UNIVERSITY OF CALIFORNIA SAN DIEGO

The role of exosomal Clic1 in metabolism

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in

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by

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DEDICATION

This thesis is dedicated to my parents and my best friends who always provide their support and encouragement to me.

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ABSTRACT OF THE THESIS

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Clic1, Chloride Intracellular Channel 1, is a ubiquitously expressed chloride channel protein known to be involved in various biological processes. In our previous studies, we have found that Clic1 is involved in olanzapine-induced hyperphagia and may play a role in food intake regulation. Also, it has been found that Clic1 is highly expressed in exosomes. Exosomes are small extracellular vesicles sized that are secreted from many different types of cells and act as information vehicles by transporting proteins and RNAs to the recipient cells. They can freely diffuse in all body fluids and be taken up by different organs. In recent years, it has been shown

that exosomes play an important role in inter-organ crosstalk in the homeostatic control of metabolism. The objective of this thesis is to discuss the potential role of exosomal Clic1 in the regulation of metabolic processes. In addition to literature review, we performed experiments by checking if the level of Clic1 expression would be affected by diet type and food deprivation. High-fat-diet fed mice had elevated plasma-derived exosomal Clic1 levels, but this effect was not significant. Fasting resulted in an approximately 2-fold increase in exosomal Clic1, and glucose injection resulted in a trend in decreased exosomal Clic1 expression, but these effects were also not statistically significant, and further additional samples need to be studied to determine a more consistent result. Taken together, this study suggests that future studies are needed to further investigate the potential role of exosomal Clic1 as a regulator of feeding behavior and a coordinator of metabolism.

INTRODUCTION

Chloride intracellular channels (Clics)

Chloride ions are the principal anions involved in cell proliferation and cell apoptosis in human body (Lathrop and Loeb 1915; Heimlich and Cidlowski 2006). Chloride channels are a major type of anion channel. They play a significant role in the regulation of various fundamental cellular physiological processes and are involved in the occurrence of many pathological disorders (Gururaja Rao, Patel, and Singh 2020). Chloride channels perform diverse functions in living organisms, including GABA and glycine signaling, membrane potential stabilization, pH maintenance, cell volume regulation, and NaCl fluid transport (Suzuki, Morita, and Iwamoto 2005; Vardi and Zhang 2010).

Chloride intracellular channel (Clic) proteins are a highly conserved family of chloride channels consisting of six distinct paralogues (Clic1-6) in mammals (Littler et al. 2010). A unique feature of Clic proteins is that they are a family of "metamorphic" proteins that can undergo reversible transitions between different folded conformations (Murzin 2008). Clic proteins were first discovered in 1989 by Landry et al. while purifying chloride channels related to cystic fibrosis. Among four major purified proteins, a protein with a molecular mass of 64 kilodaltons, named p64, was subsequently identified as a ubiquitous component of chloride channels (Redhead et al. 1992). The primary sequence of p64, now renamed as Clic5B, showed no significant homology to any known proteins (Landry et al. 1993). Clic1 protein is the first cloned human member of the Clic family (Valenzuela et al. 1997), and the Clic1 gene is found to be located in the major histocompatibility complex (MHC) class III region on chromosome 6p21.3 (Lehner et al. 2004).

The function and distribution of Clic1

Clic1 is expressed ubiquitously, but its biological and physiological functions are still not completely understood. Previous studies show that Clic1 is involved in the regulation of cell cycle (Valenzuela et al. 2000). For example, in Chinese hamster ovary (CHO-K1) cells, the chloride ion conductance of Clic1 in the plasma membrane was only detected in the G2/M phase of the cell cycle, and the inhibition by chloride channel blocker indanyloxyacetic acid 94 (IAA-94) arrested the cells in the G2/M phase (Valenzuela et al. 2000). Other studies have shown that Clic1 plays a role in the activation of microglia (Novarino et al. 2004), the production of reactive oxygen species (ROS) (Milton et al. 2008), and the regulation of osteoblast differentiation (Yang et al. 2009).

The distribution and expression patterns of Clic1 are tissue- and cell-type specific, indicating that Clic1 has different functions in different tissues (Ulmasov et al. 2007). In adult mouse tissues, three patterns of Clic1 expression have been reported. In a variety of simple columnar epithelia, including stomach, small intestine, colon, bile ducts, pancreatic ducts, airway, epididymis tail, and kidney proximal tubule, Clic1 exhibits an apical-polarized pattern of expression; in the basal cells of some stratified squamous epithelia of the upper gastrointestinal tract, such as the non-keratinizing epithelia of the esophagus and the forestomach, Clic1 exhibits a non-polarized pattern of expression; in the skeletal muscle fibers, Clic1 is expressed throughout the cytoplasm (Ulmasov et al. 2007).

The dimorphism of Clic1

Clic proteins are dimorphic proteins that can exist in both soluble, globular form as well as membrane-associated ion channel form (Littler et al. 2010; Argenzio and Moolenaar 2016). Cytosolic soluble Clics contain a conserved “Clic module” of approximately 240 residues whose

structure appears similar to the omega-class of glutathione-S-transferases (GSTs) and exhibit glutaredoxin-like glutathione-dependent oxidoreductase enzymatic activity (Dulhunty et al. 2001; Harrop et al. 2001; Al Khamici et al. 2015; Argenzio and Moolenaar 2016). Although an amphipathic motif consisting of β-strand 1, α-helix 1, and β-strand 2 in the glutaredoxin-like N-domain is commonly regarded to form the transmembrane region of Clics, the structure of the ion channel form remains unclear (Fanucchi, Adamson, and Dirr 2008).

Soluble Clic1 spontaneously inserts into preformed phospholipid vesicles to form a chloride-selective ion channel in the absence of a leader sequence targeting the membrane (Tulk, Kapadia, and Edwards 2002). However, the mechanism behind the transition between the soluble enzymatic form and the membrane-associated form of Clics remains controversial. Oxidative conditions can trigger a reversible non-covalent dimerization of Clic1 monomers whereby the glutaredoxin-like domain in Clic1 monomers undergoes a significant conformational change, exposing a new hydrophobic surface (Littler et al. 2004). During this transition from monomer to dimer, a critical intramolecular disulfide bond is formed between Cys-24 and Cys-59 in the N-terminal of Clic1, which stabilizes the dimer structure (Littler et al. 2004). The resulting Clic1 dimer can then successfully form chloride channels in the membrane. Other studies suggest that Clic1 monomers are oxidized and attached to the lipid bilayer first, followed by conformational changes that further promote its insertion into the membrane (Goodchild et al. 2009). The acidic environment at the surface of the membrane also primes the transmembrane region of Clic1 in favor of its insertion into the membrane, whereby the structure of Clic1 becomes destabilized to form partially unfolded intermediates of soluble Clic1 that contain exposed hydrophobic surfaces (Fanucchi, Adamson, and Dirr 2008). Furthermore, the activity of Clic1 as an ion channel is known to be highest at acidic pH and lowest at pH 7.0 (Tulk, Kapadia, and Edwards 2002).

Clic1 Knockout (KO) mice

Clic1 KO mice are viable and show no obvious phenotype in unstressed laboratory conditions (Chalothorn et al. 2009). However, upon various challenges, Clic1 KO mice display some phenotypic differences compared with WT littermates.

Clic1 participates in inflammatory processes by regulating macrophage phagosomal functions such as pH and proteolysis. Clic1 is expressed on the phagosomal membranes of macrophages and of bone marrow dendritic cells (BMDCs), and phagosomal acidification is impaired in both Clic1 KO macrophages and BMDCs (Jiang et al. 2012; Salao et al. 2016). As a result, the proteolytic activity of macrophage and BMDCs phagosomes is reduced in Clic1 KO mice (Jiang et al. 2012; Salao et al. 2016). When challenged with serum from K/BxN mice that causes WT mice to develop arthritis (Monach et al. 2007), Clic1 KO mice are protected from developing this inflammatory condition, strongly suggesting that Clic1 is involved in pro-inflammatory responses (Jiang et al. 2012). In a similar inflammatory model of human demyelinating disease, multiple sclerosis (MS), Clic1 KO mice developed milder experimental autoimmune encephalomyelitis (EAE) disease due to a reduced capacity of Clic1 KO BMDCs to elicit the initial local response before secondary amplification of the immune response occurs at more distal sites (Salao et al. 2016). In addition, the absence of Clic1 attenuates some aspects of acute inflammatory injury in both acute pancreatic and acute kidney injury models and is associated with suppression of local production of reactive oxygen species (ROS) (Ulmasov et al. 2017).

Importantly, to date, there are no specific publications describing a role for Clic1 in metabolism. However, Ulmasov et al. (2017) reported that female Clic1 KO mice weigh

significantly less than the wild-type control, raising the potential for Clic1 to play a role in the regulation of body weight.

Overview of exosomes

Clic1 is found in small extracellular vesicles (EVs) from multiple cell types and tissues. EVs are lipid membrane-bound nanoparticles containing lipids, nucleic acids, and proteins that are secreted by cells into the extracellular space (Zaborowski et al. 2015). EVs have various subtypes, including microvesicles (MVs), exosomes, and apoptotic bodies, which are differentiated based upon their biogenesis, release pathways, size, content, and function (Doyle and Wang 2019). Exosomes were first discovered in the maturation of reticulocytes and subsequently found to function as antigen-presenting vesicles (Johnstone et al. 1987; Raposo et al. 1996). Exosomes are small vesicles (30-150 nm) that are generated via endosomal pathway and released via exocytosis (Raposo and Stoorvogel 2013; Doyle and Wang 2019). Exosomes are enriched with various proteins, including chaperones (HSP70, HSP90) and tetraspanins (CD9, CD63, and CD81) (Moreno-Gonzalo et al. 2014). Early endosomes are originated by the inward budding of plasma membranes and function as a major sorting station (Ziello, Huang, and Jovin 2010; Doyle and Wang 2019). The early endosomes mature into multivesicular endosomes (MVEs), by which small intraluminal vesicles containing specifically sorted molecules are formed by the inward budding of the delimiting membrane of early endosomes (Zhang et al. 2015; Guay and Regazzi 2017). The MVEs then fuse with lysosomes for degradation or with the plasma membrane for the release of the intraluminal endosomal vesicles as exosomes (Colombo, Raposo, and Théry 2014; Zhang et al. 2015; Guay and Regazzi 2017). The mechanism of how the cargos are sorted into exosomes is not well understood yet. Post-translational modifications, including ubiquitination, sumoylation,

and phosphorylation, are found in exosomal proteins and possibly play a role in the sorting process (Moreno-Gonzalo et al. 2014). miRNAs with GGCU (hEXO) motif are observed to be enriched in hepatocyte exosomes, and this motif directly binds to synaptotagmin-binding cytoplasmic RNA-interacting protein (SYNCRIP), directing the sorting of exosomal miRNAs in hepatocytes (Santangelo et al. 2016). The endosomal sorting complexes required for transport (ESCRT) proteins are found to be involved in the generation of MVEs and the secretion of the exosomes. The ESCRT complex consists of approximately thirty proteins that assemble into four complexes, ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III, named according to the order of their recruitments (Hanson and Cashikar 2012). ESCRT-0 initiates the sorting of cargos into MVEs and recruits ESCRT-I; ESCRT-I and ESCRT-II sort the cargos and promote the formation of intraluminal vesicles; ESCRT-III drives membrane budding and vesicle scission (Hanson and Cashikar 2012; Guay and Regazzi 2017). ESCRT-independent mechanisms also exist, and these mechanisms vary between different cell types. For example, tetraspanin protein CD-63 plays a role in the exosome formation of human melanoma cells (Verweij et al. 2011), whereas in oligodendroglial cell lines, MVE budding depends on an enzyme that produces ceramide, sphingomyelinase (Trajkovic et al. 2008).

Exosomal Clic1

Clic1 is one of the top 50 most commonly identified proteins in exosome preparations (Basso and Bonetto 2016) (**Table 1**). High levels of exosomal Clic1 are potential biomarkers of various types of cancer (Chang et al. 2009; Tang et al. 2011; Tang et al. 2013; Mathivanan et al. 2010; Setti et al. 2015; Zhao et al. 2019). Overexpressed Clic1 is found in the conditioned medium of many different cancer cell lines (Setti et al. 2015; Mathivanan et al. 2010). In addition, the

plasma level of CLIC1 is significantly higher in patients with various cancers, including nasopharyngeal carcinoma (NPC) (Chang et al. 2009) and ovarian cancer patients (Tang et al. 2011; Tang et al. 2013).

Glioblastoma cells express high levels of Clic1, and they secrete Clic1 via EVs (Setti et al. 2015). Functionally, Clic1-containing EVs derived from glioblastoma cells can promote the proliferation of recipient glioblastoma cells in vitro and enhance the growth of tumors in vivo (Setti et al. 2015). Furthermore, the silencing of Clic1 in glioblastoma cells significantly impairs the growth-stimulating effects of the EVs they produce on recipient glioblastoma cells or tumors (Setti et al. 2015).

High levels of exosomal Clic1 are also associated with chemotherapy resistance in cancer development. Vincristine is a chemotherapy medication used to treat a number of types of cancer. An elevated expression of Clic1 is observed in vincristine-resistance human gastric cancer cell lines, and siRNA-mediated silencing of intracellular Clic1 expression can reverse the resistance to vincristine (Zhao et al. 2019). Furthermore, exosomes derived from vincristine-resistance cell lines can promote the resistance to vincristine in recipient cells, possibly through the upregulation of the expression intracellular Clic1 and apoptosis-related factors (Zhao et al. 2019). This study also demonstrates that the exosomal expression level of Clic1 mirrors the intracellular expression level (Zhao et al. 2019).

The role of exosomes in intercellular communication

Exosomes were originally thought to simply provide a way for cells to get rid of unneeded or unwanted material. However, it has since been found that exosomes have an important role in intercellular communication (Raposo and Stoorvogel 2013). Exosomes are secreted by various cell

types and play an important role in inter-organ crosstalk by transporting bioactive molecules, including proteins, mRNAs, and miRNAs (Guay and Regazzi 2017) (**Figure 1**). After being released from a cell, exosomes can then be taken up by recipient cells via a variety of processes (Guay and Regazzi 2017). Exosomes can bind to the receptors on the cell membrane and trigger intercellular signaling, as observed in mouse peritoneal macrophages (Miyanishi et al. 2007). Secondly, exosomes can be taken up by plasma membrane fusion, as seen in various tumor cells (Parolini 2009). In addition, exosomes can also be internalized into the cells via various endocytic pathways and then fuse with the endosomal membrane (Mulcahy, Pink, and Carter 2014).

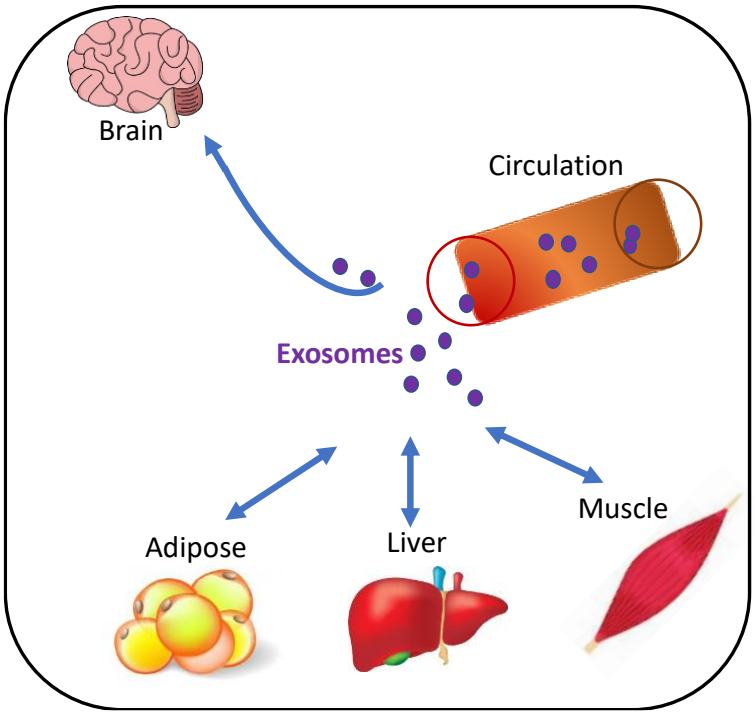

Figure 1. Schematic diagram of inter-organ cross talk mediated by exosomes.

Exosomes perform a critical function in the coordination between metabolic organs

Exosomes participate in cell-cell communication and cell maintenance and play an important role in inter-organ crosstalk (Doyle and Wang 2019) and the development of obesity-related metabolic diseases (Zhang, Yu, and Tian 2016).

Exosomes secreted from adipose tissue contain adipokines, including Interleukin-6 (IL-6) and monocyte chemoattractant 1 (MCP-1) that can impact the insulin sensitivity of liver and muscle cells (Kranendonk et al. 2014). The miRNA profiles in obese visceral adipocyte exosomes are distinct from lean visceral adipocyte exosomes with 55 differentially expressed mature miRNAs detected, but further investigation is needed to determine the contribution of these miRNAs on the development of metabolic disease (Ferrante et al. 2014). Exosomes from adipose tissue can also be taken up by monocytes and can trigger these immune cells to change their inflammatory state, which can also impact insulin sensitivity (Deng et al. 2009).

Skeletal muscle cells secrete exosomes to modify muscle homeostasis and communicate with key metabolic tissues during diet-induced insulin resistance (Aswad et al. 2014). It has been found that muscle exosomes can be taken up by pancreatic β-cells and affect the size and gene expression of β-cells (Jalabert et al. 2016). In addition, exosomes derived from pancreatic β-cells can either act in an autocrine manner or be taken up in a paracrine manner by other β-cells, immune cells, or endothelial cells, thereby regulating glucose homeostasis and insulin resistance (Guay and Regazzi 2017).

Importantly, further understanding of the role of exosomes derived from peripheral metabolic organs will elucidate mechanisms of development of metabolic diseases and may be candidates for diagnosis and treatment or novel biomarkers of metabolic diseases (Müller 2012).

Exosomes as messengers between the periphery to the brain

Exosomes are thought to be released by all cells in the body and can serve as messengers between the periphery and the brain. While the blood-brain barrier (BBB) provides a selective barrier to protect the brain, exosomes are able to cross this barrier, but the specific mechanisms are still under investigation. In theory, exosomes could either undergo transcytosis like some viruses or pass through the intercellular spaces between the endothelial cells (Jarmalavičiūtė and Pivoriūnas 2016). One hypothesis says that exosomes internalized by brain endothelial cells at first may release specific substances that increase the intercellular permeability of brain endothelial cells, promoting the subsequent passage of exosomes across the BBB (Jarmalavičiūtė and Pivoriūnas 2016). However, experimental evidence is lacking. In an in vitro study, exosomes were found to cross a monolayer of brain microvascular endothelial cells (BMECs) majorly via the transcellular route, where the exosomes go through endocytosis, MVE formation, and exocytosis (Chen et al. 2016). Also, exosomes crossed the BMEC monolayer under inflammatory conditions with TNF-α activated BMECs but not under normal conditions with untreated BMECs (Chen et al. 2016). Different types of exosomes cross the BBB at different transport rates and by different mechanisms. Banks et al. (2020) investigated the interaction between 10 exosome populations derived from mouse, human, cancerous, and non-cancerous cell lines with BBB. The transport rates of each exosome type in different brain regions were mostly homogeneous, whereas four exosome types exhibited a significantly higher transport rate in the olfactory bulb (Banks et al. 2020). The enhancing effect of wheat germ agglutinin (WGA) on the transports of five types of exosomes across BBB suggested that absorptive transcytosis is involved in the passage (Banks et al. 2020). The inhibition effect of mannose 6-phosphate (M6P) on the transport of NIH-3T3-

derived exosomes across BBB suggested that M6P receptor is involved in their passage (Banks et al. 2020).

The ability of peripherally-derived exosomes to impact gene expression in the brain has been demonstrated by multiple groups. Alvarez-Erviti et al. (2011) demonstrated effective delivery of siRNA to the brain via systemic injection of exosomes in mice. They engineered dendritic cells to express an exosomal membrane protein, lysosome-associated membrane protein 2 (Lamp2b). They then fused Lamp2b to a rabies virus glycoprotein (RVG) peptide that is specific to the central nervous system resulting in exosomes were targeted exclusively to the brain. These exosomes loaded with GAPDH siRNA were injected intravenously, and specific silencing of GAPDH gene expression was observed in the brain (Alvarez-Erviti et al. 2011). The therapeutic potential of this approach was then demonstrated by injection of siRNA-loaded RVG exosomes that successfully silenced the expression of brain expression of Beta-secretase (BACE1), a gene implicated in Alzheimer's-related (Alvarez-Erviti et al. 2011). Other studies have also delivered exosomes to the brain through the intranasal route (Zhuang et al. 2011). Exosomes derived from a mouse lymphoma cell line were loaded with the anti-inflammatory drug curcumin and delivered intranasally to mice. These curcumin-loaded exosomes were subsequently taken up by microglial cells, where they attenuated the severity of myelin oligodendrocyte glycoprotein (MOG) peptide-induced experimental autoimmune encephalomyelitis (EAE) (Zhuang et al. 2011). In another study, exosomes from brain endothelial bEND.3 cells were found to be capable of crossing the BBB of zebrafish (Danio rerio) embryos and delivering anticancer drugs into the brain (Yang et al. 2015). Doxorubicin delivered in bEND.3 exosomes effectively attenuated cancer development in the zebrafish xenotransplant brain cancer model (Yang et al. 2015).

Once exosomes reach the brain, they can also directly impact neurogenesis and neurocircuit development (Sharma et al. 2013). Exosomes from human-induced pluripotent stem cell-derived neurons can enhance the proliferation and differentiation of human primary neural cultures in vitro (Sharma et al. 2019). These findings have also been validated in vivo, where exosomes derived from primary neuronal cultures were injected directly into the dentate gyrus of postnatal P4 and promoted cell growth in this region (Sharma et al. 2019). Further studies showed that cells with loss of function of MECP2 have significantly impaired brain development, but treatment with control exosomes can 'rescue' aspects of this loos of function cellular phenotype, including cellular density and neuronal firing rate (Sharma et al. 2019). These studies identified a novel role for exosomes in neural circuit development leading to enhanced neural progenitor proliferation, neuronal differentiation, and circuit connectivity.

Exosomes can also cross the BBB in a bi-directional manner and deliver signals from the brain to the periphery. Most recently, a study using rats identified that a fluorescently tagged protein expressed selectively in brain tissue could be recovered in small EVs in their blood (Gómez-Molina et al. 2018). This route of brain-to-periphery signaling, mediated by a differential molecular cargo of small extracellular vesicles, provides a mechanism by which the brain might communicate to the rest of the body.

Potential role of exosomes as regulators of food intake

Food intake is a highly regulated process requiring the integration of many signals from both the periphery and central nervous system. The specific role of exosomes in the regulation of food intake is not yet clear, but some studies have suggested exosomes may play a role in coordinating this process. Notably, after 48-hour fasting, the number of exosomes in the plasma

of wild-type C57BL/6 mice approximately doubles, suggesting that exosomes may play a role in communication regarding nutritional status (Zou et al. 2018). In an exciting study, exosomes have been shown to function as regulators of food intake and body weight by acting on the appetite-suppressing pro-opiomelanocortin (POMC) neurons in the hypothalamus (Gao et al. 2019). Firstly, adipocyte-derived EVs were successfully transferred into POMC neurons in vitro. Then, EVs from adipocytes of obese mice were administered to lean mice resulting in increased appetite and weight gain. Conversely, adipocyte-derived EVs from lean mice decreased food intake and weight when administered to obese mice (Gao et al. 2019). It has previously been established that the activation of the mammalian target of rapamycin (mTOR) signaling pathway suppresses the activity of POMC neurons (Albert and Hall 2015). Mechanistically, it is proposed that EVs secreted by visceral adipocytes of obese mice significantly enhanced mTOR activity in hypothalamic POMC neurons. Whereas EVs from lean mice significantly reduced mTOR activity in the hypothalamus of obese mice (Gao et al. 2019). The level of metastasis-associated lung adenocarcinoma transcript-1 (MALAT1) in adipocyte EVs was found to be significantly higher in obese mice compared to lean mice, and the overexpression of MALAT1 was shown to significantly enhance mTOR activity in hypothalamic POMC neurons (Gao et al. 2019). Furthermore, inhibition of mTOR activation by rapamycin blocked the effect of obese adipocyte-derived EVs on food intake and body weight of lean mice, confirming the importance of the mTOR signaling pathway on exosome regulation of food intake in POMC neurons (Gao et al. 2019).

Additional studies have implicated other cell types in the exosomal regulation of food intake. For example, milk-derived exosomes have high levels of miRNA-148a and are thought to act on dopaminergic neurons to increase food intake (Melnik 2015; Melnik and Schmitz 2017). Milk-derived miRNA-148a suppresses the expression of DNA methyltransferase 1 (DNMT1),

resulting in elevated fat mass and obesity-associated (FTO) protein expression increased *FTO* promoter demethylation (Melnik 2015). FTO enhances the activity of the dopaminergic midbrain circuitry critically involved in reward signaling from food intake (Melnik and Schmitz 2017). FTO also plays an important role in regulating ghrelin levels, a key mediator of ingestive behavior. Therefore, milk-derived exosomes have high levels of miRNA-148a, which upregulates the expression of ghrelin and dopamine receptor D2 (DRD2) which both lead to increased food intake.

In summary, these studies identify a role for exosomes in relaying the nutritional status between the CNS and periphery and in the coordinated regulation of food intake.

Preliminary data suggesting Clic1 expression may be important in food intake

Olanzapine is a typical antipsychotic drug known to has side effects of increasing food intake and body weight. In a previous study from the Osborn lab, they investigated the underlying mechanisms driving olanzapine-induced hyperphagia in mice (Perez-Gomez 2018). In these studies, they treated mice with olanzapine or co-treated with the drug minocycline (Mino), which specifically blocked Olanzapine-induced hyperphagia. When they sequenced the hypothalamus from these mice treated with olanzapine, they observed significant upregulation of Clic1, suggesting it may play a role in food intake regulation (**Figure 2**).

Further evidence for the potential role of Clic1 in food intake regulation is provided by the study of Clic1 KO mice. Ulmasov et al. (2017) reported that the body weights of Clic1 KO female mice were significantly lower than their wild-type littermates, while no differences in body weight of male WT and KO mice were observed (**Figure 3**). The reasons for this sex difference are not clear, and food intake data was not reported for this study.

To determine which cell types secrete exosomes containing Clic1, we used the exosome database Exocarta (Mathivanan et al. 2010). Clic1 is found in exosomes from many cell types, including macrophages, neuronal cells, and cancer cells in mouse, human and other species (**Table 1**).

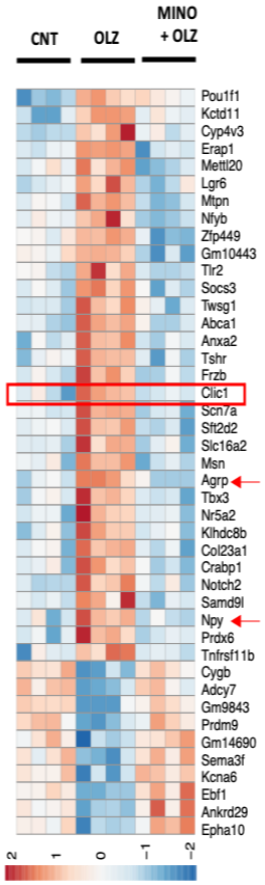

Figure 2. The RNA-seq data of the hypothalamus of mice upon olanzapine treatment (Perez-Gomez 2018). The expression of Clic1 was upregulated.

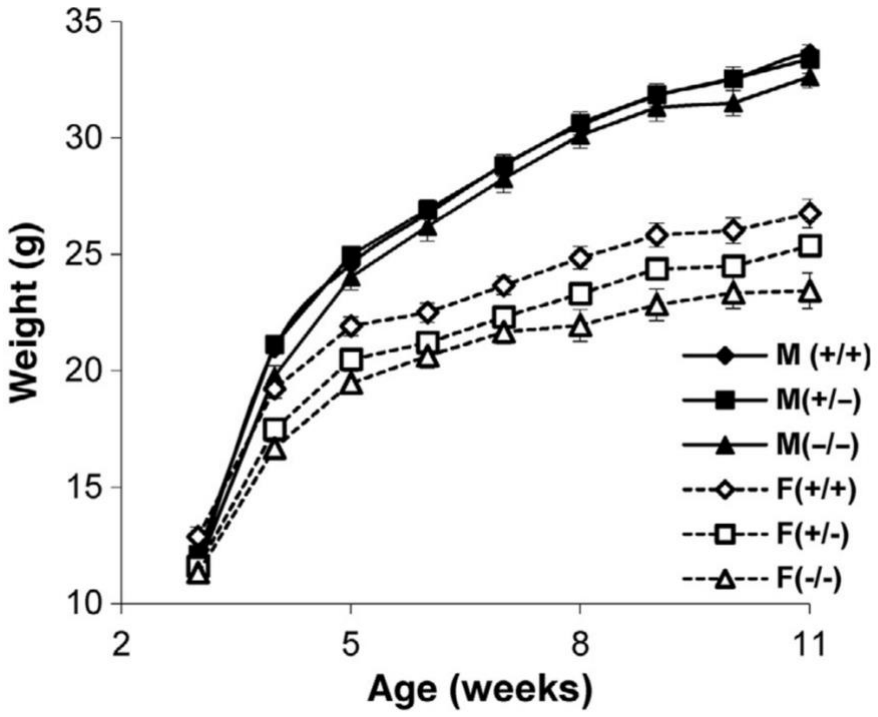

Figure 3. Female Clic1 KO mice have a lower body weight than wild-type mice. The average body weight of male (M) and female (F) wild-type and Clic1 KO mice, as shown in Ulmasov et al. 2017.

Table 1. Cell types where exosomal Clic1 has been identified in multiple organisms. Data was extracted from the Exocarta exosome database.

Species	Tissue/Cell Type	PMID Reference
Human	B Cell	20458337
Human	Bladder Cancer Cells	20224111
Human	Breast Cancer Cells	19415654
Human	Colorectal Cells	25890246, 23161513
Human	Hepatocellular Carcinoma Cells	26054723
Human	Melanoma Cells	25950383
Human	Nasopharyngeal Carcinoma Cells	25857718
Human	Neuroblastoma Cells	25944692
Human	Ovarian Cancer Cells	23333927
Human	Platelets	25332113
Human	Prostate Cancer Cells	25844599
Human	Squamous Carcinoma Cells	20124223
Human	Thymus	23844026
Human	Tracheobronchial Cells	19190083
Human	Urine	15326289, 19056867, 21595033, 22418980
Mouse	Fibroblasts	23260141
Mouse	Macrophages	23658846
Mouse	Mast Cells	17486113
Mouse	Pancreatic Cells	19351151
Mouse	Mov Neurological Cells	15210972
Rat	Reticulocytes	21828046
Cow	Milk	23459212
Cow	Serum	23459212

Research objective and hypothesis of this thesis

The primary objective of this thesis is to review and discuss the current findings of the potential role of exosomal Clic1 in metabolism. Many previously published studies have established that exosomes are key coordinators between metabolic organs, and Clic1 is highly expressed in exosomes and implicated in multiple diseases. Preliminary data from the Osborn lab established that Clic1 is expressed in the hypothalamus and plays a role in food intake. In this thesis, in addition to the literature review, I will study the expression of Clic1 in exosomes on different diets and nutritional statuses. The hypothesis of this thesis is that exosomal Clic1 acts as a messenger between metabolic organs to relay information related to nutritional status and metabolic health.

RESULTS

Characterization of exosomes derived from mouse plasma

To examine whether high-fat-diet (HFD) feeding had any influence on exosomal Clic1 expression, we extracted exosomes from the plasma of mice fed normal chow (NC) or HFD. Exosomes were characterized using the Nanosight to determine particle size and concentration per sample (**Figure 4**).

The expression of exosomal Clic1 shows no significant difference between different diets

We then assessed Clic1 abundance in these plasma-derived exosomes by immunoblotting (**Figure 5A**). Components of ESCRT complex, ALG-2-interacting protein X (ALIX) and tumor susceptibility gene 101 protein (TSG101) (Raiborg and Stenmark 2009) were used as loading controls. The average exosomal Clic1 abundance was slightly higher in plasma from obese HFD-fed mice compared with lean NC-fed controls, but these changes were not statistically significant. (p-value =0.18) (**Figure 5B**).

The expression of exosomal Clic1 shows no significant difference between different nutritional status

We next examined whether the deprivation of nutrition has any influence on exosomal Clic1 expression. The ‘fed’ group received NC ad libitum while the ‘fasted’ group, were deprived of food for 24 hours prior to sacrifice. We also included a group where we fasted the mice for 23 hours followed by glucose injection and sacrificed them after 30-45 mins. We extracted the exosomes from the plasma of mice and compared the exosomal expression of Clic1 of fed, fast,

and fast + glucose group (**Figure 6A**). Upon fasting, the levels of Clic1 increased approximately 2-fold, but this effect was highly variable between samples and thus not statistically significant (**Figure 6A-B**). The injection of glucose slightly decreased Clic1 levels, but this effect was also not statistically significant (**Figure 6B**).

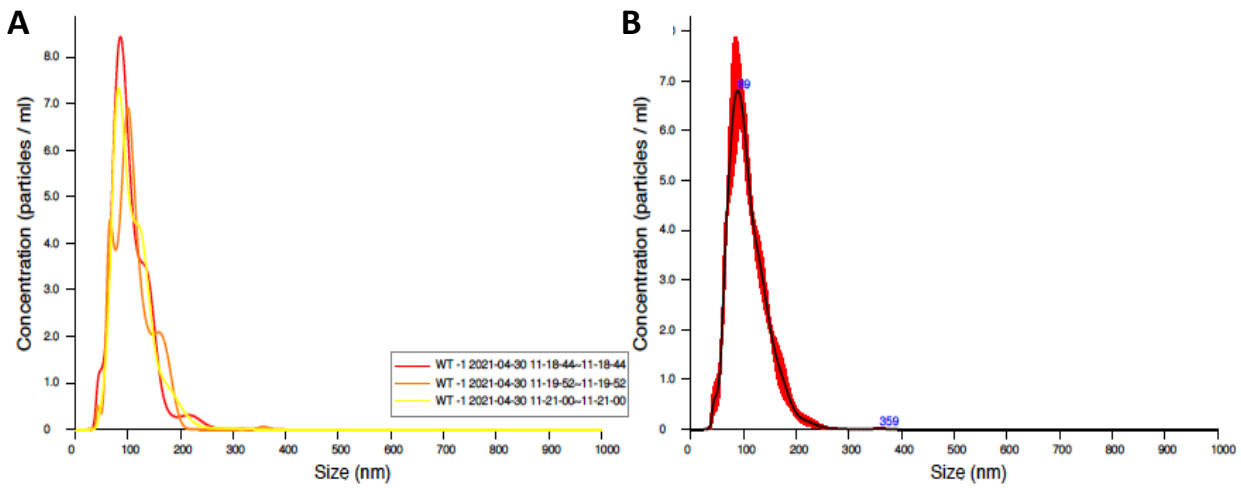

Figure 4. Characterization of plasma derived exosomes. (A) Determination of particle size from 3 replicates (orange, red and yellow) of one exosome sample from plasma from WT mice. (B) Mean particle size for this sample was 107.8 +/- 36.2 nm. The concentration was 4.98e+10 +/- 1.84e+09 particles/ml.

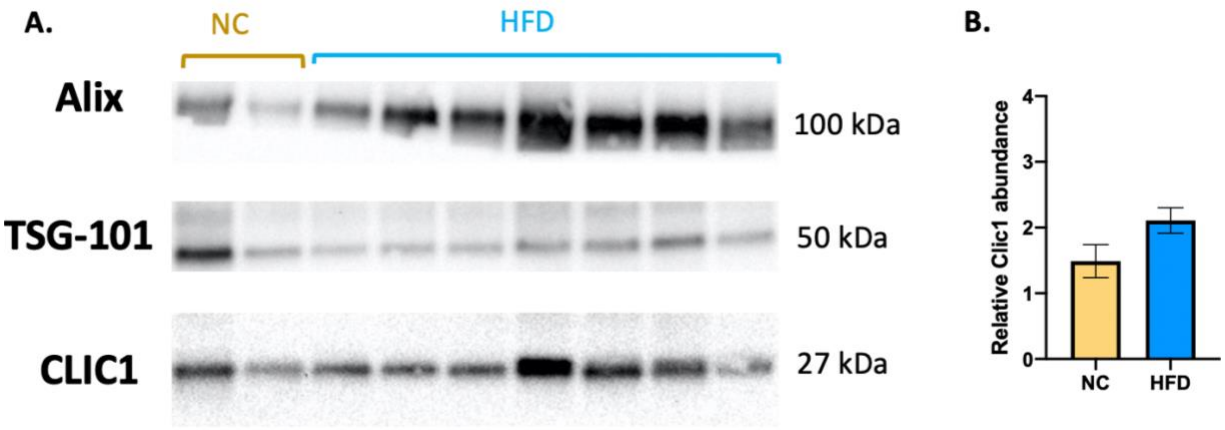

Figure 5. Clic1 abundance in plasma derived exosomes from lean, NC fed mice and obese, HFD-fed mice. (A) Western blot. (B) The quantification of Western blot by ImageJ. $P > 0.05$. (NC, n=2; HFD, n=7)

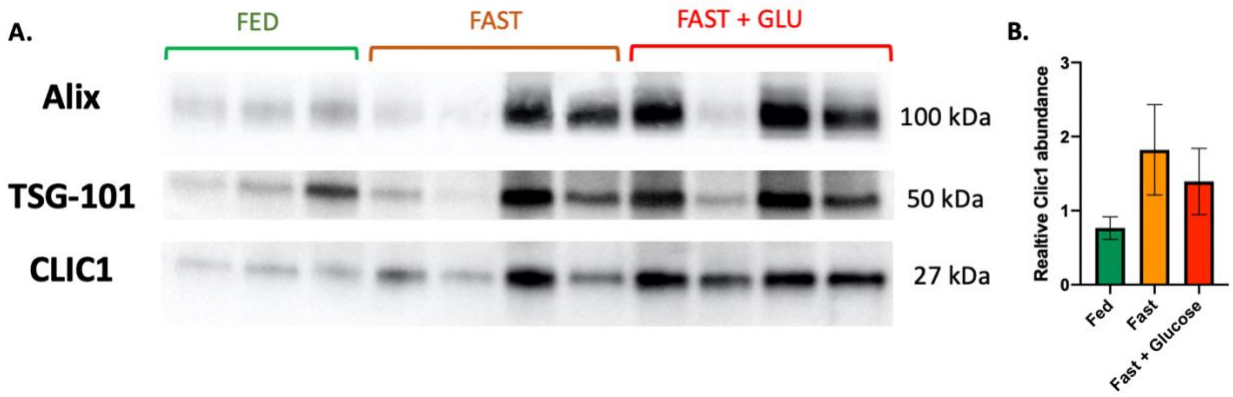

Figure 6. Clic1 abundance in plasma-derived exosomes from mice either fed ad libitum (Fed), fasted (Fast) and fasted with glucose (Fast+ Glu). (A) Western blot. (B) The quantification of Western blot by ImageJ. $P > 0.05$. (n=3-4 mice per group)

DISCUSSION

Clic1 is abundantly expressed in exosomes, but its role in inter-organ crosstalk is yet to be studied. Our preliminary data suggested a possible role of Clic1 in food intake regulation, and we hypothesized that exosomal Clic1 might communicate between the CNS and the periphery to relay information related to the metabolic and nutritional state.

In these studies, we compared the expression level of exosomal Clic1 in plasma from NC-fed lean mice and HFD-fed obese mice. Obese mice had elevated plasma-derived exosomal Clic1 levels compared with lean controls ($p= 0.18$) (**Figure 5A-B**). This effect was not significant in this pilot study but suggested that further replicates may be needed to observe potential significant changes in Clic1 exosomal abundance.

We next compared the expression levels of plasma-derived exosomal Clic1 in *ad libitum* fed, fasted, and fasted mice treated with glucose. Fasting resulted in an approximately 2-fold increase in exosomal Clic1 (**Figure 6A-B**). In the ad libitum fed state, Clic1 levels were very consistent between the three replicates. However, upon fasting, Clic1 levels were variable between the replicates, and further additional samples need to be studied to determine a more consistent result. This trend in increased Clic1 in the fasted state is in line with our hypothesis that increased Clic1 expression is associated with hunger and drives a pro-feeding response. Glucose injection resulted in a trend in decreased exosomal Clic1, suggesting re-feeding may normalize this effect. Further studies are needed to determine whether, with increased replicates, these effects would be more consistent between groups.

Importantly, some recent studies reported that exosomes could alter food intake and body weight of mice by acting on pro-opiomelanocortin (Pomc) neurons in the arcuate nucleus, which promote “fullness/satiety” (Yaswen et al. 1999; Gao et al. 2019). The arcuate nucleus also contains

Agouti-related protein (Agrp) expressing neurons that promote food intake (Ollmann 1997; Schwartz 1997). In future work, hypothalamic cells will be cultured and then treated with exosomes from fed and fasted WT and Clic KO mice to determine the effect on neuropeptide expression, including Agrp, Npy, and Pomc. In addition, a time-course analysis will be conducted to quantify plasma-derived exosomal Clic1 after various lengths of fasting.

The role of exosomal Clic1 in crosstalk between other organs also remains to be studied. Clic1 is highly expressed in macrophages which play a significant role in the development of inflammation-induced insulin resistance (Osborn and Olefsky 2012). In future work, we will determine whether exosomal Clic1 may impact insulin sensitivity of muscle (L6 myocytes), hepatocytes (HepG2), or adipocytes (3T3l1 adipocytes). Each cell type will be treated with plasma-derived exosomes from WT or Clic1KO mice, and levels of phosphorylated Akt will be assayed by Western blot.

In conclusion, these pilot studies suggest that exosomal Clic1 is a potential regulator of food intake and may play a role in inter-organ crosstalk in metabolism. Further studies are needed to determine how consistent these changes and whether they reach statistical significance.

MATERIALS AND METHODS

Animals

All Experiments were carried out with the approval of, and in accordance with, the Animal Care Program and Institutional Animal Care and Use Committee at the University of California San Diego.

C57BL6 mice were purchased from Jackson laboratories (stock number 00664). Mice were housed on a 12:12-h light/dark cycle and had ad libitum access to normal chow 'NC' (catalog no. 7912, irradiated; Envigo Teklad) and water. 10-week-old WT mice were divided into two groups and fed either NC or HFD (60% calories from fat, Research Diets, D12492) for 12 weeks before sacrifice and plasma extraction. In the second study, 12-week-old mice were divided into three groups and either fed ad libitum, fasted for 24 hours, or fasted for 23 hours, followed by intraperitoneal injection of glucose (2mg/kg) and sacrificed one hour later for plasma extraction.

Exosome extraction and characterization

Blood samples were centrifuged at 3,000 g for 15 mins at 4°C. The supernatant was collected and again centrifuged at 3,000 g for 15 mins at room temperature. The supernatant was collected, diluted in PBS, and centrifuged at 16,500 g for 40 mins at 4 °C. The supernatant was collected, filtered by 0.22 μm sterile syringe filter (BioPioneer, USA), and ultracentrifuged at 120,000 g for 3.5 h at 4 °C. The supernatant was decanted, and the exosome pellets were resuspended in 200 μL of PBS. The exosomes were characterized using NanoSight NS300 (Malvern Panalytical, UK).

Immunoblotting

Exosomal protein concentrations were measured using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA). Equal amounts of proteins were separated on Criterion™ XT Bis-Tris Precast Gels (Bio-Rad Laboratories, USA) by SDS-PAGE under reducing conditions and transferred onto Whatman Protran nitrocellulose transfer membranes (Cytiva, USA) by electroblotting. The nitrocellulose membranes were blocked with 5% BSA in TBST at room temperature for one hour and incubated overnight at 4 °C with primary antibodies, including antibodies against Clic1 (sc-81873, Santa Cruz), ALIX (2171S, Cell Signaling Technology), and TSG-101 (sc-7964, Santa Cruz). Subsequently, the nitrocellulose membranes were then incubated with secondary antibodies at room temperature for one hour. The immunoblots were visualized using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, USA), and pictures were acquired using Image Lab Software (Bio-Rad Laboratories, USA).

Statistics

All data are expressed as mean ± standard error of the mean (SEM) and analyzed using an unpaired Student's t-test or One-way ANOVA. The statistical difference was considered significant if $P < 0.05$.

This thesis, in whole, is currently being prepared for submission for publication of the material. Charlotte Chenxin Yan, Rizaldy Zapata, Dinghong Zhang, Olivia Osborn. The thesis author was the primary investigator and author of this material.

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