The differential expression of miRNAs in breast cancer cell-lines upon autophagy induction

by

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ABSTRACT

Autophagy is a catabolic process of self-digestion that occurs at basal levels in all cells, degrading old and damaged components such as proteins and organelles. Autophagy is upregulated in response to cellular stress, including the types imposed on cancer cells, and its role in these circumstances is often to facilitate cell survival. However, the molecular mechanisms regulating autophagy in response to cellular stress are still not well understood. Other components of the cell that are regulated in response to cellular stress are small non-coding portions of RNA termed microRNAs (miRNAs) that act to control protein levels in the cell by degrading mRNA targets or repressing their translation.

The overall objective of this research was to identify candidate miRNA regulators of autophagy. My hypothesis was that miRNAs that regulate autophagy will demonstrate differential expression in response to cell stresses that induce autophagy. To test this hypothesis, I conducted miRNA expression profiling by Illumina sequencing in untreated BT-474 breast cancer cells and in BT-474 cells treated with a high dose of tamoxifen, a known inducer of autophagy. 113 distinct miRNAs were found to be significantly differentially expressed ($p<0.05$ and >1.5 fold) between the tamoxifen treated sample and control, and the differential expression of a subset of these miRNAs was validated using QRT-PCR. Using the online miRNA resource, TargetScan, 27 of the differentially expressed miRNAs were found to have potential autophagy-related targets, and of these, seven were selected to undergo further expression analysis in two additional breast cancer cell-lines using an additional autophagy inducing treatment, nutrient deprivation. Four of the selected miRNAs demonstrated similar patterns of differential expression in all three breast cancer cell-lines under the two different autophagy-inducing conditions. These four miRNAs consistently showed decreased expression when autophagy was induced, and have the potential to be direct regulators of the autophagy pathway upon cell stress induction in breast cancer cells. Understanding the mechanisms underlying the regulation of stress-induced autophagy may provide insight into the role autophagy plays in breast cancer and reveal potential targets to alter the process for clinical benefit.

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1 INTRODUCTION

1.1 Cellular stress and autophagy

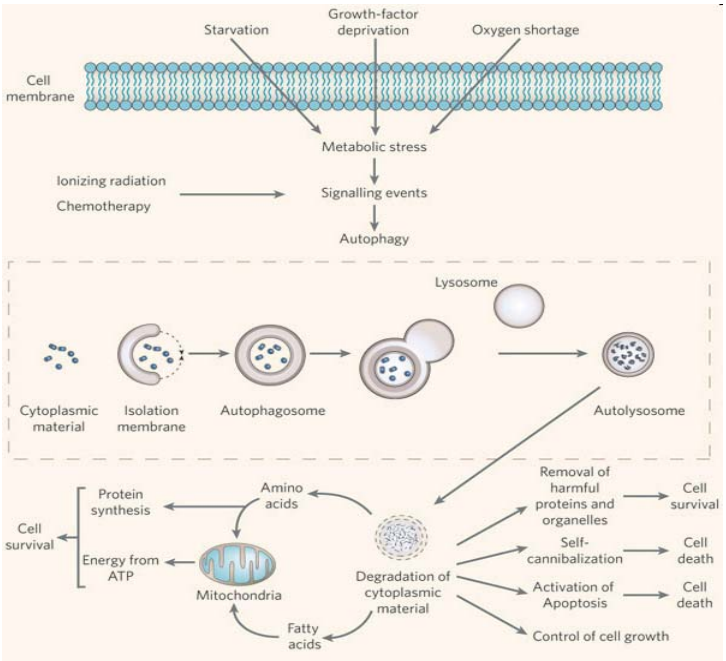
Cell stress can come in many different forms from both the environment, as well as within the cell. Environmental cues can include starvation, high temperatures, low oxygen, and hormonal stimulation, while intracellular stress may involve damaged organelles, accumulation of mutant proteins, or microbial invasion (Valko et al, 2005; Levine and Kroemer, 2008). Cellular stress can directly result in improper protein folding, increases in mutation frequencies, and DNA damage within the confines of the cell (Rockwell et al, 2001). These types of insults to the cells of the body and accumulation of damaged components have been implicated in a number of diseases including liver, muscle and heart disease, many types of cancer, inflammatory bowel disease (Hampe et al, 2007; Cadwell et al, 2008) and certain neurodegenerative disorders such as Parkinson's (Alam et al, 1997) and Huntington's disease (Anne et al, 2007; Levine and Kroemer, 2008).

Normally in instances of severe cellular stress, an organism can avoid potentially life-threatening consequences by initiating death in damaged cells via the apoptotic pathway. Apoptosis, a type of programmed cell death, is a normal occurrence in the lifespan of an organism, especially in early development, and functions to remove both non-essential and damaged cells. In the case of irreversibly damaged cells, there are proteins within the cell, such as p53, which are activated upon receiving information on the status of the injured cell (Levine, 1997). This activation can lead to the induction of apoptotic protein activity that results in cellular events such as nuclear chromatin condensation, cytoplasmic shrinking, dilated endoplasmic reticulum, and membrane blebbing that eventually lead to cell death (Kerr et al, 1972). By eliminating the cells that are beyond repair, the organism can avoid more negative repercussions. However, in terms of cell viability, apoptosis is an extreme resolution to cell stress. There is another process within the cell that works to avoid an accumulation of damaged proteins and organelles, and this important mechanism is known as autophagy.

Autophagy, a term originating from Greek roots literally meaning “self-eating”, is a catabolic (breakdown) process of self-digestion that, under most conditions, promotes cell survival by degrading and recycling internal components. Three forms of autophagy have been described - macroautophagy, microautophagy and chaperone-mediated autophagy – that differ in terms of their physiological functions and mode of cargo delivery (Reggiori and Klionsky, 2002; Wang and Klionsky, 2003). This thesis focuses on the most studied form, macroautophagy, which will be referred to simply as autophagy for the remainder of this thesis.

Autophagy is an evolutionarily conserved process that involves the formation of a double-membrane within the cell that elongates and engulfs a bulk portion of the cytoplasm. Although the process is thought to be nonspecific, the engulfed cytoplasm often contains old or damaged cellular organelles and aberrant proteins (Levine and Klionsky, 2004). Following cytoplasmic engulfment, the double membrane fuses to form a vesicle, termed an autophagosome, which in turn fuses with the lysosome, an acidic organelle, to form an autolysosome. The acid hydrolases within the lysosomes degrade the components of the autophagosome into their elemental forms (free nucleotides, amino acids and fatty acids), which can then be recycled back into the cytoplasm of the cell, to make new proteins as well as provide building blocks for ATP generation (Figure 1.1) (Noda and Ohsumi, 1998; Levine and Klionsky, 2004).

Figure 1.1 - Mechanism of Cellular Macroautophagy

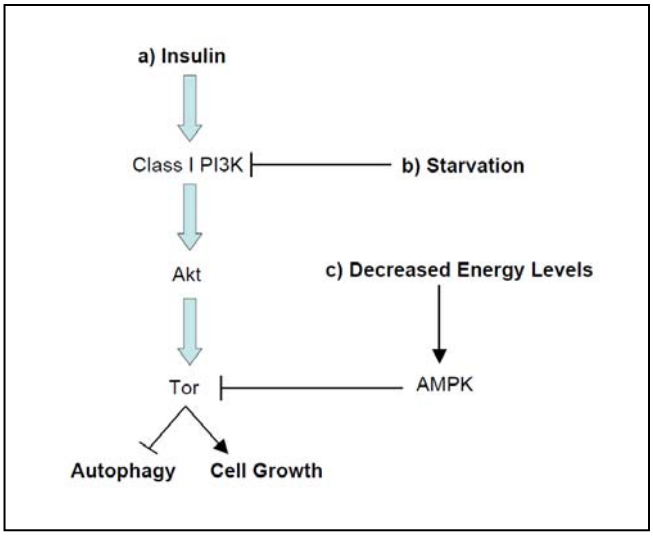


Autophagy occurs at low levels in virtually all cells to perform homeostatic functions such as the turnover of misfolded proteins and damaged organelles. However, in situations of high cellular stress and damage-inducing conditions when the survival of the cell is threatened, autophagy is up-regulated and performs at a higher capacity to facilitate survival or possibly to remove damaged proteins and organelles (Figure 1.1). The mechanism by which stress regulates this pathway is not well understood (Levine and Klionsky, 2004). Autophagy is therefore seen as an important tool in everyday cellular housekeeping duties as well as a survival mechanism of the cell. Additionally in extreme cases of stress or starvation, often in the absence of properly functioning apoptosis, this type of degradation within the cell presumably can't keep up to its energy demands and ultimately results in what is sometimes called autophagic cell death, as the cell literally "eats" itself beyond sustainability (Clark, 1990; Bursch et al, 2000).

1.2 The autophagy pathway

Autophagy involves a complex pathway of cellular events, containing many different proteins. The process can be divided into several distinct steps: induction and signaling, autophagosome nucleation, membrane expansion and vesicle completion, autophagosome targeting, docking and fusion with the lysosome, and degradation and export of materials to the cell's cytoplasm (Melendez and Neufeld, 2008; Levine and Yuan, 2005). One of the key proteins known to regulate autophagy is TOR kinase, which inhibits the autophagic process in the presence of growth factors and

Figure 1.2 - Regulation of the Autophagic Pathway



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abundant nutrients - when the cell doesn't need to break down internal components to synthesize energy (Noda and Ohsumi, 1998). Also involved in autophagy inhibition are the class I PI3K/Akt signaling molecules that link receptor tyrosine kinases to TOR activation (Figure 1.2) (Takeuchi et al, 2005; Debnath et al, 2003). Some activators of autophagy include the proteins AMPK (Meley et al, 2006), which responds to low ATP levels to represses TOR kinase, and eIF2a (eukaryotic Initiation Factor 2 alpha), which responds to nutrient starvation (Levine and Kroemer, 2008). The cellular checkpoint protein involved in apoptosis, p53, is also involved in autophagy regulation. The p53 protein can positively control autophagy, at least in some cases through a stress-induced regulator termed DRAM (Crighton et al, 2006). The protein DRAM, or damage-regulated autophagy monitor, is a downstream transcriptional target of p53 that when activated has been shown to induce autophagy and is critical in p53-induced cell death (Crighton et al, 2006). More recently it was shown that the cytoplasmic form of p53 might instead act as a negative regulator of autophagy, suggesting a more complex model that may be dictated by the specific nature of the stress signal (Tasdemir et al, 2008).

Downstream of TOR kinase there are more than 20 genes (in yeast), termed autophagy-related (*atg*) genes that encode proteins that are essential to the execution of autophagy (Klionsky et al, 2003). One of the major breakthroughs in studying the autophagic process came from the initial identification of these genes in yeast (Tsukada and Ohsumi, 1993; Thumm et al, 1994; Harding et al, 1995). Homologues of these genes have been found in mammals and include *atg8* (known as *MAP1LC3B* or *LC3* in mammals) and *atg6* (known as *beclin-1* in mammals), both of which are needed for proper autophagosome formation (refer to Table 1.1). A uniform nomenclature was agreed upon for homologues of *atg* genes in multiple species; essentially a species identifier precedes "*atg*". Table 1 lists *atg* genes conserved between yeast and human, and briefly describes their role(s) in autophagy that are further detailed elsewhere (Klionsky et al, 2003; Meijer et al, 2007).

Table 1.1 - Core autophagy genes found in yeast and humans

Yeast Name	Human Homologue	*Role in autophagy	References
ATG1	*Ulk1*, *Ulk2*	Atg1 is a serine/threonine protein kinase; involved in the regulation of autophagy induction; may regulate subcellular re-distribution of mammalian Atg9 that takes place following nutrient starvation	Matsuura et al, 1997; Kametaka et al, 1998; Kamada et al, 2000; Young et al, 2006
ATG3	*atg3*	Functions as an ubiquitin-conjugating-like enzyme that covalently attaches Atg8 to phosphatidylethanolamine	Ichimura et al, 2000; Tanida et al, 2002
ATG4	*atg4* (*4A,4B,4C,4D*)	Atg4 is a cysteine protease that cleaves the C-terminus of Atg8 to expose a glycine residue for subsequent conjugation	Kirisako et al 2000; Hemelaar et al, 2003; Tanida et al, 2004a
ATG5	*atg5*	Atg5 is covalently attached to Atg12 to facilitate autophagosome formation (Atg12 conjugation system)	Kametaka et al, 1996; Mizushimi et al, 1998; Kuma et al, 2004; Shao et al, 2007
ATG6	*beclin-1*	Atg6 is a component of the class III phosphotidylinositol-3-kinase complex that is required for autophagosome formation (forms a complex with Atg14)	Kametaka et al, 1998; Liang et al, 1999; Liang et al, 2000; Kihara et al, 2001
ATG7	*atg7/GSA7*	Atg7 functions as an ubiquitin-activating-like enzyme; it activates both Atg8 and Atg12 before conjugation	Kim et al, 1999; Tanida et al, 2006; Shao et al, 2007
ATG8	*MAP1LC3B, MAP1LC3A, MAP1LC3C, GABARAP, GATE16*	Atg8 is involved in autophagosome formation and is used as a marker for autophagosomes; lipidation of LC3 paralogues is involved in the closure of autophagosomes.	Kirisako et al, 1999; Kirisako et al 2000; He et al, 2003; Fujita et al, 2008
ATG9	*atg9* (*9A* and *9B*)	Atg9 is a transmembrane protein that may be involved in delivering membrane to the forming autophagosome	Noda et al, 2000; Lang et al, 2000
ATG10	*atg10*	Atg10 functions as an ubiquitin-conjugating-like enzyme that covalently attaches Atg12 to Atg5 to aid in autophagosome formation (Atg12 conjugation system)	Mizushima et al, 1998; Boya et al, 2005; Shao et al, 2007
ATG12	*atg12*	Atg12 is conjugated to an internal lysine of Atg5 through its C-terminal glycine to facilitate autophagosome formation (Atg12 conjugation system)	Mizushimi et al, 1998; Shao et al, 2007; Boya et al, 2005
ATG13	*atg13*	Induction (modulates Atg1 response)	Funakoshi et al, 1997; Kamada et al, 2000; Chan et al, 2009
ATG14	*atg14/barkor*	Autophagosome formation	Itakura et al, 2008; Sun et al, 2008

Yeast Name	Human Homologue	*Role in autophagy	References
ATG16	*atg16* (*16L1* and *16L2*)	Atg16 binds Atg5 and homo-oligomerizes to form a tetrameric complex for autophagosome formation	Mizushima et al, 1999; Kuma et al, 2002; Mizushima et al, 2003

1.2.1 Methods of quantifying autophagy

The formation of autophagosomes was first noted in dying cells through observational techniques (de Duve, 1963). Since this time a number of assays have been designed in order to both observe the process of autophagy within cells and quantify its activity. LC3 (Atg8) is an especially important autophagy protein as it can be used in the laboratory to monitor autophagy in a variety of ways. This protein exists in two forms within the cell: LC3-I and LC3-II. LC3-I, the form most commonly found within the cytoplasm, is predominant under normal conditions. When autophagy is induced, LC3-I is converted to LC3-II by Atg4-mediated C-terminus cleavage (Tanida et al, 2004) and lipidated with phosphatidylethanolamine (PE), which allows it to be inserted into the autophagosome membrane (Kabeya et al, 2000). The LC3-II form is the only Atg protein in higher eukaryotes that is known to be associated with the fully formed autophagosome and can be found on both the outer and inner surfaces of the autophagosome membrane (Mizushima and Yoshimori, 2007). LC3 conversion can be visualized via Western blot analysis, and comparing the levels of LC3-II to appropriate controls can indicate the relative number of autophagosomes between samples (Kabeya et al, 2000; Kirisako et al, 1999). Another method used to visualize LC3 in the cell is by tagging its N-terminus with a fluorescent marker, such as green fluorescent protein (GFP) (Kabeya et al, 2000; Mizushima and Yoshimori, 2007). This allows one to view the localization of LC3 within the cell; in normal conditions LC3 (LC3-I) appears diffuse in the cell, but under autophagy-inducing conditions, LC3 appears as localized structures (puncta) which represent its incorporation into autophagosomes (LC3-II) (Kabeya et al, 2000). Other methods of identifying the incidence of cellular autophagy include observing the formation of autophagosomes through electron microscopy, staining with monodansylcadaverine (MDC) and quantifying autophagy gene transcript levels (Biederbick et al, 1995; Klinosky et al, 2008; Zhu et al, 2009; Nara et al, 2002; Kouroku

et al, 2007). MDC stains for acidic compartments within cells and correlates well to the increase in autolysosomes when autophagy is induced, however it is not a specific marker for autophagy (Biederbick et al, 1995; Klionsky et al, 2008). There have also been a number of studies that have correlated the increase in transcript levels of certain genes in the autophagy pathway, including LC3, to an induction of the autophagy process (Zhu et al, 2009, Nara et al, 2002; Kouroku et al, 2007; Kuma et al, 2004; Young et al, 2009).

Autophagy is a dynamic, multi-step process that can be monitored at various steps (seen in figure 1). It is important to distinguish between measurements that monitor the number of autophagosomes (steady-state) versus those that measure flux through the autophagic pathway. Autophagic flux refers to the complete process of autophagy, including the delivery of cargo to the lysosomes and subsequent breakdown of its components (Klionsky et al, 2008). Simply observing an increase in autophagosomes or core proteins in the autophagy pathway may be a result of an increase in the cellular mechanism or could be indicative of a downstream block and the inability of the components of the autophagosome to be degraded (Klionsky et al, 2008). Recently, Klionsky and 231 other researchers published a comprehensive set of autophagy-monitoring guidelines to assist in the interpretation of various assays (Klionsky et al, 2008). Most researchers agree that the best way to measure autophagy in an *in vitro* or *in vivo* system is to use a combination of these techniques.

1.3 Autophagy and breast cancer

What effect does altering the autophagy process have on cancer cells? There has been a dramatic increase in research dedicated to answering this question during the last decade. A cancerous cell is one in which the normal machinery malfunctions, potentially resulting in an increase in proliferation, evasion of cell death, and insensitivity to anti-growth signals, among other possible consequences. Breast cancer is one of the most prevalent forms of the disease in women and affects millions of people worldwide (American Cancer Society, 2007). Today, breast cancer, like many other forms of cancer, is considered to be an outcome of both environmental and genetic factors leading to

development of cancer cell traits, including self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis, impairment of DNA repair mechanisms, and metabolic transformation (Hanahan and Weinburg, 2000; Rockwell et al, 2001; Tennant et al, 2009). The precise mechanisms responsible for these cancer cell traits are still not fully understood but many of the molecules involved are beginning to be elucidated.

In 1999, the first direct association between a core autophagy gene and human cancer was discovered by Liang et al, who found that the autophagy gene *beclin-1* was monoallelically deleted in 40-75% of sporadic human breast and ovarian cancers (Liang et al, 1999), suggesting a possible tumour suppressor function for this autophagy protein. Since then, there have been an increasing number of scientific papers published on the relationships between autophagy and cancer, examining not only *beclin-1*, but several of the other *atg* genes required for autophagy (refer to Table 1.1). While studies to elucidate the role of autophagy in tumorigenesis have been carried out in various cancer types, predominantly in cancer cell lines, here I will mainly describe studies conducted specifically in breast cancer.

Establishing exactly what the link is between breast cancer cells and autophagy is an important research area that has increased in recognition over the past few years. The solution to this quandary is more complicated than it might seem at first glance. There is experimental evidence that supports the theory that autophagy acts as a tumour suppressor and should therefore be stimulated to reduce the occurrence of cancer in breast cells (Liang et al, 1999; Qu et al, 2003; Karantza-Wadsworth et al, 2007; Mathew et al, 2007). However there is other evidence supporting the idea that the survival effects of autophagy may help breast cancer cells endure and subsequently proliferate, particularly in adverse environmental conditions, and thus autophagy should be inhibited to help reduce breast cancer (Paglin et al, 2001; Abedin et al, 2007; Qadir et al 2008; Samaddar et al, 2008; Apel 2008). This leads to the question of whether the process of autophagy acts to help or hinder cancerous breast cells. The answer to this could help

determine whether we should attempt to therapeutically activate or inhibit autophagy and if so, at what stage of tumour development. Below I describe the supporting evidence for the roles autophagy may play in cancer and discuss how these opposing roles can be reconciled.

1.3.1 Tumour suppressor role

Over the past 10 years several genetic links have emerged between autophagy defects and cancer, providing increasing support for the concept that autophagy functions as a tumour-suppressor pathway (Liang et al, 1999; Mathew et al, 2007; Marino et al, 2007; Karantza-Wadsworth et al, 2007). In addition, an overlap between the regulation of autophagy and signaling pathways that regulate tumour formation has been established. Several autophagy-inducing genes involved in the upstream inhibition of TOR signaling pathways including PTEN, TSC1, and TSC2 are known tumour suppressors that act to decrease the incidence of cancer (Arico et al, 2000; Inoki et al, 2003; Levine and Kroemer, 2008). TOR-activating oncogene products on the other hand, such as class I PI3K and Akt, inhibit autophagy (Figure 1.2), and when over-expressed increase the likelihood of developing cancer (Blommaart et al, 1997). The PI3K pathway is particularly relevant to breast tumorigenesis as a recent large-scale sequence analysis of genes mutated in human breast cancer identified mutations in multiple PI3K pathway components (Wood et al. 2007). Lastly, the apoptosis checkpoint protein p53 is the most commonly mutated tumour suppressor gene in human breast cancers, and is known to be involved in regulating autophagy in DNA-damaged cells (Bursch et al, 2000; Crighton et al, 2006; Tasdemir et al, 2008).

The first core *atg* gene discovered that is linked to human breast cancer, as mentioned previously, is *beclin-1*, located on chromosome 17q21. *Beclin-1* is required for autophagosome formation in a complex with class III PI3K and was found to be monoallelically deleted in human ovarian, breast, and prostate cancers (Liang et al 1999; Aita et al, 1999). Experiments have shown that many breast carcinoma cell lines, although polyploid for chromosome 17, exhibit deletions of one *beclin-1* allele, and human breast tumours show decreased *beclin-1* protein levels compared to normal

adjacent tissue (Liang et al, 1999). In mouse knock-out studies, it was shown that *beclin-1* heterozygous mice have hyperproliferative, pre-neoplastic changes in mammary cells (Qu et al, 2003) and are more prone to the development of spontanteous lung and liver tumours, as well as lymphoma (Qu et al, 2003, Yue et al, 2003). These findings suggest that the mono-allelic deletions of *beclin-1* in human breast cancers are likely mechanistically important in tumorigenesis. Karantza-Wadsworth et al (2007) showed that allelic loss of *beclin-1* resulted in attenuated and delayed autophagy induction compared to wild-type, and also sensitized mammary epithelial cells (iMMECs) to metabolic stress (*in vitro* ischemia) as well as accelerated lumen formation in mammary acini. Autophagy defects activated the DNA damage response and promoted gene amplification and drug resistance *in vitro*. In allograft mouse mammary tumours *in vivo*, allelic loss of *beclin-1* activated the DNA damage response and, when combined with defective apoptosis, promoted mammary tumorigenesis (Karantza-Wadsworth et al, 2007).

A study by Mathew et al in 2007 used similar experimental methods to confirm that monoallelic deletion of *beclin-1* decreases the likelihood of cell survival *in vitro*. The Mathew (2007) study also analyzed cells harboring a homozygous deletion of another essential autophagy gene, *atg5*, and its effects on cell survival. They found that the *atg5* deficiency in iBMK (immortalized baby mouse kidney epithelial cells) cell-lines impaired survival of the cells and promoted DNA damage under metabolic stress *in vitro,* but increased tumourigenicity in allograft mouse models *in vivo* (Mathew et al, 2007). Marino et al (2007) generated mutant mice deficient in *atg4C*/*autophagin-3*, whose gene product is a member of the mammalian Atg4 family of cysteine proteases. These mutant mice appeared both viable and fertile, with no apparent abnormalities; however upon further analysis these mice were found to have decreased autophagy in the diaphragm when starved and an increased susceptibility to developing fibrosarcomas when exposed to chemical carcinogens (Marino et al, 2007). Together, these findings suggest that defective autophagy increases the susceptibility to DNA damage and genomic instability.

In addition to autophagy's cell autonomous mechanism as a suppressor of genomic damage, a non-cell-autonomous tumor suppressor function for autophagy has been proposed. In apoptosis-defective iBMK cells, autophagy inhibition by AKT activation or *beclin-1* knockdown resulted in necrotic cell death *in vitro* and *in vivo* (Degenhardt et al, 2006). This cell death by necrosis was associated with inflammation, providing a potential non-cell-autonomous mechanism by which autophagy defects resulted in the observed acceleration in tumour growth (Degenhardt et al, 2006). Inflammatory infiltration and cytokine production are found in necrotic tumours and are thought to foster the growth of such tumours (Balkwill, 2004). Therefore, promoting autophagy may also act to restrict necrosis and inflammation, and such tumor suppressor effects would ultimately discourage breast cancer progression.

The notion that decreased levels of autophagy can lead to a higher probability of tumour formation is consistent with the anti-ageing theory of autophagy, as it has been shown that both the formation of autophagosomes and their removal by lysosomal fusion decrease with age (Terman, 1995, Cuervo et al, 2005), negatively correlating with the incidence of most types of cancer initiation, including breast cancer. The first genetic evidence linking autophagy and longevity was found by Melendez et al using *C. elegans* models where specific mutations in genes related to the insulin-like signaling pathway doubled the lifespan of the worms, however when autophagy was blocked in these mutated worms, their lifespan returns to normal values (Melendez et al, 2003). This suggests that the activation of autophagy is one of the downstream effectors that lead to a prolonged existence (Melendez et al, 2003, Cuervo et al, 2004). A study by Bergamini et al in 2003 also described an association between ageing and autophagy. They proposed that caloric restriction and decreased levels of IGF-1, both known factors in life longevity, may act by stimulating an increase in the basal levels of autophagy (Bergamini et al, 2003). Decreased basal levels of autophagy would allow for the accumulation of damaged or non-functioning components of the cell. Normally in humans, these events would result in DNA instability and often lead to the activation of the apoptotic pathway and eventually cell death (Moll and Zaika, 2001; Yen and Klionsky, 2008). However, it has been shown that a large percentage of breast cancer cells have defective or inactive

apoptotic proteins (such as p53), leading to an inhibition of this programmed cell death. The accumulation of increased sources of oxidative stress because of decreased levels of both autophagy and apoptosis may result in damaged DNA or other known initiators of cancer (Karantza-Wadsworth et al, 2007). Although this evidence so far comes from different *in vitro* models, if applied to the human population, it may help explain why cancer is more prevalent in the elderly than in younger populations who have higher levels of autophagy.

With this information, one might instinctively think that the link between autophagy and breast cancer is fairly obvious; autophagy must play a tumour suppressor role and when not functioning properly it may lead to chromosomal instability (Karantza-Wadsworth et al, 2007; Mathew et al, 2007) and eventually cancer cell development. Therefore therapeutically targeting pre-cancerous cells to upregulate components of autophagy might be a method of decreasing the initiation of the disease in breast tissue. However an important distinction is required between preventing the initiation of a cancerous tumour and treating a tumour that has already been established. Most successful cytotoxic chemotherapy agents utilized by oncologists in treating cancers (including breast cancer) actually promote significant DNA damage, leading to activation of the apoptosis pathway in cancerous cells. In fact, there are a number of drugs either already in clinical use or in the development stage for the treatment of already-established breast tumors, that promote DNA damage in tumour cells, and also have an effect on autophagy (Table 1.2). There is increasing evidence to suggest that up-regulating autophagy in already-established cancerous cells would aid in tumour survival and perhaps resistance to chemotherapy treatment by degrading damaged organelles, preventing apoptosis, and/or by providing additional energy the tumour cells would otherwise lack. This leads to the second role - that increasing autophagy in established cancerous cells promotes tumour viability. In this context, therapeutic strategies employing autophagy inhibition may be warranted, with the resulting cytotoxic effects on the tumour outweighing any possible tumor-promoting side effects secondary to DNA damage.

1.3.2 Tumour survival role

As described above, autophagy is an important process for maintaining cell viability under conditions of stress within the cell. The proliferation of breast tumour cells puts the rapidly dividing cells under tremendous stress and without an adequate blood supply the ability to properly sustain nutrition and energy decreases exponentially. If sufficient energy cannot be supplied to the proliferating cells, then it would seem the appropriate response would be for the cancerous cells to stop dividing and die off as a result of decreased nutrition. However, this is often not the case in breast tumours and it has therefore been suggested that perhaps the increase in autophagy levels in breast tumour cells leads to enhanced survival properties (Abedin et al, 2007). This mechanism seems very plausible from a functional standpoint as the autophagic pathway can be used to break down proteins and organelles to provide the basic elements needed to assemble new proteins crucial to the growth of the cell, as well as energy to perform the necessary functions. During the initial phase of tumour formation, new blood vessels have not yet been created and the nutritional demands of tumour cells are likely to surpass the supply from normal vasculature (Harris, 2002). Applying the autophagic survival mechanism to these rapidly growing cancer cells, which have outgrown their vascular supply and face oxygen shortage or metabolic stress, would allow them to continue to grow and divide with little to no outside resources (Maiuri et al, 2007). In support of this general concept, Debnath et al. used a three-dimensional mammary epithelial cell (MCF10A) culture model to show that during detachment induced apoptosis (termed *anoikis* [Frisch and Francis, 1994]) the incidence of autophagy increased dramatically (Debnath et al, 2002; Fung et al, 2008). It was demonstrated further that knockdown of core autophagy genes using siRNA techniques resulted in an increased ability of the detached cells to undergo apoptosis (Fung et al, 2008; Debnath, 2008). These findings led the researchers to speculate that autophagy may contribute to the survival of tumour cells lacking matrix contact either early on in carcinoma development or later during dissemination and metastasis (Fung et al, 2008; Debnath, 2008).

A study by Abedin et al (2007) showed in human breast adenocarcinoma MCF7 cells that, upon DNA damage, autophagy significantly delayed the apoptotic response by

the cell resulting in extended cell viability. This delay occurred even in the presence of camptothecin (CPT), a drug that binds topoisomerase I, resulting in DNA damage and the upregulation of p53 expression, normally leading to apoptosis of the cell. When the researchers down-regulated autophagy-related proteins (Beclin-1 and Atg7) they unmasked a caspase-dependant apoptotic response to DNA damage, which eventually led to cell death (Abedin et al, 2007). Therefore it was concluded that in the presence of DNA damage induced by CPT, the subsequent increase in autophagy led to an increased cell survival and delayed cell-death (Abedin et al, 2007).

1.3.3 Induction of autophagy by tamoxifen

Autophagy also appears to have a pro-survival role in the cellular response to the endocrine therapy agent, tamoxifen, possibly implicating the pathway in the high incidence of tamoxifen resistance in breast cancer patients. Approximately 30-50% of women treated with anti-estrogen therapy (a type of endocrine therapy for estrogen-positive breast cancers) do not initially respond or their breast cancer cells ultimately acquire resistance during treatment (Clark et al, 2001); therefore it is crucial that we understand how resistance occurs in breast cancer patients. In 1996, Bursch et al. showed that high-dose cytotoxic levels of tamoxifen (10^{-6} M) resulted in an increase in autophagy, which they proposed aided MCF-7 cell death. A more recent functional study by Qadir et al. (2008) employed small interfering RNAs (siRNAs) to knock down three different autophagy-related proteins, Atg5, Atg7, and Beclin-1 in three different estrogen receptor positive (ER+) breast cancer cell-lines (MCF-7, T47D, and MCF7-HER2) in the presence of tamoxifen (2.5-5.0 uM) treatment. This study showed that autophagy knockdown using Atg7 or Beclin-1 siRNAs resulted in enhanced mitochondrial depolarization and reduced cell viability even in breast cancer cells with reduced sensitivity (T47D) or resistance (MCF7-HER2) to tamoxifen. Thus, cellular sensitization to tamoxifen can be enhanced when autophagy gene function is knocked down, perhaps due to increased cytotoxic effects of tamoxifen that lead to increased apoptosis and significantly reduced cell viability. Similar findings were made by Samaddar et al. (2008) who demonstrated that 4-hydroxytamoxifen (1-5 uM) induces autophagy in ER+ breast cancer cells that do not die. They further showed that autophagy facilitates the

development of anti-estrogen resistance, allowing the cells to survive in culture despite the presence of toxic drug concentrations (Samaddar et al, 2008; Schoenlein et al, 2009). Reduction of autophagy by 3-MA treatment or Beclin-1 RNAi in combination with 4-hydroxytamoxifen resulted in increased cell death, indicated by increased cleavage of caspase-9 and the caspase-6 substrate Lamin A (Samaddar et al, 2008). While further studies are required to uncover the molecular pathways involved, these results suggest that autophagy may represent a general mechanism responsible for avoiding or delaying tamoxifen-induced apoptosis and that autophagy knockdown may be useful in a combination therapy setting to sensitize breast cancer cells to high dose tamoxifen therapy that is used in the treatment of late stage or recurrent breast cancer.

The induction of autophagy by irradiation, a common treatment modality for breast cancer, has also been demonstrated. An early report by Paglin et al. (2001) described the accumulation of acidic vesicular organelles following radiation treatment in MCF7 cells and associated these with a protective autophagy response. In a more recent study, Apel et al. (2008) showed that breast cancer cells (MDA-MB-231) can be re-sensitized to radiation by autophagy inhibition. They hypothesized that autophagy protects the cells against radiation damage by providing catabolites required for repair processes, and possibly by physically containing the toxic molecules, thereby preventing cytoplasmic acidification (Apel et al, 2008). Their proposed model was that in situations where autophagy was inhibited, the increased need for catabolite supplies for enhanced DNA repair in radio-resistant cells could not be fulfilled, resulting in induction of the apoptotic pathway and eventually cell death (Apel et al, 2008). Their results supported this model, in that cancer cell-lines that had previously been resistant to radiation treatment became re-sensitized following inhibition of the autophagic pathway (Apel et al, 2008). The increased number of recent findings designating the autophagic pathway as a mechanism that can delay activation of apoptosis in breast cancer cells (Abedin et al, 2007; Apel et al, 2008, Qadir et al, 2008, Samaddar et al, 2008) have strengthened the notion that autophagy has a primary tumour survival role in established breast cancer cells.

1.3.4 Autophagy and its potential role in drug resistance

Resistance to therapeutic drugs is a negative outcome in chemotherapy and a major issue in the clinical treatment of breast cancers. There are several therapeutic drugs that have been shown to alter the levels of autophagy in breast cancer cells – at least in cell-lines (Table 1.2). A more thorough list of clinically relevant drugs demonstrated to modulate autophagy in a variety of other cancer cell types can be found in a recent review by Høyer-Hansen and Jäättelä, 2008, and includes treatments such as HDAC inhibitors, angiogenesis inhibitors, Imatinib, HIV protease inhibitors, and Resveratrol among others. Since these drugs affect multiple cellular processes (eg. autophagy, cell division), the extent that autophagy modulation plays a role in their therapeutic effects remains to be determined. Many of the drugs listed in Table 1.2 and in Høyer-Hansen and Jäättelä (2008), like tamoxifen and rapamycin, were found to promote autophagy. Thus, recent data implicating the induction of autophagy in response to certain chemotherapy drugs warrants further investigation in relation to chemoresistance. This is true especially since much of the incriminating data come from breast cancer cell-lines only, or limited work with mouse models, both of which have their own shortcomings and can be difficult to apply directly to human patients (Wagner, 2004; Jessani et al, 2005). If these results are in fact applicable to humans, then inhibiting the autophagy pathway in breast cancer cells in combination with autophagy-inducing treatments may actually increase the cytotoxicity of these therapies, thereby increasing cellular apoptosis and reducing the emergence of therapy resistance. However, as described above, in apoptosis-defective iBMK cells, autophagy inhibition by AKT activation or Beclin-1 knockdown resulted in cell death by necrosis that was associated with inflammation and accelerated tumor growth (Degenhardt et al, 2006). Thus, the apoptotic competence of a cell and likely other factors may have an important impact on potential autophagy-related therapeutic strategies.

Table 1.2 - Treatments shown to have autophagy-modulating effects in breast cancer cells.

Agent	Target	Step of autophagy affected	Effect on autophagosomes & autolysosomes (& methods)	Effect on Autophagic Flux	References
Tamoxifen	Estrogen Receptor; Other	Induction	Increase in autophagosomes (punctate GFP-LC3)	Induce*	Bursch et al, 1996; Qadir et al, 2008; Samaddar et al, 2008
Radiation	DNA	Induction	Increase in autophagosome and autolysosome formation (EM, GFP-LC3, acridine orange and LAMP-1)	ND	Paglin et al, 2001; Apel et al, 2008
Camptothecin	Topoisomerase I; leads to DNA damage	Induction	Increase in autophagosomes (punctate GFP-LC3) & increase in association of mitochondria with autophagic vesicles	ND	Abedin et al, 2007
mTOR inhibitors (eg. Rapamycin)	mTOR	Induction	Increase in autophagosome and autolysosome formation (EM, punctate DsRed1-LC3 & GFP-LC3, MDC, Lamp-1)	Induce	Noda et al, 1998; Hoyer-Hansen et al, 2005; Kim et al, 2006, Abedin et al, 2007
EB1089	Ca2+ mobilization; leads to AMPK activation and mTor inhibition	Induction	Increase in autophagosome and autolysosome formation (EM, punctate DsRed1-LC3, GFP-LC3, MDC)	Induce	Høyer-Hansen et al, 2005; Demasters et al, 2006
Chloroquine	Lysosomal pH	Fusion/ Degradation	Increase in number of autophagosomes (EM and punctate GFP-LC3)	Inhibit*	Amaravadi et al, 2007 (lymphoma model)
Microtubule-targeting agents e.g. vincristine	Tubulin	Fusion	Increase in number of autophagosomes (punctate GFP-LC3); Decrease in fusion of autophagosomes with	Inhibit	Groth-Pedersen et al, 2007

Agent	Target	Step of autophagy affected	Effect on autophagosomes & autolysosomes (& methods)	Effect on Autophagic Flux	References
			lysosomes (lack of GFP-LC3 and Lamp2 colocalization)		

***** Gorski laboratory; effect demonstrated in breast cancer cell lines using western blot-based LC3 flux assay (unpublished). ND = Not Demonstrated

1.3.5 Reconciling dual roles

The compelling experimental data supporting autophagy as both a tumour suppressor and tumour survival mechanism give rise to the idea that the role of autophagy changes depending on the tumour type, and more importantly, the stage of development. Under normal conditions, autophagy would act as a tumour suppressor and, through its homeostatic housekeeping role, prevent an accumulation of potentially harmful agents. If autophagy was not working properly or efficiently, such as in the monoallelic deletion of *beclin-1*, deficient components would not be properly broken down and could potentially damage the cell or cause genetic instability, leading to development of cancer (Mathew et al, 2007; Karantza-Wadsworth et al, 2007; Liang et al, 1999). Therapeutically up-regulating missing/faulty components in the pathway of autophagy defective cells would therefore likely decrease the incidence of tumour formation. However, most gene/protein abnormalities associated with autophagy are discovered after a tumour has been initiated. According to this dual-function model, now that the cell has become cancerous, autophagy may instead play a role in tumor promotion or tumor survival. At this point, cancerous cells can amplify the autophagic pathway, taking advantage of its cell-survival properties and preventing apoptosis. By breaking down proteins and organelles in the cytoplasm to produce additional energy, the cell is able to flourish in its surroundings despite little to no additional nutrients or blood supply (Noda and Ohsumi, 1998). At this stage, therapeutically targeting components in the autophagy pathway might act to decrease the ability of the cancer cells to survive. As described above, combining this type of autophagy inhibition with cytotoxic autophagy-inducing therapeutics such as

tamoxifen may help increase the sensitivity of the cells to the toxic treatment and decrease the likelihood of resistance. In both contexts, tumor suppressor vs. tumor survivorship, autophagy is conferring a cyto-protective function: at pre-cancerous stages, this helps to prevent cancer initiation, but in already-established cancer cells or tumours, this helps to promote cancer survival. However, there still remains the third possibility – that autophagy may also act to kill breast cancer cells in a therapeutic context in established cancers. Again it should be noted that most findings to date resulted from *in vitro* cell culture studies and applying these conclusions to mouse models and then to human patients is currently speculative at best.

1.4 MicroRNAs

The differential expression of microRNAs (miRNAs) is another phenomenon seen in cells undergoing stress conditions (Babar et al, 2008; Marsit et al, 2006; Kulshreshtha et al, 2007). MiRNAs are 19-25 nucleotides in length and are the small non-coding portions of RNA that function by binding to corresponding target messenger RNA (mRNA) molecules. MiRNAs modulate gene expression via the RNAi interference pathway, resulting either in target degradation or repression, depending on the degree of complementary between the miRNA sequence and mRNA target site (Alvarez and Miska, 2005). Less than 2% of the human genome is comprised of known and predicted protein coding transcripts; the abundance of non-protein coding transcripts, like miRNAs, are progressively seen as having important regulatory roles in normal cellular functions and can be dysregulated in disease (Pang et al, 2007). Although specific functions and mRNA targets of miRNAs have only been assigned to a few dozen miRNAs, much experimental evidence suggests that these non-coding RNAs participate in the regulation of an immense spectrum of biological processes.

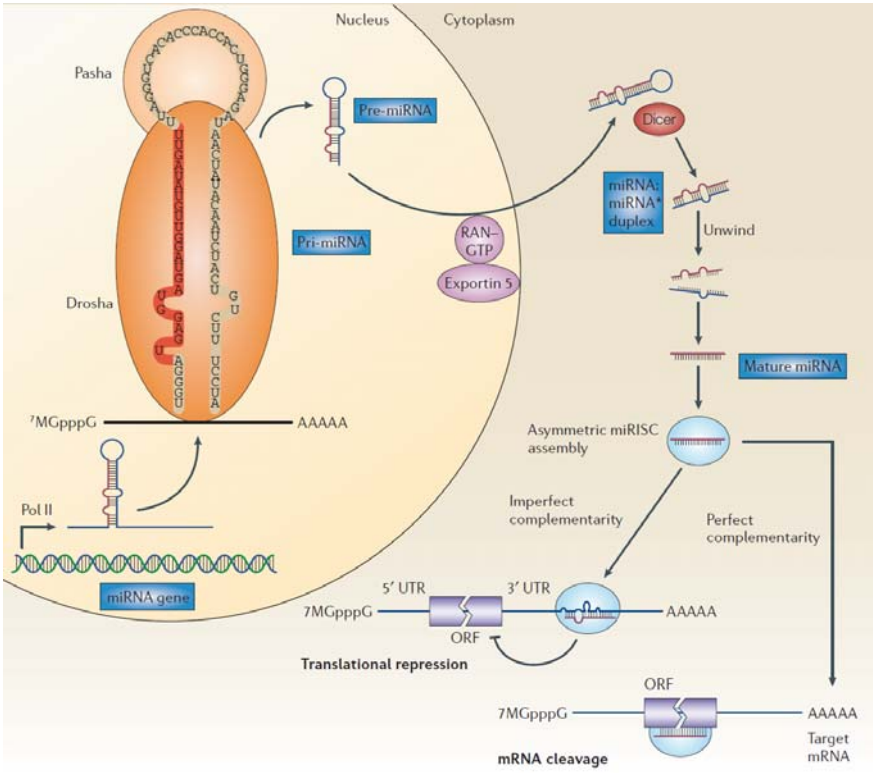
MiRNAs and their targets involve complex regulatory networks since a single miRNA can potentially bind to and regulate many different messenger RNA targets (Lim et al, 2005). Conversely, several different miRNAs can bind to and cooperatively control a single mRNA target (Lewis, 2003; Lee, 2004). A mature miRNA contains a ‘seed

region'; an area on the molecule that binds to its mRNA target, which often includes nucleotides 2 - 7 in the 5' end. The seed region primarily defines the specificity of the miRNA toward the 3' untranslated-region (UTR) of the target mRNA. Most miRNAs bind with imperfect complementarity to the 3'UTR of the corresponding mRNAs, which often results in the translational repression of that messenger RNA (Meister and Tuschl, 2004). However in rare occurrences, the miRNA binds with perfect complementary to the target sequence on the mRNA, resulting in the degradation of the messenger RNA. Since most miRNAs bind with imperfect complementary to their mRNA counterparts, each miRNA can have a few hundred predicted mRNA targets, however only a small amount of these interactions have been experimentally validated (Lewis et al, 2005). There are thought to be anywhere between 500 and 1000 different miRNAs in humans, although some researchers believe that number should be much higher (Bentwich, 2005), which target at least half of all messenger RNA targets.

1.5 Synthesis of microRNAs

MiRNA genes can be found throughout the genome, either individually or as part of gene clusters. The majority of miRNA genes are located in intergenic regions or in antisense orientation to annotated genes, and therefore contain their own promoter and form independent transcription units (Lagos-Quintana et al, 2001; Mourelatos et al, 2002; Lee et al, 2002). The other miRNA genes are mainly found in intronic regions and may be transcribed as part of the annotated gene within which they reside (Lee et al, 2002). MiRNA genes are transcribed in the nucleus via RNA Polymerase II, either from their own promoter or from the promoter of the gene in which they reside, forming long primary miRNAs (pri-miRNAs) that contain a 5' CAP structure and a polyadenylated 3' end and may contain one or more miRNAs (Denli et al, 2004; Gregory et al, 2004).

Figure 1.3 - MiRNA synthesis and method of transcriptional regulation



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The pri-miRNAs fold into a stem-loop structure, from which mature miRNAs are generated via two sequential processing steps (Figure 1.3). This stem-loop or hairpin structure is first recognized and cleaved by Drosha, a nuclear RNase III endonuclease, in conjunction with the double-stranded RNA binding protein, the Di-George syndrome critical region gene 8 (DGCR8) (Han et al, 2004).

The cleavage results in the formation of a 60-100nt RNA hairpin intermediate known as a nucleotide precursor miRNA (pre-miRNA) that contains a two nucleotide 3' overhang. The pre-miRNA is then transported to the cytoplasm via the nuclear export factor, Exportin-5 along with its cofactor, Ran-GTP (Lund et al, 2004; Yi et al, 2003). In the cytoplasm, the pre-miRNA encounters a second RNase III endonuclease known as Dicer which, along with its double-stranded RNA binding partner, transactivating response RNA binding protein (TRBP), cleaves the pre-miRNA, forming a double-

stranded RNA. Next Dicer and TRBP recruit hAgo2 (human argonaute protein), to form a RNA-induced silencing complex (RISC). As only one of the two miRNA strands is able to guide the RISC complex to the 3' UTR of the target mRNA, the alternate RNA strand (the passenger strand) is discarded from the complex and usually degraded (Matranga et al, 2005; Winter et al, 2009). The mature miRNA strand then directs the RISC to target mRNAs potentially leading to translational repression or direct degradation, depending on the level of complementarity. The binding of a single miRNA at a single site on the 3' UTR of a mRNA is often not sufficient to block translation; instead several miRNAs, either identical or different, must bind for translational repression to occur (Bartel, 2004).

The mechanism by which miRNAs translationally repress their mRNA targets is still not well understood. The available data suggest that there are a number of processes that could account for the repression. It has been shown that most miRNA-repressed mRNAs undergo deadenylation and consequent destabilization (Wu et al, 2006); however Wu et al also showed that miRNAs lacking a poly-A tail are still subject to translational repression without miRNA destabilization, and thus deadenylation events appear to occur in addition to translational repression. Recent data indicate that miRNA-mRNA pairs are concealed from the translational machinery in distinct cytoplasmic sites known as processing bodies (P-bodies). P-bodies are cytoplasmic foci that function as storage sites for non-translating mRNAs and proteins involved in both mRNA decay and translational repression (Chan and Slack, 2006). Messenger RNAs can accumulate at these locations and are destined for storage or decay. The argonaute family proteins, namely Ago2 can be detected in P-bodies in a miRNA-dependant manner (Sen and Blau, 2005). Relocation of the mRNA to the P-bodies inhibits translation due to the lack of translational machinery and since mRNA degradation enzymes also reside in the P-bodies, this relocation may account for the reported decrease in mRNA levels (Pillai, 2005). Interestingly, in a 2006 study, Bhattacharyya et al. found that miRNA repression of CAT-1, a high-affinity amino-acid transporter, can be relieved under different types of cellular stress conditions such as cell starvation. This derepression required the binding of an AU-rich-element binding protein, HuR, to the 3' UTR of the mRNA (Bhattacharyya et al, 2006). They therefore proposed that proteins interacting with the 3'UTR of mRNA

molecules can act as modifiers, altering the potential of some miRNAs to repress gene expression, though there has been no further direct evidence of this to date.

1.6 MicroRNA involvement in cellular stress and cancer

In 1962, the first genes involved in the cellular response to stress were discovered and termed 'heat shock proteins' (Ritossa, 1962; Hirsch, 2006). Since then, studies have exposed entire networks of proteins that are induced under cellular stress conditions including hypoxia, starvation, heat, UV irradiation and oxidizing agents (Hirsch et al, 2006; Kregel, 2002). Regardless of the stress involved, the cell's general defense mechanism includes sensors that recognize the perturbation, transducers that amplify the signal, and effectors that alter the cell to counteract the stress (Babar et al, 2008; Kultz, 2005). MiRNAs were first recognized as having a role in the response of plant cells to stressors such as dehydration, salt stress, UV-B radiation, phosphate deficiency, as well as oxidative and mechanical stress (Fujii, 2005; Chiou, 2007; Jung et al, 2007). A number of studies have recently emerged in mammals, showing miRNA expression profiles are altered in response to a number of cell stressor stimuli including folate and arsenic exposure (Marsit et al, 2006), drug treatments (Pogribny et al, 2007), hypoxia (Kulshreshtha, 2007), cardiac stress (van Rooij, 2006) and radiation exposure (Weidhaas et al, 2007). Most of these studies described global changes of miRNA levels as a response to cellular stress, however some found crucial roles for specific miRNAs in modulating the stress response under both *in vitro* and *in vivo* conditions (Babar et al, 2008).

MiRNAs are well suited for a role in cellular stress for several reasons:

1- Since they are post-transcriptional gene regulators, they may be able to function as 'quick responders' to cellular perturbations (Babar et al, 2008)
2- Since miRNAs regulate numerous targets, they have the capacity to efficiently coordinate a stress response involving numerous genes.

3- miRNAs may be less susceptible to certain types of stress due to their small size and high stability (Babar et al, 2008), and are therefore less likely to be compromised in a potentially toxic environment.

Given that cellular stressors such as hypoxia and starvation are prevalent in many cancerous tumour microenvironments, miRNA regulation of stress may play an important role in tumour proliferation and/or survival. In fact, a number of miRNA profiling studies have shown that many miRNAs are abnormally expressed in clinical cancer samples. The first link found between miRNA levels and cancer was discovered by Calin et al, who found that the miR15a/16-1 cluster was deleted in the majority of chronic lympocytic leukemia (CLL) cases and that more than half of known human miRNAs reside in particular genomic regions that are prone to alteration in cancer cells (Calin et al, 2002; Calin et al, 2004). Another study by Zhang et al, reported that a significant percentage of miRNA genes show loss or gain of copy number in several different types of cancer (Zhang et al, 2006).

Aberrant gene expression in many different cancers has now been associated with abnormal levels of expression for mature and/or precursor miRNA sequences compared with the corresponding normal tissues. Differential miRNA expression has been implicated in a number of different cancers including breast, lung, ovarian, colorectal, and cervical cancer and miRNAs have been proposed to contribute to oncogenesis (Iorio et al, 2005; Michael et al, 2003; Takamizawa et al, 2004; Esquela-Kerscher and Slack, 2006). It should be noted that not all differentially expressed miRNAs in cancerous cells are considered directly involved in cancer progression and/or tumourigenesis, as many changes occur in these cells that in a direct or indirect manner may influence miRNA expression (Calin and Croce, 2006). Nevertheless, researchers have already found several specific miRNAs that may function directly as either tumour suppressors or oncogenes (termed oncomirs) (Esquela-Kerscher and Slack, 2006). MiRNAs that acted as tumour suppressors would be expected to have a decreased expression in cancerous cells as compared to normal cells. For example, a report by Cimmano et al. showed that miR-15a and miR-16-1 negatively regulate the anti-apoptotic oncogene BCL-2 (Cimmino et al,

2005). It has also been shown that these two miRNAs have decreased expression in leukemia which may cause the increased expression of BCL-2 (Calin et al, 2005). MiRNAs can also potentially act as oncogenes, and in this role, would most likely be up-regulated in cancerous cells. Over the past few years, the role of miRNAs in disease has gathered much attention and their potential as oncomirs/tumor suppressors are currently being investigated.

In summary, miRNAs belong to a relatively new, exciting area of research. They have been implicated in numerous biological processes including apoptosis, cell division, developmental timing, aging, neuronal patterning, metabolism and, as detailed above, cancer (Cimmino et al, 2005; Calin et al, 2002; Wang et al, 2008; Mersey et al, 2005) . The miRNA genes are encoded in regions once considered 'junk' DNA, such as introns of coding and non-coding genes. This field is relatively new and there is still much information to be discovered regarding miRNAs, their targets and their potential role in cellular processes. It is this potential that drew my interest and led me to investigate whether miRNAs may play a regulatory role in the cellular mechanism known as autophagy.

1.7 Rationale, hypothesis and aims

The mechanisms by which cellular stresses regulate autophagy are not well understood. Recent studies have emerged demonstrating the differential expression of many different miRNAs in response to cellular stresses that are also known to induce autophagy; namely nutrient deprivation, hypoxia, and radiation (Kulshreshtha et al, 2007; Pogribny et al, 2007; Ishii et al, 2006; Zhu et al, 2009). In 2009, a study by Zhu et al implicated a specific miRNA (miR-30a) as a direct regulator of *beclin-1*, a core gene in the autophagy pathway. *Beclin-1* is involved in autophagosome formation during autophagy induction and miR-30a was shown to demonstrate decreased levels of expression when autophagy was induced. The Zhu study identified miR-30a as having a direct suppression effect on *beclin-1* in a number of different cancer cell lines including

glioblastoma (T98G), lung cancer (H1299), and breast cancer (MDA-MB-468) (Zhu et al, 2009).

My hypothesis is that miRNAs that regulate autophagy will demonstrate differential expression in response to cell stresses that induce autophagy. I decided to focus my study on one tissue type since miRNA expression is thought to be tissue specific (Lagos-Quintana et al, 2002), and examine a number of different breast cancer cell lines for candidate miRNA regulators of autophagy. As previously mentioned, autophagy may be involved in both tumour survival and chemotherapy resistance in some cancers. Linking miRNA expression to the induction of autophagy in cancerous cells undergoing cellular stress may illuminate an alternate pathway to modulate autophagy levels in tumours.

Two Aims were developed in order to test this hypothesis:

Aim 1 – Identify candidate miRNAs that are differentially expressed during autophagy-inducing conditions

In the first part of this aim the breast cancer cell-line BT-474 was exposed to high-dose levels of tamoxifen in order to induce autophagy. Tamoxifen concentration and length of exposure was optimized by observing the differential levels of both core autophagy gene transcripts and candidate miRNAs (including miR-30a) via qRT-PCR in the treated sample and an untreated control sample. Once the optimal treatment condition was found, RNA from each sample (treated and control) was isolated and sent to the Genome Sciences Centre's Library Construction and Sequencing core for miRNA library preparation and sequencing. The miRNA sequencing plus subsequent analyses identified the different miRNAs expressed within each sample and the abundance of each, allowing me to determine the specific miRNAs exhibiting significant differences ($p<0.05$, >1.5 fold change) in abundance upon autophagy induction.

Aim 2 – Validate the differential expression of select miRNAs and identify those that could potentially regulate the autophagy pathway

Following the Illumina sequencing and bioinformatic analysis, I validated the observed differential expression of 20 selected miRNAs by qRT-PCR analysis, utilizing an aliquot of the same RNA sample that was used for sequencing. Sequences of all differentially expressed miRNAs were entered into the online database, TargetScan, to identify genes that are candidate targets for each miRNA. The resulting gene lists were searched for autophagy-related genes and their corresponding miRNAs. Seven potential autophagy-targeting miRNAs were selected for further analysis based on their significance of differential expression (low p-value) and ability to target more than one autophagy gene. QRT-PCR was again be employed to discover whether the selected miRNAs were also differentially expressed in two other breast cancer cell-lines exposed to two autophagy-inducers, tamoxifen and starvation. The miRNAs that were differentially expressed in all three cell-lines under both autophagy-inducing conditions were considered good potential candidates for regulating autophagy in breast cancer cells under conditions of stress.

2 MATERIALS AND METHODS

2.1 Tissue culture, cell lines and reagents

The three different breast cancer cell-lines used in this study, BT-474 (ER +, HER-2 over-expressing), MDA-MB-361 (ER+, HER-2 over-expressing), and SKBR3 (ER-, HER-2 over-expressing), were obtained from the M. Bally laboratory (BCCRC) but originated from ATCC. Each cell line was grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 100 units/ml penicillin, 100ug/ml streptomycin, 10mM HEPES, 10ug/ml insulin, 1x non-essential amino acids (all from Invitrogen Canada, Burlington ON, Canada) and 10% heat-inactivated fetal bovine serum (HI-FBS) (Sigma-Aldrich, Munich, Germany). This modified medium with 10% HI-FBS was referred to as PINK.

2.1.1 Tamoxifen treatment

Prior to tamoxifen treatment, cell-lines were passaged once in phenol-red free media (DMEM #31053-28) supplemented with 2mM L-Glutamine, 1mM sodium pyruvate and the ingredients specified in PINK (referred to as F-10). Cells were then plated at different densities: 32,500 cells/cm^2 (MDA-MB-361), 25,000 cells/cm^2 (SKBR3) and 10,000 cell/cm^2 (BT-474), in a 6-well (9.6cm^2 area per well) plate containing 2ml of OPTI-MEM (Invitrogen Canada) with 4% charcoal-stripped FBS (DCC-FBS). After 48 hours of incubation, 0.5ml of OPTI-MEM containing tamoxifen was added to the 2ml of OPTI in 6-well plates containing the cells. Tamoxifen Citrate (TAM) was purchased from Sigma (product #T9262) and diluted to 1000uM stock concentration in 100% Ethanol. The final concentrations of Tamoxifen in the 6-well plates were 0uM, 2.5uM and 5uM respectively. Cells remained in Tamoxifen treated wells for 24, 48, 72 and 96 hours before being extracted for further analysis. All concentrations and time periods were tested in triplicate wells for each experiment.

2.1.2 Nutrient starvation

Cell lines were passaged in F-10 and plated at densities as described above using OPTI-DCC. After 48 hours of incubation, media from the wells was extracted using aspiration and in those samples subjected to starvation, the media was replaced with Earl's Balanced Salt Solution (EBSS) with $NaHCO_3$ (Sigma-Aldrich, Munich, Germany). The media from the control wells was replaced with OPTI-DCC. The cells remained in the media for 2, 4 or 6 hour intervals, after which the cells were isolated for further analysis (below).

2.2 Cell lysis and RNA isolation

Media was extracted from the wells using an aspirator and then cells were washed twice using 2ml of 1x Phosphate Buffered Saline (PBS) pH 7.4 (Invitrogen, Canada). After each wash, media was again aspirated from the wells. 700ml of QIAzol Lysis Reagent (Qiagen, Canada) was then added to each well to lyse and detach cells from the well surface. The QIAzol solution containing the detached cells was then extracted from each well using a 1ml 28-guage syringe and deposited into a 1.5ml RNAse-free microfuge tube. To reduce cell clumping, each sample of cells was passed through the syringe a minimum of five times to disperse clumps. Cell lysates from experiments were collected and passed through a miRNEasy Mini Kit column (Qiagen, Canada) to isolate purified RNA as per manufacturer's protocol. Some cell lysates were stored at -80°C prior to RNA isolation. The RNA was quantified using a ND8000 Nanodrop Spectrophotometer.

2.3 QRT-PCR

2.3.1 Analysis of autophagy transcript levels

First-strand synthesis of cDNA from isolated RNA samples was carried out using Invitrogen's Superscript III Reverse Transcriptase and Oligo dTTP. As per manufacturer's recommendations, 250ng of total RNA was used for cDNA synthesis.

Real-Time Quantitative PCR was carried out using Invitrogen's Platinum SYBR Green PCR Master Mix. Forward and reverse primers for specific core autophagy genes (MAP1LC3B, Beclin-1, Atg5, Atg7, Ulk-1 and Ulk-2) were used at a final concentration of 10uM (Table 2.1). The house-keeping gene β-Actin was used as a reference gene to calculate transcription expression values, also at a concentration of 10uM (Table 2.2). Samples were run in triplicate on an ABI 7900 HT-Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The data generated were analyzed using Applied Biosystem's SDS Software (version 2.2). Using the comparative Ct method (or ΔΔCT method) the relative amount of target material was quantified compared to the β-Actin reference gene (Kubista et al, 2006). CT values generated for each autophagy gene under treated conditions were normalized to the untreated control sample and the values were plotted on a bar graph. The data shown in the results section with respect to the qRT-PCR analysis is the average of triplicate samples from 3 independent experiments.

Table 2.1 – Autophagy gene forward and reverse primer sequences

Autophagy Genes	**Direction of primer**	**Primer sequences (5' – 3')**
MAP1LC3B	F	GAACGATACAAGGGTGAGAAGC
	R	AGAAGGCCTGATTAGCATTGAG
Beclin-1	F	GGAGAGGAGCCATTTATTGAAA
	R	AGAGTGAAGCTGTTGGCACTTT
Atg5	F	CGGCGGCAAGAAATAATG
	R	CCCAACATCCAAGGCACTAC
Atg7	F	TGATCCTGAAGATGGGGAAA
	R	TCCGGGTAGCTCAGATGTTC
Ulk-1	F	CGTTGCAGTACTCCATAACCAG
	R	GGGGAAGGAAATCAAAATCC
Ulk-2	F	TAATCTGCGAGGTCTCCACC
	R	TCACAAATACTGCTTGGAAAGG

Table 2.2 – QRT-PCR Reference Primer Sequence

Reference Primer	Direction of primer	Primer sequences (5' – 3')
ACTB	F	CTGGAACGGTGAAGGTGACA
	R	AAGGGACTTGTAACAATGCA

2.3.2 Analysis of miRNA levels

Due to the short length of miRNAs, the isolated RNA from our samples was first elongated with *E. coli* poly A polymerase to generate a poly-A tail at the 3' end of each RNA molecule using the High-Specificity miRNA 1st strand cDNA synthesis kit (Stratagene) as per the manufacturer's protocol. Following polyadenylation, the RNA was used as a template for 1st-strand cDNA synthesis via reverse transcription. The High-Specificity miRNA 1st strand cDNA synthesis kit (Stratagene) was again used for this procedure and the steps taken were as detailed by the manufacturer's protocol. As per the manufacturer's recommendations, 250ng of total RNA was used for cDNA synthesis. Real-Time Quantitative PCR was carried out using Stratagene's miRNA QPCR Master Mix with SYBR green Dye. A universal miRNA reverse primer from the High-Specificity miRNA 1st strand cDNA synthesis kit (Stratagene) was used to bind to a 5' tag added to RNA during cDNA synthesis. Forward primers specific to 23 known mature strand miRNA sequences were used in this study (Table 2.3) Forward primers for miRNAs were diluted to a final concentration of 3.125uM in 5uM TE (Tris + EDTA).

Table 2.3 – MiRNA mature strand primer sequences

miRNA	Primer Sequence (5' – 3')
hsa-miR-15b	TAGCAGCACATCATGGTTTACA
hsa-miR-18a	GGTAAGGTGCATCTAGTGCAGATAG
hsa-miR-19a	TGTGCAAATCTATGCAAAACTGA
hsa-miR-20a	CGGTAAAGTGCTTATAGTGCAGGTAG
hsa-miR-26b	CGCTTCAAGTAATTCAGGATAGGT

miRNA	Primer Sequence (5' – 3')
hsa-miR-30a	TGTAAACATCCTCGACTGGAAG
hsa-miR-30b	GGTGTAAACATCCTACACTCAGCT
hsa-miR-98	GACGCTGAGGTAGTAAGTTGTATTGTT
hsa-miR-99a	ACCCGTAGATCCGATCTTGTG
hsa-miR-101	CCGGTACAGTACTGTGATAACTGAA
hsa-miR-106b	CCTAAAGTGCTGACAGTGCAGAT
hsa-miR-122	TGGAGTGTGACAATGGTGTTT
hsa-miR-125b-1	TCCCTGAGACCCTAACTTGTGA
hsa-miR-126	TCGTACCGTGAGTAATAATGCG
hsa-miR-130b	AGTGCAATGATGAAAGGGCAT
hsa-miR-141	GCTAACACTGTCTGGTAAAGATGG
hsa-miR-192	GCTGACCTATGAATTGACAGCC
hsa-miR-211	CCTTTGTCATCCTTCGCCT
hsa-miR-224	GCAAGTCACTAGTGGTTCCGTT
hsa-miR-301a	GCAGTGCAATAGTATTGTCAAAGC
hsa-miR-339	CCTGTCCTCCAGGAGCTCA
hsa-miR-429	CGCTAATACTGTCTGGTAAAACCGT
hsa-miR-454	CCTAGTGCAATATTGCTTATAGGGT

The miRNAs hsa-let-7a and hsa-miR-16 were used as reference genes for normalization of expression levels (Table 2.4) (Davoren et al, 2008).

Table 2.4 – Reference miRNA primer sequences

Reference Primer	Sequence (5' – 3')
hsa-let-7a	TGAGGTAGTAGGTTGTATAGTT
hsa-miR-16	TAGCAGCACGTAAATATTGGCG

Samples were run in triplicate on an ABI 7900 HT-Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The data generated were analyzed using Applied Biosystem's SDS Software (version 2.2).

2.4 Illumina sequencing library

Total RNA was isolated and first underwent qRT-PCR analysis to confirm differential expression of Atg genes as described above. A 10ug aliquot of the same RNA samples was sent to the Genome Sciences Centre's Library Core and Illumina sequencing platform.

2.4.1 MiRNA library preparation

The protocol used by the GSC's Library Core to prepare miRNA sequencing libraries was as follows: 10ug of total RNA from each sample was size fractioned on a 10% TBE-Urea gel, and a 15-30nt fraction (typical miRNA size) was excised. The resulting RNA was then extracted from the gel slice and precipitated. The precipitate was washed, air-dried and re-suspended in DEPC water. A 20 nt 5' adapter was then ligated to the RNA overnight using T4 RNA ligase. The 5'adaptor-ligated RNA was then size fractioned on a 10% TBE-Urea gel. The 40-60 nt fraction was separated from excess adapters and unligated RNAs by once again excising ligation products of the desired size. 3' RNA adaptors were subsequently added to the precipitated RNA samples and the newly ligated RNA was again size fractioned (60-95 nt) on a 10%TBE-Urea gel. After separation and precipitation of the desired product, the RNA was converted to single-stranded cDNA using Superscript II reverse transcriptase (Invitrogen, Canada) and Illumina's small RNA RT-Primer kit, as per the manufacturer's instructions. The single-stranded cDNA was then PCR-amplified with Hotstart Phusion DNA Polymerase (BioRad) in 10-15 cycles using Illumina's small RNA primer set containing primer sequences homologous to the 5' and 3' adapters as well as being complementary to the oligonucleotides of the flow cell surface of the Illumina platform. The PCR products were then gel purified, precipitated, resuspended and quantified using an Agilent DNA 1000 chip (Agilent) and diluted to a 15nM stock. For further detail on the Illumina protocol, please refer to Morin et al, 2010.

2.4.2 Bridge amplification

Once converted to cDNA, the single-stranded DNA fragments were loaded onto a single flowcell lane, bound by their 5' and 3' adaptor molecules. Flowcell lanes are designed to present the DNA in a manner that facilitates access to enzymes while ensuring high stability of surface-bound template and low non-specific binding of fluorescently labeled nucleotides (www.invitrogen.com). Next, unlabeled nucleotides along with DNA Polymerase were added to initiate solid-phase bridge amplification. The enzyme incorporates nucleotides to build double-stranded bridges on the solid phase substrate. The double-strand bridges then underwent denaturation, leaving single-stranded templates anchored to the substrate (side-by-side identical copies with one adaptor anchoring it to the surface of the flowlane) and this process was repeated until several million dense clusters of double stranded DNA were generated in each channel of the flow cell (Morin et al, 2010; www.invitrogen.com).

2.4.3 Illumina sequencing

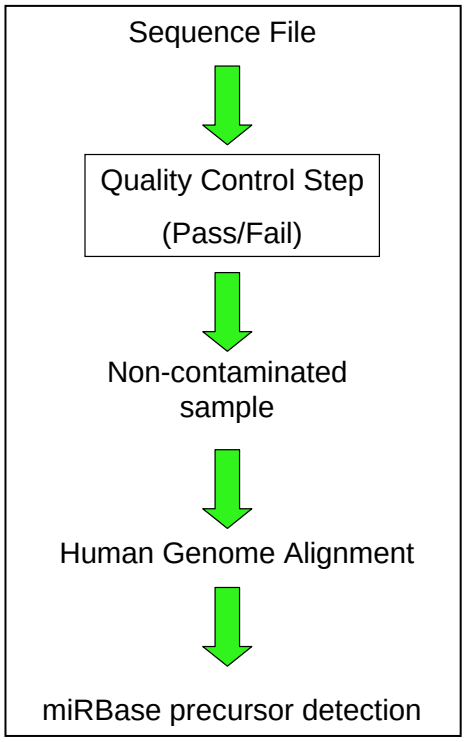
The Illumina platform uses four proprietary fluorescently-labeled modified nucleoside triphosphates along with primers and DNA polymerase to sequence the tens of millions of clusters present on the flow cell surface. The sequences undergo laser excitation that emits a fluorescent signal from each cluster which is captured after each nucleotide addition step and allows each base to be identified in order, and the flourophore is then chemically cleaved to allow further extension reactions. The sequencing cycles are repeated to determine all the bases in a particular fragment, one base at a time. An overlay of all images collected during this process is used to produce full-length sequence reads (www.invitrogen.com).

2.5 Analysis of sequencing data

A version of in-house software, mirna code, was used to analyze the millions of raw sequences generated by the Illumina platform (Morin et al, 2008). The large number

of sequences generated by the Illumina sequencing initially undergo a quality control step, where a small subset of the reads are checked for contamination from other genomes. A pass/fail system is employed and those samples are found to be contaminated, do not undergo further analysis (Figure 2.1) (Crieighton et al, 2009). After the quality control step, the remaining reads are matched to sequences within the human genome and can include miRNA precursor sequences, Introns, Coding Exons, etc. The in-house mirna code uses the known miRNA precursor sequences from the online database miRBase (version 13) (http://microrna.sanger.ac.uk/sequences/index.shtml), which currently contains over 700 recognized miRNA sequences (Griffiths-Jones, 2004; Griffiths-Jones et al, 2006; Griffiths-Jones et al, 2008), to match the small RNA reads to known miRNA sequences (Morin et al, 2008; Creighton et al, 2009).

Figure 2.1 –Method of analyzing sequencing data



2.5 Statistical analysis

2.5.1 Differential miRNA expression

After Illumina sequencing and library normalization, miRNAs that were found to be common between the two samples, as well as those that were present in one sample but not the other, were examined for differential expression. To be considered differentially expressed, the p-value had to be greater than 0.05 and there had to be a 1.5 fold difference between the abundance of the miRNAs. The p-value was calculated using Fisher's Exact Test, a statistical assessment used in the analysis of contingency tables (Fisher, 1922; Agresti, 1992). This test examined the miRNA abundance within each

sample, taking the total miRNAs derived into account. The absolute values were entered into the online Fisher's test resource (http://www.langsrud.com/fisher.htm) and the resulting p-values were recorded.

2.5.2 QRT-PCR comparisons

To determine whether there was a statistical significance between the autophagy gene or miRNA transcript levels during qRT-PCR analysis, a 2-tail t-test was used to compare the normalized expression values from 3 independent experiments. As described above, the CT values from the qRT-PCR for each autophagy gene transcript or miRNA were compared to an internal reference gene (actin and let-7a respectively) and then normalized to the transcript level of the untreated control sample for each particular autophagy transcript or miRNA. The normalized expression of each control sample was set at a value of 1.0 in order to compare fold differences between samples. The p-value was generated by using a 2-tail t-test, comparing normalized transcript levels from 3 independent experiments, and a difference of 0.05 or less was considered statistically significant in this analysis (as indicated in the Results section).

2.6 TargetScan

TargetScan, an online miRNA target prediction program, was used to generate potential gene targets for the differentially expressed miRNAs found via Illumina sequencing. The latest version of TargetScan (version 5.1) was used in this analysis and compared to previous releases, 5.1 considers site conservation, many more genomes, and uses more sensitive measures to detect conservation (Friedman et al, 2009).

3 RESULTS

3.1 Tamoxifen exposure increases autophagy transcript levels in BT-474 cells

A number of different cellular stressors have the ability to induce autophagy in different cell types. For this study, tamoxifen was selected as the initial cellular stress since high doses of tamoxifen induce autophagy consistently in many different breast cancer cell-lines regardless of estrogen receptor status (Bursch et al 1996; Bilir et al, 2001; Qadir et al, 2008; Sammadar et al, 2008; Lam et al, in preparation). The BT-474 breast cancer cell-line (ER+, HER2+), was chosen for this experiment based on the observation that it demonstrated a consistent and robust induction of autophagic flux following treatment with tamoxifen (Lam et al, in preparation).

It was shown previously that upon autophagy induction, the expression level of several autophagy genes increases compared to untreated controls (Klionsky et al, 2008; Zhu et al, 2009; Lomonaco et al, 2009; Lam et al, in preparation). The correlation between induction of autophagy and increased expression of autophagy genes has been reported at both the transcript level (via RT-PCR) and protein level (via western blot) (Klionsky et al, 2008). To determine whether autophagy gene transcript levels were upregulated in the BT-474 cell-line in response to tamoxifen and to optimize treatment conditions, quantitative reverse transcription polymerase chain-reaction (qRT-PCR) was employed to measure the level of mRNA expression of various core genes in the autophagy pathway. The autophagy genes selected for analysis included Atg5, Atg7, Beclin-1 and MAP1LC3B, all of which encode proteins that are active at early stages in the autophagic process in either the isolation membrane formation or autophagosome membrane elongation (Klionsky et al, 2003; Meijer et al, 2007). To optimize treatment conditions that result in maximal differential expression of Atg gene transcript levels, two different concentrations of tamoxifen, 2.5 and 5.0uM, were introduced to the BT-474 cells over four different time periods of exposure: 24, 48, 72 and 96 hours (Qadir et al, 2008). The levels of autophagy gene transcripts were measured at each tamoxifen concentration and period of exposure and compared to their respective untreated controls.

Similar to 3 other autophagy gene transcript levels explored in this analysis, the fold difference in transcript level of MAP1LC3B at 5.0uM of TAM after 24 and 72hrs was found to be significant compared to the control (p-value<0.05) (Figure 3.1).

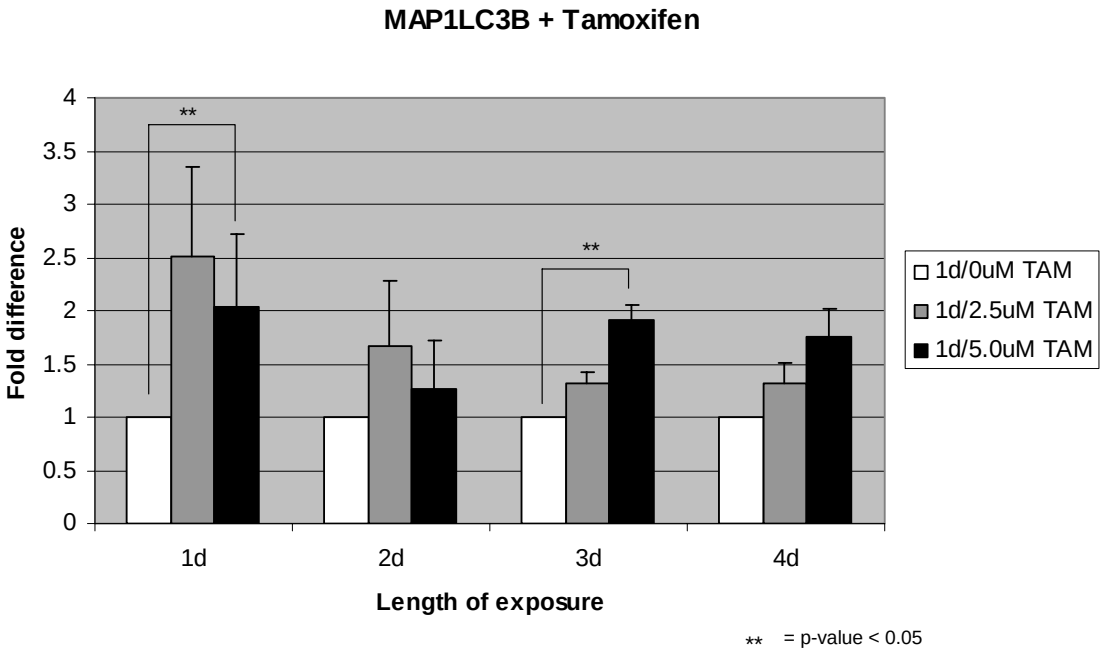


Figure 3.1 – MAP1LC3B transcript levels in BT-474 cells exposed to different TAM concentrations for various time periods. The bar graphs show the expression of MAP1LC3B transcript levels relative to the internal endogenous control actin(β) and normalized to the control sample of 0uM tamoxifen (TAM) for each time period. Length of exposure to 0, 2.5 and 5.0uM TAM is plotted on the x-axis (d=day) and transcription expression fold differences are plotted on the y-axis. Error bars represent standard error of 3 independent experiments.

Overall, the tamoxifen treatment that led to the most consistent significant increase (p-value <0.05) in all four autophagy gene transcript levels (Beclin-1, LC3, Atg5 and Atg7) was 24 hours of 5.0uM tamoxifen exposure (Figure 3.2). It was therefore decided that this condition was optimal for detecting autophagy gene transcript changes in the BT-474 breast cancer cell-line.

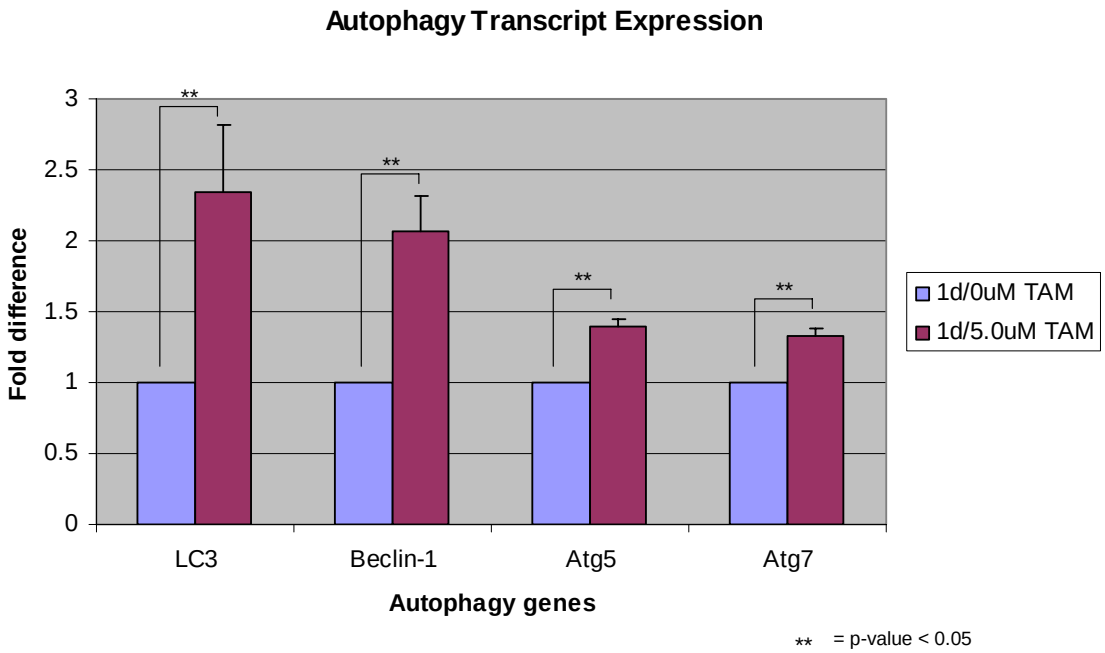


Figure 3.2 – Autophagy gene transcript levels after 24hr tamoxifen exposure. The bar graphs show the fold difference in expression of MAPLC3B, Beclin-1, Atg5 and Atg7 transcript levels in BT-474 cells at 5.0uM of TAM normalized to 0uM TAM control conditions after 24hrs of exposure. The transcript levels were calculated relative to the endogenous control β-actin. Error bars represent standard error of 3 independent experiments.

3.2 Tamoxifen exposure results in differential expression of miR-30a, miR-101 and miR-211 in BT-474 cells

To assess whether the autophagy-inducing drug tamoxifen was able to elicit differential expression of potential autophagy-regulating miRNAs, qRT-PCR was again employed to examine miRNA expression levels. Based on a previous study, miR-30a was chosen as a positive control for assessing miRNA differential expression under autophagy-inducing conditions (2.5 and 5.0uM TAM) as it has been experimentally shown to regulate *beclin-1*(Zhu et al, 2009). To determine whether autophagy-induction via tamoxifen correlates with the differential expression of miRNAs and at what concentration and duration of exposure, additional miRNAs were selected to undergo testing. Currently there are a number of miRNA prediction databases based on algorithms that can match specific genes to miRNAs that may potentially regulate them based on the 'seed region' of the miRNA and corresponding 3' UTR sequence of the mRNAs (Lewis et al, 2005; Friedman et al, 2009). The particular database used in this research project

was TargetScan (www.targetscan.org; Lewis et al, 2005; Friedman et al, 2009) and the four core human autophagy genes analyzed by qRT-PCR were entered into this program, along with several other known autophagy genes/regulators. Several different miRNAs were identified as potential regulators of the autophagy pathway (Table 3.1).

Table 3.1 – Candidate miRNA regulators of autophagy genes as determined by TargetScan

Autophagy gene	Role in Autophagy	Candidate miRNAs
MAP1LC3B (Atg8)	Autophagosome formation (marker for autophagosome membrane)	hsa-miR-211, hsa-miR-204
BECLIN-1 (Atg6)	Component of class III PI-3K complex that is required for autophagosome formation	hsa-miR-30(a-e)
ATG5	Part of the Atg12 conjugation system needed to facilitate autophagosome formation	hsa-miR-629, hsa-miR-30(a-e)
ULK-1	Involved in regulation and vesicle formation	hsa-miR-320 (a-d), hsa-miR-520 (a-e), hsa-miR-372, hsa-miR-373, hsa-miR-302(a-e)
ULK-2	Involved in regulation and vesicle formation	hsa-miR-9
Atg4B	Cleaves the C-terminus of Atg8 to expose a glycine residue for subsequent conjugation	hsa-miR-449(a,b), hsa-miR-34 (a, c-5p)
Atg4D	Cleaves the C-terminus of Atg8 to expose a glycine residue for subsequent conjugation	hsa-miR-101, hsa-miR-125
Atg12	Conjugated to Atg5 to facilitate autophagosome formation	hsa-miR-146 (a,b-5p)
mTOR	Negative regulator of autophagy induction	hsa-miR-101, hsa-miR-579, hsa-miR-1244, hsa-miR-144

As evident from Table 3.1, miR-30a is a potential regulator of *beclin-1*, which has been experimentally demonstrated (Zhu et al, 2009) and, interestingly, is also a potential regulator of Atg5. Two additional miRNAs were selected to undergo qRT-PCR analysis,

miR-101 and miR-211. Mir-101 was selected based on its paradoxical nature as a potential regulator of both *mTOR*, a negative regulator of the autophagy pathway, and *Atg4d*, a gene involved in autophagosome formation. The other miRNA, miR-211, was selected because it was identified as a potential regulator of MAP1LC3B (*Atg8)*, one of the core autophagy genes that showed increased transcript levels following tamoxifen exposure (Figure 3.2).

To investigate whether tamoxifen had an impact on the levels of the chosen miRNAs, qRT-PCR was used to measure their levels under the same treatment conditions described in section 3.1. Given that miRNAs are transcribed either separately or within genes, processed within the nucleus and then exported into the cytoplasm (Lee et al, 2002; Lund et al, 2004), their expression levels can be quantified via qRT-PCR (Krichevsky et al, 2003; Nelson et al, 2004). In order to identify the optimal concentration of tamoxifen and length of exposure for miRNA differential expression, both 2.5 and 5.0uM of tamoxifen were introduced to the cells for 24, 48, 72 and 96 hours. All three miRNAs demonstrated significant (p-value <0.05) decreased expression after 24 hours of exposure to 5uM of tamoxifen (Figure 3.3).

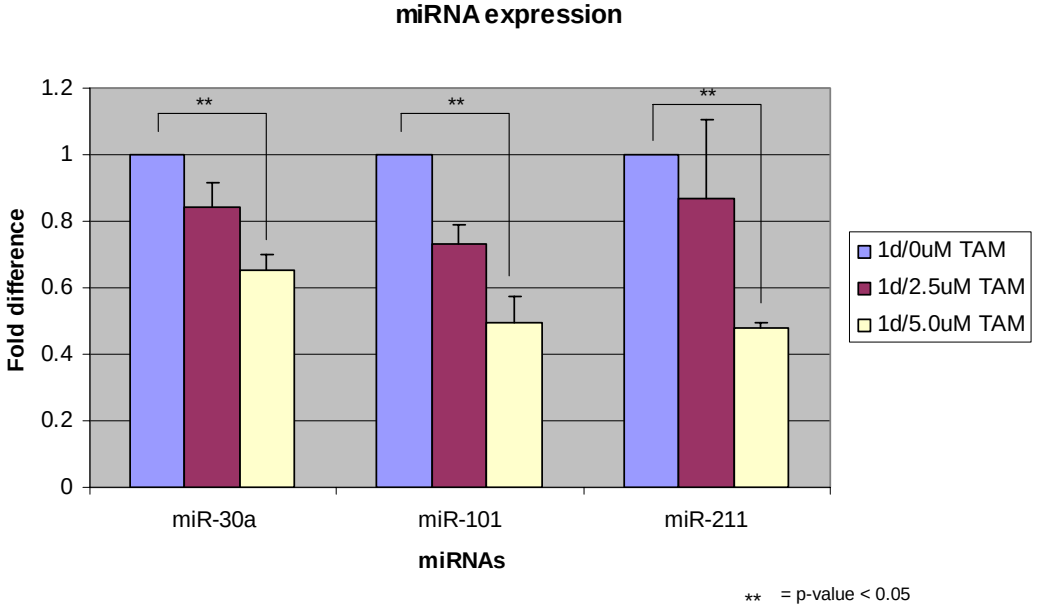


Figure 3.3 – MiRNA expression levels after 24 hours of exposure to 5.0uM tamoxifen. The bar graphs show the fold difference in expression of miR-30a, miR-101 and miR-211 at 2.5 and 5.0uM of TAM normalized to 0uM TAM control conditions after 24hrs of exposure. The transcript levels were calculated relative to the endogenous control let-7a. The three miRNAs are plotted on the x-axis and the fold difference is represented on the y-axis. Error bars represent standard error of 3 independent experiments.

Based on these findings and those described in section 3.1, it was decided that 24 hours of exposure to 5.0uM of tamoxifen was optimal for the induction of both autophagy transcript and miRNA changes. Two RNA samples processed in parallel were prepared from the BT-474 cells: one sample treated with 5.0uM tamoxifen to induce increased levels of autophagy, and one control sample that was not exposed to tamoxifen. These two samples were then sent to the Genome Sciences Centre's Library Construction and Sequencing Core for miRNA library preparation, sequencing and analysis.

3.3 Results of Illumina sequencing and miRNA analysis

Illumina's Genome Analyzer platform utilizes massive parallel sequencing of millions of fragments of genetic data using Clonal Single Molecule Array technology and reversible terminator-based sequencing chemistry (www.illumina.com). The two RNA samples were initially run on a gel to isolate the portion of RNA within the parameters of typical miRNA size (15-30nt); the resulting RNA was then labeled with 5' and 3' adaptors. These small RNAs were then purified, underwent RT-PCR to convert to cDNA, and enriched by 10-15 cycles of PCR before sequencing occurred (Morin et al, 2010). This library preparation step along with the Illumina sequencing strategy employed is detailed further in Material and Methods, section 2.4. To ultimately determine the identity and abundance of different miRNAs within each sample, the Illumina platform was used to sequence the cDNA fragments within the allotted size (miRNA + adaptors), resulting in the generation of millions of raw DNA sequence reads.

To identify the specific miRNAs that corresponded to the reads generated from Illumina sequencing of the two samples, mirna code, a GSC in-house analysis program was used which matched raw reads to the sequences of known miRNAs taken from a recent version of the miRBase (version 13), an online miRNA database that contains over 700 recognized miRNA sequences (Griffiths-Jones, 2004; Griffiths-Jones et al, 2006; Griffiths-Jones et al, 2008). Before matching occurred, however, mirna code analyzed the reads to ensure sequences were not contaminated, as detailed further in Materials and Methods section 2.4.

After the filtering analysis, it was determined that there were more than 6.4 million reads in both the control and treatment samples. Of those reads, 30% and 44% were able to align to known sequences in the human genome respectively (Table 3.2), while the remainder that did not match were most likely adapter-adapter dimers. In addition to matching the reads to known miRNAs, the sample sequences were also matched against known coding exons, introns, and UTR exons (Table 3.2)

Table 3.2 – Analysis of two BT-474 samples sequenced by the Illumina platform

	Control (0uM TAM)		Treated (5.0uM TAM)	
	Number	**%**	**Number**	**%**
Total Reads After Filtering	6,431,789		6,930,670	
Total Reads Aligned	1,952,970	30.36%	3,072,277	44.32%
Aligned reads corresponding to miRNAs	1,825,667	93.48%	2,893,670	94.18%
Aligned reads corresponding to Coding Exons	2,591	0.13%	3,318	0.10%
Aligned reads corresponding to Introns	30,959	1.58%	50,790	1.65%
Aligned reads corresponding to UTR Exons	22,792	1.16%	34,244	1.11%
Aligned reads corresponding to Unknown*	65,601	3.35%	90,309	2.93%

*Unknown refers to RNA that aligns to sequences of the human genome but the alignment coordinates don't match that of any annotated feature

Of the reads that were able to align to known sequences, the majority (>93%) in both the control and treated samples matched to known miRNAs from the miRBase catalogue. This high proportion of miRNA raw sequences is likely due to high quality in library preparation steps detailed previously. The remainder of the reads (<7%) aligned to other various genetic components as described in the table above and in the figure below (Table 3.2; Figure 3.4).

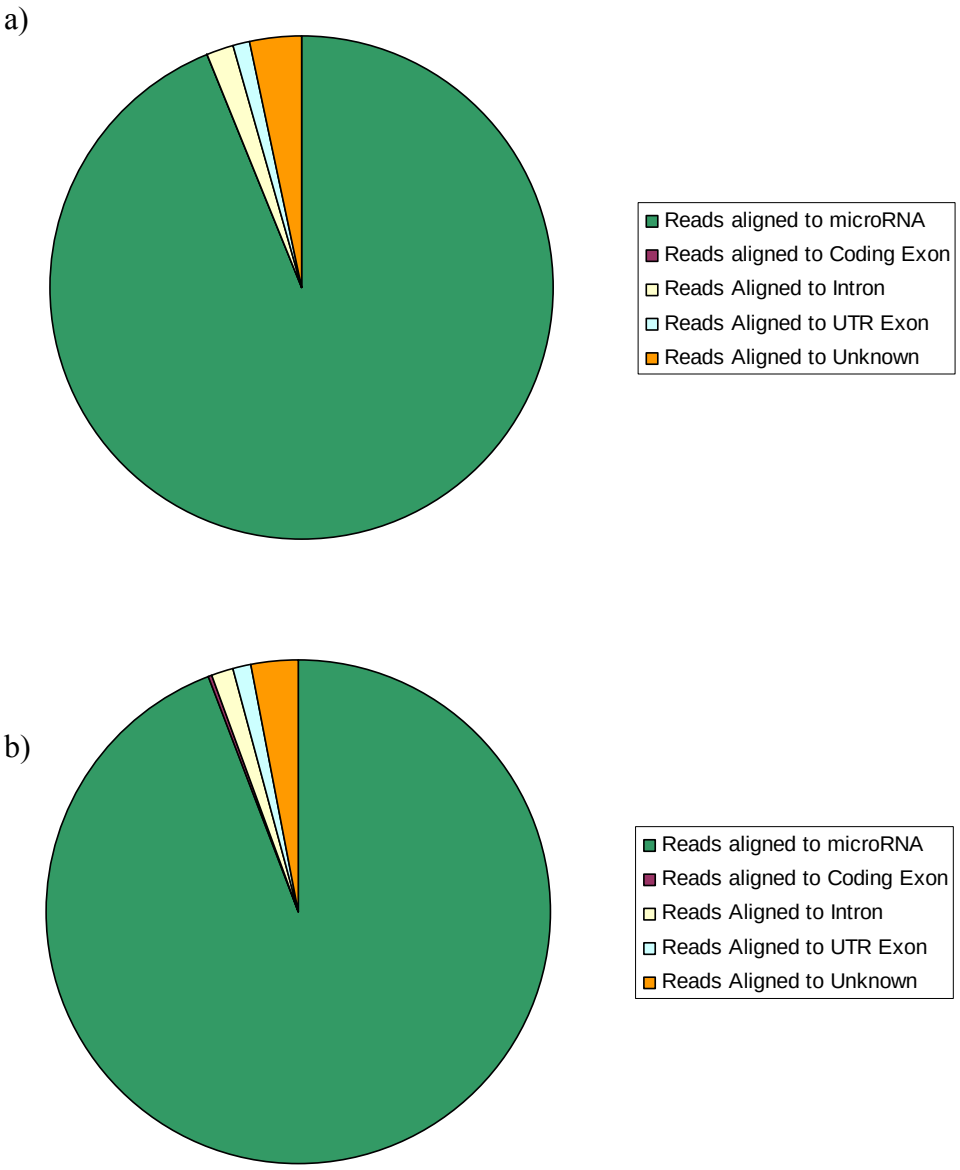


Figure 3.4 – Proportion of reads that aligned to sequences of the human genome. Pie charts represent the proportion of reads that matched to various sequences in the human genome via the mirna code analysis program. (a) Representation of the proportion of reads that aligned to miRNAs, Coding Exons, Introns, UTR Exons and Unknown in the control sample (0uM TAM). (b) Representation of the proportion of reads that aligned to miRNAs, Coding Exons, Introns, UTR Exons and Unknown in the tamoxifen treated sample (5.0uM TAM). For both samples, the largest proportion of filtered sequences mapped to known miRNAs in miRBase.

The latest version of the mirna code analysis program also has the ability to identify and take into account miRNAs that can potentially be counted as multiple reads based on whether they map to more than one location in the genome, a phenomenon known as cross-mapping. The miRNAs which had the potential to cross-map were subtracted from the differential expression analysis, but not from the total number of miRNA read counts (1220450 and 2033063 for the control and treatment sample respectively) (Table 3.3). These reads were matched to specific miRNAs and the abundance of sequences that matched to each one was calculated. It was discovered that there were 357 and 352 distinct miRNA sequences identified in the control and treated samples respectively. Of this total there were 31 and 30 miRNAs within each respective sample that were not found in their counterpart and these were taken into account in the final selection of miRNA candidates (Table 3.3).

Table 3.3 – Analysis of different miRNAs and their abundance in Control and Treated samples

	Control	5uM TAM	Total
Total # miRNAs sequenced*	1,220,450	2,033,063	
# of different miRNAs identified*	357	352	
# miRNAs present in control but absent with TAM	31		
# miRNAs present with TAM but absent in Control		30	
# miRNAs that are differentially expressed			113
Increased relative to control			27
Decreased relative to control			85
# miRNAs with potential autophagy-related targets			27
# miRNAs selected for further study			7

** Includes miRNAs that may have undergone cross-mapping*

3.4 Illumina sequencing identifies known microRNAs that are differentially expressed following tamoxifen treatment in BT-474 cells

The miRNAs that were present in each sample were aligned and then normalized in order to compare the abundance of each particular miRNA between samples. For this type of sequence-based profile, the total number of valid sequence reads for each sample may be used as the scaling factor for normalization (Creighton et al, 2009). To accomplish this, one sample is used as the reference, and the values of the other sample are divided by a scaling factor with the assumption that the total small RNA population remains fairly constant between samples (Creighton et al, 2009). The difference between the absolute number of reads between the treatment and control sample (2033063/1220450) was equal to 1.67 and so the abundance of each treatment sample miRNA, since containing the greater number of reads, was divided by 1.67. The normalized values were then utilized in the subsequent analysis of differential expression.

To determine whether the difference in the amount of each particular miRNA between the two samples was significant ($p<0.05$), a Fisher's Exact Test was employed. A Fisher's Exact test is a statistical assessment used in the analysis of contingency tables (Fisher, 1922; Agresti, 1992). The P-value for each pair of miRNAs (along with miRNAs present in one sample but not the other) was calculated and those that were deemed significant as per the 0.05 cutoff were selected for further analysis. There were 302 unique miRNAs within the samples that were not considered cross-mapped (including the unique miRNAs present in only one sample but not the other) (Appendix). Due to the large abundance of miRNAs within each sample, a relatively large proportion (50%) of the 302 miRNAs, were deemed significantly altered. In order to narrow down the candidate miRNA pairs that were considered differentially expressed between the two conditions, another criterion was added. A 1.5 fold difference between the samples was used as the second criterion, as similar studies in the literature have used this particular criterion as a cutoff (Guruju et al, 2008; Tian et al, 2008). When this second parameter was put in place, only 113 (or 38%) of the miRNAs were significantly altered as per our

criteria, with the majority of them (75%) having decreased expression in the 5.0uM TAM treatment group (Table 3.3).

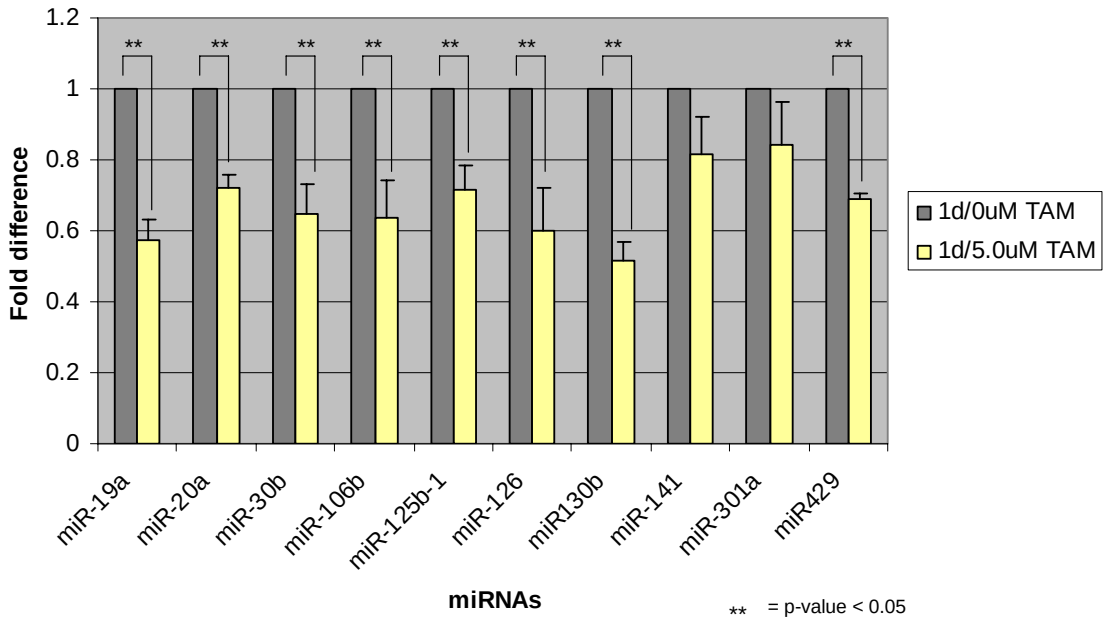
3.5 Validation of differentially expressed miRNAs by qRT-PCR

It was demonstrated previously that there is reliable concordance between high throughput sequencing techniques and qRT-PCR when measuring differential expression of microRNAs (Pradervand et al, 2010; Linsen et al, 2009). In order to validate the differential expression results I obtained using Illumina sequencing, qRT-PCR was conducted for 20 apparently differentially expressed miRNAs using an aliquot of the same RNA sample used for the miRNA library preparation and sequencing (0uM TAM control and 5.0uM TAM treated samples). From the Illumina sequencing data, 10 of the selected miRNAs demonstrated a decreased expression with tamoxifen exposure, while the other 10 demonstrated an increase (Table 3.4). The results of the qRT-PCR are shown in Figure 3.5. Ultimately 15 of the 20, or 75% of the miRNAs demonstrated a similar significant differential expression pattern ($p\text{-value}<0.05$) when tamoxifen was introduced. Consistent with the results of the Illumina sequencing, 8 of the 10 miRNAs that showed reduced expression with tamoxifen exposure by sequencing also demonstrated a significant ($p<0.05$) decreased expression via qRT-PCR analysis (Figure 3.5a). The other two miRNAs also demonstrated a decrease in expression, but were not deemed to be significant. As well, 7 of the 10 miRNAs that showed increased levels in the treated sample via Illumina sequencing also showed a significant ($p<0.05$) increase in expression using qRT-PCR (Figure 3.5b). From this analysis we concluded that in terms of miRNA differential expression patterns, the Illumina Sequencing and QRT-PCR strategies yielded similar findings. However, it should be noted that the 20 miRNAs selected from the Illumina sequencing results, all had a p-value less than 0.05; therefore we would expect that a maximum of 5%, or 1 miRNA, might demonstrate an altered differential expression if qRT-PCR could replicate the Illumina results exactly. Since 5 (25%) of the miRNAs demonstrated a differential expression that was not significant, we concluded that there were fundamental differences between the two methods of analyzing miRNA differential expression, which I discuss further in the Discussion and Conclusions section.

Table 3.4 – 20 miRNAs from Illumina sequencing selected to undergo validation of differential expression via qRT-PCR

miRNAs	Control	Treated (TAM)	Ratio TAM/Control	P-value	Candidate Autophagy Targets
miR-19a	41	0	0	5.76E-14	None
miR-20a	566	106	0.187	3.14E-87	Atg16L1, Atg2b, Atg2a
miR-30b	1184	210	0.177	9.24E-187	Atg5, Bec-1
miR-106b	1572	343	0.218	3.34E-215	Atg4b, Bec-1, Ulk-1
miR-125b-1	141	29	0.204	1.96E-21	Atg4d, Ulk-3
miR-126	108	9	0.083	5.33E-25	None
miR-130b	3728	771	0.207	0	Atg16L1, Ulk-1
miR-141	291	35	0.119	3.02E-57	None
miR-301a	343	34	0.098	2.01E-72	Atg16L1, Atg2b, Ulk-1
miR-429	623	90	0.144	5.40E-11	Ulk-2, Ambra-1
miR-15b	1242	6805	5.480	0	Atg9a
miR-18a	0	154	0	2.68E-26	None
miR-26b	10211	18202	1.783	0	Ulk-1, Ulk-2
miR-98	2628	10137	3.857	0	Atg4b, Atg16L1, Ulk-2
miR-99a	1313	7647	5.824	0	Frap-1
miR-122	21	55	2.620	4.99E-04	None
miR-192	4202	6991	1.664	1.47E-101	None
miR-224	1047	4764	4.550	0	None
miR-339	421	789	1.873	2.95E-52	None
miR-454	172	329	1.915	2.86E-09	Atg16L1, Atg2b, Ulk-2

a)



b)

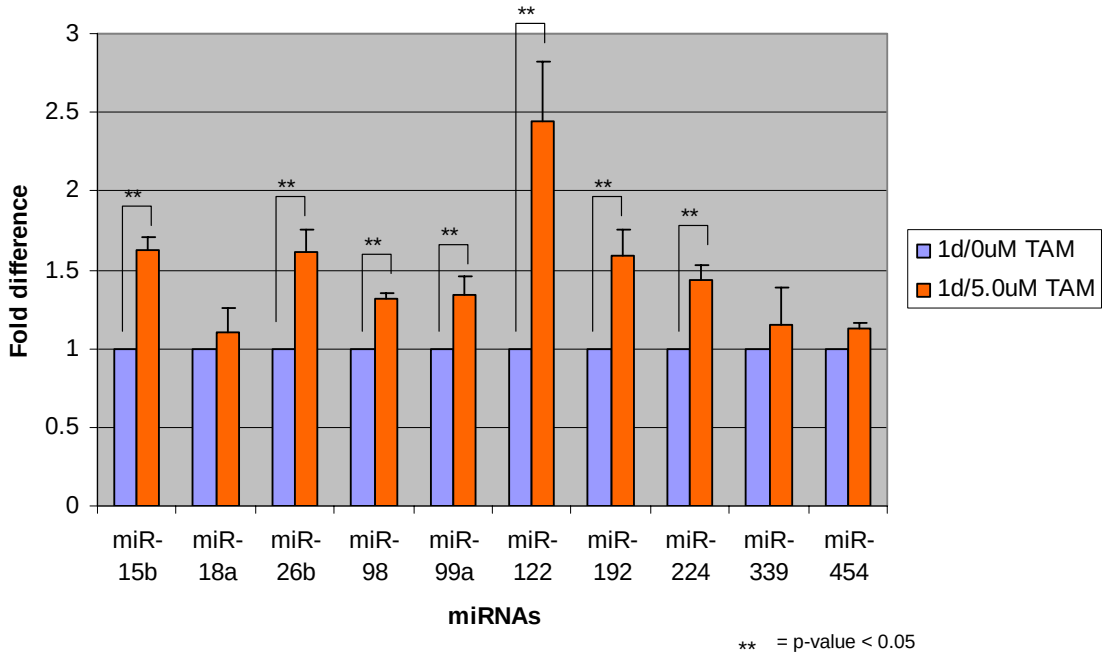


Figure 3.5 - Validation of differential expression of 20 miRNAs by qRT-PCR. The bar graphs show the fold difference in expression levels of 20 miRNAs in BT-474 cells at 5.0uM of TAM normalized to 0uM TAM control conditions after 24hrs. The BT-474 sample was an aliquot of the original RNA sent for Illumina sequencing analysis. The transcript levels were calculated via qRT-PCR relative to the endogenous internal control let-7a. The 10 miRNAs in each graph are plotted on the x-axis and the fold difference in expression is represented on the y-axis. (a) 8 out of 10 miRNAs demonstrated a significant decrease in expression level in the 5.0uM tamoxifen treated sample as compared to the control ($p<0.05$). (b) 7 of these 10 miRNAs demonstrated a significant increase in expression level in the 5.0uM tamoxifen treated sample as compared to the control ($p<0.05$). Overall 15 of 20 (75%) miRNAs demonstrated a change consistent with that observed by sequence analysis. Error bars represent standard error of 3 independent experiments.

3.6 Twenty-seven differentially expressed microRNAs are potential regulators of the autophagy pathway

In order to identify candidate gene targets of the 113 differentially expressed miRNAs, each miRNA sequence was entered into the miRNA prediction database TargetScan (www.targetscan.org). This online resource can identify potential miRNA regulators of target genes of interest as described above in section 3.2 (Table 3.1), and it can also identify candidate gene transcript targets using a miRNA as input to the program (Lewis et al, 2005; Friedman et al, 2009). Since miRNAs typically bind their mRNA targets with imperfect complementary, each miRNA entered into the database typically results in a list of about 25-50 genes it could potentially target. Therefore each of the 113 differentially expressed miRNAs was entered into the program and the resulting list of potential gene targets for each miRNA was searched for known autophagy gene regulators. The miRNA target gene list in TargetScan is normally ranked from most likely candidate to least likely based on site-type, 3'pairing, local AU and position contribution (Lewis et al, 2005; Friedman et al, 2009), however this ranking system was not taken into consideration in this preliminary search for autophagy gene targets. Autophagy targets included core genes within the autophagy pathway as well as known positive or negative regulators of the process. Of the 113 miRNAs entered into TargetScan, 27 miRNAs were found to potentially target autophagy genes or regulators as illustrated in Table 3.5

Table 3.5 – MiRNAs that potentially regulate autophagy pathway

miRNA	# in Control	# in Treated (TAM)	TAM/Control	ATG Genes
hsa-mir-106b	1572	343	0.218	Atg4b, Bec-1, Ulk-1
hsa-mir-130b	3728	771	0.207	Atg16L1, Ulk-2
hsa-mir-125b-1	141	29	0.204	Atg4d, Ulk-3
hsa-mir-15a	776	118	0.152	Atg9a
hsa-mir-15b	1242	6805	5.479	Atg9a
hsa-mir-181c	157	28	0.179	Atg2b
hsa-mir-200c	28197	6672	0.237	Ambra1
hsa-mir-20a	566	106	0.187	Atg16L1, Atg2b, Atg 2a, Ulk-1, Bec-1

miRNA	# in Control	# in Treated (TAM)	TAM/Control	ATG Genes
hsa-mir-20b	13	20	1.520	Atg16L1, Atg2b, Atg 2a, Ulk-1, Bec-1
hsa-mir-29c	5853	704	0.120	Atg9a
hsa-mir-301a	343	34	0.098	Atg16L1, Atg2b, Ulk-1
hsa-mir-30b	1184	210	0.177	Atg5, Bec-1
hsa-mir-34a	51	8	0.153	Atg4b, Atg9
hsa-mir-424	170	369	2.170	Atg9a
hsa-mir-429	623	90	0.144	Ulk-2, Ambra-1
hsa-mir-454	172	329	1.915	Atg16L1, Atg2b, Ulk-2
hsa-mir-629	61	103	1.688	Atg5
hsa-mir-874	14	3	0.214	Atg16L1
hsa-mir-96	332	90	0.271	Atg16L1, Atg9a, Frap1
hsa-mir-98	2628	10137	3.857	Atg4b, Atg16L1, Ulk-2
hsa-mir-99a	1313	7647	5.824	Frap1 (mTOR)
hsa-let-7g	536	0	0	Atg4b, Ulk-2
hsa-let-7i	1058	0	0	Atg4b, Ulk-2
hsa-mir-19a	41	0	0	Atg16L1
hsa-mir-100	0	6	0	Frap1 (mTOR)
hsa-mir-26b	10211	18202	1.783	Ulk-1, Ulk-2
hsa-mir-454	172	329	1.915	Atg16L1, Atg2b, Ulk-2

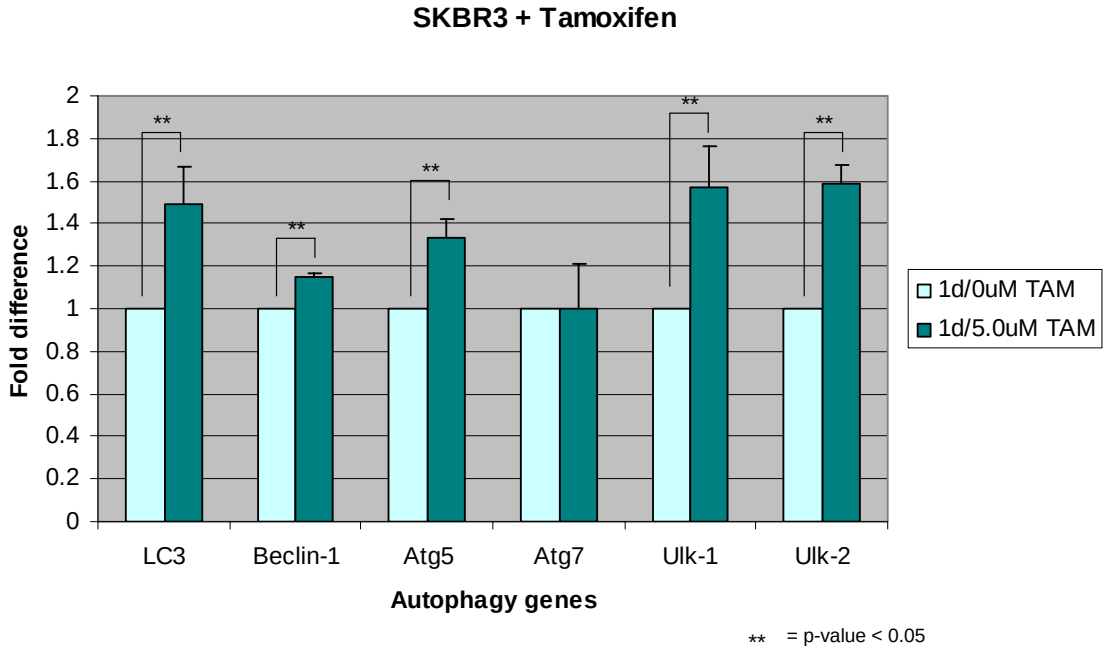
To further investigate whether these miRNAs were potential regulators of autophagy genes, 7 miRNAs from the list of 27 were selected for further analysis (highlighted in red in Table 3.5). These seven were selected based on high significant fold difference between the control and treatment samples (TAM/Control in Table 3.5) and potential to regulate more than one component of the autophagy pathway. An exception was made for miR-99a as it only potentially regulated Frap1 (mTOR) (Table 3.5). Since mTOR is a potent negative regulator of autophagy and miR-99a was over-expressed in the treated sample, it warranted further investigation. Five of the seven miRNAs selected showed decreased expression in the 5.0uM tamoxifen treated sample as compared to the control, while the other two showed increased expression levels (Table 3.5).

3.7 Four miRNAs – miR-20a, miR-30b, miR-106b and miR-125b-1, demonstrate significantly decreased expression in three different breast cancer cell-lines under multiple autophagy-inducing conditions.

To test whether the 7 putative autophagy-related miRNAs demonstrate similar patterns of differential expression in multiple breast cancer cell lines when exposed to alternate autophagy-inducers, miRNA expression was measured using qRT-PCR in 3 different breast cancer cell lines under two autophagy-stimulating conditions. The two autophagy-inducing conditions used were treatment with tamoxifen and nutrient starvation (using EBSS media), both of which have been shown to stimulate an increase in autophagic flux (Zhu et al, 2009; Lam et al, in prep; Hannigan and Gorski, unpublished results). The three breast cancer cell-lines used were BT-474 (ER+, HER2+), MDA-MB-361 (ER+, HER2+), and SKBR3 (ER-, HER2+); these cell-lines were chosen based on their demonstrated ability to undergo increased autophagy under both starvation and tamoxifen treatment.

In order to determine optimal autophagy transcript-inducing concentrations and lengths of exposure (to both tamoxifen and starvation), a qRT-PCR assay was conducted. The transcript levels of six core autophagy genes (Beclin-1, LC3, Atg5, Atg7, Ulk-1 and Ulk-2) were compared under treatment (0, 2.5 and 5.0uM of tamoxifen and 2, 4, 6h starvation) and control conditions and those conditions that elicited the greatest difference in transcript expression were selected (similar to section 3.1). In all three breast cancer cell-lines it was demonstrated that 24h/5.0uM of tamoxifen or 4 hours of starvation significantly increased most autophagy gene transcript levels. Examples of SKBR3 with exposure to 5.0uM tamoxifen (TAM) and MDA-MB-361 for 4 hours of starvation are illustrated below (Figure 3.6)

a)



b)

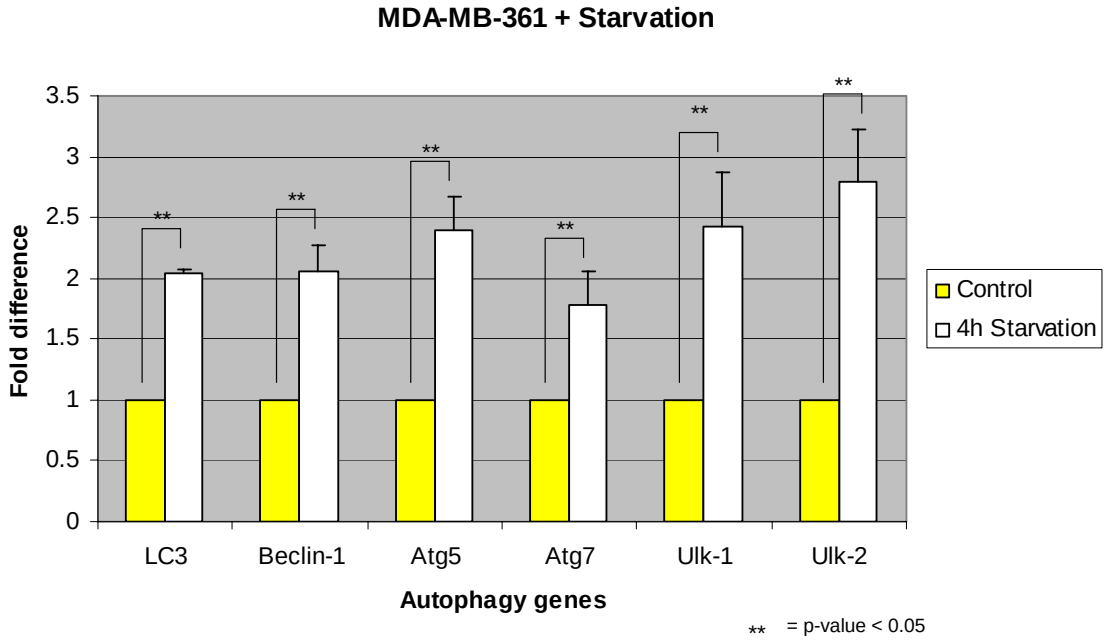


Figure 3.6 – Autophagy gene transcript expression levels in 2 different breast cancer cell lines under two autophagy-inducing conditions. The bar graphs show the expression of MAP1LC3B, Beclin-1, Atg5, Atg7, Ulk-1 and Ulk-2 normalized to their respective control conditions. The transcript levels were calculated via qRT-PCR relative to the endogenous internal control β actin. (a) SKBR3 cells treated with 5.0uM TAM were normalized to the untreated control sample and 5 of the 6 autophagy genes showed significant increases in their transcript levels ($p<0.05$). (b) MDA-MB-361 cells were starved for 4 hours. The transcript levels of the autophagy genes were normalized to the fed control and, as demonstrated above, all 6 of the autophagy gene transcript levels show a significant increase in their level ($p<0.05$). Error bars represent standard error of 3 independent experiments.

To investigate whether the seven selected miRNAs (miR-20a, miR-30b, miR-98, miR-99a, miR-106b, miR-125b-1 and miR-429) showed similar patterns of differential expression in the three cell-lines and two autophagy-inducing conditions, each cell-line was exposed to 5.0uM of tamoxifen for 24 hours or, in a separate experiment, underwent starvation for 4h. A qRT-PCR assay was performed to determine the relative miRNA levels in each cell-line compared to untreated control cells. A summary of the results is described below.

A) BT-474 Cell-Line

Using the same culture and treatment conditions employed for the Illumina sample sequencing (24 hours of 5.0uM of tamoxifen exposure), the seven miRNAs demonstrated a similar pattern of differential expression as that determined by sequence analysis. Namely, miR-20a, miR-30b, miR-106b, miR-125b-1 and miR-429 all demonstrated a significant ($p<0.05$) decreased expression as compared to their counterpart levels under control conditions in the independently isolated RNA (Figure 3.7). Although miR-98 and miR-99a show a slight increase in expression under conditions consistent with sequencing results, only the levels of miR-99a were found to be statistically significant ($p>0.05$) (Figure 3.7).

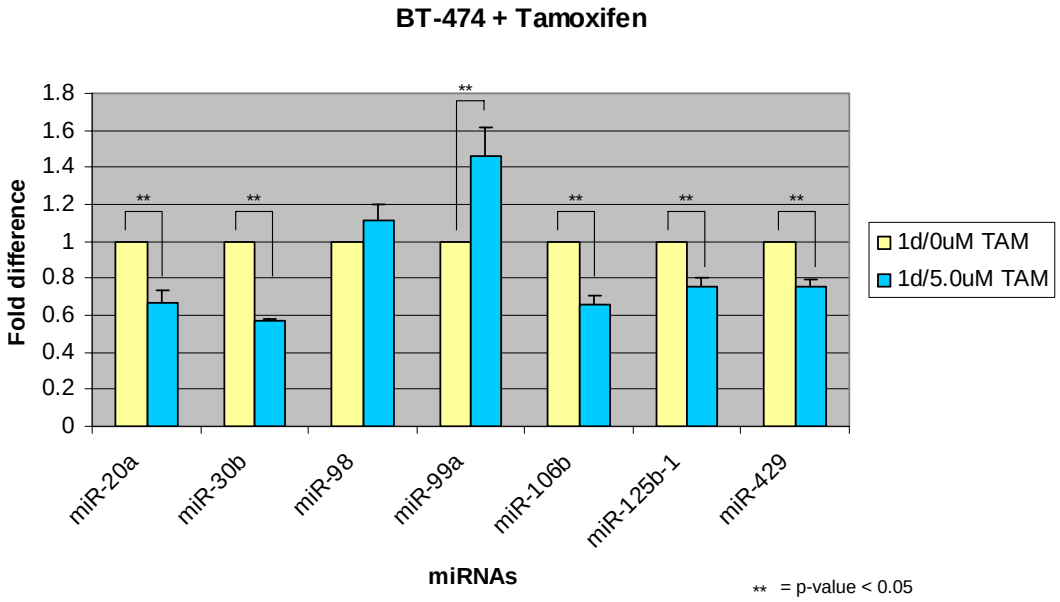


Figure 3.7 – MiRNA expression levels in the BT-474 cell-line following TAM exposure. The bar graphs show the expression of miR-20a, miR-30b and miR-98, miR-99a, miR-106b, miR-125b-1 and miR-429 in BT-474 cells at 5.0uM of TAM normalized to 0uM TAM control conditions after 24hrs of exposure. The transcript levels were measured using qRT-PCR relative to the endogenous internal control let-7a. The seven miRNAs are plotted on the x-axis and the fold difference is represented on the y-axis. Error bars represent standard error of 3 independent experiments.

As indicated in the figure below, the five miRNAs that demonstrated decreased expression via Illumina sequencing under tamoxifen exposure (miR-20a, miR-30b, miR-106b, miR-125b-1 and miR-429) also showed significantly ($p<0.05$) lower levels upon starvation, while only miR-98 showed a significant increase in miRNA levels (Figure 3.8). The decreased expression in the level of miR-99a was considered significant ($p<0.05$) but was in the opposite direction as expected from the Illumina sequencing results (Figure 3.8).

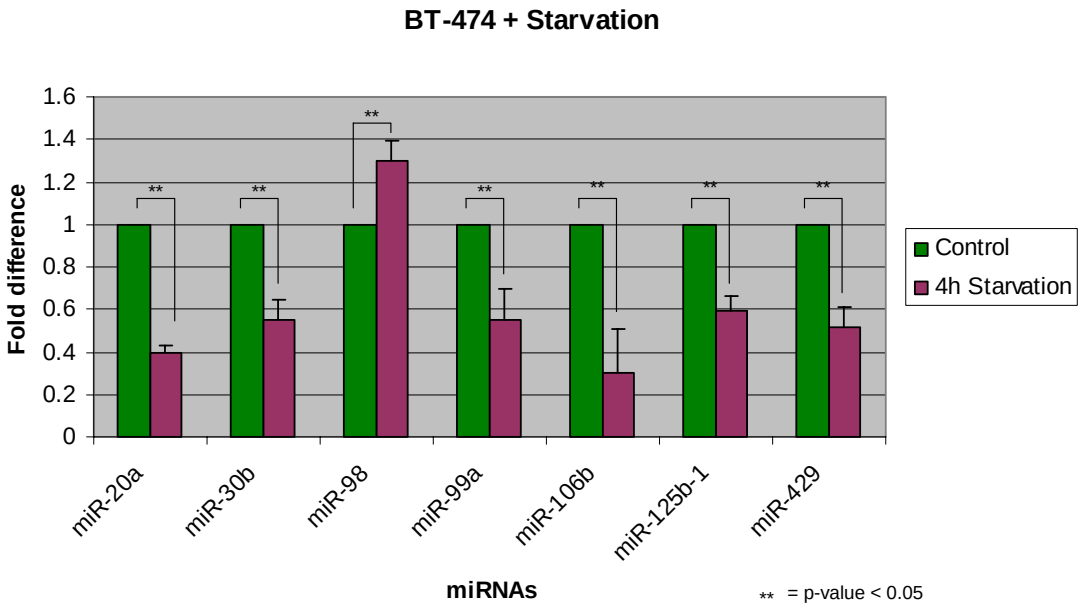


Figure 3.8 – MiRNA expression level in BT-474 cell-line following 4h of nutrient deprivation. The bar graphs show the expression of miR-20a, miR-30b and miR-98, miR-99a, miR-106b, miR-125b-1 and miR-429 in BT-474 cells after 4h of nutrient starvation normalized to fed control conditions. The transcript levels were measured by qRT-PCR and determined relative to the endogenous internal control let-7a. The seven miRNAs are plotted on the x-axis and the fold difference is represented on the y-axis. Error bars represent standard error of 3 independent experiments.

B) MDA-MB-361 Cell-Line

With the introduction of 5.0uM of tamoxifen for 24 hours to MDA-MB-361 cells, six of the seven selected miRNAs demonstrated an expression profile similar to the findings obtained using Illumina sequencing in BT-474 cells. Mir-20a, miR-30b, miR106b and miR-125b-1 all showed a significant ($p<0.05$) decrease in expression following tamoxifen treatment as compared to the control, while miR-98 and miR-99a both showed an increase in expression (Figure 3.9). Contrary to the profile in BT-474 cells, however, miR-429 showed an increase in expression level following tamoxifen treatment, though it was not considered significant (Figure 3.9).

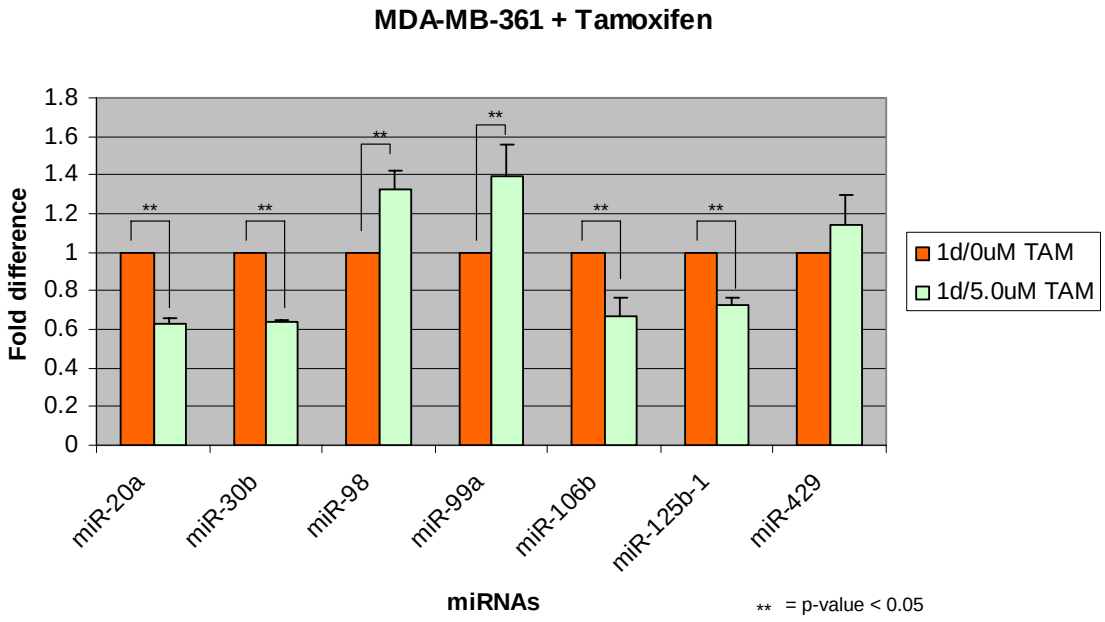


Figure 3.9 – MiRNA expression in TAM treated MDA-MB-361 cells. The bar graphs show the expression of miR-20a, miR-30b and miR-98, miR-99a, miR-106b, miR-125b-1 and miR-429 in MDA-MB-361 cells at 5.0uM of TAM normalized to 0uM TAM control conditions after 24hrs of exposure. The transcript levels were measured by qRT-PCR relative to the endogenous internal control let-7a. The seven miRNAs are plotted on the x-axis and the fold difference is represented on the y-axis. Error bars represent standard error of 3 independent experiments.

In the MDA-MB-361 breast cancer cell-line undergoing starvation-induced autophagy, the five miRNAs that showed a decreased expression in the BT-474 cells (miR-20a, miR-30b, miR-106b, miR-125b-1 and miR-429) all showed significantly lower expression ($p<0.05$) when compared to their fed controls (Figure 3.10). However, the two miRNAs that were predicted to show increased expression (miR-98 and miR-99a) based on findings in BT-474 cells, instead also showed significant decreased levels as compared to the fed control (Figure 3.10).

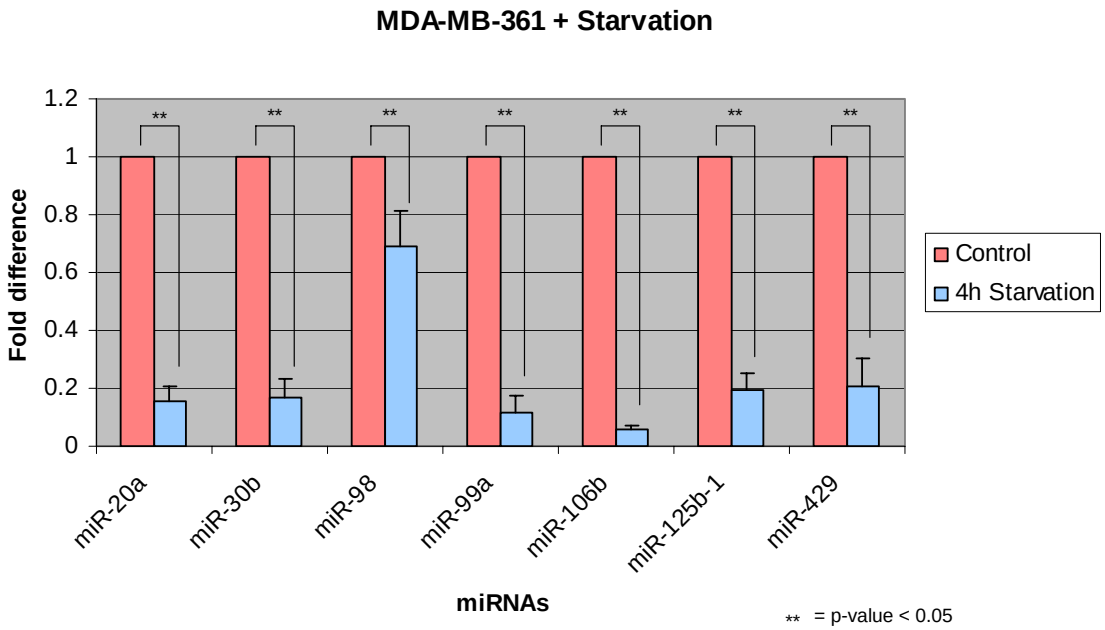


Figure 3.10 – MiRNA expression level in MDA-MB-361 cells following 4h of nutrient deprivation. The bar graphs show the expression of miR-20a, miR-30b and miR-98, miR-99a, miR-106b, miR-125b-1 and miR-429 in BT-474 cells after 4h of nutrient starvation normalized to fed control conditions. The transcript levels were measured by qRT-PCR and determined using the endogenous internal control let-7a. The seven miRNAs are plotted on the x-axis and the fold difference is represented on the y-axis. Error bars represent standard error of 3 independent experiments.

C) SKBR3 Cell-Line

After 24 hours of tamoxifen exposure, five of the miRNAs (miR-20a, miR-30b, miR-106b, miR-125b-1 and miR-429) showed significant decreased expression ($p < 0.05$) in SKBR3 cells (Figure 3.11), consistent with the expression profiles in BT-474 cells. The other two miRNAs, miR-99a and miR-98, however did not show a significant difference in expression levels between the control and tamoxifen treated sample (Figure 3.11)

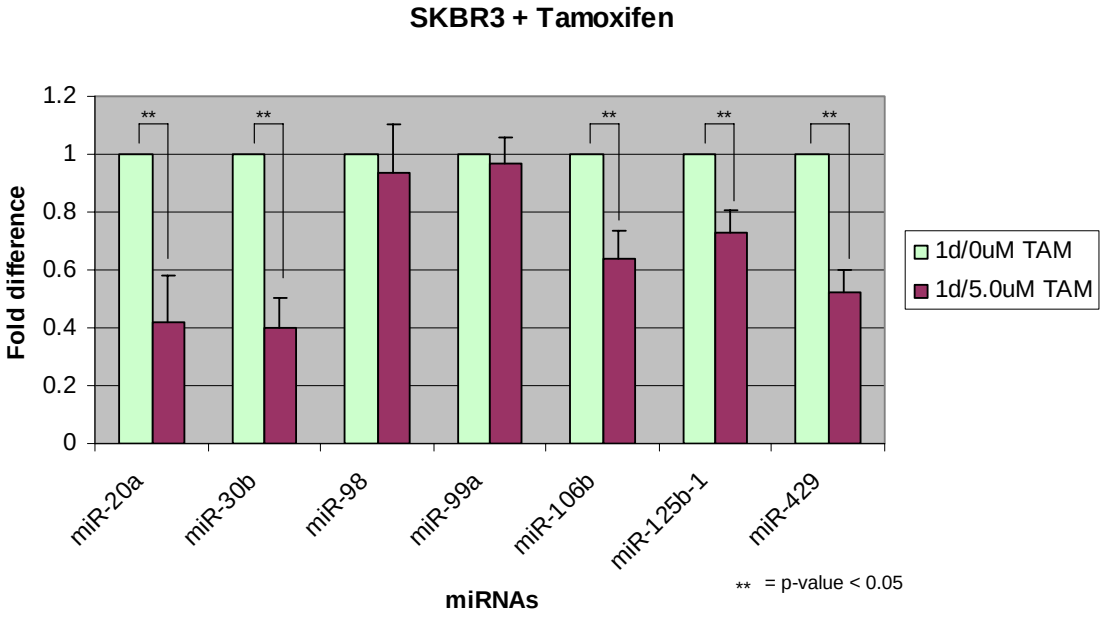


Figure 3.11 – MiRNA expression in TAM treated SKBR3 cells. The bar graphs show the expression of miR-20a, miR-30b and miR-98, miR-99a, miR-106b, miR-125b-1 and miR-429 in SKBR3 cells at 5.0uM of TAM normalized to 0uM TAM control conditions after 24hrs of exposure. The transcript levels were measured by qRT-PCR with let-7a as the endogenous internal control. The seven miRNAs are plotted on the x-axis and the fold difference is represented on the y-axis. Error bars represent standard error of 3 independent experiments.

Additionally, after a four-hour starvation period, only four miRNAs showed a pattern of differential expression that was similar to that observed in the sequenced BT-474 cells. Mir-20a, miR-30b, miR-106b, and miR-125b-1 all demonstrated significant ($p<0.05$) decreased expression as compared to the control. However, none of the other three miRNAs, miR-98, miR-99a and miR-429, demonstrated a significant difference in expression level ($p>0.05$) as compared to the control (Figure 3.12).

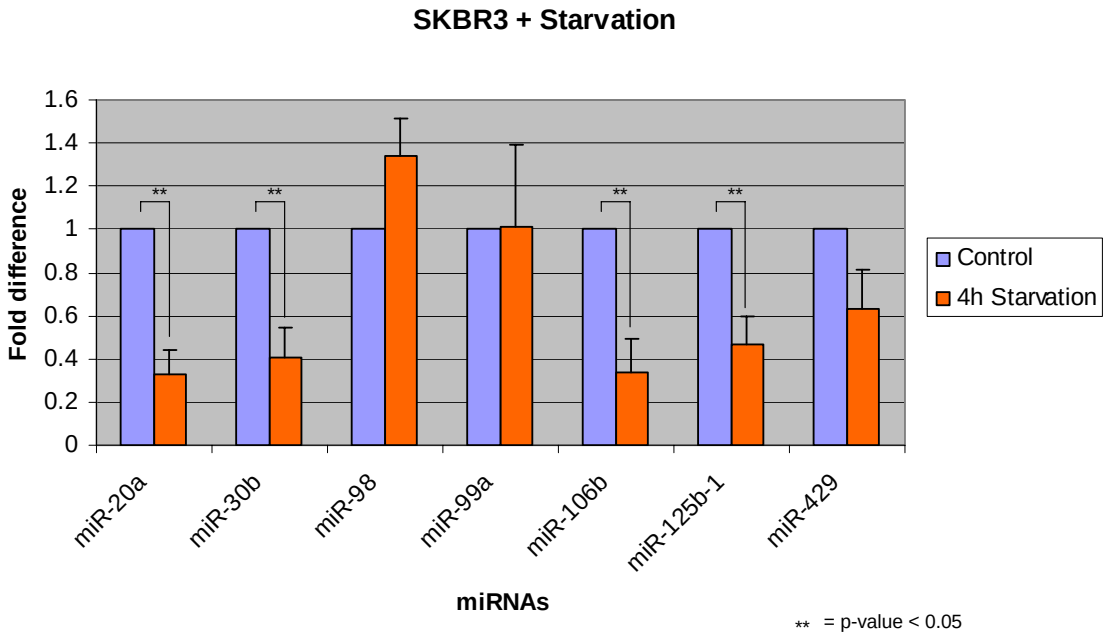


Figure 3.12 – MiRNA expression level in SKBR3 cells following 4h of nutrient deprivation. The bar graphs show the expression of miR-20a, miR-30b and miR-98, miR-99a, miR-106b, miR-125b-1 and miR-429 in BT-474 cells after 4h of nutrient starvation normalized to fed control conditions. The transcript levels were quantitated by qRT-PCR using let-7a as the endogenous internal control. The seven miRNAs are plotted on the x-axis and the fold difference is represented on the y-axis. Error bars represent standard error of 3 independent experiments.

In summary, four of the original seven miRNAs that were found to be differentially expressed in the BT-474 cell-line Illumina sequencing analysis were found to be similarly differentially expressed in a total of 3 breast cancer cell-lines undergoing 2 different autophagy-inducing conditions (Table 3.6). The four miRNAs: miR-20a, miR-30b, miR-106b and miR-125b-1, all show decreased expression when autophagy is induced, and represent good candidates for potential regulators of autophagy levels.

Table 3.6 – Summary of differential expression patterns of miRNAs in three breast cancer cell-lines exposed to two autophagy-inducing conditions

Cell Line	Treatment	miR-20a	miR-30b	miR-98	miR-99a	miR-106b	miR-125b-1	miR-429
BT-474 Sequencing	TAM vs. Control							
BT-474 (QRTPCR)	TAM	+	+	-	-	+	+	+
	Starvation	+	+	+	-	+	+	+
MDA-MB-361	TAM	+	+	+	+	+	+	-
	Starvation	+	+	-	-	+	+	+
SKBR3	TAM	+	+	-	-	+	+	+
	Starvation	+	+	-	-	+	+	-

(+) = significant differential expression ($p<0.05$) in the same direction as Illumina sequencing data

(-) = differential expression that was not considered significant ($p>0.05$) or pattern of expression was in the opposite direction as Illumina sequencing data

4 DISCUSSION AND CONCLUSIONS

The main objective of my study was to discover miRNAs that demonstrated differential expression when autophagy was induced in breast cancer cells. The longer term goal of this research is to identify miRNAs that are involved in regulating the autophagy process in breast cancer. The results from my study included a number of findings that are relevant to the process of autophagy and the regulation of miRNAs in the context of breast cancer. Though previous studies have shown that high-dose tamoxifen can induce autophagy in a number of different breast cancer cell-lines (Bursch et al, 1996, Qadir et al, 2008; Samaddar et al, 2008; Lam et al, in prep), there are no published reports that indicate this particular breast cancer drug increases the transcript level of core autophagy genes in BT-474, MDA-MB-361 or SKBR3 cell-lines. Tamoxifen was also shown to have an effect on the abundance of many different miRNAs, and this study is the first sequence-based miRNA expression analysis, to my knowledge, that has been associated with autophagy induction and breast cancer. Of the more than 700 miRNAs discovered to date in the human genome, only miR-30a has been functionally linked to a particular gene in the autophagy pathway. I hypothesized at the beginning of this experiment that I would see the differential expression of certain miRNAs under autophagy-inducing conditions. Ultimately four miRNAs were discovered in this study that demonstrated a consistent and significant differential expression in all three breast cancer cell-lines under 2 different autophagy-inducing conditions. These four miRNAs, miR-20a, miR-30b, miR-106b and miR-125b-1, represent novel candidates for future functional studies to elucidate whether they can directly regulate components of the autophagy pathway.

4.1 Methods for analyzing miRNA expression and the advantages of Illumina sequencing

Since miRNAs have been discovered to play a role in regulating the expression of many genes, miRNA expression analysis studies have become more popular. To date, miRNAs have been experimentally linked to different cancers and neurodegenerative

diseases, among other human disorders (Calin et al, 2002; McManus, 2003; Gregory and Shikhattar, 2005; Hebert et al, 2008). The three major technological options to analyze miRNA expression are high-throughput sequencing, microarray based hybridization techniques and Quantitative Real Time Polymerase Chain Reaction (QRT-PCR) (Pradervand et al, 2010; Sato et al, 2009). QRT-PCR has traditionally been seen as the gold-standard as it is a relatively inexpensive assay and demonstrates high sensitivity to miRNA expression levels. However, the cost of sequencing genetic material has decreased considerably over recent years and sequencing technology is starting to become more common for miRNA analysis studies. Despite being seen as a more time consuming and expensive technique, the Genome Sciences Centre's Illumina platform was used to sequence the miRNA libraries prepared from the two samples. The advantages of high-throughput sequencing are that it can analyze a large amount of data at once, is considered more sensitive and accurate than microarray-based techniques and has the potential of identifying novel miRNAs within samples (Pradervand et al, 2010; Linsen et al, 2009). Though the detection of novel miRNAs was not undertaken in this study, the reads generated by the Illumina Sequencer have the potential to be analyzed in the future for undiscovered miRNAs, as discussed further in the Future Directions section (5.1).

4.2 Analysis of miRNA library sequencing data

An important aspect of high-throughput sequencing in determining miRNA differential expression is the algorithm used for analyzing the raw sequence reads and matching them to sequences of known miRNAs. In this study, mirna code, a GSC software program was used to identify the known miRNA sequences within the raw reads generated by the Illumina platform. Prior to the sequences being matched to the human genome, the reads undergo a quality control check, where a small subset of the reads is checked for contamination from other genomes. This check is based on a pass/fail system, and if no contamination is present, the reads continue on for analysis (Figure 2.1). Ultimately the reads were aligned to known miRNA precursors, coding exons, introns, and UTR exons of the human genome. Of interest in this study were the reads

that matched to miRNA precursor sequences. Using miRBase, an online resource containing more than 700 miRNA sequences (Griffiths-Jones, 2008), the raw reads were matched to the known miRNA sequences via mirna code, identifying the miRNAs within each sample and the abundance of each. One potential problem with this type of alignment is accounting for possible cross-mapping between miRNAs. Similar to cross-hybridization in microarray experiments, miRNA sequence reads can be inadvertently mapped to multiple miRNAs, known as cross-mapping. Cross-mapping can occur when a specific miRNA has a very similar sequence (within a few base pair changes) to other members of its family and can align to more than one miRNA (de Hoon et al, 2010). When this occurs, there is the potential for counting one miRNA multiple times per sample. To account for this, the newest version of mirna code, allows a maximum of 3nt changes between the raw reads and known miRNAs and identifies miRNA reads that align to the genomic coordinates of two or more different microRNAs, isolating them from those miRNAs that match only to a single location (de Hoon et al, 2010). These cross-mapped miRNAs were then excluded from further analysis.

4.3 Potential reasons why miRNA expression fold change varies between sequencing and qRT-PCR assays

To determine whether the differential expression results obtained by the Illumina sequencing analysis could be validated, qRT-PCR analysis was conducted on an aliquot of the same RNA samples sent for sequencing. As described in the results section (3.6), 20 differentially expressed miRNAs were examined using this established method. As shown in Figure 3.5, the pattern of miRNA differential expression was found to be the same and statistically significant ($p<0.05$) for 75% (15/20) of the selected miRNAs. While the pattern of changes in miRNA expression were consistent using the two miRNA expression analysis methods, the fold expression difference was less as measured by qRT-PCR compared to sequencing.

One possible explanation as to why this difference in the magnitude of the differential expression varies, may reside with the different methods of analyzing the raw data

between the two techniques. After the raw sequence data is received from the Illumina sequencing platform, the reads are matched to known precursor miRNA sequences using the mircode miRNA software and the online database, miRBase, as described previously. Nucleotide precursor miRNA sequences or pre-miRNAs described in the introduction are double-stranded hairpin intermediates that are transported from the nucleus into the cytoplasm where they are further processed by RNAse III endonuclease and then associate with the RNA-induced silencing complex (RISC) (Figure 1.3). The final mature miRNA is single-stranded and therefore one of the two pre-miRNA strands (the passenger or 'star' strand) is discarded and usually degraded during this process (Khvorova et al, 2003;Winter et al, 2009). Because the analysis software is set to match the raw library reads to the entire pre-miRNA structure, it can pick up mature miRNAs as well as passenger strands within the sequences (Creighton et al, 2009). One of the limitations of mirna code to date is that it doesn't differentiate between these categories of evolving miRNAs. Therefore a passenger strand that will eventually be degraded and has little functional consequence would likely be categorized in the same manner as a functional mature miRNA strand. Alternatively the primers used in the qRT-PCR analysis are for the mature miRNA sequences and therefore will only detect mature strand miRNAs within the samples.

Another explanation as to why the qRT-PCR analysis of miRNAs shows a similar pattern of expression as the Illuman sequencing results but less dramatic fold changes, may reside with the differences in dynamic range and the limitations associated with the technology. Sequencing the miRNA reads within the two samples is a very sensitive measure of the absolute abundance of each miRNA. Quantitative RT-PCR, on the other hand, measures the fluorescence emitted by the primers binding to specific miRNAs within the samples. Since this analysis is image-based, relying on the detection of fluorescent signals, it has inherently greater limitations than sequencing in differentiating the abundance of miRNAs between samples. Therefore this may be another reason why the patterns of miRNA differential expression are similar between the two techniques but the exact fold differences vary.

4.4 The list of differentially expressed miRNAs derived using Illumina sequencing and potential future applications

Tamoxifen is a current treatment method for certain forms of breast cancer and can have varying effects on the cells of the breast tissue. Our analysis looked at one particular human breast cancer cell-line, BT-474 and the effects high-dose tamoxifen had on the miRNA content of those cells. The result of the high-throughput sequencing and analysis was a list of 302 miRNAs that were expressed in the control and tamoxifen exposed sample in the BT-474 breast cancer cell-line (Appendix). This type of technology can give a relatively precise indication of how miRNA expression changes with the addition of tamoxifen to breast cancer cells. There were 113 miRNAs discovered in this study that were considered differentially expressed as per two specific parameters – a p-value less than 0.05 and at least a 1.5 difference in fold change. Relevant to our objective was the 27 miRNAs that were found to potentially target regulators of the autophagy pathway. The online resource TargetScan was useful for predicting potential gene targets for the miRNAs, however for each individual miRNA the program often generated 50 or more possible targets, which were ranked by order of likelihood of matching, as indicated previously. With the emergence of miRNA studies in many different aspects of human disease, the raw sequences or list of differentially expressed miRNAs generated by this study, have the potential to be valuable to others. There are over 700 known miRNAs that exist in the human genome (Griffiths-Jones, 2008), however only very few have been experimentally linked to their gene transcript targets. The screen of differentially expressed miRNAs in BT-474 with exposure to tamoxifen done in this study has the potential to be useful in other aspects of studying this disease beyond the potential regulation of the autophagy pathway. For example, these results could be used to possibly narrow down candidate miRNAs that regulate an alternate pathway or provide experimental evidence to warrant further studies examining how tamoxifen affects miRNAs in breast cancer cells. Similar concentrations of tamoxifen (uM), have been found to induce apoptosis and decrease proliferation in both *in vitro* and *in vivo* studies (Gelmann, 1996; Perry et al, 1995; Cameron et al, 2000) and affect signaling pathways such as protein kinase C (Chen et al, 1998), calmodulin (O'Brian et al, 1990), c-myc (Kang et al, 1996) and MAP kinases (Mandlekar et al, 2000).

4.5 Prioritization of candidate autophagy-associated miRNAs

It has been shown that miRNAs can regulate a number of different gene transcript targets (John et al, 2004; Esquela-Kerscher and Slack, 2006), and therefore it was important in this study to select miRNAs involved specifically in the autophagy process for further analysis. The observation that each differentially expressed miRNA had a relatively large number of genes that were considered potential targets did present an issue in this study: how could it be determined which miRNAs that showed differential expression were most likely related to autophagy and not some other process? The twenty-seven miRNAs selected as potential regulators of autophagy-related genes also had other gene targets according to TargetScan. Thus, one of the key criteria selected for prioritizing the twenty-seven miRNAs for further analysis was to determine which miRNAs matched to at least two genes in the autophagy pathway. Having this criterion in place increased the probability that the miRNA regulated the autophagy process. Of the twenty-seven differentially expressed miRNAs discovered in this study, twelve had at least two candidate autophagy pathway targets. The seven miRNAs that were ultimately selected from these 12 were chosen due to the particular early role their target Atg genes had in the autophagy pathway. To further investigate whether expression of these miRNAs correlated with changes in autophagy and were not solely a result of tamoxifen exposure (which could have many different affects), autophagy was induced by a second means (nutrient deprivation) and miRNA expression was examined in three different breast cancer cell lines as detailed in the Results section. As illustrated in Table 3.6, the nutrient deprivation condition resulted in a similar pattern of miRNA expression as found by sequencing in all three breast cancer cell-lines. It has been demonstrated previously in breast cancer cells, among others, that nutrient deprivation results in an increase in autophagy through inhibition of mTOR (Levine and Kroemer, 2008). There were some variations in the miRNA differential expression between the three cell-lines and this proved to be a useful strategy for determining whether the differential expression of the seven miRNAs was correlated with an induction of autophagy.

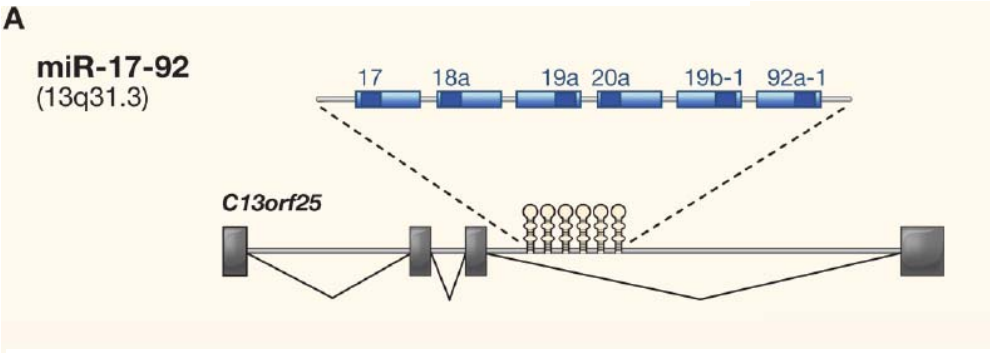
4.6 The four candidate autophagy-regulating miRNAs: miR-20a, miR-30b, miR-106b and miR-125b-1

In this study it was important that miRNAs considered candidates for future investigation were not only differentially expressed in the BT-474 cell samples sent for Illumina sequencing, but demonstrated similar patterns in expression across 3 different breast cancer cell lines under 2 autophagy-inducing conditions. Four of the miRNAs (miR-20a, miR-30b, miR-106b and miR-125b-1) consistently demonstrated decreased expression in response to both autophagy-inducing conditions (tamoxifen and starvation) in all three breast cancer cell-lines tested. The other three miRNAs (miR-429, miR-98, miR-99a) demonstrated differing expression patterns between conditions and/or cell-lines. It was expected that not all seven selected miRNAs would show the same differential expression in all three breast cancer cell lines and this result could be due to a number of factors including no actual association with the induction of autophagy, and cell-line or treatment specific expression differences. For example, the cytotoxic properties of tamoxifen have a number of different effects on breast cancer cells and since miRNAs are thought to regulate the expression of genes in many different cellular processes, the altered expression of specific miRNAs may be due to a change in an alternate cellular pathway responding to tamoxifen treatment.

4.7 Potential role(s) of four miRNAs (miR-20a, miR-30b, miR-106b and miR-125b-1) in cancer

The four miRNAs identified in this study as having possible autophagy-regulating effects in multiple breast cancer cell-lines have been identified previously as having a role in different cancers. MiR-20a is encoded within the microRNA-17-92 cluster along with five other miRNAs (miR-17, miR-18a, miR-19a, miR-19b-1, and miR-92-1), which are tightly grouped within an 800bp region of human chromosome 13 (13q31.3). This particular cluster is located in the third intron of a 7kb primary transcript known as C13orf25 (Figure 4.1) (Ota et al, 2004). In our analysis, all 6 miRNAs belonging to this cluster demonstrated decreased expression upon tamoxifen exposure, however only the expression levels of miR-19a and miR-20a were considered significantly different.

Figure 4.1 – The six miRNAs encoded in the miR-17-92 cluster



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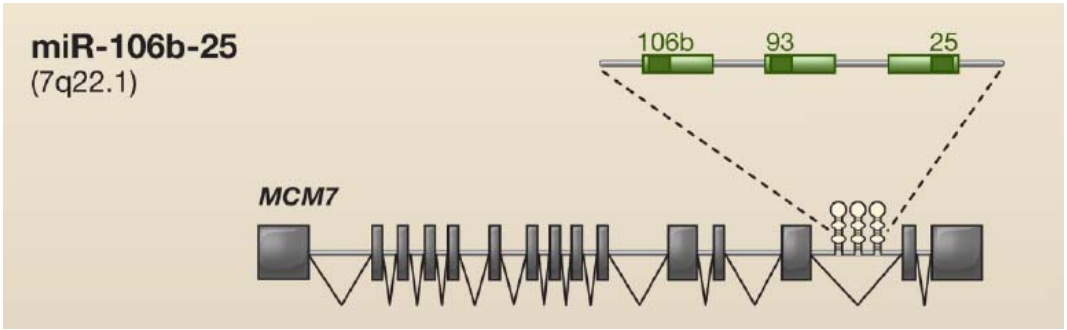
The expression of this miRNA cluster is highly conserved in all vertebrates, yet C13orf25 is non-protein coding, suggesting that the sole function of this transcript is to produce these six miRNAs (Mendell, 2008). The miRNAs within the miR-17-92 cluster are thought to contribute to heart, lung, blood vessel and immune system development in mammals, and are found to be highly expressed in tumor cells (Lu et al, 2007; Pickering et al, 2009; Ota et al, 2004). This cluster was first linked to cancer pathogenesis when it was discovered that the human genome locus encoding these miRNAs (13q31.3) underwent amplification in several types of lymphoma and solid tumors (Mendell, 2008). Since then, expression profiling studies have revealed a widespread overexpression of these miRNAs, including miR-20a in diverse tumor subtypes including solid tumors such as those derived from lung, colon, breast, pancreas, prostate and stomach (Petrocca et al, 2008; Volinia et al, 2006). This particular cluster has been implicated in cellular proliferation and apoptosis by targeting the E2F family of transcription factors (Pickering et al, 2009; O'Donnell et al, 2005), and down-regulating the tumor suppressor p21 (Inomata et al, 2009; Fontana et al, 2008) as well as pro-apoptotic protein BIM (Fontana et al, 2008). It should be noted that, under genotoxic stress, it has been shown that a member of the E2F family, E2F1, has the capacity to regulate both DNA damaged-induced apoptosis (Stevens and La Thangue, 2004) and induce specific autophagy genes (Polager et al, 2008).

While overexpression of miR-20a remains predominant in the literature in promoting tumorigenesis, there is also some evidence that loss-of-function of this miRNA and others in the mir-17-92 cluster might be advantageous for breast cancer cells. It was revealed that the miR-17-92 cluster was deleted in 21.9% of breast cancers in a recent genome-wide analysis (Zhang et al, 2006), and that the introduction of some of these miRNAs back into breast cancer-cell lines reduced the proliferation of the cancer cells (Hossain et al, 2006). The seemingly conflicting role of the miR-17-92 cluster, and by extension miR-20a, seems to play in different cancers may be due to many factors including specific gene targets and differential tissue expression.

Interestingly it has been hypothesized that ancient duplications of the miR-17-92 cluster that codes for miR-20a, gave rise to two paralogs: the miR-106b-25 and miR-106a-363 clusters (Mendell, 2008). The miR-106b-25 cluster encodes miR-106b, another one of the four miRNAs found to be differentially expressed under autophagy-inducing conditions in this study. This particular miRNA cluster codes for miR-93 and miR-25 along with miR-106b and is located in a 515bp region at chromosome 7q22. The cluster is located in the thirteenth intron of the host protein-coding gene MCM7 (Figure 4.2), a type of highly conserved mini-chromosome maintenance protein (MCM), which is essential for the initiation of eukaryotic genome replication (Mendell, 2008; Nakatsuru et al, 1995). In our Illumina sequencing analysis, it was shown that miR-93, along with miR-106b, demonstrated a significant decreased expression ($p<0.05$), whereas the differential expression of miR-25 was not considered significant.

Figure 4.2 – The three miRNAs encoded in the miR-106b-25 cluster



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Due to its similarity with miR-17-92, the miR-106b cluster is implicated in many of the same tumorigenesis roles, such as the suppressive effects on the E2F family of transcription factors (O'Donnell et al, 2005) as well as the regulation of p21 (Kan et al, 2009; Ivanovska et al, 2008).

There is less material in the literature surrounding the other two differentially expressed miRNAs in this experiment: miR-125b-1 and miR-30b. Studies that have examined miR-125b-1 suggest that this miRNA acts as a tumor suppressor in both breast and oral cancers (Hoffman, 2009; Henson, 2009). MiR-125b-1 is located at chromosome 11q24.1 and has been shown to demonstrate decreased expression in both breast and ovarian cancers (Iorio et al, 2005; Iorio et al, 2007). A study by Mattie et al in 2006 established that miR-125b-1, along with 125a, are downregulated in HER2-amplified and HER2-overexpressing breast cancers (Mattie et al, 2006). A different study by Scott et al the following year found that overexpression of either miRNA was able to decrease HER2 and HER3 mRNA and protein levels, which led to a reduction in anchorage-dependant growth, cell motility, and invasiveness (Scott et al, 2007).

MiR-30b is located on chromosome 8 (8q24.22) and there is some evidence that the amplification of miR-30b may play a role in medulloblastoma (Lu et al, 2009). Lu et al recently identified a novel recurrent region of genomic amplification in medulloblastoma cell lines that encompasses the location of miR-30b (8q24.22-q24.23), but concluded that the functional relevance of elevated miR-30b levels required further study (Lu et al, 2009).

The literature connecting the expression of these four miRNAs to certain cancers and cancer-regulating mechanisms is just a small example of recent material on the hundreds of known miRNAs. Since Calin et al first linked miRNA expression to the regulation of cancer in 2002, the amount of resources put into this field and experimental results derived, has grown significantly. This study is one of the first however to link the expression of specific miRNAs to the induction of autophagy, an important cellular

mechanism in both normal cell homeostasis and a recognized role in cancerous cells. Like many studies attempting to make a novel association between the expression of miRNAs and the regulation of a cellular mechanism, there are both strengths and weaknesses to the methods of experimentation, as well as limitations in the interpretation of the results.

4.8 Summary of strengths

In this study I was fortunate to have access to one of the Genome Sciences Centre's Illumina Genome Analyzers, a high-throughput sequencer that, among other functions, has the ability to comprehensively sequence small RNA expression. The advantages of this technology are that the platform can sequence millions of gene fragments with high sensitivity and reliability, providing a more complete view of the miRNA transcriptome than previous microarray techniques, as well as the potential for discovering novel miRNAs (Pradervand et al, 2010; Morin et al, 2008; Linsen et al, 2009). Therefore using the Illumina technology as part of this study strengthens the miRNA differential expression results derived from the two samples and the sequencing results themselves can act as a potential resource for identifying novel miRNAs in future studies. With the costs associated with high-throughput sequencing decreasing, this method of genetic analysis is becoming more popular in the miRNA research field.

Though not yet fully developed, the newest version of the analysis software (mirna code) has the ability to take the problems associated with cross-mapping between miRNAs into account, as detailed above. Cross-mapping or cross-hybridization is one of the limitations associated with microarray assays that monitor the differential expression of miRNAs, decreasing their sensitivity (Pradervand et al, 2010; Willenbrock, 2009). Cross-mapping can alter the absolute quantities of miRNAs, possibly skewing differential expression results. The Illumina sequence data derived in this experiment was originally analyzed using an older version of the software that did not account for this cross-mapping phenomenon, but was re-analyzed when the new program was implemented. There were not any major differences between the differential expression patterns

between the lists of miRNAs that emerged from the two different versions of the analysis program, however a number of miRNA pairs that were considered significantly differentially expressed (p-value <0.05 and 1.5 fold change) using the old program had altered significance values.

The Zhu et al experiment is the only published report with similar goals as this study – to discover if miRNAs play a role in regulating the autophagy pathway under cell-stress conditions. Zhu et al, however investigated differential miRNA levels using microarray techniques in three different cancer cell-line models, all of which came from different tissue sources (Zhu et al, 2009). One of the strengths of my study was that the four identified miRNAs that were associated with autophagy regulation were observed under more than one autophagy-inducing condition using three different cell-lines from the same tissue (breast). Since some reports indicate that miRNA expression may be tissue-specific (Lagos-Quintana et al, 2002), the advantage of our approach is that we exclude the variability of miRNA levels in other tissues. Cancer is a very diverse disease, encompassing many different types and tissues; therefore miRNAs that may regulate autophagy in one cancer or cell-type might not have the same effect in another.
There are a number of sources of cellular stress that induce autophagy. To prioritize candidate regulators of autophagy in this study, the miRNAs examined had to show a similar pattern of differential expression under the two autophagy-inducing conditions (tamoxifen and starvation). This increases the possibility that the miRNA differential expression is related to autophagy and not another aspect of the treatments.

4.9 Summary of weaknesses and limitations

MiRNAs have the potential to regulate many different gene targets. In this study we were able to associate the differential expression of four different miRNAs with the induction of autophagy in breast cancer cells. However, without the addition of functional analysis, we cannot conclude whether these four miRNAs repress their predicted autophagy targets. The miRNA gene target ranking lists were not taken into consideration for selecting miRNAs and therefore it is highly possible that any one of the twenty-seven

miRNAs selected as 'candidate regulators of the autophagy pathway', regulate the expression of a gene or genes involved in different cellular processes. It should be noted that 3 of the 4 miRNAs (miR-20a, miR-30b and miR-106b) all contained at least one autophagy gene target within the top 25% of the gene ranking list, while both autophagy-related targets of miR-125b-1 were in the bottom 25%. It should also be recognized that the drug tamoxifen can have many different effects on the cell and the differential expression of many of the miRNAs may be due to a separate cellular effect rather than the induction of autophagy, as described above (Section 4.4).

Another shortcoming of this study was that Illumina sequencing was conducted on just one cell line under one autophagy inducing condition. It would be valuable to use the Illumina platform to sequence the miRNAs in all three breast cancer cell-lines in both tamoxifen and starvation-induced autophagy conditions. The significantly differentially expressed miRNAs that were common in all three cell-lines and both conditions could then be validated for expression level and examined further. Such a strategy might have resulted in a greater number of miRNAs that were potential regulators of the autophagy pathway or at least aided in prioritizing the candidates. Also, in relation to prioritizing candidate miRNAs, it would be useful to test whether any of the other twenty miRNAs found with possible autophagy-related targets were also differentially expressed in the three cell-lines under both autophagy-inducing conditions. QRT-PCR analysis was conducted on the seven miRNAs that were considered to have the greatest probability of regulating the process based on the number of potential autophagy gene targets and low p-value, but it could be argued that there were other candidates among the 20 unselected miRNAs worthy of further analysis.

There were also limitations surrounding the software used in the analysis of the Illumina sequencing data. As described above, the mirna code program matches raw sequence reads to the precursor sequences of known miRNAs. The pre-miRNA sequence contains both the mature and passenger strand and will potentially count both as matches to a particular miRNA, even though the passenger strand is often seen to have little functional relevance and is almost immediately degraded after it is released from the

RISC complex (Matranga et al, 2005; Winter, 2009). This may alter the absolute values of the functional miRNAs within samples and may be one of the explanations as to why there were differences in fold expression observed between the sequencing and qRT-PCR analysis, as qRT-PCR only takes the mature strand into account. This is simply a limitation of the analysis software and as it evolves in the future, it should be able to differentiate between the mature and passenger strands of miRNAs to give a more stringent read count. However, as noted in section 4.3, this is just one explanation as to why we see fold expression differences between the two miRNA expression analysis techniques.

5 FUTURE DIRECTIONS

5.1 Sequence analysis

Illumina platform sequencing is a novel technological tool for elucidating miRNA expression profiles and the corresponding software to analyze the data hasn't evolved as fast as the technology. One of the current limitations of this analysis software is that it has not developed to a point where it can accurately identify novel miRNAs within samples, which theoretically can be achieved by direct observational methods such as Illumina, and validation of the folding potential of flanking genomic sequences (Berezikov et al, 2006a). In each of the two samples in this sequenced study, there was a proportion of reads generated (~3%) that did not match to known sequences. Within this group of sequences that map to a location in the genome not currently associated with any notable reads, there may be novel miRNAs that have yet to be discovered. Fortunately the results of the Illumina sequencing remain, and in the future these 'unknown' sequences that mapped to the human genome can be analyzed for their potential to contain novel miRNAs.

5.2 Functional analysis

A step that could be taken in the future to determine whether these differentially expressed miRNAs play a role in autophagy regulation in breast cancer would be functional analysis. Functional studies could include both knocking down and over-expressing the identified miRNAs in the three breast cancer cell-lines and observing the resulting effects on autophagy levels. MiRNA inhibitors, sometimes referred to as antagomirs (Meister and Tuschl, 2006; Zhu et al, 2009), are 15-30 nucleotide anti-sense strands that bind to their complement miRNA sequences within cells, rendering them unable to regulate their mRNA target. Since the four selected miRNAs all possibly target autophagy genes and most miRNAs suppress expression of their gene targets, it would be expected that inhibiting these miRNAs with antagomirs would cause an increase in the

autophagy transcript levels, potentially increasing autophagy. MiRNA precursors or miRNA mimics are small, chemically modified RNA molecules designed to mimic endogenous mature miRNAs (Ji et al, 2008; Zhu et al, 2009). Upon addition to the cells, miRNA mimics essentially over-express the specific miRNA and cause an increase in mRNA target suppression. In this case, it would be expected that increasing the levels of the four selected miRNAs would cause increased repression of their autophagy mRNA transcript targets, leading to a decrease in autophagy. Autophagy levels in these experiments could be determined in a number of ways including observing MDC staining levels, which identify acidic compartments within cells, observing cellular distribution of a GFP-tagged MAP1LC3B, and measuring p62 levels to monitor autophagy flux (Klionsky et al, 2008). One strategy to confirm direct binding to predicted targets is a luciferase assay. In this assay, vectors are constructed encoding a luciferase (renilla or firefly) gene upstream of an autophagy gene with either a wild-type 3'UTR containing the miRNA binding sequence of interest or a mutant form that the miRNA cannot bind (Ender et al, 2008).These two vectors are added to the cells of interest and the suspect miRNA mimic is added. The luciferase activity within the cells is then measured. If the miRNA binds to the wild-type 3'UTR target, the luciferase activity would be decreased relative to the mutated 3'UTR (Ender et al, 2008; Zhu et al, 2009).

5.3 Using miRNAs to control the homeostatic level of autophagy

The miRNA analysis in this study focuses on the regulation of autophagy under cellular stress conditions in cancer cells, when autophagy is induced to elevated levels. Autophagy is also important in homeostatic maintenance within the cell, potentially as a tumor suppressor mechanism, and it could be argued that the identified miRNAs, along with others, may be acting to maintain this homeostatic level of autophagy within cells. It could be the induced stress within the cellular environment that results in the dysregulation (in this case, decrease) of miRNA levels that subsequently lead to the derepression of Atg trancripts levels and an increase in autophagy. Alternatively, these miRNAs may be repressing the autophagy mRNA transcripts under homeostasis by accumulating in the P-bodies (Chan and Slack, 2006). Under cell stress conditions, as

demonstrated in the Bhattacharyya study (2006), the miRNAs may release their autophagy transcript targets, allowing the mRNAs to return to the cytoplasm, leading to a subsequent increase in autophagy. It would be interesting to examine whether these same 4 miRNAs play a role in regulating the autophagy pathway in normal breast cells under both homeostatic and stress conditions. A relatively simple experiment to determine whether any of the four miRNAs can regulate homeostatic levels of autophagy might include adding miRNA inhibitors or miRNA mimics to normal breast cell-lines and observing the effects on the resulting autophagy levels. It might be expected that the introduction of miRNA inhibitors (decreasing levels of the 4 miRNAs) would result in a similar pattern of autophagy induction as tamoxifen exposure or nutrient starvation.

5.4 In vivo analysis

For my results to be investigated further in terms of applicability to solid tissues or pre-clinical potential, the *in vitro* work done in this study requires not only additional functional experiments, but *in vivo* testing in breast cancer models. Presently, monitoring autophagic flux *in vivo* is one of the least developed areas in autophagy research and there are limitations to the different approaches (Klionsky et al, 2008). In future studies, researchers could modulate the suspect miRNA levels through introduction of miRNA inhibitors (antagomirs) and mimics into the breast tissue of mouse models and observe the effects on autophagy levels and the progression and treatment response of the breast cancer. A recent study by Ma et al (2010) used mouse models to observe the functional effects of miR-10b in breast cancer metastasis. Previously it had been discovered that miR-10b is highly expressed in metastatic cancer cells *in vitro* and also in metastatic breast tumors from patients (Ma et al, 2007). MiR-10b was discovered to target Hox10, repressing its transcripts, leading to an increase in RHOC – a well characterized pro-metastatic protein. In the 2010 study, Ma et al implanted 4T1 cells, mouse mammary tumor cells, into the mammary fat pad of immunocompromised mice, leading to the development of breast tumors that were highly metastatic. Intravenous doses of antagomirs – a class of chemically modified anti-oligonucleotides – to miR-10b led to a decrease in 10b expression (65%) and a corresponding increase in Hox10 and decreased

ability of the tumor to metastasize (Ma et al, 2010). A similar approach to the Ma et al study could be taken for further analysis of the four identified miRNAs. Since these miRNAs have shown negative regulation in breast cancer cells under stress conditions, the introduction of miRNA mimics could be introduced to the mammary fat pads of mice with breast cancer, potentially decreasing the levels of autophagy in the tumor, thereby making breast cancer treatment strategies more effective.

The process of autophagy has become more prevalent in the study of cancer over the last decade due to its potential tumor suppressor, tumor survival and possible drug resistance roles. A proliferating cancerous tumor often grows more rapidly than the body can provide it with adequate blood supply. As a result, these tumor masses are often hypoxic and deprived of sufficient nutrients, yet continue to survive and develop. It has been known for some time that hypoxia and nutrient deprivation are among the cellular stress conditions that induce autophagy, and have been the subject of many comprehensive reviews (Levine and Klionsky, 2004; Klionsky, 2007; Mauiri et al, 2007; Rubinsztein et al, 2007). However the exact mechanism by which these cellular stresses regulate autophagy is still not well understood and understanding more about this process could lead to insight into the role autophagy plays in cancer as well as a mechanism to potentially regulate the catabolic process.

5.5 Role of tamoxifen in treating breast cancer in a clinical setting and potential link to autophagy

Since 1971, tamoxifen has been used in the clinic as the gold standard treatment of both early and advanced estrogen-receptor (ER) positive breast cancers (Jordan, 1993). Today it is mainly used in pre- and post-menopausal women with ER+ invasive breast cancer (Visvanathan et al, 2009). It acts as a selective estrogen receptor modulator (SERM), competing for the receptor antagonistically with the estrogen hormone, resulting in recruitment of co-repressors and thereby reducing the levels of ER gene transcription (Osborne and Schiff, 2005). In this study and others (Bursch et al, 1996, Qadir et al, 2008, Sammadar et al, 2008; Lam et al, in prep), high-dose tamoxifen has been shown to induce autophagy in breast cancer cell-lines. The mechanism by which

tamoxifen induces autophagy is not well understood and is thought to be related to its cytotoxic properties (Qadir et al, 2008); although recent evidence supports the theory that sterol accumulation in breast cancer cells leads to an induction of autophagy (de Medina et al, 2009). If the induction of autophagy by tamoxifen is to be applied in a clinical setting, it could mean that high-dose tamoxifen treatment given to women with ER positive breast cancer not only targets ER receptors but its cytotoxic or sterol accumulation effects may also increase autophagy levels within these cancerous cells. High-dose tamoxifen treatments are still given to women with advanced forms of breast cancer, in cases of cancer relapse and in some adjuvant settings (Mandlekar and Kong, 2001; Coleman, 2003; Vsivanathan et al, 2009). According to the autophagy-related tumor survival hypothesis detailed in the introduction, the cytotoxicity of the drug may be upregulating the catabolic and energy synthesis characteristics of the autophagy pathway, possibly leading to resistance to the drug or eventual cancer relapse. In a study by Qadir et al (2008), it was shown that tamoxifen was more efficient in destroying breast cancer cells when autophagy gene function was knocked down by siRNA, through mitochondrial-mediated apoptosis (Qadir et al, 2008). This study, and others, suggests that the repression of autophagy may be useful in combination with chemotherapy drugs in order to sensitize breast-cancer cells to the treatments (including tamoxifen) (Qadir et al, 2008; Samaddar et al, 2008, Lam et al, in preparation).

5.6 Clinical manipulation of autophagy

Most of the information connecting chemotherapy drug use to an induction of autophagy is from *in vitro* studies and very few are from clinical tumor samples or *in vivo* settings. In the clinical setting, there is currently no therapy that directly addresses autophagy's role as a tumor suppressor or survival mechanism in patients. A number of cancer treatment options currently used by clinicians have been found to induce autophagy in tumors (Table 1.2), causing some researchers to speculate that treatment resistance and cancer relapse may be related to the induction of autophagy (Qadir et al, 2007; Sammadar et al, 2008; Abedin et al, 2007; Katayama et al, 2007). Thus, combining autophagy inhibition with current chemotherapy methods may increase their efficacy.

The only known clinically-relevant inhibitor of autophagy is hydroxychloroquine, which is currently being used in clinical trials in combination with chemotherapy agents (http://clinicaltrials.gov/ct2/results?term=hydroxychloroquine+and+cancer&recr=&rslt=&type=&cond=&intr=&outc=&lead=&spons=&id=&state1=&cntry1=&state2=&cntry2=&state3=&cntry3=&locn=&gndr=&rcv_s=&rcv_e=&lup_s=&lup_e=). In this study, I was able to determine that there is a connection between autophagy gene expression levels in breast cancer cells and the expression of miRNAs. MiRNAs are not currently being used as clinical therapeutics, however there is potential to pharmacologically modulate expression of miRNAs during processing or at the level of transcription via small molecule drugs, though additional research into this field is required (Verma and Weitzman, 2005; Pfeifer and Lehmann, 2010). Therefore if it can be further shown that miRNAs functionally regulate the autophagy pathway in breast cancer cell-lines under cell stress conditions, then it provides researchers and clinicians with additional and plausible targets to manipulate autophagy levels in cancer models or patients to prevent the initiation of, or more effectively treat, breast cancer.

REFERENCES

Abedin MJ, Wang D, McDonnell MA, Lehmann U, and Kelekar A. Autophagy delays apoptotic death in breast cancer cell following DNA damage. Cell Death and Differentiation 2007; 14, 500–510.

Agresti, A. A Survey of Exact Inference for Contingency Tables. Statistical Science 1992; 7(1): 131-153.

Aita VM, Liang XH, Murty VV, Pincus DL, Yu W, Cayanis E, Kalachikov S, Gilliam TC, Levine B. Cloning and genomic organization of beclin I, a candidate tumor suppressor gene on chromosome 17q21. Genomics 1999; 59:59-65.

Alam ZI, Jenner A, Daniel SE, Lees AJ, Cairns N, Marsden CD, Jenner P, Halliwell B. Oxidative DNA damage in the parkinsonian brain: an apparent selective increase in 8-hydroxyguanine levels in substantia nigra. J Neurochem. 1997; 69(3):1196-203.

Alvarez-Garcia I and Miska EA. MicroRNA functions in animal development and human disease. *Development* 2005; 132(21): 4653-4662.

Amaravadi RK, Yu D, Lum JJ, Bui T, Christophorou MA, Evan GI, Thomas-Tikhonenko A, Thompson CB. Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. J Clin Invest 2007; 117:326-36.

American Cancer Society (2007). “Cancer Facts and Figures 2007”. Retrieved on 2009/03/11.

Anne SL, Saudou F, and Humbert S. Phosphorylation of Huntingtin by Cyclin-Dependent Kinase 5 Is Induced by DNA Damage and Regulates Wild-Type and Mutant Huntingtin Toxicity in Neurons. The Journal of Neuroscience 2007; 27(27):7318-7328.

Apel A, Herr I, Schwarz H, Rodemann HP, Mayer A. Blocked Autophagy Sensitizes Resistant Carcinoma Cells to Radiation Therapy. Cancer Res 2008; 68: (5): 1485-94.

Arico S, Petiot A, Bauvy C, Dubbelhuis PF, Meijer AJ, Codogno P, Ogier-Denis E. The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. J Biol Chem. 2000; 276(38): 35243-6.

Armstrong LJ, Gorski SM. “Breast Cancer and Autophagy.” Breast Cancer: Causes, Diagnosis and Treatment. Eds. Romero ME and Dashek LM. Nova Science Publishing, 2010. At Press.

Azad MB, Chen Y, Henson ES, Cizeau J, McMillan-Ward E, Israels SJ, Gibson SB. Hypoxia induces autophagic cell death in apoptosis-competent cells through a mechanism involving BNIP3. Autophagy. 2008; 4(2):195-204.

Babar IA, Slack FJ, Weidhaas JB. miRNA modulation of the cellular stress response. Future Oncol. 2008 Apr; 4(2):289-98.

Balkwill F. Cancer and the chemokine network. Nature Rev Cancer. 2004; 4: 540-550.

Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116(2): 281-97.

Bentwich I, Avniel A, Karov Y, Aharonov R, Gilad S, Barad O, Barzilai A, Einat P, Einav U, Meiri E, Sharon E, Spector Y, Bentwich Z. Identification of hundreds of conserved and nonconserved human microRNAs. Nat Genet. 2005; 37(7): 766-70.

Berezikov E, Cuppen E, Plasterk RH. Approaches to microRNA discovery. Noat Genet. 2006a; 38: S2-S7.

Bergamini E, Cavallini G, Donati A, Gori Z. The anti-ageing effects of caloric restriction may involve stimulation of macroautophagy and lysosomal degradation, and can be intensified pharmacologically. Biomed Pharmacother. 2003; 57(5-6):203-8.

Bhattacharyya SN, Habermacher R, Marine U, Closs EI, Filipowicz W. Relief of microRNA-mediated translational repression in human cells subjected to stress. Cell 2006; 125(6): 1111-24.

Biederbick A, Kern HF, Elsasser HP. Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles. Eur J Cell Biol. 1995; 66:3-14.

Bilir A, Altinoz MA, Erkan M, Ozman V, Aydiner A. Autophagy and nuclear changes in FM3A breast tumor cells after epirubicin, medroxyprogesterone and tamoxifen treatment in vitro. Pathobiology 2001; 69: 120-126.

Bjorkoy G, Lamark T, Pankiv S, Overvatn A, Brech A, Johansen T. Monitoring autophagic degradation of p62/SQSTM1. Methods Enzymol. 2009; 452: 181-97.

Blommaart, Krause U, Schellens JP, Vreeling-Sindelárová H, Meijer AJ. The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit in isolated rat hepatocytes. Eur. J. Biochem1997; 243: 240–246.

Boya P, González-Polo RA, Casares N, Perfettini JL, Dessen P, Larochette N, Métivier D, Meley D, Souquere S, Yoshimori T, Pierron G, Codogno P, Kroemer G. Inhibition of macroautophagy triggers apoptosis. Mol Cell Biol. 2005; 25(3):1025-40.

Bursch, W. The autophagosomal-lysosomal compartment in programmed cell death. Cell Death Differ 2001; 8:569–81.

Bursch W, Ellinger A, Gerner C, Fröhwein U, Schulte-Hermann R. Programmed cell death (PCD). Apoptosis, autophagic PCD, or others? Ann. N. Y. Acad. Sci. 2000; 926: 1 – 12.

Bursch W, Ellinger A, Kienzl H, Török L, Pandey S, Sikorska M, Walker R, Hermann RS. Active cell death induced by the anti-estrogens tamoxifen and ICI 164 384 in human mammary carcinoma cells (MCF7) in culture: the role of autophagy. Carcinogenesis 1996; 17:1595-1607.

Bursch W, Hochegger K, Torok L, Marian B, Ellinger A, Hermann RS. Autophagic and apoptotic types of programmed cell death exhibit different fates of cytoskeletal filaments. J. Cell Sci. 2000; 113:1189–1198.

Cadwell K, Liu JY, Brown SL, Miyoshi H, Loh J, Lennerz JK, Kishi C, Kc W, Carrero JA, Hunt S, Stone CD, Brunt EM, Xavier RJ, Sleckman Bp, Li E, Mizushima N, Stappenbeck TS, Virgin HW4th. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. Nature. 2008; 456:259-63.

Calin GA and Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006; 6(11): 857-66.

Calin GA, Dumitru CD, Shimizu M, Bichi R, Zupo S, Noch E, Aldler H, Rattan S, Keating M, Rai K, Rassenti L, Kipps T, Negrini M, Bullrich F, Croce CM. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proc Natl Acad Sci USA. 2002; 99(24): 15524-9.

Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, Wojcik SE, Iorio MV, Visone R, Sever NI, Fabbri M, Iuliano R, Palumbo T, Pichiorri F, Roldo C, Garzon R, Sevignani C, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med. 2005; 353(17): 1793-801.

Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, Yendamuri S, Shimizu M, Rattan S, Bullrich F, Negrini M, Croce CM. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. Proc Natl Acad Sci USA 2004; 101: 2999-3004.

Cameron DA, Keen JC, Dixon JM, Bellamy C, Hanby A, Anderson TJ, Miller WR. Effective tamoxifen therapy of breast cancer involves both antiproliferative and pro-apoptotic changes. Eur J Cancer 2000; 36: 845–851.

Chan EY, Longatti A, McKnight NC, Tooze SA. Kinase-inactivated ULK proteins inhibit autophagy via their conserved C-terminal domains using an Atg13-independent mechanism. Mol Cell Biol. 2009; 29(1): 157-71.

Chan SP and Slack FJ. MicroRNA-mediated silencing inside P-bodies. RNA Biol. 2006; 3(3): 97-100.

Chen J, Lozach J, Garcia EW, Barnes B, Luo S, Mikoulitch I, Zhou L, Schroth G, Fan JB. Highly sensitive and specific microRNA expression profiling using BeadArray technology. Nucleic acids Res. 2008; 36: e87.

Cheng AL, Chuang SE, Fine RL, Yeh KH, Liao CM, Lay JD, Chen DS. Inhibition of the membrane translocation and activation of protein kinase C, and potentiation of doxorubicin-induced apoptosis of hepatocellular carcinoma cells by tamoxifen. Biochem Pharmacol. 1998; 55: 523–531.

Chiou TJ. The role of micro RNAs in sensing nutrient stress. Plant Cell Environ. 2007; 30(3): 323-332.

Chu CT. Autophagic stress in neuronal injury and disease. J Neuropathol Exp Neurol 2006;65:423–32.

Cimmino A, Calin GA, Fabbri M, Iorio MV, Ferracin M, Shimizu M, Wojcik SE, Aqeilan RI, Zupo S, Dono M, Rassenti L, Alder H, Volinia S, Liu CG, Kipps TJ, Negrini M, Croce CM. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc Natl Acad Sci USA. 2005; 102(39): 1394409.

Clarke PG. Developmental cell death: morphological deversity and multiple mechanisms. Anat. Embryol. 1990; 181:195-213.

Clark R, Leonessa F, Welch JN, Skaar TC. Cellular and molecular pharmacology of antiestrogen action and resistance. Pharmacol Rev 2001; 53:25-71.

Coleman RE. Current and future status of adjuvant therapy for breast cancer. Cancer. 2003; 97(3 Suppl): 880-6.

Crighton D, Wilkinson S, O'Prey J, Syed N, Smith P, Harrison PR, Gasco M, Garrone O, Crook T, Ryan KM. DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. Cell 2006; 126: 121-134.

Cuervo AM. Autophagy: in sickness and health. Trends Cell Biol. 2004;14(2):70-7.

Cuervo AM, Bergamini E, Brunk UT, Droge W, French M, Terman A. Autophagy and aging: The importance of maintaining "clean" cells. Autophagy 2005; 1:131-40.

Davoren PA, McNeill RE, Lowery AJ, Kerin MJ, Miller N. Identification of suitable endogenous control genes for microRNA gene expression analysis of human breast cancer. BMC Mol Biol. 2008; 9:76-87.

Debnath J. Detachment-induced autophagy during anoikis and lumen formation in epithelial acini. Autophagy 2008; 4(3):351-3.

Debnath J, Mills KR, Collins NL, Reginato MJ, Muthuswamy SK, Brugge JS. The role of apoptosis in creating and maintaining luminal space within normal and oncogeni-expressing mammary acini. Cell 2002; 111:29-40.

Debnath J, Walker SJ, Brugge JS. Akt activation disrupts mammary acinar architecture and enhances proliferation in an mTOR-dependent manner. J Cell Biol. 2003; 163(2):315-26.

Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, Mukherjee C, Shi Y, Gélinas C, Fan Y, Nelson DA, Jin S, White E. Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell 2006; 10: 51-64.

Demasters G, Di X, Newsham I, Shiu R, Gewirtz DA. Potentiation of radiation sensitivity in breast tumor cells by the vitamin D3 analogue, EB 1089, through promotion of autophagy and interference with proliferative recovery. Mol Cancer Ther. 2006; 5(11): 2786-97.

Denli AM, Tops BB, Pasterk RH, Ketting RF, Hannon GJ. Processing of primary microRNAs by the Microprocessor complex. Nature 2004; 432(7014): 235-40.

Doebele C, Bonauer A, Fischer A, Scholz A, Reiss Y, Urbich C, Hofmann WK, Zeiher AM, Dimmeler S. Members of the microRNA-17-92 cluster exhibit a cell intrinsic anti-angiogenic function in endothelial cells. Blood. 2010; [Epub ahead of print].

de Duve C. Lysosomes. J. & A. Churchill, London 1963.

Ender C, Krek A, Friedlander MR, Beitzinger M, Weinmann L, Chen W, Pfeffer S, Meister G. A human snoRNA with microRNA-like functions. Mol Cell. 2008; 32: 519-528.

Esquela-Kerscher A and Slack FJ. Oncomirs – microRNAs with a role in cancer. Nat Rev Cancer 2006; 6(4):259-69.

Filigheddu N, Gregnanin I, Porporato PE, Surico D, Perego B, Galli L, Patrignani C, Graziani A, Surico N. Differential expression of microRNAs between eutopic and ectopic endometrium in ovarian endometriosis. J Biomed Biotechnol. 2010; 2010: 369549-78.

Fischer RA. On the interpretation of X^2 from contingency tables, and the calculation of P. Journal of the Royal Statistical Society 1922. 85(1): 87-94.

Fontana L, Fiori ME, Albini S, et al. Antagomir-17-5p abolishes the growth of

therapy-resistant neuroblastoma through p21 and BIM. *PLoS One.* 2008; 3(5):e2236.

Friedman RC, Farh KKH, Burge CB, Bartel DP. Most Mammalian mRNAs Are Conserved Targets of MicroRNAs. Genome Res. 2009; 19(1): 92-105.

Fujii H, Chiou TJ, Lin SI, Aung K, Zhu JK. A miRNA involved in phosphate-starvation response in *Arabidopsis*. Curr Biol. (2005); 15(22): 2038-2043.

Fujita N, Hayashi-Nishino M, Fukumoto H, Omori H, Yamamoto A, Noda T, Yoshimori T. An Atg4B mutant hampers the lipidation of LC3 paralogues and causes defects in autophagosome closure. Mol Biol Cell. 2008; 19(11):4651-59.

Funakoshi T, Matsuura A, Noda T, and Ohsumi Y. Analyses of APG13 gene involved in autophagy in yeast, *Saccharomyces cerevisiae*. Gene 1997; 192:207-213.

Fung C, Lock R, Gao S, Salas E, Debnath J. Induction of autophagy during extracellular matrix detachment promotes cell survival. Mol Biol Cell. 2008; 19(3): 797-806.

Gelmann EP. Tamoxifen induction of apoptosis in estrogen receptor-negative cancers: New tricks for an old dog? J Natl Cancer Inst 1996; 88: 224–226.

Gills JJ, Lopiccolo J, Tsurutani J, Shoemaker RH, Best CJ, Abu-Asab MS, Borojerdi J, Warfel NA, Gardner ER, Danish M, Hollander MC, Kawabata S, Tsokos M, Figg WD, Steeg PS, Dennis PA. Nelfinavir, A lead HIV protease inhibitor is a broad-spectrum, anti-cancer agent that induces endoplasmic reticulum stress, autophagy, and apoptosis in vitro and in vivo. Clin Cancer Res 2007; 13:5183-94.

Gregory RI and Sheikhattar R. MicroRNA biogenesis and cancer. Cancer Res. 2005; 65: 3509-3512.

Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, Shiekhattar R. The Microprocessor complex mediates the genesis of microRNAs. Nature 2004; 432(7014): 235-40.

Griffiths-Jones S. The microRNA Registry. Nucleic Acids Research 2004; 32: D109-D111.

Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Research 2006; 34: D140-D144.

Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ. miRBase: tools for microRNA genomics. Nucleic Acids Research 2008; 36: D154-158.

Groth-Pedersen L, Ostenfeld MS, Hoyer-Hansen M, Nylandsted J, Jaattela M. Vincristine induces dramatic lysosomal changes and sensitizes cancer cells to lysosome-destabilizing siramesine. Cancer Res 2007; 67:2217-25.

Guruju MR, Zhou Y, Infanger DW, Braga VA, Peterson J, Sharma RV, Davisson RL. Identification of Differentially-Expressed MicroRNAs in the Paraventricular Nucleus (PVN) Following Myocardial Infarction (MI). The FASEB Journal 2008; 22: 952-7.

Hampe J, Franke A, Rosenstiel P, Till A, Teuber M, Huse K, Albrecht M, Mayr G, De La Vega FM, Briggs J, Günther S, Prescott NJ, Onnie CM, Häsler R, Sipos B, Fölsch UR, Lengauer T, Platzer M, Mathew CG, Krawczak M, Schreiber S. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. Nat Genet. 2007; 39(2):207-11.

Han J, Lee Y, Yeom KH, Kim YK, Jin H, Kim VN. The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev. 2004; 18(24): 3016-27.

Hanahan D and Weinburg RA. The Hallmarks of Cancer. Cell 2000; 100: 57-70.

Hannigan AM and Gorski SM. Macroautophagy: The key ingredient to a healthy diet? Autophagy 2009; 5(2): 140-151.

Harding TM, Morano KA, Scott SV, Klionsky DJ. Isolation and characterization of yeast mutants in the cytoplasm to vacuole protein targeting pathway. J Cell Biol 1995;131(3):591-602.

Harris AL. Hypoxia – a key regulatory factor in tumour growth. Nat. Rev. Cancer 2002; 2: 38-47.

Hay N and Sonenberg, N. Upstream and downstream of mTOR. Genes Dev 2004; 18: 1926–1945.

He H, Dang Y, Dai F, Guo Z, Wu J, She X, Pei Y, Chen Y, Ling W, Wu C, Zhao S, Liu JO, Yu L. Post-translational modifications of three members of the human MAP1LC3 family and detection of a novel type of modification for MAP1LC3B. J Biol Chem. 2003; 278(31):29278-87.

Hebert SS, Horre K, Nicolai L, Papadopoulou AS, Mandemakers W, Silahtaroglu AN, Kauppinen S, Delacourte A, De Strooper B. Loss of microRNA cluster miR-29a/b-1 in sporadic Alzheimer's disease correlates with increased BACE1/beta-secretase expression. Prob Natl Acad Sci USA. 2008; 105(17): 6415-20.

Hemelaar J, Lelyveld VS, Kessler BM, Ploegh HL. A single protease, Apg4B, is specific for the autophagy-related ubiquitin-like proteins GATE-16, MAP1-LC3, GABARAP, and Apg8L. J Biol Chem. 2003; 278(51):51841-50.

Henson BJ, Bhattacharjee S, O'Dee DM, Feingold E, Gollin SM. Decreased expression of miR-125b and miR-100 in oral cancer cells contributes to malignancy. Genes Chromosomes Cancer. 2009; 48(7): 569-82.

Hippert MM, O'Toole PS, and Thorburn A. Autophagy in Cancer: Good, Bad, or Both? Cancer Res 2006; 66(19): 9349-9351.

Hirsch C, Gauss R, Sommer T. Coping with stress: cellular relaxation techniques. Trends Cell Biol 2006; 16(12): 657-663.

Hofmann MH, Heinrich J, Radziwil G, Moelling K. A short hairpin DNA analogous to miR-125b inhibits C-Raf expression, proliferation, and survival of breast cancer cells. Mol Cancer Res. 2009; 7(10): 1635-44.

de Hoon MJ, Taft RJ, Hshimoto T, Kanamori-Katayama M, Kawaji H, Kawano M, Kishima M, Lassman T, Faulkner GJ, Mattick JS, Daub CO, Carninci P, Kawai J, Suzuki H, Hayashizaki Y. Cross-mapping and the identification of editing sites in mature microRNAs in high-throughput sequencing libraries. Genome Res. 2010; 20(2): 257-64.

Hossain A, Kuo MT, Saunders GF. Mir-17-5p regulates breast cancer cell proliferation by inhibiting translation of AIB1 mRNA. Mol Cell Biol. 2006; 26(21): 8191-201.

Høyer-Hansen M, Bastholm L, Mathiasen IS, Elling F, Jäättelä M. Vitamin D analog EB1089 triggers dramatic lysosomal changes and Beclin 1-mediated autophagic cell death. Cell Death Differ. 2005;12(10):1297-309.

Høyer-Hansen M, Bastholm L, Szyniarowski P, Campanella M, Szabadkai G, Farkas T, Bianchi K, Fehrenbacher N, Elling F, Rizzuto R. Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2. Mol. Cell, 2007; 25: 193–205.

Høyer-Hansen M and Jäättelä M. Autophagy: an emerging target for cancer therapy. Autophagy. 2008; 4(5):574-80.

Ichimura Y, Kirisako T, Takao T, Satomi Y, Shimonishi Y, Ishihara N, Mizushima N, Tanida I, Kominami E, Ohsumi, M. A ubiquitin-like system mediates protein lipidation. Nature 2000. 408, 488–492.

Inoki K, Zhu T, Guan KL. TSC2 mediates cellular energy response to control cell growth and survival. Cell 2003; 115(5):577-90.

Inomata M, Tagawa H, Guo YM, Kameoka Y, Takahashi N, Sawada K. MicroRNA-17-92 down-regulates expression of distinct targets in different B-cell lymphoma subtypes. Blood. 2009; 113(2): 396-402.

Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, Magri E, Pedriali M, Fabbri M, Campiglio M, Ménard S, Palazzo JP, Rosenberg A, Musiani P, Volinia S, Nenci I, Calin GA, Querzoli P, Negrini M, Croce CM. MicroRNA gene expression deregulation in human breast cancer. Cancer Res. 2005; 65(16): 7065-70.

Iorio MV, Visone R, Di Leva G, Donati V, Petrocca F, Casalini P, Taccioli C, Volinia S, Liu CG, Alder H, Calin GA, Ménard S, Croce CM. MicroRNA signatures in human ovarian cancer. Cancer Res 2007; 67:8699-707.

Ishii H and Saito T. Radiation-induced response of micro RNA expression in murine embryonic stem cells. Med Chem. 2(6): 555-563.

Itakura E, Kishi C, Inoue K, Mizushima N. Beclin 1 forms two distinct phosphatidylinositol 3-kinase complexes with mammalian Atg14 and UVRAG. Mol Biol Cell. 2008; 19(12):5360-72.

Ivanovska I, Ball AS, Diaz RL, Magnus JF, Kibukawa M, Schelter JM, Kobayashi SV, Lim L, Burchard J, Jackson AL, Linsley PS, Cleary MA. MicroRNAs in the miR-106b family regulate p21/CDKN1A and promote cell cycle progression. Mol Cell Biol. 2008; 28(7): 2167-74.

Iwai-Kanai E, Yuan H, Huang C, Sayen MR, Perry-Garza CN, Kim L, Gottlieb RA. A method to measure cardiac autophagic flux in vivo. Autophagy 2008; 4(3): 322-9.

Jessani N, Niessen S, Mueller BM, Cravatt BF. Breast cancer cell lines grown in vivo: what goes in isn't always the same as what comes out. Cell Cycle. 2005; 4(2):253-5.

Ji Q, Hao X, Meng Y, Zhang M, Desano J, Fan D, Xu L. Restoration of tumor suppressor miR-34 inhibits human p53-mutant gastric cancer tumorspheres. BMC Cancer. 2008; 8: 266.

John B, Enright AJ, Aravin A, Tuschl T, Sander C, Marks DS. Human MicroRNA Targets. PLoS Biol. 2004; 2(11): e363.

Jordan VC. “Fourteenth Gaddum Memorial Lecture. A current view of tamoxifen for the treatment and prevention of breast cancer”. Br J Pharmacol. 1993; 110(2): 507-517.

Jung HJ and Kang H. Expression and functional analyses of microRNA417 in *Arabidopsis thaliana* under stress conditions. Plant Physiol Biochem. 2007; 45(10-11): 805-811.

Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 2000; 19:5720-8.

Kamada Y, Funakoshi T, Shintani T, Nagano K, Ohsumi M, Ohsumi Y. Tor-mediated induction of autophagy via an Apg1 protein kinase complex. J Cell Biol 2000; 150:1507-13.

Kametaka S, Matsuura A, Wada Y, and Ohsumi Y. Structural and functional analyses of *APG5*, a gene involved in autophagy in yeast. Gene 1996; 178, 139–143.

Kametaka S, Okano T, Ohsumi M, and Ohsumi Y. Apg14p and Apg6/Vps30p form a protein complex essential for autophagy in the yeast, *Saccharomyces cerevisiae*. J. Biol. Chem. 1998; 273, 22284–22291.

Kan T, Sato F, Ito T, Matsumura N, David S, Cheng Y, Agarwal R, Paun BC, Jin Z, Olaru AV, Selaru FM, Hamilton JP, Yang J, Abraham JM, Mori Y, Meltzer SJ. The miR-106b-25 polycistron, activated by genomic amplification, functions as an oncogene by suppressing p21 and Bim. Gastroenterology. 2009; 136(5): 1689-700.

Kang Y, Cortina R, Perry RR. Role of c-myc in tamoxifeninduced apoptosis estrogen-independent breast cancer cells. J Natl Cancer Inst 1996; 88: 279–284.

Karantza-Wadsworth V, Patel S, Kravchuk O, Chen G, Mathew R, Jin S, White E. Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. Genes & Dev. 2007 21: 1621-1635.

Karim MR, Kanazawa T, Daigaku Y, Fujimura S, Miotto G, Kadowaki M. Cytosolic LC3 ratio as a sensitive index of macroautophagy in isolated rat hepatocytes and H4-II-E cells. Autophagy 2007; 3:553-60.

Katayama M, Kawaguchi T, Berger MS, Pieper RO. DNA damaging agent-induced autophagy produces a cytoprotctive adenosine triphosphate surge in malignant glioma cells. Cell Death Differ. 2007; 14: 548-58.

Kavacs AL, Rith A, Seglen PO. Accumulation of autophagosomes after inhibition of hepatocytic protein degradation by vinbalstine, leupeptin or a lysosomotropic amine. Exp Cell Res 1982; 137:191-201.

Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer 1972; 26 (4): 239–57.

Khvorova A, Reynolds A, Jayasena SD. Functional siRNAs and miRNAs exhibit strand bias. Cell 2003; 115: 209-216.

Kihara A, Kabeya Y, Oshumi Y, Yoshimori T. Beclin-phosphatidylinositol 3-kinase complex functions at the trans-Golgi network. EMBO Rep 2001; 2:330-5.

Kim J, Dalton VM, Eggerton KP, Scott SV, and Klionsky DJ. Apg7p/Cvt2p is required for the cytoplasm-to-vacuole targeting, macroautophagy, and peroxisome degradation pathways. Mol. Biol. Cell 1999; 10:1337–1351.

Kim KW, Mutter RW, Cao C, Albert JM, Freeman M, Hallahan DE, Lu B. Autophagy for cancer therapy through inhibition of pro-apoptotic proteins and mammalian target of rapamycin signaling. J Biol Chem. 2006; 281(48):36883-90.

Kimura S, Noda T, Yoshimori T. Dissection of the autophagosome maturation process by a novel reporter protein, tandem fluorescent-tagged LC3. Autophagy 2007; 3: 452-60.

Kirisako T, Baba M, Ishihara N, Miyazawa K, Ohsumi M, Yoshimori T, Noda T, Ohsumi Y. Formation process of autophagosome is traced with Apg8/Aut7p in yeast. J Cell Biol. 1999;147(2):435-46.

Kirisako T, Ichimura Y, Okada H, Kabeya Y, Mizushima N, Yoshimori T, Ohsumi M, Takao T, Noda T, Ohsumi Y. The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. J Cell Biol. 2000; 151(2):263-76.

Klionsky et al. Guidelines for the use and interpretation of assays for monitorig autophagy in higher eukaryotes. Autophagy 2008; 4(2): 139-40.

Klionsky DJ, Cregg JM, Dunn Jr. WA, Emr SD, Sakai Y, Sandoval IV, Sibirny A, Subramani S, Thumm M, Veenhuis M, Ohsumi Y. A unified nomenclature for yeast autophagy-related genes. Dev. Cell 2003; 5:539-545.

Kouroku Y, Fujita E, Tanida I, Ueno T, Isoai A, Kumagai H, Ogawa S, Kaufman RJ, Kominami E, Momoi T. ER stress (PERK/eIF2a phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. Cell Death Differ. 2007; 14:230-9.

Kregel KC. Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerace. J. Appl Physiol. 2002; 92(5): 2177-2186.

Krichevsky AM, King KS, Donahue CP, Khrapko K, Kosik KS. A microRNA array reveals extensive regulation of microRNAs during brain development. RNA 2003; 9: 1274-1281.

Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonák J, Lind K, Sindelka R, Sjöback R, Sjögreen B, Strömbom L, Ståhlberg A, Zoric N. The real-time polymerase chain reaction. Mol Aspects Med. 2006; 27(2-3): 95-125.

Kulshrehtha R, Ferracin M, Wojcik SE, Garzon R, Alder H, Agosto-Perez FJ, Davuluri R, Liu CG, Croce CM, Negrini M, Calin GA, Ivan M. A microRNA signature of hypoxia. Mol Cell Biol. 2007; 27(5): 1859-1867.

Kultz D. Molecular and evolutionary basis of the cellular stress response. Annu Rev Physiol. 2005; 67: 225-257.

Kuma A, Hatano M, Matsui M, Yamamoto A, Nakaya H, Yoshimori T, Ohsumi Y, Tokuhisa T, Mizushima N. The role of autophagy during the early neonatal starvation period. Nature 2004; 432:1032–1036.

Kuma A, Mizushima N, Ishihara N, Ohsumi Y. Formation of the approximately 350-kDa Apg12-Apg5.Apg16 multimeric complex, mediated by Apg16 oligomerization, is essential for autophagy in yeast. J Biol Chem. 2002; 277(21):18619-25.

Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. Science 2001; 294: 858-862.

Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. Cur Biol. 2002; 12(9): 735-39.

Lam J, Qadir M, Leung A, and Gorski SM. Autophagy inhibition decreases survival of ER- and Her2+ SKBR3 breast cancer cells (manuscript in preparation).

Lang T, Reiche S, Straub M, Bredschneider M, Thumm M. Autophagy and the cvt pathway both depend on AUT9. J Bacteriol. 2000; 182(8):2125-33.

Lee Y, Jeon K, Lee JT, Kim S, Kim VN. MicroRNA maturation: stepwise processing and subcellular localization. EMBO Journal 2002; 21: 4663-4670.

Lesfargues EY, Coutinho WG, Redfield ES. Isolation of two human tumor epithelial cell lines from solid breast carcinomas. J Natl Cancer Inst. 1978; 61(4):967-78.

Levine AJ. p53, the cellular gatekeeper for growth and division. *Cell* 1997; 88: 323-331.

Levine B. Cell biology: autophagy and cancer. Nature. 2007; 446(7137): 745-7.

Levine B and Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev Cell. 2004; 6(4):463-77.

Levine B and Kroemer G. Autophagy in the pathogenesis of disease. Cell 2008; 132:27-42.

Levine B and Yuan J. Autophagy in cell death: an innocent convict? J Clin Invest. 2005; 115(10):2679-88.

Lewis BP, Burge CB, Bartel DP. Conserved Seed Pairing, Often Flanked by Adenosines, Indicates that Thousands of Human Genes are MicroRNA Targets. Cell 2005; 120:15-20.

Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. Cell 2003; 115: 787-798.

Liang XH, Jackson S, Seaman M et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 1999; 402:672-6.

Liang XH, Yu J, Brown K, Levine B. Beclin 1 contains a leucine-rich nuclear export signal that is required for its autophagy and tumor suppressor function. Cancer Res. 2001; 61(8):3443-9.

Lim LP, Lau NC, Garret-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM. Microarray analysis shows that some mircoRNAs downregulate large numbers of target mRNAs. Nature. 2005; 433(7027): 769-73.

Linsen SE, de Wit E, Janssens G, Heater S, Chapman L, Parkin RK, Fritz B, Wyman SK, de Bruijn E, Voest EE, Kuersten S, Tewari M, Cuppen E. Limitations and possibilities of small RNA digital gene expression profiling. Nat. Methods. 2009; 6: 474-476.

Lomonaco SL, Finniss S, Xiang C, Decarvalho A, Umansky F, Kalkanis SN, Mikkelsen T, Brodie C. The induction of autophagy by gamma-radiation contributes to the radioresistance of glioma stem cells. Int J Cancer. 2009; 125(3): 717-22.

Lu Y, Ryan SL, Elliott DJ, Bignell GR, Futreal PA, Ellison DW, Bailey S, Clifford SC. Amplification and overexpression of Hsa-miR-30b, Hsa-miR-30d and KHDRBS3 at 8q24.22-q24.23 in medulloblastoma. PLoS One. 2009; 4(7): e6159.

Lu Y, Thompson JM, Wong HY, Hammond SM, Hogan BL. Transgenic over-expression of the microRNA miR-17-92 cluster promotes proliferation and inhibits differentiation of lung epithelial progenitor cells. Dev Biol 2007; 310: 442-453.

Lu Z, Luo RZ, Lu Y, Zhang X, Yu Q, Khare S, Kondo S, Kondo Y, Yu Y, Mills GB, Liao WS, Bast RC Jr. The tumor suppressor gene ARHI regulates autophagy and tumor dormancy in human ovarian cancer cells. J Clin Invest 2008; 118(12): 3917-29.

Lund E, Guttinger S, Calado A, Dahlberg JE, Kutay U. Nuclear export of microRNA precursors. Science 2004; 303(5654): 95-8.

Ma L, Reinhardt F, Pan E, Soutschek J, Bhat B, Marcusson EG, Teruya-Feldstein J, Bell GW, Weinberg RA. Therapeutic silencing of miR-10b inhibits metastasis in a mouse mammary tumor model. Nat Biotechnol. 2010; 28(4): 341-7.

Ma L, Teruya-Feldstein J, Weinberg RA. Tumor invasion and metastasis intiated by microRNA-10b in breast cancer. Nature 2007; 449: 682-688.

Maiuri MC, Zalckvar E, Kimchi A, and Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat. Rev. Mol. Cell Biol 2007; 8: 741-752.

Mandlekar S and Kong AT. Mechanisms of tamoxifen-induced apoptosis. Apoptosis 2001; 6: 469-477.

Mandlekar S,Yu R, Tan TH, Kong AN. Activation of caspase-3 and c-Jun NH2-terminal kinase-1 signaling pathways in tamoxifen-induced apoptosis of human breast cancer cells. Cancer Res 2000; 60: 5995–6000.

Mariño G, Salvador-Montoliu N, Fueyo A, Knecht E, Mizushima N, López-Otín C. Tissue-specific autophagy alterations and increased tumorigenesis in mice deficient in Atg4C/autophagin-3. J Biol Chem. 2007;282(25):18573-83.

Marsit CJ, Eddy K, Kelsey KT. microRNA responses to cellular stress. Cancer Res. 2006; 66(22): 1084-1088.

Mathew R, Karantza-Wadsworth V, and White, E. Role of autophagy in cancer. Nat. Rev. Cancer 2007; 7: 961-967.

Mathew R, Kongara S, Beaudoin B, Karp CM, Bray K, Degenhardt K, Chen G, Jin S, White E. Autophagy suppresses tumor progression by limiting chromosomal instability. Genes Dev. 2007; 21(11):1367-81.

Matranga C, Tomari Y, Shin C. Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell 2005; 123(4): 607-20.

Mattie MD, Benz CC, Bowers J, Sensinger K, Wong L, Scott GK, Fedele V, Ginzinger D, Getts R, Haqq C. Optimized high-throughput microRNA expression profiling provides novel biomarker assessment of clinical prostate and breast cancer biopsies. Mol Cancer. 2006; 5:24.

McManus HT. MicroRNAs and cancer. Semin Cancer Biol. 2003; 13: 253-258.

De Medina P, Silvente-Poirot S and Poirot M. Tamoxifen and AEBS ligands induced apoptosis and autophagy in breast cancer cells through the stimulation of sterol accumulation. Autophagy 2009; 5(7): 1066-1067.

Meijer WH, van der Klei IJ, Veenhuis M and Kiel JAKW. ATG Genes Involved In Non-Selective Autophagy are Conserved from Yeast to Man, But the Selective Cvt and Pexophagy Pathways also Require Organism-Specific Genes. Autophagy 2007; 3: 2, 106-116.

Meister G and Tuschl T. Mechanisms of gene silencing by double-stranded RNA. Nature 2004; 431(2006): 343-9.

Meléndez A and Neufeld TP. The cell biology of autophagy in metazoans: a developing story. Development. 2008;135(14):2347-60.

Melendez A, Tallóczy Z, Seaman M, Eskelinen EL, Hall DH, Levine B. Essential role of autophagy genes in dauer development and lifespan extension in C. elegans. Science 2003; 301:1387 – 1391.

Meley D, Bauvy C, Houben-Weerts JH, Dubbelhuis PF, Helmond MT, Codogno P, Meijer AJ. AMP-activated protein kinase and the regulation of autophagic proteolysis. J. Biol. Chem 2006; 281(46): 34870-34879.

Mendall JT. miRiad roles for the miR-17-92 cluster in development and disease. Cell 2008; 133(2): 217-22.

Menzies FM, Ravikumar B, and Rubinsztein DC. Protective roles for induction of autophagy in multiple proteinopathies. Autophagy 2006; 2: 224–225.

Mersey BD, Jin P, Danner DJ. Human microRNA (miR29b) expression controls the amount of branched chain alpha-ketoacid dehydrogenase complex in a cell. Hum Mol Genet. 2005; 14(22): 3371-7.

Michael MZ, O'Connor SM, van Holst Pellekaan NG, Young GP, James RJ. Reduced accumulation of specific microRNAs in colorectal neoplasia. Mol Cancer Res. 2003; 1(12): 882-91.

Mizushima N and Klionsky D. Protein turnover via autophagy: implications for metabolism. Annu. Rev. Nutr. 2007; 27: 19-40.

Mizushima N, Kuma A, Kobayashi Y, Yamamoto A, Matsubae M, Takao T, Natsume T, Ohsumi Y, Yoshimori T. Mouse Apg16L, a novel WD-repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate. J Cell Sci. 2003; 116(Pt 9):1679-88.

Mizushima N, Noda T, Ohsumi Y. Apg16p is required for the function of the Apg12p-Apg5p conjugate in the yeast autophagy pathway. EMBO J. 1999; 18(14):3888-96.

Mizushima N, Noda T, Yoshimori T, Tanaka Y, Ishii T, George MD, Klionsky DJ, Ohsumi M, Ohsumi Y. A protein conjugation system essential for autophagy. Nature. 1998; 395(6700):395-8.

Mizushima N, Sugita H, Yoshimori T, Ohsumi Y. A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy. J Biol Chem. 1998; 273(51):33889-92.

Mizushima N and Yoshimori T. How to interpret LC3 immunoblotting. Autophagy 2007; 3: 542-5.

Moll UM and Zaika A. Nuclear and mitochondrial apoptotic pathways of p53. FEBS Lett. 2001; 493(2-3):65-9.

Morin RD, Zhao Y, Prabhu AL, Dhalla N, McDonald H, Pandoh P, Tam A, Zeng T, Hirst M, Marra M. "Preparation and Analysis of microRNA Libraries using the Illumina Massively Parallel Sequencing Technology." RNAi and microRNA-Mediated Gene Regulation in Stem Cells. Eds. Zhang B and Stellwag EJ. Springer: Methods in Molecular Biology Vol.650; 2010 [In Press].

Mourelatos Z, Dostie J, Paushkin S, Sharma A, Charroux B, Abel L, Rappsilber J, Mann M, Dreyfuss G. miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes Dev. 2002; 16: 720-728.

Nakatsuru S, Sudo K, Nakamura Y. Isolation and mapping of a human gene (MCM2) encoding a product homologous to yeast proteins involved in DNA replication. Cytogenet Cell Genet**.** 1995; 68(3-4): 226–30.

Nara A, Mizushima N, Yamamoto A, Kabeya Y, Ohsumi Y, Yoshimori T. SKD1 AAA ATPase-dependent endosomal transport is involved in autolysosome formation. Cell Struct Funct. 2002; 27:29-37.

Nelson PT, Baldwin DA, Scearce LM, Oberholtzer JC, Tobias JW, Mourelatos Z. Microarray-based, high-throughput gene expression profiling of microRNAs. Nat. Methods 2004; 1: 155-161.

Nixon RA. Autophagy, amyloidogenesis and Alzheimer disease. J Cell Sci 2007;120:4081–91.

Noda T, Kim J, Huang WP, Baba M, Tokunaga C, Ohsumi Y, Klionsky DJ. Apg9p/Cvt7p is an integral membrane protein required for transport vesicle formation in the Cvt and autophagy pathways. J Cell Biol. 2000; 148(3):465-80.

Noda T and Ohsumi Y. Tor, a Phosphatidylinositol Kinase Homologue, Controls Autophagy in Yeast. J Biol Chem 1998; 273 (7): 3963-3966.

O'Brian CA, Ioannides CG, Ward NE, Liskamp RM. Inhibition of protein kinase C and calmodulin by the geometric isomers cis- and trans-tamoxifen. Biopolymers 1990; 29: 97–104.

O'Day E. Lal A. MicroRNAs and their target gene networks in breast cancer. Breast Cancer Res. 2010; 12(2):201 [Epub ahead of print].

O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature.* 2005; 435(7043):839-843.

Osborne CK and Schiff R. Estrogen-receptor biology: continuing progress and therapeutic implications. J Clin Oncol. 2005; 23: 1616-22.

Ota A, Tagawa H, Karnan S, Tsuzuki S, Karpas A, Kira S, Yoshida Y, Seto M. Identification and characterization of a novel gene, C13orf25, as a target for 13q31-q32 amplification in malignant lymphoma. Cancer Res 2004; 64: 3087-3095.

Paglin S, Hollister T, Delohery T, Hackett N, McMahill M, Sphicas E, Domingo D, Yahalom J. A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. Cancer Res 2001; 61(2): 439-44.

Pang KC, Stephan S, Dinger ME, Engström PG, Lenhard B, Mattick JS. RNA2b 2.0 – an expanded database of mammalian non-coding RNAs. Nucleic Acids Res. 2007; 35:D178-82.

Perry RR, Kang Y, Greaves B. Effects of tamoxifen on growth and apoptosis of estrogen-dependent and -independent human breast cancer cells. Ann Surg Oncol 1995; 2: 238–245.

Petrocca F, Visone R, Onelli MR, Shah MH, Nicoloso MS, De Martino I, Iliopoulos D, Pilozzi E, Liu CG, Negrini M, Volinia S, Alder H, Ruco LP, Baldassarre G, Croce CM, Vecchione A. E2F1-regulated microRNAs impair TGFbeta-dependant cell-cycle arrest and apoptosis in gastric cancer. Cancer Cell 2008; 13: 272-286.

Pickering MT, Stadler BM, Kowalik TF. miR-17 and miR-20a temper an E2F1-induced G1 checkpoint to regulate cell cycle progression. *Oncogene.* 2009; 28(1):140-145.

Pillai RS, Bhattacharyya SN, Artus CG, Zoller T, Cougot N, Basyuk E, Betrand E, Filipowicz W. Inhibition of translational initiation by let-7 miRNA in human cells. Science 2005; 309: 1573-1576.

Pogribny IP, Tryndyak VP, Boyko A, Rodriguez-Juarez R, Beland FA, Kovalchuk O. Induction of microRNAome deregulation in rat liver by long-term tamoxifen exposure. Mutat Res. 2007; 619(1-2): 30-37.

Pursiheimo JP, Rantanen K, Heikkinen PT, Johansen T, Jaakkola PM. Hypoxia-activated autophagy accelerates degradation of SQSTM1/p62. Oncogene. 2009; 28(3):334-44.

Qadir MA, Kwok B, Dragowska W H , To K H, Le D, Bally MB and Gorski SM. Macroautophagy inhibition sensitizes tamoxifen-resistant breast cancer cells and enhances mitochondrial depolarization. Breast Cancer Res Treat 2008; 112(3):389-403.

Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y. Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J. Clin. Invest 2003; 112: 1809–1820.

Reggiori F, Klionsky DJ. Autophagy in the eukaryotic cell. Eukaryot. Cell. 2002; 1:11–21.

Ritossa F. A new puffing pattern induced by heat shock and DNP. Drosophila Experientia 1962; 18: 571-573.

Rockwell S, Yuan J, Peretz S and Glazer PM. Genomic instability in cancer. Novartis Found Symp 2001; 240:133–142.

Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A. Identification of mammalian microRNA host genes and transcription units. Genome Res. 2004; 14(10A): 1902-10.

Rubinsztein DC, Gestwicki JE, Murphy LO and Klionsky DJ. Potential therapeutic applications of autophagy. Nature 2007; 6: 304-312.

Samaddar JS, Gaddy VT, Duplantier J, Thandava SP, Shah M, Smith MJ, Browning D, Rawson J, Smith SB, Barrett JT, Schoenlien PV. A role for macroautophagy in protection against 4-hydroxytamoxifen-induced cell death and the development of antiestrogen resistance. Mol Cancer Ther 2008; 7(9): 2977-87.

Sato F, Tsuchiya S, Terasawa K, Tsujimoto G. Intra-Platform Repeatability and Inter-Platform Comparability of MicroRNA Microarray Technology. PLoS ONE 2009; 4(5): e5540.

Schoenlein PV, Periyasamy-Thandavan S, Samaddar JS, Jackson WH, Barrett JT. Autophagy facilitates the progression of ERalpha-positive breast cancer cells to antiestrogen resistance. Autophagy 2009; 5(3): 400-403.

Scott GK, Goga A, Bhaumik D, Berger CE, Sullivan CS, Benz CC. Coordinate suppression of ERBB2 and ERBB3 by enforced expression of micro-RNA miR-125a or miR-125b. J Biol Chem. 2007; 282: 1479-1486.

Shao Y, Gao Z, Feldman T, Jiang X. Stimulation of ATG12-ATG5 conjugation by ribonucleic acid. Autophagy 2007; 3(1):10-6.

Sun Q, Fan W, Chen K, Ding X, Chen S, Zhong Q. Identification of Barkor as a mammalian autophagy-specific factor for Beclin 1 and class III phosphatidylinositol 3-kinase. Proc Natl Acad Sci U S A. 2008; 105(49):19211-6.

Takamizawa J, Konishi H, Yanagiswaw K, Tomida S, Osada H, Endoh H, Harano T, Yatabe Y, Nagino M, Nimura Y, Mitsudomi T, Takahashi T. Reduced expression of

the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res. 2004; 64(11): 3753-6.

Takeuchi H, Kondo Y, Fujiwara K, Kanzawa T, Aoki H, Mills GB, Kondo S. Synergistic Augmentation of Rapamycin-Induced Autophagy in Malignant Glioma Cells by Phosphatidylinositol 3-Kinase/Protein Kinase B Inhibitors. Cancer Research 2005; 65: 3336-3346.

Tanida I, Minematsu-Ikeguchi N, Ueno T, Kominami E. Lysosomal turnover, but not a cellular level, of endogenous LC is a marker for autophagy. Autophagy 2005;1:84-91.

Tanida I, Sou YS, Ezaki J, Minematsu-Ikeguchi N, Ueno T, Kominami E. HsAtg4B/HsApg4B/ autophagin-1 cleaves the carboxyl termini of three human Atg8 homologues and dilapidates microtubule-associated protein light chain 3- and GABAA receptor-associated protein-phospholipid conjugates. J Biol Chem 2004a; 279: 36268-76.

Tanida I, Tanida-Miyake E, Komatsu M, Ueno T, Kominami E. Human Apg3p/Aut1p homologue is an authentic E2 enzyme for multiple substrates, GATE-16, GABARAP, and MAP-LC3, and facilitates the conjugation of hApg12p to hApg5p. J Biol Chem. 2002; 277(16):13739-44.

Tanida I, Ueno T, Kominami E. Human light chain 3/MAP1LC3B is cleaved at its carboxyl-terminal Met121 to expose Gly120 for lipidation and targeting to autophagosomal membranes. J Biol Chem 2004; 279: 47704-10.

Tanida I, Sou YS, Minematsu-Ikeguchi N, Ueno T, Kominami E. Atg8L/Apg8L is the fourth mammalian modifier of mammalian Atg8 conjugation mediated by human Atg4B, Atg7 and Atg3. FEBS J. 2006; 273(11):2553-62.

Tasdemir E, Maiuri MC, Galluzzi L, Vitale I, Djavaheri-Mergny M, D'Amelio M, Criollo A, Morselli E, Zhu C, Harper F, Nannmark U, Samara C, Pinton P, Vicencio JM, Carnuccio R, Moll UM, Madeo F, Paterlini-Brechot P, Rizzuto R, Szabadkai G, Pierron G, Blomgren K, Tavernarakis N, Codogno P, Cecconi F, Kroemer G. Regulation of autophagy by cytoplasmic p53. Nat Cell Biol. 2008;10(6):676-87.

Tennant DA, Duran RV, Boulahbel H, Gottlieb E. Metabolic transformation in cancer. Carcinogenesis. 2009; Mar 25 [Epub ahead of print].

Terman, A. The effect of age on formation and elimination of autophagic vacuoles in mouse hepatocytes. Gerontology 1995; 41(Suppl 2): 319 – 326.

Thompson, CB. Apoptosis in the pathogenesis and treatment of disease. Science 1995; 267(5203): 1456–62.

Thumm M, Egner R, Koch B, Schlumpberger M, Straub M, Veenhuis M, Wolf DH. Isolation of autophagocytosis mutants of Saccharomyces cerevisiae. FEBS Lett 1994; 349:275–280.

Tian Z, Greene AS, Pietrusz JL, Matus IR, Liange M. MicroRNA – target pairs in the rat kidney identified by microRNA microarray, proteomic, and bioinformatic analysis. Genome Res. 2008; 18: 404-411.

Toogood PL. Inhibition of protein–protein association by small molecules: approaches and progress. J. Med. Chem 2002; 45: 1543–1558.

Tsuchihara, Fujii S, Esumi H. Autophagy and cancer: Dynamism of the metabolism of tumor cells and tissues. Cancer Lett. 2008; [Epub ahead of print].

Tsukada M and Ohsumi Y. Isolation and characterization of autophagy-defective mutants of Saccharomyces cerevisiae. FEBS Lett 1993; 333:169–174.

Valko M, Morris H, and Cronin MT. Metals, toxicity and oxidative stress. Curr Med Chem. 2005;12(10):1161-208.

van Rooij E, Sutherland LB, Liu N, . A signature pattern of stress responsive microRNAs that can evoke cardiac hypertrophy and heart failure. Proc Natl Acad Sci. 2006; 103(48): 18255-18260.

Verma IM and Weitzman MD. Gene Therapy: Twenty-first century medicine. Annu Rev Biochem. 2005; 74: 711-738.

Visvanathan K, Chlebowski RT, Hurley P, Col NF, Ropka M, Collyar D, Morrow M, Runowicz C, Pritchard KI, Hagerty K, Arun B, Garber J, Vogel VG, Wade JL, Brown P, Cuzick J, Kramer BS, Lippman SM; American Society of Clinical Oncology. American society of clinical oncology clinical practice guideline update on pharmacologic interventions including tamoxifen, raloxifen, and aromatase inhibition for breast cancer risk reduction. J Clin Oncol. 2009; 27(19): 3235-58.

Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, Prueitt RL, Yanaihara N, Lanza G, Scarpa A, Vecchione A, Negrini M, Harris CC, Croce CM. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci USA. 2006; 103(7): 2257-61.

Wagner-Ecker M, Schwagner C, Wirkner U, Abdollahi A, Huber PE. MicroRNA expression after ionizing radiation in human endothelial cells. Radiat Oncol. 2010; 5(1): 5-25.

Wagner KU. Models of breast cancer: *quo vadis*, animal modeling? Breast Cancer Res 2004; 6:31-38.

Wang CW, Klionsky DJ. The molecular mechanism of autophagy. Mol. Med. 2003; 9:65–76.

Wang WX, Rajeev BW, Stromberg AJ, Ren N, Tang G, Huang Q, Rigoutsos I, Nelson PT. The expression of microRNA miR-107 decreases early in Alzheimer's disease and may accelerate disease progression through regulation of beta-site amyloid precursor protein-cleaving enzyme 1. J Neurosci. 2008; 28(5): 1213-23.

Willenbrock H, Salomon J, Sokilde R, Barken KB, Hansen TN, Nielsen FC, Moller S, Litman T. Quantitative miRNA expression analysis: comparing microarrays with nex-generation sequencing. RNA. 2009; 15: 2028-2034.

Winter J, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol. 2009; 11: 228-234.

Yam JC, Kwok AK. Ocular toxicity of hydroxychloroquine. Hong Kong Med J. 2006; 12(4):294-304.

Yen WL, Klionsky DJ. How to live long and prosper: autophagy, mitochondria, and aging. Physiology (Bethesda). 2008; 23:248-62.

Yi R, Qin Y, Macara IG, Cullen BR. Exportin-5 mediates the nuclear export of pr-microRNAs and short hairpin RNAs. Genes Dev. 2003; 17(24): 3011-6.

Yormitsu T, Klionsky DJ. Autophagy: Molecular machinery for self-eating. Cell Death Differ 2005; 12:1542-52.

Young AR, Chan EY, Hu XW, Köchl R, Crawshaw SG, High S, Hailey DW, Lippincott-Schwartz J, Tooze SA. Starvation and ULK1-dependent cycling of mammalian Atg9 between the TGN and endosomes. J Cell Sci. 2006; 119(Pt 18):3888-900.

Yue Z, Jin S, Yang C, Levine AJ, Heintz N. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc Natl Acad Sci USA 2003;100(25):15077-82.

Yu L, Wan F, Dutta S, Welsh S, Liu Z, Freundt E, Baehrecke EH, Lenardo M. Autophagic programmed cell death by selective catalase degradation. Proc Natl Acad Sci U S A. 2006;103(13): 4952-7.

Zhang L, Huang J, Yang N, Greshock J, Megraw MS, Giannakakis A, Liang S, Naylor TL, Barchetti A, Ward MR, Yao G, Medina A, O'brien-Jenkins A, Katsaros D, Hatzigeorgiou A, Gimotty PA, Weber BL, Coukos G. microRNAs exhibit high frequency genomic alterations in human cancer. Proc Natl Acad Sci USA 2006; 103:9136-9141.

APPENDIX

miRNA	1d/0uM TAM	1d/5.0uM TAM	Ratio of TAM/Control	P-Value	Autophagy Genes
hsa-mir-130b	3728	771	0.20672175	0.00E+00	Atg16L1, Ulk-2
hsa-mir-15b	1242	6805	5.479379405	0.00E+00	Atg9a
hsa-mir-185	6927	3447	0.497575659	0.00E+00	
hsa-mir-190b	2850	781	0.273978359	0.00E+00	
hsa-mir-191	17538	4824	0.275057139	0.00E+00	
hsa-mir-193a	10179	3511	0.344904062	0.00E+00	
hsa-mir-200c	28197	6672	0.236637057	0.00E+00	Ambra1
hsa-mir-203	13438	5700	0.424170263	0.00E+00	
hsa-mir-21	574066	674954	1.175743714	0.00E+00	
hsa-mir-224	1047	4764	4.550211897	0.00E+00	
hsa-mir-23a	5676	1543	0.271866415	0.00E+00	
hsa-mir-23b	23559	787	0.033423539	0.00E+00	
hsa-mir-26b	10211	18202	1.78262611	0.00E+00	Ulk-1, Ulk-2
hsa-mir-27a	2334	1665	0.713226503	0.00E+00	Ulk-1, Ulk-2
hsa-mir-29c	5853	704	0.120312936	0.00E+00	Atg9a
hsa-mir-320a	17422	2744	0.157519882	0.00E+00	
hsa-mir-342	3205	7708	2.405111773	0.00E+00	
hsa-mir-423	5543	25157	4.538604552	0.00E+00	
hsa-mir-93	17487	6967	0.398414014	0.00E+00	
hsa-mir-98	2628	10137	3.857353786	0.00E+00	Atg4b, Atg16L1, Ulk-2
hsa-mir-99a	1313	7647	5.824299611	0.00E+00	Frap1
hsa-let-7i	1058	0	0	0.00E+00	Atg4b, Ulk-2
hsa-mir-345	1118	100	0.089445438	4.69E-241	
hsa-mir-375	24274	34896	1.437579648	1.86E-230	
hsa-mir-107	4567	2307	0.50505514	2.26E-219	
hsa-mir-106b	1572	343	0.217884841	3.34E-215	Atg4b, Bec-1, Ulk-1
hsa-mir-148b	884	80	0.090768689	3.23E-190	
hsa-mir-30b	1184	210	0.177010843	9.24E-187	Atg5, Bec-1
hsa-mir-148a	1268	251	0.198341487	3.42E-186	
hsa-mir-16-2	846	81	0.095553574	1.43E-178	
hsa-let-7g	536	0	0	7.53E-174	Atg4b, Ulk-2
hsa-mir-22	994	193	0.193978241	2.77E-148	
hsa-mir-128-1	1371	409	0.29830929	7.52E-143	
hsa-mir-15a	776	118	0.152015557	3.06E-134	Atg9a
hsa-mir-205	781	163	0.208545777	3.20E-111	
hsa-mir-187	522	51	0.098653268	7.79E-110	
hsa-mir-618	348	9	0.025810448	2.94E-98	
hsa-mir-25	33839	43202	1.276686557	2.50E-94	
hsa-mir-425	797	212	0.265967438	1.65E-93	
hsa-mir-499	631	137	0.216366	1.45E-87	
hsa-mir-20a	566	106	0.187257993	3.14E-87	Atg16L1, Atg2b, Atg 2a, Ulk-1, Bec-1
hsa-mir-210	1981	1119	0.564947843	7.09E-75	
hsa-mir-301a	343	34	0.097763656	2.01E-72	Atg16L1, Atg2b, Ulk-1

miRNA	1d/0uM TAM	1d/5.0uM TAM	Ratio of TAM/Control	P-Value	Autophagy Genes
hsa-mir-27b	1422	2744	1.929895483	8.16E-69	
hsa-mir-1287	6	253	42.21556886	8.36E-62	
hsa-mir-141	291	35	0.119348931	3.02E-57	
hsa-mir-744	1084	2105	1.942240979	3.51E-54	
hsa-mir-331	280	38	0.134730539	2.95E-52	
hsa-mir-374b	296	45	0.15172358	4.03E-52	
hsa-mir-192	4202	3408	0.810991059	4.27E-41	
hsa-mir-660	1114	643	0.576763887	1.16E-40	
hsa-mir-96	332	90	0.270543251	3.90E-39	Atg16L1, Atg9a, Frap
hsa-mir-183	542	222	0.409881344	4.65E-39	
hsa-let-7e	4385	3770	0.859762801	1.80E-29	
hsa-mir-181a-1	871	52	0.059811491	4.63E-26	Atg2b
hsa-mir-181c	157	28	0.179259316	4.63E-26	Atg2b
hsa-mir-126	108	9	0.083166999	5.33E-25	
hsa-mir-193b	442	213	0.482293332	9.50E-25	
hsa-mir-1975	160	33	0.205838323	3.98E-24	
hsa-mir-590	118	15	0.126864914	2.09E-23	
hsa-mir-17	511	274	0.536695689	4.58E-23	Atg16L1, Atg2b, Atg 2a, Ulk-1, Bec-1
hsa-mir-652	135	26	0.195165225	1.43E-21	
hsa-mir-125b-1	141	29	0.203847624	1.96E-21	Atg4d, Ulk-3
hsa-mir-30d	2594	3632	1.400052632	1.08E-20	
hsa-mir-1307	484	269	0.555500569	3.28E-20	
hsa-mir-196a-2	101	14	0.136360941	1.37E-19	
hsa-mir-340	80	6	0.074850299	2.17E-19	
hsa-mir-339	421	789	1.873213194	3.67E-19	
hsa-mir-190	82	7	0.080327151	4.70E-19	
hsa-mir-24-2	67	7	0.098310841	6.53E-18	
hsa-mir-504	76	7	0.086668768	2.14E-17	
hsa-mir-181d	324	162	0.500850152	2.64E-17	
hsa-mir-625	91	14	0.157925906	9.83E-17	
hsa-mir-421	400	228	0.568862275	5.40E-16	
hsa-mir-29b-2	127	35	0.27346881	1.47E-15	
hsa-mir-424	170	369	2.169778091	5.28E-14	Atg9a
hsa-mir-19a	41	0	0	5.76E-14	Atg16L1
hsa-mir-1978	818	625	0.763509656	3.55E-12	
hsa-mir-197	168	74	0.438408896	6.92E-12	
hsa-mir-324	113	38	0.333845583	1.27E-11	
hsa-mir-338	1	44	44.31137725	1.40E-11	
hsa-mir-362	189	92	0.487913063	2.97E-11	
hsa-mir-140	787	610	0.775323559	4.70E-11	
hsa-mir-429	623	90	0.144173931	5.40E-11	Ulk-2, Ambra-1
hsa-mir-1270	19	89	4.695871415	1.03E-10	
hsa-mir-1908	35	1	0.03421728	1.78E-10	
hsa-mir-34a	51	8	0.152635905	6.00E-10	Atg4b, Atg9
hsa-mir-186	190	101	0.532618973	1.46E-09	
hsa-mir-454	172	329	1.914775101	2.86E-09	Atg16L1, Atg2b, Ulk-2

miRNA	1d/0uM TAM	1d/5.0uM TAM	Ratio of TAM/Control	P-Value	Autophagy Genes
hsa-mir-29a	6639	8067	1.215102556	5.02E-09	
hsa-mir-125a	869	716	0.823439427	6.79E-09	
hsa-mir-125b-2	43	8	0.181033282	6.49E-08	
hsa-mir-1301	354	558	1.576508001	1.25E-07	
hsa-mir-296	33	4	0.108873163	1.48E-07	
hsa-mir-30a	268	180	0.672535526	2.21E-07	
hsa-mir-149	70	25	0.350727117	2.79E-07	
hsa-mir-26a-2	2	31	15.26946108	4.40E-07	
hsa-mir-505	83	35	0.418440228	6.93E-07	
hsa-mir-33a	65	25	0.386918471	2.40E-06	
hsa-mir-766	46	13	0.286383754	2.58E-06	
hsa-mir-1224	17	0	0	3.24E-06	
hsa-mir-452	343	259	0.754176778	3.80E-06	
hsa-mir-589	15	55	3.672654691	1.00E-05	
hsa-mir-31	34	8	0.228953857	1.50E-05	
hsa-mir-502	111	64	0.577223931	2.77E-05	
hsa-mir-199b	1	20	19.76047904	3.01E-05	
hsa-mir-451	36	11	0.316034597	5.80E-05	
hsa-mir-16-1	23	4	0.15620932	6.66E-05	
hsa-mir-182	284	219	0.771696045	6.82E-05	
hsa-mir-200b	846	768	0.908112852	8.99E-05	
hsa-mir-877	69	34	0.485984553	9.92E-05	
hsa-mir-1306	45	17	0.385894877	1.00E-04	
hsa-mir-151	273	212	0.77646904	1.30E-04	
hsa-mir-532	100	177	1.766467066	1.42E-04	
hsa-mir-221	71	37	0.522897866	1.50E-04	
hsa-mir-18a	93	55	0.59236366	1.98E-04	
hsa-mir-132	14	1	0.085543199	3.00E-04	
hsa-mir-139	234	183	0.783049286	4.00E-04	
hsa-mir-200a	492	666	1.353390779	5.70E-04	
hsa-mir-942	21	5	0.256629598	6.52E-04	
hsa-mir-196a-1	12	1	0.099800399	1.20E-03	
hsa-mir-7-1	39	80	2.042069707	1.25E-03	
hsa-mir-664	76	46	0.598802395	1.40E-03	
hsa-mir-501	49	25	0.501038739	1.50E-03	
hsa-mir-128-2	24	8	0.349301397	2.18E-03	
hsa-mir-195	45	23	0.518962076	2.20E-03	
hsa-mir-2110	70	44	0.633019675	3.50E-03	
hsa-mir-18b-2	52	29	0.552740673	3.56E-03	
hsa-mir-497	67	43	0.643489141	5.50E-03	
hsa-mir-874	14	3	0.213857998	5.77E-03	Atg16L1
hsa-mir-24-1	18	6	0.332667997	7.57E-03	
hsa-mir-629	61	103	1.688426426	7.80E-03	Atg5
hsa-mir-1-2	15	37	2.4750499	7.86E-03	
hsa-mir-624	9	1	0.133067199	8.70E-03	
hsa-mir-99b	2189	2603	1.18912472	9.93E-03	
hsa-mir-1294	9	1	0.133067199	1.00E-02	

miRNA	1d/0uM TAM	1d/5.0uM TAM	Ratio of TAM/Control	P-Value	Autophagy Genes
hsa-mir-142	8	1	0.149700599	1.00E-02	
hsa-mir-363	105	79	0.752780154	1.00E-02	
hsa-mir-369	6	0	0	1.00E-02	
hsa-mir-92b	1646	1974	1.19905996	1.20E-02	
hsa-mir-222	20	8	0.389221557	1.30E-02	
hsa-mir-378	24	11	0.449101796	1.70E-02	
hsa-mir-1286	0	7	0	2.00E-02	
hsa-mir-215	1	9	8.982035928	2.29E-02	
hsa-mir-92a-2	1	9	8.982035928	2.30E-02	
hsa-mir-1249	7	1	0.171086399	3.00E-02	
hsa-mir-130a	7	1	0.171086399	3.00E-02	
hsa-mir-1826	22	43	1.95971693	3.00E-02	
hsa-mir-188	13	4	0.322432059	3.00E-02	
hsa-mir-100	0	6	0	3.00E-02	Frap1
hsa-mir-615	22	11	0.489929232	4.00E-02	
hsa-mir-33b	10	3	0.299401198	5.00E-02	
hsa-mir-1291	4	0	0	5.00E-02	
hsa-mir-1323	4	0	0	5.00E-02	
hsa-mir-30e	328	310	0.945669636	5.20E-02	
hsa-mir-1293	0	5	0	6.00E-02	
hsa-mir-10a	2	9	4.491017964	7.00E-02	
hsa-mir-491	11	4	0.38105607	7.00E-02	
hsa-mir-489	7	2	0.256629598	9.50E-02	
hsa-mir-320b-2	5	13	2.51497006	1.03E-01	
hsa-mir-935	5	1	0.239520958	1.10E-01	
hsa-mir-1179	3	0	0	1.10E-01	
hsa-mir-582	3	0	0	1.10E-01	
hsa-mir-486	0	4	0	1.20E-01	
hsa-mir-1269	41	62	1.518913393	1.40E-01	
hsa-mir-548k	45	35	0.771789754	1.50E-01	
hsa-mir-1247	6	2	0.299401198	1.60E-01	
hsa-mir-1248	6	2	0.299401198	1.60E-01	
hsa-mir-579	9	4	0.399201597	1.60E-01	
hsa-mir-641	9	4	0.399201597	1.60E-01	
hsa-mir-887	9	4	0.399201597	1.60E-01	
hsa-mir-374a	21	14	0.684345595	1.70E-01	
hsa-mir-1259	10	5	0.479041916	2.00E-01	
hsa-mir-144	4	1	0.299401198	2.00E-01	
hsa-mir-1914	4	1	0.299401198	2.00E-01	
hsa-mir-1974	4	1	0.299401198	2.00E-01	
hsa-mir-628	4	1	0.149700599	2.00E-01	
hsa-mir-122	21	33	1.568291987	2.20E-01	
hsa-mir-548e	1	5	4.790419162	2.20E-01	
hsa-mir-484	94	86	0.917314308	2.30E-01	
hsa-mir-153-2	2	0	0	2.30E-01	
hsa-mir-495	2	0	0	2.30E-01	
hsa-mir-627	2	0	0	2.30E-01	

miRNA	1d/0uM TAM	1d/5.0uM TAM	Ratio of TAM/Control	P-Value	Autophagy Genes
hsa-mir-642	2	0	0	2.30E-01	
hsa-mir-892a	2	0	0	2.30E-01	
hsa-mir-103-1	13	22	1.70428374	2.39E-01	
hsa-mir-194-2	33	26	0.798403194	2.41E-01	
hsa-mir-1304	11	19	1.741970604	2.70E-01	
hsa-mir-92a-1	592	694	1.172317527	2.88E-01	
hsa-mir-361	122	117	0.957102189	3.00E-01	
hsa-mir-32	33	28	0.834694248	3.10E-01	
hsa-mir-1266	3	7	2.395209581	3.50E-01	
hsa-mir-29b-1	7	4	0.513259196	3.70E-01	
hsa-mir-500	25	21	0.838323353	3.79E-01	
hsa-mir-20b	13	20	1.520036849	3.90E-01	
hsa-mir-28	4	2	0.598802395	4.30E-01	
hsa-mir-455	16	23	1.459580838	4.30E-01	
hsa-mir-18b-1	2	5	2.694610778	4.57E-01	
hsa-mir-1226	1	0	0	4.80E-01	
hsa-mir-1256	1	0	0	4.80E-01	
hsa-mir-1274b	1	0	0	4.80E-01	
hsa-mir-134	1	0	0	4.80E-01	
hsa-mir-136	1	0	0	4.80E-01	
hsa-mir-1976	1	0	0	4.80E-01	
hsa-mir-433	1	0	0	4.80E-01	
hsa-mir-449a	1	0	0	4.80E-01	
hsa-mir-483	1	0	0	4.80E-01	
hsa-mir-487b	1	0	0	4.80E-01	
hsa-mir-543	1	0	0	4.80E-01	
hsa-mir-552	1	0	0	4.80E-01	
hsa-mir-611	1	0	0	4.80E-01	
hsa-mir-632	1	0	0	4.80E-01	
hsa-mir-653	1	0	0	4.80E-01	
hsa-mir-675	1	0	0	4.80E-01	
hsa-mir-767	1	0	0	4.80E-01	
hsa-mir-1262	5	3	0.598802395	4.90E-01	
hsa-mir-7-2	3	5	1.596806387	4.90E-01	
hsa-mir-155	0	2	0	5.00E-01	
hsa-mir-573	0	2	0	5.00E-01	
hsa-mir-330	236	277	1.172231808	5.10E-01	
hsa-mir-1295	6	4	0.598802395	5.30E-01	
hsa-mir-335	160	165	1.032934132	5.40E-01	
hsa-mir-765	4	7	1.796407186	5.50E-01	
hsa-mir-944	4	7	1.796407186	5.50E-01	
hsa-mir-1910	7	5	0.684345595	5.70E-01	
hsa-mir-101-2	2	1	0.299401198	6.10E-01	
hsa-mir-1284	2	1	0.598802395	6.10E-01	
hsa-mir-137	2	1	0.299401198	6.10E-01	
hsa-mir-513c	2	1	0.299401198	6.10E-01	
hsa-mir-643	2	1	0.299401198	6.10E-01	

miRNA	1d/0uM TAM	1d/5.0uM TAM	Ratio of TAM/Control	P-Value	Autophagy Genes
hsa-mir-659	2	1	0.598802395	6.10E-01	
hsa-mir-940	2	1	0.598802395	6.10E-01	
hsa-mir-671	9	7	0.798403194	6.20E-01	
hsa-mir-196b	67	68	1.009920458	6.70E-01	
hsa-mir-320d-2	3	2	0.798403194	6.70E-01	
hsa-mir-450b	11	15	1.360914535	7.00E-01	
hsa-mir-152	15	14	0.918163673	7.10E-01	
hsa-mir-542	12	16	1.29740519	7.10E-01	
hsa-mir-1296	3	5	1.596806387	7.30E-01	
hsa-mir-146b	5	4	0.838323353	7.40E-01	
hsa-mir-184	16	20	1.27245509	7.40E-01	
hsa-mir-769	16	20	1.27245509	7.40E-01	
hsa-mir-1292	6	8	1.397205589	7.90E-01	
hsa-mir-503	77	81	1.049848355	8.10E-01	
hsa-mir-103-2	421	460	1.092352113	8.93E-01	
hsa-mir-365-1	38	40	1.055783171	9.10E-01	
hsa-let-7d	15734	9343	0.593778757	1.00E+00	
hsa-mir-106a	1	2	2.395209581	1.00E+00	
hsa-mir-10b	15	15	0.998003992	1.00E+00	
hsa-mir-1180	14	14	1.026518392	1.00E+00	
hsa-mir-1255a	16	17	1.085329341	1.00E+00	
hsa-mir-1276	6	7	1.19760479	1.00E+00	
hsa-mir-1278	4	4	1.047904192	1.00E+00	
hsa-mir-138-2	2	2	0.898203593	1.00E+00	
hsa-mir-1468	1	1	0.598802395	1.00E+00	
hsa-mir-146a	1	1	0.598802395	1.00E+00	
hsa-mir-1977	100	111	1.107784431	1.00E+00	
hsa-mir-206	1	2	1.796407186	1.00E+00	
hsa-mir-212	1	2	1.796407186	1.00E+00	
hsa-mir-301b	1	1	1.19760479	1.00E+00	
hsa-mir-30c-1	18	19	1.064537591	1.00E+00	
hsa-mir-30c-2	1	1	1.19760479	1.00E+00	
hsa-mir-320c-1	1	1	0.598802395	1.00E+00	
hsa-mir-320d-1	1	1	1.19760479	1.00E+00	
hsa-mir-328	2	3	1.497005988	1.00E+00	
hsa-mir-450a-1	1	1	1.19760479	1.00E+00	
hsa-mir-508	2	3	1.497005988	1.00E+00	
hsa-mir-509-3	1	2	2.395209581	1.00E+00	
hsa-mir-522	1	1	0.598802395	1.00E+00	
hsa-mir-548b	2	2	0.898203593	1.00E+00	
hsa-mir-551b	2	2	0.898203593	1.00E+00	
hsa-mir-556	2	2	1.19760479	1.00E+00	
hsa-mir-584	1	1	0.598802395	1.00E+00	
hsa-mir-616	1	2	1.796407186	1.00E+00	
hsa-mir-760	1	2	2.395209581	1.00E+00	
hsa-mir-885	1	1	0.598802395	1.00E+00	
hsa-mir-1201	0	1	0	1.00E+00	

miRNA	1d/0uM TAM	1d/5.0uM TAM	Ratio of TAM/Control	P-Value	Autophagy Genes
hsa-mir-1236	0	1	0	1.00E+00	
hsa-mir-1258	0	1	0	1.00E+00	
hsa-mir-1264	0	1	0	1.00E+00	
hsa-mir-1303	0	1	0	1.00E+00	
hsa-mir-196a-1	0	1	0	1.00E+00	
hsa-mir-219-1	0	1	0	1.00E+00	
hsa-mir-320c-2	0	1	0	1.00E+00	
hsa-mir-323	0	1	0	1.00E+00	
hsa-mir-346	0	1	0	1.00E+00	
hsa-mir-382	0	1	0	1.00E+00	
hsa-mir-449b	0	1	0	1.00E+00	
hsa-mir-492	0	1	0	1.00E+00	
hsa-mir-494	0	1	0	1.00E+00	
hsa-mir-510	0	1	0	1.00E+00	
hsa-mir-548j	0	1	0	1.00E+00	
hsa-mir-561	0	1	0	1.00E+00	
hsa-mir-574	0	1	0	1.00E+00	
hsa-mir-599	0	1	0	1.00E+00	
hsa-mir-610	0	1	0	1.00E+00	
hsa-mir-651	0	1	0	1.00E+00	
hsa-mir-663b	0	1	0	1.00E+00	
hsa-mir-888	0	1	0	1.00E+00	
hsa-mir-1229	0	1	0	1.00E+00	

* highlighted in pink – the seven miRNAs chosen for further analysis