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LC3I and LC3II as Autophagy Markers for the Development and Improvement of Products and Techniques used in Research

by

Caitlin J. Williams

Thesis

Submitted to the Graduate School

Of Wayne State University,

Detroit, Michigan

In partial fulfillment of the requirements

For the degree of:

Master of Science

2013

Major: Biochemistry and Molecular Biology

Approved By:

____

Advisor Date

Dedication

For my husband.

Acknowledgements

I'd like to acknowledge Enzo Life Sciences for allowing me to perform my research and Mike Mullenix for going over my thesis, giving me notes. Also, I'd like to thank my committee members Dr. Robert Akins, Dr. Chunying Li and Dr. Jianjun Wang.

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Chapter 1: Importance of Autophagy

1.1 Introduction

Autophagy is an intracellular process that keeps the cell intact while disposing of no longer functional organelles, misfolded/degraded proteins, as well as bacteria and viruses (2), using lysosomes to remove the unwanted cellular debris. Autophagy is a normal function of all cells required to maintain homeostasis by breaking down unnecessary cell components into useful metabolites. Autophagy also serves as a cell survival mechanism allowing cells to survive with limited nutrients and avoid mechanisms of apoptosis (7). Autophagy can be broken down into selective and non-selective. Non-selective autophagy is the random sequestration of cellular debris into autophagosomes without the use of selective proteins to bring the debris to the autophagosome. There are three main types of autophagy known as Chaperone mediated autophagy (CMA), microautophagy, and macroautophagy (8). Microautophagy was once described as clearly non-specific, working as a phagocytic mass that engulfed cytoplasmic components without specificity, however it is believed now that specificity is possible in microautophagy, yet the mechanism has yet to be determined (9). Chaperone mediated autophagy is more clearly defined than microautophagy but, is different from macroautophagy in that single proteins are fed across the lysosomal membrane with the help of chaperon proteins that bind to the target protein. One such example is hsc70 which binds to a specific motif on the target protein (10, 11). Macroautophagy is most often what is described when the blanket term autophagy is described (2, 11). Macroautophagy results in the formation of a double walled membrane around the various degradation targets. For the purpose of this publication the term autophagy will be used to refer to the process of macroautophagy.

1.2 The Process of Autophagy

Autophagy can be described as a five step process that progresses from initiation, to elongation, to maturation, to autophagosome-lysosome fusion and ends with degradation. These processes are regulated by autophagy-related genes or Atg (2).

In mammalian cells autophagy results in the formation of double membrane vesicles mostly from the ER. Ulk1 is a protein that is believed to be involved in this process by responding to cell stressors such as starvation (2, 12, 13). Ulk1 is a Serine/Threonine kinase that allows autophagy to begin in response to mTOR deactivation due to cellular stress (26). It is believed that Ulk1 complex associates with mTOR and Atg13; this complex allows for mTOR to phosphorylate Ulk1 under nutrient rich conditions (13). Under conditions of cellular stress, such as nutrient deprivation, mTOR will dissociate from Ulk1 leaving it free to associate with the ER to initiate formation of the autophagosome (13). At initiation an omegasome forms in the ER membrane, which produces the beginnings of the phagophore (14). The exact location of the beginnings of the phagophore is disputed. One site of initiation is the ER (12). Also involved in the initiation of the membrane formation is vesicular protein sorting 34 or Vps34. Vps34 complexes with Beclin-1 to increase the ability of Vps34 to produce high levels of phosphatidyl inositol triphosphate (PI3P) (12). PI3P is important to the elongation stage and recruiting the other Atg proteins by being recruited to the ER by Atg14L (12). The elongation process involves Atg7 activating Atg12, transferring it to Atg10. Atg10 allows Atg12 to link to lysine 130 of Atg5. Atg12-Atg5 complexes with Atg16L, and is believed to help form the curve of the membrane by later recruiting LC3II and dissociates once the membrane is formed (12).

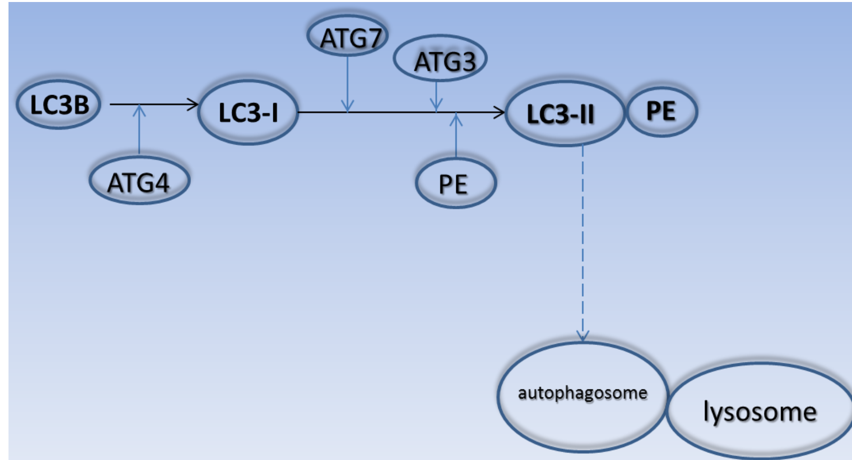

Figure 1.1 Autophagy Pathway, specific to LC3: ATG4 cleaves LC3B, leaving a glycine residue available, this becomes LC3I. LC3I is activated by ATG7, transferred to ATG3 and conjugated to PE to become LC3II. LC3I stays in the cytosol. LC3II is then translocated into the membrane of the autophagosome. This is then degraded in the lysosome. (4)

Microtubule-associated protein light chain 3 (LC3), is normally in the cytosol as a full-length protein. LC3 is a family of three proteins, LC3A, LC3B and LC3C. LC3B was the first discovered (31). LC3A and LC3C have less information available. Looking at LC3B during autophagy, LC3 is cleaved by Atg4 and becomes LC3I. A carboxyterminal glycine is exposed and is activated by Atg7. Atg3 then conjugates LC3I to phosphatidlyethanolamine (PE) at the carboxyterminal glycine to form LC3II (12). LC3II is then brought into the forming membrane by the Atg5-Atg12-Atg16L complex, to continue the elongation cycle. LC3II is distributed in the membrane and helps with selecting the cargo to go into the autophagosome and fusion of the membranes (12). The autophagosome combines with the lysosome to begin the process of degradation of the contents of the autophagosome. After degradation the degradation products are then recycled back out to the cytosol (12). LC3II in the cytoplasm has the PE removed by Atg4B, which allows for LC3I to be recycled (2).

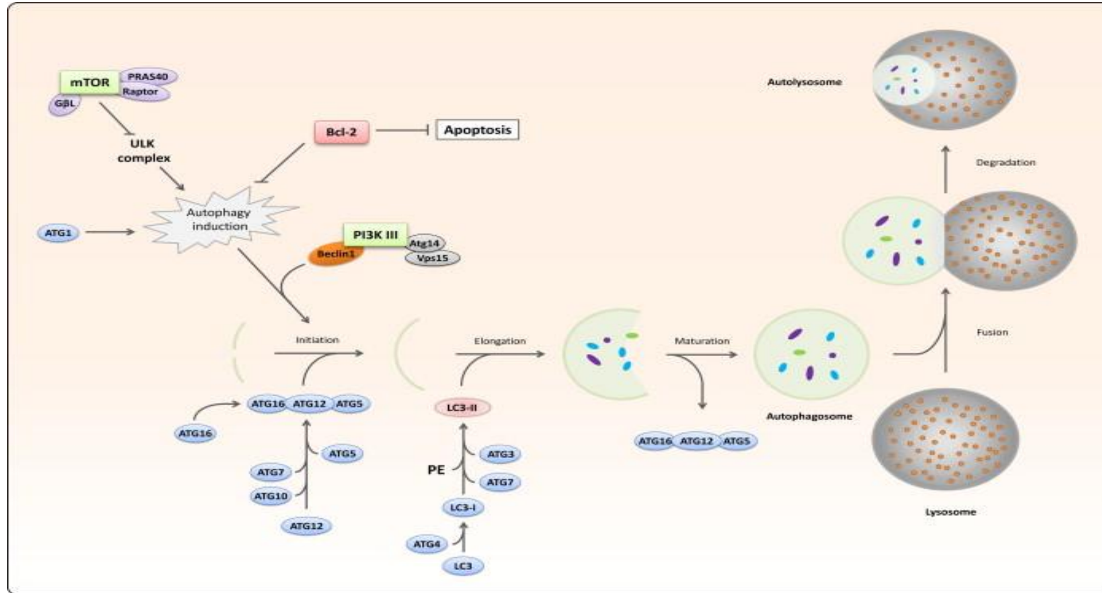

Figure 1.2: The process of Autophagy: From initiation, elongation and maturation, then to autophagosome-lysosome fusion and lysosome degradation (22).

1.3 Autophagy: Selective or Non-selective

Autophagy was initially thought to be a non-selective process that simply engulfs cellular debris without concern for the contents. Autophagy is now also known to be a selective process. Proteins that interact with components of the cell to be removed by autophagy interact with proteins bound in the autophagosome membrane and providing an anchor forming autophagosome. The proteins that mediate interaction with LC3 can be known as autophagy receptors, since they connect with the debris to be removed in the autophagosome. The interaction occurs through a specific amino acid sequence that is known as the LC3-interacting region (LIR) or the LC3 recognition sequence (LRS) (14). One example of selective autophagy termed aggrephagy involves removal of misfolded or unneeded proteins by putting them in ubiquitinated aggregates that are attached to the autophagosome for removal by the lysosome (14). Two known autophagy receptors are p62 and neighbor of BRCA1 gene (NBR1) which can bind ubiquitinated protein aggregates through an ubiquitin-

associated domain (UBA) and LC3 through the LIR. The p62 receptor has many other ubiquinated targets such as bacteria, peroxisomes, damaged mitochondria and viruses (14). Phosphorylation and ubiquitination play a part in the regulation of selective autophagy such as in the case of p62 recognizing ubiquitinated protein aggregates and bringing those to the LC3 in the autophagosome to be degraded by the lysosome. Both NBR1 and p62 are degraded during autophagy. Suppression of autophagy causes a buildup of p62 in the cells. Autophagy helps maintain homeostasis in the cells, and the importance of selective autophagy has become more apparent that as various types of cytosolic debris have been linked to degradation through autophagy receptors.

1.4 Importance of Autophagy in Disease States

Autophagy has grown as an interest in research concerning disease states such as different cancer types and neurological disease. Cells use autophagy to maintain homeostasis, under normal conditions this allows the cells to remain viable, thus enabling them to stave off apoptosis. It has been found that in cancerous cells autophagy is also used to maintain cells that may otherwise be sent to death, in the low oxygen and low nutrient environment. Autophagy can work for the cell to survive a type of cell death that is initiated when the cell detaches from the extracellular matrix, called anoikis (16). Autophagy is a way for the cancer cells to survive and metastasize in the increased presence of blood flow enabled by angiogenesis or rather the process of vascularization to a tumor (16).

Autophagy is also used to target cancer cells, through the use of autophagy inhibiting chemotherapeutic agents to destroy the tumors by inhibiting the autophagy survival mechanisms (16). It has been shown that autophagy takes part in cell death induction in

apoptosis capable cells and it becomes the main death inducing pathway in apoptosis deficient cells (16).

Autophagy appears to be impaired in neurodegenerative diseases such as Parkinsons and Alzheimers disease. In Alzheimers and Parkinsons disease autophagosomes accumulate and there are endosomal-lysosomal abnormalities found in Alzheimers disease (4). Deficiencies in autophagy and the resulting inability to remove the misfolded proteins may contribute to neurological disease.

Autophagy may also contribute in other diseases such as cardiovascular disease and diabetes. In heart disease autophagy may play either a protective role or may contribute to cell death by either preserving cells that are damaged enabling salvage of the cells or by pushing cells to apoptosis after extended or overproducing autophagy (27). In type 2 diabetes impaired autophagy is indicated in β cells, where ubiquitinated proteins accumulate, the mitochondria are swollen and the ER is distended (24).

1.5 LC3 as a Marker for Autophagy

Microtubule-associated protein 1 light chain 3 (MAP1 LC3) is very commonly noted in papers concerning autophagy as a process or autophagy in diseased cells. Up regulation of LC3 can serve as a marker for autophagy. LC3 localization within the cell also serves to monitor the progression of autophagy. In autophagy, the carboxy-terminal region of LC3 is cleaved off exposing a glycine residue forming LC3I. LC3I is then modified with a phosphatidylethanolamine (PE) to form LC3II. The LC3II becomes bound in the inner and outer membrane of the autophagosome. This process allows for the autophagosome formation to be monitored and therefore LC3 becomes an important marker to study autophagy. LC3I is a cytosolic form of LC3 that is soluble. As mentioned before LC3II is

membrane anchored through its PE modification. These differences allow for the separation of LC3I and LC3II through cellular fractionation. Western blotting is a method that can be used to show that autophagy has been activated and usually with an increase in LC3II. LC3 can be tracked using fluorescent microscopy to show the autophagosome formation. The use of fractionated lysates is also valuable to the study of autophagy. Currently the antibodies used to study autophagy are insufficient or not useful. Development of antibody that binds specifically to LC3I and LC3II would enable researchers to separately detect both forms of LC3 proteins in western blotting and immunohistochemistry. Both antibodies would also enable the development of ELISA to quantify LC3 levels in biological samples.

1.6 Conclusion

Autophagy is a contributor in maintaining homeostasis within the cells. Autophagy provides a mechanism for cell survival and cell death. During cellular stressors cell survival is extended through autophagy, yet may lead to apoptosis if extended for long periods of time or if overproduced in cells. Autophagy is responsible for the degradation of mis-folded proteins, damaged or old organelles, bacteria and viruses. Autophagy was originally thought to be a non-selective process. Certain types of autophagy are considered non-selective; autophagy is also a selective process. In selective autophagy proteins that have a region called the LC3 interacting region select the protein or cellular debris and anchor it in the autophagosome. The autophagosome selects the protein or cellular debris and anchors it in the autophagosome. The autophagosome then fuses with the lysosome for degradation of the cargo. Autophagy can enable diseased cells to survive and grow through the stress of a condition such as in cancer. In other diseases autophagy is impaired and resulting in accumulation of cellular debris leading loss of cell function. LC3 is an important autophagy

marker in that it can be used to follow and analyze the selection of protein and cellular structures into the autophagy pathway and to the formation of autophagosomes within the cells. Products for analyzing autophagy and more specifically LC3 can be improved. New reagents and assays are envisioned to aid the researcher in the further understanding of the role of autophagy in health and disease.

Chapter 2: Autophagy Processes Enable Production of LC3II Specific Lysate

2.1 Introduction

Autophagy is a tightly controlled process of degradation within the cell, enabling the cell to mitigate stress and maintain homeostasis. Autophagy can be induced by the following conditions: nutrient deprivation, infection, hypoxia and by treatment with chemical inhibitors of the proteins within the pathway (2, 8). Autophagy pathway research includes many disease states, such as cancer (1), diabetes, cystic fibrosis and cardiovascular disease. Autophagy research also includes its role in immunity (2). Autophagy activation/dysfunction is found in diseases that produce protein aggregates, such as Huntington's, Parkinson's and Alzheimer's.

The ability to detect LC3 proteins and differentiate different LC3 Protein derivatives is important for the advancement of autophagy research. At this time we are looking at LC3 products to produce in order to be able to separate and reveal quantitative differences between the two LC3 proteins found approaching the end of autophagy, LC3I and LC3II. LC3I is known to be found at 18kDa and LC3II is known to be found at 18kDa, due to the positive charged PE which changes the charge of the protein and therefore runs faster on the gel.

Chloroquine is a lysomotrophic drug that is most notably used as an antimalarial drug and is being researched as a tumor treatment. Chloroquine is a late stage autophagy inhibitor, shown to cause accumulation of autophagic vacuoles. This effect is thought to be the result of the choloroquine on the lysosomes pH, it causes the acidic lysosomes to be basic and therefore lack the degradation properties. By preventing degradation chloroquine treatment causes the termination of autophagy after the formation of the autophagolysosome. With the accumulation of autophagolysosomes the cells eventually enter apoptosis.

The ability to be able to produce a lysate that is singular in either form of the protein is invaluable to being able to quantitate the separate proteins and perhaps be able to tell levels and stage of autophagy within the cell sample where the cells are in late stage autophagy. The intention of my project was to create a protocol for isolating the membrane fraction of cells that would be easy for researchers/customers. I have used chloroquine in order to activate autophagy in cells leading to an accumulation of LC3II, in order to harvest the cells, fractionate the lysate and obtain LC3II rich lysates. Cancer cell lines have been chosen, from different organisms, as it is known that cancer cells use autophagy to survive (16). The protocol would not require the use of an ultracentrifuge and could be provided in a kit format. We intend to use this protocol to isolate LC3II from cells to create a cell lysate that can be sold and to be used as a control in an immunoassay kit. To produce such lysates cells would have to be induced to enter autophagy and stalled before reaching lysosome fusion prior to degradation. Techniques for the separation of LC3II and also LC3I are described herein.

2.2 Materials and Methods

2.2.1 Cell culture

Cell lines Neuro 2A, HeLa and 3T3 were from ATCC and cultured in DMEM (Gibco, Life Technologies, Grand Haven NY) with 10% fetal bovine serum (PAA, Dartmouth, MA) and penicillin/streptomycin (Gibco, Life Technologies, Grand Haven NY, USA). RatC6 cells were from ATCC and were cultured with 10% Horse Serum (Sigma, St. Louis, MO, USA), 2.5% fetal bovine serum and penicillin/streptomycin. SH-SY5Y cells were from ATCC and were cultured in DMEM+F12K (Gibco, Life Technologies, Grand Haven, NY, USA) with 10% fetal bovine serum and penicillin/streptomycin. All cells were kept in a 37°C incubator with 5% CO_2. All cells were plated into 15cm plates.

Cells were treated with chloroquine (Sigma, St. Louis, MO,USA) at a concentration of 100μM once the plates were approximately 70-75% confluent. After addition of the chloroquine to fresh media the plates were returned to the incubator for four hours. Plates that were not treated were also returned to the incubator after a media change. After four hours the cells were removed from the plates using trypsin (Gibco, Life Technologies, Grand Haven, NY, USA). Cell pellets were collected in 50mL conical centrifuge tubes and centrifuged at 800rpm. Cell pellets were then frozen down at -20°C.

2.2.2 Lysate Fractionation

Cell pellets were thawed on ice, buffer A was added and the pellet resuspended (28). This suspension was rotated at 4°C for 10 mins. Then it was centrifuged at 2000xg for 10 mins at 4°C. This supernatant was saved and labeled as the cytosol fraction. The pellet was then washed with 0.5mL 1X PBS twice. The remaining pellet was suspended in buffer B (28) and incubated on ice for 30 mins with vortexing every 5 mins. This was then centrifuged at 7000xg for 10 mins at 4°C. This supernatant was then saved and labeled as the membrane fraction (28). Protein concentration was determined using the BioRad DC Protein Assay (Hercules, CA, USA).

2.2.3 Western Blotting

Cytosol and membrane fraction lysates were mixed with NuPAGE LDS sample buffer (Life Technologies, Grand Haven, NY) and 1M DTT (Sigma, St. Louis, MO,USA) and boiled for 10 mins in a heat block. They were then loaded onto an 18% Tris-HCl Criterion SDS-PAGE gel (BioRad, Richmond, CA) and transferred by tank transfer to a Protran nitrocellulose membrane, 0.45μM (Whatman, Piscataway, NJ). Membranes were blocked overnight at 4°C on a rocker with 5% Non-fat milk and PBS+0.05%Tween20. Following the transfer the membranes were blotted with two clones of LC3B, mAb (5F10) and LC3B, mAb (2G6) primary antibodies

(Enzo Life Sciences, Farmingdale, NY, USA). Secondary antibodies were goat anti-mouse HRP conjugates (Jackson Immunoresearch Laboratory, West Grove, PA, USA). The signal was detected using enhanced chemiluminescence western blotting detection system (Thermo Scientific, Rockford, IL, USA).

2.3 Results

2.3.1 Cell Lysate Fractionation and Protein Concentrations

The initial protocol used $1x10^6$ or $2x10^6$ cells/mL of each lysate buffer, and resulted in protein concentrations below 1.0 mg/mL. Protein concentrations below 1.0 mg/mL do not provide enough protein in the cytosol fraction to allow LC3 detection by western blotting. Protein concentrations at or above 2.0mg/mL provide sufficient protein for detection of LC3. Initial attempts to detect LC3 in the cytosol fractions were unsuccessful, suggesting a change to the protocol. (This is shown in Figure 2.2 below).

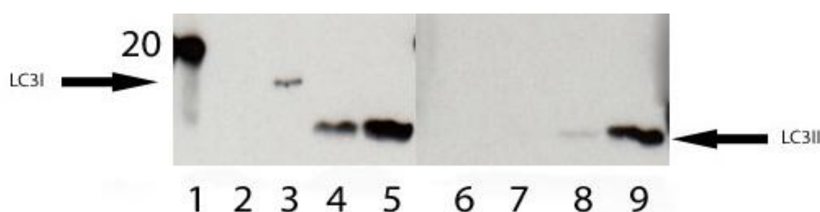

Figure 2.1: Membrane fractions of Neuro 2a (lanes 1-5) and HeLa (Lanes 6-9) cell lysates. Lanes 2 and 6 show $1x10^6$cells/mL for the control lysate and lanes 3 and 7 show $2x10^6$cells/mL for the control lysate. Lanes 4 and 8 show $1x10^6$cells/mL for the drug treated lysate and lanes 5 and 9 show $2x10^6$cells/mL for the chloroquine treated lysate. Lane 1 is the molecular weight marker. Using LC3B, mAb (5F10).

The first attempt to optimize the protocol was to use half the amount of lysis buffer in order to increase protein concentrations in the cytosolic fraction. Decreasing the amount of buffer in the lysis procedure enabled preparation of a control lysate for the membrane fraction. The membrane fraction control lysate was used to further improve the procedure.

To further improve performance of the western blots, the number of Neuro 2a cells were increased to enable the detection of LC3 through volume present. The increased cell contribution enabled the detection of LC3 in both the cytosolic and membrane fractions. The resulting protocol allowed preparation of a control lysate with protein concentration of 2.0 mg/mL for use in later experiments. The protocol was successful in fractionating HeLa cells; however LC3 detection was less intense in western.

2.3.2 Use of Different Detergents in Lysate Buffer

Fractionated lysates were initially made with the use of the detergent Detergent A alone and in combination of Detergent B, to determine if the detergents together would better fractionate LC3I in the cytosol and LC3II in the membrane fraction. Previous experiments increasing or decreasing the concentration of detergent in lysis buffers made with single detergents did not make a difference. When combining Detergent A and Detergent B for lysis of Neuro 2a cells a large difference in fractionation was observed when compared to single detergents. Detergent A alone in lysis buffer provided more complete fractionation when compared to combining Detergent A with Detergent B. The addition of Detergent B causes LC3I to appear in the membrane fraction rather than maintain segregation in the cytosol. This was completed in HeLa cells and Neuro 2a cells and is illustrated below by Neuro 2 a cells. The results were confirmed in both Neuro 2a cells and HeLa cells (Fig. 2.2).

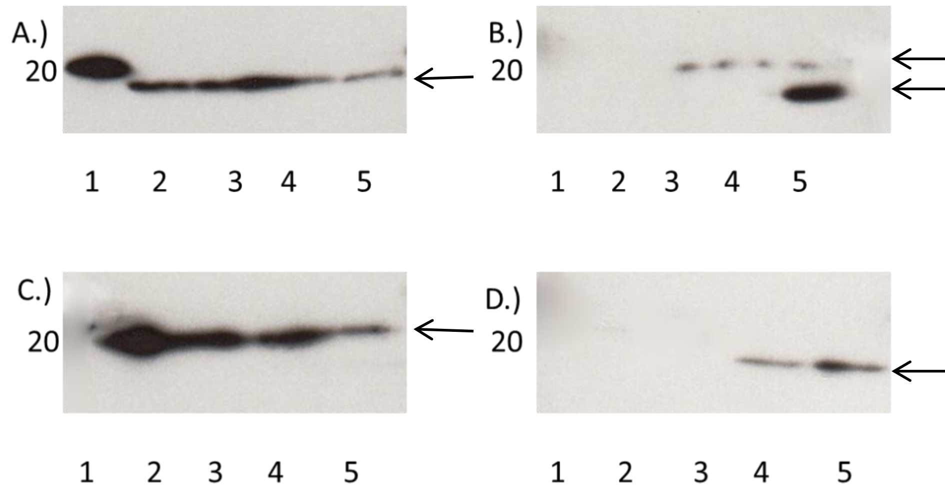

Figure 2.2: Lysate fractionation of Neuro 2a cells with different detergent additions in control cells and chloroquine treated cells with $1x10^6$cells and $2x10^6$cells: A.) Detergent A and Detergent B Cytosol Fraction; B.) Detergent A and Detergent B Membrane Fraction; C.) Detergent A Cytosol Fraction; D.) Detergent A Membrane Fraction. Lane 1: MW Marker, Lane 2: $1x10^6$ cells control, Lane 3: $1x10^6$ cells treated, Lane 4: $2x10^6$ cells control, Lane 5: $2x10^6$ cells treated. Using LC3B, mAb (5F10)

2.3.3 Western Blotting to Detect LC3 Lysate Fractionation

Western blotting was used to determine if the lysates prepared previously had properly fractionated during the lysate procedure. LC3I is expected in the cytosol fraction only, while LC3II is expected in the membrane fraction only. Fractionation was also tested using several different cell types. Fractionation of LC3I and LC3II could be found in Neuro 2a cells (Figure 2.3), 3T3 cells (Figure 2.3) and HeLa Cells. HeLa cells also produced a fractionated lysate, however LC3 levels were generally lower resulting in less intense bands by western. 3T3 cells produced a lysate that was fractionated in the drug treated lysate. 3T3 control cell lysates had LC3I in the membrane fraction, suggesting that the fractionation was not complete for this cell line, or at least in the control lysate condition.

Figure 2.3: Complete Fractionation of LC3I and LC3II in Neuro 2a cells (left) and 3T3 Cells (right). Western Blotting of Neuro 2a and 3T3 cell fractionated lysate with LC3B, mAb (5F10) antibody. Lane 1: MW Marker, Lane 2: Cytosol Control Lysate, Lane 3: Cytosol +Chloroquine Lysate, Lane 4: Membrane Control Lysate, Lane 5: Membrane+Chloroquine Lysate. LC3I is found at 18kDa and LC3II is found at 16kDa. Using LC3B, mAb (5F10)

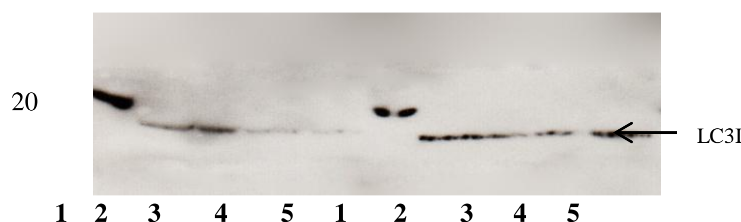

Figure 2.4: Lack of Fractionation of LC3I and LC3II in SH-SY5Y cells (left) and Rat C6 Cells (right). Western Blotting of SH-SY5Y and Rat C6 cell fractionated lysate with LC3B, mAb (5F10) antibody.
Lane 1: MW Marker, Lane 2: Cytosol Control Lysate, Lane 3: Cytosol +Chloroquine Lysate, Lane 4: Membrane Control Lysate, Lane 5: Membrane+Chloroquine Lysate. LC3I is found at 18kDa and LC3II is found at 16kDa.

A complete lack of autophagy activation or fractionation was found in SH-SY5Y cells and C6 cells. SH-SY5Y cell fractionated lysates showed only LC3I (figure 2.4) throughout several attempts and this was in all fractions. C6 cell fractioned lysates also showed only LC3I (figure 2.4) through several attempts at fractionation and the initial attempts actually produced no signal for LC3 in western blot (not shown).

2.4 Conclusions

It is possible to utilize the lysate fraction procedure using two detergent containing buffers to produce fractionated lysates without the use of an ultracentrifuge, in different cell types. The procedure is easy to follow and understand. The procedure may need to be further optimized in order to eliminate "carry over" of LC3I into the membrane fractions. SH-SY5Y and C6 cells may not participate in autophagy as much as other cell types, therefore treatment with chloroquine would be futile. Experiments indicate control lysates in sample buffer at 2.0mg/mL

can be consistently produced and at a reasonable cost. The fractionation procedure only adds one more step when compared to other control lysates that are produced as Enzo Life Sciences Products. The simple protocol is suitable for use in a kit and could be included in a total LC3 kit to allow for LC3II specificity.

2.5 Discussion

The production of fractionated lysates specifically containing LC3II may not be easily achieved in all cell types. A possible solution to working through this for researchers with interests other than HeLa, Neuro 2a or 3T3 cells may be to transfect LC3II into the cells of choice in order to produce the increase in LC3II. However, the control lysates successfully produced can be used as a control for this same researcher to test their transfection results. Another treatment could be used in conjuction with chloroquine to first initiate autophagy and then stop autophagy at the autophagolysosome stage. In working to produce antibodies for LC3I and LC3II these lysates will be instrumental in showing if a specific antibody will show a positive result in the cytosol, as would be expected in LC3I, or in the membrane fraction, as would be expected in LC3II. A control lysate for western blotting could be made using the Neuro 2a fractionated cell lysate. This lysate could be easily reproduced as a manufacturing method.

The protocol that has been set up is simple and could be followed by anyone with a scientific background. Unlike other fractionation methods, the protocol does not require the use of an ultracentrifuge, and makes the method useful in more laboratories. The procedure is simple enough to provide a sample fractionation protocol to be included in assay kits. Enzo Life Sciences intends to develop ELISA kit that can detect LC3I and/or LC3II specifically. The two fractionation buffers can be included to enable the differential detections of LC3I and LC3II. Such an assay will provide a powerful quantitative tool for autophagy research.

Chapter 3: LC3I and LC3II Monoclonal Antibody Development for the Production of an LC3I and LC3II Specific Product

3.1 Introduction

The immune system protects the body from foreign organisms that invade it. The immune system has to be able to distinguish between viruses and the body's own healthy tissue. The specificity of the immune response is provided by proteins and cells circulating the body. Different mechanisms contribute to the immune response and can be put into two divisions, adaptive immunity and non-adaptive immunity (29). The immune response is not enhanced with repeated exposures to foreign organisms (29). Non-adaptive immunity reacts the same way to foreign material every time it comes into contact with it (29). Adaptive immunity provides an enhanced response with repeated exposures (29). Examples of non-adaptive immunity include macrophages and natural killer cells and their direct destruction of offending organisms (29). Adaptive immunity uses lymphocytes which manufacture cell surface receptors or secrete proteins that bind to the foreign organisms (29). One of the secreted protein types are antibodies. Antibodies bind to foreign organisms, neutralize them or mark them for removal by other cell types (29). Any molecule that can be bound by an antibody is called an antigen (29). Any molecule that is used to induce the immune response is known as an immunogen (29).

The molecular structure of antibodies can be described as Y-shaped (29). Each antibody is made up of two copies of a polypeptide called heavy chains and two polypeptides called light chains (29). There are five classes of antibodies, IgG, IgM, IgA, IgE, and IgD (29). The classes differ in function and structure mostly driven by the type of heavy chain polypeptide and the number of immunoglobulin structures covalently associated (29). IgG antibodies have a single immunoglobulin structure and are the most abundant in serum (29). Each arm of the immunoglobulin structure has an antigen binding site, making the molecule bivalent (29). The two arms of the immunoglobulin structure are domains known as Fab domains, for fragments

that are antigen binding (29). There is a third domain that is made up of the bottom of the two heavy chains and this is known as the Fc domain, for fragment that crystallizes (29). The area between these is called the hinge and allows for movement to be able to bind different antigen configurations (29). The two heavy chains in the antibody are identical and are about 55kDa. The two light chains are also identical and are about 22kDa (29). The four chains are held together by disulfide bridges and noncovalent bonds (29). The other classes of immunoglobulins are described in Figure 3.1.

Component	IgG	IgM	IgA	IgE	IgD
Heavy Chain	gamma	mu	alpha	epsilon	delta
Light Chain	kappa or lambda	k or l	k or l	k or l	k or l
# of Y units	1	5	1,2 or 3	1	1
Conc. In Serum	8-16 mg/mL	0.5-2 mg/mL	1-4 mg/mL	10-400 ng/mL	0-0.4 mg/mL

Table 3.1: Classes and Characteristics of Immunoglobulin classes (29)

Monoclonal antibodies are antibodies that are specifically produced by a clonal cell population such that each antibody molecule in a given preparation is identical (29). These antibodies are highly useful when it comes to research methods such as ELISA, western blot, immunoprecipitation, and immunohistochemistry. Monoclonal antibodies are produced using hybridoma cells (29). Hybridoma cells are created through the fusion of myeloma cells and plasma B lymphocytes from the spleen of mice which have been immunized with a specific antigen (29). After fusion, the cells are then transferred to HAT (hypoxanthine-aminopterin-thymidine) media. Aminopterin blocks DNA de novo synthesis preventing survival of myeloma cells that have not fused with B cells (29). The myeloma cells do not survive because die because they cannot produce nucleotides using the salvage pathway (29). B cells that have not fused will die due to a short lifespan (29). Hybridomas are immortal because they inherit the myeloma cells ability to replicate along with the B cell ability to use the salvage pathway (29). The hybrids or

Hybridoma cells are grown up and serial diluted into 96-well plates in order to get to achieve concentrations of a single cell per well. The plates are then incubated to allow cell growth checked for antibody production by immunoassay. Cells producing antibody are selected and grown up in larger culture. This process is termed limited dilution cloning and can be done at least a couple of times to ensure that the resulting antibody is "monoclonal" or from a single clone (29). Hybridomas can continue to be grown in cell culture and the antibody can be purified from the tissue culture supernatants. The hybridomas can also be grown in cell culture and then injected into the peritoneal cavity of mice (29). Growth within the peritoneal cavity will cause the formation of ascites fluid rich in concentration of the antibody (29). Antibodies can be purified from the ascites fluid once it is harvested from the mice.

Monoclonals that specifically bind for the LC3I and LC3II, late autophagy, proteins specifically would be a valuable resource in the growing research interest of autophagy. Autophagy is becoming more significant in the study of many diseases including cancer, diabetes and cardiovascular disease. While there are LC3I specific antibodies on the market we believe that we can create a superior and preferred antibody, this along with being able to create the antibody at Enzo Life Sciences to be sold as a product. An LC3II specific antibody would be very beneficial for direct detection of autophagy induction LC3I specific monoclonal antibodies would be useful in determining where the cells are in the process of autophagy.

1.)LC3B

mpsektfkqr rtfeqrvedv rlireqhptk ipviierykg ekqlpvldkt kflvpdhvnm selikiirrr lqlnanqaff llvnghsmvs vstpisevye sekdedgfly mvyasqetfg mklsv-removed to form

2.)LC3I

mpsektfkqr rtfeqrvedv rlireqhptk ipviierykg ekqlpvldkt kflvpdhvnm selikiirrr lqlnanqaff llvnghsmvs vstpisevye sekdedgfly mvyasqetfg

3.)LC3II Peptide

Figure 3.1: A Comparison of LC3 Proteins with the Immunogens: Full LC3 Protein, shown in 1, LC3I Whole Protein shown in 2 and LC3II Peptide shown in 3. Mklsv is removed to form the LC3I whole protein.

In order to achieve a monoclonal antibody to LC3I and LC3II, LC3I recombinant whole protein and a peptide for LC3II were selected as the antigens. Mice at the University of Michigan were immunized with the antigen linked to the carrier protein KLH on a schedule. Two separate test bleeds from the tail of the mice were taken and tested against the antigen plated on a multiwell plate to check for the mouse with the best antibody titer, or rather the best antibody response. The mouse with the best titer was then euthanized and the spleen cells were used to make the hybridoma. Cells were grown and the hybridoma supernatants were then tested against the same antigen. The supernatants that tested positive were selected and the cells brought to Enzo Life Sciences to be grown and undergo serial dilution cloning to select the best clones to expand. The selected hybridomas were then grown and transferred gradually to a serum free media to be grown in disposable bioreactor flasks. The supernatants were harvested from the disposable bioreactors and stored at -20°C. The harvested supernatants were purified on Protein G columns. The IgG concentration of each antibody preparation was determined by absorbance at 280nm. Fractions containing concentrations of at least 1.0 mg/mL of IgG were retained and

were dialyzed against 1X PBS. The final concentration of the antibody was again determined by absorbance. The final antibody preparation was tested using previously prepared lysates to show the detection of LC3I and LC3II by western blot.

3.2 Materials and Methods

3.2.1 Immunogen Selection and Immunization Schedule

Proteins and peptides were chosen for LC3I and LC3II to serve as immunogens. Three, 6 week old Balb/c mice were injected with 10μg, 20 μg and 50 μg of each immunogen. These immunogens were mixed with Freund's adjuvant, the initial intraperitoneal injection was complete Freund's and the following injections were incomplete Freund's. Two weeks after the initial injection a second injection is given to the mice. One week after this a tail bleed is taken. Four weeks after the second injection a third injection is given, one week after this injection another tail bleed is taken. The tail bleeds were screened in ELISA against the LC3I or LC3II immunogens for the mouse showing the best response to the antigen. The mouse which showed the best response to the antigen was given a final tail vein boost. (Note: All animal work was completed by the Hybridoma Core at University of Michigan.)

3.2.2 Cell Culture

Hybridoma cells were cultured in IMDM (Gibco, Life Technologies, Grand Haven, NY, USA) with 20% fetal bovine serum (PAA, Dartmouth, MA, USA), 5% BriClone (QED Bioscience Inc., San Diego, CA, USA) and penicillin/streptomycin (Gibco, Life Technologies, Grand Haven NY, USA). For initial cloning purposes additions of HAT Media Supplement (50x) Hybri-Max (Sigma-Aldrich, St. Louis, MO, USA) was added and later changed to HT Media Supplement (50X) Hybri-Max (Sigma-Aldrich, St. Louis, MO, USA) after hybridoma out

growth. Cells were then adapted to standard media additions of 15% fetal bovine serum (PAA, Dartmouth, MA,USA) with penicillin/streptomycin (Gibco, Life Technologies, Grand Haven NY, USA). For antibody production, cells were adapted to PFHM II Media (Gibco, Life Technologies, Grand Haven, NY, USA) and put into the disposable bioreactor or CELLINE Integra flasks (Integra Biosciences, New Haven, NH). (Note: All work completed at Enzo Life Sciences by me.)

3.2.3 Hybridoma Clone Selection using ELISA

Ninety six-well plates (Corning, Corning, NY,USA) were coated with either LC3I-BSA conjugate protein or LC3II-BSA protein conjugate (Enzo Life Sciences, Farmingdale, NY,USA) at a concentration of 0.1 μg/well overnight at 4°C. The plates were blocked with an assay buffer (PBS, 0.05% Tween 20, 0.1% BSA) solution at approximately 200μL/well and incubated at room temperature for 1 hour. One hundred μL of each hybridoma supernatant was added to separate wells and incubated for 1 hour at room temperature. Peroxidase-conjugated Affinipure Goat anti-Mouse IgG Fcγ Fragment specific (Jackson Immuno Research Laboratories, West Grove, PA, USA) was used as a secondary antibody at a dilution of 1:2000 and incubated at room temperature for 1 hour. TMB substrate (Neogen, Lexington, KY, USA) was added to the plate and allowed to develop at room temperature for approximately 20 minutes. HCl (1N) was added to stop the development and after this the plate was read at 450nM on a spectrophotometer. (Note: All work completed at Enzo Life Sciences by me.)

3.2.4 Antibody Isotyping and SDS-PAGE Charaterization

Antibodies were characterized using a mouse specific isotyping kit (AbD Serotec, Inc, Raleigh, NC). The kit provides developer vials with anti-mouse lambda and anti-mouse kappa in blue micro particles as well as strips that have immobilized goat anti-mouse antibodies that are synonymous with typical mouse antibody isotypes. Common antibody isotypes are IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA. There are also immobilized goat anti-mouse antibodies to lambda and kappa light chains. The antibody sample can be in tissue culture supernatant, ascites, or purified.

Antibodies were further characterized by SDS-PAGE on Tris-HCl Criterion Gel (BioRad, Richmond, CA, USA) at 1μg and 5μg for each antibody. The gel was then stained with Imperial Protein Stain (Thermo Scientific, Rockford, IL, USA) for 1hr at room temperature on a shaker, and then destained with dH_2O overnight on the shaker at room temperature. (Note: All work completed at Enzo Life Sciences by me.)

3.2.5 Antibody Purification

Supernatants were collected from the disposable bioreactor, filtered and then frozen at $-20^{o}C$. Drip columns were made using a 50% slurry Protein G resin (Pierce, Rockford, IL, USA) packed with 2mLs of resin. Protein G IgG binding buffer (Pierce, Rockford, IL, USA) was mixed 50:50 with the cell culture supernatant, centrifuged, filtered and added to the column, the flow through was saved. Binding buffer was used to wash the column until the wash registered below 0.05 mg/mL on the nanodrop spectrophotometer. IgG Elution buffer (Pierce, Rockford, IL, USA) was used to elute the antibody into 10 fractions each containing 100 μL of 1M Tris, pH 9.0 neutralizing buffer. The fractions were tested for IgG concentration by absorbance at 280nm. All fractions that were 1.0 mg/mL and above were collected and combined. The combined

fractions were dialyzed overnight at 4°C in 3L of 1X PBS. A final concentration, after dialysis and syringe filtration, was determined by absorbance at 280nm. (Note: All work completed at Enzo Life Sciences by me.)

3.2.6 Western Blotting

Neuro 2a fractionated cell lysates were separated by SDS-PAGE using 18% Tris-HCl Criterion Gels (BioRad, Richmond, CA, USA). These gels were transferred to Protran nitrocellulose membrane, 0.45µM (Whatman, Pistcataway, NJ, USA) through tank transfer. Membranes were blocked with 5% non-fat milk and PBS+0.05%Tween20 solution overnight at 4°C on a rocker. Several dilutions of the purified antibodies were used to determine the most effective concentration for the primary antibodies. Monoclonal antibody LC3B 5F10 (Enzo Life Sciences, Farmingdale, NY, USA) was used as a control antibody to determine the effectiveness of the newly developed antibodies. Secondary antibodies were goat anti-mouse HRP conjugates (Jackson Immuno Research Laboratories, Bar Harbor, Maine, USA). The signal was detected using enhanced chemiluminescent western blotting detection system (Thermo Scientific, Rockford, IL, USA). (Note: All work completed at Enzo Life Sciences by me.)

3.3 Results

3.3.1 Tail Bleed Screen

A tail bleed screening ELISA was completed with the LC3II immunized mice, one mouse was selected however, on the day that the mouse was to be euthanized for hybridoma fusion, the final LC3II mouse was found dead. The bleed results can be found in Figure 3.1. The final LC3II fusion was performed with excess spleen cells left over and frozen during the first LC3II fusion.

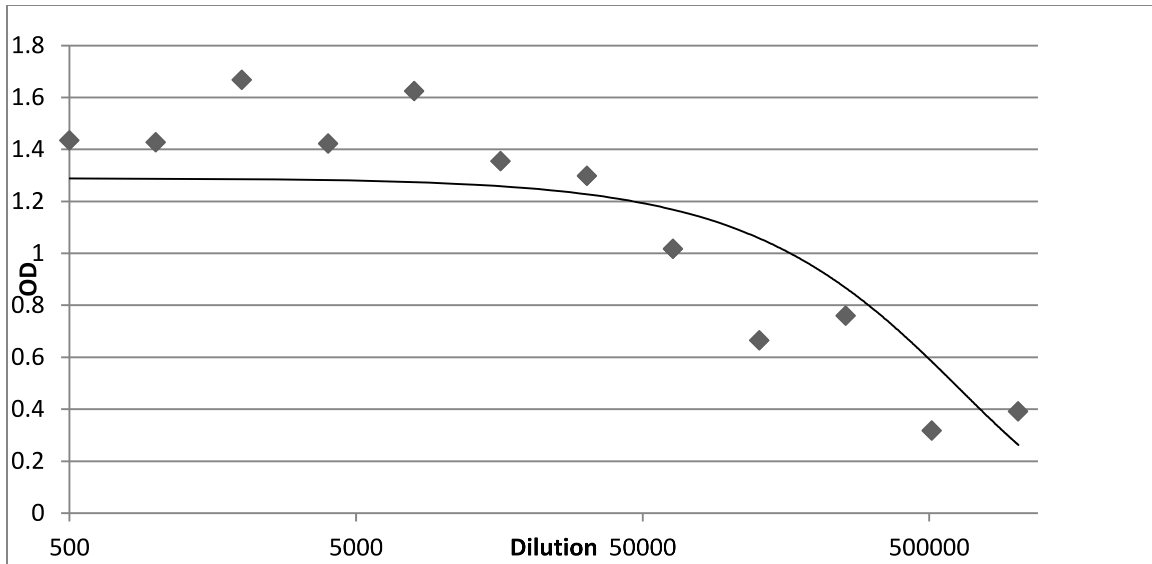

Figure 3.2: Mouse 3 Bleed Results. This graph shows titer data for mouse 3 against LC3II peptide-PE. The mouse has a titer at approx. 32000 or 1/32000, which was the cutoff concentration considered significant.

3.3.2 LC3I and LC3II Clone Selection and Subcloning

For LC3I, six plates were used to perform the initial hybridoma screen and nine clones were pulled from these plates for OD readings that were at least double the background, determined using media, PBS, and secondary only blanks. These nine clones were moved to a 24-well plate to grow a larger amount of cells. These were then screened in the same manner and five of those clones were moved on to limiting dilution subcloning in a 96-well plate to try to obtain outgrowth from a single cell that produces the antibody and assure a monoclonal culture. The clones that grew in the wells were again screened with ELISA and determined to not be reactive. The 24-well clones that these had come from tested positive and were subcloned again, using limiting dilution. None of the resulting clones produced antibody detectable in the screening assay.

The final mouse for LC3I was euthanized and the fusions were again screened, this fusion produced 140 clones that showed high OD readings and were pulled to a 24-well plate to

expand. The 140 clones were screened again and the highest eight clones were pulled for limiting dilution in a 96-well plate. The subclones were screened along with the supernatants from the 24-well cultures to confirm the subclones maintained the same reactivity to the antigen. The positive clones were found to have decreased signal relative to the 24-well culture supernatant. Two clones from the subcloning were found to have a high OD in the screening assay. These were subcloned again; upon the second subcloning no reactivity was found. Another attempt at subcloning was attempted and one subclone was found to provide a high OD, but it did not maintain reactivity once grown in larger culture.

In the LC3II project, the first clones were all producing very high OD readings, such that no differentiation between any of the clones was possible. Subcloning was completed and again, all of the supernatants from the clones produced the same high readings. The results indicated nonspecific binding in our screening assay. To improve specificity, we performed a test with the ELISA to determine if a blocking buffer change needed to be made in our ELISA screen. Blocking the plates had previously been eliminated from the procedure due to stable OD readings in LC3I plates without blocking; a blocking comparison study was designed. It was also determined that PBS, instead of an assay buffer, was used inconsistently with the secondary antibody at times. It was determined that using PBS instead of an assay buffer largely increases the background in the secondary only and the media only blanks.

Once we had re-optimized the assay the LC3II clones from the second fusion were tested and five clones were selected and subcloned. Two of the parent clones had subclones that tested positive in the screening ELISA. After a second subcloning five of these were expanded to be transferred to our regular IMDM hybridoma media. This is shown in Figure 3.3 below.

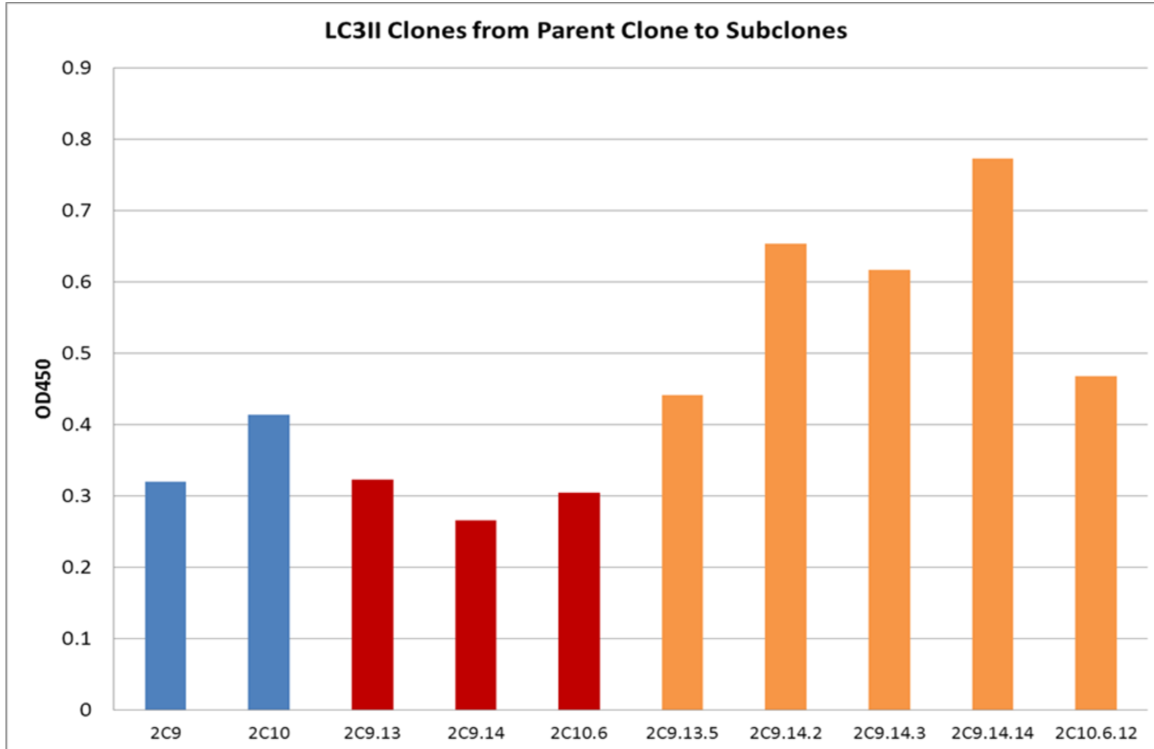

Figure 3.3: LC3II Clones from Parent Clone to Subclones: The above graph shows the initial parent clones after fusion, 2C9 and 2C10, through the first and second subcloning. After each subcloning the cells gain another number in the naming process.

3.3.3 Isotype of LC3 Antibodies

All LC3 antibodies were characterized by their isotype, using supernatants collected from disposable bioreactor flasks, which were diluted 1:1000 in a 1% BSA solution. Once mixed in the developer tube, the strips were added to the tube and allowed to sit for 5-10 minutes. The antibody isotypes are indicated in table 3.2.

Antibody	Heavy Chain	Light Chain
LC3II 2C9.13.5	IgG1	Kappa
LC3II 2C9.14.14	IgG1	Kappa
LC3II 2C9.14.2	IgG1	Kappa
LC3II 2C9.14.3	IgG1	Kappa
LC3II 2C10.6.12	IgG1	Kappa
LC3I 6C5	IgG3b	Kappa
LC3I 1A3	IgG3b	Kappa

Table 3.2: Isotypes of LC3 Antibodies

3.3.4 LC3II Supernatant Purification

The five LC3II subclones were transferred to disposable bioreactors after they were step-wise transferred to a serum free hybridoma media. Supernatants were collected from the bioreactor and saved at -20°C until they were thawed for purification. Out of the five subclones four were found to have met or exceeded1.0 mg/mL and were kept. The concentrations of the fractions and their final concentrations after dialysis can be found in Table 3.3.

Fraction #	2C9.14.3	2C9.13.5	2C10.6.12	2C9.14.2	2C9.14.14
1	0.01	0.01	0.00	-0.12	0.04
2	0.00	0.01	0.01	0.00	0.08
3	0.17	0.00	0.37	0.03	3.26
4	0.44	0.58	2.56	1.65	6.56
5	0.50	4.22	2.68	2.42	5.00
6	0.40	4.14	2.14	1.85	2.59
7	0.28	2.74	0.99	1.23	1.20
8	0.18	1.55	0.59	0.76	0.68
9	0.14	0.84	0.26	0.39	0.37
10	0.10	0.47	0.18	0.30	0.29
Volume of Culture Supe Purified	x	63.5 mL	12 mL	9.5 mL	31.5 mL
Volume after Dialysis	x	4.25 mL	3.5mL	3.5 mL	5 mL
Conc. After Dialysis	x	3.05mg/mL	1.98 mg/mL	1.59 mg/mL	3.53 mg/mL
Total Conc. Of Antibody	x	12.96 mg	6.93 mg	5.56 mg	17.65 mg

Table 3.3: Antibody Concentrations for LC3II Hybridomas. Four of the five clones produced IgG levels that were about 1.0 mg/mL, these are highlighted, which were saved and dialyzed in PBS, concentrations were determined using a nanodrop spectrophotometer.

The four LC3II clones, that produced the most purified antibody, were analyzed for purity by SDS-PAGE on a 4-15% Tris-HCl Criterion Gel at concentrations of 1μg and 5μg. This gel was then stained with Imperial Protein Stain and found to have both the heavy chain and light chains at the approximate molecular weights of 55kDa for the heavy chain and 22kDa for the light chain. This is shown in Figure 3.4.

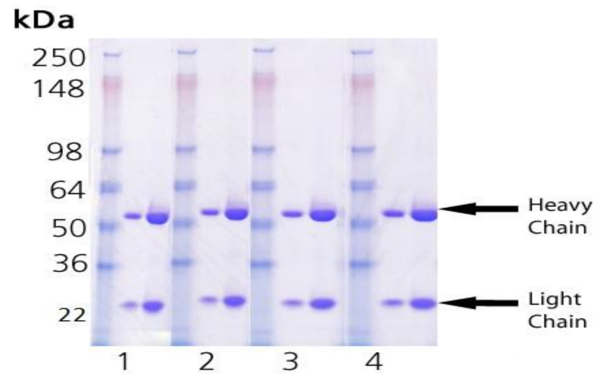

Figure 3.4:Characterization of LC3II Antibodies using SDS-PAGE. 1 represents LC3II 2C9.13.5, 2: LC3II 2C10.6.12, 3: LC3II 2C9.14.2, 4: LC3II 2C9.14.14

3.3.5 LC3 II Western Blotting

The four LC3II antibodies were diluted to 1.0 mg/mL and used at several different subsequent dilutions in a western blot of the Neuro 2a fractionated lysate that was previously prepared. Each antibody was tested at dilutions of 1:250, 1:500 and 1:1000. Western blots included the positive control antibody used previously, mAb (5F10). The control antibody detected LC3II in all experiments. None of the LC3II antibodies were capable of detecting LC3II in the western blots. Another attempt was made with Neuro 2a fractionated lysates blocking the blot with 2% BSA and using BSA to incubate the primary and secondary antibodies; this was also unsuccessful producing no reaction. Previously made HeLa Fractionated lysates were also tested with the new LC3II antibodies and the control antibody. Again, the antibodies did not detect LC3II in the western blots.

3.4 Conclusion

The LC3I monoclonal antibodies have been developed. Initial screening assays showed LC3I reactivity. LC3I hybridomas have been difficult to stabilize in culture. Instability of hybridoma cells could contribute to non-producing cells outgrowing producing cells and losing

LC3I reactivity. The LC3I project will continue through different subcloning techniques to determine if stability can be achieved.

LC3II monoclonal antibodies were developed. LC3II hybridomas were screened and subcloned. LC3II antibodies have not been found to react in western blotting. LC3II antibodies may recognize the peptide but, not the intact protein.

3.5 Discussion

The attempt at development of LC3 monoclonal antibodies has been unsuccessful. In the time following the fusion of spleen cells and myeloma cells, the newly formed hybridomas can be unstable. Hybridomas can stop making antibodies during the subcloning process, when cells that don't produce antibody may outgrow the antibody producing clones. Subcloning is the most important and challenging stage of hybridoma development (29), if successful it results in stable antibody producing cells. LC3I antibody development will continue through further subcloning using the two previously frozen clones that were previously known to produce LC3I specific antibodies. We will test those for current LC3I reactivity and expand the positive cultures. Expanded cells will be considered for antibody production and were frozen down to preserve the cells. Cells were sent to The University of Michigan for subcloning by their hybridoma core for subcloning, additionally the cells will be subcloned at Enzo Life Sciences using a semi-solid media. The semi-solid media enables subcloning by colony selection and achieves subcloning in a single round. All subclones will then be considered for ascites production in mice to accelerate antibody production.

The LC3II development was initially much more successful when compared to the LC3I development. The subcloning was successful and produced clones that had reactivity with the LC3II peptide in ELISA. For at least four of the five clones of LC3II, there was a substantial

amount of antibody purified from the cell culture supernatant. This was however unsuccessful at producing a result in western blot.

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ABSTRACT

LC3I and LC3II as Autphagy Markers for the Development and Improvement of Products and Techniques used in Research

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Autophagy is an intracellular process that functions to maintain homeostasis in the cell by degrading misfolded proteins, old or nonfunctioning organelles, and outside invaders such as bacteria or viruses. This process can be split into three different types, microautophagy, chaperone mediated autophagy and macroautophagy. Macroautophagy is the most commonly studied form and is believed to be regulated by Atg proteins, as well as cargo proteins that bring debris to the autophagosome. Macroautophagy is characterized by 5 steps including initiation, elongation, maturation, autophagosome-lysosome fusion and lysosome degradation. Autophagy has been found to be involved in diseases, such as cancer and neurological disease. LC3I and LC3II function in late stage autophagy before lysosome fusion. The study of a complex process such as autophagy requires the development of tools to enable precise and quantitative research. The ability to produce antibodies to key autophagy proteins, methods of cellular fractionation and fractionated control cell lysates provides product development opportunities for Enzo Life sciences and new research tools for researchers. To support these needs, methods of cell fractionation were developed along with antibodies specific to LC3I and LC3II. Cell fractionation methods were optimized to enable separation of LC3I and LC3II. The optimized

methods were shown to be effective on multiple cell lines with the use of western blotting. Due to the difficulty in achieving stable hybridomas monoclonal antibodies that bind both LC3I and LC3II haven't been identified. The LC3II antibody development failed to produce antibodies in western blotting. Efforts are ongoing to identify stable hybridomas producing antibodies to LC3. The antibodies should recognize both LC3I and II. Combining the use of these antibodies with fractionated cell lysate methodology should allow quantitative detection of LC3II by ELISA. Such a tool will be an important tool for autophagy researchers.

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Master of Science, Wayne State University (2010-2013)
Major: Biochemistry and Molecular Biology
Advisor: Dr. Robert Akins

Publications:

1.) Aurora A is differentially expressed in gliomas, is associated with patient survival in glioblastoma and is a potential chemotherapeutic target in gliomas, Lehman NL, O'Donnell JP, Whiteley LJ, Stapp RT, Lehman TD, Roszka KM, Schultz LR, **Williams CJ**, Mikkelsen T, Brown SL, Ecsedy JA, Poisson LM., Cell Cycle. 2012 Feb 1;11(3):489-502

Employment:
Enzo Life Sciences, Manufacturing, Ann Arbor, MI (2011-current)
Position: Associate Manufacturing Scientist

Henry Ford Health System, Department of Pathology, Detroit, MI (2010-2011)
Position: Research Assistant