THE MOLECULAR MECHANISMS OF RAPAMYCIN-INDUCED AUTOPHAGY AND APOPTOSIS IN T-47D BREAST CARCINOMA CELLS

by

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DEDICATION

This Thesis is dedicated to

my mother and my father

my grandmother

my wife; Fatimah, my son; Ismail, my daughter; Isra'a, and my new baby daughter; Asrar

my sisters and my brother, Mohammed

and all my family

Thank you for being my source of inspiration.

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1p36.2	Chromosome 1, short arm region 3, band 6, sub-band 2
12q15	Chromosome 12, long arm band 1, sub-band 5
3MA	3-methyladenine
Akt	Akt8 virus oncogenes cellular homolog
Ambra1	Autophagy/beclin-1 regulator 1
Ap-1	Activator protein 1
Apaf-1	Apoptotic protease activating factor-1
ARF6	ADP-ribosylation factor 6
ASCT2	ASC amino acid transporter 2
ASR	Age standardized rate
ATCC	American Type Culture Collection
Atg	Autophagy-related gene
Bad	Bcl-xL/Bcl-2-associated death promoter
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma extra large
Bif-1	Bax-interacting factor 1
bp	base pair
BRCA1	Breast cancer susceptibility gene 1
BRCA2	Breast cancer susceptibility gene 2
BSA	Bovine serum albumine
c-Jun	Member of AP-1 family of transcription factors
CMA	Chaperone-mediated autophagy
CO_2	Carbon Dioxide
Ct	Threshold cycle
CTSB	Cathepsin B
dATP	Deoxyadenosine triphosphate
DDIT4	DNA-damage-inducible transcript 4
Deptor	DEP-domain-containing mTOR-interacting protein
DFF40	DNA fragmentation factor 40
DFF45	DNA fragmentation factor 45

Diablo	Direct inhibitor of apoptosis-binding protein
DISC	Death inducing signaling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dUTP	Deoxyuridine triphosphate
e. g.	Example guide
EDTA	Ethylene diaminetetraacetic acid
eEF2K	Eukaryotic elongation factor 2 kinase
eIF4E	Eukaryotic translation inhibition factor 4E
EM	Endoplasmic reticulum
erb-B	erythroblastic leukemia viral oncogene homolog 2
ERK1/2	Extracellular-signal-regulated kinase 1/2
ERKs	Extracellular signal-related kinases
FADD	Fas-associated death domain adaptor protein
Fas	Tumor necrosis factor superfamily receptor 6
FAT	FRAP-ATM-TRAPP domain
FBS	fetal bovine serum
FIP200	Focal adhesion kinase family interacting protein of 200 kDa
FKBP12	FK506-binding protein of 12 kDa
FoxO1	Forkhead box protein O1
FRAP	FKBP12-rapamycin associated protein
FRB	FKBP12-rapamycin binding domain
g	Gram
G_1 phase	First gap phase
G_2 phase	Second gap phase
GABARAP	Gamma aminobutyric acid receptor-associated protein
GAP	GTPase-activating protein
GAS5	Growth arrest-specific transcript 5
GFP	Green fluorescence protein
GR	Glucocorticoid receptor
h	Hour
H_2O_2	hydrogen peroxide
HBOC	Heredity breast-ovarian cancer syndromes
HOPS	Homotypic vacuole fusion and protein sorting

HRT	hormone replacement therapy
HUVEC	Human umbilical vein endothelial cells
i. e.	id est (Latin)
IAP	Inhibitor of apoptosis
IC_{50}	Inhibition concentration 50
IGF-1	Insulin-like growth factor 1
IL-6	Interleukin-6
IRS1	Insulin-receptor substrate 1
kDa	Kilo Dalton
LAMP1	Lysosomal-associated membrane protein 1
LAMP2	Lysosomal-associated membrane protein 2
LC3	Microtubule-associated protein 1 light chain 3
LKB1	Liver kinase B1
LOH	Loss of heterozygosity
M phase	Mitotic phase
M	Molar
MAP-K	Mitogen-activated protein kinase
MCF-7	Human hormone sensitive and invasive breast cancer cell line
MDC	Monodansylcadaverine
mg	Milligram
min	Minute
ml	Milliliter
MLIAP	melanoma inhibitor of apoptosis protein
mLST8	Mammalian lethal with Sce13 protein 8
mm	Millimeter
mM	Millimolar
mRNA	Messenger RNA
mSin1	Mammalian stress-activated protein kinase interacting protein 1
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
Myc	Myelocytomatosis viral oncogene homolog [avian]
NAIP	Neuronal apoptosis inhibitory protein

NAP1L1	Nucleosome assembly protein 1-like 1
NCR	National cancer registry
NF-κB	Nuclear factor –kappa-B
nm	Nanometer
NR3C1	Nuclear receptor subfamily 3, group C, member 1
NUTF2	Nuclear transport factor 2
OD	Optical density
p53	Tumor suppressor protein 53
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCD4	Programmed cell death 4
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent protein kinase 1
PE	Phosphatidylethanolamine
PHLDA1	Pleckstrin homology-like domain, family A, member 1
PI(3,4,5)P3	Phosphatidylinositol (3,4,5) triphosphate
PI(4,5)P2	Phosphatidylinositol (4,5) biphosphate
PI3K	Phosphoinositide-3-kinase
PIKK	PI 3-kinase-related kinases
PKB	Protein kinase B
PKC	Protein kinase C
PKCα	Protein kinase Cα
PP2A	Protein phosphatase 2A
PRAS40	Proline-rich Akt substrate 40 kDa
PRR5	Proline-rich protein 5
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
Rab7	RAS oncogene family
RABEP1	Rabaptin, RAB GTPase binding effector protein 1
RAFT	Rapamycin and FKBP12 target
RAPT	Rapamycin target
Raptor	Regulatory-associated protein of mTOR
RB	Retinoblastoma
RD	Repressor domain

Rheb	Ras homolog enriched in brain
RICTOR	Rapamycin-insensitive companion of mTOR
RNA	Ribonucleic acid
RPL3	Ribosomal protein L3
rpm	Revolution per minute
rRNA	Ribosomal RNA
RSK1	p90 ribosomal S6 kinase 1
RTKs	Receptor tyrosine kinases
SC4MOL	Sterol-C4-methyl oxidase-like
SCAR	S6K1 Aly/Ref-like target
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS_PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEP	Sirolimus effector protein
SGK1	Serum and glucocorticoid-induced protein kinase 1
SLC1A5	Solute carrier family 1 “neytral amino acid transporter”, member 5
Smac	Second mitochondria-derived activator of caspases
SMase	Sphingomyelinase
STK11	Serine/threonine kinase 11
T-47D	Human hormone sensitive early stage breast cancer cell line
TBE	Tris-borate-ethylene-EDTA
TCR	T cells receptor
TDAG51	T cell death associated gene 51
TEM	Transmission electron microscope
TIF-1A	Transcription inhibition factor 1A
TNFR	Tumor necrosis factor receptor
TNFRSF1A	Tumor necrosis factor receptor superfamily, member 1A
TNFRSF6	TNF receptor superfamily, member 6
TNFSF10	TNF ligand superfamily member 10
TNFSF13	Tumor necrosis factor ligand superfamily, member 13
TOP2A	Topoisomerase (DNA) II Alpha 170kDa
TRAIL	TNF-related apoptosis-inducing ligand
tRNA	Transfer RNA
TSC	Tuberous sclerosis complex

TUNEL	TdT-mediated dUTP Nick-End Labeling
U/ml	Unit per milliliter
ULK	Unc-51-like kinase
UPS	Ubiquitin-proteasome system
USA	United States of America
UV	Ultraviolet
UVRAG	Ultraviolate radiation resistance-associated gene
V	Volt
v/v	Volume/volume
w/v	Weight/volume
WHO	World Health Organization
XIAP	X-linked inhibitor of apoptosis
µl	Micro liter
µg/ml	Microgram/milliliter
µM	Micromolar

LIST OF SYMBOLS

%	Percentage
°C	Degree Celsius
α	Alpha
β	Beta
γ	Gamma
Δ	Delta

MEKANISME MOLEKUL AUTOFAGI DAN APOPTOSIS YANG DIARUH OLEH RAPAMISIN DALAM SEL KARSINOMA PAYU DARA T-47D

ABSTRAK

Autofagi adalah laluan degradasi lisosomal suatu konservasi evolusi yang mengakibatkan degradasi protein dan seluruh organel, dengan itu ia memainkan peranan penting dalam proses homeostatik kitaran semula protein dan organel. Autofagi adalah mekanisme biologi penting yang membolehkan kemandirian sel dan mengaruhkan kematian sel-sel yang telah rosak. Terdapat banyak bukti menunjukkan bahawa autofagi mara ke kematian sel autofagik apabila proses autofagi dirangsang secara berlebihan. Perkaitan fungsi antara apoptosis (kematian sel jenis I) dan kematian sel autofagik (kematian sel jenis II) pernah dijelaskan. Walau bagaimanapun, perkaitan molekul dan keadaan laluan molekul yang menentukan pilihan di antara autofagi dan apoptosis adalah tidak diketahui buat masa ini. Autofagi dikawal atur oleh laluan kinase rapamisin manusia (mTOR). Diketahui bahawa mTOR boleh merencatkan proses autofagi dan seterusnya mendorong perkembangan tumor. Sebagai satu strategi, rapamisin yang diketahui sebagai perencat mTOR digunakan sebagai pengaruh autofagi dan 3-metiladenina (3MA) digunakan sebagai perencat autofagi. Dalam kajian ini, ekspresi gen global yang sistematik telah dijalankan untuk mengkaji proses autofagi yang diaruh rapamisin dan kesan rapamisin apabila proses autofagi direncatkan. Kajian ini juga menunjukkan bahawa rapamisin mampu mengaruh autofagi dalam sel karsinoma payu dara T-47D. Walau bagaimanapun, apabila autofagi direncat oleh 3MA, rapamisin menunjukkan kesan apoptosis dalam sel payu dara ini. Pemerhatian ini disokong sepenuhnya oleh pelbagai kaedah yang meggunakan teknik mikroskop. Di

samping itu, rapamisin mempunyai nilai *Growth inhibition* (GI_{50}), *total growth inhibition* (TGI) dan *lethal concentration* (LC_{50}) yang rendah dalam sel T-47D apabila proses autofagi direncat oleh 3MA, berbanding dengan sel yang dirawat hanya dengan rapamisin ataupun 3MA. Keputusan ini menunjukkan bahawa apoptosis adalah mod kematian sel yang efektif jika dibandingkan dengan kematian sel autofagi. Ekspresi gen *PHLDA1 (pleckstrin homology-like domain, family A, member 1*) ditingkatkan dalam kedua-dua proses autofagi dan apoptosis serta perencatan gen ini mengurangkan aktiviti kedua-duanya. Keputusan ini menyokong bahawa *PHLDA1* mampu menjadi perantur dan mungkin mengawal atur kedua-dua laluan autofagi dan apoptosis, dan kedua-dua proses ini boleh menggunakan laluan atau komponen laluan yang sama. Selain daripada itu, ekspresi gen *RICTOR* (*rapamycin-insensitive companion of mTOR*) ditingkatkan dalam apoptosis dan perencatan gen ini mampu merencatkan aktiviti apoptosis. Ini menunjukkan bahawa *RICTOR* juga memainkan peranan dalam apoptosis yang diaruh oleh rapamisin dalam sel kanser payu dara T-47D. Sebagai kesimpulan, kajian ini memberi suatu gambaran baru tentang mekanisme molekul autofagi dan apoptosis yang diaruh rapamisin dalam sel karsinoma payu dara T-47D dan seterusnya menyumbang kepada kajian semasa yang meneroka persimpangan di antara laluan autofagi dan apoptosis. Keputusan kajian ini juga mampu menjadi landasan atau idea dalam rawatan kanser payu dara, iaitu kemungkinan penggunaan perencat autofagi dan terapi gen yang menggunakan *PHLDA1*.

THE MOLECULAR MECHANISMS OF RAPAMYCIN-INDUCED AUTOPHAGY AND APOPTOSIS IN T-47D BREAST CARCINOMA CELLS

ABSTRACT

Autophagy is an evolutionarily conserved lysosomal degradation pathway that leads to degradation of proteins and entire organelles and subsequently plays a crucial role in the homeostatic process of recycling proteins and organelles. Autophagy is an important biological mechanism that enables cell survival and to induce death of damage cells. There is increasing evidence that autophagy progresses to autophagic cell death when the process is over-stimulated. Functional relationships have been described between apoptosis (Type I cell death) and autophagic cell death (Type II cell death). However, the molecular relationships and the circumstances of which molecular pathways dictate the choice between autophagy and apoptosis are currently unknown. Autophagy is regulated by the mammalian target of rapamycin (mTOR) kinase pathway. The mTOR are known to inhibit the autophagy process and subsequently lead to tumor development. As a strategy, rapamycin, a known inhibitor of mTOR, was used as an autophagy inducer and 3-methyladenine (3MA) as a classical inhibitor of autophagy. In the present study, a systematic global gene expression was investigated in rapamycin-induced autophagy and the effects of rapamycin when autophagy process is inhibited. The findings have demonstrated that rapamycin was capable of inducing autophagy in T-47D breast carcinoma cells. However, when autophagy was inhibited by 3MA, rapamycin appeared to induce apoptosis in these breast cells. This observation was fully supported by various methods using appropriate microscopic techniques. Furthermore, rapamycin produced lower growth inhibition (GI_{50}), total growth inhibition (TGI) and lethal

concentration (LC_{50}) values in T-47D cells when the autophagy process was inhibited by 3MA, compared to cells treated either with rapamycin or 3MA alone, indicating that apoptosis is an effective mode of cell death compared to autophagic death. The *PHLDA1* (pleckstrin homology-like domain, family A, member 1) gene was found to be up-regulated in both autophagy and apoptosis and silencing this gene appeared to reduce both activities. These findings strongly support that *PHLDA1* mediates and possibly regulates both autophagy and apoptosis pathways and that these two processes can utilize common pathways or pathway components. On the other hand, the *RICTOR* (rapamycin-insensitive companion of mTOR) gene was found to be up-regulated in apoptosis and silencing this gene has shown to inhibit apoptotic activity, indicating that *RICTOR* may also play a role in rapamycin-induced apoptosis in T-47D breast cancer cells. In conclusion, this study provides novel insights into the molecular mechanisms of rapamycin-induced autophagy and apoptosis in T-47D breast carcinoma cells and will definitely make contribution to the currents studies which explore the intersections between both autophagy and apoptosis pathways. The results of this study may also open up avenues to explore or ideas in the treatment of breast cancer, namely the possible utilization of autophagy inhibitors and gene therapy using *PHLDA1*.

CHAPTER 1

INTRODUCTION

1.1 Breast Cancer

Cancer is one of the leading causes of death worldwide, especially in the economically developing world. According to the Globocan 2008[1], there were approximately 12.7 million cancer cases and 7.6 million cancer deaths worldwide in 2008 (Jemal *et al*, 2011). In 2010, a total of 1,529,560 new cancer cases and 569,490 deaths from cancer were reported to occur in the United States (Jemal *et al*, 2010). Breast cancer is the most commonly diagnosed malignancy among women, and the second only to lung cancer as a cause of cancer deaths in women worldwide (Jemal *et al*, 2010). In the past decade, the worldwide incidence of breast cancer has significant geographical difference; it is highest in developed countries in northern Europe and North American, intermediate in Mediterranean countries and South America, and lowest in Asia and Africa (Ferlay *et al*, 2001). The incidence and mortality rates of breast cancer has remained higher in developed countries compared to developing countries (Althuis *et al*, 2005). Recently, there is a massive increase in the annual incidence of breast cancer among the countries where its incidence was low, especially in the Asian countries (Baig *et al*, 2011; Parkin *et al*, 2005; Pathy *et al*, 2011). The proposed contributing factors to the increase include changes in reproductive factors, environmental exposures, and differences in lifestyle such as dietary and physical activity (Hisham and Yip, 2004; Pathy *et al*, 2011).

In 2000, there were 1.05 million cases of breast cancer reported worldwide, with 372,969 deaths (Ferlay *et al*, 2001). In 2002, the number increased to 1.15 million cases, and it is the most prevalent cancer in the world (Parkin *et al*, 2005). Among Malaysian women, breast cancer is the most frequent cause of death (Hisham and Yip, 2004; Lim and Halimah, 2003). In 2003, there were 3,738 female breast

[1] Globocan is a unique software program which provides access to information on the incidence and prevalence of, and mortality from 26 major cancers for all the countries in the world (http://apps.who.int/bookorders/anglais/detart1.jsp?sesslan=1&codlan=1&codcol=76&codcch=12).

cancer cases that were reported, making it the most commonly diagnosed cancer in Malaysian women (Lim and Halimah, 2003). In the year 2006, there were 3,525 female breast cancer cases registered with the National Cancer Registry (NCR) of Malaysia, accounted for 16.5% of all cancer cases registered (Omar *et al*, 2006). Generally, the Age Standardised Rate (ASR) of female breast cancer was 47.4 per 100,000 Malaysian women (Baig *et al*, 2011).

There are many risk factors associated with development of breast cancer, for example: sex, age, lack of childbearing or breastfeeding, higher hormone levels, the long-term use of hormone replacement therapy (HRT), race, economic status, a high-fat diet, alcohol intake, radiation and dietary iodine deficiency (2002; Aceves *et al*, 2005; Boffetta *et al*, 2006; Chen *et al*, 2002; Chlebowski *et al*, 2006; Giordano *et al*, 2004; Stoddard *et al*, 2008; Venturi, 2001; Yager and Davidson, 2006). Moreover, the family history of women with a first-degree relative with breast cancer has a risk two to three times. The risk factor further increases if the relative was affected at an early age and/or had bilateral disease (Skolnick and Cannon-Albright, 1992). Recently, smoking tobacco may increase the risk of breast cancer with the greater the amount of smoking and the earlier in life smoking commences the higher the risk (Xue *et al*, 2011).

In addition to the risk factors mentioned above, at the genetic levels, some oncogenes such as *HER-2* (human epidermal growth factor receptor 2); also known as proto-oncogene *Neu*, or *erb-B* (erythroblastic leukemia viral oncogenes homolog 2), *c-myc* and B-cell lymphoma 2 (*Bcl-2*); and some tumor suppressor genes including multiple tumor suppressor 1 (*MTS1*; known as *p16*), retinoblastoma (*RB*), *p53*, breast cancer susceptibility gene 1 (*BRCA1*) and breast cancer susceptibility gene 2 (*BRCA2*) are involved in breast cancer (Weinberg, 1996). Approximately, up

to 10% of breast cancer is hereditary (Carroll *et al*, 2008). Most women who have breast cancer do not have the genes associated with hereditary breast-ovarian cancer syndromes (HBOC); the exception is women and men who are carriers of *BRCA1* and *BRCA2* mutations. Mutation in either of *BRCA1* and *BRCA2* genes gives a woman an increased lifetime risk of developing breast cancers of between 60 and 85% (Wooster and Weber, 2003). In particular, carriers of the *BRCA1* and *BRCA2* genes are at a 30-40% increased risk for breast cancer, depending on in which portion of the protein the mutation occurs (Venkitaraman, 2002). Inherited mutations in *BRCA1* and *BRCA2* genes are associated with a high risk of developing breast cancers in women of different age and ethnic groups (de Jong *et al*, 2002; Farooq *et al*, 2011). Although most of *BRCA1* and *BRCA2* studies have been focused on the Caucasian populations, the allelic frequency of higher penetrance[2] genes in Asian population may be higher than in Caucasian population (Farooq *et al*, 2011; Toh *et al*, 2008). Other mutations that lead to breast cancer have been experimentally associated with estrogen exposure (Cavalieri *et al*, 2006).

Breast cancers have shown loss of heterozygosity (LOH) at one or more of a large number of chromosomal loci, including 1q, 3p, 6q, 16q, 17p, and 18q in 50% or more tumors and at 1p, 7q, 8q, 9q, 11p, 13q, 15q, 17q, and 22q in about 30% of tumors (Callahan and Campbell, 1989; Coles *et al*, 1992; Cropp *et al*, 1990). These findings suggest that the mutations of a number of tumor suppressor genes and oncogenes are implicated in breast cancer (Callahan and Campbell, 1989). These mutations are either inherited or acquired after birth, which allow uncontrolled division of cells, lack of attachment and metastasis to distant organs (Dunning *et al*, 1999).

[2] Penetrance is the likelihood a given gene will result in disease. For example, if half (50%) of the people with the neurofibromatosis (NF) gene have the disease NF, the penetrance of the NF gene is 0.5 [Dictionary of Medicine].

Breast epithelial cells, like other normal cells, require the balance of cell proliferation with a type of cell death called apoptosis which occurs in healthy breast cells at varying rates in response to changes in both extrinsic and intrinsic pathways (Wu, 1996). The breast normal cells are protected from apoptosis by several protein clusters and pathways. One of the protective pathways is PI3K/Akt pathway (Simstein *et al*, 2003). Alterations in the genetics of apoptosis mechanisms may result in an increase in cell numbers, subsequently preservation of genetically altered cells, leading to the process of tumorigenesis (Furth, 1999). Another programmed cell death, called autophagy, has a wide involvement in metabolic equilibrium and homeostasis, makes it an important target in human cancers (Liang and Jung, 2010). The first association between autophagy and breast cancer was made on the basis of observations that dysregulation of *Beclin-1* expression induces autophagy and subsequently suppresses breast cancer tumor cell growth (Liang *et al*, 1999). Inactivation of autophagy-specific genes, such as *Beclin-1*, results in increased tumorigenesis in mice, and compel expression of this gene inhibits the formation of human breast cancers in mouse models (Levine, 2007).

The breast cancer cell lines have become major experimental models, not only for breast cancer research but for dissecting basic molecular mechanisms controlling diverse aspects of epithelial cell biology (Sutherland *et al*, 1999). The human breast carcinoma cell line T-47D and two other breast cancer cell lines, namely MCF-7 and MDA-MB-231, account for more than two-thirds of all reporting studies on mentioned breast cancer cell lines, as recorded in a Medline-based survey (Lacroix and Leclercq, 2004). The T-47D cell line was isolated from a pleural effusion of a 54 year old female patient with an infiltrating ductal carcinoma of the breast (Keydar *et al*, 1979). In the two last decades, many studies have shown some

of the features of T-47D breast cancer cells, focusing on expression of nuclear and cell surface receptors, signal transduction molecules and cell cycle regulatory molecules, since these are components of known oncogenic pathways in breast cancer (Daly *et al*, 1994; deFazio *et al*, 1997; Douglas *et al*, 1997; Hall *et al*, 1990; Musgrove *et al*, 1995; Roman *et al*, 1992). Furthermore, the usefulness of the T-47D breast cancer cells as an investigative tool led to its adoption in autophagy and apoptosis related-studies (Ait-Mohamed *et al*, 2011; Bruning *et al*, 2010; Mathivadhani *et al*, 2007; Mooney *et al*, 2002).

1.2 Programmed Cell Death (PCD)

Cell death refers to any form of death of a cell mediated by intracellular death process (Engelberg-Kulka *et al*, 2006). The process of cell death in multicellular organisms has been documented many times during the past 150 years (Peter *et al*, 1997; Vaux, 2002). Since the first description of the term “programmed cell death”, which date back to 1964, many attempts have been made to classify cell death categories based on morphological characteristics (Galluzzi *et al*, 2012b; Lockshin and Williams, 1964). Thus, in 1973 it has proposed a classification of cell death modalities, including cell death type I (apoptosis), cell death type II (autophagic cell death), and cell death type III (necrosis), depending on the morphological and biochemical features (Clarke, 2002; Gozuacik and Kimchi, 2007; Kroemer *et al*, 2005; Schweichel and Merker, 1973). The molecular pathways that mediate cell death have been investigated and the biochemical assays for monitoring cell death have become laboratory routine. A systematic classification of cell death categories based on biochemical rather than morphological characteristics has got to be adopted (Galluzzi *et al*, 2012b).

The Nomenclature Committee on Cell Death (NCCD) 2012 has recommended appropriate use of cell death-related terminology, including extrinsic apoptosis, caspase-dependent or -independent intrinsic apoptosis, autophagic cell death, regulated necrosis and mitotic catastrophe. According to the NCCD, extrinsic apoptosis refers to instances of apoptotic cell death that are induced by extracellular stress signals that are stimulated by specific transmembrane receptors. However, intrinsic apoptosis can be triggered by intracellular stress conditions; such as DNA damage and oxidative stress, leading to cell death process that is mediated by mitochondrial outer membrane permeabilization (MOMP) (Galluzzi *et al*, 2012b). The term “autophagic cell death” has been used to indicate instances of cell death that are accompanied by a massive cytoplasmic vacuolization, which indicates increased autophagy process that can be suppressed by the inhibition of the autophagic pathway (Galluzzi *et al*, 2012b).

Furthermore, NCCD proposed recommendations for the suitable use of cell death-related terminology, including *programmed cell death*, for the physiological instances of cell death that occur in the context of embryonic development and tissue homeostasis; *regulated cell death*; for cell death that occurs in the molecular mechanisms mediating like inhibition by targeted pharmacological and/or genetic interventions; and *accidental cell death*; for cell death that triggered by extremely harsh physical conditions like freeze-thawing cycles (Galluzzi *et al*, 2012b).

Autophagy and apoptosis are very important physiological mechanisms that control the development, homeostasis, and elimination of unwanted and malignant cells (Hockenbery *et al*, 1990; Wang and Klionsky, 2003). Apoptosis can start with autophagy, autophagy can end with apoptosis, and obstruction of caspase activity can cause a cell to default from apoptosis to autophagy (Lockshin and Zakeri, 2004). The

relationship between autophagy and apoptosis is varied and complex. These two pathways can be almost linked by specific molecular triggers, either in a positive, regulated manner which balances cell proliferation, or in a negative, unregulated manner which results in tumor formation (Gozuacik and Kimchi, 2004).

1.3 Apoptosis

The term “apoptosis” is derived from the Greek word used to describe the “falling off leaves from a tree” (Malcolm, 2007). The chronological history of apoptosis is described in Table 1.1 (Vaux, 2002). Apoptosis is a mode of cell death that occurs in response to cytotoxic compounds, cell dysfunction and infection. This cellular process can be initiated by either extrinsic or intrinsic stimuli. It is of fundamental importance in the development, growth, health and tissue homeostasis of multicellular organisms (Dunn *et al*, 2007).

Apoptosis is defined by biochemical events, including activation of intracellular proteases and internucleosomal DNA fragmentation, leading to morphological changes including cell membrane blebbing, cell shrinkage and chromatin condensation that characterized cell death (Kerr *et al*, 1972). Apoptosis in mammalian cells is mediated by a family of cysteine proteases known as caspases (Alnemri *et al*, 1996).

The molecular events of apoptosis can be divided into three steps: initiation by an apoptosis-inducing agent, activation of the caspases by a signal transduction cascade, and proteolytic cleavage of cellular components (Simstein *et al*, 2003). In apoptosis, there are many death and survival genes which are regulated by extracellular factors. The initial steps of apoptosis occurs in the membrane during

Table 1.1 Timeline, a history of apoptosis

Year	Event
1842	The cell death occurring normally during vertebrate development was recognized (Vaux, 2002).
1951	The first examination of cell death as a normal part of vertebrate development was described (Glucksmann, 1951).
1961	The developmental cell death was featured by transmission electron microscope (Bellairs, 1961).
1966	The cell death was found to be active process through linking of developmental and hormonally regulated cell death (Tata, 1966)
1972	The term "Apoptosis" was used to describe vertebrate cell deaths (Kerr *et al*, 1972).
1980	Chromatin condensation and DNA degradation were discovered as some characters of apoptosis (Wyllie, 1980).
1985	Targets of cytotoxic T cells (CTL) killing was displayed the characteristic features of apoptosis (Clouston and Kerr, 1985).
1986	Withdrawal of growth factor found to activate apoptosis (Duke and Cohen, 1986). First cell death gene (ced-3), now known as caspase-8, which is essential for apoptosis in the worm was recognized (Ellis and Horvitz, 1986).
1988	The Bcle-2 was discovered as the first identification of component of the apoptosis mechanism (Vaux *et al*, 1988).
1989	Fas/Apo-1 (CD95) was known to signal apoptosis when crosslinked by antibodies (Trauth *et al*, 1989; Yonehara *et al*, 1989).
1991	P53 was found to cause apoptosis via the mechanism that can be blocked by Bcl-2 (Chiou *et al*, 1994; Yonish-Rouach *et al*, 1991).
1993	Bax, a Bcl-2 family member, was promoted caspase activity (Oltvai *et al*, 1993).
1994	The first inhibitor of apoptosis (IAP) gene, p35, was identified in baculoviruses (Birnbaum *et al*, 1994).
1995	Several mammalian IAP genes have been discovered (Roy *et al*, 1995; Uren *et al*, 1996).
1996	Increased release cytochrome c to cytosol was suggested that mitochondria may function in apoptosis by releasing cytochrome c (Liu *et al*, 1996).
1997	Apaf-1 and caspase-9 were identified in human (Li *et al*, 1997b; Zou *et al*, 1997). Cytochrome c was revealed to be a molecule capable of activating Apaf-1 (Liu *et al*, 1996).
1998	Apoptosis was found to mediate by death receptors belong to TNF receptor superfamily in the extrinsic pathway (Schulze-Osthoff *et al*, 1998). Bax was found to increase the membrane's permeability and initiate the caspase pathway for apoptosis (Marzo *et al*, 1998; Narita *et al*, 1998).

Table 1.1 *Continued*

Year	Event
1999	Bak was found to be a proapoptotic membrane of the Bcl-2 family (Gross *et al*, 1999).
2000	Smac/Diablo, the second protein in the apoptosis link, along with cytochrome c, that promotes apoptosis by activating caspases, was identified as inhibitors of mammalian IAPs (Du *et al*, 2000; Verhagen *et al*, 2000).
2001	Smac/Diablo was found to displace caspase-9 from IAPs (Srinivasula *et al*, 2001).
2003	Phagocytes lacking the phosphatidylserine receptor (PSR) were found to be defective in removing apoptotic cells (Li *et al*, 2003). The cytotoxic T lymphocytes (CTLs) were found to be able to kill target cells via the extrinsic pathway, and FasL/FasR interaction was found to be the predominant method of CTL-induced apoptosis (Brunner *et al*, 2003)
2007	Golgi-anti-apoptotic protein (GAAP), a new regulator of apoptosis, was described to be expressed in all human tissues tested, inhibited apoptosis induced by intrinsic and extrinsic apoptotic stimuli (Gubser *et al*, 2007). The first parapoxvirus apoptosis inhibitor, ORFV125, is identified as a new antiapoptotic member of the Bcl-2 family (Westphal *et al*, 2007).
2009	B-cell lymphoma 2 interacting mediator of cell death (BIM) is a new mediator of tumor cell death, either apoptosis or autophagy, in response to novel oncogene-targeted therapeutics (Gillings *et al*, 2009).
2010	Overexpression of a pro-apoptotic Par-4 protein was found to sensitize TRAIL-induced apoptosis via inactivation of NF-kappaB and Akt signaling pathways (Lee *et al*, 2010)
2011	Par-4 was found to be a novel specific caspase-3 cleavage site, and the cleaved fragment of Par-4 retains proapoptotic activity (Chaudhry *et al*, 2012).

ceramide generation through activation of sphingomyelinase (SMase) and downstream signaling involving Bcl-2 family members, the inhibitor of apoptosis (IAP) family of proteins, the transcription factor nuclear factor-κB (NF-κB), members of the mitogen-activated protein kinase (MAPK) family, such as p42/44MAPKs (extracellular signal-related kinases [ERKs]), SAPK/JNK, and p38MAPK, and caspases (Baldwin, 2001; Ballif and Blenis, 2001; Deveraux and Reed, 1999; Fadeel *et al*, 1999; Kaufmann and Earnshaw, 2000; Liu *et al*, 1999; Reed, 2001). Apoptosis process is determined by the balance between pro-apoptotic and anti-apoptotic regulators expressed in the cells (Simstein *et al*, 2003).

The family of mammalian apoptotic proteases, which are known as caspases, are classified as activator (initiator) caspase (e.g. caspases-2, -8, -9, -10 and -12), which cleave and activate downstream effector caspases, or executioner (effector) caspases (e. g. caspases-3, -6, and -7), which cleave various cellular proteins substrates within the cell, to trigger the apoptosis pathway (Earnshaw *et al*, 1999; Fan *et al*, 2005). The initiator caspases (such as caspase-2, -8, -9, and -10) are activated by formation three of caspase-activating complexes including: DISC (Death Inducing Signaling Complex), which activates caspases-8 and 10; Apoptosome, which activates caspase-9; and PIDDosome, which activates caspase-2 (Park, 2012). There are two known pathways that activate the caspases cascade; the extrinsic pathway, which is independent of mitochondria and is induced by death receptor-protein complexes that cleave procaspase-8, and the intrinsic pathway, which involves the Bcl-2 family of proteins and the release of cytochrome c from mitochondria (Figure 1.1) (Gross *et al*, 1999; Simstein *et al*, 2003).

The extrinsic apoptosis signaling pathway is mediated by the activation of death receptors, which belong to the tumor necrosis factor receptor (TNFR) gene

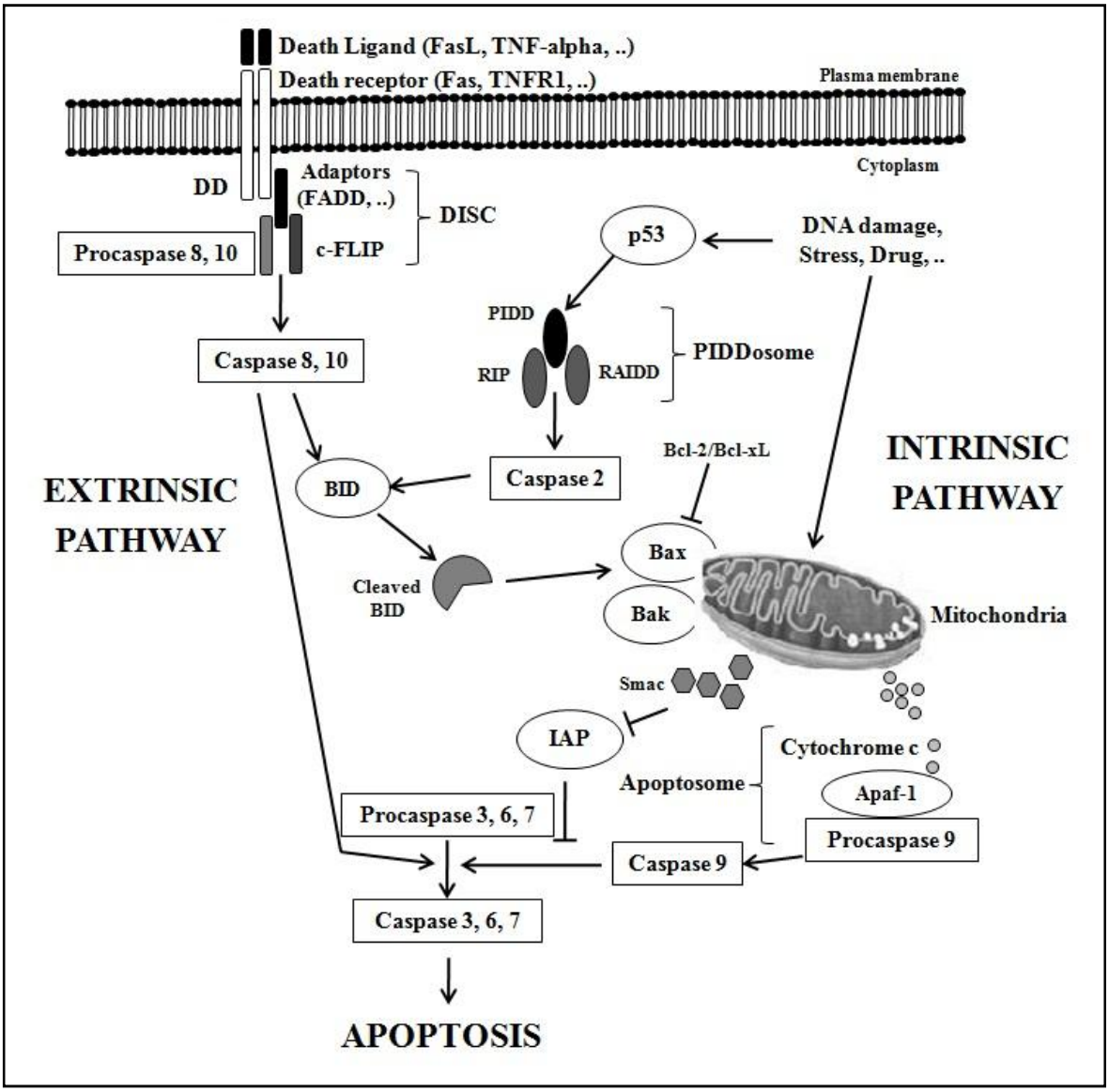

Figure 1.1 The intrinsic and extrinsic apoptotic pathways.

family such as TNFR-1, Fas/CD95, and the TRAIL receptors DR-4 and DR-5 (Ashkenazi, 2002). Ligands, including TNF ligands, TNF ligand superfamily member 10 (TNFSF10), Fas ligand and TRAIL (TNF-related apoptosis-inducing ligand), interact with their specific death receptors, recruiting Fas-associated death domain adapter protein (FADD) and thereby forming the death inducing signaling complex (DISC) (Sartorius *et al*, 2001). This complex mediates activation of pro-caspase-8 and pro-caspase-10, leading to the activation of the executioner caspases-3, -6, and -7 which in turn cleave a number of protein substrates (Hengartner, 2000; Schulze-Osthoff *et al*, 1998). Active caspase-3 or caspase-7 eventually leads to the characteristic morphological and biochemical features of apoptosis. Both caspase-3 and -7 cleaves DNA fragmentation factor 45 (DFF45) which subsequently releases active DFF40; the inhibitor's associated endonuclease, which is responsible for the degradation of chromosomes into nucleosomal fragments (Widlak and Garrard, 2005; Wolf *et al*, 1999).

The intrinsic apoptosis signaling pathway is mediated by mitochondria through release of cytochrome c and Smac (second mitochondria-derived activator of caspases)/Diablo (direct inhibitor of apoptosis-binding protein) from the mitochondrial intermembrane space to the cytosol, responding to apoptotic stimuli including DNA damage, γ-irradiation, and serum deprivation (Gross *et al*, 1999; Hengartner, 2000). DNA damage induces the expression of PIDD (p53-induced protein with death domain) which binds to the adaptor protein RAIDD (receptor interacting protein (RIP)-associated Ich-1/Ced-3 homologous protein with a death domain) and precaspase-2 forming the PIDDosome and leads to the activation of caspase-2 (Jang *et al*, 2010a; Jang *et al*, 2010b). Caspase-2 is involved in Bid cleaved and Bax translocation, which results in cytochrome c release during DNA damage

(Bouchier-Hayes, 2010; Lassus *et al*, 2002; Tinel and Tschopp, 2004). Cytochrome c contributes to the formation of the apoptosome which consists of cytochrome c, apoptotic protease activating factor-1 (Apaf-1) and dATP. Subsequently, the apoptosome recruits the initiator pro-caspase-9, leading to the activation of caspase-9 which finally mediates the activation of caspase-3 and caspase-7 (Denault and Salvesen, 2002; Earnshaw et al, 1999). Smac, another mitochondrial proapoptotic factor, acts by inhibiting the inhibitors of apoptosis (IAPs) from blocking caspase activity. IAPs are a family of proteins with antiapoptotic activity by directly inhibiting caspases. Currently, eight human IAPs have been identified such as X-linked IAP (XIAP), IAP-like protein-2 (IAP-2), cellular inhibitor of apoptosis-1 and 2 (c-IAP-1 and c-IAP-2), melanoma inhibitor of apoptosis protein (MLIAP), neuronal apoptosis inhibitory protein (NAIP), survivin and apollon (Salvesen and Duckett, 2002). XIAPs and c-IAP1/2 block cytochrome c-induced activation of caspase-9, thus preventing the activation of caspase-3, -6 and -7. In addition, they bind to and inhibit the enzymatic activity of caspase-3 leading to blocking downstream apoptotic events by the initiator caspase (Deveraux *et al*, 1998).

The B-cell lymphoma 2 (Bcl-2) family members play an important role in the regulation of mitochondrial-linked apoptosis (Tsujimoto, 1989). Activated proapoptotic Bcl-2 subfamilies such as Bax and Bak form homo-oligomer which creates pores on the mitochondrial membrane and subsequent release cytochrome c from the mitochondria. Cytochrome c binds to and activates Apaf-1 protein in the cytoplasm leading to the formation of apoptosome (Hengartner, 2000) The antiapoptotic Bcl-2 family members such as Bcl-2 and Bcl-xL which counteract the action of proapoptotic Bcl-2 subfamilies such as Bax, Bak, and Bcl-2 homolog (BH)3-only family (e.g. Bid) and therefore can inhibit mitochondrial proapoptotic

events (Tsujimoto, 1998). The intrinsic apoptotic signals coming from the inside of the cell frequently have their origin within the nucleus, being a consequence of DNA damage, which in most cases results in the activation of the p53 transcription factor which stimulates expression of proapoptotic Bcl-2 members and represses antiapoptotic Bcl-2 and Bcl-xL (Hoffman *et al*, 2002; Wu *et al*, 2001).

Interestingly, in addition to mitochondria and the nucleus, the endothelium reticulum and lysosomes also have been implicated in apoptotic pathways and seemingly hundreds of proteins are part of an extremely fine-tuned regulatory network consisting of pro- and antiapoptotic factors (Gewies, 2003).

Apoptosis is a very important developmental pathway which serves as a defense mechanism to remove unwanted and potentially dangerous cells that have been infected by virus and cancer cells. However, it is important to mention that the inappropriate activation of apoptosis may contribute to the pathogenesis of many diseases such as cancer, neurodegenerative disorders, autoimmune disease, acquired immunodeficiency syndrome and resistance to chemotherapy (Vinatier *et al*, 1996).

Therefore, different attempts are being directed towards identifying the crucial steps in the apoptosis process, the main purpose being to design therapeutic approaches that control cell death by altering (stimulating or inhibiting) signaling molecules in the pathway. Methods to quantify apoptosis and to distinguish it from necrosis have been developed[3]. The explosion of interest in apoptosis and cell death studies has resulted in the development of a diversity of different apoptosis detection methods. There are a wide variety of new techniques available, but each technique has its advantages and disadvantages which may make it acceptable to use for one

[3] Necrosis refers to the morphology usually associated with accidental cell death which occurs when cells are exposed to a serious physical or chemical insult, while apoptosis is seen when cell death is programmed or physiologically regulated (Martin D, Lenardo M (2001). Morphological, biochemical, and flow cytometric assays of apoptosis. *Curr Protoc Mol Biol* **Chapter 14:** Unit 14 13.)

application but not suitable for another application (Watanabe *et al*, 2002). Historically, the study of apoptosis was first based on cell morphology using transmission electron microscopy (TEM): chromatin condensation, cellular shrinkage, break-up of the cell and its engulfment (Bellairs, 1961). Several approaches have been used, including appearance of the characteristic 180 base pairs DNA ladder banding pattern on agarose gels (Wyllie, 1980). Subsequently, marked progress in biochemistry, molecular biology and genetics provided researchers various methods for apoptosis detection, such as the TUNEL (TdT-mediated dUTP Nick-End Labeling) assay, flow cytometry, DNA fragmentation ELISA (Ito *et al*, 1996; Kressel and Groscurth, 1994; Salgame *et al*, 1997). Subsequently, the caspase family of proteases has been identified as common mediators of the cell suicide pathway that can be detected using various types of caspase activity assays (Gurtu *et al*, 1997). Caspase activation can be detected in different ways including western blot and immunohistochemistry (Elmore, 2007; Talasz *et al*, 2002). Some of these assay systems have been described as measuring the early stages of apoptosis when the pro-apoptotic stimuli trigger activation of the molecular machinery of apoptosis, leading the molecular executioner machinery becomes totally activated as shown by the ability of the cytosolic extracts of cells to induce apoptotic changes in nuclei (Lazebnik *et al*, 1993; Solary *et al*, 1993). On the other hand, some assays systems have been described as measuring the late stages in the overall process of apoptosis when the hallmarks of apoptosis become evident including morphologic changes and DNA fragmentation (Saraste and Pulkki, 2000). Since much remains incompletely understood about the molecular pathways of apoptosis, and it is probably best to carry out more than one of the basic techniques to confirm an investigation of apoptotic cells (Muppidi *et al*, 2004a).

1.4 Autophagy

Two primary intracellular protein degradation pathways are known in eukaryotic cells: the ubiquitin-proteasome system (UPS) and the lysosome (Figure 1.2) (Luo *et al*, 2010). The differences between these two major protein degradation systems depend on their functional significance and the type of substrates they take in for degradation (Gao *et al*, 2009). In the proteasome system, the UPS catalyses the rapid degradation of abnormal proteins and short-lived regulatory proteins leading to control a diversity of essential cellular processes (Ciechanover, 1998). In the lysosomal protein degradation pathway, the degradation of extracellular materials is mediated by endocytosis, whereas the degradation of intracellular long-time cytoplasmic proteins and damaged organelles is mediated by three types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA), which are classified based on their transport of cytoplasmic materials into the lysosome for degradation (de Duve and Wattiaux, 1966; Gao *et al*, 2009; Meijer and Codogno, 2004).

The term autophagy literally comes from Greek, meaning “self-eating” (Gao *et al*, 2009; Liu *et al*, 2010a; Tan *et al*, 2009). Interestingly, although autophagy was first described more than 50 years ago, the molecular understanding of it has just started in the past decade. The chronological history of autophagy research is described in Table 1.2 (Klionsky, 2007). In the year 1957, Clark observed a process of bulk segregation of mitochondria within membrane-bounded compartments termed “dense bodies”, which were subsequently shown to include lysosomal enzymes (Clark, 1957). The same bodies containing the cellular components and lysosomal hydrolases was observed and reported between the years 1959 and 1962 (Ashford and Porter, 1962; Novikoff, 1959; Novikoff and Essner, 1962). One year

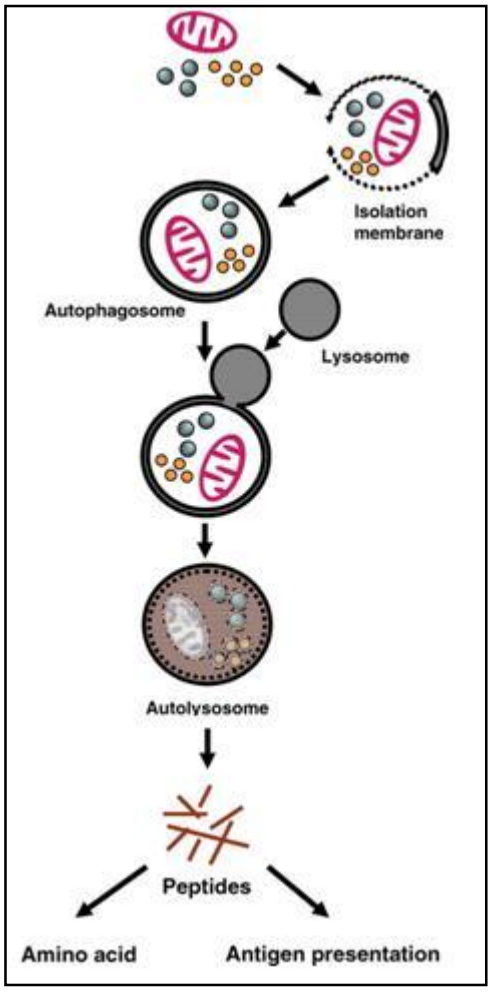

Figure 1.2 The autophagy pathway is one of essential primary intracellular protein degradation systems in eukaryotes (Luo *et al*, 2010).

Table 1.2 Timeline, a history of autophagy

Year	Event
1955	The lysosome was described (de Duve *et al*, 1955).
1963	The morphological process was described, and the name "autophagy" was coined.
1967	Glucagon was found to induce autophagy (Deter *et al*, 1967).
1973	The selective sequestration of an organelle was demonstrated (Bolender and Weibel, 1973).
1977	Amino acids were found to inhibit autophagy (Mortimore and Schworer, 1977). The autophagy function was suggested as a cell-death mechanism (Beaulaton and Lockshin, 1977).
1982	The first biochemical analysis of autophagy was carried out, and 3-methyladenine (3MA) was identified as an inhibitor (Caro *et al*, 1988; Gordon and Seglen, 1982; Seglen and Gordon, 1982).
1988	The amphisome was identified as a convergence point between autophagy and endocytosis (Gordon and Seglen, 1988).
1992	The morphology of autophagy was showed in yeast (Takeshige *et al*, 1992)
1993	The first screen was reported to identify yeast autophagy mutants (Tsukada and Ohsumi, 1993). Different methods were used to isolate autophagy mutants (Klionsky *et al*, 2003).
1995	The stimulatory role of rapamycin was documented (Blommaart *et al*, 1995).
1997	A stimulatory role for PI3K was found (Blommaart *et al*, 1997). The yeast Atg1 was cloned (Matsuura *et al*, 1997) Thirty more Atg genes were identified in yeasts (Kabeya *et al*, 2007; Kawamata *et al*, 2005; Klionsky *et al*, 2003; Stasyk *et al*, 2006).
1998	The first mammalian autophagy gene was identified, and the conservation of Atg12-Atg5 conjugation was shown (Mizushima *et al*, 1998).
1999	BECN1/Atg6 was identified as a Bcl2-interacting protein and tumor suppressor (Liang *et al*, 1999).
2000	LC3 assays were developed for monitoring autophagy in higher leukaryotes (Kabeya *et al*, 2000).
2002	The protective role of autophagy was shown in Huntington's disease (Ravikumar *et al*, 2002)

Table 1.2 *Continued*

Year	Event
2003	Deficient autophagy was found to contribute of tumorigenesis *in vivo* (Qu *et al*, 2003; Yue *et al*, 2003). The relationship between autophagy and longevity was identified (Bergamini, 2006; Melendez *et al*, 2003).
2004	The evidence of a physiological role for autophagy in neonatal mice was provided (Kuma *et al*, 2004). The evidence of a role for autophagy in innate and adaptive immunity was provided (Dengjel *et al*, 2005; Gutierrez *et al*, 2004; Liu *et al*, 2005). The cell death was shown to depend on autophagy proteins (Clarke *et al*, 2009; Shimizu *et al*, 2004; Yu *et al*, 2004). First crystal structure of autophagy protein was determined (Sugawara *et al*, 2004).
2006	The role for basal autophagy in preventing neurodegeneration was demonstrated (Hara *et al*, 2006; Komatsu *et al*, 2006).
2008	The relationship between mTOR-independent pathways and autophagy was uncovered (Wei *et al*, 2008)
2009	The mitochondria-anchored receptor Atg32 was identified as a mediator in degradation mitochondria via selective autophagy (Okamoto *et al*, 2009).
2010	The endoplasmic reticulum membrane, the mitochondrial outer membrane and the plasma membrane have found to contribute to autophagosome formation (Hailey *et al*, 2010; Ravikumar *et al*, 2010).

later, in 1963, de Duve established this research area when he described the term “autophagy” to describe the presence of single- or double-membrane vesicles that contain parts of the cytoplasm and organelles in various states of disintegration, at the Ciba Foundation symposium on lysosomes (de Duve, 1963). In 1966, De Duve and Wattiaux suggested that the sequestering membranes are derived from preformed membranes, such as smooth endoplasmic reticulum (de Duve and Wattiaux, 1966). In 1967, glucagon was found to induce autophagy (Deter *et al*, 1967). In 1977, insulin was found to inhibit autophagy (Pfeifer, 1977). In 1982, biochemical analysis of autophagy was carried out and the pharmacological reagent 3-methyladenine (3MA) was identified as an autophagy inhibitor (Seglen and Gordon, 1982). In 1992, the evidence that protein kinase and phosphatase can regulate autophagy was made known (Holen *et al*, 1992).

In 1997, Holen and colleagues identified the first autophagy-related gene Atg1 in *Saccharomyces cerevisiae* (Matsuura *et al*, 1997). The morphology of autophagy in yeast was similar to that documented in mammals (Takeshige *et al*, 1992). In 1998, the first mammalian autophagy gene was identified, and the Atg12-Atg5 conjugation system was described (Mizushima *et al*, 1998). In 2000, microtubule-associated protein 1 light chain 3 (LC3) detection assays were used for monitoring autophagy in higher eukaryotes (Kabeya *et al*, 2000). Three years later, it was discovered that deficient autophagy contributes to tumorigenesis *in vivo* (Qu *et al*, 2003; Yue *et al*, 2003).

Autophagy occurs as a housekeeping mechanism in normal growing conditions (Espert *et al*, 2007). The functions of autophagy are enabling cell survival and inducing of damaged cells death (Yousefi and Simon, 2007). Autophagy is included in several physiological processes involving the response to starvation, cell

growth control, cell death, differentiation/ development, toxic stimuli and chemotherapy (Kondo *et al*, 2005; Kroemer and Jaattela, 2005; Levine and Klionsky, 2004). In addition to the essentiality of autophagy for adaptation and survival during starvation conditions, autophagy has been shown to play a role in tumor suppression (Kuma *et al*, 2004; Qu *et al*, 2003; Scott *et al*, 2004; Yue *et al*, 2003). Decreased levels of autophagy have been associated with tumor development (Qu *et al*, 2003; Yue *et al*, 2003). The discovery of over 30 Atg genes in yeast has been essential in the molecular understanding of this process, and many of whose mammalian orthologues have also been discovered (Espert *et al*, 2007; Roy and Debnath, 2010).

Autophagy pathway is a multi-step process characterized by induction, vesicle nucleation, extension and completion of an isolation membrane to form an organelle called autophagosomes (Roy and Debnath, 2010). The final stages of autophagy are docking, and fusion with a lysosome, degradation and recycling (Yoshimori and Noda, 2008). The core autophagy machinery is composed of four major functional groups: [1] the Atg1-Atg13-Atg17 kinase complex, [2] the, class III phosphoinositide-3-kinase (PI3K) complex I; including Class III PI3K (the mammalian orthologue of vascular protein sorting 34; Vps34), p150 (the mammalian orthologue of Vps15), beclin-1 (the mammalian orthologue of Atg6, also called Vps30) and Atg14, [3] two ubiquitin-like conjugation systems; Atg12 and Atg8; and [4] Atg9 and its cycling system (Yang and Klionsky, 2010). A fifth group set of core components includes protein needed for the last step of autophagy process when the single-membrane intravascular vesicles and their cargo break down, and permeases enzymes release these degradation products back into the cytosol for re-use (Epple *et al*, 2001; Teter *et al*, 2001; Yang *et al*, 2006b). The schematic illustration of autophagy process is shown in Figure 1.3.

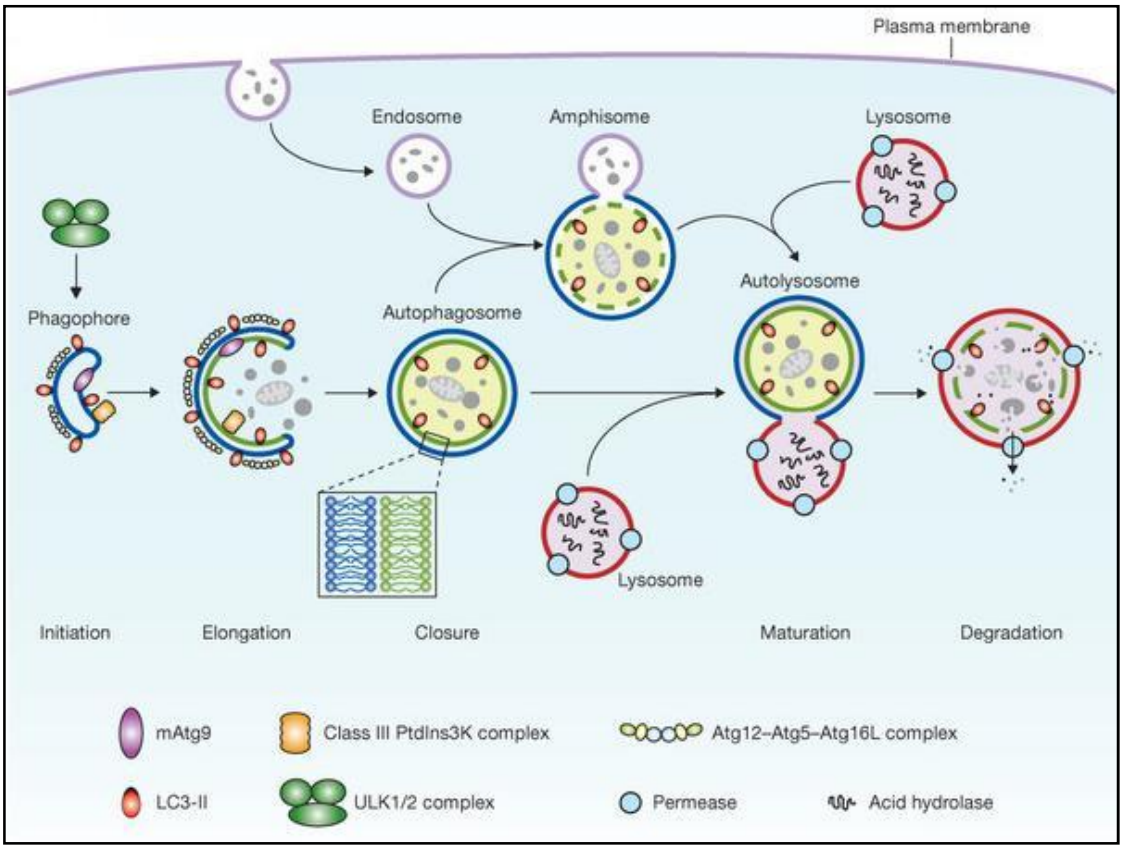

Figure 1.3 Schematic illustration of autophagy (Yang and Klionsky, 2010).

Autophagy can be induced by a variety of different stimuli such as nutrient deprivation, hypoxia, cytokines, etc, and the induction of autophagy is mostly associated with the mammalian target of rapamycin complex 1 (mTORC1) which is a central controller of cell growth (Wullschleger *et al*, 2006). The unc-51-like kinases (ULKs; the mammalian orthologs of Atg1), which exists in a large complex with mammalian Atg13 (mAtg13), focal adhesion kinase family interacting protein of 200 kDa (FIP200; the mammalian homolog of Atg17), and the recently identified Atg101, played a crucial role in induction of autophagy (Chan *et al*, 2009; Ganley *et al*, 2009; Hara and Mizushima, 2009; Jung *et al*, 2009; Mercer *et al*, 2009).

The early stages of nucleation of the phagophore are required to form a class III PI3K complex which consists of the PI3K class III protein, Beclin-1,and p150 (Simonsen and Tooze, 2009). Recent studies have shown different binding molecules positively regulate Beclin-1 activity and regulate different steps of autophagosome formation and maturation, including ultraviolet (UV) radiation resistance-associated gene (UVRAG), Atg14L, and autophagy/beclin-1 regulator 1 (Ambra1) (Fimia *et al*, 2007; Itakura *et al*, 2008; Liang *et al*, 1999; Matsunaga *et al*, 2009). The tumor suppressor, UVRAG, Bax-interacting factor 1 (Bif-1), and probably Ambra1associated with Beclin-1 to activate autophagy (Takahashi *et al*, 2007).

The next stage of phagophore membrane elongation requires two ubiquitin-like systems (Figure 1.4) (Ohsumi and Mizushima, 2004). In the first system of ubiquitin-like conjugation, the ubiquitin-like protein Atg12 firstly activates by Atg7, a ubiquitin-activated enzyme (E1)-like protein, and then transfers by Atg10, a ubiquitin carrier protein (E2)-like protein, to Atg5 through a covalent bond (Gao *et al*, 2009). The Atg5-Atg12 complex interacts with Atg16 to form a large multimeric