Understanding extracellular vesicles biology through the study of protein dynamics using pSILAC-based quantitative proteomics

Tan, Chee Fan

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**NANYANG
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**UNDERSTANDING EXTRACELLULAR
VESICLES BIOLOGY THROUGH THE
STUDY OF PROTEIN DYNAMICS USING
PSILAC-BASED QUANTITATIVE
PROTEOMICS**

**TAN CHEE FAN
INTERDISCIPLINARY GRADUATE PROGRAM
NTU INSTITUTE FOR HEALTH TECHNOLOGY
(HEALTHTECH NTU)**

2019

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TAN CHEE FAN

**INTERDISCIPLINARY GRADUATE PROGRAM
NTU INSTITUTE FOR HEALTH TECHNOLOGY (HEALTHTECH NTU)**

**A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement of the degree of
Doctor of Philosophy**

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Abbreviation

α -MSH	Alpha-melanocortin stimulating hormone
ABB	Ammonium Bicarbonate
ACN	Acetonitrile
AF4	Asymmetric-flow field-flow fractionation
AGC	Automatic gain control
AgRP	Agouti-related protein
Arc	Arcuate nucleus
BAT	Brown adipose tissue
BCA	Bicinchoninic acid
BCAS	Bilateral carotid artery stenosis
CA-074 Me	CA-074 methyl ester
CART	<u>Cocaine- and amphetamine-regulated transcript</u>
CL	Cell lysate
Ctsl-I	Cathepsin L inhibitor II
db	Diabetes
DHA	Dorsal hypothalamic area
DiR'	1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindotricarbocyanine Iodide
DMN	Dorsomedial nucleus
DTT	Dithiothreitol
emPAI	Exponentially modified protein abundance index
ESCRT	Endosomal sorting complexes required for transport
EVs	Extracellular vesicles
FA	Formic acid
FBS	Fetal bovine serum
GABA	Gamma aminobutyric acid
GI	Gastrointestinal
H/L	Heavy to light
HCD	Higher energy collisional dissociation cell
HPLC	High performance liquid chromatography
IAA	Iodoacetamide
ICAT	Isotope-code affinity tag
iLVS	Intraluminal vesicles

ISEV	International Society of Extracellular Vesicles
iTRAQ	Isobaric tags for relative and absolute quantitation
Jak	Janus-activated kinase
LC-MS/MS	Liquid chromatography coupled tandem mass spectrometry
LHA	Lateral hypothalamic area
m/z	Mass to charge
MCH	Melanin-concentrating hormone
MC4R	Melanocortin 4 receptor
MISEV	Minimal Information for Studies of Extracellular Vesicles
MVBs	Multivesicular bodies
MVs	Microvesicles
NPY	Neuropeptide Y
NTA	Nanoparticle tracking analysis
Ob	Obesity
Ob-R	Leptin receptor
OXs	Orexins
PD	Proteome Discoverer
Pep-A	Pepstatin A
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus
qPCR	Quantitative PCR
SEC	Size exclusion chromatography
sEVs	Small EVs
SILAC	Stable isotope labelling with amino acids in cell culture
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
STAT	Signal transducers and activators of transcription
TEM	Transmission electron microscopy
TMT	Tandem mass tags
UniProtKB	UniProt Knowledgebase
VMN	Ventromedial nucleus
WAT	White adipose tissue
XIC	Extracted ion chromatogram

Summary

Extracellular vesicles (EVs) are cell-derived membranous vesicles that are secreted by all cell types of eukaryotic and prokaryotic origins. These vesicles can be classified into endosomal-derived exosome, plasma membrane-derived microvesicles or apoptotic bodies. In particular, small EVs such as exosomes and microvesicles were shown to play an important role in intercellular communication as they contained an array of biological molecules such as proteins, nucleic acids and lipids. Our lab has also previously demonstrated the role of these small EVs in hypoxia-induced cancer progression, neurodegenerative disease and cardiovascular disease. As research on EV-mediated intercellular communication continues to grow exponentially, a pertinent issue to address is the origin of these vesicles. Regarding the vesicle's origin, the endo-lysosomal system is an important juncture where MVBs formation occurs and eventually the MVBs would fuse to the lysosome for degradation or be transported to the plasma membrane for secretion. Owing to the secretory nature of EVs, I postulated that proteins essential for EV biogenesis or EV-mediated intercellular communication have a higher synthesis rate to replace those that had been secreted out. Therefore, the objective of my PhD thesis is to develop a system biology approach driven by pSILAC-based quantitative proteomics methodology to study the protein dynamics in EVs and examine their role in EV biogenesis and intercellular communication.

The PhD thesis is divided into two components. In the first component, I had established a pSILAC-based quantitative proteomics methodology to study EV biogenesis by profiling the newly synthesized proteome of the EVs and lysosome derived from mHypoA 2/28 adult hypothalamus cell line. I had also postulated that the preferential sorting of newly synthesized proteins into the EVs instead of the lysosome may reveal a divergent role of these proteins in regulating the fate of MVBs. Hierarchical clustering analysis identified a group of newly synthesized cathepsin proteins that accumulated preferentially to the EVs. The unexpected localization of these newly synthesized cathepsin proteins to the EVs suggested their potential function in regulating the

destination of MVBs. Functional characterization of the cathepsin proteins revealed a role of cathepsin D in EV biogenesis. In particular, I found that the EVs fractions contained only newly synthesized cathepsin D and cellular cathepsin D was localized to the endosome-enriched fraction. Chemical inhibition of cathepsin D with pepstatin-A promoted EVs secretion in mHypoA 2/28 cells and this was accompanied by an upregulation of Rab7, Rab27B and Rab35 cellular expression. These Rab GTPases are involved in EVs secretion. Pepstatin A inhibition also increased the cellular and EVs protein expression of CD63 but reduced CD81 expression. This suggested a perturbation to the EVs biogenesis pathway that led to an increased in EVs secretion as well as a change to the EV subpopulation secreted. Taken together, I have demonstrated the usage of pSILAC quantitative proteomics in the study of EV biogenesis through examining the spatial distribution of the newly synthesized proteins between the EVs and lysosome fractions from mHypoA 2/28 cells.

The second component of this PhD thesis focused on understanding the role of hypothalamus-derived EVs in energy homeostasis. The mHypoA 2/28 hypothalamic cell originated from POMC neurons, which are regulated by leptin to maintain energy homeostasis. In recent years, a study had shown the hypothalamus ability to secrete exosomes to regulate biological function, hence indicating an alternative route where neuronal cells could propagate leptin mediated activity. As the arcuate nucleus is located adjacent to the median eminence, I postulated that EVs, which has the ability to cross the blood brain barrier, could enter the systemic circulation easily to promote intercellular communication. Therefore, I utilised the pSILAC-based quantitative proteomics methodology to establish a new mode of intercellular communication through mHypoA 2/28-derived EVs. I found that leptin treatment promoted additional protein sorting into the mHypoA 2/28 EVs as well as an increased in EVs secretion. Gene ontology analysis revealed that newly synthesized EV proteins from leptin-treated mHypoA 2/28 to be involved in biological processes related to peptidyl-tyrosine auto-phosphorylation, neuron projection development and extracellular matrix reorganization; functioned in kinase activities; and enriched in the basement membrane, extracellular matrix and lysosome. In contrast, newly synthesized proteins that preferentially sort to

EVs derived from basal mHypoA 2/28 hypothalamus cells were involved in biological process such as cellular response to LDL particle stimulus, negative regulation of Wnt signalling pathway, proteolysis involved in cellular protein catabolic process and cellular response to glucose stimulus; performed molecular functions such as threonine-type endopeptidase activity, integrin binding, enzyme binding and hydrolase activity; and enriched in cellular location such as focal adhesion, lysosome and extracellular exosome. Subsequently, I studied the spatiotemporal distribution and physiological functions of the mHypoA 2/28 EVs in C57bl/6J mice. Interestingly, EVs from both conditions displayed similar results. They were accumulated in the liver and spleen 72 h post injection. Mice injected with either type of EVs displayed a decreased in blood glucose level and body weight in the first 24 h after EVs injection. Gene analysis of the tibialis anterior muscle from mice injected with EVs from basal mHypoA 2/28 cells showed a small but significant increase in GLUT4 expression (2.26-fold). This indicated that mHypoA EV may be involved in the regulation of glucose homeostasis through its action on skeletal muscle and this process is independent of leptin action. Based on the gene ontology analysis mentioned above, it seems that EVs from leptin-treated mHypoA 2/28 cells may play an alternative role instead by acting as a leptin-mediated paracrine messenger to regulate neuronal connection in the central melanocortin system rather than as a messenger for inter-organ communication. Nonetheless, this study had established a functional role of mHypoA 2/28 EVs in the regulation of systemic blood glucose homeostasis.

In conclusion, I have adopted a discovery-driven approach and successfully applied a pSILAC-based quantitative proteomic methodology to perform proteome-wide analysis of newly synthesized proteins in EVs. This is a novel approach in the study of EV biogenesis and intracellular communication and offers a new perspective in the understanding of EVs biology. The finding in this thesis could contribute towards the development of an EV-based therapeutic treatment against metabolic diseases such as hyperglycaemia.

Chapter 1

General introduction

1. General Introduction

1.1. Introduction

1.1.1. Discovery of extracellular vesicles

Extracellular vesicles (EVs) are cell-derived membranous vesicles that contain cytosolic materials which are released out into the extracellular milieu ^[1]. The secretion of EVs into the extracellular milieu seem to be an evolutionary conserved process as it occurs in both eukaryotic and prokaryotic cells ^[2]. In multicellular organisms, EVs are produced by many cell types and can be collected from bio-fluid such as blood ^[3], urine ^[4], saliva ^[5], cerebrospinal fluid ^[6] and semen ^[7].

Study on EVs first gained prominence through the study of transferrin receptor removal during reticulocyte maturation in 1983 ^[8,9]. These MVB-derived secreted vesicles were subsequently described as exosomes ^[10]. It was only about a decade later that other function of exosome was further explored. Raposo et al. first demonstrated the role of endosomal-derived EVs from B-cells and dendritic cells in antigen presentation through the stimulation of CD4+ T cells in vitro ^[11]. This study proved that secreted exosomes were not merely a mean of waste disposal by the cell but rather a functional unit that has a biological effect.

Nonetheless, research on EV biology only begins to gain more traction upon the discovery of mRNA and miRNA-containing EVs in 2007. mRNA-containing EVs were proven to be functional when mRNA in EVs isolated from mouse MC/9 cells were translated into proteins in HMC-1 human mast cells after co-incubation of the MC/9 EVs with HMC-1 cells ^[12]. This landmark study brought about a new perception of EVs as messengers carrying a cargo of proteins, nuclei acids and lipids to initiate molecular changes in distant recipient cells. Since then, research on EVs has garnered increasing attention in the scientific committee, with publication growing from 16 papers in 1998 to over 2482 papers in 2018 (Figure 1.1) and in the process establishing EVs as an important mediator of intercellular communication ^[13]. The rapid development in EV research has also resulted in the formation of the International Society of Extracellular Vesicles (ISEV) for EVs researchers. In a collaborative effort, the

ISEV community published the Minimal Information for Studies of Extracellular Vesicles guideline in 2014 (MISEV2014) ^[14] to facilitate systematic research on EV. The guideline was updated in 2018 (MISEV2018) ^[15]. The application of EVs for therapeutic or biomarker purposes has also seen a tremendous growth ^[16]. In particular, an exosomal RNA-based diagnostic test, ExoDxTM Prostate(IntelliScore) was recently developed to distinguish high grade prostate cancer from low grade prostate cancer in a non-invasive urine EVs sample analysis ^[17].

Figure 1.1: Number of exosome-related publications per year since 1987. Literature search was performed on the PubMed database using the keyword “exosome”. Selected prominent discovery in EV research was highlighted in the chart.

1.1.2. Functions of EVs

The effect of EVs on its recipient cells has been demonstrated in both physiological and pathological settings and was comprehensively reviewed by Yáñez-Mó et al. ^[18]. In addition, our lab has also published several studies and established the role of EVs in hypoxia-induced cancer progression, neurodegenerative disease and cardiovascular disease ^[3, 19-21]. Tumour hypoxia occurs when the rate of tumour growth far exceeds the blood supply in the

tumour microenvironment, and this promotes the alteration of EVs proteome and rate of secretion that enhances cancer progression. Park et al. first established that hypoxia-treated A431 cells secreted EVs induced angiogenesis in vivo through the chorioallantoic membrane assay ^[19]. Furthermore, iTRAQ-based quantitative proteomics analysis of these hypoxic A431 EVs revealed an increased in angiogenic factors expression that may explain the EV's angiogenic capacity ^[19]. Additionally, the proteomic analysis also revealed an alteration of the MMP-13-TIMP proteolytic system, in which the expression of MMP-13 was increased while TIMP-2 and -4 expression were decreased, thereby promoting cancer invasiveness ^[19]. Subsequently, Park et al. also demonstrated the role of cancer EVs in immunomodulation ^[20]. Firstly, hypoxic cancer EVs were able to induce a greater chemotactic response of RAW264.7 cells in a Boyden chamber assay as well as the recruitment of F4/80⁺CD206^{high} M2-like macrophage to the site of tumour mass. Furthermore, these EVs promoted M2-like polarization of bone marrow – derived myeloid cells which are known to promote cancer progression ^[22].

Myocardial infarction is caused by restricted blood flow to part of the heart which deprived the cardiac cells of oxygen. Inflammatory response set in quickly after myocardial infarction in an attempt to repair the damaged cardiac cells but prolong inflammation usually lead to necrosis and irreversible damage to the cardiac cells ^[23]. Cheow et al. compared plasma-derived EVs from patients with myocardial infarction against patients with stable angina and identified novel EVs markers that are upregulated in myocardial infarction ^[3]. These proteins were involved in biological processes such as complement activation, lipid metabolism and platelet activation. Concomitantly, fibrinogen components in myocardial infarction EVs were downregulated and indicated a suppression of coagulation activities by the damaged cells and was suggested by the authors as a potential therapeutic target.

Lastly, Gallart-Palau et al. examined brain-derived EVs from bilateral carotid artery stenosis (BCAS) mice against brain-derived EVs from early Alzheimer disease or mixed dementia patients and found similar proteins group were associated with brain ischemia ^[21]. Moreover, comparison of brain-derived EVs

with serum-derived EVs from BCAS mice identified a common set of proteins that were involved in neuronal damage. Taken together, the result indicated that vascular dysfunction is implicated in dementia and serum EVs associated with BCAS condition could serve as biomarker for idiopathic dementia.

1.1.3. Classification of EVs

Vesicles secreted by the cells are heterogeneous and generally classified based on their origin, size and composition (Table 1.1). As mentioned earlier, exosome originated from iLVs that are formed through inward budding of the endosomal membrane. These MVBs are then transported outward and fused to the plasma membrane for secretion. Due to the structure of the MVBs, the size of exosome is typically limited to between 30-150 nm ^[24]. Common cargoes found in the exosome included the nuclei acid (mRNA, miRNA and other non-coding RNA) ^[12, 20], tetraspanins (CD9, CD63, CD81) ^[25] and the endosomal sorting complexes required for transport (ESCRT) component (TSG101 and Alix) ^[26].

The second type of vesicle secreted by the cells are microvesicles (MVs). The MVs are formed through the direct budding of plasma membrane and have a diameter of 50-1000 nm ^[27]. They also contain nuclei acid ^[28, 29] but have distinctive protein marker such as CD40 ^[30]. Lastly, apoptotic bodies (>1000 nm) are released by dying cells and they are subjected to clearance by the phagocyte and contain cellular debris such as nuclear fragment ^[31]. Recently, apoptotic bodies have been proposed to promote intercellular communication in immune-modulation via its miRNA content ^[32]. Likewise, the apoptotic bodies also contain nuclei acids ^[33], as well as proteins such as annexin V ^[29] and histone proteins ^[34].

Table 1.1: Types of extracellular vesicles secreted by eukaryotic cells.

Type of EVs	Size	Origin	Content
Exosome	30-150 nm	Endosome	mRNA ^[12] , non-coding RNA ^[20] , tetraspanins (CD9, CD63, CD81) ^[25] , ESCRT component (Alix, TSG101 ^[26])
Microvesicles	50-1000 nm	Plasma membrane	mRNA ^[29] , non-coding RNA ^[28] , CD40 ^[30] , tetraspanins, TSG101
Apoptotic bodies	>1000 nm	Cytoplasmic fragment	miRNA, nuclear fragment, annexin V ^[29] , histone ^[34]

1.1.4. EVs isolation and characterization

Despite the clear distinction between exosomes and MVs in term of their biogenesis origin, isolation of pure exosome or small MV (<200 nm) fraction remained challenging due to their similarity in size and density^[35]. The ISEV committee has published several position papers as a guideline to standardize EVs research and ensure accuracy in the characterization of EVs and reproducibility of EVs functional studies^[14, 15]. In the below section, I highlighted the recommended EVs nomenclature for different methods of isolation, which is summarized in Table 1.2, along with the techniques used for global and single particle characterization of isolated EVs.

1.1.4.1. Method of EVs isolation

1.1.4.1.1. Ultracentrifugation

The classical method for EVs isolation from conditioned media or biological fluid is ultracentrifugation. A typical ultracentrifugation protocol would include a series of centrifugation consisting of an initial spin at 2000 x g to remove cell

and cellular debris, followed by a 10,000-16,000 x g spin to remove larger microvesicles and lastly the EVs are isolated by ultracentrifugation at 100,000-200,000 x g for 1-2 h. The EV pellet is usually subjected to two washing steps to reduce the amount of non-vesicles contaminant such as protein aggregates [36]. Serial ultracentrifugation allows a high recovery of EVs that are heterogeneous but contain non EV-component. A variant of the ultracentrifugation methodology is the density gradient ultracentrifugation where EVs are isolated based on their density. The EVs sample is loaded on top of a sucrose or iodixanol gradient and centrifuged at 200,000 x g for a longer time period of between 16-18 h [37, 38]. The addition of a density gradient allow better removal of contaminating protein aggregates as they have a different density from the EVs (exosome and smaller MV: 1.15–1.19 g/ml, protein aggregate: 1.22 g/ml) [35, 36, 39].

1.1.4.1.2. Size exclusion method

EVs can be separated based on their size or molecular weight by passing the sample through a barrier such as filter membrane (micro- and ultra-filtration) or gel beads (size exclusion chromatography). Filter membrane of different pore sizes (0.1, 0.22 and 0.45 μm) can be used to isolate EVs of a particular size range from the filtrate [40] and the isolated EVs can be further concentrated with a molecular weight cut-off filter. A drawback of the filtration method is the loss of sample [41] and protein contamination due to filter clogging [42].

In size exclusion chromatography (SEC), the sample is applied onto a column packed with polymer beads of various sizes (e.g. Sepharose CL-2B) and the vesicles are eluted at different time-points based on their hydrodynamic radius [43]. This method allows the efficient removal of contaminating proteins and unlike ultracentrifugation, minimal pressure is applied to the sample during elution, hence allowing a better retention of the EVs integrity. However, the SEC is unable to handle large sample volume, as such it is usually coupled to a ultrafiltration step and this had been shown to handle large sample volume (>100 ml) and facilitate optimal isolation of EVs that are functional [44].

Recently, a new technique known as asymmetric-flow field-flow fractionation (AF4) has been applied to EVs isolation. The AF4 is able to separate nanoparticles based on their hydrodynamic size and diffusion coefficient ^[45] and it can be coupled to a dynamic light scattering or UV absorbance device to measure hydrodynamic size and protein concentration respectively ^[46]. The AF4 is able to separate EVs at an impressive resolution of 1 nm and with this technology a new class of nano-sized structure, known as the exomere (35 nm), was identified in conditioned media of B16 melanoma cells ^[47].

1.1.4.1.3. EV precipitation

Another method for EVs isolation is EV precipitation with polymer. Polyethylene glycol is a commonly used polymer for EVs isolation ^[35] and it is a quick, simple and scalable method for EV isolation. Due to the ease of usage, numerous commercial kits were developed based on the principle of polymer precipitation, which included the ExoQuick from System Biosciences and miRCURY Exosome Isolation kit from Qiagen. The main drawback of the EV precipitation method is their inability to remove protein contaminants and the polymer may affect functional studies as it reduces cell viability ^[48].

1.1.4.1.4. Immunoaffinity isolation

Immunoaffinity capturing of EVs provides high specificity as it is based on the EV membrane protein composition. The transmembrane tetraspanins (CD9, CD63 and CD81) are popular targets for EVs pulldown and allow the identification of different subtype of EVs ^[49, 50]. The protocol is fairly straightforward as the EV samples are first purified, concentrated and then mixed and captured by antibody-coupled to magnetic bead and lastly they are eluted out with appropriate buffer ^[51]. The drawback of this method is that some antibodies may not be available and certain elution buffer may damage the EVs integrity and affect downstream functional studies.

1.1.4.2. EVs characterization

After EVs isolation, the sample is characterized for the presence of EVs and popular methods such as western blot analysis, single particle tracking analysis and electron microscopy imaging are used by researchers for EV

characterization ^[52]. For EV protein marker analysis, the MISEV recommended the analysis of three categories of markers. In category 1, transmembrane or proteins localized to the plasma membrane or endosome of the eukaryotes that represent the EVs should be probed and example of such proteins included the tetraspanins (CD9, CD63 and CD81) ^[25] and the MHC molecule ^[49]. In the second category, the presence of EV-related cytosolic protein that has membrane protein binding capability should be determined and this include ESCRT component such as TSG101 and Alix, flotillin and syntenin-1 proteins ^[49]. The third category of proteins are probed to determine the EVs purity. EVs isolated from plasma or conditioned media contained large amount of non EV material such as lipoproteins ^[53] and serum albumin ^[54]. Expression of other cellular protein markers such as annexin V (apoptotic bodies), GM130 (Golgi), Calnexin (endoplasmic reticulum) and VDAC (mitochondria) can be measured to indicate the absence of organelle contamination.

Global quantification of EV protein composition should be supplemented with single vesicle analysis to provide comprehensive analysis of the isolated EVs. The MISEV guideline recommended the visualization of single EVs at high resolution and profiling the biophysical properties of EVs. As small EVs (<200 nm) are below the diffraction limit of optical microscopy ^[55], non-optical method such as electron microscopy was used to visualize single EV particle. Transmission electron microscopy (TEM) is a well-established method for EV visualization but required the usage of heavy metal such as uranyl acetate as a contrasting agent for the biological membrane ^[56]. Furthermore, staining EVs with nanogold-coupled antibodies facilitate the detection of proteins in the EVs ^[57]. The sample preparation steps typically resulted in the EV collapsing into a cup-shape morphology in TEM. Subsequently, the usage of cryo-EM showed that the cup-shaped morphology of EV from TEM imaging was in fact an inaccurate portrayal of the EV morphology. The vitrification process in Cryo-EM imaging helps to preserve the EVs morphology and reveal a round-shaped vesicle that is encapsulated by a lipid bilayer ^[58].

Table 1.2: Pros and cons of commonly used EV isolation method.

EVs isolation method	Remark	MISEV category
Ultracentrifugation 1. Serial centrifugation 2. Density gradient ultracentrifugation	Pro: Able to process large sample volume, low cost if ultracentrifuge is available. Con: Unable to segregate different vesicles of similar size and density, serial centrifugation is damaging to exosome, low throughput.	1. High recovery, low specificity. 2. Low recovery, high specificity.
Size exclusion method 1. Ultra-filtration 2. Size-exclusion chromatography (SEC) 3. Asymmetric-flow field-flow fractionation (AF4)	Pro: Fast processing step when compared to ultracentrifugation. Able to remove contaminating protein from sample (SEC). EVs separation at high resolution (AF4). Con: Filter membrane will be clogged up (Ultrafiltration). Unable to process large sample volume (SEC, AF4).	1. High recovery, low specificity. 2. Low recovery, high specificity. 3. Low recovery, high specificity.
EV precipitation 1. Polyethylene glycol	Pro: Fast processing step, high throughput and simple protocol. Con: unable to remove contaminant proteins, polymer can cause cell death.	1. High recovery, low specificity.
Immunoaffinity isolation 1. Magnetic Bead Capture	Pro: Highly specificity. Con: Limited availability of antibodies, elution buffer may damage EVs integrity.	1. High specificity, low recovery.

In recent years, newer technology such as single particle tracking has become a popular choice for the characterisation of EVs [52]. Nanoparticle tracking analysis (NTA) is an optical-based methodology that can quantify the size and concentration of the EVs in a solution. The measurement is based on the light scattering property and Brownian motion of the EVs. These parameters are captured by video recording light microscopy and processed by the NTA software to provide information on the particle size and concentration [59].

1.1.5. EV biogenesis

The exosome (30-150 nm) originated as iLVs in the MVBs [1]. The endo-lysosomal system consists of an intricate and dynamic network of membranous compartment that transits from early endosome to late endosome and finally fused with the plasma membrane for secretion. Numerous mechanisms of MVBs formation have been discovered and they can be broadly divided into endosomal sorting complexes required for transport (ESCRT)-dependent or ESCRT-independent methods. In the following section, these mechanisms are discussed.

1.1.5.1. ESCRT-dependent biogenesis

The ESCRT system is the most well-studied mechanism for MVB formation and consists of 4 complexes (ESCRT-0, I, II and III) and their accessory proteins that sequestered ubiquitinated proteins into the iLVs [60]. The ESCRT-0 complex contains two subunits, hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) and signal transducing adaptor molecule 1/2 (STAM1/2) and they initiate MVB formation by linking ubiquitinated proteins to the endosomal membrane. Both Hrs and STAM contain ubiquitin interaction motif while the Hrs protein also contains a FYVE domain that binds to the phosphatidylinositol 3-phosphate of the early endosome membrane and a clathrin binding and coiled-coil domains that binds to STAM1/2 [61-63].

Hrs protein also contains two proline-rich regions that recruit the ESCRT-I complex by binding to the ubiquitin E2 variant domain of the tumour susceptibility gene 101 (TSG101) protein [64]. The recruitment of ESCRT-I complex to the endosomal membrane promotes more binding of ubiquitinated

proteins and recruits the ESCRT-II complex through the interaction between VPS28 (ESCRT-I) and VPS36 (ESCRT-II) ^[65]. The GLUE domain of VPS36 also binds to phosphatidylinositol 3-phosphate and ubiquitinated proteins. Together, ESCRT-0, I and II complexes form a stable oligomer that promote the sequestration of ubiquitinated protein into the iLVs via the interaction with endosome membrane component, phosphatidylinositol 3-phosphate.

Subsequently, ESCRT-II complex recruits and promotes the transient assembly of the ESCRT-III complex that enables membrane remodelling and inward budding of the endosomal membrane to form vesicles ^[66]. Lastly membrane scission is mediated by the ESCRT accessory protein, ATPase VPS4 ^[67]. These MVBs eventually fuse with the lysosome for protein degradation but in some cases the MVBs are transported along the microtubule to the plasma membrane for secretion ^[68]. Lysosome-mediated protein degradation requires the ESCRT machinery for sequestration of ubiquitinated proteins at the endosomal membrane, followed by inward budding of the membrane into iLVs for degradation ^[69, 70]. On the other hand, exosome formation seems to involve only a subset of the ESCRT machinery ^[26] and non-ubiquitinated proteins are sorted into the vesicle as well ^[71], which suggests that MVBs targeted for EV secretion can utilise other mode of MVB formation. It was recently discovered that syntenin-1, Alix and syndecan proteins played a role in ESCRT-III-dependent MVB formation ^[72, 73]. MVB formation is first initiated by the trimming of heparan sulphate sidechain on the syndecan protein by heparanase. This resulted in syndecan clustering that bring together the syndecan cargo and stimulate the binding of Alix protein to syndecan via syntenin-1 adaptor protein. The Alix protein then recruits the ESCRT-III machinery for MVB formation. On the other hand, heparanase promotes cargo sorting of CD63 but not CD9, CD81 or flotillin-1 into the exosome.

1.1.5.2. ESCRT-independent biogenesis and microvesicles biogenesis

Exosome biogenesis can also occur independent of the ESCRT machinery ^[74] through the conversion of endosomal membrane sphingomyelin into ceramide by the neutral type II sphingomyelinase (SMPD3) that promotes negative curvature of the endosomal membrane to form iLVs ^[75]. Tetraspanin enriched

micro-domain has also been suggested to promote exosome biogenesis as various tetraspanin knockout mouse model showed either a reduction in exosome number ^[76] or change in cargo content ^[71, 77].

The mechanism for MVs biogenesis is distinctive from exosome biogenesis as molecular cargoes are transported to the plasma membrane for budding and release rather than through the MVBs ^[78]. The process of MVs biogenesis has been shown to require the action of small GTPase such as ADP-ribosylation factor 6 (ARF6)^[79] and Ras-related protein Rab-22A (Rab22A)^[80]. Additionally, formation of MVs requires the activity of acid sphingomyelinase (SMPD1) rather than SMPD3, as illustrated in P2X7-dependent MVs biogenesis in glial cells ^[81]. Lastly, arrestin domain-containing protein 1 mediates the relocation of TSG101 from the endosome to the plasma membrane and facilitates ESCRT-dependent microvesicles biogenesis ^[82].

1.1.6. EVs secretion

The Rab GTPases are the largest family of GTPase and are known to regulate the secretion of different exosome population ^[83]. Among the 70 Rab GTPases, Rab7, 11, 27a/b and Rab35 are shown to be associated with exosome secretion. The late endosome marker, Rab7 is involved in the secretion of the syntaxin-1, Alix and syndecan-containing exosomes in MCF-7 cells and Rab7 knockdown resulted in enlarged endosomes and reduced exosome secretion ^[72]. GTP- and GDP- locked Rab11 mutation in K562 cells resulted in a decreased in transferrin receptor, protein Lyn and heat shock protein 70 expression in K562 exosome and a reduction in exosomal acetyl-cholinesterase activity, therefore illustrating the role of Rab11 in exosome secretion ^[84]. Rab27a and Rab27b are the most studied GTPase for exosome secretion. In HeLa cells, Rab27a and Rab27b are found at the MVB membrane while Rab27b is localized to the perinuclear region ^[85]. Knockdown of Rab27a and Rab27b proteins reduce exosome secretion and result in differential MVB phenotype but do not alter the exosome content and size. Rab27a silencing results in increased MVB size while Rab27b silencing causes an accumulation of MVB at the perinuclear region. Furthermore, the role of Rab27 on exosome secretion is demonstrated in in-vivo condition ^[86, 87]. Lastly, Rab35 silencing in Oli-neu cells results in a

decreased in proteolipid protein-containing exosomes and an accumulation of intracellular MVBs ^[88].

Subsequently, after the MVBs are transported to the plasma membrane, the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein mediates the fusion of MVB (v-SNARE) onto their targeted membrane region (t-SNARE). SNARE proteins such as YKT6, syntaxin 5 and Vamp7 have been demonstrated to facilitate the final docking and fusion of MVB to the plasma membrane for exosome release ^[89-91]. The action of SNARE protein is a regulated process. For example, in VAMP7 mediated MVB-plasma membrane fusion, the ATPase N-ethylmaleimide-sensitive factor (NSF) acted as a chaperone that catalysed the disassembly of v-SNARE and t-SNARE connection and a dominant-negative NSF mutant resulted in decreased exosome secretion ^[89, 92]. In contrast, MVs relied on a different set of GTPase proteins for secretion. The GTPase RhoA and its downstream ROCK-LIMK signalling pathway is required for MV formation in EGF-stimulated HeLa, MDAMB231 and U87 cancer cells ^[93].

1.1.7. Fate of MVBs: Degradation or Secretion

Matured MVBs can either fuse to the lysosome for degradation or be transported to the plasma membrane for release. As the outcome of these two processes are vastly different, a checkpoint must be in place for regulatory control. Indeed, when lysosomal function is compromised, it is accompanied by an increased in exosome secretion ^[94], indicating that lysosome degradation and exosome secretion are regulated together. Recently, several mechanisms have been discovered to regulate these two processes. Post-translational modification of TSG101 has been shown to cause a decreased in exosome secretion. ISGylation of TSG101 with ISG15 results in a reduction in MVB numbers but promotes protein aggregation and degradation in lysosome ^[95]. In contrast, tetraspanin 6 promotes exosomal secretion rather than lysosomal degradation of amyloid precursor protein fragment and overexpression of tetraspanin 6 results in decreased fusion of the autophagosome and lysosome, leading to an accumulation of the amyloid precursor protein fragment in the cells ^[96]. Nonetheless, the regulatory mechanism to determine the fate of MVBs remain

poorly defined given the complexity of MVB biogenesis that created different MVBs subpopulation and extracellular vesicles.

1.1.8. Leptin signalling in the central nervous system and energy homeostasis

Leptin, a member of the interleukin-6 cytokine family, is a hormone produced by the WATs to promote satiety. The physiological function of leptin was initially observed through a parabiosis experiment in which a leptin deficient (ob/ob) mouse joined to its wildtype compatriot was not only able to reverse its obese phenotype but also reduce food consumption tremendously ^[97]. The human leptin is encoded by the obesity (ob) gene located on chromosome 7 and translated into a 163 amino acid long, 16kDa protein that shared 84% homology to the mouse leptin ^[98]. The corresponding leptin receptor (Ob-R) is a single transmembrane receptor encoded by the diabetes (db) gene ^[99]. Ob-R has five isoforms (a, b, c, d and e) generated from alternative splicing of the db gene; of which Ob-Ra-d are transmembrane receptors while the Ob-Re is a soluble receptor ^[100]. Ob-Rb, which has the longest cytoplasmic domain among the five isoforms, is thought to be the main effector of leptin signalling and is highly expressed in the hypothalamus ^[101].

The concentration of circulating leptin is directly proportional to the adipose tissue mass, making it a key indicator of our body energy status. Irrespective of the adipose tissue mass, plasma leptin concentration does fluctuate over a short-term period owing to various physiological factors. Feeding activity affects leptin expression due to a nutrient sensing mechanism in the adipose tissue. Elevated level of intracellular glucose and fatty acids promote the hexosamine biosynthetic pathway and increase the expression of leptin ^[102]. Thus, secretion of leptin follows a diurnal rhythm which coincide with meal time and generally plasma leptin level peaks between midnight till early morning and plateau in the afternoon ^[103]. Other factors such as starvation ^[104], exercise ^[105] and insulin concentration ^[106] also affect leptin expression.

The hypothalamus is one of the key regions in the brain in regulating energy homeostasis. Situated inferior to the thalamus and surrounding the floor of the

third ventricle, the hypothalamus contains several Ob-Rb-expressing nuclei ^[107] (Figure 1.2) that mediate leptin actions. Seminal studies on hypothalamus nuclei lesion had identified lateral hypothalamic area (LHA) as the hunger center and ventromedial nucleus (VMN) and paraventricular nucleus (PVN) as the satiety center ^[108]. These nuclei contain second-order neurons that receive signals from the arcuate nucleus (Arc). Due to its proximity to the median eminence (circumventricular organ), the Arc is able to sense metabolic signal such as plasma leptin earlier than the other nuclei; hence neurons within the Arc are considered first-order neurons.

Figure 1.2: Leptin signalling occur in the hypothalamus. The hypothalamus consists of a group of nuclei that regulate the body energy homeostasis. The arcuate nucleus is located above the median eminence that allowed them to sense peripheral signal from the bloodstream. Leptin acts on the central melanocortin system to regulate energy homeostasis. (AgRP, agouti-related peptide; α -MSH, alpha melanocyte stimulating hormone; ARC, arcuate nucleus; BAT, brown adipose tissue; DHA, dorsal hypothalamic area; DMN, dorsomedial nucleus; GABA, gamma aminobutyric acid; LHA, lateral hypothalamic area; MCH, melanin concentrating hormone; MC4R, melanocortin 4 receptor; NPY, neuropeptide Y; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus; VMN, ventromedial nucleus.)

Within the Arc, leptin activates the anorexigenic pro-opiomelanocortin (POMC) neurons but antagonize the action of the orexigenic Neuropeptide Y (NPY)/ Agouti-related protein (AgRP) neurons ^[109]. Upon leptin induction, the POMC neurons secrete Alpha-melanocortin stimulating hormone (α -MSH), a neuropeptide derived from the post-translational cleavage of POMC. α -MSH activates the central melanocortin system, which consist of the POMC neurons, melanocortin receptor expressing neurons (PVN/VMH) and the brain stem ^[110]. The net effect of an activated central melanocortin system is a reduction in food intake with an increase in energy expenditure.

The NPY/AgRP neurons are activated by hunger signals such as ghrelin and reduced plasma glucose level. NPY is a neurotransmitter that act on the LHA and promotes the expression of orexigenic peptides such as orexin and melanin-concentrating hormone (MCH) that stimulate of food intake ^[111]. The NPY/AgRP neurons also secrete AgRP, which compete with α -MSH to antagonize the central melanocortin system. Additionally, NPY/AgRP neurons send GABAergic projections to the POMC neurons to inhibit their activity. At the cellular level, the Ob-Rb does not have any intrinsic enzymatic activity and rely mainly on the Janus-activated kinase/signal transducers and activators of transcription (Jak/Stat) signalling pathway for leptin activity. Upon activation, Jak2 auto-phosphorylate itself and also phosphorylate the tyrosine residues (Tyr985, 1077, 1138) found on the cytoplasmic tail of Ob-Rb ^[112, 113]. pTyr1138 recruits and phosphorylates STAT3 to promote homo-dimerization. The dimerized pSTAT3 is a transcription factor that translocate into the nucleus and promotes the expression of POMC in the POMC neurons but inhibit the expression of NPY in the NPY/AgRP neurons.

On the other hand, pSTAT3 also promotes the expression of suppressor of cytokine signalling 3 (SOCS3) which prevents prolonged leptin activity through the inhibition of pTyr985 and JAK2 phosphorylation ^[114]. Leptin-mediated signal transduction is a time-controlled event, regulated by several negative feedback mechanisms. Other than SOCS3, phosphatases such as protein-tyrosine phosphatase 1b (PTP1B) ^[115] and tyrosine phosphatase (TCPTP) ^[116] inhibit leptin signalling by dephosphorylating Jak2 and STAT3 respectively.

Separately, leptin also mediates its action through the PI3K/AKT and MAPK/ERK signalling cascade ^[117].

1.1.9. Mass spectrometry-based quantitative proteomics

1.1.9.1. General workflow of quantitative proteomics

Liquid chromatography coupled tandem mass spectrometry (LC-MS/MS) instrumentation is the state of the art quantitative proteomics technology which has greatly facilitated the quantification and identification of proteins in a high throughput manner that is well suited for discovery-driven research ^[118]. In MS-based quantitative proteomics, a bottom-up/shotgun approach dependent on enzymatic-cleaved peptide is widely used as it circumvent certain limitations such as protein ionization and fragmentation in the MS ^[119]. Typically, the protein sample is cleaved by a sequence-specific protease such as trypsin and the peptides are fractionated to reduce complexity for liquid chromatography coupled tandem mass spectrometry (LC-MS/MS) analysis. This additional fractionation step helps to improve protein identification.

The main feature of the LC-MS/MS include the usage of a high performance liquid chromatography (HPLC) to separate the peptides and as the peptides elute out of the column, an electrospray ionized the peptides to produce gaseous multiple-charged ions ^[120]. When introduced into the tandem MS, ionized peptides are sampled through the first MS to derive the precursor ion spectrum based on mass to charge (m/z) ratio ^[121]. The MS software algorithm then utilises a data –dependent top10 method to select the most abundant precursor ions for fragmentation in the collision chamber. The fragment ions are further analysis by a second MS for protein identification ^[122]. The raw data are mapped to a protein database such as UniprotKB ^[123] for protein identification through the usage of popular peptide search engine like Mascot ^[124] and Sequest ^[125].

In our lab, the Q-Exactive mass spectrometer from Thermo Scientific is the mass analyser of choice for high accuracy quantitative proteomics analysis as it offers high resolution and accurate mass capabilities that allowed the detection of low abundant proteins from highly complex sample ^[126]. The Q-Exactive

contained a fast switching quadrupole that is placed before an Orbitrap ion trap mass analyser to provide ion selection ability for fragmentation in the higher energy collisional dissociation cell (HCD). Ions selected for fragmentation is based on a data-dependent Top10 method that select the most abundant precursor ion for analysis. The fragmented ions are then injected into the Orbitrap analyser for analysis and detection is based on the differential oscillation frequencies along the electrode ^[127].

1.1.9.2. Type of quantification in MS-based proteomics

Absolute and relative quantitation are available in MS-based quantitative proteomics but absolute quantitation is not as widely used due to the requirement for expensive reagents such as synthesis of peptide standards and time-consuming protocols ^[128]. Relative quantitation does not require the usage of peptide standard for quantitation and instead compare peptides quantity between two or more samples from different conditions or treatments. Both label-free and label-based strategies have been developed for relative quantitation and they are listed in Figure 1.3 and will be discussed in the following section.

In label-free relative quantification, spectra of two or more samples are analysed to determine the protein abundance for comparison. Protein quantitation are determined by either spectral counting or precursor ion intensity. Spectral counting is based on the principle that a highly-abundant and/or large protein will have more peptides for identification as compared to a lowly-abundant and/or small protein and this method compares the MS/MS spectra of the chosen peptide in different samples ^[129]. Exponentially modified protein abundance index (emPAI) is a widely used spectral counting methodology that is implemented in the Mascot search engine ^[130]. It is calculated as 10 to the power of (number of experimentally observed peptides over number of observable peptide) minus 1. The alternative label-free quantification method is based on analysing the intensity of the peaks corresponding to the peptide precursor ions in the MS1 chromatogram, i.e. extracted ion chromatogram (XIC) ^[131].

Figure 1.3: Methods for relative quantification in quantitative proteomics.

Relative quantification can be categorized into label-based or label free methodology. In label-free method, quantification is done by spectral counting or precursor ion intensity (extracted ion chromatogram [XIC]). For label-based method, labelling can be done metabolically in method such as stable isotope labelling with amino acids in cell culture (SILAC) and Neucode. Protein/peptides can also be chemically labelled after sample preparation and some of these methods are isotope-code affinity tag (ICAT), isobaric tags for relative and absolute quantitation (iTRAQ) and tandem mass tags (TMT).

Label-based quantitative proteomics are routinely employed in our lab to study a wide range of biological questions. These labelling methodologies can be done either in-vivo (metabolic labelling) or on enzymatically cleaved peptides (chemical labelling). SILAC is a form of metabolic labelling that utilised non-radioactive amino acids to label cellular proteins ^[132]. Typically, labelled arginine or lysine (Figure 1.4) is used and the proteins are cleaved by trypsin to ensure that all peptides are labelled. The labelling process consists of two phases; adaptation phase and experimental phase. In adaptation phase, cells are grown in media containing labelled amino acids over several passages to obtain >95% incorporation of heavy amino acids. During experimental phase, cells, which are grown under different heavy amino acids, are subjected to differential treatment and the proteins are collected and combined for protein isolation, digestion and MS analysis. Quantification is done by taking the ratio

of the heavy peptide over the lighter peptide. Pulsed-SILAC (pSILAC) is a variant of the classical SILAC method and facilitates the study of protein synthesis rate rather than the concentration of the proteome^[133]. pSILAC do not have an adaptation phase and the labelled amino acids are introduced into the cell culture medium together with the treatment for a short period of time. Protein that are regulated by the treatment will incorporate these heavy amino acids during protein synthesis. The mass differences are detected by MS/MS and the rate of de novo protein synthesis can be determined by the heavy (labelled) to light (unlabelled) (H/L) isotope ratio, also known as the protein synthesis ratio.

Figure 1.4: Various arginine and lysine isotopes that are used in pSILAC-based proteomics. Lysine and arginine are commonly used amino acids used in SILAC labelling as they are cleaved by trypsin. This ensure that all trypsin-digested peptides are labelled for MS/MS analysis. Heavy carbon (¹³C) and nitrogen (¹⁵N) atoms are used to create the heavy amino acid. This give rise to L-Lysine (15N2) [+2Da] (A), L-Lysine (13C6) [+6Da] (B) and L-Lysine (13C6, 15N2) [+8 Da] (C) and L-Arginine (15N4) [+4Da] (D), L-Arginine (13C6) [+6Da] (E) and L-Arginine (13C6, 15N4) [+10Da] (F). # indicate the labelled atoms. Image of chemical structure obtained from Cambridge Isotope Laboratories, USA)

Chemical labelling of peptides is an alternative method for label-based quantification, and it is well-suited for cells that are very sensitive to changes in medium composition, i.e. not suitable to the SILAC medium or for samples that

are not amenable, such as clinical samples. iTRAQ and TMT compounds are typically used for peptide labelling and they contained isobaric tag that has a reactive moiety towards the primary amine group or cysteine. They are introduced to tryptic-digested peptides of different samples and then pooled together for MS/MS analysis ^[134, 135]. During CID, part of the isobaric tag is cleaved to create unique reporter ions that have different masses corresponding to a given sample which are detected by the second MS for relative quantitation.

1.1.9.3. EVs Proteomics

The combination of high resolution MS-based proteomics with various EV isolation methodology has allowed better characterization and understanding of EV subpopulations and their functional role in intercellular communication ^[136]. Through label-free proteomics profiling of EVs isolated by differential ultracentrifugation, flotation into density gradient and immunoaffinity isolation, Jowal et al. were able to characterized different EVs subpopulations secreted by human dendritic cells and the small EVs (sEVs) were categorized into 4 groups; 1) sEVs enriched in CD63, CD81 and CD9; 2) sEVs enriched in CD9 but absence of CD63 and CD81; 3) sEVs that do not contained the three tetraspanins; 4): sEVs enriched in ECM or serum proteins ^[49]. Moreover, the authors determined that commonly used exosome markers such as flotillin-1, MHC proteins and HSP70 were present in all types of EVs and therefore should not be used as exosome/sEV protein markers.

In another recent study, asymmetric flow field-flow fractionation was applied to EVs isolation from over 20 cell lines and based on the EV's hydrodynamic properties, three categories of EVs were determined which consisted of small exosome (60-80 nm), large exosome (90-120 nm) and a new class of nanoparticles known as the exomeres (~35 nm) ^[47]. Label-free MS-based proteomics analysis of these three types of EVs revealed that while both the small and large exosomes contained ESCRT components and Rab proteins, these proteins were not presented in the exomeres and indicated a different biogenesis mechanism for the exomeres.

To a lesser extent, label-based proteomics analysis has been utilised in characterizing EVs subpopulations. Jimenez et al. demonstrated the usage of iTRAQ labelling in distinguishing the content of small EVs and large MVs in colon adenocarcinoma cells ^[137]. By comparing the iTRAQ protein ratio, the authors found that small EVs, rather than the large MVs, are enriched in adhesion proteins that facilitate tumour cell migration. Separately, TMT-based quantitative proteomics was applied in the study of SKBR3B-derived EVs isolated by 10,000 x g, 100,000 x g and Optiprep gradient ultracentrifugation separately ^[138]. Sector vector machine cluster analysis on the TMT protein ratio of the three samples allowed the prediction on whether the proteins are of exosomal origin or protein contaminant.

1.1.10. Objective and overview of project

Due of their nano-sized dimension, small EV has a very high protein to lipid ratio ^[139] and this can enhance their intercellular signalling capacities. In recent years, EVs have indeed been found to partake in intercellular communication and as discussed earlier, our lab has established the role of EVs in hypoxia-induced cancer progression, neurodegenerative disease and cardiovascular disease ^[3, 19-21]. As research on EVs-mediated intercellular communication continue to grow exponentially, a pertinent issue to address is the origin of these secreted vesicles. Classically, vesicles secreted by the cells are categorized into endosome-originated exosomes, plasma membrane shredded microvesicles (MV) and apoptotic bodies. While exosomes and MVs are of different origins, purity of the isolated vesicles (vesicles that are <200 nm) has been hard to achieve due to the similarity in their size and density. Furthermore, Jowal et al. and Zhang et al. demonstrated that even among the small EVs, they have different protein content and possibly different origin. Regarding the vesicle's origin, the endo-lysosomal system is an important juncture where MVB formation occurs. The matured MVBs goes on to either fused to the lysosome for degradation or transported to the plasma membrane for secretion. As the outcome of these two processes are vastly different, a regulatory mechanism is required for these processes. Indeed, when lysosomal function is compromised, it is accompanied by an increase in exosome secretion ^[94], indicating that lysosome degradation and exosome secretion are regulated

concurrently. However, the mechanism that regulates the balance between EVs secretion and lysosomal degradation remains poorly elucidated.

At this moment, proteomics studies are widely used in the characterization of EVs subpopulation and their functional role but these studies have mainly focuses on the protein content and quantity ^[136]. Owing to the secretory nature of EVs, I believed that proteins essential for EVs biogenesis or intercellular communication have a higher synthesis rate to replace those that were secreted out. To the best of my knowledge, study on protein synthesis rate in EV proteins at a proteome-wide level has not been done and such information would provide a new perspective in the understanding of EVs biology. Therefore, the objective of my PhD thesis is to develop a system biology approach driven by pSILAC-based quantitative proteomics methodology to study the protein dynamics in EVs and their role in EVs biogenesis and intercellular communication.

This PhD work will be divided into two components. In the first component, I demonstrated the usage of pSILAC-based quantitative proteomics in the study of EVs biogenesis by profiling the newly synthesized proteome of the EVs and lysosome derived from mHypoA 2/28 adult hypothalamus cell line. I postulated that preferential sorting of newly synthesized proteins to the EVs instead of lysosome may reveal a divergent role of these proteins in regulating the fate of the mature MVBs toward EV biogenesis or lysosomal degradation. In this study, a combination of ultrafiltration and ultracentrifugation methodology were applied to isolation EVs from the conditioned media, while lysosome were isolated from a commercial kit. Gene ontology analysis on the proteomics data were carried out to identify newly synthesized proteins that preferentially localized to the EVs over the lysosome. Functional characterization of the protein candidate was conducted, and chemical inhibition of the protein candidate revealed their role in modulating EVs secretion.

The second component of this PhD work is focused on understanding the role of hypothalamus-derived EVs in energy homeostasis. The mHypoA 2/28 hypothalamic cell originated from POMC neurons that are regulated by leptin

to maintain energy homeostasis ^[109, 140]. In recent years, study had shown the hypothalamus ability to secrete exosomes to regulate aging process ^[141]. This indicated that neuronal cells have an alternative mode for intercellular communication other than the typical neuronal signal transduction pathway. Therefore, I seek to understand the role of EVs from leptin-treated mHypoA 2/28 cells in energy homeostasis. This would represent a novel mechanism in which the hypothalamus regulates energy homeostasis as previous studies mainly focused on hypothalamus-mediated signal transduction activities in energy homeostasis.

Chapter 2

Developing pSILAC-based quantitative proteomics for the study of EVs biogenesis

2. Developing quantitative proteomics approach to study EVs biogenesis

2.1. Chapter Summary

Cells are known to secrete EVs into the extracellular milieu and they can be categorized into MVBs-originated exosomes (30-150 nm), MVs (100-1000 nm) that shed from the plasma membrane and lastly apoptotic bodies (>1000 nm) from dying cell ^[1]. In particular, exosomes and MVs have been shown to promote intercellular communication in numerous physiological and pathological settings through the transfer of cytosolic materials such as proteins, mRNA and miRNA from the host to the recipient cells ^[12, 142-145].

Exosome originated as iLVs derived from the MVB in the endo-lysosomal system ^[1], which consists of an intricate and dynamic network of membranous compartment. These MVBs eventually fused to the lysosome for protein degradation or to the plasma membrane for exosome secretion ^[68]. As the outcome of these processes are vastly different despite the shared pathway, studies have been done to delineate these two processes. In lysosome-mediated protein degradation, the ESCRT machinery is crucial for sequestration of ubiquitinated proteins at the endosomal membrane and inward budding of the membrane into iLVs for degradation ^[69, 70]. In contrast, formation of exosomes seems to involve only a subset of the ESCRT machinery ^[26] and non-ubiquitinated proteins can be sorted into the vesicle as well ^[71]. An alternative ESCRT-dependent mechanism based on the interaction between the syntenin-1, Alix and syndecan proteins has been shown to promote the biogenesis of CD63+ exosomes as well ^[72, 146].

Exosome biogenesis can also occur independent of the ESCRT machinery ^[74] through the conversion of sphingomyelin to ceramide by the neutral type II sphingomyelinase (SMPD3) that promotes negative curvature of the endosomal membrane to form iLVs ^[75]. Tetraspanin enriched micro-domain has also been suggested to promote exosome biogenesis as various tetraspanin knockout mouse model resulted in either a reduction in exosome number ^[76] or change in cargo content ^[71, 77].

Recent findings have suggested an intricate link between lysosome status and EVs secretion where inhibition of lysosome activity has been linked to increase EVs secretion ^[147, 148]. However, the mechanism that regulates the balance between EVs secretion and lysosomal degradation remained poorly elucidated. Due to the secretory nature of EVs, it is likely that some of the proteins essential for EVs biogenesis have to be constantly synthesized to replace those that were secreted out. I postulated that preferential sorting of newly synthesized proteins to the EVs instead of lysosome may reveal a divergent role of these proteins in promoting EV biogenesis (Figure 2.1).

Figure 2.1: Schematic diagram illustrating MVBs formation and their eventual destination of either lysosome degradation or EV secretion.

Proteins cargos in the iLVs are derived from either plasma membrane, cytosol or newly synthesized proteins from the trans-golgi networks (A). Proteins are sorted into iLVs through the invagination of the endosomal membrane and this resulted in the appearance of MVBs (B). The matured MVBs can either fuse to the lysosome for protein turnover by the proteases or transported to the plasma membrane for secretion (C). MVBs destined for secretion are fused to the plasma membrane and the iLVs are releases as EVs. (EVs, extracellular vesicles; iLVs, intraluminal vesicles; MVBs, multivesicular bodies)

To the best of my knowledge, study of EVs protein synthesis rate at a proteome-wide level is not well-studied but such information would provide a new perspective in the understanding of EVs biogenesis. It was demonstrated in neuronal cells that lysosomal activities and EVs secretion are tightly linked as these cells were able to utilise EVs as an alternative mode for cargo disposal^[147]. Hence, I had utilised the mHypoA 2/28 adult hypothalamus cell line as an in-vitro model for this study. A pSILAC-based quantitative proteomics strategy was established to profile the newly synthesized proteome of the EVs, lysosome and total cell lysate of the mHypoA 2/28 cells. Heavy amino acids, arginine and lysine, were introduced to the cells for a short period of 24 h prior to harvesting. Lysosome was isolated using a commercial kit while the EVs was isolated through a series of ultrafiltration and ultracentrifugation steps. This isolation method would offer a high recovery of EVs but with low specificity as per the MISEV guideline and allow us to capture larger spectrum EVs for analysis. EVs isolated through this method is heterogeneous in nature and was therefore were referred to as small EVs.

Comparative proteomics analysis of the three samples showed that positive regulation of exosome secretion is an active process that required increased protein synthesis. Moreover, known EVs biogenesis related proteins such as Alix, syntenin-1 and the tetraspanins (CD9, CD81) had increased protein synthesis rate. More importantly, I demonstrated that newly synthesized proteins that accumulated preferentially to the EVs played an important role in their biogenesis. A group of cathepsin proteins were found to be actively synthesized and localized to the EVs fraction. Cathepsin proteins are the main lysosomal proteinases that function mainly toward protein degradation and recycling^[149]. The unexpected localization of these newly synthesized cathepsin proteins to the EVs suggest their potential function in regulating the destination of MVBs. In particular, the EVs fraction contained only newly synthesized cathepsin D protein (Ctsd), while the lysosome contained a mixture of pre-existing and newly synthesized Ctsd. Mature Ctsd proteins also preferentially localized to the endosome-enriched fraction during Optiprep ultracentrifugation. Chemical inhibition of Ctsd resulted in an increased in cellular gene expression of Rab7, Rab 27B and Rab35. This inhibition also

increased the cellular and EVs protein expression of CD63 but reduced the expression of CD81. This suggest a perturbation to the EVs biogenesis pathway that result in different EV subpopulation being secreted. Moreover, NTA measurement revealed a higher particle count in conditioned media collected from Ctsd-inhibited mHypoA 2/28 cells.

Taken together, I had demonstrated that pSILAC quantitative proteomics facilitated the study of EV biogenesis through examining the spatial distribution of the newly synthesized proteins between the lysosome and EVs fractions from mHypoA 2/28 cells.

2.2. Materials and methods

2.2.1. Reagents

All reagents used in the experiment were purchased from Sigma-Aldrich, USA, unless otherwise stated.

2.2.2. Cell Culture and pSILAC treatment

mHypoA-2/28 hypothalamic cell (*CELLutions* Biosystems, Canada) was grown in DMEM containing unlabelled “light” $^{12}\text{C}_6$, $^{14}\text{N}_2$ -L-lysine (146 mg/l) and $^{12}\text{C}_6$ -L-arginine (84 mg/l) (GE Hylcone, USA), supplemented with 5% EV-depleted fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin/streptomycin (Nacalai Tesque, Japan) at 37 °C in a 5% CO_2 humidified incubator. EVs-depleted FBS was obtained through ultracentrifugation at 200,000 x g for 18 h at 4 °C. For pSILAC treatment, upon reaching 80% confluency, the cells were washed with PBS twice and incubated in SILAC-DMEM (Cambridge Isotope Laboratories, USA) which contained 5% dialysed EV-depleted FBS (Gibco, USA), 1% penicillin/streptomycin and heavy $^{13}\text{C}_6$ -L-Arginine $^{13}\text{C}_6$ -Hydrochloride (84 mg/l) and $^{13}\text{C}_6$ -L-Lysine $^{15}\text{N}_2$ -Hydrochloride (146 mg/l) (Cambridge Isotope Laboratories, USA) for 24 h. The conditioned media was then collected for EVs isolation, while the mHypoA-2/28 cells were harvested for either total cell lysate or lysosome isolation. The mHypoA-2/28 cell was tested negative for mycoplasma contamination ^[150].

2.2.3. EVs Isolation

The EVs isolation protocol was based on our previous published article ^[20]. For each EVs isolation, 200 ml of conditioned media was collected from twenty 100 mm culture dishes with an approximate cell density of 60 million, unless otherwise stated. The conditioned media was centrifuged at 2000 x g for 20 min at 4 °C to remove cellular debris. The supernatant was then concentrated with a VivaSpin 20 centrifugal concentrator, 10kDa MWCO (Sartorius AG, Germany) at 4000 x g, 4 °C. Thereafter, the concentrated media was centrifuged at 16,000 x g for 30 min at 4 °C to remove the microvesicles. The supernatant was collected and diluted in 3 ml PBS to reduce the viscosity. The diluted supernatant was ultra-centrifuged at 100,000 x g for 16 h, 4 °C, in a Ti55 rotor (Beckman Coulter, USA). The EV pellet was subjected to two PBS washes and reconstituted in PBS containing protease inhibitor for immediate usage or was stored at -20 °C.

2.2.4. Lysosome Enrichment

Lysosome isolation was performed using the lysosome enrichment kit (Thermo Scientific, USA), as per manufacturer protocol. Briefly, the mHypoA-2/28 cell pellet was re-suspended in 800 µl buffer A and incubated on ice for 2 min. The cells were then lysed through probe sonication and mixed with 800 µl Buffer B. The sample was centrifuged at 500 x g for 10 min at 4 °C to remove cellular debris. 1500 µl of the sample lysate was then added to 500 µl of Optiprep cell separation media (60%). This solution was overlaid onto a discontinuous Optiprep density gradient (17%, 20%, 23%, 27% and 30%) and ultra-centrifuged at 145,000 x g for 2 h, 4 °C. Five equal volume were collected, washed and stored in PBS at -20 °C till further usage.

2.2.5. In-solution digestion and HPLC fractionation

mHypoA 2/28 cells were lysed with 8M Urea in 100mM Ammonium Bicarbonate (ABB), pH8, supplemented with protease inhibitor. Protein concentration was measured by Bradford assay. 200 µg of protein lysate was reduced in 20 mM dithiothreitol (DTT) for 1 h at room temperature and followed by alkylation with 55 mM iodoacetamide (IAA) in the dark for 45 min, at room temperature. The proteins were then digested with sequencing

grade modified trypsin (1:50), (Promega, USA) overnight at 37 °C and the reaction was subsequently quenched in 0.5% acetic acid (Merck, USA). The peptides were desalted using the Sep-Pak C18 1cc Vac Cartridge 50 mg (Waters Corp, USA) and dried with the Eppendorf Concentrator plus (Eppendorf, Germany). Subsequently, the dried peptides were reconstituted with 0.02% NH₄OH in HPLC water for fractionation. High pH reversed phase high performance liquid chromatography (RP-HPLC) was performed using the Prominence™ HPLC system (Shimadzu, Japan), with the XBridge™ BEH C18 column (130 Å pore size. 4.6 x 250 mm, 5 µm particle size). The mobile phase was comprised of 0.02% NH₄OH in HPLC water (A) and 0.02% NH₄OH in 80% acetonitrile (ACN) (B). A 60 min HPLC gradient consisting of 5 min of 3-10% (B), 40 minutes of 10-35% (B), 5 minutes of 35-70% (B) and 70-100% (B) was established for peptide separation. 60 fractions of sample were collected and combined into 16 fractions in a concatenated manner. The fractionated samples were dried and stored in -20 °C prior to LC-MS/MS analysis.

2.2.6. In gel digestion

Exosomal and lysosomal proteins were extracted in reducing laemmli sample buffer. 200 µg of protein samples were resolved in 12% SDS-PAGE at 120 V for 90 min. Protein bands were visualized through staining with 0.2% Coomassie blue solution. For each gel sample, the gel lane was cut into five equal parts and the gel fractions were further diced into 1 mm² gel pieces. The gel pieces were washed in alternating buffer consisting of either 100 mM ABB or 50% ACN in 100 mM ABB to remove impurities. The gel pieces were then dehydrated with 100% ACN and dried in the vacuum concentrator. The proteins were then reduced in 10 mM of DTT at 60 °C for 1 h, followed by alkylation with 55 mM of IAA at room temperature for 45 min, in the dark. The gel pieces were then subjected to washes again to remove excess DTT and IAA. After dehydrating with 100% ACN and dried in the vacuum concentrator, sequencing-grade modified trypsin was added to the gel pieces and protein digestion was performed at 37 °C, overnight. The digested peptides were extracted with 50% ACN and 5% acetic acid, dried in the vacuum concentrator and stored in -20 °C prior to LC-MS/MS analysis.

2.2.7. LC-MS/MS and data analysis

The peptides were reconstituted in 0.1% formic acid (FA) and 3% ACN for LC-MS/MS analysis in the Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer, coupled with the UltiMate™ 3000 RSLCnano System (Thermo Scientific Inc, USA). For each analysis, 2 µg of sample was injected to the system. The peptides were first concentrated with a Nano-Trap Columns 75-100 µm I.D. x 2 cm (Thermo Scientific, USA) and then separated on a Dionex EASY-Spray 75 µm x 10 cm column packed with PepMap C18 3 µm, 100 Å (Thermo Fisher Scientific, USA). The mobile phase buffers used were 0.1% formic acid (A) and 0.1% formic acid in ACN (B) and a 60 min gradient was used for peptide separation.

The samples were ionized and injected into the Q-Exactive mass spectrometer with an EASY nanospray source (Thermo Fisher Scientific, Inc.) at an electrospray potential of 1.5 kV. A full MS scan (350–1,600 m/z range) was acquired at a resolution of 70,000, with a maximum ion accumulation time of 100 ms. Dynamic exclusion was set as 30 s. The HCD spectral resolution was set to 35,000. Automatic gain control (AGC) settings of the full MS scan and the MS2 scan were 3E6 and 2E5 respectively. The top 10 most intense ions above the 5,000-count threshold was selected for fragmentation in HCD, with a maximum ion accumulation time of 120 ms. Isolation width of 2 was used for MS2. Single and unassigned charged ions were excluded from MS/MS. For HCD, the normalized collision energy was set to 28% and the under fill ratio was defined as 0.3%.

2.2.8. Database search

Raw data generated from two biological replicates for each condition were analyzed using the Proteome Discoverer (PD) 2.2 software (Thermo Fisher, San Jose, CA). Protein identification was done by mapping against the UniProt Knowledgebase (UniProtKB) for mouse proteins (downloaded on 16 Mar2017, 87,463 sequences and 38,788,886 residues), using the SequestHT and Mascot search engine. The Proteome Discoverer's workflow included an automatic target-decoy search tactic along with the Percolator to score peptide spectral matches from both Mascot and SequestHT searches to estimate the false

discovery rate (FDR). The Percolator parameters are set to maximum delta Cn = 0.05; target FDR (strict) = 0.01; target FDR (relaxed) = 0.05, validation based on q-value ^[151].

For SILAC quantitation, the Spectrum Files RC node was used for spectrum recalibration and the peak feature detection setting in the Minora Feature Detector was set as Minimum Trace Length: 5, Minimum number of isotopic peaks: 2 and Maximum Δ RT of Isotope Pattern Multiplets: 0.2 minutes. Feature mapper was set to True for retention time alignment, with a maximum retention time shift allowed of 10 minutes and Precursor abundance was based on intensity. The normalization mode was set as total peptide amount and the scaling mode was based on all average. The search parameters also included full trypsin digestion with a maximum of two missed cleavage and precursor mass tolerance and fragment mass tolerance was set at 10 ppm and 0.02 Da respectively.

Carbamidomethylation (+57.02) at cysteine was set as fixed modification, oxidation (+15.99) at methionine, deamidation (+0.98) at asparagine and glutamine and heavy $^{13}\text{C}_6$ -L-Arginine $^{13}\text{C}_6$ -Hydrochloride (+6.02) and $^{13}\text{C}_6$ -L-Lysine $^{15}\text{N}_2$ -Hydrochloride (+8.01) were set as dynamic modifications. Acetylation (+42.01) at protein N-terminus was set as dynamic modification too. Maximum dynamic modifications were set at 4.

2.2.9. Data Analysis

Quantification of the H/L ratio of each protein is based on precursor ion quantification. The protein abundance is determined by the MS signal intensities of the heavy isotope labelled protein over its unlabeled counterpart. Using the default setting, PD 2.2 uses the largest chromatographic peak and quantified the heights of this peak at the apex rather than the integrated peak area for quantification. The ratio calculation was set as Pairwise ratio based and the p-value was calculated by ANOVA (background based). The maximum fold change allowed was set to 100.

The reported H/L ratio has a range of 0.01 to 100, in which 0.01 represent

proteins with only non-labelled peptides and 100 represent proteins that either contain only labelled peptides or proteins with labelled peptides that has abundance value of more than 100 times of its non-labelled counterpart. The datasets were manually filtered by selecting for proteins with $q\text{-value} < 0.05$ and appear in both biological replicates. Gene ontology analysis was performed using the DAVID Bioinformatics v6.8 ^[152]. Pearson correlation coefficient analysis between the proteomics biological replicates was computed with the R-package; “ggplots2”.

2.2.10. Western blot analysis

Protein concentration was quantified using the bicinchoninic acid (BCA) or Bradford assay. Thereafter, laemmli sample buffer was added to the protein samples and boiled at 95 °C for 10 min. The protein samples were resolved in a 12% SDS-PAGE and transferred onto a 0.22 µm PVDF membrane. Subsequently, the membrane was blocked in 5% skimmed milk in Tris-buffered saline and Tween 20 (TBST) and then probed with primary antibodies overnight at 4 °C. The antibodies used included Alix (#2171, mouse, monoclonal), flotillin-1 (#18634, rabbit, monoclonal), GM130 (#12480, rabbit, monoclonal), LAMP1 (#9091, rabbit, monoclonal), EEA1 (#3288, rabbit, monoclonal), Rab7 (#9367) and Rab11 (#5589, rabbit, monoclonal) from Cell Signaling Technologies (Danvers, MA, USA), TSG101 (sc-136111, mouse, monoclonal), CD9 (sc-18869, rat, monoclonal), Cathepsin D (sc-377124, mouse, monoclonal), Cathepsin L (sc-390367, mouse, monoclonal) and VDAC1 (sc-58649, mouse, monoclonal) from Santa Cruz Biotechnology (Dallas Tx, USA), Cathepsin B (ab214428, rabbit, monoclonal), CD63 (ab217345, rabbit, monoclonal) and CD81 (ab109201, rabbit, monoclonal) from Abcam (Cambridge, UK) and HRP-AffiniPure Anti-Mouse IgG (H+L) (115-035-003, goat, polyclonal) and HRP-AffiniPure Anti Rabbit IgG (H+L) (111-035-003, goat, polyclonal) from Jackson ImmunoResearch Laboratories (Pennsylvania, USA). The HRP-Anti Rat IgG antibody was kindly gifted by Prof. James Tam’s lab (SBS, NTU, Singapore).

2.2.11. Nanoparticle tracking analysis

The particle number and size distribution of EVs collected from mHypoA-2/28 were characterized with the Nanosight NS300 (Malvern Panalytical, UK) equipped with a 488 nm blue laser and a sCMOS camera. The samples were diluted 200-fold with PBS for analysis using the default protocol according to the manufacturer's software (NanoSight NS300 User Manual). The instrument parameter was set as follow: camera level 5, slider shutter 100, slider gain 200, FPS 32.5, temperature 24 °C, viscosity 0.906-0.910 cP, syringe pump speed 100, capture time 60 s and detect threshold 3. Calculation of particles quantity and size distribution were based on 3 biological replicates.

2.2.12. Transmission electron microscopy

mHypoA 2/28-derived EVs were 20-fold diluted in PBS and a 7 µl volume added onto a glow discharged carbon-coated grid and incubated for 1 min. Thereafter, 2% uranyl acetate was added to the sample and incubated for 1 min. Excess uranyl acetate was blotted off with filter paper. The grid was air-dried for 10 min and subsequently imaged using the T12 Icorr transmission electron microscopy (TEM) at 120 kV (FEI Company, USA).

2.2.13. Cathepsin inhibition assay

mHypoA 2/28 cells were seeded on to twenty 100 mm dishes and upon reaching 80% confluency, the cells were washed with PBS twice, and treated with 10 µM CA-074ME, C5857 (Sigma Aldrich, USA), 10 µM Cathepsin L Inhibitor I (Santa Cruz Technologies, USA) or 20 µM of pepstatin A (Santa Cruz Technologies, USA) in 1% exosome-depleted FBS-DMEM for 24 h and the conditioned media was collected EVs isolation.

2.2.14. Proteinase K assay

50 µg of EVs was supplemented with 5 mM CaCl₂ and incubated in either PBS (control), 10 µg/ml of Proteinase K, 1% Triton X-100 or 10 µg/ml of Proteinase K with 1% Triton X-100 for 1 h at 37 °C. The treatment was stopped with the addition of laemmli Buffer supplemented with β-mercaptoethanol and the sample was heated at 95 °C for 15 min prior to immunoblotting.

2.2.15. RNA Isolation and RT PCR

Total RNA was isolated using the NucleoSpin® RNA II kit (Macherey- Nagel GmbH, Germany). RNA concentration was determined with the NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). First-strand cDNA was synthesized using the RevertAid RT Reverse Transcription kit (ThermoFisher Scientific, USA) as per manufacturer's protocol. Quantitative PCR (qPCR) reactions were performed using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) with the KAPA SYBR® FAST qPCR kit (KAPA Biosystems, USA) under the following condition; initial denaturation at 95°C for 3 minutes, followed by 40 cycle of denaturation at 95 °C for 10 s, primer annealing at 60 °C for 30 s and final extension at 72°C for 30 s.

Each sample was performed in triplicate and relative quantification was determined using Rab11 forward 5'-GAGCTTTTGCAGAGAAGAATGGT-3', Rab11 reverse 5'-TTCTGACAGCACTGCACCTT-3'; Rab27A forward 5'-AGAGAGTGGTGTACAGAGCCA-3', Rab27A reverse 5'-TTTCACAGTACGCGTGCATC-3'; Rab27B forward 5'-TTGGGACACTGCTGGACAAG-3', Rab27B reverse 5'-TGCCTGCAGTTGACTCATCC-3'; Rab35 forward 5'-CGTCAATGTGGAAGAGATGTTCA-3', Rab35 reverse 5'-GCAGCAGCGTTTCTTTCGTT-3'.

2.3. Results

2.3.1. Characterization of EVs and lysosomes for pSILAC-based quantitative proteomics

2.3.1.1. Characterization of EVs isolated from mHypoA 2/28 cells

In accordance to the MISEV guidelines ^[15], global and single EV characterization were performed on EVs isolated from the mHypoA-2/28 cells. EVs were isolated from conditioned media through a series of ultrafiltration and ultracentrifugation steps as adapted from the method described by Théry et. al. ^[36] (Figure 2.2). First, the conditioned media were pre-cleared of cellular debris by centrifugation at 1000 x g for 20 min at 4 °C. Next, the media was concentrated with a 10kDa molecular cut off filter and followed by centrifugation at 16,000 x g for 30 min at 4 °C to remove larger vesicles such as microvesicles. Lastly, the EVs were isolated through ultracentrifugation at 100,000 x g for 16 h at 4 °C.

Immunoblotting analysis of the isolated mHypoA 2/28 EVs revealed the presence of exosome-like vesicles as several known exosome markers such ESCRT-associated protein Alix, ESCRT-I protein TSG101, flotillin 1 and tetraspanin CD9 were identified (Figure 2.3). There was minimal cellular contamination during the EVs preparation as both VDAC1 (mitochondrial marker) and GM130 (endoplasmic reticulum marker) proteins were only present in the cell lysate but not in the EVs.

Figure 2.2: Diagram illustrating the procedure of EVs isolation from mHypoA-2/28 cells. 200 ml of conditioned media from approximately 60 million mHypoA 2/28 were collected for EVs isolation.

Figure 2.3: Immunoblotting analysis of vesicles isolated from mHypoA 2/28 cells. Ponceau S staining showed equal loading of proteins for analysis (left). 50 µg of cell lysate (CL) and EV proteins were used to probe for known exosomal markers (Alix, TSG101, flotillin 1, and CD9), mitochondrial marker (VDAC) and golgi marker (GM130). Molecular weight markers are indicated on the left of the blot (right).

A **B**

Figure 2.4: Physical Characterization of mHypo 2/28 EVs. Wide field and closed up TEM images obtained from negative staining of mHypoA 2/28 EVs. The scale bar represents 200 nm. (A) Nanoparticle tracking analysis of mHypoA 2/28 derived EVs reported a mean diameter of 158.8 ± 1.3 nm. (B)

TEM imaging of isolated EVs revealed a cup-shaped morphology that is typical of TEM-imaged exosome^[153] and the size of these vesicles were generally below 200 nm (Figure 2.4 a). Nanoparticle tracking analysis further confirmed that majority of the isolated particles to be within 200 nm and have a mean size of 158.8 nm, which is also the typical size of EVs isolated by ultracentrifugation (Figure 2.4 b)^[154]. Taken together, exosome-like vesicles were isolated through our isolation protocol and based on the particle size and isolation methodology, I have denoted these vesicles as small EVs.

2.3.1.2. Lysosome isolation from mHypoA-2/28 cells

To obtain lysosome for mass spectrometry analysis, mHypoA 2/28 cells were homogenized and the organelles were separated via ultra-centrifugation on a discontinuous Optiprep density gradient (15%, 17%, 20%, 23%, 27% and 30%). Five equal volume of the density gradient were obtained (Figure 2.5 a) and presence of lysosome were validated by immunoblotting analysis.

Figure 2.5: Lysosome isolation via Optiprep density gradient separation.

Discontinuous Optiprep density gradient (15%, 17%, 20%, 23%, 27% and 30%) separating organelles based on density and five fractions were collected for immunoblotting analysis. (A) Equal volume from each fraction were loaded for immunoblotting analysis and lysosomal (LAMP1) and endosomal (EEA-1 (early), Rab7 (late), Rab11 (recycling)) protein markers were probed. Molecular weight markers are indicated on the left of the blot (B).

The lysosomal-associated membrane protein 1 (LAMP-1) is a major component of the lysosomal membrane and their expression signified the presence of lysosome ^[155]. Immunoblotting analysis indicated that LAMP-1 expression was enriched at the top fraction of the density gradient (F1, Figure 2.5 b). Further analysis on various endosomal marker such as early endosome antigen-1 (EEA-1) and Rab7 protein expression showed that the early and late endosomes was found at the lower fractions (F3 and F4, Figure 2.5 b). The recycling endosome (Rab11) was located in fraction 1 and 3 (Figure 2.5 b).

2.3.2. Identification and quantification of newly synthesized protein using pSILAC based quantitative proteomics

After establishing the methodology for EVs and lysosome isolation, I utilised a pSILAC-based quantitative proteomics methodology to profile the proteome of EVs, lysosome and total cell lysate in mHypoA 2/28 cell line to facilitate the identification and study of novel newly synthesized proteins that preferentially localized to the EVs over the lysosome. I postulated that these newly synthesized proteins to play an important role in EVs biogenesis and will further our understanding on the process of EVs biogenesis and how they are distinguished from the lysosomal degradation pathway.

An overview of the experimental workflow is illustrated in Figure 2.6. Briefly the adult hypothalamus cell line, mHypoA 2/28, was first cultured in DMEM containing unlabelled ¹²C₆, ¹⁴N₂-L-lysine and ¹²C₆-L-arginine [light]. Upon reaching 80% confluency, the media was switched to SILAC-DMEM containing labelled ¹³C₆-L-Lysine ¹⁵N₂-hydrochloride (K8) and ¹³C₆-L-Arginine ¹³C₆-hydrochloride (R6) [heavy] for 24 h. Newly synthesized proteins will be labelled with the heavy amino acid (R6K8). Conditioned media were then collected for EVs enrichment while the labelled cells were harvested for lysosome isolation or cellular protein lysate. After sample preparation, the tryptic peptides were analysed with the Q-Exactive mass spectrometer, where the Orbitrap mass analyser coupled with the C-trap provides sub-ppm mass accuracy that allows identification of the light and heavy SILAC doublet ^[156]. Based on the H/L ratio, the protein synthesis rate

is defined. This ratio has a range from 0.01 to 100, with the score of 0.01 representing stable proteins with no de novo synthesis and the score of 100 representing the presence of only newly synthesized protein, i.e. not unlabelled protein or protein with an H/L ratio above 100.

Figure 2.6: Schematic diagram illustrating the workflow of pSILAC-based quantitative proteomics study of EV biogenesis through H/L ratio analysis.

Raw data files generated from 2 biological replicates (three injections) per condition were processed using the Proteome Discoverer software v2.2, with the Mascot and SequestHT search engine. 546, 1545 and 7327 proteins were identified in the EVs, lysosome and total cell lysate sample respectively (FDR at peptide level <1%) (Figure 2.7 a). A cut off value of H/L ratio >1.5 was used to denote proteins with increased protein synthesis rate. In the cell lysate sample, 3037 proteins have a H/L ratio <1.5 and 3025 proteins have a H/L ratio >1.5, while in the lysosome sample, 620 proteins have a H/L ratio <1.5 and 663 proteins have a H/L ratio >1.5 and lastly, the EVs contained 305 proteins that have a H/L ratio <1.5 and only 87 proteins that have a H/L ratio >1.5 (Figure 2.7 b). Although this data suggest that only 23% of the EVs proteome had increased synthesis rate, this percentage might have been skewed lower by possible serum contaminants found within the EVs proteome [49]. 68

proteins were identified as possible serum contaminants as they had H/L ratio of 0.01 in the EVs proteome and were absence in both the cell lysate and lysosome proteome (Cluster 3, Figure 2.10).

(A)

	Identified Proteins	Quantified Proteins
Cell Lysate	7327	6062
Lysosome	1545	1125
EV	546	392

(B)

(C)

(D)

(E)

Figure 2.7: pSILAC-based quantitative proteomics analysis of mHypoA 2/28 cell lysate, lysosome and EVs. Summary of proteins identified in the mHypoA cell lysate, lysosome and EV proteome respectively. Proteins were considered as quantified if they were identified in both biological replicates and proteins with large variation were omitted (A). The quantified proteins were further subcategorized to actively synthesized protein (H/L ratio > 1.5) or pre-existing protein (H/L ratio < 1.5) (B). Pearson's correlation coefficients

between two biological replicates from the EV, lysosome and cell lysate proteomes. Chart is plotted using R programme with ggplot2 package (C-E). Subsequently, only quantified proteins that were detected in both the biological replicates were considered for further analysis, unless otherwise indicated. The Pearson's correlation coefficient between the two biological replicates of the EVs, lysosome and cell lysate data were 0.896, 0.900 and 0.868 respectively, indicating a high reproducibility from the proteome dataset (Figure 2.7 c-e). In addition, I identified 24 of the top 25 commonly exosome proteins in the Exocarta^[157] as well as 73 of the top 100 EVs proteins in Vesiclepedia^[158] in our EVs proteome dataset, which further proved the enrichment of EVs from our sample preparation steps (Table 2.1).

Table 2.1: List of proteins from the EVs proteome dataset that matches with the database from Exocarta (A) and Vesiclepedia (B).

A					B				
Proteins quantified in the pSILAC dataset that matches the top 25 proteins in Exocarta					Proteins quantified in the pSILAC dataset that matches the top 100 EV protein in Vesiclepedia				
ACTB	ACTG1	ALB	ALDOA	ANXA2	A2M	ACLY	ACTB	ACTN1	ACTN4
ANXA5	CD63	CD81	CD9	CFL1	AHCY	ALB	ALDOA	ANXA1	ANXA2
EEF1A1	EEF2	ENO1	GAPDH	HSP90AA1	ANXA5	C3	CCT2	CCT3	CCT4
HSP90AB1	HSPA8	LDHA	MSN	PDCD6IP	CCT6A	CD63	CD81	CD9	CDC42
PGK1	SDCBP	YWHAE	YWHAZ		CFL1	CLIC1	CLTC	EEF1A1	EEF2
					EIF4A1	ENO1	FASN	FLNA	FLOT1
					FN1	GAPDH	GNAI2	GNAS	GNB1
					GNB2	GSN	HIST1H4A	HSP90AA1	HSP90AB1
					HSPA8	ITGB1	KPNB1	KRT1	KRT10
					LDHA	LDHB	LGALS3BP	MYH9	PDCD6IP
					PFN1	PGK1	PKM	PRDX1	RAB5C
					RAB7A	RAC1	RALA	RAN	RAP1B
					SDCBP	TCP1	TFRC	TLN1	TP11
					TSG101	TUBB4B	UBA1	VCP	YWHAE
					YWHAZ	YWHAQ	YWHAZ		

2.3.3. Extracellular vesicles biogenesis is an active process that required newly synthesized protein

One of the main postulations of this thesis was whether the proteins in the EV cargo are from existing cytosolic protein or it is a regulated process in which the proteins are newly synthesized by the cells and then sorted into the EVs to maintain EVs biogenesis. To evaluate this hypothesis, I first carried out gene ontology analysis on the three proteome dataset. Based on the H/L ratio (actively synthesized protein: H/L ratio > 1.5; slow turnover protein: H/L ratio

< 1.5), the respective proteome dataset were functionally annotated using the DAVID Bioinformatics tool ^[152] to elucidate the relevant biological process associated with the dataset. In particular, the analysis revealed a subset of actively synthesized proteins ($H/L > 1.5$) from both the cell lysate and EVs proteome that were able to promote positive regulation of exosomal secretion (GO: 1903543). In the cellular proteome, I identified newly synthesized charged multivesicular body protein 2a (Chmp2a), tumour susceptibility gene 101 (TSG101), vacuolar protein sorting-associated protein 4A (VPS4a), vacuolar protein sorting-associated protein 4B (VPS4b) and hepatocyte growth factor-regulated tyrosine kinase substrate (Hgs) proteins that are all part of the ESCRT machinery that are involved in MVBs formation for exosome biogenesis ^[69, 70]; the Alix (Pdc61p), syndecan-1 (Sdc1) and syntenin-1 (Sdcbp) proteins that are involved in heparanase-mediated exosome biogenesis ^[72, 146] and lastly ras-related protein Rab-7a (Rab7a) that is involved in exosome release ^[72, 159] (Table 2.2). Similarly, the EVs proteome also contained newly synthesized Alix, Rab7a and syntenin-1 protein (Table 2.3).

The ESCRT machinery consist of five protein complexes and several accessory proteins and play an important role in the formation of MVBs ^[60]. Therefore, further H/L ratio analysis on all the ESCRT machinery proteins was done to better understand the protein dynamics of these proteins in all the three samples. The H/L ratio was $\log_2$ transformed for better data representation and H/L ratio > 0.6 would represent actively translating proteins being sorted/packaged to exosomal cargo. 28 of the 31 proteins in the ESCRT complex were identified in the mHypoA-2/28 cell lysate (Figure 2.8), with CHMP4A, VPS22 and VPS37D absent from the dataset. Among the identified 28 proteins in the cell lysate, 22 of these proteins are actively translated. This suggest that the ESCRT complexes require active protein synthesis to maintain their cellular functions.

Next, HypoA 2/28 EVs were enriched in proteins from ESCRT-I as compared to the other ESCRT complexes, with TSG101 and VPS28 showing increased protein synthesis. The EVs also contained newly synthesized CHMP4B (ESCRT-III) and Alix. Taken together, this indicate that only a

subset of ESCRT-related proteins is packaged into the EVs during EV biogenesis. In particular, CHMP4B, ALIX and TSG101 proteins were found to have increased synthesis in all three datasets. The Bro1 domain in Alix binds to CHMP4B ^[160, 161] while the C-terminus of Alix contained a proline-rich region that binds to N-terminus UBC-like and proline-rich domain of TSG101 ^[162]. This suggest that the Alix-mediated association of the ESCRT-1 and ESCRT-III complex is packaged into the EVs during membrane scission ^[163], hence these proteins have to be constantly synthesized to maintain its function.

Lately, alternative mechanisms for MVB formation were reported. The syntenin-syndecan-Alix complex ^[72] was also recently proposed as an ESCRT-dependent mechanism that facilitate exosome biogenesis. Heparanase activity in the endosome promotes the trimming of the heparan sulphate group on the syndecan protein which in turn facilitate the clustering and sorting of Alix-ESCRT complex into the ILVs via syntenin-1 adaptor protein ^[146].

Table 2.2: List of newly synthesized proteins found within the cellular proteome that are involved in the positive regulation of exosomal secretion (GO:1903543).

Cell Lysate				
Accession	Gene name	Protein Name	Unique Peptide	I/L ratio
A0A1B0GT75	Chmp2a	Charged multivesicular body protein 2a	5	5.67
Q3UMA3	Hgs	Hepatocyte growth factor-regulated tyrosine kinase substrate	12	1.84
Q80Y09	Pdcd6ip	ALIX	48	1.61
P51150	Rab7a	Ras-related protein Rab-7a	19	1.55
P18828	Sdc1	Syndecan-1	3	100.00
O08992	Sdcbp	Syntenin-1	5	24.62
Q3UK08	Tsg101	Tumor susceptibility gene 101	10	1.51
Q3TDX2	Vps4a	Vacuolar protein sorting-associated protein 4A	5	3.20
P46467	Vps4b	Vacuolar protein sorting-associated protein 4B	7	2.13

Table 2.3: List of newly synthesized proteins found within the EV proteome that are involve in the positive regulation of exosomal secretion (GO:1903543).

EV

Accession	Gene name	Protein Name	Unique Peptide	H/L ratio
Q80Y09	Pdcd6ip	ALIX	21	1.64
P51150	Rab7a	Ras-related protein Rab-7a	5	5.64
O08992	Sdcbp	Syntenin-1	7	2.73

The dataset revealed that both Alix and syntenin-1 to have increased synthesis in the EVs (Alix: 5.64, syntenin-1: 2.72) and lysosome (Alix: 1.93, syntenin-1: 10.80) fractions as well (Table 2.4 a). The syndecan-1 protein is usually cleaved into syndecan-1 C-terminal fragment when inserted into the exosome^[73], the truncated form of syndecan-1 maybe the reason why it was not detected in the EVs proteome dataset. Nonetheless, cellular syndecan-1 has an H/L ratio of 100 which indicate that syndecan-1 underwent active protein synthesis to replenish the cleaved syndecan-1 that was required to support exosome biogenesis.

Next, the tetraspanins (CD9, CD63 and CD81) were also shown to promote ESCRT-dependent and -independent MVB formation^[25, 164-166]. Hence, I analysed the protein synthesis rate of these tetraspanins. The dataset showed that both CD9 and CD81 were actively synthesized in the ribosome and sorted to the EV cargo (CD9: 2.40, CD81: 10.34) and lysosome (CD9: 3.41, CD81: 30.61) fractions whereas CD63 had a lower synthesized rate (CD63 in EVs: 0.19, CD63 in lysosome: 0.73) (Table 2.4 b). As CD63 is localized at the MVBs while CD9/CD81 are located at the plasma membrane^[154], this suggest that plasma membrane-associated EVs (CD9/CD81) may have a higher secretion rate as compared to MVB-associated EVs (CD63). In support of this view, a recent study demonstrated that both CD9⁺ and CD81⁺ EVs displayed a much higher budding rate than CD63⁺ EVs^[167].

Figure 2.8: H/L ratio analysis of the ESCRT protein complex. The H/L ratio of proteins from the ESCRT-0 (A), ESCRT-I (B), ESCRT-II (C) and ESCRT-III (D) complexes and the ESCRT accessory proteins (E) was compared between the mHypoA 2/28 cell, lysosomes and EVs. The y-axis represents the fold induction that was normalized against pre-existing proteins. Error bars indicate S.E.M of two biological replicates (n = 2; Two-way ANOVA; *p<0.05; **p<0.01, ***p<0.001; **red** * = Cell lysate vs. Lysosome; **blue** * = Cell lysate vs EVs; **green** * = Lysosome vs EVs). *EV's TSG101 is found in one biological replicate only.

Lastly, both acid and neutral sphingomyelinase have been reported to promote EVs biogenesis and secretion through the enzymatic cleavage of endosomal sphingomyelin into ceramide at its optimal pH ^[75, 81]. The mHypoA-2/28 cells contain all three sphingomyelinases, SMPD1, 2 and 3. SMPD1 was found in the EVs fraction and SMPD3 was found in the lysosome fraction respectively (Table 2.4 c). Taken together, initial pSILAC-based quantitative proteomics analysis suggested that proteins involved in EVs biogenesis are being constantly synthesized and MVBs formation and EVs biogenesis required active protein synthesis to maintain its function.

Table 2.4: H/L analysis of ESCRT dependent and independent mechanism for MVB formation. The Alix-syntenin-syndecan (A), tetraspanins-enriched domain (B) and ceramide-based EV biogenesis (C) are alternate mode of MVB formation.

(A)

Alix-Syntenin-Syndecan Axis

		EVs		Lysosome		Cell lysate	
Gene Name	Protein Name	H/L ratio	Unique peptide	H/L ratio	Unique peptide	H/L ratio	Unique peptide
Pdcd6ip	Alix	5.64	21	1.93	23	1.61	48
Sdcbp	Syntenin-1	2.73	7	10.8	7	24.62	5
Sdc1	Syndecan 1	-	-	-	-	100	3

(B)

Tetraspanin-enriched domain

		EV		Lysosome		Cell lysate	
Gene Name	Protein Name	H/L ratio	Unique peptide	H/L ratio	Unique peptide	H/L ratio	Unique peptide
Cd9	CD9	2.40	2	3.41	3	2.13	4
Cd63	CD63	0.19	1	0.73	5	1.53	4
Cd81	CD81	10.34	3	30.61	2	7.43	3

(C)

Ceramide-based EV biogenesis

		EV		Lysosome		Cell lysate	
Gene Name	Protein Name	H/L ratio	Unique peptide	H/L ratio	Unique peptide	H/L ratio	Unique peptide
SMPD1	Sphingomyelin phosphodiesterase	0.01	1	-	-	16.24	1
SMPD2	Sphingomyelin phosphodiesterase 2	-	-	-	-	1.56	7
SMPD3	Sphingomyelin phosphodiesterase 3	-	-	100	1	0.88	1

2.3.4. Hierarchical clustering analysis reveal the preferential location of newly synthesized Cathepsin protein in the EVs

I postulated that the preferential localization of newly synthesized proteins to the EVs rather than the lysosome and total cell lysate to play an essential role in EVs biogenesis. To identify these proteins, hierarchical clustering analysis was applied to the proteomic dataset. Clustering is based on proteins identified in the EVs proteome and match against the corresponding protein in the other two datasets. Distance and agglomeration parameters were set to “Pearson correlations” and “Ward Method” (Figure 2.9). Clustering analysis of the proteins across the three sub-proteomes revealed 12 clusters with differential expression profile. More importantly, cluster 7, 8, 11 and 12 contained highly synthesized EVs protein with differential expression in the other compartment.

22 proteins were grouped in Cluster 7 and they are highly synthesized proteins found in the EVs and cell lysate proteome only (Figure 2.10 a). Gene ontology (GO) analysis indicated that some of these proteins are involved biological processes such as axon guidance (GO:0007411), proteolysis (GO:0006508), cell adhesion (GO:0007155) and extracellular matrix (GO:0030198) and function in protein binding (GO:0005515), calcium ion binding (GO:0005509) and protein C-terminus binding (GO:0008022). While these proteins are highly synthesized in the EVs proteome, the GO analysis

suggest that they are involved in extracellular matrix remodelling rather than in EVs biogenesis (Figure 2.10 b).

Next, Cluster 8 (Figure 2.11 a) consisted of 11 proteins that are exclusively found in the EVs proteome only. The GO analysis showed that some of the proteins are involved in vesicle-mediated transport (GO:0005975) and carbohydrate metabolic process (GO:0016192) (Figure 2.11 b). However, while the GO analysis suggested possible targets for our study, the p-value associated with the analysis was not statistically significance, as such was not pursued further.

Figure 2.9: Hierarchical clustering analysis was applied to the proteomic dataset. Clustering parameters are; clustering distance = Pearson correlation and agglomeration parameter = Ward Method. Heatmap was plotted with Complex Heatmap from R package. ■ signified the absence of protein in the dataset.

Figure 2.10: Cluster 7 contained 22 proteins that are highly synthesized in the EVs and cell lysate but absence in the lysosome. Heatmap representing Cluster 7 (A). Bar chart illustrating functional category enrichment analysis for proteins in Cluster 7 using DAVID bioinformatics tools. The y-axis shows enriched gene ontology (GO) categories and the x-axis represents enrichment scores for each term (Enrichment score: p-value < 0.05, modified Fisher Exact P-value). Orange bars = ‘Biological process’ terms in GO; green bars = ‘Cellular component’ terms in GO; purple bar = ‘Molecular function’ terms in GO; n = number of proteins clustered in each GO term.

Figure 2.11: Cluster 8 contained 11 proteins that are exclusive to the EVs proteome. Heatmap representing Cluster 8 (A). Bar chart illustrating functional category enrichment analysis for proteins in Cluster 8 using DAVID bioinformatics tools. The y-axis shows enriched gene ontology (GO) categories and the x-axis represents enrichment scores for each term (Enrichment score: p-value < 0.05, modified Fisher Exact P-value). Orange bars = ‘Biological process’ terms in GO; green bars = ‘Cellular component’ terms in GO; purple bar = ‘Molecular function’ terms in GO; n = number of proteins clustered in each GO term.

Figure 2.12: Cluster 11 contained 18 proteins that had newly synthesized proteins preferentially localized to the EVs. Heatmap representing Cluster 11 (A). Bar chart illustrating functional category enrichment analysis for proteins in Cluster 11 using DAVID bioinformatics tools. The y-axis shows enriched gene ontology (GO) categories and the x-axis represents enrichment scores for each term (Enrichment score: p-value < 0.05, modified Fisher Exact P-value). Orange bars = ‘Biological process’ terms in GO; green bars = ‘Cellular component’ terms in GO; purple bar = ‘Molecular function’ terms in GO; n = number of proteins clustered in each GO term.

Figure 2.13: Cluster 12 contained 9 proteins that are newly synthesized proteins in all three sub-proteomes. Heatmap representing Cluster 12 (A). Bar chart illustrating functional category enrichment analysis for proteins in Cluster 11 using DAVID bioinformatics tools. The y-axis shows enriched gene ontology (GO) categories and the x-axis represents enrichment scores for each term (Enrichment score: p-value < 0.05, modified Fisher Exact P-value). Orange bars = 'Biological process' terms in GO; green bars = 'Cellular component' terms in GO; purple bar = 'Molecular function' terms in GO; n = number of proteins clustered in each GO term.

In Cluster 11 (Figure 2.12 a), 18 proteins were identified in all three of the sub-proteome datasets, with the newly synthesized proteins preferentially localized to the EVs. Most of these proteins are localized to the extracellular exosome (GO: 0070062) (17 proteins) and lysosome (GO: 0005764) (14 proteins). Interestingly, most of these proteins are lysosome-associated enzymes that function in hydrolase activity (GO: 0016787), peptide binding (GO:0042277) and peptidase activity (GO: 0008233). They are also involved

in biological processes such as proteolysis (GO: 0006508), glycosaminoglycan metabolic process (GO:0030203), lysosome organization (GO:0007040) and carbohydrate metabolic process (GO:0005975) (Figure 2.12 b). Given that these proteins are found in both EVs and lysosome and yet there is a preferential enrichment of the newly synthesized proteins in the EVs, this would suggest their potential role in EVs biogenesis and could thereby play a crucial role in delineating the EVs biogenesis from lysosome degradation pathway. I further discussed on this cluster of proteins and elucidated their role in EVs biogenesis in the next section. Lastly, cluster 12 (Figure 2.13 a) had a set of 9 proteins that are newly synthesized in all three-proteome dataset. Some of these proteins are involved in antigen processing and presentation of peptide antigen via MHC class I (GO:0002474), negative regulation of neuron projection development (GO:0010977) and positive regulation of T cell mediated cytotoxicity (GO:0001913) and perform function such as peptide binding (GO:0042277) (Figure 2.13 b).

2.3.5. Characterization of newly synthesized cathepsin proteins in EVs

Cathepsin proteins are the main lysosomal proteases that function mainly towards protein degradation and recycling ^[149]. The cathepsin proteins are also involved in several physiological activities such as MHC-II antigen presentation ^[168], prohormone processing in secretory vesicles ^[169] and cathepsin mediated apoptosis ^[170]. They are classified based on the key catalytic group within its active site. For example, cathepsin B (Ctsb), cathepsin L (Ctsl) and cathepsin Z (Ctsz) are cysteine proteases, while cathepsin D (Ctsd) belongs to the group of aspartyl protease and cathepsin A (Ctsa) is a serine protease. Cysteine and serine residues in the protease's active site execute nucleophilic attack on the amide carbonyl carbon to catalyse the substrate protein cleavage while aspartyl protease utilised a water molecule as a nucleophile for protein hydrolysis ^[171, 172]. The mHypoA 2/28 cell contained several cathepsin proteins which included Ctsa, Ctsb, Ctsd and Ctsz that were identified in cluster 11 (Figure 2.14 a). Ctsl was also identified in the dataset, with a different expression profile as compared to the rest of the cathepsin proteins and was sorted to cluster 12.

For precursor ion quantification, the Proteome Discoverer algorithm calculates the protein and peptide group ratios based on their abundances instead of the PSM ratios. The quantification ratio distribution for the five cathepsin proteins across the three datasets are highlighted in Figure 2.14 b-e. Interestingly, the Ctsd aspartic protease in EVs are all newly synthesized while there is a mixture of newly synthesized and pre-existing Ctsd in the lysosome and cell lysate. On the other hand, lysosome-derived Ctsl are newly synthesized while EVs and cell lysate contained a mixture of newly synthesized and pre-existing Ctsl.

(A)

Gene Name	Protein Name	Type of proteases	Cluster	EVs		Lysosome		Cell Lysate	
				H/L ratio	Unique Peptide	H/L ratio	Unique Peptide	H/L ratio	Unique Peptide
Ctsa	Cathepsin A	Serine	11	6.64	7	-0.15	4	-0.19	13
Ctsb	Cathepsin B	Cysteine	11	3.99	2	-1.42	6	-0.40	16
Ctsd	Cathepsin D	Aspartyl	11	6.64	5	0.66	10	0.40	17
Ctsl	Cathepsin L	Cysteine	12	5.24	6	6.64	3	2.24	14
Ctsz	Cathepsin Z	Cysteine	11	5.67	2	-0.95	1	-0.77	8

(B)

Cathepsin A

(C)

Cathepsin B

(D)

Cathepsin D

(E)

Cathepsin L

(F)

Cathepsin Z

Figure 2.14: List of Cathepsin proteins identified in the three proteomic datasets. Cathepsin A, B, D, L and Z were identified (A). Quantification ratio distributions chart illustrating the distribution and spread of the ratios of all peptides used for quantification for each cathepsin protein. The ratio is displayed as log₂-fold change (y-axis). Blue line in the chart represent the median of the distribution and blue circles represent the ratio of the peptide groups used for calculating the protein ratio (B-F).

Further investigation was done to elucidate the role of Ctsb, Ctsc and Ctsl in EVs biogenesis. First, I determined their intracellular localization through immunoblotting analysis of organelles obtained from the lysosomal separation density gradient that was established in Figure 2.5 (Figure 2.15). Both mature and pro-Ctsb were found in all five fractions with mature Ctsb having the highest expression in F1 (correspond to the lysosomal fraction). Pro-Ctsl was also found in all fraction but mature Ctsl was particularly enriched in F1 too.

Figure 2.15: Location of Cathepsin B, L and D along the lysosomal density gradient. mHypoA 2/28 cell lysate was separated on a density gradient and five fractions were collected. 20 μ l of each fraction was used for immunoblotting analysis. Molecular weight markers are indicated on the left of the blot

Lastly, only mature Ctsc was identified in the gradient and it was found to be enriched in F3 and F4 which correspond to the early and late endosome fractions. The distribution pattern of the cathepsin protein along the lysosomal density gradient together with their respective H/L ratio across three proteomic datasets suggested a differential role of Ctsc from Ctsb and Ctsl. The preferential enrichment of these newly synthesized Ctsc in the EVs and their enrichment in the endosomal fraction suggest a potential function in EVs biogenesis and could thereby play a crucial role in regulating EVs secretion and lysosomal degradation.

Figure 2.16: Proteinase K protection assay to determine the localization of EVs cathepsin protein. Immunoblotting analysis revealed that Ctsb and Ctsd are localized to the lumen of the EVs while pro-Ctsl is localized to the surface of the EVs. Molecular weight markers are indicated on the left of the blot. Red box indicates the protein band for mature Cathepsin D.

Next, to investigate the localization of the cathepsin proteins within the EVs, proteinase K protection assay was conducted on mHypoA 2/28 derived EVs [173] (Figure 2.16). The EVs membrane protects EV luminal proteins against proteinase K digestion and this protection is lost when the membrane is solubilised by detergent prior to digestion. Membranous EV proteins will be digested by proteinase K even in the absence of detergent. Alix was used as the control for EVs lumen proteins while CD9 as the control for EVs membrane protein. The result indicated that both Ctsb and Ctsd are EVs luminal proteins as they have similar profile as Alix. Whereas pro-Ctsl and the EV surface CD9 protein, were sensitive to proteinase K digestion even in the absence of Triton X-100, indicating that it may be on the EV surface membrane.

Subsequently, I seek to determine whether the preferential sorting of certain newly synthesized cathepsin proteins into EVs over the lysosome is a cell-specific phenomenon. I applied pSILAC-based quantitative proteomic comparative analysis on the EVs and lysosome derived from A431 human epidermoid carcinoma cells and A549 human lung carcinoma cells. Similar to the expression profile in mHypoA 2/28 cells, newly synthesized Ctsd in both A431 and A549 cell lines preferentially localized to the EVs over the lysosome (Table 2.5) which suggest a conserved function of Ctsd in EVs biology across species and cell types.

Table 2.5: pSILAC-based quantitative proteomic comparative analysis of cathepsin proteins turnover ratio derived from the EVs and lysosome proteome of the A431 human epidermoid carcinoma cells and A549 human lung carcinoma cells.

			A431 EVs		A431 Lysosome		A549 EVs		A549 Lysosome	
Gene Name	Protein Name	Type of proteases	H/L ratio	Unique Peptide	H/L ratio	Unique Peptide	H/L ratio	Unique Peptide	H/L ratio	Unique Peptide
Ctsb	Cathepsin B	Cysteine	2.80	11	6.64	1	-	-	0.696884	1
Ctsd	Cathepsin D	Aspartyl	3.18	22	1.05	10	3.47	13	0.844787	9
Ctsl	Cathepsin L	Cysteine	-	-	-	-	3.53	9	6.643856	1
Ctsa	Cathepsin A	Serine	3.23	9	1.10	2	0.61	6	-0.545824	1
Ctsz	Cathepsin Z	Cysteine	-	-	0.42	1	3.40	2	-	-

2.3.6. Chemical inhibition of cathepsin proteins modulate EVs secretion.

Small molecule inhibitors against cathepsin proteolytic activities have been widely used to understand the various roles of cathepsin functions. Here, I utilised three different inhibitors to study the role of cathepsin in EV biogenesis (Figure 2.17). The second inhibitor used in this study is the Cathepsin L inhibitor II (Ctsl-I) that reversibly inhibit Ctsl with an IC_{50} value of 0.85 nM ^[175]. The last inhibitor used is Pepstatin A (Pep-A) which is a peptide inhibitor of Ctsd and have an IC_{50} value of 30 nM ^[176]. The inhibition study was conducted by treating mHypoA 2/28 with media containing 10 μ M of CA-074Me, 10 μ M of CTSL-I or 20 μ M of Pep-A for 24 h and thereafter both cell This included the CA-074 methyl ester (CA-074

Rab GTPases are known to regulate both ESCRT-dependent (Rab7 and Rab27) and ESCRT-independent (Rab7 and Rab35) EVs biogenesis and secretion [83]. Therefore, gene expression analysis on Rab GTPases were conducted to understand whether cathepsin inhibition affects EVs secretion in mHypoA 2/28. RT-qPCR analysis of cathepsin inhibited mHypoA 2/28 cells revealed that Ctsb inhibition increased the expression of Rab7 and Rab27b, Ctsl inhibition resulted in increased Rab7 expression and Ctsd inhibition resulted in an increased in Rab7, Rab27b and Rab35 expression (Figure 2.18).

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Figure 2.18: Gene expression analysis of Rab proteins after chemical inhibition of cathepsin proteolytic activities. mHypoA 2/28 cells were incubated in media containing 10 μ M of Ctsb inhibitor (CA-074Me), 20 μ M of Ctsd inhibitor (Pepstatin A (Pep-A)) or 10 μ M Ctsl inhibitor II (CTSL-I) for 24 h and collected for gene expression analysis of Rab proteins. β -actin was used as the reference gene and fold change was calculated using the $e^{-\Delta\Delta CT}$ method. Error bars indicate S.E.M of three biological replicates (n = 3, Student T-test, *p<0.05; **p<0.01).

Additional Bonferroni's multiple comparison test was applied and revealed that the increased particle count observed in either Ctsl-I or Pep-A treatment were of statistical significance (Figure 2.19 a). The EV particle count per non-treated mHypoA 2/28 cell had a mean value of 1657 and the lower quantile (Q1), median (Q2) and upper quantile (Q3) are 1012, 1326 and 2801 respectively, while the EV particle count from Ctsb-inhibited mHypoA 2/28 has similar value with a mean of 1352 and the Q1, Q2 and Q3 were 1096, 1363 and 1572. The EV particle count was higher in the two other conditions and the mean, Q1, Q2 and Q3 value were 2890, 2060, 2402 and 3929 for the EVs derived from Ctsl-inhibited mHypoA 2/28 and 2714, 1687, 3023 and 3277 for the EVs derived from Ctsd-inhibited mHypoA 2/28. Further analysis was done to profile the size distribution of the EVs isolated from the cathepsin inhibited cells treatment and the higher particle count from Ctsl and Ctsd inhibited cells were contributed by the increased release of particles between the size of 100-150 nm (Figure 2.19 b).

(A)

(B)

Figure 2.19: Characterization of EVs particle count and size distribution with Nanoparticle tracking analysis (NTA). Box plots showing EV concentration from control, CA-074 Me, Ctsl-I and Pep-A treated mHypoA 2/28 cells measured by NTA. Concentration of EVs was quantified by particle count that was normalized against total cell count for each condition. Red dot represents the mean particle count for each treatment. Error bars indicate S.E.M of three biological replicates (One-way ANOVA, $**p<0.01$) (A). Comparison of the size distribution of EVs from the various treatment, Bin size is 50 nm. Number of particles in each bin size is normalized against total cell count. Error bars indicate S.E.M of three biological replicates (Two-way ANOVA, $*p<0.05$, $**p<0.01$) (B).

Subsequently, western blot analysis on known EVs markers such as Alix, CD63, CD81 and syntenin-1 was conducted on cathepsin-inhibited mHypoA 2/28 EVs to examine if the treatment resulted in a change in EVs subpopulation (Figure 2.20). Interestingly, when treated with Ctst-I and Pep-A, mHypoA secrete EVs that are enriched in CD63, while inhibition of Ctstb, Ctstd or Ctstl resulted in a decrease of CD81 expression in EVs. Ctstd inhibition also resulted in a decreased in cellular CD81 expression. The EV and cellular expression of Alix and syntenin-1 remained largely unchanged after cathepsin inhibition.

Figure 2.20: Western blot analysis of known EVs marker Alix, CD63, CD81 and Syntenin-1 following 24 hours of chemical inhibition against cathepsin B, D, and L in mHypoA2/28 cells. Molecular weight markers are indicated on the left of the blot. (CL: Cell lysate; EV: Extracellular Vesicles; Red box indicate the band of interest.)

2.4. Discussion

The exponential growth in EVs research in last two decades (Figure 1.1) have highlighted their importance in the biology of intercellular communication. Encapsulated by a lipid bilayer, the EVs act as protective carrier and transport a

wide repertoire of biological molecules such as proteins, RNA, and lipids across the lymphatic and circulatory system and mediate biological responses at distant site ^[1, 143]. Previously, our lab has also established the role of EVs in hypoxia-induced cancer progression, neurodegenerative disease and cardiovascular disease ^[3, 19-21]. Despite the growing knowledge of EVs functions, the mechanism and regulation of their biogenesis and secretion remain poorly understood. A key component of EVs biogenesis is the formation of MVBs which is a shared process with the lysosome degradation system. In this chapter, I had illustrated the usage of a pSILAC-based quantitative proteomic methodology to study EVs biogenesis. Through profiling of the newly synthesized proteome of the EVs, lysosome and total cell lysate from mHypoA 2/28 adult hypothalamus cell line, I was able to study the spatial distribution of these newly synthesized proteins. I postulated that preferential localization of newly synthesized proteins into the EVs over the lysosome may play an important role in EV biogenesis.

First, I demonstrated through gene ontology analysis that the regulation of EV biogenesis and secretion is an active process that required constant synthesis of the involved proteins. In the dataset, I identified higher protein synthesis ratio in several ESCRT proteins such as Chmp2a, TSG101, VPS4a, VPS4b, HGS and Alix; protein involved in MVB formation such as syntenin-1 and syndecan as well as Rab7a that is involved in exosome secretion. This finding indicated that the application of pSILAC-based quantitative proteomics in studying EVs biogenesis was appropriate. Thereafter hierarchical cluster analysis was performed on the three-proteome dataset and I identified a cluster of proteins that were newly synthesized and preferentially localized to the EVs (Figure 2.12). In particular, I found numerous newly synthesized cathepsin proteins in this cluster and subsequently focus on Ctsb, Ctsc and Ctss for further analysis. I first established the cellular distribution of the three cathepsin proteins. Mature Ctsc was enriched in endosomal fraction while mature Ctsb and Ctss were enriched in lysosomal fraction (Figure 2.15). Newly synthesized pro-cathepsin proteins are glycosylated with a mannose-6-phosphate tag in the endoplasmic reticulum and transported from the Golgi apparatus to the endo-

lysosomal system via the mannose-6-phosphate receptor (M6PR) and become active as the endosome acidified ^[155]. While the lysosome is thought to be the terminal storage site for acid hydrolase, certain cathepsins such as Ctsd was shown to accumulate in the endosome which correlate with the finding ^[177, 178]. Moreover, cathepsin found in the endosome are known to mediate proteolytic activities indicating that they are not transiently transported across the endosome compartment ^[179].

Next, I established that Ctsb and Ctsd but not pro-Ctsl was found in the EVs luminal side Figure 2.16. Other than sorting into the endosome via the M6PR route, numerous M6PR-independent sorting mechanism of cathepsin into endosome has been discovered. The type 1 transmembrane protein SEZ6L2 was identified as a M6PR-independent receptor that facilitated Ctsd transportation to the endosome ^[180]. The endocytosis motif containing SEZ6L2 is a binding partner of Ctsd and siRNA knockdown of SEZ6L2 in N1E-115 neuroblastoma cells resulted in reduced endocytosis of exogenous pro-Ctsd and increased exocytosis of pro-Ctsd due to absence of SEZ6L2-mediated Golgi to endosome transportation. Another mechanism involving the LDL receptor and LDL receptor-related protein 1 (Lrp1) was established in the transportation of non-phosphorylated Ctsb and Ctsd to the lysosome in a secretion-recapture manner ^[181]. On the other hand, pro-Ctsl was found to co-localize with CD63 in MVBs and could self-aggregate at the membrane of dense vesicle for secretion ^[182, 183]. Therefore, the assortment of M6PR dependent and independent mechanism for sorting cathepsin into the endosomal compartment may explain the differential localization of Ctsb, Ctsd and pro-Ctsl in the EVs.

Subsequently, I performed chemical inhibition studies against the cathepsin proteins to study their role in EV biogenesis. Chemical inhibition of the three cathepsins individually resulted in increased expression of Rab7. Rab7 is involved in late endocytic vesicle trafficking ^[184, 185] and vesicle secretion in a ESCRT-dependent ^[146] or independent manner ^[136, 186]. Ctsb or Ctsd inhibition also resulted in increased expression of Rab27b, a Rab GTPases located at the perinuclear region and is involved in ESCRT-dependent EVs

secretion ^[85]. Finally, Ctsd inhibition also resulted in the upregulation of Rab35, a Rab GTPase that is involved in fast endosome recycling pathway ^[187] as well as tethering late endosome to the plasma membrane in an ESCRT-independent manner ^[75, 88]. These results suggested two things: 1) The increased in Rab GTPases upon cathepsin inhibition indicated an increased in EVs secretion. 2) Each cathepsin may regulate different subpopulation of MVBs for secretion. Additionally, found that the upregulation of Rab GTPase gene expression from Ctsd and Ctstl inhibition translated into increased EVs secretion as determined by nanoparticle tracking analysis and this is due to increased of particles between the size of 100-150 nm (Figure 2.19). The inhibition of Ctsd and Cstl also changes the composition of the EVs resulting in more CD63⁺ EVs and reduced CD81⁺ EVs.

Based on the results, I concluded that Ctsd and to a certain extent Ctsb may play a greater role in EV biogenesis than Ctstl. This is because Ctstl is predominantly localized to the lysosome fraction and pro-Ctstl instead of mature Ctstl was enriched in the EVs. Moreover, pro-Ctstl showed minimal proteolytic activity and secreted pro-Ctstl are only active in the presence of glycosaminoglycan at the extracellular matrix ^[188]. The increase in EVs secretion and Rab7 expression from Ctstl inhibition maybe explain by the perturbation of the lysosome status. Recent studies have shown that the inhibition of lysosome activities with chemical compound such as Bafilomycin A had resulted in the perturbed cells using the EVs secretion pathway as an alternative method for cellular waste disposal ^[148, 189, 190]. As an essential protease in the lysosomal system ^[191], the inhibition of Ctstl likely resulted in dysregulation of lysosomal activity and hence the increased in EVs secretion could be a coping mechanism by the cells to remove accumulated cellular waste.

Ctsb inhibition in mHypoA 2/28 caused an increased in cellular Rab7 and Rab27b gene expression but this did not translate into increase EVs secretion. The inhibition may have affected the change in protein content in the EVs rather than the secretion rate instead. Whereas newly synthesized Ctsd seem to have a major role in EV biogenesis. To begin with, mature Ctsd are

enriched in the endosome fraction and the mHypoA 2/28 EVs contained only newly synthesized Cttd while pre-existing Cttd are found in the lysosome instead. This suggest that the actively synthesized Cttd are sorted to the EVs while some of the Cttd proteins are transported to the lysosome for storage and housekeeping activities which is why pre-existing Cttd proteins are found in the lysosome dataset. Moreover, chemical inhibition of Cttd activity increased the cellular gene expression of Rab GTPases that are involved in ESCRT dependent or independent EVs secretion ^[83, 136], increased CD63⁺ EVs secretion, all of which are indicative of the potential role of Cttd in EV biogenesis and secretion.

Literature search revealed mannose-6 phosphate receptor (M6PR) ^[192], prosaposin (PSAP) ^[193] and ceramide as intercellular binding partners of Cttd ^[194]. There are two type of M6PR proteins and both cation dependent and independent M6PRs are involved in the transportation of Cttd from the Golgi to the lysosome, while a subset of M6PRs residing at the plasma membrane are involved in the recapturing of extracellular Cttd via endocytosis. ^[195, 196]. PSAP is a precursor to the sphingolipid activator proteins (saposins) that are involved in the hydrolysis of sphingolipid ^[197]. Pro-Cttd can bind and form complexes with PSAP in the endoplasmic reticulum ^[198]. Under acidic condition, PSAP catalysed the activation of pro-Cttd to Cttd and this allowed the activated Cttd to process PSAP into saposin, with saposin D being the dominant form ^[199]. Saposin D acts as a cofactor in acid ceramidase (ASAH1)-mediated degradation of ceramide into sphingosine ^[200] and also in acid sphingomyelinase(SMPD1)-mediated hydrolysis of sphingomyelin into ceramide ^[201]. Moreover, SMPD1-derived ceramide is an intracellular binding partner of Cttd and enhances Cttd's proteolytic activity ^[194].

Table 2.6: List of proteins that are associated with Cttd interactions and activities.

Gene Name	Protein Name	EVs		Lysosome		Cell Lysate	
		H/L ratio	Unique Peptide	H/L ratio	Unique Peptide	H/L ratio	Unique Peptide
PSAP	Prosaposin	6.54	7	0.68	7	-0.21	20
ASAH1	Acid ceramidase	100*	2	1.08	8	-0.48	15
SMPD1	Acid sphingomyelinase	-6.64	1	-	-	4.02	1
IGF2R	Cation-independent mannose 6-phosphate receptor	-5.67	10	6.64	14	2.90	54
M6PR	Cation-dependent mannose 6-phosphate receptor	-	-	0.47	3	0.14	5

* *ASAH1* proteins was found in one of the two biological replicates in the EVs proteome.

I was able to identify some of these proteins in the dataset (Table 2.6). Protein synthesis ratio analysis showed that M6PR and IGF2R are enriched in the lysosomal proteome and this is in agreement with the literature that the M6PRs are involved in sorting Cttd towards the lysosome. This also indicated that the sorting of Cttd into the mHypoA 2/28 EVs is not highly to be dependent on M6PR pathway. It is likely that the sorting of Cttd requires the action of PSAP as newly synthesized PSAP were enriched in the EVs fractions. As mention earlier in the chapter, acid sphingomyelinase are involved in MV biogenesis at the plasma membrane ^[81] and the association of Cttd with PSAP, ASAH1 and SMPD1 suggested the role of Cttd in sphingolipid metabolism and possible a ceramide-based EV biogenesis at the plasma membrane. The reduction in CD81+ EVs from Cttd chemical inhibition further illustrate this point (Figure 2.20) as CD81 was reported to localized to the plasma membrane ^[167]. The absence of Cttd activity may have diverted the EVs secretion pathway towards the endosomal-based CD63+ EVs secretion instead as indicated by our data. Cathepsin D is able to mediate sphingomyelin metabolism through their cleavage of prosaposin to saposin D. The reaction maybe amplified by the generation of ceramide which in turn enhance cathepsin D's proteolytic activity.

Figure 2.21: Schematic diagram illustrating the potential role of Ctsd in mediating sphingolipid metabolism during EV biogenesis.

Based on the current finding and understanding of sphingolipid metabolism in EV biogenesis, I postulated that Ctsd is involved in regulating sphingomyelin metabolism to facilitate EV generation. Both pro-Ctsd and PSAP cross-activate each other leading to the generation of saposin D which in turn acts as cofactor for SMPD1 and ASAH1 in sphingomyelin conversion to ceramide and ceramide to sphingosine respectively (Figure 2.21). Moreover, ceramide is known to enhance Ctsd's proteolytic activities, as such I believed that Ctsd can act as an important feed forward catalyst to regulate ceramide production in EV biogenesis. Going forward, establishing the role of Ctsd in SMPD1-driven EV biogenesis is pertinent as it would represent a new pathway for EVs secretion, given that the current mode of ceramide-dependent EV biogenesis are mediated by neutral sphingomyelinase. Characterization of the EVs generated from this pathway can help to further classify the heterogeneity of secreted EVs and delineate their functions toward intercellular communication or waste disposal for cell homeostasis.

Chapter 3

**Elucidating the role of EVs from leptin treated mHypoA 2/28
cells through pSILAC-based quantitative proteomics**

3. Elucidating the role of EVs from leptin treated mHypoA 2/28 cells through pSILAC-based quantitative proteomics

3.1. Chapter summary

Energy homeostasis is a biological process that helps to maintain the human body weight through the regulation of food intake, energy expenditure and energy storage. This allowed our body weight to remain relatively stable despite the daily fluctuation in food intake and energy output. Specifically, the hypothalamus is the master regulator of energy homeostasis as they response to peripheral signals such as nutrient level, hormonal changes and neuronal inputs and initiate catabolic and anabolic reactions ^[202]. The leptin hormone is secreted by the white adipose tissue and acts on the hypothalamus to promote catabolic processes and maintain the long-term regulation of energy homeostasis^[203]. Leptin activates the anorexigenic POMC neurons found within the arcuate nucleus and stimulate the central melanocortin system which eventually lead to a reduction in food intake coupled with an increased in energy expenditure^[204].

As mentioned earlier, a study had demonstrated the hypothalamus's ability to secrete EVs with regulatory function ^[141]. This indicated an alternative route where neuronal cells can propagate its signal upon stimulation. For that reason, I seek to establish a new mode of communication by the POMC neurons through EV-mediated intercellular communication upon leptin stimulation to promote energy homeostatic function. Given that the arcuate nucleus is located adjacent to the median eminence, I postulated that EVs secreted by the POMC neurons can get into the systemic circulation easily to promote intercellular communication, given that EVs are able to pass through the blood brain barrier ^[205, 206]. In this study, I continued with the usage of mHypoA 2/28 hypothalamic cell because 1) mHypoA 2/28 cell is derived from the POMC neuron of C57bl/6J mouse, which enable the study of leptin-mediated EV functions; 2) the mHypoA 2/28 cells were demonstrated to secrete EVs in our earlier study which may have potential functions other than waste disposal. In addition, I proposed that leptin signalling will stimulate de-novo synthesis of leptin-regulated proteins and these proteins will be actively sorted into the EVs for secretion and regulate energy homeostasis at distant organs (Figure 3.1).

Figure 3.1: Schematic diagram illustrating the potential role of mHypoA 2/28 (POMC neurons) EVs upon from leptin stimulation. (AgRP, agouti-related peptide; α -MSH, alpha melanocyte stimulating hormone; ARC, arcuate nucleus; BAT, brown adipose tissue; CART, cocaine- and amphetamine-regulated transcript; DHA, dorsal hypothalamic area; DMH, dorsomedial nucleus; EVs, extracellular vesicles; GABA, gamma aminobutyric acid; LHA, lateral hypothalamic area; MCH, melanin concentrating hormone; MC4R, melanocortin 4 receptor; NPY, neuropeptide Y; OXs, orexins; POMC, pro-opiomelanocortin; PVH, paraventricular nucleus; VMH, ventromedial nucleus; WAT, white adipose tissue.)

(Figure was adapted from Lopez et al. ^[207])

Similarly, a pSILAC-based quantitative proteomics methodology was utilised to profile the newly synthesized EVs proteome derived from leptin-treated mHypoA-2/28 cells. I found that leptin treatment promoted additional protein loading into the mHypoA 2/28 EVs as well as increased the number of EVs secreted. Newly synthesized proteins from EV_Leptin are involved in biological processes related to peptidyl-tyrosine auto-phosphorylation, neuron projection development and extracellular matrix reorganization, functioned in kinase activities [cyclin-dependent protein serine/threonine kinase activity and protein tyrosine kinase activity] and lastly, they are

enriched in basement membrane, extracellular matrix and lysosome. In contrast, newly synthesized proteins that preferentially sort to EVs derived from basal mHypoA 2/28 hypothalamus cells (EV_Ctrl) were involved in biological process such as cellular response to LDL particle stimulus, negative regulation of Wnt signalling pathway, proteolysis involved in cellular protein catabolic process and cellular response to glucose stimulus, performed molecular functions such as threonine-type endopeptidase activity, integrin binding, enzyme binding and hydrolase activity and enriched in cellular location such as focal adhesion, lysosome and extracellular exosome. Taken together, leptin stimulation increased protein sorting into EV_Leptin and these proteins performed a different function as compared to EV_Ctrl.

Subsequently, the isolated EVs were injected into C57bl/6J mice to study their spatiotemporal distribution and physiological functions. The EVs from both conditions were found to accumulate in the liver and spleen 72 h post injection, which are the site of EVs clearance through the reticulo-endothelial system. Blood glucose level, body weight and food intake were also measured at 0, 1, 6, 12, 24, 48 and 72 h after EVs injection. In the EV_Ctrl group, blood glucose level was significantly decreased by 2.77 and 3.57 mmol/l at 6 and 12 h post-injection respectively. Furthermore, each mouse in the EV_Ctrl group lost an average of 6.3% and 6.7% of their weight at 12 and 24 h post injection respectively. Whereas the blood glucose level of the mice in the EV_Leptin group were significantly decreased by 3.5 mmol/l at 12 h post injection and the mice lost 5.67% and 6.33% of their weight at 12 and 24 h post injection respectively.

Given that EVs treatment regulate glucose homeostasis, the tibialis anterior muscle and pancreas from basal EVs-treated mice were harvested for analysis of gene related to glucose homeostasis. Gene analysis revealed that EVs treatment resulted in a small but significant increase in GLUT4 expression in the tibialis anterior muscle (2.26-fold). This indicated that mHypoA EV might be involved in the regulation of glucose homeostasis through its action on

skeletal muscle. In contrast, EVs treatment did not affect the expression of insulin, glucagon and somatostatin.

Taken together, I have established a functional role of mHypoA 2/28 EVs in modulating glucose homeostasis and these findings suggested that small extracellular vesicles can be an alternative treatment strategy to combat metabolic syndrome such as hyperglycaemia.

3.2. Methods and Materials

3.2.1. Optimization of leptin dosage for treatment on mHypoA 2/28

mHypoA 2/28 cells were seeded at a density of 100,000 cells/well. After reaching 80% confluency, cells were serum-starved overnight in serum-free DMEM. Thereafter the cells were treated with 5 or 10 nM of leptin (R&D Systems, USA) for 5, 10, 15 and 30 min and the cells were lysed for protein and gene analysis.

3.2.2. Western blot analysis

Western blot analysis was conducted as indicated by the methodology in Chapter 2. The antibodies used are pSTAT 3 (#9145, rabbit, monoclonal) and STAT3 (#9139, rabbit, monoclonal) from Cell Signaling Technology (Danvers, MA, USA) and HRP-AffiniPure Anti-Mouse IgG (H+L) (115-035-003, goat, polyclonal) and HRP-AffiniPure Anti Rabbit IgG (H+L) (111-035-003, goat, polyclonal) from Jackson ImmunoResearch Laboratories (Pennsylvania, USA).

3.2.3. pSILAC treatment, EVs isolation and LC-MS/MS analysis

For the pSILAC experiment, the mHypoA 2/28 cell was grown in 100 mm petri dish, Upon reaching 80% confluency, the cells were washed with PBS twice and then treated with 10 nM leptin or control (Tris-HCl, pH8) in pSILAC-DMEM, supplemented with 10% dialysed FBS (Gibco, USA), 1% penicillin/streptomycin (Nacalai Tesque, Japan) and heavy $^{13}\text{C}_6$ -L-Arginine $^{13}\text{C}_6$ -Hydrochloride (84 mg/l) and $^{13}\text{C}_6$ -L-Lysine $^{15}\text{N}_2$ -Hydrochloride (146 mg/l) (Cambridge Isotope Laboratories, USA) for 24 h. The conditioned media was then collected for EVs isolation. EVs isolation, protein sample preparation and

LC-MS/MS analysis were performed in accordance to the methodology in Chapter 2.

3.2.4. Mice housing

All mouse experiments conducted were approved by the NTU Institutional Animal Care and Use Committees (IACUC ARF-SBS/NIE-A18018) and performed in accordance to the approved guidelines and regulations of the Animal Facility Center of the School of Biological Sciences, Nanyang Technological University, Singapore. 6-weeks old C57bl/6J male mice were purchased from InVivos Pte Ltd, Singapore and housed under a standard 12 h light/12 h dark cycle with food and water given ad libitum.

3.2.5. EVs injection

The EVs were labelled with 1 μ M of 1,1'-Diocadecyl-3,3',3'-Tetramethylindotricarbocyanine Iodide (DiR') lipophilic carbocyanine dye (Thermo Scientific, USA) for 1 h at room temperature and excess dye were removed through ultracentrifugation with a density gradient (0.6, 0.7 0.8 and 2.0M sucrose). 50 μ g of DiR' labelled EVs in 100 μ l of PBS solution was injected into each mouse via the retro-orbital venous sinus route under isoflurane anaesthesia (3-5%).

3.2.6. Food intake, body weight and blood glucose measurement

Body weight and food pellet weight were measured using the Precision Balances PL6001E (Mettler Toledo, Canada). Blood glucose measurement was performed using the Accu-Chek Active blood glucose meter (Roche, Switzerland), with blood collected through the tail vein. All measurements were taken at the following time-point; before EVs injection, 1, 6, 12, 24, 48 and 72 h.

3.2.7. In-vivo imaging of DiR' labelled mHypoA 2/28 EVs spatiotemporal distribution in C57bl/6J mice

In-vivo imaging was done using the IVIS Spectrum In Vivo Imaging System (PerkinElmer, UK). The mice were anaesthetized prior to each imaging and the images were taken at the time-point coinciding with the above-mentioned

measurement. At the endpoint, the mice were sacrificed and the organs (brain, heart, stomach, gastrointestinal (GI) tract, adipose tissue and skeletal muscle) were harvested for imaging. The image capture parameters were; exposure 2 s, excitation wavelength 710 nm; emission wavelength 760 nm and epifluorescence. The images were processed with the Living Image Software for IVIS (Perkin Elmer, USA).

3.2.8. Nanoparticle tracking analysis

The particle number and size distribution of EVs collected from basal or leptin-treated mHypoA-2/28 were characterized with the Nanosight NS300 (Malvern Panalytical, UK) equipped with a 488 nm blue laser and a sCMOS camera. The samples were diluted 200-fold with PBS for analysis using the default protocol according to the manufacturer's software (NanoSight NS300 User Manual). The instrument parameter was set as follow: camera level 5, slider shutter 100, slider gain 200, FPS 32.5, temperature 24 °C, viscosity 0.906-0.910 cP, syringe pump speed 100, capture time 60 s and detect threshold 3. Calculation of particles quantity and size distribution were based on 3 biological replicates.

3.2.9. RNA extraction from tissue sample and RT-PCR

50mg of tissue was snap frozen with liquid nitrogen and grinded into fine powder with a pre-cooled stainless-steel grinder. 1 ml of TRizol™ reagent (Thermo Scientific, USA) was added to the grinded sample and incubated for 5 min at room temperature. The sample was centrifuged at 12,000 x g for 10 min at 4 °C to remove cellular debris and extracellular matrix. Thereafter, 200 µl of chloroform was added to the sample, mixed and incubated for 2 min and centrifuged at 12,000 x g for 15 min at 4 °C. Phase separation occurred and the upper aqueous RNA phase was transferred to a new centrifuge tube. Equal volume of 70% ethanol was added to the aqueous RNA solution. The solution was mixed and added to the NucleoSpin® RNA extraction kit (Macherey-Nagel, Germany) for clean-up. The sample was desalted with 350 µl of membrane desalting buffer and then treated with 95 µl DNase reaction mixture for 15 min at room temperature. The sample was then washed with Buffer RAW2 once and Buffer RA3 twice as recommended by the manufacturer's protocol. The RNA sample was eluted with DNase-free water and kept at -80

°C till further analysis. RT-PCR was performed on three biological replicate and relative quantification was determined using the comparative Ct method, and the results were normalized to β -actin mRNA. All primers were commercially obtained (IDT, USA) and the primer sequences of the target genes are as follow: Glut1 forward: 5'-TACGGGGTCTTAAGTGCGTC-3', Glut1 reverse 5'-ACTCCTCAATAACCTTCTGGGG-3'; Glut4 forward: 5'-CTACGCCACCATAGGAGCTG-3', Glut4 reverse 5'-TACTGGAAACCCATGCCGAC-3'; Insulin forward: 5'-AAACCCACCCAGGCTTTTGT-3', Insulin reverse 5'-CTCCAACGCCAAGGTCTGAA-3'; Somatostatin forward: 5'-ACCCCAGACTCCGTCAGTTT-3', Somatostatin reverse 5'-GTACTTGGCCAGTTCCTGTTTC -3'; Glucagon forward: 5'-CCACTCACAGGGGCACATTCA -3', Glucagon reverse 5'-GTTTGGCAATGTTGTTCCGGT -3'.

3.3. Results

3.3.1. mHypoA 2/28 hypothalamus cell is responsive to leptin treatment

The Jak/Stat signalling pathway is up-regulated upon leptin treatment and served as a good indicator of leptin activity in the hypothalamus ^[208]. Upon leptin activation, Jak2 auto-phosphorylate itself and also phosphorylate the tyrosine residues on the leptin receptor. The phosphorylated tyrosine residues would recruit and phosphorylate and dimerized STAT3 resulting in the translocation of pSTAT3 into the nucleus for transcriptional activity. At the cellular level, pSTAT3 expression is strongly upregulated upon leptin treatment, and served as a good indicator of leptin activity in the hypothalamus. I showed that mHypoA 2/28 cells were responsive to leptin treatment (5 nM and 10 nM) and showed an increased in pSTAT3 expression after 5 min of leptin exposure which dropped back to basal level after 30 min (Figure 3.2). Subsequently, 10 nM of leptin was used for stimulation of EVs secretion in mHypoA 2/28 cells.

Figure 3.2: Leptin induction of mHypoA 2/28 hypothalamic cells at various time-points. (a) Western blot analysis of pSTAT3 expression in response to 5 nM and 10 nM of leptin induction for 5-, 10-, 15- and 30-min. Molecular weight markers are indicated on the left of the blot.

3.3.2. Identification and quantification of newly synthesized protein in EVs derived from leptin-treated mHypoA 2/28 using pSILAC-based quantitative proteomics analysis

Given the close proximity of the arcuate nucleus to the median eminence ^[109], I seek to establish a role of POMC neurons-derived EVs in energy homeostatic upon leptin stimulation. Therefore, I conducted a pSILAC-based quantitative proteomics methodology to profile protein cargo of the EVs produced by mHypoA 2/28 adult hypothalamus cells after leptin treatment. The cells were first grown to 80% confluence under standard culture and later subjected to 10nM leptin treatment in SILAC-DMEM supplemented with heavy lysine and arginine for 24 h. The EVs were isolated through ultracentrifugation and processed for tandem mass spectrometry analysis. A total of 200 µg of EVs-derived proteins were purified for the proteomic analysis for each condition.

Raw data files generated from 2 biological replicates (three injections per biological replicate) sample were processed using the Proteome Discoverer™

software v2.2, with the Mascot and SequestHT search engine. The analysis achieved high-confidence protein identification (FDR at peptide level <1%) with good reproducibility as the Pearson's correlation coefficient between the two biological replicates of the EV_Ctrl and EV_Leptin were 0.896 and 0.85 respectively (Figure 3.3 a and b). 701 proteins were identified in EVs derived from leptin-treated mHypoA 2/28 cells (EV_Leptin) which is more than the 546 proteins identified in EVs from basal mHypoA 2/28 cells (EV_Ctrl) (Figure 3.3 c). After initial data sorting to remove proteins that lacked a measurable H/L ratio or those that appeared in only one of the two biological replicates, 392 proteins in EV_Ctrl and 510 proteins in EV_Leptin were quantified. Similar to previous chapter, a cut off value of H/L ratio >1.5 was used to denote proteins with increased protein synthesis rate. EV_Ctrl contained 305 proteins that had an H/L ratio < 1.5 and 87 proteins that had an H/L ratio > 1.5 while EV_Leptin contained 387 proteins that have an H/L ratio <1.5 and 123 proteins that have an H/L ratio >1.5. (Figure 3.3 d).

Figure 3.3: pSILAC-based quantitative proteomics analysis of EVs derived from leptin-treated mHypoA 2/28 hypothalamic cells. Scatter plot illustrating the Pearson's correlation coefficients between two biological replicates from the EV_Ctrl (A) and EV_Leptin (B). Chart is plotted using R programme with ggplot2 package. Summary of proteins identified in EVs derived from control or leptin treated mHypoA 2/28 cells. Proteins were considered as quantified if they were identified in both biological replicates and proteins with large variation was omitted (C). The quantified proteins were further subcategorized to newly synthesized protein (H/L ratio > 1.5) or pre-existing protein (H/L ratio < 1.5) (D). Venn diagram illustrating the newly synthesized EVs proteins (H/L ratio ≥ 1.5) detected in the EV_Ctrl and EV_Leptin datasets (E). EVs particle number was determined with nanoparticle tracking analysis. Box plots showing EV concentration from EV_Ctrl and EV_Leptin. Concentration of EVs was quantified by particle count that was normalized against total cell count for each condition. Error bars indicate S.E.M of three biological replicates (Unpaired T-test, ***p<0.001) (F).

Next, leptin treatment of mHypoA 2/28 cells was found to increase the synthesis and loading of proteins into the EVs as 72 of the 123 newly synthesized proteins were found in EV_Leptin only whereas 36 of the 87 newly synthesized proteins were found in EV_Ctrl (Figure 3.3 e). Lastly, leptin treatment promoted a 4-fold increase in mHypoA 2/28 EVs secretion (Mean particle count: EV_Ctrl = 274, EV_Leptin = 1192, normalized against cell count) as measured by NTA (Figure 3.3 f). Taken together, this suggested that

hypothalamus-derived EVs may possibly act as messenger to regulate leptin-mediated events.

Interestingly, several proteins displayed 100% incorporation of heavy isotope-labelled amino acids (H/L ratio = 100) in the EV_Ctrl and/or EV_Leptin dataset. For example, the EV_Ctrl dataset contained proteins such as arylsulfatase A (Arsa), β 2 microglobulin (B2m), hexosaminidase A (Hexa), heat shock protein 90 beta family member 1 (Hsp90b1), laminin Subunit Alpha 5 (Lama5), alpha-N-acetylgalactosaminidase (Naga) and slit guidance ligand 2 (Slit2) that were present in both the basal mHypoA 2/28 cells and EVs but were not found or newly synthesized in the EVs derived from leptin-treated mHypoA 2/28 (Table 3.1). On the other hand, the EV_Leptin dataset also contained proteins such as cyclin-dependent kinase (CDK1, 2, 4, 5, 6, 9, 12, 13, 16, 17), fibroblast growth factor receptor 1 (Fgfr1), tyrosine-protein kinase Yes (Yes1), arylsulfatase B (Arse), heterogeneous nuclear ribonucleoprotein F (Hnrnpf), sphingomyelin phosphodiesterase (Smpd1), tetraspanin 9 (Tspan9), emilin 1, biglycan (Bgn), carboxypeptidase Q (Cpq), cathepsin C (Ctsc) and carboxypeptidase E (Cpe) that showed 100% incorporation of heavy amino acids in EVs from leptin-treated mHypoA 2/28 cells (Table 3.2).

Table 3.1: Highly synthesized proteins that preferentially loaded into EVs derived from basal mHypoA 2/28 hypothalamus cells.

Gene	<u>Cell Ctrl</u>		<u>EV Ctrl</u>		<u>Cell Leptin</u>		<u>EV Leptin</u>	
	H/L ratio	Unique Peptide	H/L ratio	Unique Peptide	H/L ratio	Unique Peptide	H/L ratio	Unique Peptide
PsmA6	0.82	15	1.53	12	1.19	12	0.94	10
PsmB2	0.91	10	1.56	6	1.19	9	0.20	4
Ifitm2	-	-	1.63	2	-	-	-	-
Rab7a	1.55	19	1.64	5	-	-	0.27	4
YwhaQ	0.98	17	1.80	3	1.53	15	0.26	1
Plscr1	2.28	8	1.81	1	2.68	6	-	-
Lrp1	6.99	149	1.81	54	9.49	116	1.47	93
Eif4a2	0.72	8	1.87	2	1.32	9	0.01	5
Rack1	1.29	23	1.94	9	2.07	20	1.37	14
Cd9	2.13	4	2.40	2	3.04	3	-	-
Vps28	1.25	11	2.61	3	2.53	5	-	-
Arf1	1.15	4	3.06	4	2.04	4	0.01	4
Arf2	-	-	3.06	4	-	-	0.01	4
Arf3	-	-	3.06	4	-	-	0.01	4
Col6a1	9.26	23	4.26	2	14.37	21	-	-
Arpc1b	0.94	14	4.66	4	1.44	8	0.66	5
Man2b1	-	-	5.54	7	0.00	1	0.28	7
Tln1	1.12	125	5.95	35	1.70	88	0.91	63
Cd81	7.43	3	10.34	3	8.58	3	0.76	3
Igsf8	15.94	5	13.60	5	16.87	5	0.23	4
PsmB8	0.64	8	15.82	4	-	-	-	-
Plscr3	1.19	7	20.37	2	1.97	6	0.70	2
Ppp2ca	1.50	1	28.76	2	2.05	1	0.01	5
Ppp2cb	1.61	1	28.76	2	2.19	1	0.01	5
H2-K1	11.64	8	35.85	2	0.00	1	-	-
Tsku	-	-	56.01	1	0.84	2	-	-
Arsa	0.53	4	100.00	2	1.31	1	-	-
B2m	11.89	2	100.00	1	13.83	1	-	-
Cystm1	-	-	100.00	1	-	-	-	-
Galt	-	-	100.00	1	-	-	0.01	1
Hexa	0.25	5	100.00	3	1.84	3	-	-
Hsp90b1	0.89	55	100.00	1	1.44	44	0.01	2
Katnal1	-	-	100.00	1	-	-	-	-
Lama5	54.49	6	100.00	5	100.00	8	-	-
Naga	0.53	4	100.00	1	1.74	1	-	-
Slit2	10.00	3	100.00	1	100.00	4	-	-

Table 3.2: Highly synthesized proteins that preferentially loaded into EVs derived from leptin-treated mHypoA 2/28 hypothalamus cells.

Gene	Cell Ctrl		EV Ctrl		Cell Leptin		EV Leptin	
	H/L ratio	Unique Peptide	H/L ratio	Unique Peptide	H/L ratio	Unique Peptide	H/L ratio	Unique Peptide
Gm6498	5.86	2	-	-	0.00	3	1.51	2
Glpr2	1.42	6	1.32	2	2.04	5	1.61	2
Itih3	0.01	4	-	-	0.01	5	1.65	6
Hspa8	1.09	15	0.08	8	1.56	9	1.71	17
Lanc1	1.55	8	0.01	2	1.68	6	1.82	1
Cct7	1.05	1	-	-	1.37	2	1.91	8
Vps37b	3.40	5	-	-	4.38	6	1.92	2
Ftl1	2.98	10	1.23	7	3.57	9	1.98	5
Bpgm	100.00	3	0.64	3	0.00	1	2.09	2
Apom	-	-	1.40	2	-	-	2.12	3
Actn1	0.17	2	0.52	5	1.38	36	2.31	6
Fasn	1.09	103	0.06	6	1.43	74	2.44	35
Gnas	2.02	16	0.01	1	3.97	12	2.56	2
Psm4	0.60	13	0.91	8	1.11	11	2.72	8
Eif3b	1.04	45	0.28	2	1.59	35	4.50	5
Gnb4	0.19	5	-	-	7.84	1	4.71	1
Gnb1	2.87	8	0.05	2	3.98	8	5.14	3
Nell2	-	-	0.01	2	-	-	6.15	1
Bmp1	-	-	0.71	2	0.00	1	8.78	5
Rab11a	-	-	0.06	4	2.33	1	10.16	4
Rab11b	1.50	1	0.06	4	1.80	1	10.16	4
Cd63	1.53	4	0.17	1	1.87	3	11.67	3
Col5a2	9.23	31	-	-	85.69	31	12.96	11
Fn1	2.85	79	-	-	5.04	57	14.15	1
gag-pro-pol	-	-	-	-	-	-	21.03	2
Ruvb12	0.79	24	-	-	1.17	21	25.11	2
Thbs3	26.04	2	-	-	0.00	1	31.82	5
Fbn1	17.21	26	-	-	20.65	16	38.32	3
Hprt	0.80	9	-	-	-	-	38.82	1
Hprt1	0.80	9	-	-	1.37	7	38.82	1
Gja1	14.89	11	-	-	16.65	6	41.05	4
Gusb	0.29	6	-	-	0.00	3	47.52	11
Cdk12	4.91	5	-	-	2.65	4	100.00	1
Cdk5	0.80	9	-	-	1.67	4	100.00	1
Cdk9	3.41	8	-	-	4.35	6	100.00	1
Fgfr1	4.72	5	-	-	100.00	4	100.00	1
Fgfr2	-	-	-	-	-	-	100.00	1
Fgr	-	-	-	-	-	-	100.00	1
Blk	-	-	-	-	-	-	100.00	1
Cdk1	2.09	22	-	-	3.10	17	100.00	1
Cdk13	5.26	8	-	-	3.69	9	100.00	1
Cdk14	-	-	-	-	100.00	3	100.00	1
Cdk15	-	-	-	-	-	-	100.00	1
Cdk16	4.74	9	-	-	7.43	5	100.00	1
Cdk17	23.10	4	-	-	100.00	3	100.00	1
Cdk18	-	-	-	-	3.24	1	100.00	1
Cdk2	1.64	14	-	-	0.46	13	100.00	1
Cdk20	-	-	-	-	-	-	100.00	1
Cdk3	-	-	-	-	-	-	100.00	1
Cdk4	27.77	19	-	-	29.29	8	100.00	1
Cdk6	100.00	2	-	-	-	-	100.00	1
Fgfr3	-	-	-	-	0.00	1	100.00	1
Fgfr4	-	-	-	-	-	-	100.00	1
Fyn	67.05	3	-	-	-	-	100.00	1
Hck	-	-	-	-	-	-	100.00	1
Ick	-	-	-	-	-	-	100.00	1
Lck	-	-	-	-	-	-	100.00	1
Lyn	-	-	-	-	-	-	100.00	1
Mak	-	-	-	-	-	-	100.00	1
Yes1	7.45	5	-	-	2.86	3	100.00	1
Siae	-	-	-	-	-	-	100.00	1
Arsb	0.79	5	-	-	1.62	5	100.00	1
Hnrnpf	1.37	11	-	-	1.86	11	100.00	1
Smpd1	16.24	1	0.01	1	14.10	1	100.00	1
Tspan9	100.00	1	-	-	100.00	1	100.00	1
Emilin1	100.00	8	-	-	100.00	7	100.00	3
Bgn	10.71	14	-	-	15.69	10	100.00	4
Cpq	0.51	2	-	-	2.54	1	100.00	5
Ctsc	0.38	5	-	-	1.43	2	100.00	6
Cpe	34.96	8	-	-	23.64	9	100.00	7
Asah1	0.72	15	-	-	2.15	12	100.00	8
Psm6	0.76	28	0.02	4	1.19	24	100.00	9

GO analysis was performed on the proteins listed in Table 3.1 and 3.2. Newly synthesized EVs proteins were found to preferentially sort into EV_Ctrl were involved in biological process such as cellular response to LDL particle stimulus (GO:0071404), negative regulation of Wnt signalling pathway (GO:0030178), proteolysis involved in cellular protein catabolic process (GO:0051603) and cellular response to glucose stimulus (GO:0071333) ; performed molecular functions such as threonine-type endopeptidase activity (GO:0004298), integrin binding (GO:0005178), enzyme binding (GO:0019899) and hydrolase activity (GO:0016787); and enriched in cellular location such as focal adhesion (GO:0005925), lysosome (GO:0005925 and extracellular exosome (GO:0070062) (Figure 3.4 a). In contrast, GO analysis of newly synthesized EVs proteins that preferentially sort into EV_Leptin suggested that these EVs played a differential role under the influence of leptin (Figure 3.4 b). These proteins are involved in biological processes related to peptidyl-tyrosine auto-phosphorylation (GO:0038083), neuron projection development (GO:0031175) and extracellular matrix reorganization (GO:0030198), function in kinase activities [cyclin-dependent protein serine/threonine kinase activity (GO:0004693) and Protein tyrosine kinase activity (GO:0004713)] and they are localized to basement membrane (GO:0005604), extracellular matrix (GO:0031012) and lysosome (GO:0005764).

Enrichment score: p-value < 0.05

Figure 3.4 Gene ontology analysis of newly synthesized EV proteins that are preferentially sorted into EV_Ctrl (A) or EV_Leptin (B). The y-axis shows enriched gene ontology (GO) categories and the x-axis represents enrichment scores for each term (Enrichment score: p-value < 0.05, modified Fisher Exact P-value). Orange bars = ‘Biological process’ terms in GO; green bars = ‘Cellular component’ terms in GO; purple bar = ‘Molecular function’ terms in GO; n = number of proteins clustered in each GO term.

3.3.3. Bio-distribution and physiological effect of EVs derived from leptin-treated mHypoA

In recent years, researches had shown that nano-sized EVs, which contained a repertoire of proteins, miRNA, RNA and lipids, partake in intercellular communication and contribute toward energy homeostasis and metabolic disorder [209, 210]. I was interested in understanding the action of hypothalamus-derived EVs on peripheral organs. The hypothalamus is the master regulator of energy homeostasis that received input from peripheral signal such as leptin to maintain adiposity [109]. Yet the role of hypothalamus-derived EVs in energy homeostasis remain undefined. Finding from this study would represent a novel mechanism in which the hypothalamus-maintained energy homeostasis and allow us to create new therapeutic target in managing obesity.

Next, I studied the bio-distribution as well as physiological effect of EVs derived from basal or leptin-treated mHypoA 2/28 cells in an animal model.

The mHypoA 2/28 hypothalamic cell line is derived from two-month old male C57bl/6J mouse ^[211] and is a POMC neuron that regulate appetite and energy homeostasis when stimulated by leptin, an orexigenic hormone ^[212].

Furthermore, C57bl/6J mouse is the standard model for diet-induced obesity and leptin resistance ^[213-215], therefore the usage of C57BL/6 mice in this study allowed us to examine the effect of mHypoA 2/28-derived EVs in regulating energy homeostasis during normal physiology.

To visualize the in vivo distribution of the EVs, a lipophilic infrared carbocyanine dye, 1, 1' Dioctadecyl-3, 3, 3', 3'-Tetramethyl-indotricarbocyanine Iodide (DiR') was used to label the EVs (Figure 3.5). The DiR' dye binds to membranous component, has high tissue penetrance, exhibits low auto-fluorescence and is also not known to cause cytotoxicity ^[216-218]. The DiR'-labelled EVs can be separated from free DiR' dye through density gradient ultracentrifugation as the labelled EVs are trapped in the 0.8 M sucrose cushion while the free dye remained at the top of the gradient. The workflow of this study is described in Figure 3.6. Three groups of mice are needed for this study which included: Group 1: Control (Depleted DiR' solution in PBS); Group 2: DiR' labelled EVs from basal mHypoA 2/28 hypothalamus cell; Group 3: DiR' labelled EVs from leptin mHypoA 2/28 hypothalamus cell.

Figure 3.5: Lipophilic infrared dye was used to label mHypoA-derived EVs to examine in vivo bio-distribution. The DiR-labelled EVs are purified through density gradient ultracentrifugation. (*DiR' chemical structure obtained from Thermo Scientific, USA*)

Previous studies on spatiotemporal distribution of EVs had shown that they had a short half-life in the serum, with only 5% of the EVs still detectable 5 min post-injection ^[219]. In the first few hours, EVs accumulated predominately in the liver, spleen and GI tract and the distribution patterns remain relatively similar up till 24 h but changes after 48 h ^[218, 219]. Based on our observation, we found that EVs were observable even after 72 h post injection (unpublished data). Therefore, I studied the bio-distribution of mHypoA 2/28 EVs in C57bl/6J mouse over a period of 72 h. 6-weeks old mice were purchased for this study and were housed under a standard 12 h light/12 h dark cycle. They were allowed to accustom to their housing for a week before the commencement of the study. 50 µg of DiR'-labelled EVs in 100 µl of PBS was injected into each anaesthetized mouse via the retro-orbital venous sinus route. This route of injection was chosen to mimic the likely movement of EVs travelling from the brain region to the peripheral organs and this is important because the route of injection affects the distribution of the EVs ^[220]. In vivo imaging was performed on the C57bl/6J mouse after EVs injection with the IVIS Spectrum In Vivo Imaging System at selected time-point 1, 6 12, 24, 48

and 72 h. Blood glucose, body weight and food intake measurement were taken before the EVs injection and after each imaging time-point. At the end point, the mice were euthanized in CO₂ chamber and organs such as brain, heart, liver, pancreas, spleen, stomach, skeletal muscles, kidney, GI tract and white adipose tissue was collected for further analysis.

Figure 3.6: A workflow diagram illustrating the study of in vivo distribution of mHypoA 2/28 EVs in C57bl/6J mouse.

3.3.4. In vivo distribution of mHypoA 2/28 EVs in C57bl/6J mouse

In this study, 50 µg of DiR'-labelled EVs was used per condition per mouse. The mice were anesthetized and imaged using the IVIS Spectrum In Vivo Imaging System at different time-point; 1, 6, 12, 24, 48, 72 h (Figure 3.7 a). As expected, the control mice (DiR'-depleted PBS solution) did not show any

fluorescence at any time-point. Conversely, injection of DiR'-labelled EVs derived from either basal or leptin-treated mHypoA 2/28 cells resulted in increasing fluorescence intensity at the abdominal region over the 72-h observation period. However, the fluorescence intensity was weaker in mice injected with EVs from leptin-treated mHypoA 2/28 cells.

(A)

(B)

Figure 3.7: In vivo imaging of DiR' labelled mHypoA 2/28 EVs in C57bl/6J mice. Representative image of live mouse 1, 6, 12, 24, 48 and 72 h post-injection with 50 μ g of DiR'-labelled EVs derived from either basal (center) [n = 3] or leptin-treated (right) mHypoA 2/28 cells [n = 3] or DiR' depleted solution (left) [n = 2] (A). Representative images of each organ 72 h after mice were injected via retro-orbital sinus with the DiR'-labelled EVs (B).

After 72 h, the mice were euthanized, and their organs were harvested for further imaging as the in vivo imaging of live mice did not provide enough clarity to examine the spatiotemporal distribution of the EVs and their uptake

by the peripheral organs. In the control mice, fluorescence was detected in the stomach and it was later revealed that food content in the stomach had auto-fluorescence and subsequently omitted for analysis (Figure 3.7 b). For the EV-injected mice, the spleen and in particular the liver showed strong fluorescence intensity, while the rest of the organs such as brain, pancreas, gonadal fat, GI tract and skeletal muscles (tibialis anterior) showed no observable fluorescence signal. A previous study had shown that EVs injected into the blood circulation are cleared by the reticulo-endothelial system which are found in the liver and spleen and this may explained the strong fluorescence intensity that was observed in the liver and spleen of the EV-treated mice ^[221].

3.3.5. mHypoA 2/28 EVs reduced blood glucose concentration in C57bl/6J mouse

Leptin are known to regulate body weight, food intake and glucose level in the biological system through the central nervous system ^[222, 223] and I seek to determine if EVs derived from leptin-treated mHypoA 2/28 cells mediate similar response. After EVs injection, blood glucose level, body weight and food intake were measured at 0, 1, 6, 12, 24, 48 and 72 h time-point. Injection of EVs derived from basal mHypoA 2/28 cells significantly decreased mean blood glucose level by 2.77 and 3.57 mmol/l at 6 and 12 h post-injection respectively (Figure 3.8 a), whereas EVs derived from leptin-treated mHypoA 2/28 cells significantly decreased mean blood glucose level by 3.5 mmol/l at 12 h post injection. Blood glucose level of control mice were elevated by 0.85 and 3.25 mmol/l at 6 and 12 h respectively instead. Next, EVs from both conditions caused body weight loss at 12 and 24 h post injection as well (Figure 3.8 b). Each mouse in the EV_Ctrl group lost an average of 6.3% and 6.7% of their weight 12 and 24 h post injection respectively while mice injected with EVs from leptin-treated mHypoA 2/28 cells lost 5.67% and 6.33% of their weight at the same time-point. Food intake between the three groups remained relatively unchanged (Figure 3.8 c).

Taken together, the data indicated that mHypoA 2/28 EVs were able to modulate blood glucose level and body weight independent of leptin influence. Given that the earlier bio-imaging of the organs of EV-injected mice did not

show significant EVs uptake other than liver and spleen, I decided to analyse the harvested pancreas and skeletal muscles from mice injected with EVs from basal mHypoA cells only to identify possible molecular changes that may account for the decreased in blood glucose level. The pancreas is an endocrine organ that plays a crucial role in glucose homeostasis through insulin secretion to promote glucose uptake in peripheral organs; glucagon promotes hepatic glycogenolysis to increase blood glucose level and somatostatin which inhibit the release of both insulin and glucagon ^[224]. Analysis of pancreatic insulin, glucagon and somatostatin gene expression level after 72 post-injection of mHypoA 2/28 EVs showed that their expressions were not significantly altered (Figure 3.11 d). Next, the skeletal muscle account for most of the glucose uptake in the body through increased glucose transporter (GLUT1 and GLUT4) expression upon insulin stimulation ^[225, 226]. The tibialis anterior muscle was obtained for analysis and EVs injection resulted in a small but significant increase in GLUT4 expression (2.26-fold) but not in GLUT1 (Figure 3.11 e). This indicate that mHypoA EV is involved in the regulation of glucose homeostasis through the acting on skeletal muscle.

Figure 3.8: Effects of mHypoA 2/8 derived EVs on the blood glucose level, body weight and food intake of C57bl/6J mouse. 50 μ g of EVs from either basal or leptin-treated mHypoA 2/28 cells was injected via retro orbital sinus into 7-week-old male C57bl/6J mice. Blood glucose level (A), body weight (B) and food intake (C) were measure pre-injection and 1, 6, 12, 24, 48 or 72 h post injection. Data are presented as mean $\pm$ SEM for blood glucose and body weight measurement. (p value < 0.05*, <0.01**, <0.001***, Two-way ANOVA) Blue asterisk represents statistical testing between EV_Ctrl and Blank while red asterisk is between EV_Leptin and Blank. Gene expression analysis of glucose regulating proteins in pancreas (D) and skeletal muscle (E) from C57bl/6J mice injected with mHypoA EVs. The organs were harvested 72 h after the injection for gene expression analysis. β -actin was used as the reference gene and fold change was calculated using the $e^{-\Delta\Delta CT}$ method. (n = 3, for each condition; Student T-test, *p<0.05).

3.4. Discussion

The human's body weight remains remarkably stable over a period of time despite the large daily variation in food intake and energy expenditure. This energy balance is governed by intricate biological processes through the maintenance of our food intake, energy expenditure and energy storage. At the systemic level, the hypothalamus is the master regulator of the energy balance [202]. They integrate nutrient, hormonal and neuronal inputs from the peripheral organs and subsequently initiate appropriate hormonal and neuronal responses

in a tissue-specific manner that control both the short- and long-term management of the energy homeostasis. Leptin hormone is an adiposity signal that maintained the long-term regulation of the energy homeostasis by regulating food intake and energy expenditure ^[204]. In the hypothalamus, leptin acts on the anorexigenic POMC neurons and activates the central melanocortin to elicit its effect ^[109, 110].

On the other, the hypothalamus was shown to have the capacity to secrete EVs and regulate biological function ^[141] and therefore illustrate an alternative route for neuronal communication. Furthermore, leptin has recently been shown to promote exosome biogenesis ^[227], therefore I postulated that leptin may promotes EVs secretion in POMC neurons to regulate energy homeostasis. The postulation was that the action of leptin signalling would induced de novo protein synthesis and these newly synthesized proteins would be sorted into the EVs for secretion and elicit its functions at distant organs and had established a pSILAC-based quantitative proteomics methodology to profile the newly synthesized EVs proteome derived from leptin treated mHypoA-2/28 cells and study their role in energy homeostasis. Leptin treatment was found to promote protein loading into the mHypoA EVs as the number of proteins identified were higher in EV_Leptin (510 proteins) over EV_Ctrl (392 proteins). This was accompanied by an increased in EV secretion upon leptin stimulation which was consistent with other study ^[227]. Gene ontology analysis also revealed that newly synthesized EV proteins from leptin-treatment mHypoA 2/28 to be enriched in biological process vastly different from EVs from basal mHypoA 2/28 cells, suggesting that they may have different functional role. Next, EVs were injected into the C57bl/6J mice to study their spatiotemporal distribution as well as physiological effect. Surprisingly, EVs from basal and leptin-treated mHypoA 2/28 exhibited showed similar uptake pattern by the peripheral organs and mice injected with EVs from either conditions promoted body weight loss and decreased in blood glucose level to a similar extent. This implied that the observed phenotypic changes such as decreased in blood glucose level were due to the intrinsic properties of the mHypoA 2/28 EVs regardless of leptin treatment, especially since the mHypoA cell line is derived from POMC neurons.

The POMC neurons displayed glucose sensing ability as its' firing rate increases with rising glucose concentration ^[228]. Exposure to glucose induces intercellular ATP production which in turn promote the closure of ATP-dependent potassium channel and the eventual depolarization of the neurons ^[229]. Furthermore, the AMP-activated protein kinase (AMPK) signalling pathway modulate POMC neuron's glucose sensitivity as the membrane potential of POMC-AMPK α 2-KO neurons remain unresponsive when external glucose concentration was altered ^[230]. Lastly, the loss of glucose sensing ability in POMC neurons resulted in systemic impaired glucose tolerance ^[229].

The mHypoA 2/28 cell, derived from POMC neuron, used in this experiment is cultured in high glucose-DMEM (4.5g/l) and may have stimulated the glucose sensing ability in mHypoA 2/28, resulting in the secretion of EVs that regulate systemic glucose homeostasis. Interestingly, GO analysis of newly synthesized proteins in EV_Ctrl identified a group of proteins, such as receptor of activated protein C kinase 1 (Rack1), galectin-1 (Lgals1) and protein phosphatase 2 catalytic subunit alpha (Ppp2ca), that are involved in cellular response to glucose stimulus (GO:0071333). Rack1 is a scaffold protein that support the dephosphorylation of the inositol-requiring enzyme 1 α (Ire1 α) by promoting IRE1 α interaction with protein phosphatase 2A in response to glucose stimulation in pancreatic β cells and the net effect is reduced insulin production ^[231]. On the other hand, Ppp2ca is a phosphatase that negatively regulate insulin signalling through dephosphorylation of key enzyme such as Akt ^[232], AMPK^[233] and the above mentioned Ire1 α to modulate glucose balance. Lastly, Lgals1 is able to regulate glucose uptake through inducing the expression of GLUT1 via the mTOR and STAT3 pathway ^[234]. From this dataset, GLUT4 instead GLUT1 expression was increased in the skeletal muscle of the C57bl/6J mouse treated with mHypoA 2/28 EVs and perhaps Lgals1 may play a similar role in the skeletal muscle by inducing the expression of a different glucose transporter. Whereas Rack1 and Ppp2ca may act in a negative feedback manner to regulate glucose uptake. In future study, it would be interesting to study if these proteins belong to different population of EVs and if they had different uptake rate by the recipient cells to modulate their functions in a sequential

manner. This would allow us to explain the initial decrease and eventual recovery of the blood glucose level upon injection of the mHypoA 2/28 EVs.

Based on the animal study, EVs from leptin-treated mHypoA 2/28 cells promoted similar effect as the EVs from basal mHypoA 2/28 cells which was a surprise as it was postulated that EVs_Leptin would promote a more potent regulation in energy or glucose homeostasis. Even so, based on the GO analysis, it seems that that EVs_Leptin may play an alternate role instead. Newly synthesized proteins from EV_Leptin were found to be involved in biological process such as neuron projection development (GO:0031175) and extracellular matrix reorganization (GO:0030198), which indicate these EVs may act as leptin-mediated paracrine messenger to regulate neuronal connection in the central melanocortin system ^[235] rather than as a messenger for inter-organ communication. Nonetheless, this study had established a functional role of mHypoA 2/28 EVs in the regulation of systemic blood glucose homeostasis. These findings suggested that small extracellular vesicles may be used as a potential treatment strategies or therapeutic target to combat metabolic syndrome such as hyperglycaemia.

Chapter 4

Conclusion

4. Conclusion

4.1 Concluding remarks and future perspective

Extracellular vesicles (EVs) are secreted by different types of cells and contained an array of cytosolic materials such as proteins, nuclei acid and lipids that are packed into a nano-sized membranous vesicle ^[1]. Research on EVs has gained a lot of traction due to their role in intercellular communication and our labs had previously unravelled the role of EVs in hypoxia-induced cancer progression, neurodegenerative disease and cardiovascular disease ^[3, 19-21]. Even as research on EVs-mediated intercellular communication continue to grow exponentially, a pertinent issue to address is the heterogeneous nature of the EVs population and their biogenesis. MVBs formation is an important step for generation of iLVs for EVs secretion but is also required for lysosomal degradation too. However, the mechanism that regulates MVBs formation towards EVs secretion and lysosomal degradation remained poorly elucidated. The overarching goal of my PhD thesis is to establish a discovery-driven quantitative proteomics approach to study EVs biogenesis and EV-mediated intercellular communication.

I established a pSILAC-based quantitative proteomics methodology to study EVs biogenesis by conducting a comparative analysis between the newly synthesized proteome of the EVs and lysosome from mHypo A 2/28 adult hypothalamus cell line. The postulation was that newly synthesized proteins that were preferentially sorted to the EVs over the lysosome may play an important role in regulating the fate of mature MVBs. The introduction of heavy amino acids such as arginine and lysine into the cell culture for a short period of time facilitated the measurement of protein synthesis rate by quantifying the ratio of the heavy isotope over the light isotope (H/L ratio) for each protein. Through H/L ratio analysis, EV biogenesis-related proteins were shown to be actively synthesized and therefore indicated that that EV biogenesis is an active process that required constant protein synthesis to maintain its function. Further hierarchal clustering analysis of the proteomic dataset identified a group of newly synthesized cathepsin proteins that are enriched in the EVs. Functional characterization of the cathepsin proteins through chemical inhibition revealed the role of Ctsd in modulating EV

biogenesis and secretion. I demonstrated that the chemical inhibition of Ctsc proteins resulted in an increase in secreted mHypoA EVs, together with a change in EV composition. Ctsc inhibition enhanced the secretion of CD63+ EVs population while at the same time reduced the secretion of CD81+ EVs. This indicated that Ctsc possibly regulate the generation of CD81+ EVs which are localized near the plasma membrane ^[167]. Additionally, it was discovered that proteins involved in sphingolipid/ceramide metabolism such as PSAP, ASAH1 and SMPD1 are enriched in EV_Ctrl. These proteins are functionally associated with Ctsc which point towards a role of Ctsc in a ceramide-based EV biogenesis at the plasma membrane. Moreover, newly synthesized Ctsc were found in the EVs of various cell types which highlighted an important and novel role of Ctsc in EVs biogenesis. Future work should focus on the downstream effect of the altered EVs population upon Ctsc inhibition, particularly in EV-associated cellular waste disposal or EV-mediated intercellular communication. Dysregulation of these functions has huge implication in certain disease such as neurological disease and a thorough understanding of EV biogenesis and the regulatory mechanism of how different EVs subpopulation are selected for secretion may provide valuable information towards exosome engineering for therapeutic purposes.

Leptin is an adipocyte hormone that control energy homeostasis through their action on the hypothalamus and the central melanocortin system to regulate food intake and energy expenditure ^[203, 204]. While it is established that leptin propagates its action through neuronal activity, I postulated that leptin is able to promote EV-mediated intercellular communication in POMC neurons to promote energy homeostatic function. Additionally, the hypothalamic arcuate nucleus, in which the POMC neurons are located, is situated near the median eminence which can facilitate the dispersal of the secreted EVs into the systemic circulation. Therefore, in Chapter 3, the established pSILAC-based quantitative methodology was applied to study the role of leptin-mediated mHypoA 2/28 EV secretion in regulating energy homeostasis. Proteomics analysis revealed that leptin stimulated the sorting of more newly synthesized proteins into mHypoA 2/28 EVs while NTA measurement indicated leptin promoted EVs secretion, and this suggested a role of leptin in utilising EVs as

a mean of communication. The bio-distribution and functional role of EVs from basal and leptin-treated mHypoA 2/28 cells were studied in C57bl/6J mouse over a period of 72 h. Observation on the effect of EVs from both conditions on C57bl/6J mice showed strong similarity. Both EVs were found to accumulate in the liver and spleen, induce body weight loss and reduce blood glucose level post-injection. This suggested that the observed effect was independent of leptin treatment and GO analysis of EVs proteins from leptin-treated mHypoA 2/28 cells indicated that these EVs may played a role in paracrine signalling instead. Nonetheless, I had demonstrated a novel mechanism in which hypothalamus cells regulate glucose homeostasis. Findings from this study will contribute to the understanding of developing EV-based therapy against hyperglycaemia in metabolic syndrome. Current treatment for hyperglycaemia in Type II diabetes patients included blood glucose-lowering drug such metformin and sulfonylurea ^[236] which managed the symptom without treating the underlying disease. EV-based therapy may provide a more holistic approach towards disease treatment given that EVs contained an array of biological molecules that include proteins, lipids and nuclei acids such as the miRNA. Therefore, EVs may have a more potent influence on the cell physiology than a single compound drug.

In conclusion, I had adopted a discovery-driven approach and successfully applied a pSILAC-based quantitative proteomic methodology to perform proteome-wide analysis of newly synthesized proteins in EVs. This is a novel approach in the study of EV biogenesis and intracellular communication and offers a new perspective in the understanding of EVs biology and the findings in this thesis could contribute towards an EV-based therapeutic treatment against metabolic diseases.

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Appendix A

Publications

Park JE, Dutta B, Tse SW, Gupta N, **Tan CF**, Low JK, Yeoh KW, Kon OL, Tam JP, Sze SK: **Hypoxia-induced tumor exosomes promote M2-like macrophage polarization of infiltrating myeloid cells and microRNA-mediated metabolic shift.** *Oncogene*, 2019

Gallart-Palau X, Serra A, Hase Y, **Tan CF**, Chen CP, Kalaria RN, Sze SK: **Brain-derived and circulating vesicle profiles indicate neurovascular unit dysfunction in early Alzheimer's disease.** *Brain Pathology*, 2019

Min ATL, Langley SR, **Tan CF**, Fang CJ, Khoo CM, Leow MK, Khoo EYH, Moreno Moral A, Pravenec M, Rotival M *et al*: **Ethnicity-specific skeletal muscle transcriptional signatures and their relevance to insulin resistance in Singapore.** *The Journal of clinical endocrinology and metabolism*, 2018

Presentation

1. Speaker and Poster presentation, **Understanding exosome biogenesis in hypothalamic cells through studying of protein dynamic using pulsed-SILAC**, American Society for Exosomes and Microvesicles, Baltimore, November 2018
2. Speaker, **An Introduction to pSILAC**, IGS Student Seminar Series, Singapore, March 2017
3. Poster presentation, **Food as an environmental influence on the epigenetics of obesity and metabolic syndrome**, 2nd Peptides and Proteins Symposium, Singapore, Dec 2016