found in the cytosolic fraction. However, the amount of biotin in the cytosolic fraction decreased following a 2-fold increase in the amount of neutravidin used. (C) Following the 2-fold increase in the amount of biotin used the same fractions probed for GAPDH and the Na/K ATPase which showed that an enrichment of membrane proteins was still absent. (B1=beads wash 1, B2=beads wash 2).

D. Elution of Proteins Bound to the Biotin-Neutravidin Complex

A silver stain analysis of the beta-mercaptoethanol elution was done to investigate whether proteins were eluting off of the beads. As illustrated in Figure 9, all the proteins seemed to be localized on the beads and there was minimal proteins found in the elutant. The lack of proteins in the elutant indicated that the elution methodology was unable to break the disulfide bond linking the proteins to the biotin complex which is required to
release the proteins from the biotin-neutravidin complex. (Experiment performed by Dr. Parveen Sharma)

![Proteins in elutant and proteins on beads](image)

**Figure 9. Silver stain analysis of biotinylation elution.**
Following a biotinylation experiment the biotin-neutravidin complex was incubated with 10% beta mercaptoethanol for 30 minutes to elute proteins. The resulting elutant and the beads were loaded on a gel and subjected to a silver stain analysis with eluted proteins in the left lane and proteins found remaining on the beads after the elution in the right lane. The silver stain analysis showed that all of the proteins remained on the beads following elution with little to no protein signal in the elutant.

**E. Plasma Membrane Protein Enrichment via Silica-Bead Extraction**

PM proteins of all four cell types were isolated by silica bead membrane extraction and eluted using 1% TX100 and 8M Urea for the hCMs, hcSMCs and hcECs, and 1% TX100 and 0.2% PPS-silent surfactant for the hfVCs. To confirm enrichment of cell surface-associated proteins in the silica-bead plasma membrane fractions of each cell type, an immunoblot analysis was performed using equal protein concentrations from the membrane depleted (MD)-fraction and plasma membrane enriched (P)-fraction isolated from each cell. Both fractions were probed for known membrane and cytoplasmic markers for each cell type. As depicted in Figure 10, all four cell types were probed for the Na/K ATPase, which produced a strong signal in the P-fraction but not in the MD-fraction. This prominent signal
P-fraction and absent signal in the MD-fraction was also seen in the hCMs and hcSMCs when probed for the PM protein the DHPR. The endothelial cell fractions were probed for the known PM protein PECAM-1 which showed a strong signal in the P-fraction and an absent signal in the MD-fraction. The hfVC fractions were probed for PMCA and as seen in Figure 10, a signal was found in the P-fraction which was absent in the MD-fraction. All cell types were also probed for the cytosolic protein GAPDH which gave a prominent signal in the membrane-depleted MD-fraction and a faint signal in the P-fraction, except for the endothelial cells which displayed a strong signal in both fractions. Both fractions of the cultured hCMs, hcSMCs, and hcECs were probed for the cytoplasmic protein UCHL1 which showed a signal in the MD-fraction but not in the P-fraction. Similarly, the hfVCs were probed for the endoplasmic reticulum protein, calnexin, which was also depleted in the membrane fraction and enriched in the MD-fraction.
Figure 10. Immunoblot validation of plasma membrane protein enrichment and cytoplasmic protein depletion via silica bead membrane extraction.

The membrane-depleted (MD) fractions and membrane-enriched pellet (P) fractions from the silica bead experiments from each cell type were probed for known cytoplasmic and plasma membrane proteins. The plasma membrane protein, the sodium-potassium ATPase (Na/K ATPase) produced a strong signal in P-fraction but not in the MD-fraction in all four cell types. The dihydropyridine receptor (DHPR) produced a strong signal in the membrane-enriched fraction of the cardiomyocytes and smooth muscle cells. Probing for the endothelial cell membrane protein, platelet endothelial cell adhesion molecule-1 (PECAM1) also produced a strong signal in the P-fraction but not in the MD-fraction. Yet there is a clear signal indicating the presence of the known cytosolic protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the MD-fraction but not in the P-fraction of all cell types except the endothelial cells. Also a signal is seen in the MD-fraction of the cardiomyocytes, smooth muscle cells and endothelial cells of ubiquitin carboxyl-terminal esterase L1 (UCHL1). The same result is seen for calnexin in the ventricular cells. Furthermore, the nuclear protein transportin-1 is also found in the MD-fraction of the primary cardiomyocytes. As expected, these cytosolic and nuclear proteins are not present in the membrane enriched fraction in each cell line. This western blot analysis indicates that the silica bead method effectively enriched for membrane proteins and depleted for most intracellular proteins.
III. IDENTIFICATION AND CHARACTERIZATION OF PLASMA MEMBRANE PROTEINS

A. Protein Identification of the Membrane Depleted and Plasma Membrane Enriched Fractions of Each Cell Type

The MD-fraction and P-fractions were analysed by liquid chromatography tandem mass spectrometry and searched against the Human IPI Database using the X!Tandem algorithm. In total 449801 spectra were identified of which 109082 matched to peptides. Following the rigorous filtering strategy depicted in Figure 4, where only proteins that had two or more unique peptides per analyzed fraction were accepted, approximately 3354 proteins were identified from all fractions of all four the cell types. These 3354 proteins corresponded to 374260 spectra and 77361 total peptides. A hierarchical clustering analysis of the MD-fraction and P-fractions was employed for each cell type, as illustrated in Figure 11. There was a distinct segregation of proteins in their respective fractions as compared to the other fractions. A set of 1624 proteins were identified in the membrane-depleted and membrane-enriched fractions of the in vitro cardiomyocytes of which 801 proteins were identified in the MD-fraction and 1055 and 920 proteins were identified in the TX100 P-fraction and urea P-fraction respectively. Approximately 77% of the total proteins identified in the MD-fraction of the hCMs clustered together and 80% of the total number of proteins identified in the TX100 and Urea fractions clustered together. A total of 1573 proteins were identified in all the fractions of the human coronary endothelial cells of which 1145 proteins were identified in the MD-fraction and 559 and 738 proteins were identified in the TX100 P-fraction and urea P-fraction respectively. Of the total proteins identified in the MD-fraction of the hcECs, approximately 76% of them clustered together and 76% of the proteins isolated in the P-fractions clustered together. A set of 1304 proteins were identified from the MD- and P-enriched fractions of the human coronary smooth muscle cells of which 778 proteins were identified in the MD-fraction and 942 and 170 proteins were identified in the TX100 P-fraction and urea P-fraction respectively. Approximately 74% of the proteins identified in the hcSMC H-fraction clustered together and 93% of the proteins identified in
the hcSMC P-fractions clustered together. A total of 2663 proteins were identified in all of the fractions isolated from the in vivo human fetal ventricular cells of which 2348 proteins were identified in the MD-fraction and 1417 and 1020 proteins were identified in the TX100 P-fraction and urea P-fraction respectively. Approximately 66% of membrane-depleted proteins and 68% of membrane-enriched proteins clustered together. There was also a distinct segregation between proteins in the TX100 membrane fraction as compared to the urea or PPS membrane fraction. Many proteins that were enriched in the TX100 P-fraction were absent in the urea P-fraction of the hCMs, hcECs, and hcSMCs, and the PPS P-fraction of the hfVCs. This phenomenon was also seen in reverse, where proteins that clustered together in the urea P-fraction and PPS P-fraction were not found in the TX100 P-fraction.

Figure 11. Hierarchical clustering of proteins found in the membrane-depleted fraction and the TX-100 and Urea or PPS-silent surfactant buffer eluted membrane fractions.
Shown here is the data obtained from all MS runs of the membrane-depleted and membrane-enriched fractions of each cell type that was clustered according to the presence of each protein in the membrane-depleted, TX100 membrane-enriched (membrane TX) and Urea membrane-enriched (membrane Urea) or PPS membrane-enriched (membrane PPS) fractions. Red indicated the presence of a protein in a fraction whereas black indicates its absence. A total of 1624, 1572, 1304 and 2663 proteins were identified in all fractions of the human cardiomyocytes (hCM), endothelial cells (hcEC), smooth muscle cells (hcSMC) and human fetal ventricular cells (hfVC) respectively. Many proteins that were enriched in the TX100 P-fraction that were absent in the Urea P-fraction of the hCMs, hcECs, and hcSMCs, and the PPS P-fraction of the hfVCs. This phenomenon was also seen in reverse, where proteins that clustered together in the Urea P-fraction and PPS P-fraction was not found in the TX100 P-fraction.
B. Solubilisation of Hydrophobic Proteins

To further assess the difference in proteins eluted by TX100 and urea or PPS, an analysis was carried out to determine the predicted number of transmembrane helices and proteins eluted by each fraction in all cell types. All the proteins eluted by 1% TX100 from the hCMs, hcSMCs, and hcECs were compared to all the proteins eluted by 8M urea from these same cell types. Figure 12A shows that there was a significant increase in the number of predicted transmembrane proteins and predicted transmembrane helices eluted in the TX100 P-fractions than the Urea P-fractions. The same assessment was done on the in vivo hfVCs comparing the TX100 and PPS unique P-fractions that showed that there was a significant increase in the number of predicted transmembrane proteins and helices eluted in the 1% TX100 fraction compared to the 0.2% PPS (Figure 12B).

**Figure 12. Transmembrane analysis of TX100 versus Urea/PPS eluted fractions.**

Depicted here is a comparison of the predicted number of transmembrane proteins and helices eluted from (A) the hCMs, hcSMCs, and hcECs by 1% TX100 and 8M urea and (B) the hfVCs by 1% TX100 and 0.2% PPS silent surfactant. (A) Significantly more predicted transmembrane proteins and predicted transmembrane helices were eluted in the TX100 P-fractions than the urea P-fractions. (B) There was a significant increase in the number of predicted transmembrane proteins and helices eluted in the TX100 fraction compared to the PPS fraction from the fetal ventricular cells.
C. Bioinformatic Characterization of Cell Surface-Enriched Proteins

Proteins identified in the membrane-enriched fractions of each cell type were further filtered according to the schematic in Figure 5 to obtain a set of proteins that had a greater potential of being enriched at the cell surface (CS). A pair wise comparison of the spectral count of TX100 vs. H and Urea or PPS vs. H was applied. Proteins that were found in both the membrane-depleted and membrane-enriched fraction had to have a 2-fold or greater spectral count in the membrane enriched fraction to be accepted into the CS-enriched dataset. Proteins found only in the membrane-enriched fraction were retained if they had a spectral count of five or more. This filtering strategy was applied to the membrane-depleted and membrane-enriched fractions of each cell type to obtain a cell surface enriched dataset for each cell type. Approximately 581, 528, 490 and 634 proteins were filtered into the cell surface enriched dataset of the hCMs, hcECs, hcSMCs, and hfVCs, respectively.

1. Analysis of the CS-Enriched Dataset from the hCMs, hfVCs, hcSMCs, and hcECs

The proteins in the cell-surface enriched datasets of all the cell-types were combined and a bioinformatic analysis was applied to characterize them. The combined cell surface-enriched dataset included 1265 proteins. Gene ontology (GO) is a controlled vocabulary that describes a gene’s subcellular localization, biological process or molecular function and is inferred from a literature reference, another database and/or a computational analysis (http://www.geneontology.org/GO.annotation.shtml). A GO analysis describing each protein’s subcellular localization was employed to characterize the proteins found in this combined CS-enriched dataset. Figure 13 illustrates that approximately 47%, 25%, 11%, and 5% of proteins had a GO-term of membrane, plasma membrane (PM), extracellular region (ECR), and cell surface (CS) respectively. All together approximately 53% of proteins had a GO-term associated with membrane and 32% had a cell-surface associated annotation (ie. PM, ECR, CS). Furthermore, 64% of proteins had intracellular annotations such as cytoskeleton (14%), mitochondrion (23%), endoplasmic reticulum (12%) and nucleus (25%). Almost 14% of proteins had both an intracellular and a membrane associated GO-term annotation. The open source PANTHER program was used to annotate the combined CS-enriched dataset for GO-terms of the biological process each
A gene ontology (GO) analysis was performed on the 1265 filtered CS-enriched proteins from all four cell types. The presence of an annotation is indicated by red or blue and the absence of an annotation is indicated by black. Approximately 47%, 25%, 11%, and 5% of proteins have a GO-term of membrane, plasma membrane (PM), extracellular region (ECR), and cell surface (CS) respectively. Many of the proteins had intracellular annotations including 14%, 23%, 12% and 25% of proteins with GO-terms of cytoskeleton, mitochondrion (mito), endoplasmic reticulum (ER), and nucleus respectively. In total 53% had a membrane-associated GO-term, 64% of proteins had an intracellular-related GO-term and 14% had one or more intracellular and membrane-associated GO-term.
Figure 14. Gene ontology-biological processes annotations of hCM, hfVC, hcSMC and hcEC CS-enriched proteins.

A gene ontology analysis was employed to describe the biological processes the 1265 CS-enriched proteins from all four cell types may be involved in. Approximately 22% of the annotated proteins had a CS-associated annotation such as cell communication, transport and cell adhesion. However, 22% of the proteins were also annotated as being involved in metabolic processes.

2. Analysis of hCM and hfVC CS-Enriched Proteins

Since the focus of my study was to uncover protein enriched at the cell surface of human cardiomyocytes a bioinformatic analysis was carried out on the \textit{in vitro} cardiomyocyte and \textit{in vivo} ventricular CS-enriched datasets. The 581 and 634 proteins in the hCM cell surface-enriched dataset and hfVC cell-surface enriched dataset respectively, were merged into a combined cardiomyocyte (CC) CS-enriched dataset of 1006 proteins. The GO database was used to match proteins within this CC CS-enriched subset to annotations describing their subcellular localization. A GO-term analysis annotated these proteins for GO-terms such as membrane, plasma membrane, cell surface, and extracellular region and, as illustrated in Figure 15, approximately 47%, 25%, 5%, and 11% of proteins had these annotations respectively. Approximately 53% of the CC CS-enriched proteins had one or more membrane-associated GO-terms. The online program TMHMM 2.0 was used to
describe the number of predicted transmembrane helices (pTMH) contained within the CC CS-enriched dataset. A total of 611 pTMHs were identified within this dataset including 24% of proteins with at least 1 predicted transmembrane helix. Altogether 42% of proteins had annotations associated with the cell-surface and/or had one or more predicted transmembrane helix. A GO-term analysis of proteins with intracellular annotations was also applied to evaluate the amount of contamination from non-CS associated proteins. Approximately 4%, 5%, 3%, and 10% of proteins had annotations of cytoskeleton, mitochondrion, endoplasmic reticulum and nucleus, respectively. Altogether approximately 18% of proteins in the CC CS-enriched dataset had an intracellular GO-term annotation. However, 68% of the proteins with an intracellular GO-term also had one or more membrane-associated GO-term and 38% of the proteins with an intracellular GO-term had one or more CS-associated annotation (ie. PM, CS or ECR). Altogether 13% of the 1006 CC CS-enriched proteins had an intracellular and membrane-associated GO-term. Subsequently a gene ontology analysis describing the biological processes was applied to the CC cell-surface enriched dataset as illustrated in Figure 16. Approximately 23% of the proteins with annotations were shown to be involved in metabolic processes, whereas 21% were found to be involved in CS-associated processes such as transport, cell communication and cell adhesion.
A total of 1006 CS-enriched proteins from the human in vitro and in vivo cardiomyocytes were annotated with membrane-associated GO-terms and intracellular GO-terms. Approximately 53% of proteins had a membrane-associated GO-term such as membrane, plasma membrane (PM), cell surface (CS), and extracellular region (ECR) whereas 18% of proteins had one or more intracellular GO-term annotations of cytoskeleton, mitochondrion (mito), endoplasmic reticulum (ER) and/or nucleus. About 13% of proteins had a membrane-associated and intracellular GO-term. Approximately 611 predicted transmembrane helices (pTMH) were found within this dataset.
Figure 16. Gene ontology analysis of the biological processes of the hCM and hfVC CS-enriched proteins.

This graph depicts a gene ontology analysis that was employed to describe the biological processes of the CC CS-enriched proteins. The top three annotations include metabolic processes, transport, and cell communication. Approximately 23% of the annotated proteins were involved in metabolic processes, whereas 21% were involved in cell-surface processes such as transport, cell communication, and cell adhesion.

D. Subtractive Proteomic Comparison

1. *In Vitro* vs. *In Vivo* Cardiomyocytes

Within my study the cell types focused on were the *in vitro* cardiomyocytes and the *in vivo* ventricular cells. A comparison was performed between the protein datasets of the hCMs and hfVCs to illustrate their similarities and differences. Figure 17A illustrates that 74% of the *in vitro* hCM proteins were also identified in the *in vivo* hfVCs, yet only 55% of the proteins found in the hfVCs were identified in hCMs. Among all the proteins found in the hCM and hfVC datasets, approximately 39% of proteins were found in both, such as sarcomeric alpha-actinin (ACTN2) and the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA2). Yet many cardiac proteins were uniquely isolated in the ventricular dataset, including NCX (SLC8A1), the cardiac isoform of myomesin (MYOM1), the cardiac troponin T (TNNT2), slow skeletal and cardiac troponin C (TNNC1) and the
cardiac ryanodine receptor (RYR2). A comparison of the CS-enriched subsets (Figure 17B) showed that out of all the 1006 proteins from the hCM and hfVC combined cell surface-enriched dataset, approximately 20% were identified in both subsets. These included known membrane proteins such as the Na/K ATPase subunit alpha-1 (ATP1A1), PMCA 4 (ATP2B4), several G-protein subunits and integrins.

2. Combined Human in vitro and in vivo Cardiomyocytes vs. Mouse Cardiomyocytes

Previous to my human cell surface cardiomyocyte study a mouse cell surface cardiomyocyte (MCM) study was conducted by a collaborative group led by Dr. Sharma that utilized the silica bead membrane extraction procedure combined with tandem MS to isolate and identify cell surface proteins of the mouse neonatal cardiomyocyte (Sharma et al. unpublished results). In the MCM study 3192 proteins were identified from the membrane-depleted and membrane-enriched fractions. The 3192 proteins were then mapped to 3163 1:1 human orthologs. A combined human cardiomyocyte proteome (CC) of all the proteins found in the hfVC and hCM datasets was compared to the 3163 mouse to human ortholog proteins uncovered in the MCM study (Figure 17C). Approximately 42% of all the proteins were found similarly in both datasets. Examples of proteins that were found similarly in both proteomes included, troponin T (TNNT2), cardiac ryanodine receptor (RYR2), alpha actinin isoforms (ACTN1, 2, and 4), DHPR (CACNA2D1), NCX (SLC8A1) and the sarcoplasmic/endoplasmic reticulum calcium ATPase (ATP2A2).
Figure 17. Subtractive proteomic comparison of the cardiomyocyte datasets.
A) A subtractive proteomic comparison was completed of the total proteins identified in the human cardiomyocyte and the human fetal ventricle cells and visualized in a Venn diagram. Approximately 39% of the total proteins identified in both proteomes are found similarly in both datasets. (B) In this Venn diagram is a comparison of the CS-enriched datasets isolated from the in vitro and in vivo cardiomyocytes. Approximately 20% of the total proteins identified altogether are found in both fractions. (C) A subtractive proteomic comparison of the combined human in vitro and in vivo cardiomyocyte proteins, to the 1:1 mouse to human ortholog data from the MCM study was completed. Almost 42% of proteins from the entire human and mouse cardiac proteome combined are found in both subsets.

3. Comparison of the Cell Surface-Enriched Datasets of All Cell Types

A subtractive proteomic comparison of the 1265 cell surface-enriched proteins from the CCs, hcSMCs and hcECs was performed. In the complete cell surface-enriched dataset, 59 CD antigens were identified. Figure 18 shows that 50% of the proteins from the CC cell
surface enriched subset were similarly found in either one or both of the endothelial or smooth muscle cell CS-enriched datasets. Approximately 18% of proteins were found similarly in all three datasets which included, for example, two isoforms of the Na/K ATPase (ATP1A1, ATP1B3), two isoforms of the PMCA (ATP2B1, ATP2B4) and several integrins. The CC, hcSMC and hcEC membrane enriched subsets consisted of 41, 32 and 44 CD antigens respectively. Approximately 50% of the identified proteins found in the entire membrane enriched dataset were found exclusively in the CC CS-enriched dataset. Unique proteins found in the CS-enriched dataset of the CC subset included, for example, the alpha2 isoform of the Na/K ATPase (ATP1A2), NCX (SLC8A1), sarcoglycans (SGCB and SGCG) as well as several CD molecules (Figure 18). Proteins unique to the coronary endothelial membrane dataset included many known endothelial PM proteins such as von Willebrand factor (VWF), vascular cell adhesion protein 1 (VCAM1), and platelet endothelial cell adhesion molecule 1 (PECAM1) (Figure 18). Only about 8% of the proteins from the entire CS-enriched dataset were found uniquely in the hcSMC CS-enriched dataset. The CD antigens, CD151 and CD63 shown in Figure 18 were a few examples of proteins unique to the membrane-enriched dataset of the hcSMCs.
Figure 18. Subtractive proteomic comparison of cell surface enriched human cardiac myocyte, coronary smooth muscle cell and endothelial cell proteins.

Shown here is a Venn diagram of the subtractive proteomic comparison of the *in vitro* and *in vivo* cardiomyocytes, the coronary smooth muscle cells and the coronary endothelial cells. Examples of proteins found in the CS-enriched dataset of only one cell-type are listed in the corresponding tables connected to that dataset. Approximately 18% of all the combined human cardiomyocyte, human coronary smooth muscle cells, and endothelial cell CS-enriched proteins were found in all three subsets. Approximately 50% of the CC CS-enriched proteins were found uniquely in that CC dataset, 20% of the hcSMC CS-enriched proteins are unique to the hcSMC dataset, and 27% of hcEC CS-enriched proteins are unique to the hcEC dataset.
IV. GENERATION OF A CANDIDATE PROTEIN DATA SET

A. Enrichment of Essential Cell-Surface Associated Proteins of the Human Cardiomyocyte

1. Enrichment of Essential Human Cardiomyocyte Proteins

A major aim of this study was to identify proteins that may be essential cell-surface proteins of the human cardiac myocyte. Therefore, a data mining strategy was developed and applied to the proteins in the CS-enriched datasets of all the cell-types, as depicted in Figure 19. To enrich for cell surface associated proteins in human cardiomyocytes, proteins in the CS-enriched dataset of the human hCMs and human hfVCs were accepted into the first subset (i.e. the CC cell surface-enriched dataset) (Figure 19, Step 1). All other proteins were removed, such as hcSMC and hcEC proteins that were not found in the hCM or hfVC dataset. Examples of proteins that were removed include von Willebrand factor (VWF), vascular cell adhesion molecule (VCAM1) and PECAM1. This data mining strategy produced a set 1006 proteins.

To further focus on heart proteins that were enriched in cardiomyocytes an enrichment factor for the other major cell types of the heart was calculated. The hcEC enrichment factor was devised by calculating the ratio of the spectral count of the proteins in the CS-enriched dataset of the endothelial cells to the average spectral count of the CS-enriched dataset of the hCMs and hfVCs if the protein was found in both cardiomyocyte datasets. If the protein was found in only one of the cardiomyocyte datasets then the hcEC enrichment factor was calculated as a ratio of the hcEC CS-enriched spectral count to the hCM or hfVC CS-enriched spectral count (based on which cardiomyocyte dataset the protein was found in). Any protein with an hcEC enrichment factor above 1 indicates that the protein was more enriched in the endothelial cells than the hCMs and/or hfVCs and therefore was removed (Figure 19, Step 2). Examples of discarded proteins included a few integrins, 2 isoforms of
PMCA and well known endothelial cell-surface proteins such as intercellular adhesion molecule-1 (ICAM1) and caveolin-1 (CAV1).

Subsequently the enrichment factor for the coronary hcSMCs was also calculated in the same manner as the hcECs. Specifically, the hcSMC enrichment factor was a ratio of the spectral count of each protein in the CS-enriched dataset of the smooth muscle cells to the average spectral count of that same protein in the CS-enriched dataset of the hCMs and hfVCs if it is found in both cardiomyocyte datasets. If the protein was only isolated in one of the cardiomyocyte datasets than the hcSMC enrichment factor was a ratio of the hcSMC CS-enriched spectral count of the protein to the spectral count of that same protein in the hCM or hfVC CS-enriched dataset (depending on which dataset it was found in). Since the smooth muscle cell is a muscle cell and any proteins enriched in its cell-surface may also be essential to other muscle cells such as cardiomyocytes, the hcSMC enrichment factor for accepted proteins was higher. Thus proteins with an hcSMC enrichment factor of 3 and above were removed which included a few ras-related proteins, a DHPR isoform and a haemoglobin subunit for example (Figure 19, Step 3). The removal of proteins enriched in the hcEC and hcSMC CS-enriched dataset reduced the set of proteins to a subset of 700.

2. Enrichment of Cell Surface Associated Proteins

The subset of 700 proteins was then mined based on their subcellular GO-term annotations and predicted transmembrane helix (pTMH) content to enrich for any proteins that may be associated with the cell surface. Proteins with GO-terms of plasma membrane, cell surface and extracellular region and any proteins with one or more pTMH were retained (Figure 19, Step 4). This data mining strategy returned a set of 264 proteins and removed many contaminating nuclear, mitochondrial and cytoskeletal proteins.

3. Enrichment of Most Abundant and Potentially Essential Cardiac Myocyte Proteins

Proteins that are conserved within related species could be considered essential, therefore the next filter to enrich for essential proteins integrates data from the MCM study conducted by Sharma et al. (unpublished data). In the MCM study 3192 proteins were identified and 1:1 human orthologs was calculated for all proteins. Only 3162 mouse proteins had a 1:1 human ortholog and these proteins were compared to the candidate dataset. Any proteins that were not found in the MCM study were removed (Figure 19,
Step 5). This filter removed several contaminants such as nuclear and mitochondrial proteins. This mining criterion returned a set of 174 proteins. The last subset of proteins was filtered based on the total CS-enriched spectral count of the hfVCs and hCMs. Proteins with a total spectral count of 5 and below were removed, which included a few mitochondrial proteins and a few unknown proteins (Figure 19, Step 6). Thus this mining strategy has enriched for the most abundant proteins in the *in vitro* and *in vivo* human cardiomyocyte and has produced a set of 167 proteins. The final set of proteins included many cell surface associated proteins such as the NCX, the alpha2 subunit of the Na/K ATPase, G-protein subunits, as well as 8 CD molecules.
Figure 19. Schematic diagram of data mining strategy to enrich for essential cell-surface associated proteins of human cardiomyocytes.

Depicted here is a schematic diagram of the data mining strategy designed to enrich for essential cell-surface, cardiomyocyte proteins. (Step 1) Proteins in the CS-enriched dataset of the human hCMs and human hfVCs were accepted into the first subset protein. Proteins such as von Willebrand factor (vWF), vascular cell adhesion molecule (VCAM) and platelet endothelial cell adhesion molecule (PECAM) were removed. (Step 2) Subsequently, any proteins with a hcEC enrichment factor above 1 was removed such as the plasma membrane calcium ATPase (PMCA) 1 & 4 isoforms, intracellular adhesion molecule 1 (ICAM1), and caveolin 1 (CAV1). (Step 3) Any proteins with an hcSMC enrichment factor of 3 and above were removed, which included a neuronal isoform of the dihydropyridine receptor (DHPR) and a haemoglobin subunit. (Step 4) Proteins were then accepted if they had a GO-term of plasma membrane (PM), cell surface (CS) or extracellular region (ECS) and/or had one or more predicted transmembrane helices (pTMH). (Step 5 & 6) Finally any proteins that were not found in the mouse cardiomyocyte (MCM) study or had a total spectral count of 5 or less were eliminated. The last three filtering steps removed many nuclear, mitochondrial and cytoskeletal proteins. A few unknown proteins were removed in the last filtering step.
B. Mining for Candidate Proteins using Bioinformatics and Literature Searches

A major aim of this study was to identify proteins that were understudied in the cardiovascular field and were enriched in cardiac cells at the cell-surface. Therefore, another data mining strategy was applied as depicted in Figure 20 to the 167 human cardiomyocyte and PM enriched proteins to isolate novel candidate PM proteins. As previously stated, there are many proteins that have plasma membrane and intracellular annotations. However this investigation focuses on proteins that are unique to the cell-surface since any future studies will assay the effect of knockdown or overexpression of a PM protein on the cell function. If a protein is found in another intracellular organelle and performs a vital function for that organelle it will alter any future assays of the plasma membrane protein. Therefore any proteins with annotations of cytoskeleton, mitochondrion, endoplasmic reticulum and nucleus were removed since these were the major contaminants. This filter removed proteins that were shown to be found in the plasma membrane and other intracellular compartments such as VDAC1 and ATP5b. To increase the likely-hood that the candidate proteins were enriched in the heart the 167 proteins were compared to the human MicroArray database developed by the Genomics Institute of the Novartis Research Foundation. Proteins that were found to have mRNA enrichments (3 fold above median, as described in the methods section) of heart and/or cardiomyocyte were accepted. This criterion removed known PM proteins such as the Na/K ATPase and 2 CD antigens, and several unknown proteins. An extensive literature search was done to determine which proteins may have been understudied in cardiac literature. Proteins found to play a role in cardiac health and disease in 10 or more journal articles were removed. This mining strategy removed some collagens and 2 CD molecules as well as an annexin (isoform A2). To target candidates that are more specific to cardiac tissue than any other tissue or cell in the body, proteins that were enriched in more than 9 other non-cardiac tissues or cells were removed from the final list. This mining criterion removed a few unknown proteins.
Figure 20. Data mining strategy to identify understudied protein candidates of interest.

Shown here is a schematic diagram of the data mining strategy employed to identify proteins that may be understudied in the cardiac field, localized only to the cell surface and enriched in the heart. (Step 1) Any proteins with annotations of cytoskeleton, mitochondrion, endoplasmic reticulum and nucleus were eliminated such as VDAC1 and ATP5b. (Step 2) Proteins that were found to have enrichments (3 fold above median) of heart and/or cardiomyocyte in the human MicroArray database were accepted. This criterion removed known plasma membrane proteins such as the Na\(^{2+}/K^+\) ATPase and CD antigens, and several unknown proteins. (Step 3) Proteins found to play a role in cardiac health and disease in 10 or more journal articles were removed such as a few collagens and CD molecules as well as Annexin A2. (Step 4) Finally any candidates that were enriched in more than 9 other non-cardiac tissues or cells were removed from the final list.
C. Candidate Proteins

The set of 9 protein candidates, listed at the top of Table 1, included isoform 1 of popeye domain-containing protein 2 (POPDC2), protein kinase C and casein kinase substrate in neurons protein 3 (PACSIN3), myeloid-associated differentiation marker (MYADM), isoform 1 of acetyl-CoA synthase-like protein (ILVBL), isoform 1 of caprin-1 (CAPRIN1), matrix metalloproteinase-14 (MMP14), isoform 2 of nebulin-related anchoring protein (NRAP), glypican-1 (GPC1) and septin-11 (SEPT11) as shown in Table 1. To further validate the plasma membrane localization of candidate proteins the cDNA constructs for candidate proteins and controls were acquired from Open Biosystems’ Human ORFeome Collection, version 1 in collaboration with Dr. Jason Moffat (University of Toronto). However, three candidate clones, namely NRAP, GPC1, and SEPT11, were not in this collection. Also the aliquots obtained for CAPRIN1 and MMP14 had different sequences in it. Therefore the PM validation was limited to four final protein candidates: POPDC2, PACSIN3, ILVBL, and MYADM.

Table 1. List of Protein Candidates.
Listed here is the nine potential protein candidates elucidated from the data mining strategies employed to isolate essential and understudied proteins candidates that are enriched at the cell-surface and in the human heart. Following the gene and protein name is a description of the number of predicted transmembrane helices (pTMH) each protein has, if the protein was found in the hCM CS-enriched dataset (hCM CS) and the hfVC CS-enriched dataset (hfVC CS), the total number of MicroArray enrichments the protein has other than heart or cardiomyocyte (total # if MicroArray Enrichment), the combined spectral count of the protein found in the hCM and hfVC CS-enriched datasets (Total hfVC + hCM SpC), and whether the protein is found in the Human ORFeome collection (ORF). The last five candidates listed in the table could not be confirmed for PM validation because they were not contained in the ORFeome collection or were contaminated by other sequences. Four final candidates at the top of the table were utilized for further confirmation of PM localization.
POPDC2 had the highest combined spectral count of the five final candidates and it had two predicted transmembrane domains. According to the MicroArray database its mRNA was not found enriched in any other tissue except for heart tissue. PACSIN3 had no predicted transmembrane domains and had 12 combined cardiomyocyte spectra. In the MicroArray database it was found enriched in the heart and the adrenal cortex. MYADM and ILVBL were enriched in seven other tissues other than the heart and each had a combined cardiomyocyte spectral count of over 20. MYADM had 8 predicted transmembrane helices and ILVBL had one.

V. CONFIRMATION OF PLASMA MEMBRANE LOCALIZATION OF CANDIDATE PROTEINS

To confirm plasma membrane localization, candidate proteins and controls were tagged and transfected into human embryonic kidney (HEK) cells and fluorescently imaged. Originally transfections were completed in mouse neonatal cardiomyocytes and human ventricular fibroblasts however transfecting into these cell types repeatedly failed. As a result, all subsequent localization studies were completed by transfecting into HEK cells which gave a 10-15% transfection efficiency. Sucrose density fractionation is a previously established technique used to separate different organelles\textsuperscript{98,99}. Sucrose density fractionation was carried out for each candidate and control with cell lysates from transfected HEKs. Fractionation was done in combination with transfected HEK fluorescence to confirm localization of protein candidates. Neuropilin 1 (NRP1) was used as the positive transfected control for PM localization because it is a known PM protein and CD antigen, and was filtered in the 167 cardiomyocyte and cell-surface enriched dataset. GAPDH was used as the negative control because it is a known cytoplasmic protein and it had approximately a 2- to 20-fold increase in the membrane-depleted fractions as compared to the membrane enriched fractions of the hCMs and hfVCs. PACSIN3, ILVBL, MYADM and NRP1 candidate cDNAs were tagged with V5 whereas POPDC2 and GAPDH were previously tagged with GPF by Dr. Parveen Sharma.
A. Sucrose Density Fractionation of Tagged and Transfected Candidate HEK Cells

HEKs were transfected twice with tagged candidate DNA and then harvested 48 hours after the final transfection. The cell lysate was layered on top of a 20-60% continuous sucrose gradient and the gradient was centrifuged for 18 hours. Thirteen equal volume fractions were collected from the bottom and a western blot analysis was performed on each fraction. As illustrated in Figure 21A NRP1, a known plasma membrane protein, was eluted in the last eight fractions of the gradient. Similarly, POPDC2 was eluted in the last six fractions and PACSIN3 was eluted in the final fractions. ILVBL eluted in the first seven fractions of the gradient whereas the signal for MYADM was consistently found in the first three fractions and fractions five, six, seven and eight. The elution profiles of NRP1, POPDC2, PACSIN3, ILVBL and MYADM were consistently seen in all three biological repeats as depicted by the average densitometry measurements in Figure 21B. As seen in Figure 21A the western blots were also probed for endogenous proteins such as the Na/K ATPase, protein disulfide isomerise (PDI), the estrogen receptor subunit beta and the alpha subunit of the coatomer protein (alpha COP). The known PM transporter the Na/K ATPase eluted in the final seven fractions which was comparable to the elution pattern of NRP1, POPDC2 and PACSIN3. However, some of the NRP1 and PACSIN3 expression also overlapped with the elution profile of PDI and alpha COP. PDI is an endoplasmic reticulum protein and it eluted in fractions two to six. Alpha COP is involved in retrograde protein transport from the Golgi to the endoplasmic reticulum and thus can be found in both organelles. Alpha COP eluted in fractions four to seven. The estrogen receptor is localized to the nucleus and eluted in the first four fractions. MYADM had a similar elution pattern to the estrogen receptor but also overlapped with the alpha COP elution profile. ILVBL`s elution pattern most resembled the elution pattern of PDI.
Figure 21. Assessment of subcellular localization of protein candidates by sucrose gradient centrifugation.

NRP1, POPDC2, PACSIN3, ILVBL and MYADM proteins were tagged and transiently transfected into human embryonic kidney (HEK) cells. Transfected HEK cells were harvested and layered on a 20-60% sucrose gradient to fractionate the various organelles and plasma membrane and 13 fractions were collected from the bottom. A) A western blot analysis was performed to investigate which fractions the transfected proteins and control endogenous proteins eluted in. NRP1, POPDC2 and PACSIN3 had an elution profile that overlapped the profile of the Na/K ATPase. However, NRP1 and PACSIN3 also eluted in a few of the same fractions as the alpha coatamer protein (alpha COP) and the endoplasmic reticulum protein, protein disulfide isomerise (PDI). MYADM eluted in fractions one to three and fractions five to eight. MYADM overlapped with the elution pattern of the beta estrogen receptor which eluted in fractions one to four, and with alpha COP which eluted in fractions four to seven. ILVBL eluted in the first seven fractions of the gradient similar to PDI which eluted in fractions two to six. B) A western blot analysis and densitometry measurement was completed for three biological repeats of the transfected HEKs subjected to sucrose gradient fractionation. Each graph represents the mean densitometry reading for all three biological repeats for each transfected protein and the error bars indicate the standard error.
B. Immunofluorescent Localization of Candidate Proteins in HEKs

The subcellular localization of each candidate was visualized by examining HEK cells that were transiently transfected with tagged candidate cDNAs and subsequently fluorescently stained for the corresponding tag as illustrated in Figure 22. The known CD antigen, NRP1 tagged to V5 was used as a positive control for plasma membrane staining whereas GAPDH tagged to GFP was used as the negative control to illustrate cytoplasmic staining. NRP1, POPDC2 and PACSIN3 exhibited clear cell surface staining around the HEK cell consistent with their elution profiles from the sucrose gradient fractionation. The NRP1, POPDC2 and PACSIN3 staining shown in Figure 22 is representative of approximately 86%, 92%, and 80% of the transfected cells in each slide respectively. The staining pattern of NRP1, POPDC2 and PACSIN3 was starkly different from that seen in GAPDH and ILVBL transfected cells, which showed prominent cytosolic staining. The HEK cells transiently transfected with MYADM displayed a vesicular staining pattern. The GAPDH, ILVBL and MYADM staining shown in Figure 22 is representative of approximately 95%, 87%, and 93% of the transfected cells in each slide respectively.

Figure 22. Fluorescent staining of candidate proteins.
HEK cells were fluorescently stained (green) against the corresponding tag for each candidate and visualized using confocal microscopy. NRP1 is a known CD antigen and thus served as the positive plasma membrane control. It showed clear staining around the cell surface. Similarly, POPDC2 and PACSIN3 had a plasma membrane staining pattern. GAPDH tagged with GFP portrayed prominent cytoplasmic staining similar to ILVBL. MYADM was found to have a vesicular staining pattern.
CHAPTER FOUR: DISCUSSION

In this study silica bead membrane isolation was combined with mass spectrometry based proteomics to isolate and identify plasma membrane proteins of the human heart. An extensive filtering strategy was utilized to isolate proteins that may be enriched at the cell surface. Cell surface-enriched proteins were characterized using bioinformatics and subtractive proteomics. An integrative data mining approach was applied to cell surface-enriched data to identify potentially novel and essential plasma membrane proteins that were found to be enriched in cardiomyocytes. Two candidate proteins, namely POPDC2 and PACSIN3, were confirmed to be localized to the plasma membrane by confocal microscopy and sucrose density fractionation of human embryonic kidney cells that were transfected with tagged candidate cDNA.

I. CHARACTERIZATION OF THE MAJOR CELL TYPES OF THE HUMAN HEART

The in vitro and in vivo cardiomyocytes, coronary endothelial cells and coronary smooth muscle cells were stained with alpha-actinin, platelet-endothelial cell adhesion molecule 1, and smooth muscle alpha-actin respectively, and were imaged using confocal microscopy, as shown in Figure 6. The coronary endothelial cells (Figure 6C) had characteristic cell surface staining of PECAM1, which is a glycoprotein shown to be densely packed on the cell surface of endothelial cells\(^{100}\). However, the alpha actinin staining of the in vitro cardiomyocytes (Figure 6A) was disorganized and atypical of cardiomyocytes\(^{102}\). Alpha actinin is the major structural protein in striated muscle found at the Z-disk in sarcomeres, where it cross-links actin filaments from adjacent sarcomeres and forms a lattice-like structure to stabilize the sarcomere\(^{102}\). This lattice is visible in the in vivo cardiomyocytes (Figure 6B) however it is absent in the in vitro cardiomyocytes. The lack of this lattice-like staining pattern is expected of the in vitro cardiomyocytes since these cells have been shown to undergo a de-differentiation following harvesting and culturing\(^{103}\). However, the hCMs were still included in this study because it has been shown
that they do produce many of the essential proteins of human cardiomyocytes such as myosin heavy chain and alpha actinin.

II. ISOLATION AND ENRICHMENT OF PLASMA MEMBRANE PROTEINS

To obtain a comprehensive protein profile of the plasma membrane of the major cell types of the human heart, two different methods of isolating PM proteins were employed. A previously described silica bead membrane isolation procedure was applied to the four different cell types. As illustrated in Figure 2, this procedure separates the plasma membrane, and any proteins associated with it, from the cell. Many proteins that may only be transiently associated with the membrane can also be separated with the PM. In order to get a more specific PM protein profile, a surface biotinylation procedure was also employed to isolate PM proteins. As described in Figure 3, this procedure is limited to isolating proteins that have a protein domain exposed to the extracellular side of the PM. As a result, this procedure would further focus on membrane proteins with an extracellular domain as well as proteins associated with the extracellular surface of the PM. Therefore, the combination of both procedures provides the opportunity to identify all proteins associated with the plasma membrane meanwhile focusing on the proteins that are in direct communication with the extracellular environment.

A. Biotinylation

To confirm enrichment of PM proteins and depletion of cytoplasmic proteins via surface biotinylation a western blot analysis was performed to probe for a known PM and cytoplasmic protein. However Figure 8A showed that although there was a depletion of the cytoplasmic protein there was a lack of a PM protein signal in the P-fraction. Further analysis illustrated that a large amount of biotin was not binding to the neutravidin (Figure 8B, left) possibly due to super saturation of the neutravidin beads with biotin. An increase in the amount of beads alleviated the problem of biotin in the flow through (Figure 8B, right), yet there was still a lack of PM protein in the P-fraction with a significant amount still present on the beads (Figure 8C). Silver stain analysis of elutant and beads following the elution showed that proteins were not being eluted off of the neutravidin beads. A 5% beta-
B. Silica Bead Membrane Isolation

Enrichment of PM proteins via the silica bead extraction methodology of the in vitro cardiomyocytes, endothelial cells, smooth muscle cells and in vivo ventricular cells was successful, as shown in Figure 10. Similarly, depletion of cytoplasmic proteins in every cell type, except the endothelial cells, was illustrated in Figure 10. The endothelial cells had significant contamination of GAPDH in its membrane-enriched fraction. Arjunan et al. also encountered significant contamination of mitochondrial and cytoplasmic proteins after silica bead extraction combined with MS analysis of mouse coronary endothelial cells\textsuperscript{104}. They suggested that the shearing force used to disintegrate tissue may be contributing to contamination of PM fractions with intracellular proteins since they observed an increased contamination using Teflon pestle homogenization as compared to ultra blade homogenization\textsuperscript{104}. Similarly, the force used to homogenize the human coronary endothelial cells in my study may have played a role in the increased contamination of cytoplasmic proteins in the membrane fraction.
III. ANALYSIS OF PLASMA MEMBRANE PROTEOMIC DATA

A. Hierarchical Clustering Analysis

Proteins isolated by silica bead extraction from the membrane-depleted and membrane-enriched fraction were identified by mass spectrometry. As a result of silica bead fractionation the membrane-depleted and membrane-enriched samples from all cell types differentially segregated into groups with a few proteins being present in both fractions (Figure 11). Interestingly, there was also a difference in membrane-enriched proteins eluted by 1% TX100 and 8M Urea, and 1% TX100 and 0.2% PPS-silent surfactant. Analysis of the predicted transmembrane helices and proteins eluted by each solubilizing agent (Figure 12) illustrated that the 1% TX100 fraction consistently had significantly more predicted transmembrane proteins and helices. The increased presence of pTMHs in the TX100 fraction was expected when comparing TX100 with urea because TX100 is a detergent molecule with a hydrophobic and hydrophilic domain. TX100 molecules surround hydrophobic proteins, such as transmembrane proteins, and bring the protein into solution by forming a micelle around it. However, 8M urea tends to denature proteins by breaking hydrogen bonds thus exposing internal hydrophobic residues to the aqueous environment. As a result, urea will more readily solubilise proteins that are hydrophilic instead of hydrophobic which is what was illustrated in this study (Figure 12A).

The elution methodology was modified to increase the number of hydrophobic proteins being resolved due to the decreased solubilisation of hydrophobic proteins by 8M Urea. PPS-silent surfactant is a mass spectrometry-compatible detergent and thus does not need to be removed prior to MS analysis. Removal of 8M urea by TCA precipitation tends to cause the loss of proteins that may irreversibly precipitate. It was shown that a greater number of proteins are digested in PPS than the 2M urea buffer the proteins are resolubilised in following TCA precipitation. Therefore, PPS-silent surfactant was used to solubilise membrane proteins extracted from the in vivo ventricular cells. A comparison of the predicted transmembrane helices and proteins resolved by TX100 as compared to PPS showed that the TX100 fraction had a significantly greater number of transmembrane
proteins and helices identified in it (Figure 12B). This difference in transmembrane protein content was expected because more TX100 molecules per unit volume were available to solubilise proteins than PPS since a 1% TX100 solution was used to elute proteins whereas only a 0.2% PPS solution was used for the PPS fraction.

B. Bioinformatic Analysis of CS-Enriched Data

An extensive filtering strategy was employed to the membrane-enriched dataset of each cell type as depicted in Figure 5 to obtain a set of proteins that are enriched at the cell surface of each cell and designated the CS-enriched dataset. A GO-term analysis of all the CS-enriched datasets was employed and, as illustrated in Figure 13, contamination of intracellular proteins from the endoplasmic reticulum, nucleus, cytoskeleton and mitochondria existed. Also, majority of the proteins in the CS-enriched datasets were involved in metabolic processes, which may also indicate a large contamination of mitochondrial proteins. Recently Van Hoof et al. used a combination of differential centrifugation and density gradient centrifugation to isolate a plasma membrane fraction from human embryonic stem cell-derived cardiomyocytes and human fetal cardiomyocytes\textsuperscript{106}. A gene ontology analysis of their plasma membrane fraction indicated that only about 24\% of proteins had GO-terms that are associated with the cell surface (ie. plasma membrane, cell surface, extracellular region)\textsuperscript{106}. There was abundant cytoskeletal, mitochondrial, nuclear and endoplasmic reticulum contamination\textsuperscript{106}. In my study, cell surface associated proteins of \textit{in vitro} and \textit{in vivo} human cardiomyocytes were identified by silica bead isolation combined with an extensive filtering strategy, which produced a set of proteins in which approximately 38\% had a cell surface associated GO-term. However, approximately 18\% of the CS-enriched dataset from the \textit{in vitro} and \textit{in vivo} cardiomyocytes had intracellular GO-term annotations of cytoskeleton, mitochondrion, endoplasmic reticulum, and nucleus. Previous studies that have employed the silica bead method also found contamination of intracellular proteins from the cytoplasm, mitochondria, cytoskeleton, nucleus and endoplasmic reticulum in the membrane-enriched fraction\textsuperscript{61-63}. Moreover, the high degree of mitochondrial contamination that was found in the CS-enriched dataset of the \textit{in vitro} and \textit{in vivo} cardiomyocytes (Figure 15) can be attributed to the high abundance of mitochondria in cardiac myocytes\textsuperscript{107}. 
Many proteomic studies that isolated a plasma membrane fraction from cells included a combination of other plasma membrane purifying strategies to get rid of intracellular contamination. For example, several studies have also included one or more sodium carbonate washes at high pH to further purify for cell surface proteins following PM isolation by silica bead extraction or another membrane isolation procedure\textsuperscript{62, 63, 66, 73}. Washing membranes with sodium carbonate has been shown to open the membrane into sheets and solubilise proteins that are peripherally bound to the membrane, thus removing proteins that are not tightly bound to the membrane\textsuperscript{73, 79}. This sodium carbonate wash was not included in the silica bead extraction procedure carried out in my study, which may have contributed to the resulting intracellular contamination. Furthermore, studies that utilize the silica bead extraction to isolate the PM have also included two or more density gradient centrifugation spins\textsuperscript{61, 62} or two or more differential centrifugation spins\textsuperscript{61, 63} to further purify the membrane fraction. In retrospect the addition of more centrifugation steps and washes with sodium carbonate may have further decreased the contamination of intracellular proteins in my silica bead isolation study.

However, not all intracellular proteins found in the CS-enriched fractions were a contamination. Many intracellular proteins were also found within the PM or associated with the plasma membrane. For example, approximately 14% of CS-enriched proteins from the hCMs, hfVCs, hcSMCs, and hcECs that have a PM annotation were also annotated with one or more intracellular annotations of mitochondria, cytoskeleton, endoplasmic reticulum, or nucleus. There is evidence which suggests the presence of proteins in the PM originally thought to be localized in an organelle. For example, the beta subunit of the mitochondrial F1 ATP synthase (ATP5b), which is responsible for the synthesis of ATP in the mitochondria\textsuperscript{108}, has been found to perform an important binding function (binding angiotensin) in the PM of endothelial cells\textsuperscript{109}. Another class of mitochondrial proteins, called the voltage-dependent anion channels have also been shown to be localized in the PM\textsuperscript{110}. There is also evidence in the literature of proteins that move from the PM to another intracellular compartment, or vice versa, to carry out its cellular function\textsuperscript{111}. For example, the nuclear protein histone deacetylase 3 (HDAC3) has been shown to shuttle out of the nucleus\textsuperscript{111}, localize to the plasma membrane and interact with a PM associated kinase, the c-Src\textsuperscript{112}. Another example includes caveolin-1 which was found to migrate from the caveolae directly to the endoplasmic reticulum when cholesterol is oxidized and to transport
cholesterol from the ER to the plasma membrane\textsuperscript{113}. Furthermore, there are proteins that are essential in trafficking other proteins from intracellular compartments, such as the ER and Golgi network, to the plasma membrane, and as a result can be found in many intracellular compartments as well as the PM\textsuperscript{114}. Clearly, many proteins that are localized in the PM may also have intracellular annotations.

C. Subtractive Proteomic Comparison

1. \textit{In Vitro} vs. \textit{In Vivo} Cardiomyocytes

Subtractive proteomics was used to investigate the similarities and differences between the various subsets of data from the different cell types. A comparison of the entire set of proteins identified from the \textit{in vitro} cardiomyocytes and the \textit{in vivo} ventricular cells showed that approximately 39\% of proteins were similarly found in both cell types. Although it is expected that \textit{in vitro} and \textit{in vivo} cardiomyocytes would be very comparable in protein content, Durr et al. also found approximately a 40\% difference between \textit{in vitro} and \textit{in vivo} endothelial cells from lung microvasculature. The differences between the \textit{in vitro} and \textit{in vivo} cardiomyocytes may be attributed to several circumstances. Firstly, the \textit{in vitro} cardiomyocytes have been isolated from adult ventricular tissue\textsuperscript{103} whereas the \textit{in vivo} cardiomyocytes have been isolated from fetal tissue. Thus the cells are from different stages of human development. It has been suggested that when cells undergo differentiation during the development of tissues there are significant changes in gene and protein expression. For example, it has been shown that the differentiation of an \textit{in vitro} mouse myoblast cell line, called C2C12 cells, causes a change in approximately 16\% of genes\textsuperscript{115}. Significant changes in protein expression have also been found during the differentiation of this same myoblast cell line\textsuperscript{116} as well as in bovine myoblasts\textsuperscript{117}. Furthermore, dynamic protein expression has also been seen during the development of other cells and tissues such as adipocytes\textsuperscript{118} and embryonic lung tissue\textsuperscript{119}. Therefore the varying protein expression between the \textit{in vivo} and \textit{in vitro} cardiomyocytes may be caused by the different stages of development the cells were extracted from. It has also been shown that when cardiomyocytes are cultured they undergo a de-differentiation\textsuperscript{120}. This de-differentiation was illustrated in the hCMs by a lack of a sarcomeric pattern seen in Figure 6A and by the absence of many of the
proteins essential for contraction such as NCX, myomesin, troponins T and C. The de-differentiation of hCMs may have added to an even greater gap in stage of maturity of the hCMs as compared to the hfVCs. However, 74% of the hCM proteins were also found in the total hfVC dataset, suggesting that the in vitro cells are cardiomyocyte-like.

Another reason for the variation between the in vitro and in vivo cells may due to the differing microenvironments of each cell. It has been shown that perfusion of neonatal cardiomyocytes in a decellularized adult rat heart composed primarily of extracellular matrix can re-establish fundamental functioning of that heart\textsuperscript{121}. This group went on to show that unorganized neonatal contractile fibres developed into organized sarcomeres after 8-10 days in the extracellular matrix scaffold\textsuperscript{121}. As a result, the microenvironment of the cells may be essential to the proper functioning and development of mature cardiomyocytes. The greater difference found between the CS-enriched datasets (Figure 17B) could be attributed to the loss of proteins during biochemical fractionation of the samples and stringent filtering of the datasets to obtain a CS-enriched subset.

2. Human Cardiomyocytes vs. Mouse Cardiomyocytes

The subtractive proteomic comparison of the combined in vitro and in vivo human cardiomyocytes to the mouse cardiomyocytes showed that 42% of all of the proteins are found in both datasets. This number was expected to be higher since humans and mice share most physiological and pathological features\textsuperscript{122} including extensively documented similarities in the cardiovascular system\textsuperscript{123}. Previous proteomic studies assessing the similarities between mouse and human red blood cells\textsuperscript{124} and placenta\textsuperscript{125} have shown strong similarities between the two. Cox et al.\textsuperscript{125} reported approximately an 80% conservation of co-expressed phenotypic genes between human and mouse placental cells. The greater variation of proteins between the mouse and human cardiomyocytes identified in this current study may be, once again, attributed to the different stages of development the cells were isolated from. The mouse cardiomyocytes were isolated from neonatal cardiac tissue whereas the human in vitro and in vivo cells were extracted from adult and fetal ventricle respectively. However, many known proteins essential to cardiomyocyte function were found in both the mouse and human cardiomyocytes such as RYR2, NCX, DHPR, SERCA2 and troponin T, which suggests that many proteins vital to cardiomyocyte function were isolated.
3. Comparison of CS-Enriched Proteins

The final comparison of protein subsets was done among all the human CS-enriched datasets (Figure 18), which includes the hCM and hfVC, hcSMC and hcEC subsets. Altogether 59 CD antigens were identified in the hCM, hfVC, hcSMC and hcEC CS-enriched datasets. The CS-enriched subsets of each cell type isolated approximately 32 to 44 CD molecules. This range of the CD antigens is similar to the range of antigens found in the glycoscapture study conducted by Wollscheid et al. who identified approximately 38-53 CD molecules in the PM fractions of the various cell types they analysed. Many known PM proteins were also found in the various CS-enriched subsets, such as the Na/K ATPase and the PMCA, suggesting that the enrichment of plasma membrane proteins and the filtering strategy was successful. Moreover, the sodium calcium exchanger was found uniquely enriched in the combined cardiomyocyte PM fraction. Similarly the known endothelial PM marker, von Willebrand factor, was enriched in the coronary endothelial cell PM subset, which further solidified the success of the enrichment and filtering of PM proteins.

IV. DATA MINING STRATEGIES USED TO IDENTIFY CANDIDATE PROTEINS

A. Selection of Candidate Proteins Enriched at the Cell Surface of Cardiomyocytes

A major goal of this study was to identify a set of proteins that were found to be enriched in cardiomyocytes and the plasma membrane, and that may be vital to cardiomyocyte function. Therefore to focus on cardiomyocyte PM proteins, the data mining strategy, depicted in Figure 19, began with accepting proteins that were found only in the CS-enriched datasets of the hCMs and/or the hfVCs. This mining criterion was followed by subtracting PM proteins found enriched in the endothelial cells and smooth muscle cells. Proteins that have previously been shown to be highly enriched in endothelial cell plasma membranes such as vascular cell adhesion molecule, von Willebrand factor, PECAM1,
intracellular cell adhesion molecule-1\textsuperscript{128} and caveolin-1\textsuperscript{129}, were removed. PM proteins that are found in many other cell types such as the neuronal isoform of DHPR\textsuperscript{130} and PMCA1 and 4\textsuperscript{131}, as well as contaminating proteins such as haemoglobin, were removed. Consequently these criteria were successful in focusing the candidate dataset to proteins that may be more enriched in cardiomyocyte plasma membranes than in the PMs of any of the other major cell types of the heart.

The next data mining step focused on subtracting out proteins that did not have a cell-surface associated annotation, which effectively removed nuclear, mitochondrial and cytoskeletal proteins such as histones, cytochromes, and myosins respectively. The final strategy focused on proteins that may be vital to cardiomyocytes and therefore proteins that were not present in the mouse cardiomyocyte PM study conducted by Sharma et al. were removed. Furthermore proteins that had a combined hfVC and hCM CS-enriched spectral count of less than 5 were removed. This step effectively removed more nuclear and mitochondrial contaminants as well as a few unknown proteins, thus focusing on proteins that are more likely to be vital to cardiomyocytes. Altogether, these strategies focused in on vital PM proteins that were enriched in cardiomyocytes and depleted in the other major cell types of the heart.

B. Understudied, Cardiac-Enriched, Cell Surface-Associated PM Proteins

Another aim of this study was to identify proteins that were understudied in the cardiovascular field, had an increased likelihood of being more specific to cardiac tissue, and were enriched only at the cell surface. To fulfill these qualifications, proteins were removed if they 1) had one or more intracellular annotations such as ER, mitochondrion, cytoskeleton and nucleus 2) were not found enriched in heart tissue or cardiomyocytes in the Novartis MircoArray database 3) have been extensively studied in cardiac health and disease and 4) had mRNA enrichments in 10 or more non-cardiac tissues and cells. This bioinformatic and literature analysis effectively removed majority of the proteins that were found to be associated with the PM and found in other organelles, such as the mitochondrial
ATPase, ATP5b\textsuperscript{109}, VDAC1\textsuperscript{110}, and RYR2\textsuperscript{132}. Proteins with an mRNA transcript that were not found to be enriched in heart tissue or cardiomyocytes were removed, such as the Na/K ATPase and BCAM\textsuperscript{133}. Proteins that are not novel to cardiovascular research, such as Annexin A2\textsuperscript{134}, were removed.

The overall mining strategy has focused on ten potentially vital proteins, enriched at the cell-surface with transcripts enriched more in the heart than most other tissues or cells in humans. These candidates were listed in Table 1 and included isoform 1 of popeye domain-containing protein 2 (POPDC2), protein kinase C and casein kinase substrate in neurons protein 3 (PACSIN3), myeloid-associated differentiation marker (MYADM), isoform 1 of acetolactate synthase-like protein (ILVBL), isoform 1 of caprin-1 (CAPRIN1), matrix metalloproteinase-14 (MMP14), isoform 2 of nebulin-related anchoring protein (NRAP), glypican-1 (GPC1) and septin-11 (SEPT11). As seen in Table 1, due to unavailable cDNA constructs required for further experiments, only the top four candidates, namely POPDC2, PACSIN3, MYADM and ILVBL, were chosen for further plasma membrane validation experiments.

V. LOCALIZATION OF PROTEIN CANDIDATES

A confirmation of subcellular localization of each protein candidate was acquired by tagging candidate cDNAs and transiently transfecting them into human embryonic-293 (HEK) cells. These cells were layered on top of a continuous 20-60% sucrose density gradient to identify which subcellular fraction the tagged candidates eluted in. Sucrose gradient centrifugation was combined with confocal microscopy of HEK cells stained against the appropriate candidate tag. Electron microscopy has previously shown that sucrose density fractionation has successfully separated different cellular organelles into fractions\textsuperscript{99}. Density fractionation showed that ILVBL eluted in the first seven fractions similar to the endoplasmic reticulum protein, PDI and the nuclear protein, the beta estrogen receptor. The immunofluorescent image of ILVBL displayed a cytoplasmic staining pattern comparable to the GAPDH fluorescence. MYADM’s immunofluorescent image portrayed a
vesicular staining pattern which was similar to results collected by Dannaeus et al. who described its localization as “the nuclear envelope and intracytoplasmic membranes”\textsuperscript{135}. The elution profile of MYADM also confirmed its localization to the nucleus and intracytoplasmic membranes because it eluted in the same fractions as the nuclear protein the beta estrogen receptor and the coat protein, alpha COP, which is involved in retrograde protein transport from the Golgi to the ER.

The expression of NRP1, POPDC2 and PACSIN3 was clearly different from the expression of MYADM and ILVBL in the sucrose gradients and the confocal images. Density fractionation showed that NRP1, POPDC2 and PACSIN3 eluted in the same fraction as the endogenous plasma membrane protein the Na/K ATPase indicating that these proteins may be localized in the plasma membrane. PM localization was confirmed by confocal microscopy which showed that NRP1, POPDC2 and PACSIN3 transfected HEK cells had distinct cell surface staining as compared to GAPDH transfected cells. However, the elution profile for NRP1 and PACSIN3 extended into the same fractions as the endoplasmic reticulum protein, PDI and the coat protein, alpha COP. The presence of NRP1 and PACSIN3 protein in the same fractions as the ER protein and coat protein may have been caused by overexpression of these transfected proteins. NRP1 is a known cell membrane protein and CD molecule shown to be involved in angiogenesis\textsuperscript{136} and it belongs to the vascular endothelial growth factor family of proteins that has been implicated in cardiac disease\textsuperscript{137}. Therefore, in this study it was used as a positive control for cell surface localization. The role of PACSIN3 in the heart has not been investigated in the literature. It contains an SH3 domain which has been shown to be involved in endocytosis and clathrin coated vesicle formation\textsuperscript{138}. Previously, the overexpression of PACSIN3 in adipocytes was shown to increase the PM expression of the glucose transporter type 1 protein indicating that it may be involved in endocytosis\textsuperscript{139}. More recently however, it has been suggested that PACSIN3 may also bind to the PM transient receptor potential channel 4 from the vanilloid subfamily of proteins, to modulate its activity\textsuperscript{140}. Although PACSIN3 may be involved in vesicle trafficking there is evidence that it may also play a role at the plasma membrane\textsuperscript{140}. The POPDC family has been found to be highly expressed in mouse, rat, chicken and human heart tissue\textsuperscript{141, 142}. POPDC2 is from the same protein family as blood vessel epicardial substance protein, which concentrates at cell-cell junctions in epithelium to regulate integrity by associating with tight junctions\textsuperscript{143}. It has been proposed that POPDC2 may be involved in
chick heart development\textsuperscript{144} and an abstract published in Circulation connects POPDC2 null mice to stress-induced cardiac sinus node dysfunction\textsuperscript{145}. In this Circulation abstract, Brand et al. suggested that the stress-induced sinus node dysfunction was not due to aberrant electrical conductance but instead it was linked to degradation of the sinoarterial node tissue\textsuperscript{145}. Both PACSIN3 and POPDC2 are proteins that are understudied in cardiac health and disease, enriched in human cardiomyocytes and according to this study and the literature they may also play a role at the plasma membrane. This study therefore was successful in combining silica bead membrane isolation and mass spectrometry based proteomics to isolate and identify proteins that are understudied in the cardiac literature and are enriched at the cell surface and in human heart tissue.
CHAPTER FIVE: LIMITATIONS

This study utilized silica bead membrane extraction to isolate PM proteins of *in vitro* and *in vivo* human cardiomyocytes, coronary smooth muscle cells and coronary endothelial cells. Cells in culture are not the best representation of *in vivo* cells because cells in culture are situated in a different microenvironment. The proteins in the plasma membrane of cells in culture may differ to the PM proteins of *in vivo* cells because the expression levels of certain proteins may be reinforced by cues from the external environment. It was already shown that the *in vitro* cardiomyocytes were different in morphology and protein content from the *in vivo* cardiomyocytes and that is why *in vivo* cardiomyocytes were included in this study. Due to the difficulty associated with obtaining fresh human coronary vessels and with isolating endothelial cells and smooth muscle cells, cultured hcECs and hcSMCs were obtained commercially. PromoCell cells, however, were not cost-effective, could only be passaged a maximum of six times before they degraded and no longer represented the cell type of interest, and they multiplied at a very slow rate. Consequently a large amount of cells, required for cell surface procedures that better enrich for CS proteins and deplete for intracellular proteins such as glycocapture, could not be generated. Furthermore silica bead experiments could only be done a few times before cell degradation.

During silica bead extraction, cationic silica beads bind to the anionic PM, which is separated from the rest of the cell contents by lysis and centrifugation. Many intracellular proteins and organelles are still connected to the PM by cytoskeletal linker proteins. The high centrifugation spin removes many of the low density intracellular proteins while the high density silica pellicle travels to the bottom. As found in the data, not all intracellular contaminants were removed. Other PM isolation procedures which were more effective in removing intracellular protein contamination from the PM fraction, such as glycocapture and biotinylation, mainly focused on proteins with extracellular protein domains. Therefore, to acquire a comprehensive PM protein isolation, including proteins found on the cytoplasmic face of the PM, the silica bead extraction procedure was applied. However, in retrospect, in the silica bead procedure it may have been beneficial to include further washing steps with either sodium carbonate or another buffer following ultracentrifugation, to remove intracellular contamination. Since previous studies alluded to contamination of intracellular proteins using this procedure, cell surface biotinylation was performed on all cell types to further focus on PM proteins with
an extracellular protein domain. Numerous attempts at surface biotinylation repeatedly failed and therefore focusing on cell surface proteins with an extracellular protein domain could not be completed. Therefore, subsequent experiments and analyses were done on samples collected using colloidal silica bead methods with the potential for contamination from intracellular proteins.

Potential loss of protein was also a limitation during this study. Silica bead extraction of PM proteins of cells in culture was not complete since the basal PM was not coated with silica beads. Proteins destined for the membrane-depleted fraction may have loosely bound to the silica pellicle after cell lysis and these loosely bound proteins could have been removed in the nycodenz gradient following ultracentrifugation. Loss of proteins from the membrane enriched fractions may have occurred due to incomplete solubilisation of membrane proteins due to extreme hydrophobicity of proteins or supersaturation of the solubilising agent. Loss of analytes from all samples may have occurred during MS sample preparation due to potential irreversible acetone precipitation or marginally ineffective trypsin digestion. MS analysis biased towards the most abundant proteins since any protein that had less than 2 unique peptides per fraction were removed. However, without this filtering the potential for false positives would have increased. Further bias for abundant proteins in the CS-enriched dataset occurred due to the stringent filtering applied to the membrane-enriched fraction.

Bioinformatic analyses of proteins using the Gene Ontology database to annotate for subcellular localization and biological process, and the TMHMM 2.0 program to predict for transmembrane helices had a few limitations. GO annotations can be inferred from experimental evidence, a computational analysis, another database, or a curator who makes judgements about a gene or protein based on its association with another GO-term. As a result, GO annotations are fallible and cannot give a perfect representation of the data. The TMHMM 2.0 program makes predictions about domains within a protein based on a mathematical model and it has been reported that the program can make approximately 3% of over-predictions (ie. false positives) and 3% of under-predictions (false negatives)\textsuperscript{94}. Consequently, of the 611 predicted transmembrane helices from the CC CS-enriched dataset, approximately 18 predicted helices could be inaccurate.

Limitations also occurred when studying the various datasets uncovered during the study. The endothelial and the smooth muscle cell datasets were not extensively characterized in this study although they are vital cell-types involved in cardiovascular health and disease. The focus
of this study was human cardiac myocyte cell surface proteins and since the in vivo ventricular tissue consisted of endothelial and smooth muscle cells, the data obtained from silica bead experiments of the hcECs and hcSMCs was used in this study as a method to select against contaminating hcEC and hcSMC proteins. However, the hcEC and hcSMC datasets could provide essential information about the coronary vasculature and thus would be interesting to study further in the future.

Another limiting factor was that not all potential candidates were confirmed for plasma membrane localization. Localization experiments required access to cDNAs of proteins that could be cloned into a tag and subsequently visualised by confocal microscopy. Unfortunately, not all candidates had available cDNAs, and MMP14 and CAPRIN1 had different sequences in their respective wells. The candidates that did not have available cDNAs were available as antibodies that could be purchased from various companies. However I chose to confirm the subcellular localization of proteins using the transfection of cloned tags and I used the unavailability of cDNAs for other proteins as an exclusion criterion to select a manageable set of candidates to further study. Consequently candidates that had unavailable cDNAs were not investigated to elucidate their subcellular localization but they may still play a vital role in cardiomyocyte function at the plasma membrane.

A final limitation involved the use of human embryonic kidney cells for plasma membrane localization of cardiomyocyte proteins. The transfection of candidates into mouse neonatal cardiomyocytes and human ventricular fibroblasts was attempted several times using various different transfection reagents under several different conditions (ie. varying incubation time and concentration of reagent and DNA). However, these attempts repeatedly failed and produced slides with zero transfection efficiency. A double transfection was performed on ventricular fibroblasts in concert with the double transfection of HEK cells. The transfection of HEK cells however worked well and yielded approximately a 20% transfection efficiency. However none of the cells in the fibroblast transfection were transfected, therefore HEK cells were utilized for subsequent subcellular localization experiments. Unfortunately the HEK cells were small and had large nuclei, which made it difficult to visualize cell surface staining as compared to intracellular or nuclear staining. To account for the difficulty in distinguishing between the plasma membrane and the nuclear membrane or intracellular space, sucrose density fractionation was employed to confirm confocal microscopy results.
CHAPTER SIX: NOVEL INNOVATIONS AND FUTURE DIRECTIONS

This study has combined silica bead membrane extraction with two-dimensional tandem mass spectrometry to isolate and identify proteins enriched in the PM of human cardiomyocytes, smooth muscle cells and endothelial cells. Two proteins, understudied in the cardiac literature and enriched in cardiomyocytes and at the cell surface, have been identified using a bioinformatic and subtractive proteomic data mining strategy. Furthermore, POPDC2 and PACSIN1 were shown to have confirmed localization in the plasma membrane of human embryonic kidney cells that were transfected with tagged candidate cDNA and subsequently subjected to immunofluorescence and sucrose gradient fractionation.

Understanding the role of theses protein candidates in cardiovascular health and disease in humans will be the overall goal of any potential future studies. The next immediate steps will be to assess potential function of candidates and confirm localization of these proteins in cardiomyocytes. The former can be completed using knockdown or overexpression of each protein in combination with appropriate functional assays based on the predicted function of candidates. For example, PACSIN3 has been shown to alter the activity of a plasma membrane transient receptor potential channel\textsuperscript{140} and an appropriate functional assay to assess its function could be a membrane potential assay. It has been suggested that POPDC2 may be involved in an age-specific degradation of the sinoarterial node following stress therefore an apoptosis assay following stress would be an appropriate functional assay for this candidate protein. If a notable change in normal cell function occurs then antibodies for each protein will be obtained and endogenous immunofluorescent or immunohistochemical staining of proteins in cardiomyocytes or cardiac sections respectively can be elucidated. Furthermore, tandem affinity purification can be employed to elucidate any binding partners the protein may have. Together, these studies will characterize the candidate proteins.

Subsequently an association of that protein with current cardiac diseases can then also be predicted. A genetic screen of patients presenting with the predicted disease can be completed to test if any mutations exist in the protein of interest. If a mutation in this protein does exist a quantitative assessment of the candidate mRNA levels and protein expression in diseased cardiac tissue compared to healthy cardiac tissue can then be completed using quantitative
polymerase chain reaction and multiple reaction monitoring mass spectrometry respectively. If a mutation is not present, the candidate protein could still be essential in regulation of proteins shown to be involved in that disease. Model organisms displaying symptoms of the predicted disease can be obtained or made, to test if varying candidate expression levels change the disease phenotype. Together these studies will elucidate whether these candidate proteins play a role in cardiac health and disease.
CHAPTER SEVEN: REFERENCES


126. Kim, I. et al. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin


